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The Antidepressant Clomipramine Regulates Cortisol Intracellular Concentrations and Glucocorticoid Receptor Expression in Fibroblasts and Rat Primary Neurones

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Incubation of LMCAT fibroblasts cells with antidepressants potentiates glucocorticoid receptor (GR)-mediated gene transcription in the presence of cortisol, but not of corticosterone. We have suggested that antidepressants do so by inhibiting the LMCAT cells membrane steroid transporter and thus by increasing cortisol intracellular concentrations. We now confirm and extend this model to primary neuronal cultures. Clomipramine, a tricyclic antidepressant, increased the intracellular accumulation of ^{3}H-cortisol, but not ^{3}H-corticosterone, in LMCAT cells (+80%) and primary rat neurones (+20%). The latter finding is the first demonstration that a membrane steroid transporter is present in neurones. Moreover, verapamil, a membrane steroid transporter inhibitor, reduced the effects of clomipramine on the intracellular accumulation of ^{3}H-cortisol in LMCAT cells. Finally, clomipramine also decreased GR expression (whole-cell Western blot) in LMCAT cells (50% reduction) and primary rat neurones (80% reduction). This GR downregulation can explain the reduced GR-mediated gene transcription previously described under experimental conditions that do not elicit the effects on the LMCAT cells steroid transporter. This work further supports the hypothesis that membrane steroid transporters regulating the access of glucocorticoids to the brain *in vivo* are a fundamental target for antidepressant action.
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INTRODUCTION

Although the effects of antidepressants on the glucocorticoid hormones and their receptors could be relevant for the therapeutic action of these drugs, the molecular mechanisms underlying these effects are unclear (Holsboer, 2000; Pariante and Miller, 2001). Patients with major depression show hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis, which is thought to participate in the development of the depressive symptoms (Nemeroff, 1996). One explanation for the HPA axis hyperactivity is an impaired feedback inhibition by the endogenous glucocorticoid cortisol. This feedback is mediated by the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) in the brain (McEwen *et al*, 1997). Patients with major depression exhibit impaired HPA negative feedback in the context of elevated circulating levels of cortisol (Nemeroff, 1996), and the GR is important in the regulation of the HPA when endogenous levels of cortisol are high (de Kloet *et al*, 1998). Consistent with this, the function of GR is reduced in depressed patients (GR resistance) and antidepressants reverse these putative GR changes (Pariante and Miller, 2001; Pariante *et al*, 2002). Specifically, studies in depressed patients, animals, and cellular models have demonstrated that antidepressants increase GR (and MR) expression, enhance GR function and promote GR nuclear translocation; this, in turn, is associated with enhanced GR-mediated negative feedback by endogenous glucocorticoids, and thus with reduced resting and stimulated HPA axis activity (Holsboer, 2000; Pariante and Miller, 2001).

In vitro studies are a fundamental tool to clarify the mechanisms underlying the effects of antidepressants on the GR. These experimental systems do not contain catecholamine reuptake sites within synaptic connections (Pariante *et al*, 1997, 2001a; Okugawa *et al*, 1999; Hery *et al*, 2000; Lai *et al*, 2003). Therefore, these systems allow the study of molecular effects that are unrelated to the inhibition of

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catecholamine uptake, the mechanism believed to be crucial in the therapeutic action of antidepressants. Antidepressants increase GR function and GR expression in neuronal cell cultures (Pepin *et al*, 1989; Okugawa *et al*, 1999; Hery *et al*, 2000; Lai *et al*, 2003), fibroblasts (Pepin *et al*, 1992; Pariante *et al*, 1997, 2001a; Miller *et al*, 2002), and human peripheral blood mononuclear cells (Vedder *et al*, 1999). However, reduced GR function *in vitro* by antidepressants has also been described (Pariante *et al*, 1997, 2001a; Budziszewska *et al*, 2000; Miller *et al*, 2002).

