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Hyperforin-Containing Extracts of St John's Wort Fail to Alter Gene Transcription in Brain Areas Involved in HPA Axis Control in a Long-Term Treatment Regimen in Rats

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We previously showed that a methanolic extract of St John's wort (SJW) (*Hypericum*) and hypericin, one of its active constituents, both have delayed regulation of genes that are involved in the control of the hypothalamic–pituitary–adrenal (HPA) axis. Hyperforin, another constituent of SJW, is active *in vitro* and has been proposed to be the active constituent for therapeutic efficacy in depression. We therefore examined if hyperforin has delayed effects on HPA axis control centers similar to those of *Hypericum* and hypericin. We used *in situ* hybridization histochemistry to examine in rats the effects of short-term (2 weeks) and long-term (8 weeks) oral administration of two hyperforin preparations, fluoxetine (positive control), and haloperidol (negative control) on the expression of genes involved in the regulation of the HPA axis. Fluoxetine (10 mg/kg) given daily for 8 weeks, but not 2 weeks, significantly decreased levels of corticotropin-releasing hormone (CRH) mRNA by 22% in the paraventricular nucleus (PVN) of the hypothalamus and tyrosine hydroxylase (TH) mRNA by 23% in the locus coeruleus. Fluoxetine increased levels of mineralocorticoid (MR) (17%), glucocorticoid (GR) (18%), and 5-HT_{1A} receptor (21%) mRNAs in the hippocampus at 8, but not 2, weeks. Comparable to haloperidol (1 mg/kg), neither the hyperforin-rich CO_2 extract (27 mg/kg) nor hyperforin-trimethoxybenzoate (8 mg/kg) altered mRNA levels in brain structures relevant for HPA axis control at either time point. These data suggest that hyperforin and hyperforin derivatives are not involved in the regulation of genes that control HPA axis function.

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INTRODUCTION

Herbal extracts of *Hypericum perforatum* L. (St John's wort, SJW) are now successfully competing for status as a standard antidepressant therapy. Owing to this, great effort has been devoted to identifying the active antidepressant compounds. From a phytochemical point of view, SJW is one of the best-investigated medicinal plants. A series of bioactive compounds has been detected in the crude material, namely phenylpropanes, flavonol derivatives, biflavones, proanthocyanidines, xanthones, phloroglucinols, some amino acids, naphthodianthrones, and essential oil constituents (for a review, see Bombardelli and Morazzoni, 1995; Nahrstedt and Butterweck, 1997; Nahrstedt, 2000). Although SJW has been subjected to extensive scientific studies in the last decade, there are still many open questions about the pharmacology and the mechanism of action. In fact, the active constituents are not fully known. The pharmacological activity of SJW extracts has recently been reviewed (Butterweck, 2003; Greeson *et al*, 2001; Nathan, 1999). Reports about the antidepressant activity of SJW extracts and their constituents both *in vivo* and *in vitro* have been published (Baureithel *et al*, 1997; Butterweck *et al*, 1997, 1998, 2000, 2001a, b, 2002; Calapai *et al*, 1999; Chatterjee *et al*, 1996, 1998a, b; Di Matteo *et al*, 2000; Franklin and Cowen, 2001; Gobbi *et al*, 1999, 2001; Müller *et al*, 1997, 1998, 2001; Simmen *et al*, 1999, 2001; Singer *et al*, 1999; Wonnemann *et al*, 2001).

A characteristic feature common to both tricyclic antidepressants and SJW is the delay of 10–14 days or more before the therapeutic effect becomes evident, and the efficacy continues to increase in the following weeks. The long delay may reflect long-term central nervous system biological adaptations occurring during the daily administrations. A common biological alteration in patients with major depression is the activation of the hypothalamic–pituitary–adrenal (HPA) axis, manifested as hypersecretion of adrenocorticotropic hormone (ACTH) and cortisol and an abnormal cortisol response to dexamethasone and

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corticotropin-releasing hormone (CRH) administration (Gold *et al*, 1988; Holsboer and Barden, 1996; Holsboer *et al*, 1985; Raadsher *et al*, 1994). Correspondingly, the hyperactivity of the HPA axis in depressed patients was corrected during clinically effective therapy with antidepressant drugs (Barden *et al*, 1995; Holsboer *et al*, 1985).

