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The interaction between dopamine D_2-like and beta-adrenergic receptors in the prefrontal cortex is altered by mood-stabilizing agents

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Abstract

Several studies have suggested the involvement of biogenic monoaminergic neurotransmission in bipolar disorder and in the therapy for this disease. In this study, the effects of the mood-stabilizing drugs lithium, carbamazepine or valproate on the dopaminergic and adrenergic systems, particularly on D_2-like and β-adrenergic receptors, were studied both in cultured rat cortical neurones and in rat prefrontal cortex. *In vitro* and *in vivo* data showed that stimulation of β-adrenergic receptors with isoproterenol increased cyclic adenosine monophosphate (cAMP) levels and this effect was significantly inhibited by lithium, carbamazepine or valproate. The activation of dopamine D_2-like receptors with quinpirole decreased the isoproterenol-induced rise in cAMP in control conditions. This inhibition was observed *in vivo* after chronic treatment of the rats with carbamazepine or valproate, but not after treatment with lithium or in cultured rat cortical neurones after 48 h exposure to the three mood stabilizers. Dopamine D_2 and β_1-adrenergic receptors were found to be co-localized in prefrontal cortical cells, as determined by immunohistochemistry, but western blot experiments revealed that receptor levels were differentially affected by treatment with the three mood stabilizers. These data show that mood stabilizers affect D_2 receptor-mediated regulation of β-adrenergic signalling and that each drug acts by a unique mechanism.

Keywords: adenylate cyclase, β-adrenergic receptors, biogenic monoamines, bipolar disorder, cyclic adenosine monophosphate, dopamine D_2 receptors.
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Bipolar disorder is a common psychiatric condition affecting 1% of the world population (Goodwin and Jamison 1990). The most widely prescribed drugs for bipolar disorder are commonly referred to as mood stabilizers, and include lithium, valproate and carbamazepine. Although lithium is the prototypical medication for the treatment of bipolar disorder, there is a substantial group of patients (20–40%) who do not respond to this treatment (Gershon and Soares 1997; Maj 2000). The anti-convulsants carbamazepine and valproate, originally used to treat epileptic seizures, are an alternative or adjunctive to lithium in bipolar disorder treatment (Bowden 1996; Dunn *et al*. 1998; Post *et al*. 1998). Despite the widespread clinical use of lithium, valproate and carbamazepine, the molecular mechanism(s) underlying the mood-stabilizing effects are poorly understood (Jope 1999; Manji and Lenox 2000).

Changes of noradrenergic and dopaminergic signalling have been implicated in the pathogenesis and pharmacotherapy of bipolar disorder and related diseases like depression and schizophrenia (Avissar *et al*. 1996; Sundram *et al*. 2003;

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Abbreviations used: AC, adenylate cyclase; BSA, bovine serum albumin; β_1AR, β_1-adrenergic receptors; cAMP, cyclic adenosine monophosphate; DAD_2R, dopamine D_2 receptors; G-proteins, GTP-binding proteins; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; PKA, cAMP-dependent protein kinase; Ro-201724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; SDS, sodium dodecyl sulfate.

Wang and Goldman-Rakic 2004). Several lines of evidence indicate that bipolar disorder is associated with a dopaminergic dysregulation. Supporting this hypothesis, drugs that inhibit dopaminergic transmission exert an anti-manic action in bipolar disorder, whereas drugs that stimulate dopamine synthesis, activate dopamine receptors or inhibit dopamine re-uptake, often precipitate mania (Yatham 2002; Schatzberg 2004; Silverstone and Silverstone 2004). Interestingly, whereas conventional neuroleptics and novel anti-psychotics, which are also effective in treating acute mania (McElroy and Keck 2000), act by directly inhibiting dopamine D_2-like receptors (Seeman and Lee 1975; Creese *et al*. 1976), the mood-stabilizing drugs lithium, carbamazepine and valproate do not directly block dopamine D_2-like receptors and may therefore act by a different mechanism, such as by dampening the downstream second messenger signalling pathways activated by D_2-like receptors (Yatham *et al*. 2002). On the other hand, good evidence suggests that noradrenaline levels are higher than normal in the body fluids of patients with mania, and drugs such as lithium can blunt noradrenaline signalling through the β-adrenoreceptors. Moreover, noradrenergic drugs, such as tricyclic antidepressants, are particularly likely to promote manic switches and rapid cycling in bipolar patients (Young 2001). A β-adrenergic hyperfunction has been proposed to underlie manic episodes (Schreiber *et al*. 1991), whereas bipolar depressed patients showed a hypofuncfion of β-adrenergic receptors (Avissar *et al*. 1996).

Dopamine D_2-like and β-adrenergic receptors belong to the superfamily of GTP-binding protein (G-protein)-coupled receptors that transduce extracellular stimuli to intracellular signalling events. Agonist activation of the $G_{\alpha s}$-coupled β-adrenergic receptor stimulates cyclic adenosine monophosphate (cAMP) production, whereas acute activation of the $G_{\alpha i/o}$-coupled D_2-like dopamine receptors inhibits cAMP accumulation to provide antagonistic control on receptor-mediated adenylate cyclase (AC) activity at the cellular level (Watts 2002). Bipolar disorder is associated with an enhanced signalling activity of the cAMP cascade, as shown by higher levels of $G_{\alpha s}$ protein, stimulation of AC and increased activity of the cAMP-dependent protein kinases (PKA) (Young *et al*. 1993; Friedman and Wang 1996; Chang *et al*. 2003). Most of the components of the cAMP signalling pathways have been implicated in the actions of mood-stabilizing agents (Jope 1999; Manji *et al*. 1995; Mørk and Geisler 1995; Montezinho *et al*. 2004).

The aim of this study was to identify the mechanisms by which the mood stabilizers lithium, valproate and carbamazepine affect the interaction of dopamine D_2-like and β-adrenergic signalling, both *in vitro* and *in vivo*. The effects of the mood stabilizers on dopamine D_2 and β_1-adrenergic protein levels were studied by immunoblotting of both cultured cortical neurones and rat prefrontal cortex. The cAMP levels produced by the isoproterenol-sensitive AC, mimicking β-adrenergic activity, as well as the ability of D_2 receptor stimulation to block the increase of isoproterenol-stimulated cAMP levels, were measured *in vitro* in cultured cortical neurones and *in vivo* by microdialysis in the rat prefrontal cortex after treatment with mood-stabilizing drugs.

Materials and methods

Materials

The cAMP radioimmunoassay kits, [8-^{3}H] and [^{125}I], the polyvinylidene difluoride (PVDF) membrane, the alkaline phosphatase-linked anti-mouse and anti-rabbit secondary antibodies, the enhanced chemifluorescence (ECF) reagent and the low-fat milk were obtained from Amersham Biosciences (Little Chalfont, UK). Other reagents used in immunoblotting experiments were purchased from Bio-Rad (Hercules, CA, USA). Neurobasal medium, B27 supplement and trypsin (USP grade) were purchased from Gibco Invitrogen (Glasgow, UK). The 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, 3-isobutyl-1-methylxanthine and the bicinchoninic acid reagent were obtained from Biomol (Plymouth Meeting, PA, USA), Aldrich-Chemie (Steinheim, Germany) and Pierce (Rockford, IL, USA.), respectively. Primary mouse monoclonal and rabbit polyclonal antibodies against the dopamine D_2 receptors (sc-5303) and the β_1-adrenoreceptor (sc-568), respectively, were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cy3-conjugated anti-rabbit and cy2-conjugated anti-mouse antibodies were from Jackson Immuno Laboratories (West Grove, PA, USA). Guide cannulas and microdialysis probes were from CMA/Microdialysis AB (Stockholm, Sweden). All other reagents were from Sigma Chemical Company (Madrid, Spain) or from Merck (Darmstadt, Germany).

