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Effects of Antidepressants on the Brain/Plasma Distribution of Corticosterone

Claudia-Carolin Weber[1], Gunter P Eckert[1] and Walter E Müller*[,1]

[1]*Department of Pharmacology, Biocenter (ZAFES), University of Frankfurt, Frankfurt, Germany*

It is well established that hypothalamic–pituitary–adrenal (HPA) axis dysregulation, characterized by elevated circulating cortisol concentrations and impaired negative feedback inhibition, is associated with affective disorders. As normalization of the HPA axis function and mood-stabilizing effects occur simultaneously during antidepressant treatment, it is likely that these effects are either directly or indirectly dependent. Although data concerning the outward transport of glucocorticoids from the brain by P-glycoprotein (Pgp) are inconsistent, it has been hypothesized that antidepressants exert their clinical activity in parts by inhibiting Pgp, subsequently leading to enhanced brain glucocorticoid levels and the normalization of the HPA axis function. Here, we report on the effects of different antidepressants (amitriptyline, fluoxetine, mirtazapine, St John's wort extract) on the brain/plasma distribution of corticosterone in mice after acute and subchronic treatment. The four antidepressants exerted different effects on the corticosterone concentration in brain and plasma. Changes in corticosterone levels were highly correlated, suggesting passive diffusion between both tissues. St John's wort extract and fluoxetine elevated brain and plasma corticosterone concentrations after subchronic treatment. Mirtazapine and amitriptyline had no effect on corticosterone concentration after subchronic treatment, possibly because both are also potent antagonists at the 5-HT2 receptor, which mediates HPA axis stimulation by serotonergic stimuli. In addition, St John's wort is the only antidepressant tested which slightly elevated Pgp protein level in the brain.

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INTRODUCTION

Hypothalamic–pituitary–adrenal (HPA) axis dysregulation, characterized by elevated cortisol concentrations and changes in diurnal rhythms of cortisol secretion, are observed in many patients suffering from affective disorders (Holsboer, 2000; Gillespie and Nemeroff, 2005). Preclinical as well as clinical findings suggest that this may possibly be caused by an impaired feedback inhibition (Holsboer, 2000; Pariante, 2003).

Normalization of the HPA axis function and the mood-stabilizing effects seem to occur simultaneously during antidepressive treatment and after recovery, indicating that the two effects might be either directly or indirectly dependent. However, whether HPA axis dysfunction is cause or consequence of depression is still a matter of dispute. Nevertheless, the effects of long-term antidepressant treatment on HPA axis and feedback inhibition by glucocorticoids may be important in understanding the mechanism by which antidepressants exert their clinical activity. It has been hypothesized that antidepressants inhibit membrane glucocorticoid transporters, such as P-glycoprotein (Pgp), at the blood-brain barrier (BBB) (Schinkel *et al*, 1996) and hence enhance the glucocorticoid concentration in the brain, leading to an increased glucocorticoid receptor-mediated gene transcription and therefore to normalization of the function of the HPA axis (Pariante *et al*, 2001; Pariante *et al*, 2004b).

Pgp, a 170–180 kDa plasma membrane-associated protein, is a member of the superfamily of ATP-binding-cassette transporters (ABC transporters). At the BBB, Pgp is localized in the apical membrane of brain capillary endothelial cells and transports substrates towards the blood compartment (Schinkel *et al*, 1996). Therefore, Pgp can limit the penetration into and retention within the central nervous system (CNS) of numerous compounds, thus modulating their effectiveness and CNS toxicity (Weiss *et al*, 2003).

Data concerning the transport of glucocorticoids by Pgp, however, are controversial. Ueda *et al* (1992) found that cortisol, aldosterone and dexamethasone but not progesterone, are physiological substrates for Pgp. Another group reported that Pgp hampers the access of cortisol but not of corticosterone to mouse and human brain (Karssen *et al*,

*Correspondence: Professor Dr WE Müller, Department of Pharmacology, Biocenter (ZAFES), Marie-Curie Strasse 9, 60439 Frankfurt, Germany, Tel: +49 69 79829373, Fax: +49 69 79829374,
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2001). van Kalken *et al* (1993) demonstrated that Pgp may function as a transporter for cortisol. Uhr *et al* analyzed the transport of different glucocorticoids in mdr1a/b (−/−) mice, lacking Pgp. In these mice, corticosterone and cortisol penetration into the brain is limited by the presence of Pgp at the BBB (Uhr *et al*, 2002). If antidepressants induce the expression of Pgp or inhibit its function, they could therefore alter the distribution of glucocorticoids between brain and plasma.