We have suggested that antidepressants control GR function *in vitro* by regulating the intracellular concentration of glucocorticoids (Pariante *et al*, 2001a). Glucocorticoids are excreted from fibroblasts, leukocytes, and epithelial cells by transporters of the ATP-binding cassette family (Ueda *et al*, 1992; Kralli and Yamamoto, 1996; Shabbits *et al*, 2001; Pariante *et al*, 2001a, b). One of these transporters, the multiple drug resistance (MDR) p-glycoprotein (PGP), confers treatment resistance to tumor cells and limits the access of several drugs, including tricyclic antidepressants, to the brain (Ueda *et al*, 1992; Uhr *et al*, 2000; Shabbits *et al*, 2001). However, the mice (mdr1a) and human (MDR) PGPs, localized at the luminal membrane of the endothelial cells of the blood–brain barrier (BBB), also limit the access of cortisol and dexamethasone—but not of corticosterone—to mouse and human brain (Karssen *et al*, 2001; Meijer *et al*, 1998). We have described that the treatment of LMCAT fibroblasts with antidepressants inhibits a membrane steroid transporter that is virtually identical to PGP in its substrates and modulators (Ueda *et al*, 1992; Kralli and Yamamoto, 1996; Medh *et al*, 1998; Marsaud *et al*, 1998; Shabbits *et al*, 2001; Pariante *et al*, 2001a, b). Specifically, chemically unrelated antidepressants—the tricyclics, desipramine, amitriptyline, and clomipramine (Pariante *et al*, 2001a), and the selective serotonin reuptake inhibitors (SSRIs), citalopram, paroxetine (Pariante *et al*, 2001a), and fluoxetine (Pariante *et al*, unpublished)—enhance GR-mediated gene transcription in the presence of dexamethasone or cortisol, but not of corticosterone. Based on these results, we have proposed that potentiation of GR-mediated gene transcription in the presence of dexamethasone and cortisol is because of the antidepressants inhibiting the LMCAT cells' membrane steroid transporter and thus increasing the intracellular concentrations of these glucocorticoids. There are, however, some unanswered questions, which are addressed in the present study. First, the increased GR-mediated gene transcription only provides indirect evidence that antidepressants regulate glucocorticoid intracellular concentrations. Therefore, we have examined whether antidepressants directly increase the intracellular levels of radioactive glucocorticoids in LMCAT cells. Second, antidepressants regulate GR expression and function in the animal brain and neurones. Therefore, we have examined whether a similar, antidepressant-sensitive, membrane transport of glucocorticoids is present in cultured rat primary neurones. Third, a reduced GR-mediated gene transcription by antidepressants is present in LMCAT cells under experimental conditions that do not elicit the effects on the transporter, like in the absence of any glucocorticoid (Miller *et al*, 2002) or in the presence of corticosterone rather than cortisol (Pariante *et al*, 2001a). Therefore, we have

examined whether these inhibitory effects are because of a reduction in GR expression by antidepressants. We have used clomipramine, a tricyclic antidepressant, because it gives the largest potentiation of GR-mediated gene transcription in the presence of cortisol, and the largest inhibition of GR-mediated gene transcription in the presence of corticosterone, among the antidepressants previously tested (Pariante *et al*, 2001a).

MATERIALS AND METHODS

Materials

All chemicals, unless otherwise stated, were purchased from Sigma (UK). The LMCAT cell line—L929 mouse fibroblast cells stably transfected with the mouse mammary tumor virus-chloramphenicol acetyltransferase (MMTV-CAT) reporter gene—was generously provided by ER Sanchez (Medical College of Ohio, Toledo, OH) (Sanchez *et al*, 1994). We have used clomipramine, at 10 μM, for 24 h, as previously described (Pariante *et al*, 2001a). *In vitro* treatment with micromolar concentrations of antidepressants for at least 24 h have been previously used in studies that have investigated the *in vitro* effects of antidepressants on the GR (Pariante *et al*, 1997, 2001a; Vedder *et al*, 1999; Budziszewska *et al*, 2000; Miller *et al*, 2002; Lai *et al*, 2003) or on other molecular systems (Chen and Rasenick, 1995; Varga *et al*, 1996; Szabo *et al*, 1999; Xia *et al*, 1999; Maes *et al*, 1999). Moreover, this concentration resembles the therapeutic plasma and brain levels of tricyclic antidepressants (Hrdina and Dubas, 1981; Glotzbach and Preskorn, 1982). We have used verapamil (100 μM for 1.5–24 h) to inhibit the steroid transporters, as previously described (Pariante *et al*, 2001a, b).

LMCAT Cell Culture

LMCAT cells were maintained in 175-cm^2 flasks (Marathon, UK) at 37° with a 5% CO_2 and 95% air atmosphere. The culture medium was DMEM with 10% (v/v) charcoal-stripped, delipidated, heat-inactivated (56°, 30 min) bovine calf serum (Autogen Bioclear, UK) and 0.2 mg/ml G418 (Geneticin) antibiotic. The levels or cortisol in this medium were < 0.1 nM (Andrew Papadopoulos, personal communication). For measuring the intracellular accumulation of radioactive steroids, LMCAT cells were subcultured in 12-well plates (Marathon, UK) and grown for 48–72 h (final confluency 95%) prior to drug treatment. For Western blotting experiments, LMCAT cells were subcultured in 25 cm^2 flasks (Marathon, UK) for 48–72 h (final confluency 95%) prior to drug treatment.

Primary Cortical Neuronal Cultures

Neuronal cultures were prepared from embryonic day 18 rat embryos as previously described (Williamson *et al*, 2002). Essentially, embryos were removed and their fetal brain cortices dissected and freed of meninges. The cells were dissociated by trypsinization (0.25% (v/v) for 20 min at 37°C). Trypsinization was stopped by washing three times in Neurobasal medium (Life Technologies, UK) containing 10% (v/v) fetal calf serum (FCS) (Autogen Bioclear, UK),

and then treated with 2 Kunitz units/ml deoxyribonuclease 1 followed by trituration with fire-polished Pasteur pipettes. For measuring the intracellular accumulation of radioactive steroids, 1×10^6 primary rat brain cortical cells were plated onto poly-L-lysine (10 µg/ml)-coated glass coverslips in 12-well tissue culture plates (Marathon, UK). For Western blotting experiments, 4×10^6 neurones were plated into 25 cm² flasks (Marathon, UK). Cells were maintained in Neurobasal medium containing B27 supplement (Life Technologies, UK), 2 mM glutamine, and 20 µg/ml gentamicin solution. The levels of cortisol in this medium were 0.6–0.7 nM (Andrew Papadopoulos, personal communication). Rat brain primary cells were cultured for 7 days before being used for the treatments described.

