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Antidepressant fluoxetine enhances glucocorticoid receptor function *in vitro* by modulating membrane steroid transporters

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1 Incubation of LMCAT fibroblast cells with antidepressants potentiates glucocorticoid receptor (GR)-mediated gene transcription in the presence of dexamethasone and cortisol, but not of corticosterone. We have shown that antidepressants do so by inhibiting the LMCAT cell membrane steroid transporter (which is virtually identical to the multidrug resistance P-glycoprotein) and thus by increasing dexamethasone or cortisol intracellular concentrations. However, previous experiments with the antidepressant fluoxetine in the presence of dexamethasone have produced negative results (Pariante *et al*. (2001). *Br. J. Pharmacol*., **134,** 1335–1343).

2 We have since re-examined the effects of fluoxetine on GR-mediated gene transcription in the presence of dexamethasone. Moreover, we have examined the effects of fluoxetine on GR-mediated gene transcription in the presence of cortisol and corticosterone, and on the intracellular accumulation of radioactive cortisol and corticosterone. Finally, we have examined the effects of fluoxetine on inhibition of P-glycoprotein activity in Caco-2 cells.

3 We now find that fluoxetine (1–10 μM) enhances GR-mediated gene transcription in the presence of dexamethasone and cortisol (+140–170%), but not of corticosterone, and increases the intracellular accumulation of ^{3}H-cortisol (+5–15%), but not of ^{3}H-corticosterone. Moreover, fluoxetine (10 μM) induces approximately 30% inhibition of PGP activity in Caco-2 cells.

4 Our results show that fluoxetine, like other antidepressants, inhibits membrane steroid transporters.
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Keywords: Antidepressant; Caco-2 cells; cortisol; corticosterone; fluoxetine; glucocorticoid receptor; hypothalamic–pituitary–adrenal (HPA) axis; L929/LMCAT cells; multidrug resistance (MDR); P-glycoprotein (PGP)

Abbreviations: BBB, blood–brain barrier; GR, glucocorticoid receptor; GREs, glucocorticoid response elements; h, hour; HPA axis, hypothalamic–pituitary–adrenal axis; LMCAT cells, L929 cells stably transfected with the mouse mammary tumour virus-chloramphenicol acetyltransferase reporter gene; MDR, multidrug resistance; MMTV-CAT, mouse mammary tumour virus-chloramphenicol acetyltransferase reporter gene; MTP, microtiter plate; PGP, P-glycoprotein; SSRI, selective serotonin reuptake inhibitor

Introduction

Studies in depressed patients, animals and cellular models have demonstrated that antidepressants increase the function and the expression of the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). This, in turn, is associated with enhanced GR- and MR-mediated negative feedback by endogenous glucocorticoids on the hypothalamic–pituitary–adrenal (HPA) axis, and thus with reduced HPA-axis activity. These effects could be relevant for the therapeutic action of antidepressants, but the molecular mechanisms are still unclear (Pariante & Miller, 2001).

Glucocorticoids are excreted from fibroblasts, leukocytes and epithelial cells by transporters of the ATP-binding cassette family (Kralli & Yamamoto, 1996; de Kloet *et al*., 1998; Pariante *et al*., 2001a, b; 2003). One of these transporters, the multidrug resistance (MDR) *p*-glycoprotein (PGP), is localized at the luminal membrane of the endothelial cells of the blood–brain barrier (BBB) and limits the access of cortisol (the endogenous glucocorticoid in humans) and dexamethasone to mouse and human brain (Karssen *et al*., 2001). Corticosterone (the endogenous glucocorticoid in rodents) is not or only minimally transported by PGP (Karssen *et al*., 2001; Uhr *et al*., 2002).

We have suggested that antidepressants regulate GR function in LMCAT mouse fibroblasts by inhibiting membrane steroid transporters that are virtually identical to PGP in their substrates and modulators (Pariante *et al*., 2001a, b; 2003). Specifically, chemically unrelated antidepressants (the tricyclics desipramine, amitriptyline and clomipramine, and the selective serotonin reuptake inhibitors (SSRIs), citalopram and paroxetine) enhance GR-mediated gene transcription in the presence of dexamethasone or cortisol, but not of corticosterone (Pariante *et al*., 1997; 2001a). Based on these results, we have proposed that potentiation of GR-mediated gene transcription in the presence of dexamethasone or cortisol is due to the antidepressants inhibiting the LMCAT cell membrane steroid transporter and thus increasing the intracellular concentrations of these glucocorticoids. In further support of this model, we have recently shown that clomipramine increases intracellular concentrations of ^{3}H-cortisol, but

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not of ^{3}H-corticosterone, in LMCAT cells and primary rat neurones, thus also providing the first report of a functional membrane transport of glucocorticoids in neurones (Pariante *et al*., 2003).