MATERIALS AND METHODS

Materials

Extract of St John's wort LI 160 (containing 0.18% hypericin; 3.77% hyperforin and 5.92% flavonoids) was a kind gift of Lichtwer Pharma AG (Berlin, Germany). Fluoxetine and mirtazapine were kindly provided by Stada Arzneimittel AG (Bad Vilbel, Germany). The Correlate-EIA™ corticosterone enzyme immunoassay kit (Assay Designs Inc.) was purchased from Biotrend (Cologne, Germany). Anti-Pgp C219 was obtained from Alexis Biochemicals (Lausen, Switzerland). All other chemicals, culture media and substances were purchased in the highest grade commercially available.

Animal Study

In total, 50 male NMRI mice with body weights ranging between 25 and 35 g were supplied by Harlan–Winkelmann GmbH (Borchen, Germany). Animals were housed under standard conditions in cages (five mice per cage) and given standard chow diet and water *ad libitum*. Mice were equally devided into a control group (vehicle) and four verum groups receiving either St John's wort extract (LI160, Lichtwer), amitriptyline (Sigma), fluoxetine (Stada) or mirtazapine (Stada). All antidepressants were suspended in aqueous agarose gel (0.2%) for treatment. For the acute study, the animals were treated once and dissected 1 h after treatment. For the subchronic study, treatment was given daily for 2 weeks and the animals were killed on day 15 after a washout period.

The following doses were used for the acute study: St John's wort: 500 mg/kg; amitriptyline, fluoxetine and mirtazapine 10 mg/kg. For the subchronic study, the following doses were administered: St John's wort: 300 mg/kg; amitriptyline, fluoxetine and mirtazapine 10 mg/kg. Control mice received the weight-adjusted volume of vehicle. Treatment was given once daily by oral gavage via a pharyngeal tube (diameter 1 mm) with maximal application volume of 0.5 ml. Oral application was chosen as it is the standard application of antidepressants and St John's wort extract. Mice were weighed daily for dose adjustment.

All experiments were carried out according to the guidelines of the German Protection of Animals Act (Deutsches Tierschutzgesetz, BGBI 1998, Part I, No. 30, S. 1105 ff) by individuals with appropriate training and experience.

Blood samples were collected retrobulbarly in tubes containing 0.03 ml heparin to avoid coagulation and subsequently centrifuged to obtain the serum fraction. Mice were then sacrificed by decapitation. The plasma and weighed cerebrum samples were collected and stored at −80°C until analysis.

Sample Preparation

Brains were thawed and subsequently homogenized (1000 r.p.m., ×10) using the buffer supplied with the corticosterone EIA kit (300 mg wet weight (WW) = 2 ml buffer). The homogenates were divided into two aliquots and centrifuged.

Western Blots. From one aliquot the supernatant was removed. The pellet was resuspended in STEN buffer and recentrifuged after vortexing. The protein concentration in the supernatants was determined according to Lowry *et al* (1951).

Corticosterone EIA. Corticosteroid displacement reagent was added to the supernatants of the second brain homogenate aliquot and was subsequently diluted with the respective assay buffer. For blood corticosterone analysis, 100 µl of the plasma were mixed with steroid displacement reagent and afterwards diluted with the respective assay buffer.

Western Blots

A previously published protocol was used for Western blot analysis (Keil *et al*, 2004). Briefly, after dilution in sample buffer, 10–20 µg of protein were loaded on an acrylamide gel (Invitrogen, Karlsruhe, Germany) and examined by SDS-PAGE. The proteins were transferred on PVDF-membranes (Amersham Bioscience, Uppsala, Sweden) at 25 V for 90 min. Thereafter, membranes were saturated with 5% nonfat dry milk in TBST for 1 h. PVDF membranes were then exposed to the primary antibody (C219) over night. After treatment for 1 h with the corresponding secondary antibody (Merck Bioscience, Darmstadt, Germany), protein bands were detected by ECL reagent (Amersham Bioscience, Uppsala, Sweden).