Preparation of rat prefrontal cortical neuronal cultures

Primary rat cortical neurones were prepared as previously described (Montezinho *et al*. 2004). Cortical neurones were cultured in Neurobasal medium supplemented with 2 mM L-glutamine, 2% B27 supplement, penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were plated on poly D-lysine (0.1 mg/mL)-coated multiwell plates, at a density of 0.15×10^6 cells/cm^2, and the cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Experimental treatments on cultured cortical neurones

After 5 days in culture, cortical neurones were pre-exposed to therapeutic relevant doses of LiCl (1 mM), valproate (0.05 mM) or carbamazepine (0.5 mM), for 2 days. Non-exposed cultures served as a control. After this time period, neurones were pre-incubated with the cAMP phosphodiesterase inhibitor, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro-201724) 25 μM, 15 min (Reeves *et al*. 1987) and then treated or not (basal cAMP levels) with isoproterenol (10 μM; 15 min), or isoproterenol (10 μM; 15 min) after pre-treatment with quinpirole (10 μM, 5 min). The medium was then removed and the cells were scraped off the plates in 1 mL 50 mM Tris/4 mM EDTA buffer, pH 7.35, after washing with an ice-cold phosphate-buffered saline (PBS) solution. The extracts were boiled at 90°C for 4 min, then centrifuged at 14 000 *g* for 5 min at 4°C. Supernatant fluids were used to quantify cAMP levels. Levels of cAMP were normalized to the protein concentration using the

bicinchoninic acid assay and expressed as a percentage relative to the control cAMP levels. For western blot analysis of receptor expression, cortical neurones exposed to different treatments for 48 h were washed twice with PBS and then lysed at 4°C with lysis buffer [in mM: 50 KCl, 50 Piperazine-1,4-bis(2-ethanesulfonic acid; PIPES), 10 EGTA, 2 $MgCl_2$, pH 7.4] supplemented with 0.2% Triton X-100, 100 μM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, 1 mg/mL chymostatin, 1 mg/mL leupeptin, 1 mg/mL antipain and 5 mg/mL pepstatin. The lysates were collected in microcentrifuge tubes, sonicated for 10 s, and centrifuged at 14 000 *g* for 10 min. Pellets were resuspended in 5% sodium dodecyl sulfate (SDS) and stored at – 80°C for further use. Protein content was determined by the bicinchoninic acid assay, using bovine serum albumin (BSA) as standard.

Animals and drug treatments

Male Wistar rats, initially weighing 200–250 g, housed two per cage under a 12 h light/dark cycle were used (lights on at 06.00 hours). Food and water were available *ad libitum*. The lithium-treated group received 50 mM LiCl/kg diet for 3 weeks with free access to NaCl during the treatment period. The rats fed with the lithium-supplemented diet achieved a plasma lithium level (determined by flame photometry) of 0.84 ± 0.11 mM. The carbamazepine-treated rats were fed with 5 g carbamazepine/kg diet, whereas the intraperitoneal dosage of valproate was 250 mg/kg once daily for 3 weeks. The doses of carbamazepine and valproate used were previously shown to produce the therapeutic drug levels found in the plasma (Mørk and Jensen 2000). Control rats received standard diet, and vehicle-treated rats received intraperitoneal injections with sterile-filtered saline solution (NaCl 0.9%) once daily for 3 weeks. Ethical permissions for the studies were granted by the animal welfare committee appointed by the Danish Ministry of Justice, and all animal procedures were carried out in compliance with the EC directive 86/609/EEC and with the Danish law regulating experiments on animals.

Surgery for microdialysis

Two days before completing the treatment, rats were anaesthetized with 2 mL/kg Hypnorm/Dormicum (fentanyl citrate, 0.079 mg/mL; fluanisone, 2.5 mg/mL; midazolam, 1.25 mg/mL) and intracerebral guide cannulas (CMA/12) were stereotaxically implanted into the brain, positioning the dialysis probe tip in the prefrontal cortex (co-ordinates: 3.2 mm anterior to bregma; lateral, − 0.8 mm; 3.3 mm ventral to duramater) (Paxinos and Watson 1986). Anchor screws and acrylic cement were used for fixation of the guide cannulas. The body temperature of the animals was monitored by a rectal probe and maintained at 37°C. The rats were allowed to recover from surgery for 2 days, housed individually in cages.

Microdialysis procedure

On the day of the experiment, a microdialysis probe (CMA/12, 0.5 mm diameter, 2 mm length) was inserted through the guide cannula. The probes were connected via dual channel swivel to a microinjection pump. Perfusion of the microdialysis probe with filtered Ringer's solution [in mM: 145 NaCl, 3 KCI, 1 $MgCl_2.6H_2O$, 1.2 $CaCl_2.2H_2O$, 0.25 3-isobutyl-1-methylxanthine (IBMX), pH 7.4] was initiated shortly before insertion of the probe into the brain and continued for the duration of the experiment, at a constant flow of 1.36 μL/min. After 180 min of stabilization, the experiments were initiated. A 30 min sampling regime was used throughout the experimental period. Time points were corrected for lag time of the perfusate from the microdialysis site to the probe outlet. Basal cAMP levels were taken as the average cAMP concentration in four consecutive samples. Thereafter, the agent of interest (100 μM quinpirole, or 2.5 mM isoproterenol, or 100 μM quinpirole simultaneously with 2.5 mM isoproterenol) dissolved in Ringer's solution was infused through the probe for 30 min and seven further samples were collected. The dialysates were stored at – 80°C until cAMP determination.

Tissue preparation for immunoblotting

The rats were killed by decapitation and the brains rapidly removed. The cerebral prefrontal cortices were dissected at 4°C and homogenized in 5 volumes of 10 mM Tris (pH 7.2), 1 : 100 protease inhibitor cocktail, 0.1 mM phenylmethysulfonyl fluoride and 1% orthonovanadate. Nonidet P-40 (1%) was added for 30 min and then homogenates were centrifuged at 18 000 *g*, 4°C, for 20 min. Supernatant fluids (cytoplasmic fraction) were collected and stored at − 80°C until further use. SDS (1%) was added to the pellet for 30 min and this was further centrifuged at 18 000 *g*, 4°C, for 20 min. Supernatant fluids (membrane fraction) were collected and stored at – 80°C for further use. Protein content was determined by the bicinchoninic acid assay with BSA as standard.

Quantification of cAMP levels

The content of cAMP in cortical lysates and microdialysis dialysates was determined in duplicate using the [8-^{3}H] and [^{125}I] radioimmunoassay kits, respectively, following the manufacturer's instructions. In the microdialysis dialysates, the high-sensitivity method following acetylation of the samples was used. The lower limit of detection for cAMP was 2 fmol per tube and basal levels were at least four times the detection limit.

Immunohistochemistry

Male Wistar rats were anaesthetized with 680 mg/kg avertin intraperitoneally and perfused transcardially with PBS followed by phosphate-buffered 4% paraformaldehyde, for 10 min. The brains were removed, post-fixed overnight in the same fixative and then embedded in paraffin. Prefrontal cortex sections of 4 μm were picked up on gelatin-coated glass slides. Following xylene deparaffination and alcohol rehydration, the slides were heated in a microwave oven for antigen retrieval (Cattoretti *et al*. 1993; Shi *et al*. 1995) at 700 W for 10 min (2 × 5 min) in 0.01 citrate buffer, pH 6.0. Sections were blocked for 20 min with 5% normal swine serum in PBS supplemented with 1% BSA and 0.3% Triton X-100, and then incubated overnight at 4°C with the specific primary antibodies mouse anti-dopamine D_2 receptor and rabbit anti-β_1-adrenoreceptor diluted 1 : 100 and 1 : 500, respectively. These antibodies were previously used in immunohistochemistry (anti-dopamine D_2; Zou *et al*. 2005) and immunoblotting (anti-β_1; Dincer *et al*. 2001). Sections were thoroughly washed in PBS with 0.25% BSA and 0.1% Triton X-100. Then, secondary cy3-conjugated anti-rabbit and cy2-conjugated anti-mouse antibodies were applied to sections after dilution of 1 : 250 and 1 : 100 in PBS, respectively, and incubated for

60 min at 22–25°C. Slides were washed with PBS and then coverslipped with Dako (Carpinteria, CA, USA) fluorescent mounting medium. As negative controls, adjacent sections were incubated without primary antibody. Brain sections were analysed using the Zeiss upright fluorescence microscope equipped with the LSM 510 Meta confocal system (Carl Zeiss AG, Göttingen, Germany).