We had previously failed to demonstrate a potentiation of GR function by the SSRI fluoxetine in the presence of dexamethasone (Pariante *et al*., 2001a). Although this result had differentiated fluoxetine from all other antidepressants tested (Pariante *et al*., 2001a), in itself it was not inconsistent with the evidence, available at that time, that chronic treatment with fluoxetine does not increase GR expression in animals (Pariante & Miller, 2001) and that fluoxetine is not transported by PGP (Uhr *et al*., 2000). However, subsequent research from other laboratories has demonstrated that fluoxetine regulates GR and MR expression acutely in animals and *in vitro* (Yau *et al*., 2001; Lai *et al*., 2003). This has prompted us to reconsider our original negative findings. Therefore, we have obtained a new batch of fluoxetine and repeated the experiments looking at GR-mediated gene transcription in the presence of fluoxetine and dexamethasone. Moreover, we have conducted novel experiments examining the effects of fluoxetine on GR-mediated gene transcription in the presence of cortisol and corticosterone and on the intracellular accumulation of radioactive cortisol and corticosterone. Finally, to investigate whether the effects of fluoxetine can be generalized to other cell systems containing PGP, we have also examined the ability of fluoxetine to inhibit PGP activity in Caco-2 cells.

Methods

Materials

All chemicals, unless otherwise stated, were purchased from Sigma (U.K.).

As in our previous work, we have conducted these experiments in LMCAT cells (L929 mouse fibroblasts stably transfected with the mouse mammary tumour virus-chloramphenicol acetyltransferase (MMTV-CAT) reporter gene) (Pariante *et al*., 1997; 2001a, b; 2003). The LMCAT cell line was generously provided by E.R. Sanchez (Medical College of Ohio, Toledo, OH, U.S.A.) (Sanchez *et al*., 1994). Expression of CAT by these cells is under glucocorticoid control by virtue of several glucocorticoid response elements (GREs) residing within the MMTV promoter, which lies upstream of the CAT reporter gene (Sanchez *et al*., 1994). This cell line is an ideal tool to look at the effects of antidepressants on both GR function and membrane steroid transporters. Research by our group (Pariante *et al*., 2001a, b) and by others (Kralli & Yamamoto, 1996; Marsaud *et al*., 1998; Medh *et al*., 1998) has described that L929/LMCAT cells express a membrane steroid transporter that is virtually identical to PGP in its substrates and modulators, including in its capacity to be inhibited by H-89 and verapamil. More recently, Webster & Carlstedt-Duke (2002) have demonstrated the presence of MDR PGP mRNA in these cells. LMCAT cells have therefore the unique advantage of expressing endogenous (not transfected) membrane steroid transporters, while also allowing the direct examination of GR function. Mouse fibroblasts (and these cells in particular) have been used in a variety of studies looking at molecular determinants of GR function, including in some of the pivotal studies looking at the effects of antidepressants on the GR (Pepin *et al*., 1992; Sanchez *et al*., 1994; Pariante *et al*., 1997; 1999; 2001a, b; 2003; Budziszewska *et al*., 2000).

We have treated cells with the synthetic glucocorticoid dexamethasone (10 nM) and the endogenous glucocorticoids, cortisol (50 nM) and corticosterone (50 nM). These concentrations of endogenous hormones (cortisol and corticosterone) correspond to the plasma levels obtained during the low-basal phase of HPA-axis activity (evening in humans and morning in rodents) (Orth & Kovacs, 1998). Moreover, we have previously shown that these concentrations of glucocorticoids induce only partial GR activation and can be successfully used to elicit the effects of antidepressants and other GR modulators on GR function (Pariante *et al*., 1997; 1999; 2001a, b; 2003).

The antidepressant fluoxetine (hydrochloride, from RBI) has been used at 1–10 μM, for 24–72 h. *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; 2003; Budziszewska *et al*., 2000; Lai *et al*., 2003). Moreover, this concentration resembles the therapeutic plasma and brain levels of antidepressants (Glotzbach & Preskorn, 1982).