Intracellular Accumulation of Radioactive Glucocorticoids

The assay to measure intracellular accumulation of radioactive glucocorticoids has been developed from Bourgeois *et al* (1993). Cells were incubated with ³H-cortisol (47.0 Ci/mmol) or ³H-corticosterone (70.0 Ci/mmol) (Amersham Pharmacia Biotech, UK), for 1.5 h, at 37°C in a CO_2 incubator. After three cold washes in phosphate-buffered salt solution (PBS), cells were scraped into lysis buffer (Roche Diagnostic, UK) and then transferred to vials for liquid scintillation counting. The radioactive signal, as measured by scintillation counting, is proportional to the intracellular concentration of the radioactive glucocorticoid. Results were normalized with respect to cell number by measurement of metabolic activity by cleavage of the tetrazolium salt WST-1 (Roche Diagnostic, UK). Clomipramine directly reduces cell metabolic activity only at concentrations of 100 µM or higher (Pariante *et al*, 2001a), while cortisol does not influence cell metabolic activity for concentrations ranging 10 nM–1 µM.

The intracellular accumulation of glucocorticoids likely reflects both GR-bound and GR-unbound signal. The ability of the radioactive glucocorticoids to bind the GR during the 1.5 h incubation is influenced by the degree of dissociation that occurs between GR and the endogenous ligands. This processes is dependent on temperature and pH, and requires a period of time when the cells are incubated in steroid-free conditions (Steiner and Wittliff, 1985). Since our assay did not aim to measure GR levels, we did not include this step. Therefore, even the use of an excess of unlabelled glucocorticoids in this assay gives only an approximate measurement of the relative size of these two compartments (see Results and Discussion).

Western Blotting and Densitometry Image Analysis

The procedures for Western blotting and densitometry image analysis were previously described (Williamson *et al*, 2002). Briefly, cells were washed three times in ice-cold TBS (25 mM Tris, pH 8.0, 140 mM NaCl, and 5 mM KCl) and lysed by scraping into hot (100°C) 2× sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) sample buffer, heated to 100°C for 5 min, and then centrifuged at 15 800*g*(av) for 5 min. Proteins were resolved by SDS-PAGE using 10% (w/v) polyacrylamide. Proteins were transferred to nitrocellulose (Schleicher & Scheull,

Germany) and submerged in blocking buffer TBS–Tween (TBS containing 0.2% (v/v) Tween-20 and 3% (w/v) nonfat dried milk) for 1 h at room temperature. Blots were incubated with the primary antibody diluted in blocking buffer overnight at 4°C. The primary antibody was the rabbit polyclonal antibody 57 (GR57) against the human GR (Affinity BioReagents, UK), 0.5 µg/ml, as previously described (Pariante *et al*, 1997, 1999, 2001b). Blots were washed three times in PBS-Tween and incubated with horseradish peroxidase (HRP)-linked donkey anti-rabbit Igs (Amersham Pharmacia, UK) diluted in blocking buffer for 1 h. After a further three washes in TBS–Tween, antibody binding was detected by an enhanced chemiluminescence (ECL) system (Amersham Pharmacia, UK). Film images from ECL-developed Western blots, developed for different times in order to ensure linearity of exposure, were analysed with a GS710 scanning densitometer utilizing the Quantity One (Bio Rad, UK) quantification software.

Experimental Design

Intracellular accumulation of ³H-glucocorticoids in LMCAT cells and rat cortical neurones. To study the relation between cortisol concentrations and its transport across cell membranes, we examined the intracellular accumulation of ³H-cortisol (1, 10, 100 nM and 1 µM), alone or in the presence of verapamil (100 µM), in LMCAT cells. To study the GR-bound and GR-unbound signal, we examined the intracellular accumulation of ³H-cortisol (50 nM) or ³H-corticosterone (50 nM), alone or in the presence of an excess (30 µM) of the unlabelled glucocorticoid, in LMCAT cells and rat primary neurones. To study whether rat primary neurones express a verapamil-sensitive membrane steroid transporter, we treated cells for 24 h with verapamil (100 µM), and then examined the intracellular accumulation of ³H-cortisol (50 nM) (verapamil was continued during the incubation with the radioactive glucocorticoid).