Western blot

For western blot analysis, 60 μg of each protein sample were used, after addition of a sixfold concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, 0.012% bromophenol blue) and boiling for 5 min at 95°C. Proteins were separated by electrophoresis on 7.5% acrylamide/bisacrylamide gels and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes which were subsequently blocked for 1 h at 22–25°C in Tris-buffered saline (in mM: 137 NaCl and 20 Tris-HCl, pH 7.5) containing 0.1% Tween 20 (TBS-T) and 5% low-fat milk. Incubation with the primary antibodies (mouse anti-dopamine D_2 receptor, diluted 1 : 100, or rabbit anti-β_1-adrenergic receptor, diluted 1 : 1000, in TBS-T with 1% low-fat milk) was performed overnight at 4°C. After extensive washing in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 h at room temperature (22–25°C) with alkaline phosphatase- or horseradish peroxidase (HRP)-linked secondary antibodies (anti-mouse IgG diluted 1 : 5000 and anti-rabbit IgG diluted 1 : 10 000 in TBS-T with 1% low-fat milk). Protein immunoreactivity was visualized by chemiluminescence on a Fuji Imager 4400 chemiluminescence detection unit (Fujifilm Danmark A/S, Vedbaek, Denmark) or by chemifluorescence on a Storm 860 Gel and Blot Imaging System (Amersham Biosciences).

Statistical analysis

The levels of cAMP in the dialysates were calculated as fmol/20 μL dialysate. The average of the four basal values prior to isoproterenol and quinpirole plus isopreoterenol infusions was taken as baseline and set to 100%. The changes in cAMP levels occurred mostly during the first two samples collected after the beginning of isoproterenol and quinpirole plus isopreoterenol infusions. Therefore, the maximal release of cAMP, measured after 30 min post-infusion with different treatments, was determined and differences were analysed using one-way ANOVA, followed by the Bonferroni test for multiple comparisons, as indicated in the figure captions. However, since in some cases the extracellular accumulation of cAMP was slower, the total amount of cAMP produced during 150 min post-infusion (area under the curves) was also calculated, and the results were expressed as a percentage of the total amount of cAMP produced during 150 min post-infusion with isoproterenol in control or vehicle-treated rats. Differences were analysed using one-way ANOVA, followed by the Bonferroni test for multiple comparisons, as indicated in the figure captions. The density of the bands in the western blots was quantified using QUANTITY ONE 1D-ANALYSIS Software, then data were expressed as percentage of control or vehicle, and analysed using one-way ANOVA, followed by the Bonferroni test for multiple comparisons, as indicated in the figure captions. The data obtained were expressed as means ± SEM values and $p < 0.05$ was considered significant.

Results

Determination of intracellular cAMP levels in cerebrocortical neurones

Figure 1 shows the cAMP accumulation in cultured cortical cells pre-exposed or not (control) to lithium (1 mM), carbamazepine (0.05 mM) or valproate (0.5 mM), during

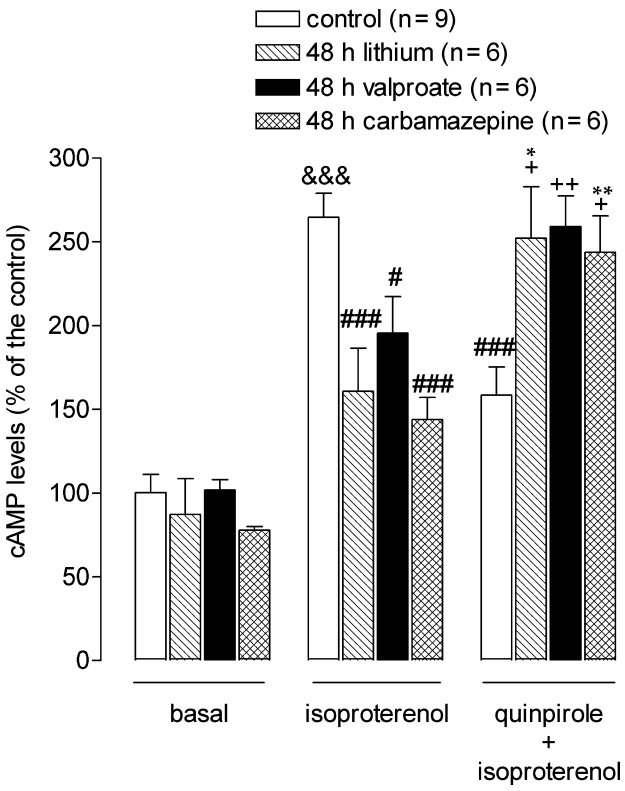


Fig. 1 Effects of lithium, valproate and carbamazepine on the intracellular cAMP levels in cortical neurones under basal conditions and after stimulation with isoproterenol or quinpirole plus isoproterenol. Neurones were pre-exposed or not (control) to 1 mM lithium, 0.05 mM carbamazepine or 0.5 mM valproate, for 48 h. After this period, neurones were incubated with Ro-201724 (25 μM, 15 min) and were then treated or not (basal) with isoproterenol (10 μM; 15 min), or with isoproterenol together with quinpirole (10 μM; 15 min). In the latter experimental conditions, the cells were pre-treated with 10 μM quinpirole for 5 min. The cAMP levels were measured as described in the Methods section. The average value of basal cAMP level for all cells tested (101.4 ± 21.0 nmol/μg protein) was set to 100%. Data are means ± SEM, for the indicated number of independent experiments, performed in duplicate. Data were analysed by one-way ANOVA, followed by posthoc Bonferroni's test for multiple comparisons. ###$p < 0.001$, #$p < 0.05$, compared with control isoproterenol-treated cells; $^{+}p < 0.05$, $^{++}p < 0.01$, compared with control quinpirole plus isoproterenol-stimulated cells; $^{\&\&\&}p < 0.001$, compared with control of untreated cells; $^{*}p < 0.05$, $^{**}p < 0.01$, compared with neurones pre-exposed to LiCl and valproate, respectively.

48 h. After pre-treatment with the mood-stabilizing agents, cells were exposed to isoproterenol (10 μM), for 15 min, in the absence or presence of quinpirole (10 μM), and then intracellular cAMP levels were determined. The average value of basal cAMP level for all cells tested (101.4 ± 21.0 nmol/μg protein) was set to 100%. Isoproterenol-stimulated cAMP production was twofold above basal levels (264.6 ± 14.3%, $p < 0.001$). Lithium, carbamazepine or valproate had no effect on the basal cAMP production, but partially inhibited isoproterenol-induced cAMP accumulation (lithium: 160.7 ± 25.6%, $p < 0.001$; carbamazepine: 143.9 ± 13.1%, $p < 0.001$; valproate: 195.4 ± 21.8%, $p < 0.05$). The agonist of dopamine D_2-like receptors, quinpirole, had no effect on the basal cAMP accumulation (data not shown) but inhibited isoproterenol-enhanced cAMP levels in untreated neurones (158.4 ± 16.8%, $p < 0.001$). In neurones pre-exposed to lithium, carbamazepine or valproate, the ability of quinpirole to reduce the isoproterenol-evoked rise in cAMP was decreased (lithium: 249.1 ± 28.4%, $p < 0.05$; valproate: 259.0 ± 18.4%, $p < 0.01$; carbamazepine: 243.8 ± 21.5%, $p < 0.05$).