We have used verapamil (100 μM for 24 h) to inhibit the steroid transporter. Verapamil is a known inhibitor of the LMCAT cell membrane steroid transporter (and of the MDR PGP), and it has been used in experiments with these cells at concentrations ranging from 10 to 100 μM (Marsaud *et al*., 1998; Medh *et al*., 1998; Pariante *et al*., 2001a, b; 2003). In particular, work conducted in LMCAT cells, by us and others, has shown that verapamil enhances GR translocation and GR-mediated gene transcription in the presence of glucocorticoids that are expelled by the transporter, and increases the intracellular concentrations of glucocorticoids that are expelled by the transporters (Marsaud *et al*., 1998; Medh *et al*., 1998; Pariante *et al*., 2001a, b; 2003). However, these studies have also shown that verapamil induces only partial inhibition of the LMCAT cell steroid transporter at concentrations of 10 and 50 μM (Marsaud *et al*., 1998; Medh *et al*., 1998), and our original dose-finding experiments in these cells have shown that verapamil (100 μM) induces a larger potentiation of GR translocation in the presence of dexamethasone than verapamil (50 μM) (Pariante *et al*., 2001b). Therefore, we have consistently used verapamil (100 μM) in our previous work (Pariante *et al*., 2001a, b; 2003) and in the present paper. Verapamil has no effect on GR-mediated gene transcription in the absence of glucocorticoids, even at 100 μM (Marsaud *et al*., 1998; Medh *et al*., 1998; Pariante *et al*., 2001a, b). Verapamil is also a well-known blocker of the L-type calcium channel, but inhibition of the LMCAT cell steroid transporter (and of the MDR PGP) is unrelated to this effect (Ford, 1996). In fact, other inhibitors of the LMCAT cell steroid transporter, like FK506, rapamycin, cyclosporin A and quinidine, have no blocking effect on calcium channels, but they all produce changes on GR function in LMCAT cells that are identical to those induced by verapamil (Marsaud *et al*., 1998; Medh *et al*., 1998). Moreover, both the S and the R enantiomers of verapamil are inhibitors of the MDR *p*-glycoprotein, while only the S enantiomer binds to calcium channels, and tests conducted on several channel blockers have found no correlation between

the calcium channel-blocking activity and the anti-MDR activity (Ford, 1996).

LMCAT cell culture

LMCAT cells were maintained in 175-cm^2 flasks (Marathon, U.K.) at 37°C with a 5% CO_2 and 95% air atmosphere. The culture medium was DMEM with 10% (v v^{-1}) charcoal-stripped, delipidated, heat-inactivated (56°C, 30 min) bovine calf serum (Autogen Bioclear, U.K.) and 0.2 mg ml^{-1} G418 (Geneticin) antibiotic. The levels of cortisol in this medium were <0.1 nM (Pariante *et al*., 2003). For the CAT assay, LMCAT cells were subcultured in six-well plates and grown for 48–72 h (final confluency 95%) prior to drug treatment. For measuring the intracellular accumulation of radioactive steroids, LMCAT cells were subcultured in 12-well plates (Marathon, U.K.) and grown for 48–72 h (final confluency 95%) prior to drug treatment.

CAT reporter cell line and CAT assay

The LMCAT cell line is stably transfected with the MMTV-CAT reporter gene. As we have previously described, the expression of CAT is dependent on the type of glucocorticoids (the affinity for the GR and the ability to be expelled by the LMCAT membrane steroid transporter) as well as the concentration and the duration of the incubation (Pariante *et al*., 1997; 1999; 2001a, b). For example, dexamethasone (10 nM) and corticosterone (50 nM) give approximately the same induction of CAT, while cortisol (50 nM) induces approximately half the CAT induction induced by corticosterone (50 nM) (Pariante *et al*., 2001a). Since corticosterone and cortisol have similar affinities for the GR (de Kloet *et al*., 1998), we have interpreted this finding as being secondary to the fact that cortisol, but not corticosterone, is expelled by the membrane steroid transporter (Pariante *et al*., 2001a).

Measurement of CAT was performed using a recently developed colorimetric enzyme immunoassay (Roche Diagnostic, U.K.), as previously described (Pariante *et al*., 2001a). The colorimetric enzyme immunoassay is based on the sandwich ELISA principle, and antibodies to CAT are prebound to the surface of the microtiter plate modules (MTP). Cell extracts were obtained by incubation in the manufacturer's lysis buffer. After centrifugation (20,000 × *g* for 10 min), supernatants were transferred to the wells of the MTP (1 h, 37°C). Next, a digoxigenin-labelled antibody was added to CAT (1 h, 37°C), followed by an antibody to digoxigenin conjugated to peroxidase (1 h, 37°C). In the final step, the peroxidase substrate ABTS was added, yielding a coloured reaction product. The absorbance of the sample was determined using the MRXII microplate reader (Dynex Technologies, Chantilly, VA, U.S.A.). The absorbance is directly correlated to the level of CAT present in the cell extract (as determined by a standard curve). Results were normalized with respect to cell number by measurement of metabolic activity by cleavage of the tetrazolium salt WST-1 (Roche Diagnostic, U.K.).