Effects of clomipramine on intracellular accumulation of ³H-cortisol and ³H-corticosterone in LMCAT cells and neurones. To study whether clomipramine inhibits membrane transport of glucocorticoids in LMCAT cells and rat primary neurones, we treated cells for 24 h with clomipramine (10 µM) and then examined the intracellular accumulation of ³H-cortisol (50 nM) or ³H-corticosterone (50 nM) (clomipramine was continued during the incubation with the radioactive glucocorticoids). We did not incubate cells with clomipramine and the radioactive glucocorticoids for 24 h, because the radioactive glucocorticoids would affect GR expression over such a long incubation; moreover, this design is equivalent to that used to examine the effects of clomipramine on GR expression. To study whether clomipramine increases the intracellular accumulation of radioactive cortisol in the presence of verapamil, we treated LMCAT cells for 24 h with clomipramine (10 µM) and then examined the intracellular accumulation of ³H-cortisol (50 nM) in the presence of verapamil (100 µM) (and clomipramine).

Effects of clomipramine on GR protein levels in LMCAT cells and neurones. To study the effects of clomipramine on

GR expression in LMCAT cells and rat primary neurones, we treated cells for 24 h with clomipramine (10 μM) and then examined GR by Western blot.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) of three or more independent experiments. Comparisons between treatment conditions and vehicle controls were conducted using *t*-test.

RESULTS

Intracellular Accumulation of ^{3}H-Glucocorticoids in LMCAT Cells and Rat Cortical Neurones

As shown in Figure 1, there was a linear relation between ^{3}H-cortisol concentrations in the media and the levels of intracellular accumulation in LMCAT cells. At all concentrations of ^{3}H-cortisol, verapamil increased the intracellular levels of the glucocorticoid hormone. Based on these results, we decided to use ^{3}H-cortisol (50 nM) for all subsequent experiments. In fact, the effects by verapamil were greater between 10 and 100 nM. Moreover, we have previously shown that this concentration of cortisol induces only partial GR activation and can be successfully used to elicit the effects of antidepressants on GR function (Pariante *et al*, 2001a). ^{3}H-corticosterone (50 nM) was used for comparison.

In a second series of experiments, we wanted to test whether the intracellular accumulation of radioactive cortisol or radioactive corticosterone would have been lowered by an excess of unlabelled glucocorticoid, because of competition at the binding with the GR. The results of the experiments in LMCAT cells are presented in Figure 2.
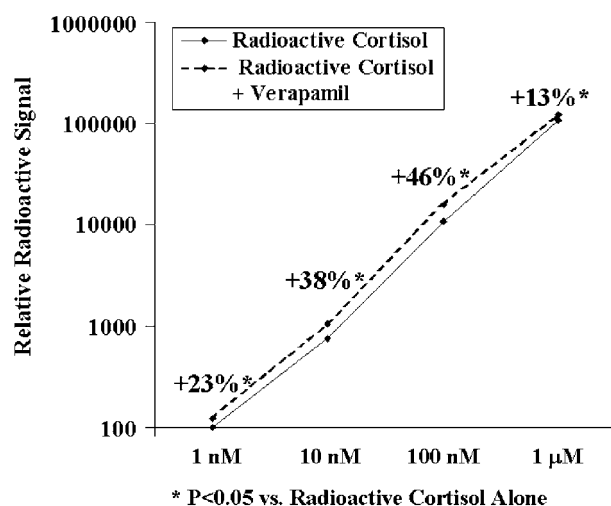
Figure 1 Intracellular accumulation of radioactive cortisol in LMCAT cells, alone or with verapamil. LMCAT cells were treated for 1.5 h with ^{3}H-cortisol (1, 10, 100 nM, and 1 μM), alone or in the presence of verapamil (100 μM). Cells extracts were analyzed for relative intracellular accumulation of ^{3}H-cortisol (fold accumulation relative to cells treated with ^{3}H-cortisol (1 nM) alone). Mean values from three independent experiments are presented in a logarithmic scale. The effects of verapamil are indicated as percentage increase compared to ^{3}H-cortisol alone. The * indicates a significant (*P*<0.05) difference between ^{3}H-cortisol plus verapamil (100 μM) and the corresponding concentration of ^{3}H-cortisol alone.
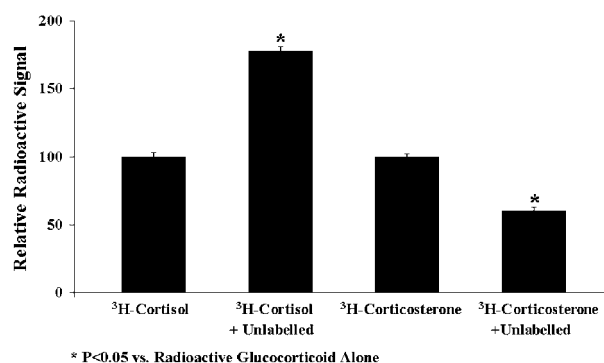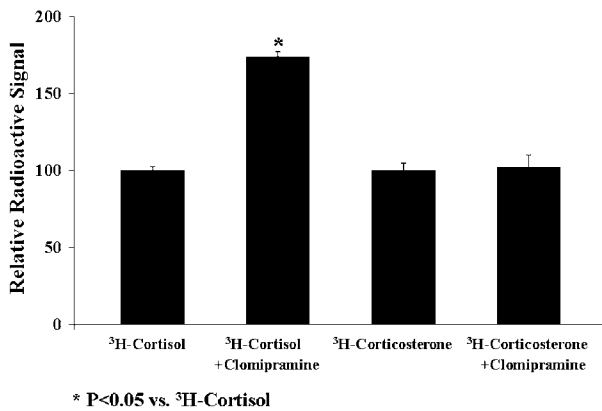
Figure 2 Intracellular accumulation of radioactive glucocorticoids in LMCAT cells, alone or with excess of unlabelled competitor. LMCAT cells were treated for 1.5 h with (1) ^{3}H-cortisol (50 nM), (2) ^{3}H-cortisol (50 nM) plus cortisol (30 μM), (3) ^{3}H-corticosterone (50 nM), and (4) ^{3}H-corticosterone (50 nM) plus corticosterone (30 μM). Cell extracts were analyzed for relative intracellular accumulation (fold accumulation relative to cells treated with the radioactive glucocorticoid alone). The results are shown as the mean ± SEM of four independent experiments. The * indicates a significant (*P*<0.05) difference compared to the radioactive glucocorticoid alone.