In an animal study designed to examine the association between long-term antidepressant administration and the possibly delayed alteration in HPA axis activity, CRH mRNA levels in the hypothalamic paraventricular nucleus (PVN) of rats were shown to be decreased following long-term (8 weeks) but not short-term (2 weeks) treatment with imipramine, the prototypic tricyclic antidepressant (Brady *et al*, 1991). The same results were found with several other antidepressant drugs selected for their distinctly different primary pharmacological actions (Brady *et al*, 1992). Based on these data, we studied the effects of short-term (2 weeks) and long-term (8 weeks) administration of a methanolic SJW extract, hypericin and imipramine on the expression of genes that may be involved in the regulation of the HPA axis (Butterweck *et al*, 2001b). Our data showed that imipramine, SJW extract, and hypericin given daily for 8 weeks, but not for 2 weeks, significantly decreased levels of CRH mRNA in the PVN. Comparable to imipramine, the SJW extract and hypericin reduced plasma ACTH and corticosterone levels after 2 weeks of daily treatment. In line with previous findings (Brady *et al*, 1991; Nestler *et al*, 1990), we found that long-term treatment with imipramine significantly decreased TH mRNA levels in the locus coeruleus, whereas SJW extract and hypericin had no effect on TH message levels. Furthermore, long-term treatment with all three agents significantly decreased 5-HT_{1A} receptor mRNA expression in CA1 of the hippocampus. As the HPA axis effects could also be demonstrated with pure hypericin, it appears that the naphthodianthrone is a possible major active principle that may contribute to the beneficial effect of SJW extract after long-term oral dosing. The results obtained with hypericin could therefore be a starting point for approaches to design CNS-active molecules with a novel mode of action.

However, besides hypericin, pharmacological research on SJW has focused on either lipophilic extracts obtained with hypercritical CO_2 that were devoid of hypericins and flavonoids, but enriched with hyperforin (Bhattacharya *et al*, 1998; Chatterjee *et al*, 1998b; Dimpfel *et al*, 1998; Franklin and Cowen, 2001; Gobbi *et al*, 2001; Simmen *et al*, 1999), or hydroalcoholic extracts that were standardized on a certain amount of hyperforin (Chatterjee *et al*, 1996, 1998b; Dimpfel *et al*, 1998; Franklin and Cowen, 2001; Gobbi *et al*, 2001; Simmen *et al*, 1999, 2001; Wonnemann *et al*, 2001). The phloroglucinol derivative hyperforin has recently become a molecule of increasing interest. Studies have demonstrated significant effects of hyperforin on various serotonergic, noradrenergic, dopaminergic, cholinergic, and opioid system activities *in vitro* (Chatterjee *et al*, 1998a; Holcomb *et al*, 1982; Müller *et al*, 1998, 2001; Neary *et al*, 2001; Simmen *et al*, 1999; Singer *et al*, 1999; Wonnemann *et al*, 2001). As the *in vitro* data have been used to argue that hyperforin is the major active principle of SJW extract, we determined in the present study whether it is active *in vivo* in a model of therapeutic efficacy. Thus, we gave daily oral administration of two preparations of hyperforin to rats in the short-term/long-term administration paradigm to determine if hyperforin had effects on the levels of the above-mentioned mRNAs in a manner similar to that of the methanolic SJW extract and hypericin. The effects were compared to those of the selective serotonin reuptake inhibitor (SSRI) fluoxetine. Fluoxetine was chosen as a reference control because it was shown in several studies that similar to the SSRI, hyperforin inhibited serotonin reuptake in *in vitro* experiments (Chatterjee *et al*, 1998a; Gobbi *et al*, 1999; Jensen *et al*, 2001; Müller *et al*, 1998; Neary *et al*, 2001; Simmen *et al*, 1999; Wonnemann *et al*, 2001).