Intracellular accumulation of radioactive glucocorticoids

The assay to measure intracellular accumulation of radioactive glucocorticoids has been developed from Bourgeois *et al*. (1993) and has been described before (Pariante *et al*., 2003). Cells were incubated with ^{3}H-cortisol (47.0 Ci mmol^{-1}) or ^{3}H-corticosterone (70.0 Ci mmol^{-1}) (Amersham Pharmacia Biotech, U.K.), for 1.5 h, at 37°C in a CO_2 incubator. After three cold washes in phosphate-buffered salt solution, cells were scraped into lysis buffer (Roche Diagnostic, U.K.) 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, U.K.). Although theoretically the intracellular accumulation of glucocorticoids reflects both GR-bound and GR-unbound signals, our data show that most of the signal is GR-unbound (Pariante *et al*., 2003).

Inhibition of PGP activity in Caco-2 cells

The Caco-2 cells (from American Type Culture Collection, Manassas, VA, U.S.A.) are derived from a human colon carcinoma and are viewed as a model of human intestinal epithelial cells. The cells were grown and plated in the same format as previously published (Ekins *et al*., 2002).

Inhibition of PGP activity in Caco-2 cells was assessed using radiolabelled digoxin as the PGP substrate. Complete inhibition of PGP-mediated transport would be expected to result in the loss of digoxin's basal-to-apical (B→A) vs apical-to-basal (A→B) transport difference. Accordingly, percentage inhibition was estimated by

$$\text{Degree of inhibition} = \left(1 - \left(\frac{i_{B\to A} - i_{A\to B}}{a_{B\to A} - a_{A\to B}}\right)\right) \times 100\%$$

where i and a are the percentages of digoxin transport in the presence and absence of the putative inhibitor, according to the direction of transport. Control digoxin transport in the absence of any inhibitor (2 wells per plate) were included on every plate (12 wells). Aliquots (25 μl) of the compartmental buffer solution containing radiolabelled digoxin were analysed by liquid scintillation counting.

Experimental design

For experiments in LMCAT cells, we prepared a stock solution of fluoxetine, 1 mg ml^{-1}, in double-distilled, filtered water; the fluoxetine concentration was confirmed by gas liquid chromatography using nitrogen phosphorous detector (GLQ-NPD) and this solution was used for all experiments. There was no difference between this new batch of fluoxetine and the old one used in our previous study leading to negative results (Pariante *et al*., 2001a), analysed by UV absorbance and GLQ-NPD. However, in the previous study, a fresh fluoxetine solution (1 mg ml^{-1}) in water was prepared for each experiment, and then a few microlitres of this solution were immediately diluted in cell culture media to the final 10 μM concentration (a similar protocol was used for amitriptyline, desipramine, clomipramine and citalopram). It is possible that in the previous experiments, the fluoxetine might have not yet completely dissolved in the water when these small volumes were taken for further dilution, thus leading to inconsistent concentrations. This hypothesis is supported by the fact that 1 mg ml^{-1} is close to the limit of solubility in water of

fluoxetine (4 mg ml^{-1}), while for all the other antidepressants this limit is much higher (25–50 mg ml^{-1}) (data from manufacturers). For the experiments with Caco-2 cells, again one stock solution of fluoxetine in water was prepared, and used for all experiments.

To study whether fluoxetine regulates GR-mediated gene transcription, we coincubated cells for 24 h with fluoxetine (1–10 μM) and dexamethasone (10 nM), cortisol (50 nM) or corticosterone (50 nM); to study whether fluoxetine is able to regulate GR-mediated gene transcription in the presence of a membrane steroid inhibitor, we coincubated cells with fluoxetine (10 μM), dexamethasone (2.5 nM) and verapamil (100 μM); we did not incubate cells with fluoxetine and the glucocorticoids for longer duration of treatment (e.g., 72 h) because this would cause maximal CAT induction, and we already know that antidepressants are unable to potentiate GR-mediated gene transcription under these circumstances (Pariante *et al*., 2001a).

To study whether fluoxetine inhibits membrane transport of glucocorticoids in LMCAT cells, we treated cells for 24–72 h with fluoxetine (10 μM) and then examined the intracellular accumulation of ^{3}H-cortisol (50 nM) or ^{3}H-corticosterone (50 nM) (fluoxetine was continued during the incubation with the radioactive glucocorticoids); we did not incubate cells with fluoxetine and the radioactive glucocorticoids for 24–72 h, because the radioactive glucocorticoids would affect GR expression over such a long incubation (Pariante *et al*., 2003).

To study whether fluoxetine inhibits PGP activity, we examined transepithelial transport of [^{3}H]-digoxin across a Caco-2 cell culture monolayer in the absence or presence of fluoxetine (10 μM) (Ekins *et al*., 2002).

Statistical analysis

Data are presented as mean ± standard error of the mean (s.e.m.) of three or more independent experiments. Data were analysed using *t*-test (for comparisons between each treatment condition and the appropriate vehicle controls).