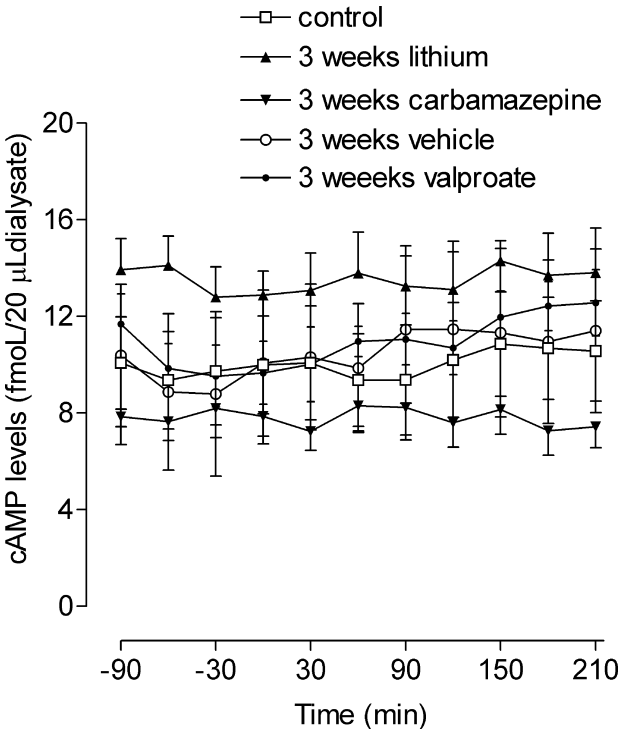


Fig. 2 Effect of chronic treatment, with doses yielding therapeutic plasma levels of lithium, carbamazepine and valproate, on basal cAMP levels in the prefrontal cortex of freely-moving rats. Extracellular cAMP levels in the dialysates were measured by radioimmunoassay analysis. The rats were treated for 3 weeks with doses yielding therapeutic plasma levels of lithium ($n = 6$) or carbamazepine ($n = 6$) in the diet, whereas others were treated with doses yielding therapeutic plasma levels of valproate ($n = 6$) by intraperitoneal injections, once daily, for 3 weeks. Control rats ($n = 6$) received standard diet, whereas vehicle-treated rats ($n = 4$) received intraperitoneal injections with saline solution, once daily, for 3 weeks.

Regulation of cAMP levels in prefrontal cortex

In order to confirm the data obtained with cultured cortical neurones, parallel *in vivo* experiments were performed on prefrontal cortex of freely-moving rats using microdialysis. Extracellular concentration of cAMP can be monitored in the prefrontal cortex of freely-moving animals by microdialysis (Masana *et al*. 1991, 1992). It has been demonstrated that a fraction of intracellular cAMP generated by activation of AC is extruded into the extracellular fluid in proportion to its accumulation in cells. Therefore, the efflux of cAMP can be used to study the cAMP second messenger system in intact brains, using *in vivo* microdialysis (Mørk and Geisler 1994).

Preliminary experiments showed that extracellular levels of cAMP declined to stable values within 180 min (data not shown). Therefore, the experiments were started 180 min after the insertion of the probes and the baseline values were stable throughout the experiment (Fig. 2). The average basal cAMP levels for all animals tested was 9.68 ± 2.10 fmol/20 μL. Average basal levels were not different in valproate-treated rats but were significantly increased in lithium-treated rats, to 13.17 ± 1.21 fmol/20 μL ($p < 0.05$), and decreased in carbamazepine-treated rats, to 7.82 ± 0.34 fmol/20 μL ($p < 0.01$) (Fig. 2).

The effect was then determined of local infusion of isoproterenol, or quinpirole together with isoproterenol, on the extracellular cAMP levels in control or vehicle-treated rats, and in lithium-, carbamazepine- or valproate-treated rats. In a preliminary series of experiments, it was confirmed that infusion of isoproterenol in the rat prefrontal cortex (500 μM, 1 mM, 2.5 mM, 5 mM and 10 mM) increased cAMP production in a concentration-dependent manner, and this effect was inhibited by quinpirole (50 μM, 100 μM, 500 μM, 1 mM and 2.5 mM) (data not shown). Accordingly, the concentrations of isoproterenol and quinpirole used in all subsequent experiments were 2.5 mM and 100 μM, respectively, which were found to be the minimal concentrations that produced an observable effect under our experimental conditions (data not shown). These concentrations of isoproterenol and quinpirole cannot be directly compared with those used in *in vitro* experiments and with extracellular levels obtained in the brain (5–15% of the initial concentrations), since the extracellular concentration achieved in brain tissue under the present conditions depends on diffusion across the dialysis membrane (Mørk and Geisler 1994).

The infusion of isoproterenol in the prefrontal cortex of control and vehicle-treated rats for 30 min increased cAMP levels to approximately 234.6 ± 12.2% (Fig. 3a)

and 272.1 ± 12.0% (Fig. 3d) of the basal values, and the extracellular amount of cAMP returned to basal values by 150 min post-infusion. In lithium-, carbamazepine- or valproate-treated rats there was a significant decrease in the isoproterenol-stimulated cAMP levels determined after 30 min of infusion (lithium: 131.9 ± 5.8%, $p < 0.01$; carbamazepine: 159.0 ± 8.5%, $p < 0.01$; valproate: 137.9 ± 8.7%, $p < 0.01$) (Figs 3b, c and e), as well as a decrease in the total evoked increase in extracellular cAMP (lithium: 85.1 ± 3.7%, $p < 0.01$; carbamazepine: 87.5 ± 1.6%, $p < 0.05$; valproate: 69.5 ± 2.4%, $p < 0.001$), when compared with the effect observed in control or vehicle-treated rats (Fig. 3f). Local infusion of the D_2-like receptor agonist, quinpirole, significantly inhibited the effect of isoproterenol on the extracellular cAMP, measured 30 min after the infusion of the agonist (control rats: 132.5 ± 5.8%, $p < 0.01$; vehicle-treated rats: 141.0 ± 9.1, $p < 0.01$) (Figs 3a and d). The total increase in extracellular cAMP evoked by isoproterenol within 150 min was also significantly decreased in the presence of quinpirole (control rats: 77.0 ± 1.6%, $p < 0.001$; vehicle-treated rats: 74.9 ± 2.7, $p < 0.001$) (Fig. 3f). As observed in cultured cortical neurones, in lithium-treated rats the activation of D_2-like receptors did not inhibit the isoproterenol-induced increase in extracellular cAMP. Instead, there was an increase in extracellular cAMP measured 30 min after the infusion (188.2 ± 19.1%, $p < 0.01$) (Fig. 3b) when compared with the amount of the cyclic nucleotide determined in control rats under the same conditions (132.5 ± 5.8%) (Fig. 3a) or in animals treated with lithium and infused only with isoproterenol (131.9 ± 5.8%) (Fig. 3b). Treatment with lithium also increased the total extracellular accumulation of cAMP induced by quinpirole and isoproterenol, and produced within 150 min (77.0 ± 1.6 vs. 94.7 ± 4.4%, $p < 0.01$) (Fig. 3f). In contrast to what was observed with cultured cortical neurones and lithium-treated rats, in carbamazepine- or valproate-treated rats, quinpirole infusion did not significantly change the isoproterenol-induced increase in extracellular cAMP produced 30 min post-infusion (Figs 3c and e) (176.9 ± 8.3% and 158.0 ± 11.7%, respectively), when compared with the effect observed in control and vehicle-treated rats under the same conditions (132.5 ± 5.8% and 137.9 ± 8.7%, respectively) (Figs 3a and d), or in animals treated with carbamazepine or valproate and infused only with isoproterenol (159.0 ± 8.5% and 137.9 ± 8.7%, respectively) (Figs 3c and e). Treatment with carbamazepine or valproate did not significantly change the total evoked extracellular accumulation of cAMP measured during 150 min following infusion with quinpirole and isoproterenol (84.7 ± 3.5% and 85.9 ± 2.2%, respectively), when compared with the values obtained in control and vehicle-treated rats, 77.0 ± 1.6% and 74.9 ± 2.7%, respectively (Fig. 3f). In carbamazepine-treated rats, the activation of D_2-like receptors did not significantly interfere with the total isoproterenol-induced increases in extracellular cAMP, although the effect occurred at a slower rate.