Blots were scanned and the band intensity was determined after background subtraction using Scion Image for windows beta 4.0.2 (Frederick, USA).

Corticosterone EIA

The Correlate-EIA™ Corticosterone kit is a competitive immunoassay for the quantitative determination of corticosterone in biological fluids. The kit uses a polyclonal corticosterone antibody to bind corticosterone in the standard or sample or an alkaline phosphatase molecule with covalently attached corticosterone. The sensitivity of the assay is 26.99 pg/ml (http://www.assaydesigns.com/products/catalog/inserts/900-097.pdf). The brain and plasma levels seen in NMRI (this paper) are within the ranges seen previously for several other mouse strains (Butte *et al*, 1972).

Samples and standards were treated according to the manufacturer's instructions. After adding the stop solution, the plate was immediately placed in a micro plate reader (1420 Wallac Victor^2, Perkin-Elmer, Rodgau-Jügesheim,

Germany). Absorbance was read at 405 nm. Each sample and standard was measured in duplicates.

The following control values were determined: Blank, nonspecific binding and total activity. A standard curve was determined on every plate.

Statistics

Data are given as mean ± SEM. Statistical analysis was performed using *t*-test against the untreated control. *p*-value $\leqslant 0.05$ was considered significant. Linear regressions and correlations were calculated using GraphPad Prism 4.02 (San Diego, USA).

RESULTS

As the data concerning transport of glucocorticoids by Pgp are conflicting, we previously analyzed the inhibition of calcein-AM uptake of cortisol and corticosterone in CEM/VLB cells (human lymphocytic leukemia cell lines) and PBCEC cells (porcine brain capillary endothelial cells). Both cell lines were used to characterize the transport of several antidepressant drugs by Pgp (Weber *et al*, 2005; Weber *et al*, 2004). Cortisol is a weak and corticosterone a slightly more potent inhibitor of the transport function of Pgp (cortisol 100 μM: 14.7 ± 3.1% (VLB); 21.0 ± 8.4% (PBCEC); corticosterone 100 μM: 54.3 ± 6.4% (VLB); 82.2 ± 9.5% (PBCEC) inhibition of calcein-AM uptake) (data not shown).

As changes in the expression of Pgp at the BBB could influence corticosterone brain levels, we additionally investigated the effects of all treatment conditions on Pgp expression. After acute treatment, we found no significant differences between the individual groups (data not shown). Only St John's wort extract significantly increased the expression of Pgp after subchronic application. The other antidepressants, that is amitriptyline, fluoxetine and mirtazapine, had no effect (Figure 1). An *in vitro* model confirmed the increase of Pgp expression by St John's wort extract. Both St John's wort extract and hyperforin increased the protein levels of Pgp in the CEM cells after 24 h incubation (St John's wort extract 10 μg/ml: + 65.5%; St John's wort extract 25 μg/ml: + 84.0%; hyperforin 0.1 μM: + 26.3%; hyperforin 1 μM: + 52.2%) (data not shown).

All animals, control groups and the four treatment groups, gained weight (about 3–4 g), during the subchronic treatment study. However, no statistically significant differences were observed between groups.

The possible effect of treatment-associated stress on the plasma or brain corticosterone levels were assessed by comparing animals treated orally under control conditions (0.2% agarose only) with animals not treated at all. There were no significant differences detectable between these groups (Figure 2).

Fluoxetine, mirtazapine and St John's wort extract, but not amitriptyline, significantly elevated plasma and brain corticosterone levels after one single oral treatment (Figures 3–6). However, in subchronic treatment, only fluoxetine and St John's wort extract significantly elevated plasma as well as brain corticosterone levels. Mirtazapine and amitriptyline showed no effect (Figure 3–6).