Results

Fluoxetine (10 μM, 24 h) increased GR-mediated gene transcription in the presence of dexamethasone (+170%), compared to cells treated with dexamethasone alone (Figure 1). Moreover, fluoxetine increased GR-mediated gene transcription in the presence of cortisol (+140%) (Figure 2). However, fluoxetine had no effect on GR-mediated gene transcription in the presence of corticosterone (Figure 2). These results closely resemble those obtained with other antidepressants (Pariante *et al*., 2001a). Fluoxetine (1 μM) induced a 16±2% potentiation of GR-mediated gene transcription in the presence of cortisol ($P<0.05$), similar to what we have already described using the same concentration of other antidepressants in the presence of dexamethasone (Pariante *et al*., 2001a).

To corroborate these findings, we examined the effects of fluoxetine on GR-mediated gene transcription in the presence of dexamethasone (2.5 nM) plus the steroid transporter inhibitor verapamil. If inhibition of the steroid transporter is the mechanism by which fluoxetine increases GR-mediated gene transcription in the presence of dexamethasone, this effect should disappear in the presence of verapamil (Pariante *et al*., 2001a). As we have previously shown for other concentrations of dexamethasone (Pariante *et al*., 2001a), verapamil (100 μM) increases GR-mediated gene transcription in the presence of dexamethasone (2.5 nM), thus inducing levels of GR-mediated gene transcription similar to that induced by dexamethasone (10 nM) alone (Figure 3). As hypothesized, we found that fluoxetine did not potentiate GR-mediated gene transcription in the presence of dexamethasone, when the membrane steroid transporter was blocked by verapamil (Figure 3). Again, these results are consistent with our previous finding that clomipramine does not potentiate GR-mediated gene transcription in the presence of dexamethasone (2.5 nM) and verapamil (Pariante *et al*., 2001a).

We also examined the effects of fluoxetine on the intracellular concentrations of radioactive glucocorticoids.

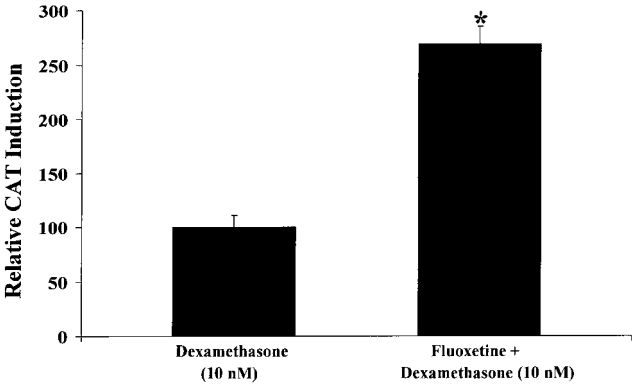

Figure 1 GR-mediated gene transcription in LMCAT cells treated with fluoxetine and dexamethasone (10 nM). LMCAT cells were treated for 24 h with dexamethasone (10 nM), alone or in coincubation with the antidepressant fluoxetine (10 μM). Cell extracts were analysed for relative CAT enzyme induction (fold induction relative to dexamethasone-treated cells). The results are shown as the mean ± s.e.m. of three independent experiments. *indicates significant ($P<0.05$) difference vs dexamethasone alone.

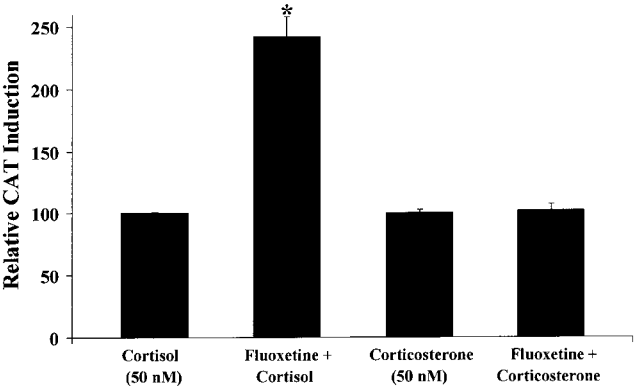

Figure 2 GR-mediated gene transcription in LMCAT cells treated with fluoxetine and cortisol (50 nM) or corticosterone (50 nM). LMCAT cells were treated for 24 h with cortisol (50 nM) or corticosterone (50 nM), alone or in coincubation with the antidepressant fluoxetine (10 μM). Cell extracts were analysed for relative CAT enzyme induction (fold induction relative to glucocorticoid-treated cells). The results are shown as the mean ± s.e.m. of three independent experiments. *indicates significant ($P<0.05$) difference vs the glucocorticoid alone.