Surprisingly, unlabelled cortisol induced almost an 80% increase, rather than a decrease, of the intracellular accumulation of radioactive cortisol. We interpreted these findings as showing that the unlabelled cortisol competes for the radioactive cortisol at the efflux system, thus increasing the intracellular accumulation of the radioactive cortisol. However, because corticosterone is not transported, the unlabelled corticosterone competes with the radioactive corticosterone at the GR binding site and results in a 40% reduction of the intracellular accumulation.

The results of the experiments conducted in primary neurones mirrored those in LMCAT cells using the same protocols, thus providing the first evidence that a membrane transport of cortisol is present in these cells. First, unlabelled cortisol induced a small increase of the intracellular accumulation of radioactive cortisol (+7 ± 4%, *P* = 0.1), while unlabelled corticosterone induced a decrease of the intracellular accumulation of radioactive corticosterone (−15 ± 3%, *P*<0.05). Second, verapamil increased the intracellular accumulation of ^{3}H-cortisol (+174 ± 15%, *P*<0.05).

Effects of Clomipramine on Intracellular Accumulation of ^{3}H-Cortisol and ^{3}H-Corticosterone in LMCAT Cells and Neurones

Clomipramine induced an increase in the intracellular accumulation of ^{3}H-cortisol, compared to cells treated with vehicle: approximately +80% in LMCAT cells (Figure 3) and +20% in neurones (Figure 4). In the presence of ^{3}H-corticosterone, clomipramine had no effect in LMCAT cells (Figure 3) and induced a small (−11%) reduction of the intracellular concentration in neurones (Figure 4). To corroborate these findings, we tested in LMCAT cells whether clomipramine would increase the intracellular accumulation of radioactive cortisol in the presence of verapamil. If inhibition of the steroid transporter is the mechanism by which clomipramine increased intracellular accumulation of ^{3}H-cortisol, this effect should decrease in

Figure 3 Intracellular accumulation of radioactive glucocorticoids in LMCAT cells, treated with vehicle or clomipramine. LMCAT cells were treated with vehicle or clomipramine (10 μM) for 24 h, and then incubated for 1.5 h with ^{3}H-cortisol (50 nM) or ^{3}H-corticosterone (50 nM). Clomipramine was continued during the 1.5 h incubation with the radioactive glucocorticoid. Cells extracts were analyzed for relative intracellular accumulation (fold accumulation relative to control cells). The results are shown as the mean ± SEM of three independent experiments. The * indicates a significant ($P < 0.05$) difference compared to control cells.
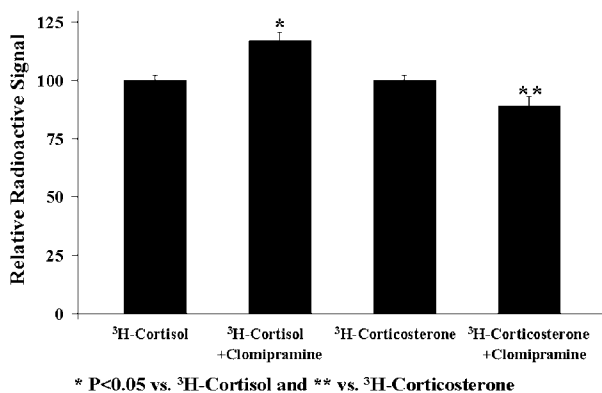