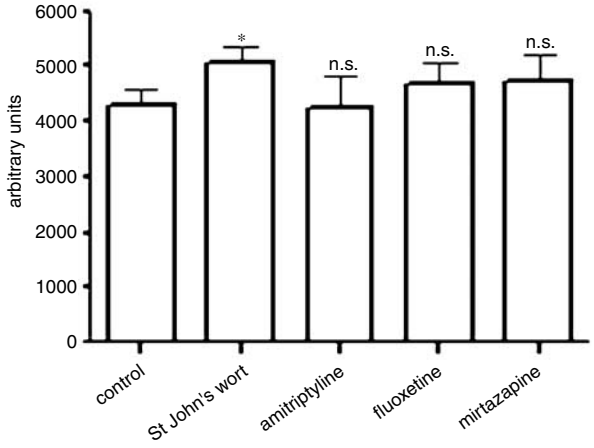


Figure 1 Expression of Pgp in brain homogenate after subchronic treatment with antidepressants. Densitometric immunoblot analysis. Data are given as mean ± SEM for $n = 10$ animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS = not significant.

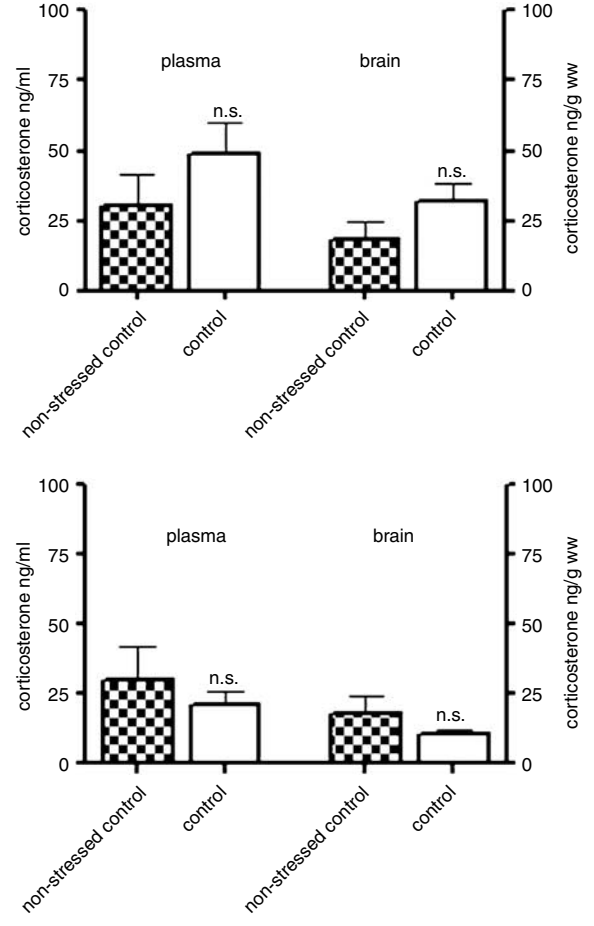


Figure 2 Corticosterone plasma and brain levels of controls without stress by oral gavage and treated controls (upper panel: acute; lower panel: subchronic). Data are given as mean ± SEM for $n = 10$ animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS = not significant.

Interestingly, over all subchronic treatment conditions, changes of plasma corticosterone always led to parallel changes of the respective brain concentrations (Figure 7). Brain concentration (ng/g WW) was always about 70% of