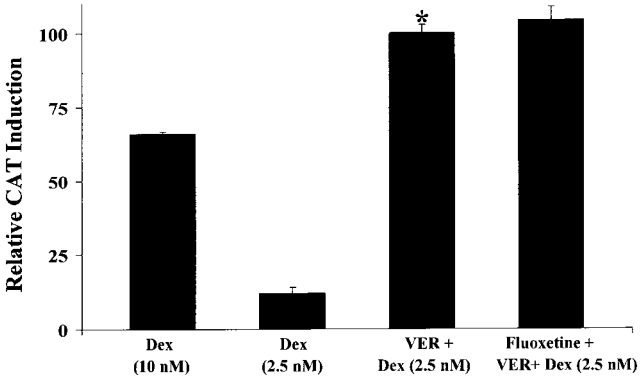

Figure 3 GR-mediated gene transcription in LMCAT cells treated with fluoxetine, verapamil and dexamethasone (2.5 nM). LMCAT cells were treated for 24 h with: (1) dexamethasone (Dex) (10 nM); (2) Dex (2.5 nM); (3) Dex (2.5 nM) in coincubation with verapamil (VER) (100 μM); or (4) Dex (2.5 nM) in coincubation with verapamil (VER) (100 μM) and fluoxetine (10 μM). Cells extracts were analysed for relative CAT enzyme induction (fold induction relative to cells treated with Dex (2.5 nM) plus verapamil). The results are shown as the mean $\pm$ s.e.m. of three independent experiments. *indicates a significant ($P<0.05$) difference vs Dex (2.5 nM).

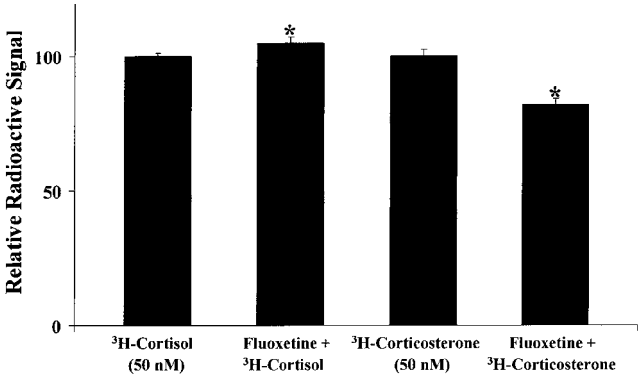

Figure 4 Intracellular accumulation of radioactive glucocorticoids in LMCAT cells, treated with vehicle or fluoxetine for 24 h. LMCAT cells were treated with vehicle or fluoxetine (10 μM) for 24 h, and then incubated for 1.5 h with ^{3}H-cortisol (50 nM) or ^{3}H-corticosterone (50 nM). Fluoxetine was continued during the 1.5 h incubation with the radioactive glucocorticoid. Cells extracts were analysed for relative intracellular accumulation (fold accumulation relative to control cells). The results are shown as the mean $\pm$ s.e.m. of five independent experiments. *indicates a significant ($P<0.05$) difference vs the glucocorticoid alone.

Fluoxetine (10 μM) induced a small but reliable and statistically significant increase in the intracellular accumulation of ^{3}H-cortisol after 24 h (+5%), and a larger increase after 72 h (+15%) (Figures 4 and 5). In the presence of ^{3}H-corticosterone, fluoxetine induced a reduction of the intracellular concentration after 24 h, and no effect after 72 h (Figures 4 and 5).

Finally, we determined the ability of fluoxetine to inhibit PGP-mediated transport in Caco-2 cells using digoxin as the prototypical substrate. In the absence of any inhibitor, marked differences in the basal–apical vs apical–basal transport of digoxin were observed, consistent with PGP-mediated efflux of digoxin. Polarized transport of digoxin was inhibited by 30% when fluoxetine (10 μM) was added to the cells (Figure 6).

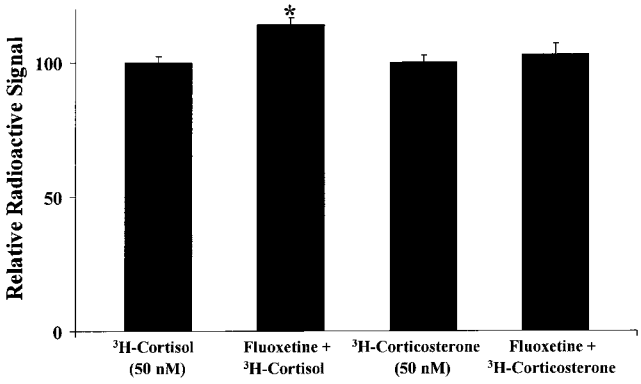

Figure 5 Intracellular accumulation of radioactive glucocorticoids in LMCAT cells, treated with vehicle or fluoxetine for 72 h. LMCAT cells were treated with vehicle or fluoxetine (10 μM) for 72 h, and then incubated for 1.5 h with ^{3}H-cortisol (50 nM) or ^{3}H-corticosterone (50 nM). Fluoxetine was continued during the 1.5 h incubation with the radioactive glucocorticoid. Cells extracts were analysed for relative intracellular accumulation (fold accumulation relative to control cells). The results are shown as the mean $\pm$ s.e.m. of three independent experiments. *indicates a significant ($P<0.05$) difference vs the glucocorticoid alone.