MATERIALS AND METHODS

Animals

Male CD rats (150–180 g, Charles River WIGA, Sulzfeld, Germany) were single housed in a 12 h light/dark cycle, with lights off at 1900, at a constant temperature of $25 \pm 1^{\circ}C$, and free access to food (Altromin 1324, Altromin Lage, Germany) and tap water. Rats were randomly assigned to the various experimental groups ($n = 8$/group) and weighed daily. The experimental procedures used in this work were officially approved by the Regierungspräsident, Münster (A 92/99). Animals were killed between 0900 and 1100; the last drug administration was the day before between 1600 and 1700. Brains were removed, frozen by immersion in 2-methyl butane at $-30^{\circ}C$, and stored at $-70^{\circ}C$ prior to sectioning. Trunk blood was collected on ice-chilled EDTA-coated (10 ml) tubes containing 500 kIU aprotinin/ml and centrifuged. Plasma was frozen at $-70^{\circ}C$.

Chronic Antidepressant Treatment

Fluoxetine hydrochloride was generously provided by Hexal (Holzkirchen, Germany), haloperidol-HCl was purchased from Sigma (Deisenhofen, Germany). A lipophilic SJW extract obtained with hypercritical CO_2 (containing about 23.7% hyperforin and 6.2% adhyperforin) and hyperforin-trimethoxybenzoate (TMB) were supplied by Indena S.p.A. (Milan, Italy).

All drugs were suspended in deionized water, and the solutions were emulsified with Tween-80 to a final concentration of 2% (Pälvimäki *et al*, 1994). Tween was also added to the deionized water received by the control groups (= vehicle). Fluoxetine (10 mg/kg), haloperidol (1 mg/kg), hyperforin-TMB (8 mg/kg), and the CO_2 extract (27 mg/kg) were dissolved with vehicle. Tween-80 was used as a solubilizer because the CO_2 extract and hyperforin-TMB are barely soluble in aqueous solutions. The final application volume of each preparation was 10 ml/kg body weight. Drug solutions were prepared fresh daily prior to use. In all experiments, substances were administered orally using the gavage technique. The oral administration route had to be chosen because (1) both the CO_2 extract, and hyperforin-TMB are barely soluble in aqueous solvents and thus must be administered as an emulsion; (2) for chronic application, i.p. injections are contraindicated (Wolfensohn and Lloyd, 1994); and (3) gavage—if performed properly—is less stressful for animals than i.p. injection (Wolfensohn and Lloyd, 1994). For consistency of the

method, fluoxetine and haloperidol were also given by gavage. Stock compounds were kept in light-tight containers at −20°C under argon atmosphere. The CO_2 extract (27 mg/kg) and the hyperforin-TMB (8 mg/kg) dosages were chosen because of their demonstrated efficacy in the forced swimming test. In preliminary experiments, the immobility times (seconds) at the optimized doses listed above were: control (213 ± 15); fluoxetine (94 ± 14, $P<0.05$ *vs* control); CO_2 extract (124 ± 11, $P<0.05$ *vs* control); and hyperforin-TMB (126 ± 14, $P<0.05$ *vs* control). The application of hyperforin as an ester appeared to be necessary because hyperforin is extremely sensitive to light and oxidation processes and is decomposed in aqueous solutions. In pharmacokinetic studies, the release of hyperforin from the ester was demonstrated (P Morrazzoni, personal communication).

Measurement of Corticosterone and ACTH

Radioimmunoassay (RIA) of corticosterone was performed using [^{125}I]corticosterone, antiserum, and standard solution in a kit from ICN Biomedical (Costa Mesa, CA, USA). The assay was adapted to rat serum conditions. Precipitation was performed using a second antibody solid phase. ACTH was measured using a kit from Diagnostic Systems Laboratories (Sinzheim, Germany). Both assays were performed according to the manufacturer's instructions. The inter- and intra-assay coefficients of variance for ACTH were 10.6 and 6.9%, respectively, with a detection limit of 10 pg/ml. For corticosterone, the inter- and intra-assay coefficients of variance were 7.2 and 4.4%, with a detection limit of 15 ng/ml.