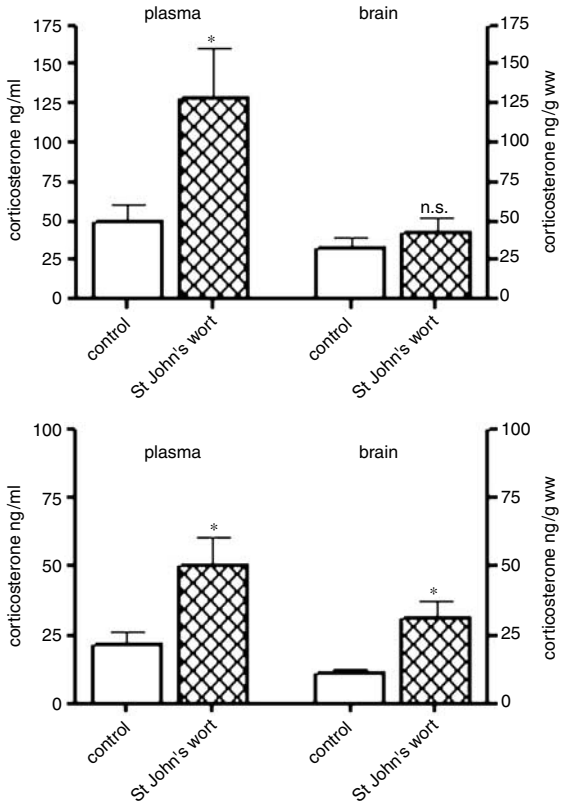
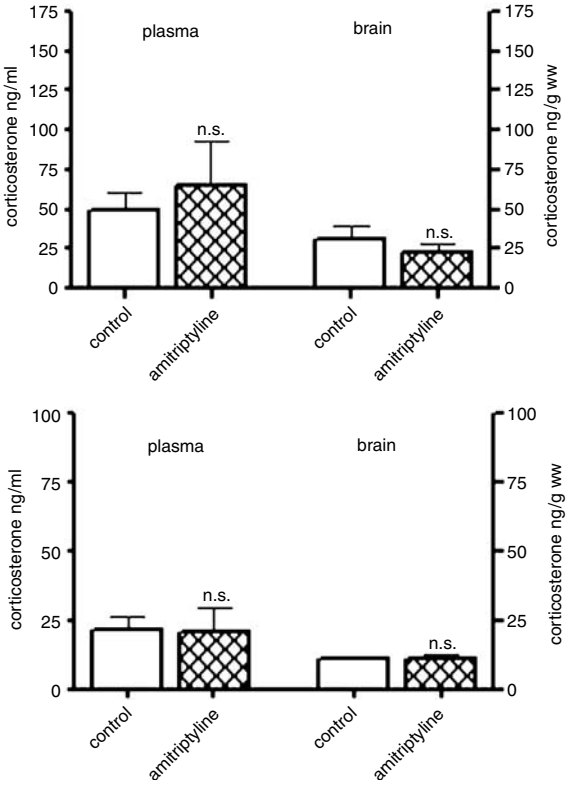


Figure 3 Corticosterone brain and plasma levels after treatment with St John's wort compared to control group (upper panel: acute; lower panel: subchronic). Data are given as mean ± SEM for $n = 10$ animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS = not significant.



the plasma levels (ng/ml) suggesting equal distribution in the extra—as well as the intracellular water space. After acute treatment, the brain concentrations after 1 h were slightly lower than, but linearly correlated to, plasma concentrations (Figure 7).

DISCUSSION

The present study shows that the brain corticosterone concentration in the mouse is a linear function of its plasma concentration and that brain levels (in ng/g WW) are usually slightly less than the plasma levels (in ng/ml). These support the assumption that corticosterone diffuses passively into the brain, mainly into the intra- and extracellular water space. Any contribution of an outward transport by Pgp, if present, can only be of minor importance. Thus, it appears rather unlikely that inhibition of Pgp by some of the antidepressants used has any relevant effect on brain levels of corticosterone in the mouse. The differences in the brain and plasma concentrations after acute treatment may be explained by the time lag it takes for diffusion into the brain.

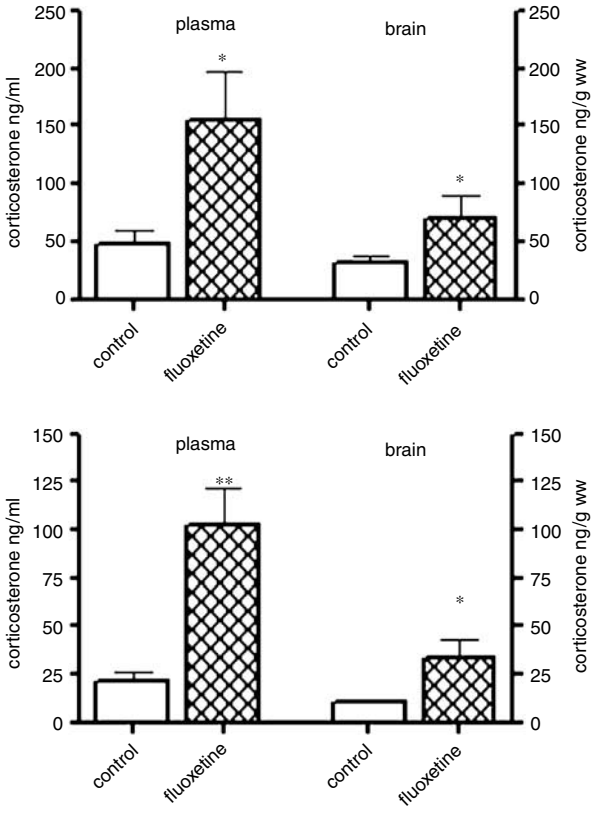


Figure 5 Corticosterone brain and plasma levels after treatment with fluoxetine compared to control group (upper panel: acute; lower panel: subchronic). Data are given as mean ± SEM for $n = 10$ animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS = not significant.