Figure 4 Intracellular accumulation of radioactive glucocorticoids in rat primary neurones, treated with vehicle or clomipramine. 1-week old rat primary neurones were treated with vehicle or clomipramine (10 μM) for 24 h, and then incubated for 1.5 h with ^{3}H-cortisol (50 nM) or ^{3}H-corticosterone (50 nM). Clomipramine was continued during the 1.5 h incubation with the radioactive glucocorticoid. Cell extracts were analyzed for relative intracellular accumulation (fold accumulation relative to control cells). The results are shown as the mean ± SEM of three independent experiments. The * indicates a significant ($P < 0.05$) difference compared to cells treated with ^{3}H-cortisol alone, and ** indicates a significant ($P < 0.05$) difference compared to cells treated with ^{3}H-corticosterone alone.

the presence of verapamil. As hypothesized, the treatment of LMCAT cells with clomipramine in the presence of verapamil induced only a small (and not statistically significant) increase of intracellular accumulation of ^{3}H-cortisol ($+17 \pm 11\%$, $P = 0.2$), compared to cell treated with verapamil alone.

Effects of Clomipramine on GR Protein Levels in LMCAT Cells and Neurones

We examined the effects of clomipramine on GR expression, assessed by whole-cell Western blot, in LMCAT cells
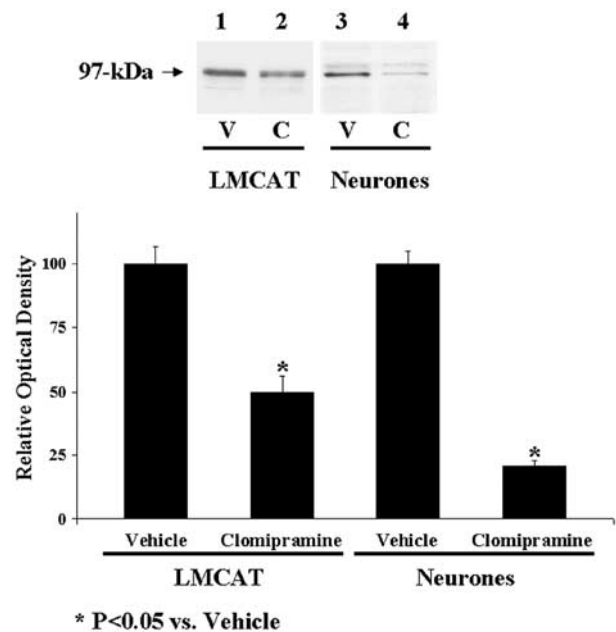
Figure 5 Western blot of the GR in LMCAT cells and rat primary neurones, treated with vehicle or clomipramine. LMCAT cells and 1-week old rat primary neurones were treated with vehicle (V) or clomipramine (C) (10 μM) for 24 h. Cells were lysed by scraping into hot (100°C) 2 × SDS-PAGE sample buffer. GR present in the lysates was analysed by Western blot using the anti-GR polyclonal antibody GR57 and a horseradish peroxidase-conjugated counterantibody. The GR57 recognized a prominent band at ~97 kDa. Top: representative Western blots. Bottom: quantitation of GR bands, based on densitometric analysis. Results are expressed as percentage of the GR signal in the vehicle-treated cells, and are presented as mean ± SEM of 3-4 independent experiments. The * indicates a significant ($P < 0.05$) difference compared to control cells.

and primary rat cortical neurones. Figure 5 shows representative Western blots, together with the results of the densitometric quantitation of the GR band from independent experiments. In both types of cells, the rabbit polyclonal antibody 57 (GR57) against the human GR recognized a prominent band at ~97 kDa, consistent with similar experiments performed with the same antibody in the cytosolic and nuclear fractions of LMCAT cells (Pariante *et al*, 1997, 1999, 2001b) and in the rat brain (O'Donnell *et al*, 1995) or with different monoclonal antibodies anti-GR (Beck *et al*, 1993; Sanchez *et al*, 1994). Compared with vehicle (lanes 1 and 3), treatment with clomipramine (10 μM, 24 h) (lanes 2 and 4) resulted in a reduction of GR levels. This was confirmed by densitometric quantitation of the GR bands, which showed a reduction in GR signal in cells treated with clomipramine: 50% reduction in LMCAT cells and 80% reduction in neurones.

DISCUSSION

This study shows that the antidepressant clomipramine inhibits a membrane steroid transporter and thus increases intracellular concentrations of cortisol, in fibroblasts and neurones. In summary: (1) clomipramine increases the intracellular concentrations of ^{3}H-cortisol, but not ^{3}H-corticosterone, in LMCAT cells and primary rat neurones and (2) the effect of clomipramine on the intracellular

accumulation of ^{3}H-cortisol in LMCAT cells is reduced in the presence of the steroid transporter inhibitor verapamil.