In Situ Hybridization Histochemistry

Guided by Nissl-stained sections collected during the cutting and by the atlas of Paxinos and Watson (1998), coronal frozen sections (15 μm-thick) were collected at the levels of the midportion of the parvocellular region of the PVN where the magnocellular nucleus is largest (−1.8 mm); dorsal hippocampus (−3.3 mm), pituitary, and locus coeruleus (−9.7 mm). Sections were thaw-mounted onto gelatin-coated slides, dried, and stored at −40°C prior to processing for *in situ* hybridization histochemistry.

The *in situ* hybridization histochemistry procedures were performed as described previously for ribonucleotide (cRNA) probes (Brady *et al*, 1991). First, tissue sections were processed by fixation with 4% formaldehyde solution, acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl, pH 8.0 solution, dehydration with ethanol, and delipidation with chloroform. Second, the antisense probes were transcribed from linearized plasmids using the Riboprobe System (Promega Biotech, Madison, WI) with ^{35}S-UTP (specific activity > 1000 Ci/mmol; New England Nuclear, Boston, MA) and T7, T3, or SP6 RNA polymerase. The cDNA probes were: a 760 bp fragment of rat CRH (a gift from Dr James Herman, University of Kentucky, Lexington, KY), 923 bp of mouse pro-opiomelanocortin (POMC) (a gift from Dr James Douglass, Vollum Institute, Portland, OR), 384 bp of rat tyrosine hydroxylase (TH) (a gift from Dr Barry Kosofsky, Harvard Medical School, Boston, MA), 900 bp of the rat serotonin 5-HT_{1A} receptor gene (a gift from Dr Paul Albert, University of Ottawa, Ontario, Canada), bases 81–528 of the rat glucocorticoid receptor (GR) (gifts from Dr Keith Yamamoto, University of California, San Francisco, CA), and 513 bases encoding the carboxy-terminal 25 amino acids of the rat mineralocorticoid receptor (MR) (gifts from Dr Jeffrey Arriza, Salk Institute, La Jolla, CA).

The radiolabeled probes were diluted in a riboprobe hybridization buffer and applied to brain sections (approximately 500 000 cpm/section). After overnight incubation at 55°C in a humidified chamber, slides containing brain sections were washed first in 20 μg/ml RNase solution and then 1 h each in 2 × SSC (50°C) and 0.2 × SSC (55 and 60°C) solutions to reduce nonspecific binding of the probe. The slides were then dehydrated with ethanol and air-dried for autoradiography.

Slides and ^{14}C plastic standards containing known amounts of radioactivity (American Radiochemicals, St Louis, MO) were placed in X-ray cassettes, apposed to film (BioMax MR, Kodak, Rochester, NY) for periods ranging from 1 to 72 h, and developed in an automatic processor (X-OMAT, Kodak).

Data Analysis and Presentation

Autoradiographic images were digitized with a solid-state camera (CCD-72, Dage-MTI) and a Macintosh computer using NIH Image software (Wayne Rasband, National Institute of Mental Health). Transmittance measurements were converted into dpm/mg plastic using the calibration curve (Rodbard equation) generated from the standards. Brain structures were identified according to the atlas of Paxinos and Watson. Light transmittance through the film at PVN (CRH in the parvocellular division), hippocampus (5-HT_{1A}, MR, GR), anterior and intermediate lobes of pituitary (POMC), and locus coeruleus (TH) was measured by outlining the structure with the mouse cursor. The average value for each animal in experimental or control groups (based on four measurements per animal) was used to calculate group means ($n = 5$–8 per group). Except for the anterior pituitary, a density-slice function was applied to each structure; in the hippocampus, it was used to select and measure transmittance confined to the cellular layers of the CA fields and dentate gyrus. Mouse cursor control was used to outline the selected structure. The average value for each animal in experimental or control groups (based on four measurements per animal) was used to calculate group means ($n = 8$ per group).