Figure 4 Corticosterone brain and plasma levels after treatment with amitriptyline compared to control group (upper panel: acute; lower panel: subchronic). Data are given as mean ± SEM for $n = 10$ animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS = not significant.

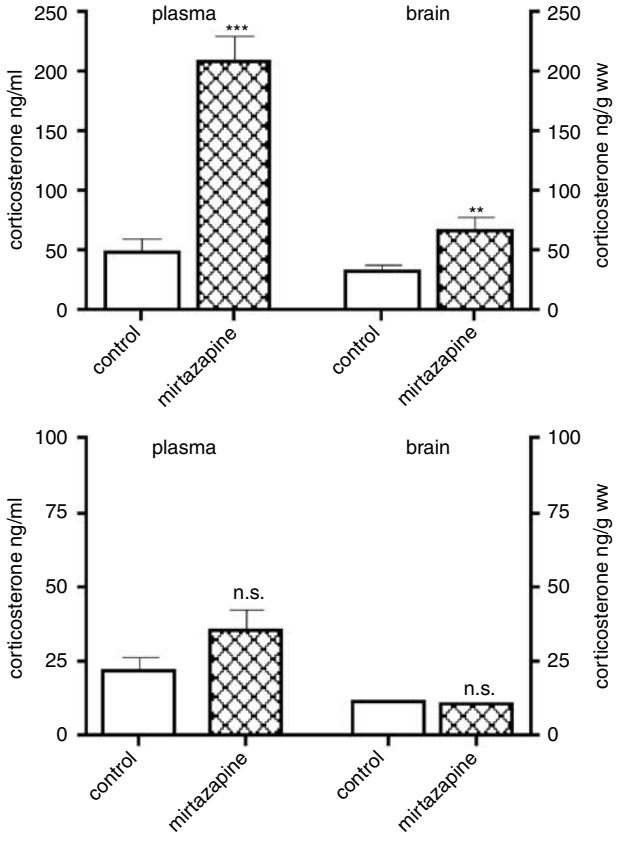


Figure 6 Corticosterone brain and plasma levels after treatment with mirtazapine compared to group (upper panel: acute; lower panel: subchronic). Data are given as mean ± SEM for $n = 10$ animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS = not significant.