This is the first report, to our knowledge, of a functional membrane transport of glucocorticoids in neurones. Human neuroblastoma cells express PGP (Kurowski and Berthold, 1998), and cells from the mouse hippocampal cell line HT22 express a membrane steroid transporter that is blocked by verapamil (Herr et al, 2000). However, no immunolabelling of PGP has been found in adult rat brain neurones (Matsuoka et al, 1999), although PGP and PGP-like transporters can be expressed at very low levels that are undetected by immunocytochemistry (Kralli and Yamamoto, 1996; Marsaud et al, 1998). Interestingly, immunoreactive and mRNA signals for another family of membrane transporters, the multidrug resistance-associated proteins (MRPs), have been found in rat neuronal cultures (Hirrlinger et al, 2002) and in dysplastic neurones from brain samples of epileptic patients (Sisodiya et al, 2002). The MRPs are organic anion transporters that participate in multidrug resistance (Leslie et al, 2001). The function and expression of MRPs are modulated by cortisol (Mulder et al, 1996) and MRP-overexpressing cells are resistant to the effects of cortisol (Bergman et al, 2001). At present, however, the nature of the cortisol transporters in rat neurones is unclear.

We can only estimate the amount of GR-bound vs GR-unbound radioactive signal in the cells (see Materials and methods). However, it seems clear that most of the signal is GR unbound. In LMCAT cells, the excess of corticosterone decreases the signal by 40%, but the 50% reduction in GR expression by clomipramine leads to no change in intracellular accumulation of radioactive corticosterone. In neurones, the excess of unlabelled corticosterone and the 80% reduction of GR expression by clomipramine lead only to a 10–15% reduction in the intracellular accumulation of radioactive corticosterone. These results corroborate data by Karssen et al (2001, 2002) in mdr1a PGP knockout mice, showing that brain labelling by a radioactive glucocorticoid is not reduced by an excess of the unlabelled glucocorticoid (except in the hippocampus), thus suggesting that the radioactive glucocorticoid in the brain of these animals is mostly unbound.

A second major finding in our study is that clomipramine reduces GR expression in LMCAT cells and primary rat neurones. These data are consistent with our previous results showing that 24–96 h of treatment with desipramine induces a 10–25% reduction in cytosolic GR binding in L929 cells; this reduction is still present after the cells have recovered for 24 h in media with no desipramine (Pariante et al, 1997). Since 24 h desipramine also induces activation and translocation of the GR from the cytoplasm to the nucleus (Pariante et al, 1997), and this is associated with a decrease of GR in the cytosolic fraction (McEwen et al, 1997), we had interpreted the desipramine-induced decrease in GR binding as representing a greater proportion of GR in the nucleus (Pariante et al, 1997). However, in the light of the present data, these results can be interpreted as an overall decrease in the total number of GRs. Similar to our study, treatment of hippocampal cell culture with antidepressants for 48 h has been shown to induce GR translocation (Okugawa et al, 1999) and downregulation of GR mRNA (Yau et al, 2001).

We suggest the following model for the in vitro effects of antidepressants on the GR. Treatment with antidepressants inhibits steroid transport, induces GR translocation, and reduces GR expression (Pariante et al, 1997, 2001a; present paper). These three effects could be mediated by the same mechanism. For example, blocking of steroid transport can increase the intracellular levels of steroids from the media—even steroid stripping of serum cannot be absolute (Miller et al, 2002)—thus leading to translocation of the GR. Data showing that PGP inhibitors induce partial GR translocation support this possibility (Prima et al, 2000). Antidepressants could also induce GR translocation through their effect on cAMP-dependent protein kinases (Rangarajan et al, 1992; Chen and Rasenick, 1995; Miller et al, 2002; Blom et al, 2002). Translocation of GR, in turn, could lead to the reduction in GR expression. In fact, GR translocation by both GR agonists and GR antagonists has been associated with a GR downregulation, which takes place over few hours and is because of a reduction in the protein half-life and an inhibition of GR mRNA synthesis; this reduction is temporary and can be followed by a subsequent upregulation (Schmidt and Meyer, 1994). Therefore, it is possible that the inhibition of the transporters precedes the GR downregulation. If cells are coincubated for 24 h with antidepressants and a glucocorticoid that is expelled by the transporter, like cortisol, an enhanced GR-mediated gene transcription is evident (Pariante et al, 2001a, b). This is because the increase in the intracellular levels of the glucocorticoid overcomes the GR downregulation, and possibly precedes the GR downregulation. However, if cells are treated in experimental conditions that do not elicit the effects on the transporter, the GR downregulation leads to a reduced GR-mediated gene transcription. For example, antidepressants give a reduction in GR-mediated gene transcription when cells are coincubated with corticosterone (Pariante et al, 2001a). Moreover, Miller et al (2002) have recently found that incubation of LMCAT cells with desipramine alone induces a small decrease in the unstimulated GR-mediated gene transcription. Finally, preincubation of cells with an antidepressant (for up to 5 days) leads to approximately 50% inhibition of GR-mediated gene transcription induced by a subsequent short treatment (1.5–2 h) with dexamethasone or corticosterone (Pariante et al, 1997; Budziszewska et al, 2000). In this case, even if the inhibition of the transporter increases the intracellular levels of dexamethasone (as suggested by our experiments with ^{3}H-cortisol), these are unable to compensate for the GR downregulation, possibly because of the short incubation, or possibly because the GR downregulation is present before the glucocorticoid is added (differently than in the coincubation experiments).