One-way ANOVAs (drug treatment) were used to compare specific mRNA levels in control *vs* treated groups. The Student–Newman–Keuls test was used for *post hoc* comparisons of mRNA levels in each hippocampal region. A criterion level of $P<0.05$ was used to determine significance.

RESULTS

Effects of Daily Antidepressant Treatment on CRH mRNA Levels in the PVN of the Hypothalamus and on POMC mRNA Levels in the Pituitary

CRH mRNA levels in the PVN were not significantly changed at 2 weeks (short-term administration), but were

significantly decreased at 8 weeks (long-term administration) after treatment with fluoxetine (21%) (Figure 1). No changes in mRNA expression of CRH in the PVN were observed for haloperidol, hyperforin-TMB, or the lipophilic CO_2 extract at either time point. No treatment effects on the expression of POMC were observed in the anterior lobe of the pituitary (Table 1).

Effects on MR, GR, and 5-HT_{1A} mRNA Levels in the Hippocampus

After 2 weeks of daily treatment, no significant changes in MR, GR, and 5-HT_{1A} receptor mRNA levels were found for any of the drugs (Table 1). Long-term treatment with fluoxetine significantly increased MR (18%) and GR (19%) receptor mRNA expression in the dentate gyrus of the hippocampus and 5-HT_{1A} receptor mRNA expression by 21 and 22% in the hippocampal fields CA1 and CA3 relative to control (Table 1). No changes in mRNA expression of MR, GR, and 5-HT_{1A} in the hippocampus were observed for haloperidol, hyperforin-TMB, or the lipophilic CO_2 extract at either time point.

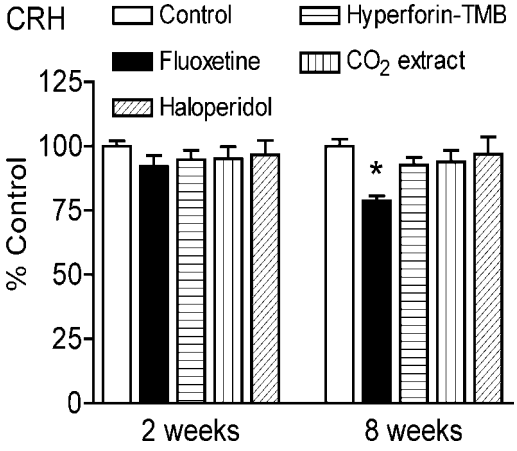

Figure 1 CRH mRNA expression in the PVN after 2 and 8 weeks of daily administration.

Effects on TH mRNA Levels in the Locus Coeruleus

No changes in TH mRNA levels were evident after 2 weeks for any treatment (Figure 2). After 8 weeks of daily fluoxetine administration, TH gene expression levels were decreased by 22% ($P<0.05$). Haloperidol, hyperforin-TMB, or the lipophilic CO_2 extract did not alter TH mRNA levels.

Effects on Hormone Levels, Body Weight, and Adrenal Gland Weight

After 2 weeks of daily treatment, fluoxetine significantly increased plasma ACTH (46%, $P<0.001$) and corticosterone (417%, $P<0.001$) levels (Figure 3a, b). Haloperidol, hyperforin-TMB, or the lipophilic CO_2 extract did not alter plasma hormone levels. No changes in hormone levels were observed after 8 weeks. Adrenal gland weights were not significantly altered by 2 or 8 weeks of chronic antidepressant treatment. Body weight was significantly decreased by fluoxetine ($\sim$10%, $P<0.05$) after 2 and 8 weeks and by haloperidol after 2 weeks (13% $P<0.05$), but not after 8 weeks (Table 2).