The activation of D_2-like receptors had no effect on basal cAMP levels under control conditions (control rats: 100.4 ± 18.4%; vehicle-treated rats: 104.0 ± 11.3%). However, in lithium-treated rats, the activation of dopamine D_2-like receptors, with quinpirole, significantly increased cAMP levels to 155.1 ± 9.3%, $p < 0.05$. In contrast, in carbamazepine- and valproate-treated rats, the infusion of quinpirole did not significantly change basal cAMP levels (114.4 ± 8.9% and 98.7 ± 13.6%, respectively, vs. 100.4 ± 18.4% and 104.0 ± 11.3%) (Fig. 4).

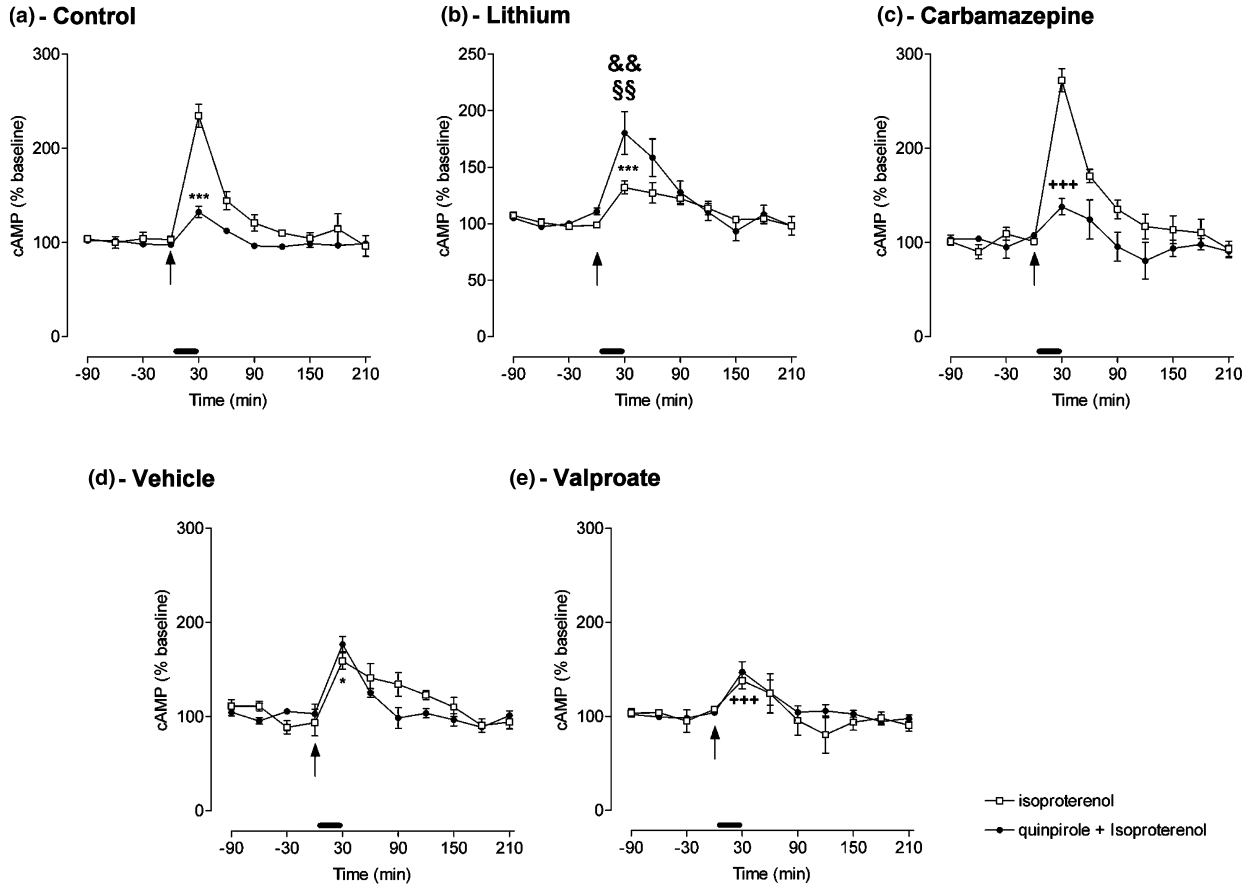
Immunohistochemistry

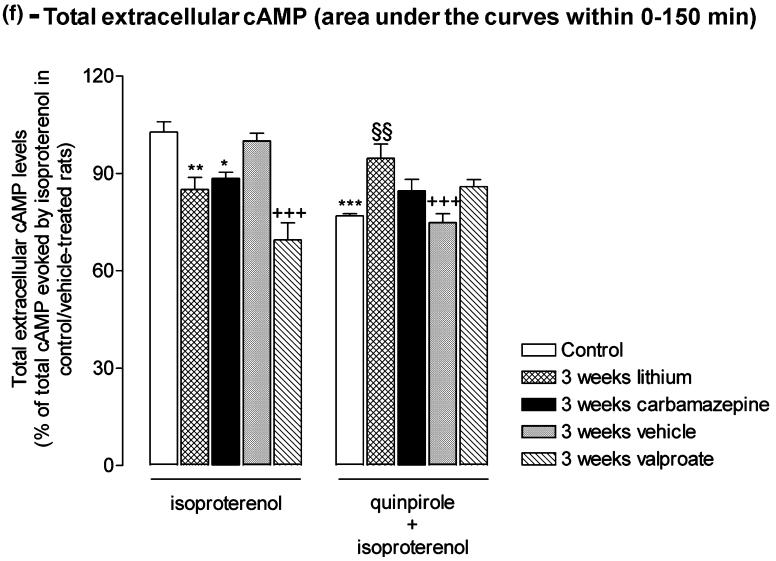
The dopamine D_2 receptor is the predominant member of the family of D_2-like dopamine receptors in the brain (Emilien *et al*. 1999) and the β_1-adrenergic receptors are the most abundant members of β-adrenergic receptors in the cerebral cortex (Rainbow *et al*. 1984). To confirm that dopamine D_2 and β_1-adrenergic receptors are co-localized in cells of the prefrontal cortex, allowing the interactions at the second messenger level, immunohistochemistry studies were performed. Double-labelling experiments confirmed the co-existence of dopamine D_2 and β_1-adrenergic receptors in the majority of the cells (Fig. 5). The prefrontal cortex of sections prepared after treating rats for 3 weeks with lithium, carbamazepine or valproate suggested a loss of dopamine D_2 receptors. In contrast, in valproate-, carbamazepine- and lithium-treated rats the β_1-adrenergic receptors seemed to be down-regulated, up-regulated or not changed, respectively (Fig. 5). Immunohistochemical controls were prepared by omitting addition of the primary antibody in some slices, and it was observed that no significant amount of secondary binding occurred.