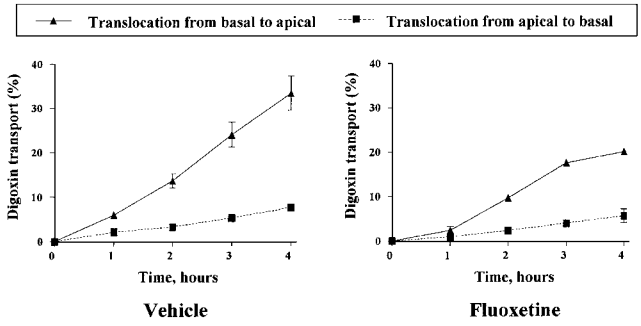

Figure 6 Transepithelial transport of [^{3}H]-digoxin across a Caco-2 cell culture monolayer in the absence or presence of fluoxetine. Translocation [^{3}H]-digoxin (5 μM), from basal to apical compartments and from apical to basal compartments, was examined across a Caco-2 cell culture monolayer, in the absence or presence of fluoxetine (10 μM). The results are shown as the mean $\pm$ s.e.m. from three independent experiments. Fluoxetine induced a 30% inhibition of polarized transport of digoxin ($P<0.05$).

Discussion

This study shows that the antidepressant fluoxetine inhibits a membrane steroid transporter and thus increases intracellular concentrations of cortisol in LMCAT fibroblasts. In summary: (1) fluoxetine potentiates GR-mediated gene transcription in the presence of cortisol (and dexamethasone) and increases the intracellular accumulation of ^{3}H-cortisol; (2) the effects of fluoxetine on GR-mediated gene transcription are blocked by verapamil, a steroid transporter inhibitor; and (3) fluoxetine has no potentiating effects on GR-mediated gene transcription in the presence of corticosterone and does not increase the intracellular accumulation of ^{3}H-corticosterone. These findings are further corroborated by the data showing that fluoxetine (10 μM) causes 30% inhibition of PGP activity in Caco-2 cells.

These results are consistent with the data we have obtained with other antidepressants. In fact, fluoxetine (10 μM) gives

140–170% potentiation of GR-mediated gene transcription in the presence of dexamethasone (10 nM) or cortisol (50 nM), and most of the other antidepressants tested, at a concentration of 10 μM, induce 80–150% potentiation (Pariante *et al*., 1997; 2001a). A smaller (15–25%) potentiation is induced by all antidepressants at a concentration of 1 μM in the presence of dexamethasone (10 nM), which is consistent with the 16% potentiation by fluoxetine (1 μM) in the presence of cortisol (50 nM) (Pariante *et al*., 1997; 2001a). However, the pharmacological mechanism leading to transporter inhibition by fluoxetine could be different from that of other antidepressants. In fact, tricyclic antidepressants as well as citalopram are also transported by PGP, while fluoxetine is not transported (Uhr *et al*., 2000; Uhr & Grauer 2003). However, not all PGP inhibitors are transported by PGP; for example, progesterone is a potent inhibitor but it is not transported (Ford, 1996). Inhibition of PGP and other membrane steroid transporters is not receptor-mediated and is related to the drugs' physiochemical properties, like lipophilicity, electric charge and ability to accept hydrogen bonds (Castaing *et al*., 2000; Ekins *et al*., 2002).

Interestingly, fluoxetine gives 5% increase in the intracellular concentration of radioactive cortisol (50 nM), but gives 140% potentiation of GR-mediated gene transcription in the presence of cortisol (50 nM). Although there seems to be a discrepancy in the magnitude of these effects, these figures are not incompatible. We have demonstrated that cortisol (100 nM) for 24 h leads to approximately 20-fold the CAT induction induced by cortisol (50 nM) for 24 h (Pariante *et al*., 2001a). This is approximately 20% potentiation of CAT activity for every 1% increase in cortisol concentrations in the media, which would result in a 100% potentiation of CAT activity for the 5% increase induced by fluoxetine in the cells. Moreover, these results are also consistent with our previous data obtained with clomipramine. In fact, compared to fluoxetine, clomipramine gives a larger increase in the intracellular concentration of radioactive cortisol (80%), and also gives a larger potentiation of GR-mediated gene transcription in the presence of cortisol (400%) (Pariante *et al*., 2001a; 2003). However, it is also possible that fluoxetine increases GR function by more than one mechanism. For example, besides increasing cortisol intracellular concentrations, fluoxetine, as other antidepressants, also increases the activity of cAMP-dependent protein kinases (Edgar *et al*., 1999; Thome *et al*., 2000), which in turn increase GR function (Rangarajan *et al*., 1992; Miller *et al*., 2002). Fluoxetine induces a larger (15%) increase of ^{3}H-cortisol intracellular concentrations after 72 h.