Is this model relevant for the effects of antidepressants on the GR and on the HPA axis in animals? Rodents have two isoforms of PGP: the mdr1a and the mdr1b. The mdr1a PGP is predominantly expressed at the BBB (Regina et al, 1998) and expels cortisol from the brain of rodents, but not corticosterone, which is the endogenous glucocorticoid in these animals (de Kloet et al, 1998; Karssen et al, 2001). However, the mdr1b PGP transports corticosterone (Wolf and Horwitz, 1992; Uhr et al, 2002). This isoform is predominantly expressed in the adrenal and the ovaries

(Lee *et al*, 2001), but is also expressed in the brain (Regina *et al*, 1998), particularly in the hippocampus (Kwan *et al*, 2002). Although mdr1b PGP has not been detected in brain capillaries (Regina *et al*, 1998), it is expressed in rat brain endothelial cells *in vitro* (Felix and Barrand, 2002). Consistent with this, mice that are knockout for mdr1a and mdr1b PGP show increased access of corticosterone to the brain (although this effect is smaller than that on cortisol) and increased negative feedback on the HPA axis by corticosterone (Uhr *et al*, 2002). Therefore, there are intriguing—but still preliminary—lines of evidence suggesting that membrane transport of corticosterone regulates HPA axis function in rodents. By inhibiting membrane steroid transport, antidepressants could directly increase the access of corticosterone to the brain, or the access of other steroids that are transported by PGP, like aldosterone (Ueda *et al*, 1992), and enhance GR (and MR) activation. Although in the brain the highest expression of PGP has been found at the BBB (Lee *et al*, 2001), our data show that this effect could also occur directly at the neuronal level. In turn, the increased access of glucocorticoids to the brain could lead to the decrease in HPA axis activity and the GR and MR downregulation described in rats after 3–9 days of treatment with antidepressants (Reul *et al*, 1993; Yau *et al*, 2001). This receptor downregulation is consistent with our data *in vitro* and, theoretically, could explain part of the lag time seen with antidepressants for the onset of their therapeutic action. Chronically, that is, after 14 days or more of treatment, antidepressants induce GR and MR upregulation in the brain (Pariante and Miller, 2001). Although catecholamines upregulate GR and MR expression (Lai *et al*, 2003), inhibition of catecholamines' uptake seems not to be relevant for the antidepressant-induced GR upregulation. In fact, desipramine increases GR expression in the rat brain even following neurotoxic lesioning of noradrenergic neurones with DSP4 (Rossby *et al*, 1995). Moreover, antidepressant-induced changes in GR expression *in vitro* are not blocked by antagonists of alpha- or beta-adrenergic receptors or of 5HT1a or 5HT2 serotonergic receptors (Okugawa *et al*, 1999; Lai *et al*, 2003). The inhibition of steroid transporters—and the increased access of glucocorticoids to the brain—could explain this GR and MR upregulation, as a compensatory mechanism following the initial downregulation or as a consequence of the reduced HPA axis activity. Consistent with our model, tricyclic antidepressants induce brain MR and GR upregulation at the same concentrations known to inhibit MDR PGP in subcutaneous tumors (10–20 mg/kg/day) (Merry *et al*, 1991; Pariante and Miller, 2001). Moreover, pretreatment with nifedipine, an MDR PGP inhibitor, prevents the hippocampal GR upregulation induced by antidepressants (Przegalinski *et al*, 1993). Inhibition of PGP and other membrane steroid transporters is not receptor mediated and is related to the drugs' physiochemical properties, that is, lipophilicity, electric charge, and ability to accept hydrogen bonds (Ford, 1996; Castaing *et al*, 2000; Ekins *et al*, 2002).

In conclusion, we have demonstrated that the antidepressant clomipramine regulates the intracellular levels of cortisol by inhibiting membrane steroid transporters, in LMCAT cells and neurones, that are similar to PGP in their substrates and modulators. In humans, the endogenous glucocorticoid, cortisol, is transported by the PGP at the BBB (Karssen *et al*, 2001). We propose that antidepressants in humans could inhibit the steroid transporters localized on the BBB and in neurones, and thus increase the access of cortisol to the brain and the negative feedback on the HPA axis. This is consistent with studies showing that treatment with GR and MR agonists (Dinan *et al*, 1997; Bouwer *et al*, 2000), including cortisol (DeBattista *et al*, 2000), has antidepressant effects in humans. Hypothetically, the increased access of cortisol to the brain could balance the reduction of GR function described in patients with major depression (Pariante and Miller, 2001). Or, perhaps, this effect could compensate for a 'reduction' of cortisol levels in the brain of depressed patients that was first (and last) described exactly 30 years ago: 'In studies comparing steroid concentration in eight sudden death controls and six suicide brains, the concentration of cortisol and corticosterone was higher in the controls, in both cerebral cortex and the hypothalamus. (...) All six suicides had a clear history of depressive illness' (Brooksbank *et al*, 1973).

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