Immunoblotting

In order to confirm the immunostaining data, the dopamine D_2 and β_1-adrenergic receptor protein levels were determined in membranes prepared from cultured rat cortical neurones and from rat prefrontal cortex, pre-treated or not with the mood-stabilizing agents, by western blot analysis, using the same anti-D_2 and anti-β_1 specific antibodies used for immunohistochemistry. A strong immunoreactivity was observed at approximately 50 kDa and 64 kDa, which are the predicted molecular weights for the dopamine D_2 and β_1-adrenergic receptor proteins, respectively (Fig. 6a). The two bands obtained with the dopamine D_2 antibody could be due to the two isoforms of the receptor, D_2 long (D_{2L}) and short (D_{2S}), which are encoded by splice variants of a single gene and detected by this antibody. Densitometry analysis of the bands at approximately 50 kDa showed that lithium, valproate and carbamazepine treatments decreased the immunoreactivity of the dopamine D_2 receptor protein band,

by 87.6 ± 3.0% ($p < 0.01$), 90.5 ± 2.7% ($p < 0.01$) and 86.5 ± 3.1% ($p < 0.01$), respectively, in membranes from cultured rat cortical neurones, when compared with the control (103.9 ± 1.7%) (Fig. 6b). Similar results were obtained in the prefrontal cortex tissue, where lithium, valproate and carbamazepine reduced D_2 protein levels by 69.6 ± 3.9% ($p < 0.01$), 97.9 ± 6.2% ($p < 0.01$) and 76.5 ± 5.0% ($p < 0.05$), respectively, when compared with control (97.9 ± 4.7%) and vehicle-treated rats (101.9 ± 4.3%) (Fig. 6c). In cultured cerebrocortical neurones, only





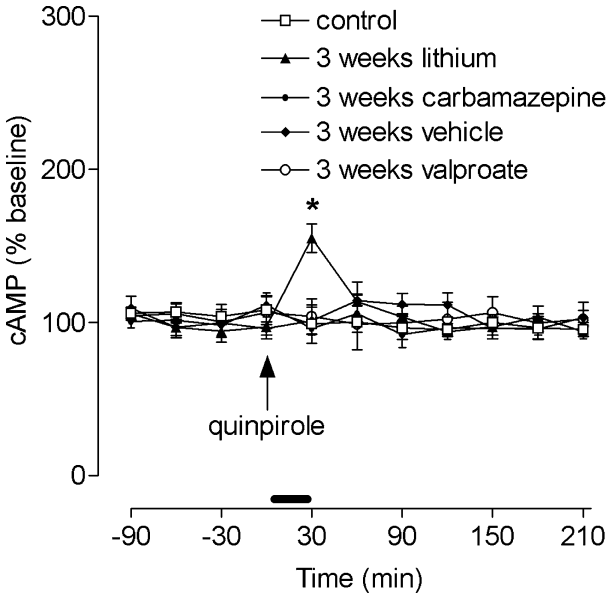


Fig. 4 Infusion of quinpirole (100 μM) in the prefrontal cortex of freely-moving rats (starting at arrow) on the extracellular cAMP levels in the prefrontal cortex of control, vehicle-, lithium-, carbamazepine- or valproate-treated rats. Baseline levels of cAMP were taken as the average cAMP content in the four consecutive samples collected from 3 h after the insertion of the probe. Thereafter, 100 μM of quinpirole, dissolved in Ringer's solution, were infused through the probe for 30 min, and six samples were collected post-infusion. Extracellular cAMP levels in the dialysates were measured by radioimmunoassay analysis and expressed as percentages of the baseline. Results are means ± SEM, from four independent experiments. The rats were treated or not with doses yielding therapeutic plasma levels of lithium, carbamazepine or valproate, for 3 weeks, as indicated in the legend of Fig. 2. Data were analysed by one-way ANOVA, followed by posthoc Bonferroni's test for multiple comparisons. $^{*}p < 0.05$ compared with the cAMP produced 30 min after the infusion of quinpirole in control rats.

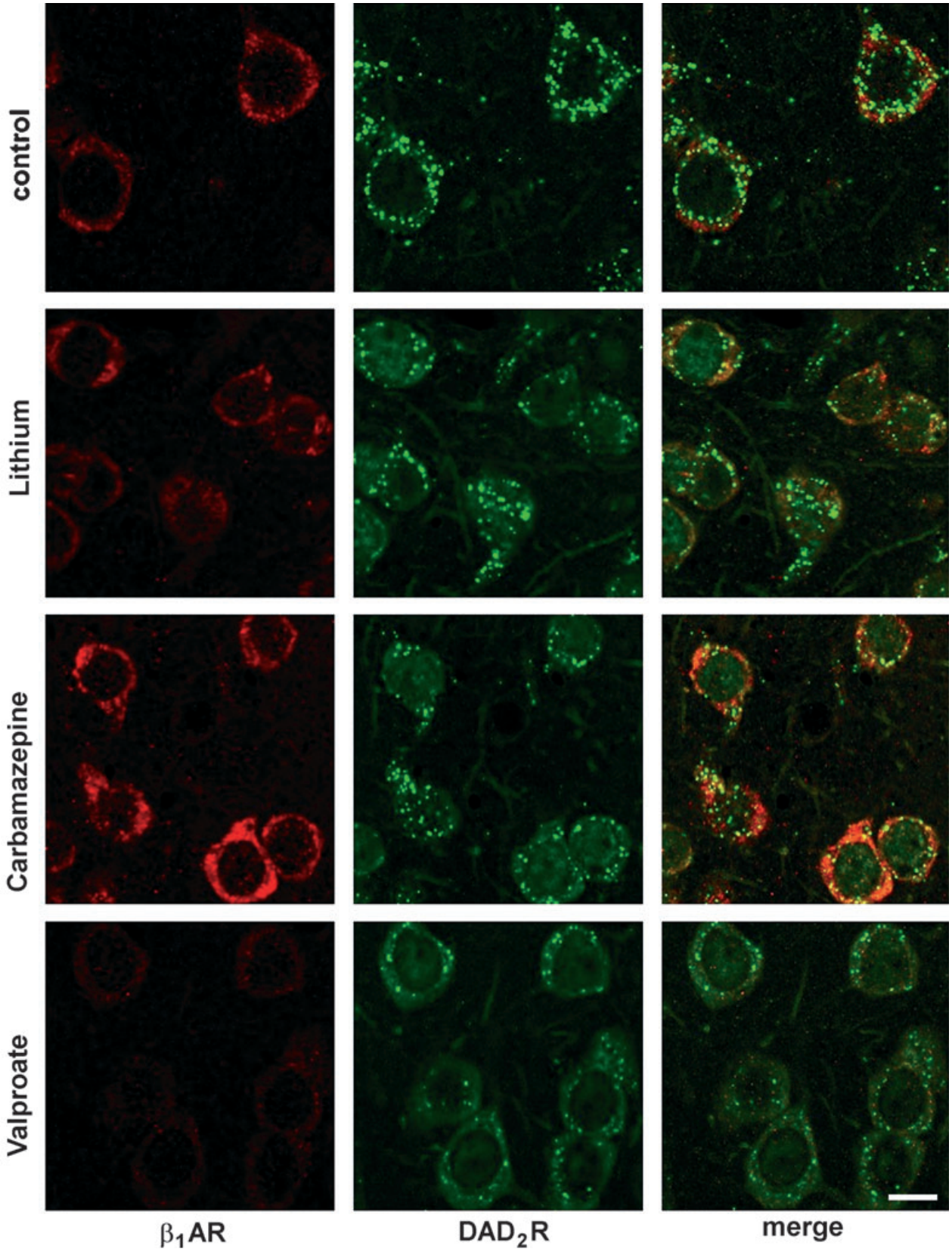
valproate had an effect on β_1-adrenergic receptors by decreasing the amount of the receptor present (101.9 ± 4.3%, $p < 0.05$), compared with control (105.6 ± 6.7%) (Fig. 6b, graph). In contrast, *in vivo* treatment of rats with carbamazepine, valproate or lithium up-regulated (129.6 ± 7.0%, $p < 0.01$), down-regulated (70.8 ± 4.4%, $p < 0.01$) or had no effect (100.4 ± 3.6%) on β_1-adrenergic receptor levels, respectively, when compared with control (101.3 ± 1.6%) and vehicle-treated (100.9 ± 4.6%) rats (Fig. 6c).