Although fluoxetine increases ^{3}H-cortisol intracellular concentrations at both 24 and 72 h, it also decreases ^{3}H-corticosterone intracellular concentrations at 24 h, while having no effect at 72 h. ^{3}H-corticosterone intracellular concentrations mostly represent GR-unbound signal (Pariante *et al*., 2003), and therefore the decreased levels could be theoretically interpreted as showing an inhibitory effect of fluoxetine on an 'uptake mechanism' for corticosterone. This hypothesis would also explain our previous findings that the intracellular concentration of ^{3}H-corticosterone is decreased by 40% in the presence of an excess of unlabelled corticosterone, an effect that does not seem to be related to competition at the GR-binding site (Pariante *et al*., 2003). Indeed, coexistence of both efflux and uptake transporters for glucocorticoids has been described in hepatocytes (Bossuyt *et al*., 1996; Lackner *et al*., 1998). If fluoxetine had the ability to increase GR function by stimulating cAMP-dependent protein kinases (besides increasing the intracellular concentrations of cortisol), as suggested above, this could explain why there was no effect of fluoxetine on GR-mediated gene transcription in the presence of corticosterone even if the intracellular levels of corticosterone were decreased. In fact, stimulation of cAMP-dependent kinases in LMCAT cells results in increased GR-mediated gene transcription even in the presence of corticosterone (Miller *et al*., 2002), and therefore these two putative opposite effects of fluoxetine–one leading to decreased corticosterone levels and the other leading to increased GR function–could result in no net effect.

These findings have a number of potential implications regarding the effects of antidepressants. Rodents have two isoforms of PGP: the mdr1a and the mdr1b. The mdr1a PGP is predominantly expressed at the BBB 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 (Uhr *et al*., 2002; Wolf & Horwitz, 1992) and this isoform is also expressed in the brain, although it has not been detected in brain capillaries (Regina *et al*., 1998). Consistent with the hypothesis that mdr1b PGP regulates the effects of corticosterone on the brain in rodents, mice that are knockout for mdr1a and mdr1b PGP show increased access of corticosterone to the brain and increased negative feedback on the HPA axis by corticosterone (Uhr *et al*., 2002). Therefore, by inhibiting membrane steroid transport *in vivo*, antidepressants could directly increase the access of corticosterone to the brain, enhance GR- and MR-mediated negative feedback by endogenous glucocorticoids on the HPA axis and thus reduce HPA-axis activity. This model is further supported by studies showing that tricyclic antidepressants reduce HPA-axis activity in rodents at the same concentrations known to inhibit MDR PGP in subcutaneous tumours (Merry *et al*., 1991; Pariante & Miller, 2001). This antidepressant-induced reduction in circulating corticosterone levels, which has been consistently demonstrated in animals treated with antidepressants (Pariante & Miller, 2001), could participate in the neuroprotective effects of these drugs (Duman *et al*., 2001).

In conclusion, we have demonstrated that the antidepressant fluoxetine, similar to other tricyclic and SSRI antidepressants, regulates the intracellular levels of cortisol by inhibiting a membrane steroid transporter that is similar to PGP in its substrates and modulators. Cortisol, the endogenous glucocorticoid in humans, is transported by the PGP at the BBB (Karssen *et al*., 2001). Moreover, we have recently described a membrane steroid transporter in primary rat neurones that also transports cortisol and is inhibited by antidepressants (Pariante *et al*., 2003). 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 glucocorticoid-mediated negative feedback on the HPA axis. This is consistent with clinical studies showing that treatment with GR and MR agonists, including cortisol, has antidepressant effects in humans (Dinan *et al*., 1997; Bouwer *et al*., 2000; DeBattista *et al*., 2000). Moreover, clinical studies have also shown that patients with major depression have reduced function of the GR (Pariante & Miller, 2001; Pariante *et al*., 2002), and possibly reduced

cortisol levels in the brain (Brooksbank *et al*., 1973), a situation that in itself may be associated to neuronal suffering and could be reverted by the increased access of cortisol to the brain (McEwen, 2000; Cotter & Pariante, 2002;). Enhanced cortisol action in the brain might prove to be a successful approach to maximize therapeutic antidepressant effects.

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