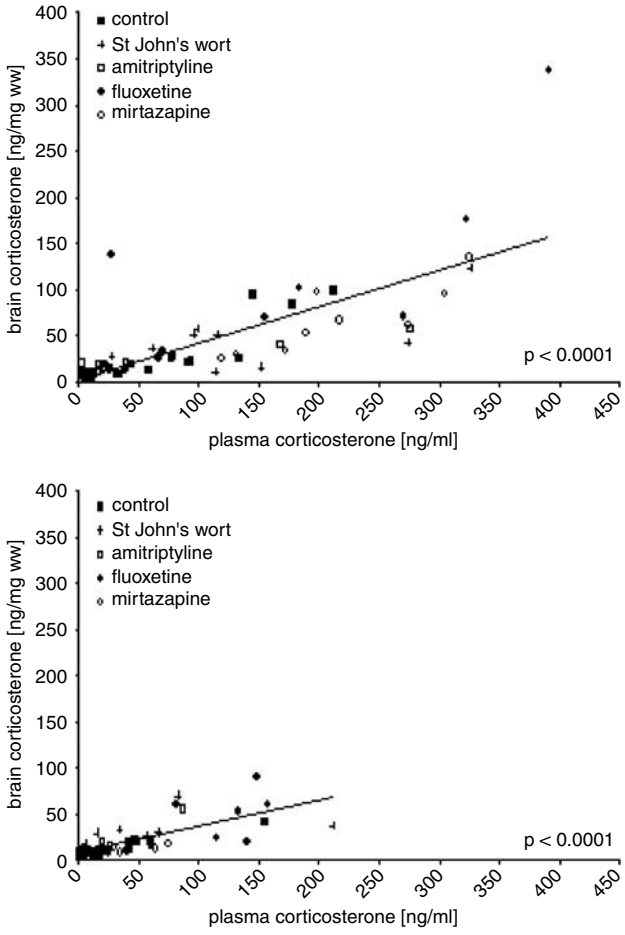


Figure 7 Correlation of brain and plasma corticosterone levels in the four treatment (St John's wort, amitriptyline, fluoxetine and mirtazapine) and control groups after acute (upper panel) and subchronic (lower panel) treatment. Linear regression was performed using GraphPad Prism (acute: slope: 0.396 ± 0.05; r^2: 0.5619 and subchronic: slope: 0.278 ± 0.05; r^2: 0.3708).

Our findings that antidepressants have different acute or chronic effects on plasma levels of glucocorticoids confirm and extent several other findings in humans or lab animals.

A previous study on the effect of fluoxetine on plasma corticosterone concentration in rats yielded inconsistent results (Stout *et al*, 2002). The data of our study, however, clearly indicate that the administration of fluoxetine enhances brain and plasma corticosterone levels after acute and subchronic treatment. These results coincide with those previously shown in resting and acutely stressed rats (Berton *et al*, 1999), female Mongolian gerbils (Hendrie *et al*, 2003), and humans (Von Bardeleben *et al*, 1989).

Furthermore, neither acute nor subchronic administration of amitriptyline influenced brain or plasma corticosterone levels. These results agree with a previous human study which showed that amitriptyline did not influence the plasma cortisol concentration in patients suffering from DSM-IV major depressive disorder (Rota *et al*, 2005). Reul *et al* (1993) in contrast, found that long-term oral treatment with amitriptyline significantly decreased basal as well as stress-induced corticosterone plasma level in rats.

Acute administration of mirtazapine led to an elevation of corticosterone in brain and plasma. However, after 14 days of treatment, no differences of corticosterone plasma levels were observed, in comparison to control animals. Previously, mirtazapine (15 ms) has been shown to decrease plasma cortisol after acute administration in healthy male subjects (Laakmann *et al*, 1999). In another study with depressed patients, mirtazapine seemed to reduce plasma cortisol after 5 weeks of treatment (Schule *et al*, 2003).

Similar to fluoxetine, St John's wort extract increased plasma as well as brain corticosterone levels after acute and subchronic treatment. These data agree with most previous findings in animals and humans (Franklin, 2005). Franklin *et al* (2004), for example, treated rats with about 300 mg/kg St John's wort extract via food pellets for two weeks. Whereas plasma corticosterone was not elevated, brain corticosterone was reduced by about 30%. The authors suggested that upregulation of Pgp by St John's wort extract at the BBB might explain their findings. In our experiments, however, oral treatment led to a substantial elevation of plasma as well as brain corticosterone. Although small Pgp upregulation in the St John's wort-treated animals were observed, they seem too small, however, to explain these results. Brain corticosterone regulation might therefore differ between mice and rat, also indicated by 25–30 times higher brain *vs* plasma levels of corticosterone in the rat (Franklin *et al*, 2004) compared to the nearly one-to-one relationship in the mouse (this work).

Antidepressants, such as reboxetine, increase cortisol secretion by enhancing serotoninergic and noradrenergic neurotransmission (Schule *et al*, 2004). Serotoninergic stimulation seems to be mediated mainly via 5-HT2A receptors (Feldman *et al*, 1998; Bagdy, 1996). This may explain why amitriptyline and mirtazapine do not enhance plasma corticosterone, since both are also potent 5-HT2A antagonists (Sanchez and Hyttel, 1999).

In summary, fluoxetine, mirtazapine, amitryptiline and St John's wort exerted in the two test regimes different effects on brain corticosterone levels. Our observations do not support the hypothesis presented by Pariante *et al* (2004a, b) and Pariante *et al* (2001) that normalization of the disturbed feedback inhibition of the HPA axis by enhancing cerebral glucocorticoid concentration represents a rather common mechanism of action of most antidepressant drugs.

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