Discussion

The effects of mood-stabilizing drugs on the dopaminergic and adrenergic systems were studied via analysis of the intracellular cAMP accumulation induced by activation of β-adrenergic and/or dopamine D_2-like receptors. The change in the levels of dopamine D_2 and β_1-adrenergic receptors under the same experimental conditions was also investigated. In the present study, we showed that there is a cross-talk between dopamine D_2-like and β-adrenergic receptor activities in the rat brain cortical region, which is differentially affected by therapeutic concentrations of the mood-stabilizing drugs lithium, carbamazepine and valproate. Indeed, *in vivo* and *in vitro* data showed that activation of dopamine D_2-like receptors inhibits β-adrenergic receptor-stimulated cAMP production. *In vitro* this inhibition was attenuated by lithium, carbamazepine and valproate, whereas *in vivo* only lithium had such an effect. Consistent with this regulatory role on AC activity, dopamine D_2 and β_1-adrenergic receptors are co-localized in the rat prefrontal cortex and their protein levels are changed by mood stabilizers, as determined by immunohistochemistry and immunoblotting, respectively.

In vivo chronic treatment with lithium increased the intracellular cAMP content in the rat prefrontal cortex by 40%. This type of effect of lithium may be attributed to the inhibition of G_i, which is the G-protein preferentially activated under basal conditions (Masana *et al*. 1991, 1992; Jope 1999; Montezinho *et al*. 2004). However, our results showing a functional cross-talk between β-adrenergic and

Fig. 3 Effect of the local infusion of isoproterenol (2.5 mM) or quinpirole (100 μM) simultaneously with isoproterenol (2.5 mM) (starting at arrow) on the maximal extracellular cAMP levels produced 30 min post-infusions [(a) Control rats; (b) lithium-treated rats; (c) carbamazepine-treated rats; (d) vehicle-treated rats; (e) valproate-treated rats] and on total cAMP levels (corresponding to the area under the curves), measured within 150 min post-infusions (f). Basal cAMP level was taken as the average cAMP concentration in the four consecutive samples collected from 3 h after the insertion of the probe, prior to isoproterenol (8.9 ± 2.6 fmol/20 μL, $n = 6$) and quinpirole plus isoproterenol infusions (9.4 ± 1.1 fmol/20 μL, $n = 4$). Thereafter, the agent of interest (2.5 mM isoproterenol or 100 μM quinpirole plus 2.5 mM isoproterenol) dissolved in Ringer's solution was infused through the probe, for 30 min, and seven samples were then collected. Extracellular cAMP levels in the dialysates were measured by radioimmunoassay analysis and expressed as percentages of the basal value (a–e), or expressed as a percentage of the percentage of the total amount of cAMP produced during 150 min post-infusion with isoproterenol in control or vehicle-treated rats (f). Results are means ± SEM, from four to six independent experiments. The rats were treated or not with doses yielding therapeutic plasma levels of lithium, carbamazepine or valproate, for 3 weeks, as indicated in the legend of Fig. 2. Data were analysed by one-way ANOVA, followed by posthoc Bonferroni's test for multiple comparisons. $^{***}p < 0.001$, compared with the cAMP produced 30 min after the infusion of isoproterenol in control rats; $^{+++}p < 0.001$, compared with the cAMP produced 30 min after the infusion of isoproterenol in vehicle-treated rats; §§$p < 0.01$, compared with the cAMP produced 30 min after the infusion of quinpirole plus isoproterenol in control rats; $^{\&\&}p < 0.001$, compared with the cAMP produced 30 min after the infusion of quinpirole plus isoproterenol in lithium-treated rats.



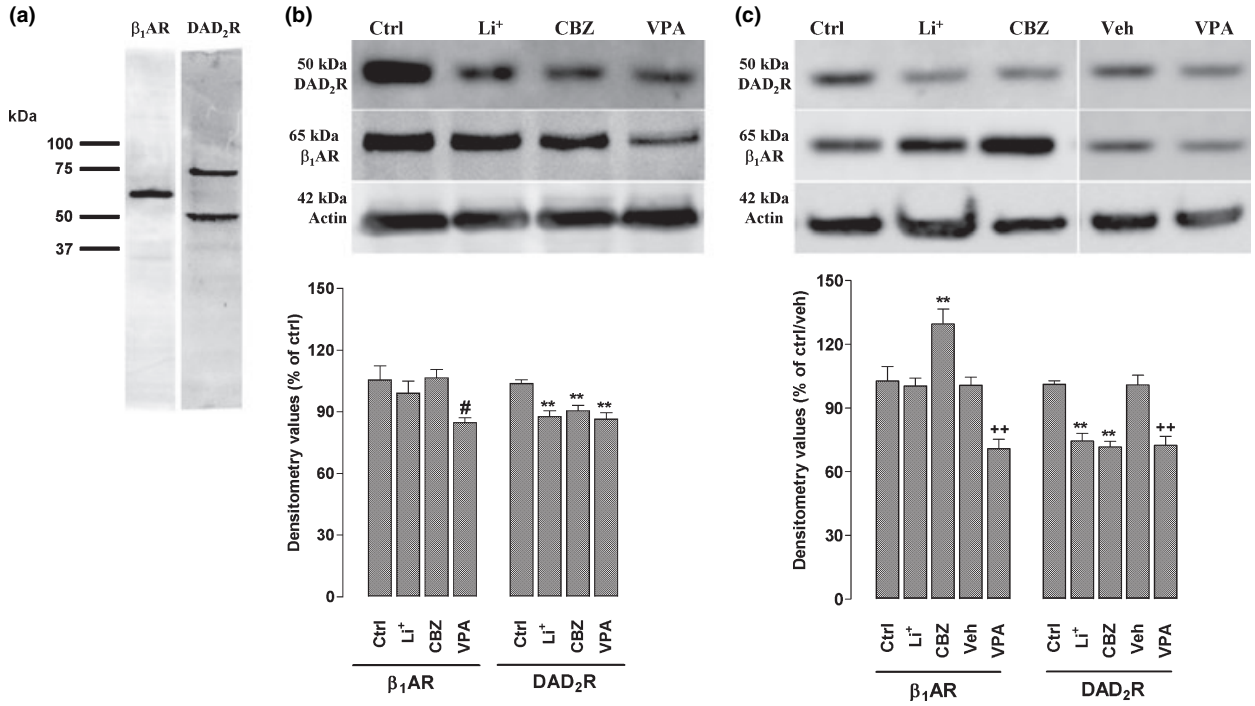


Fig. 6 Changes in β_1AR and DAD_2R protein levels following lithium, valproate or carbamazepine treatments. Representative western blots showing β_1AR and DAD_2R, and actin immunoreactivity, in protein extracts from cultured rat cortical neurones treated with lithium, valproate or carbamazepine for 48 h (a), or from the prefrontal cortex of rats treated for 3 weeks with the same drugs (b). The bottom panels represent normalized levels of β_1AR and DAD_2R immunoreactivity. The results are presented as percentage of the controls/vehicles and data are means ± SEM, from four to six independent experiments. Data are means ± SEM and were analysed by one-way ANOVA, followed by posthoc Bonferroni's test for multiple comparisons. $^{*}p < 0.05$, $^{**}p < 0.001$, compared with control rats; $^{+}p < 0.05$, $^{++}p < 0.001$, compared with vehicle-treated rats; $\#p < 0.05$, $\#\#p < 0.001$, compared with control neurones. Ctrl, control; Li^+, lithium; CBZ, carbamazepine; Veh, vehicle; VPA, valproate.

dopamine D_2-like receptors, and a selective down-regulation of the dopamine D_2 receptors by chronic treatment with lithium, indicate that the loss of these receptors, which are coupled to the inhibition of AC, may also account for the increase in the basal cAMP levels. The decrease in dopamine D_2 receptor levels following lithium treatment might also have contributed to the lack of effect of D_2 receptor stimulation on isoproterenol-evoked intracellular cAMP accumulation in the *in vitro* and *in vivo* studies. Recent evidence has implicated cortical D_2 receptors as the important sites of action of anti-psychotics. Psychotic and manic symptoms may result from the supersensitivity of a subset of cortical pyramidal neurones to stimulation of D_2 receptors (Yatham *et al*. 2002; Schatzberg 2004; Silverstone and Silverstone 2004). Therefore, part of the anti-manic action of chronic lithium treatment could be mediated by a decrease in D_2 receptor levels shown in this study. In contrast to lithium, *in vivo* chronic treatment with carbamazepine inhibited basal cAMP levels by 20%. This is in agreement with previous findings showing that carbamazepine decreases the basal concentrations of cAMP in mouse cerebral cortex and cerebellum (Palmer *et al*. 1979), as well as in rabbit CSF (Myllyla 1976). In contrast with the results obtained with lithium, the *in vivo* effects of carbamazepine on the intracellular cAMP content under resting conditions do not correlate with the down-regulation of dopamine D_2 receptors and the up-regulation of β-adrenergic receptors under the same conditions. These changes in AC-coupled receptor would be expected to increase the intracellular cAMP content. This discrepancy may be explained by the fact that the action of carbamazepine, at therapeutically relevant concentrations, is independent of the phosphodiest-

Fig. 5 Representative confocal images of double immunofluorescence β_1 adrenergic receptors (β_1AR) and dopamine D_2 receptor (DAD_2R) in rat prefrontal cortical slices obtained from control rats or rats treated for 3 weeks with doses yielding therapeutic plasma levels of lithium, carbamazepine or valproate. β_1AR immunoreactivity is revealed by red cy3 fluorescence, whereas DAD_2R-positive cells are identified by green cy2 immunofluorescence in the same sections. Scale bar, 10 μm.

erase activity and is mediated by a G_i-independent mechanism (Chen *et al*. 1996a). Thus, the inhibitory effect of carbamazepine on cAMP levels may be exerted primarily at the level of cAMP production, acting directly on AC and/or through factor(s) that are tightly associated with this enzyme (Chen *et al*. 1996a). A direct inhibitory action of carbamazepine on AC could also explain our finding that *in vivo* chronic carbamazepine treatment attenuated the isoproterenol-evoked cAMP production, despite an increase in the β_1-receptor levels and the lack of a further effect of D_2 receptor activation. The changes in receptor levels could represent a compensatory mechanism in the *in vivo* conditions, although this was not observed in cultured cortical neurones. Recently, it was proposed that the β-adrenoreceptors mediating cAMP efflux after norepinephrine stimulation are localized predominantly on glial cells in the rat brain cortex (Stone *et al*. 1990; Stone and John 1991). This is not the case in the neuronal cultures used in the present work, since they contain a very small percentage of astrocytes (Brewer *et al*. 1993). Identification of the cells that contribute to the cAMP responses observed both *in vivo* and *in vitro*, and the regulating mechanisms involved, remain to be clarified. In addition, Khan *et al*. found that approximately one-third of the total D_2 receptor binding sites in the cortex are associated with astroglia (Khan *et al*. 2001). *In vivo* chronic administration of valproate significantly reduced both the β_1-adrenoreceptor and dopamine D_2 receptor immunoreactivity in the rat prefrontal cortex. The down-regulation of both receptor types did not affect the basal levels of cAMP, but the decrease in the density of β-adrenoreceptors is likely to be reflected in the decrease in isopreterenol-evoked, receptor-mediated cAMP production. Furthermore, dopamine D_2 receptor stimulation did not further attenuate cAMP production, which could be related to the lower levels of receptor protein after chronic treatment with valproate. Supporting our findings, previous studies showed that valproate selectively down-regulates β_1-adrenergic receptors *in vitro*, in rat C6 glioma cells (Chen *et al*. 1996b).

Each of the mood-stabilizing drugs modulated dopamine D_2 and β-adrenergic receptor-regulated cAMP levels by a distinct mechanism *in vivo*. The effects of carbamazepine are most likely due to direct inhibition of AC; lithium may act by affecting dopamine D_2 receptor-mediated signalling and valproate, by down-regulating β-adrenergic transmission. Interestingly, the results obtained *in vitro* with cultured cerebrocortical neurones did not fully correlate with the *in vivo* findings. In cultured cortical neurones, lithium, valproate and carbamazepine did not significantly affect basal cAMP levels. The increase in β-adrenergic receptor-mediated production of cAMP was attenuated by all three drugs both *in vitro* and *in vivo*. This attenuation was reversed by dopamine D_2-like receptor stimulation in controls and after chronic treatment of the rats with carbamazepine or valproate, but not after treatment with lithium or in cultured cortical neurones after pre-exposure to the three mood-stabilizing drugs. Therefore, we propose that in *in vitro* conditions, the three mood-stabilizing drugs used interfere with the ability of quinpirole to blunt isoproterenol effects, which may result from the action of these drugs downstream of second messenger signalling pathways activated by D_2-like receptors (Yatham *et al*. 2002). The overall results obtained in the present work suggest that additional mechanisms are operative *in vivo* compared with *in vitro*. It can be speculated that dopaminergic and noradrenergic neurotransmitter levels released by afferent projections participate in the regulation of these monoaminergic systems. In agreement with this hypothesis, a decrease in the dopamine concentration in prefrontal cortex (Baptista *et al*. 1993) and an increase in striatum (Dziedzicka-Wasylewska *et al*. 1996) were reported after the *in vivo* intragastrical administration of lithium. In contrast, it has been reported that therapeutic concentrations of carbamazepine or valproate enhanced basal releases of dopamine in the prefrontal cortex (Ichikawa and Meltzer 1999). In addition, carbamazepine and valproate are metabolized *in vivo*, which might contribute to the differences observed in the effects of these drugs *in vitro* and *in vivo*. Moreover, the presence of glia cells expressing β-adrenergic (Stone *et al*. 1990; Stone and John 1991) and D_2 receptors (Khan *et al*. 2001) in rat brain cortex increases the complexity of the regulation of the cAMP levels *in vivo* when compared with the *in vitro* data. Lastly, the other D_2-like receptor subtypes, namely the D_3 and D_4 receptors, and the β_2- and β_3-adrenergic receptors, may also play a role in the regulation of cAMP levels, and the expression pattern of these receptors *in vitro* may be different from that observed *in vivo*. Although the *in vivo* system is rather complex, providing results that may be affected by multiple cell–cell interactions, it reflects the effects of the drugs in the brain, which is also a complex system. Comparison with data from cell culture experiments is useful, to some extent, for distinguishing the effect of drugs within the cell from those resulting from multiple cell–cell interactions.

In this study we provide evidence that the three mood stabilizers act by different mechanisms on the dopamine D_2-like and β-adrenergic receptor-mediated regulation of cAMP levels both *in vivo* and *in vitro*. Furthermore, we show that the effects of the mood stabilizers are different *in vivo* and *in vitro*, highlighting the importance of the *in vivo* studies. The different effects of the three mood-stabilizing drugs in the *in vivo* studies suggest that they have distinct mechanisms of action. This can be related to differences in the efficacy of the treatment of manic episodes in clinical cases, where lithium, but not carbamazepine or valproate, has been shown to be effective as an acute treatment (Silverstone and Silverstone 2004). In conclusion, we have shown that lithium, carbamazepine and valproate affect differentially the basal and evoked cAMP levels produced by the balance between β-adrenergic and dopamine D_2 receptors, both

in vitro and *in vivo*. However, the results did not lead to a unifying hypothesis for the mechanism of action of these drugs in bipolar disorder. A common effect on the interaction between β-adrenergic and dopamine D_2 receptors does not seem to explain the therapeutic efficacy of these drugs, suggesting that additional signalling systems are involved as well.

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