DISCUSSION

Previous work validated the long-term drug administration protocol applied to unstressed eucortisolemic rats as an appropriate model for assessing the therapeutic efficacy of antidepressant drugs (Brady *et al*, 1991, 1992). The changes in mRNA expression levels of genes involved in the HPA axis control served to correct imbalances created in these

Table 1 Expression of mRNAs in Rat Brain after Short-Term and Long-Term Antidepressant Treatment

	Control		Fluoxetine		Hyperforin-TMB		CO_2 extract		Haloperidol	
Brain region/mRNA	**2 weeks**	**8 weeks**	**2 weeks**	**8 weeks**	**2 weeks**	**8 weeks**	**2 weeks**	**8 weeks**	**2 weeks**	**8 weeks**
MR										
CA1	100 ± 4	100 ± 3	97 ± 2	103 ± 3	103 ± 4	100 ± 2	94 ± 3	99 ± 4	99 ± 2	106 ± 3
CA3	100 ± 4	100 ± 3	97 ± 2	101 ± 3	101 ± 4	99 ± 2	92 ± 4	98 ± 2	97 ± 2	105 ± 2
DG	100 ± 4	100 ± 2	100 ± 4	118 ± 2*	100 ± 5	112 ± 2	97 ± 5	110 ± 3	100 ± 3	110 ± 2
GR										
CA1	100 ± 5	100 ± 2	101 ± 3	108 ± 4	104 ± 3	104 ± 4	96 ± 3	96 ± 3	97 ± 5	99 ± 3
CA3	100 ± 3	100 ± 1	100 ± 2	108 ± 3	100 ± 2	103 ± 2	97 ± 2	97 ± 1	99 ± 4	108 ± 5
DG	100 ± 4	100 ± 2	99 ± 3	119 ± 2*	101 ± 5	104 ± 5	97 ± 3	101 ± 2	94 ± 4	104 ± 3
5-HT_{1A}										
CA1	100 ± 4	100 ± 3	102 ± 7	121 ± 3*	101 ± 5	107 ± 4	104 ± 4	105 ± 5	108 ± 4	102 ± 3
CA3	100 ± 5	100 ± 3	98 ± 7	122 ± 3*	96 ± 5	102 ± 5	98 ± 6	96 ± 4	103 ± 6	111 ± 6
DG	100 ± 4	100 ± 5	95 ± 6	106 ± 6	98 ± 5	102 ± 5	94 ± 4	96 ± 5	100 ± 3	108 ± 4
POMC										
Ant. pituitary	100 ± 8	100 ± 6	86 ± 14	102 ± 8	98 ± 3	99 ± 8	94 ± 10	99 ± 7	107 ± 7	99 ± 13

Values represent mean ± SEM based on the average of four sections for each brain region per animal ($n=8$ animals per group) and are expressed as percentage of the respective 2- or 8-week controls. Values significantly different from the respective 2- or 8-week control animals are expressed as *$P<0.05$ and **$P<0.01$, Student–Newman–Keuls *post hoc* test.

neurochemical systems by chronic immobilization stress (Butterweck *et al*, 2001b). Brady *et al* (1991, 1992) showed that long-term treatment with imipramine, fluoxetine, idazoxan, and phenelzine reduces HPA axis activity and regulates gene transcription levels in relevant structures with delayed onset, resembling the delayed onset of therapeutic efficacy of these drugs when given to patients for treatment of depression. In the current study, using *in situ* hybridization histochemistry, we have examined the effect of chronic administration of the SSRI fluoxetine, the antipsychotic drug haloperidol, a lipophilic SJW extract prepared with supercritical CO_2, and hyperforin-TMB on these same genes. Long-term (8 weeks), but not short-term (2 weeks), treatment with fluoxetine resulted in a marked decrease in CRH mRNA levels in the PVN and TH mRNA levels in the LC, and it produced an increase in hippocampal levels of MR, GR, and 5-HT_{1A} mRNA. In contrast, the negative control haloperidol (1 mg/kg), the hyperforin-rich CO_2 extract (27 mg/kg), and hyperforin-TMB (8 mg/kg) did not alter levels of gene transcription at either time point. These data suggest that hyperforin and hyperforin derivatives are not involved in the regulation of genes that control HPA axis function in a therapeutic administration paradigm.

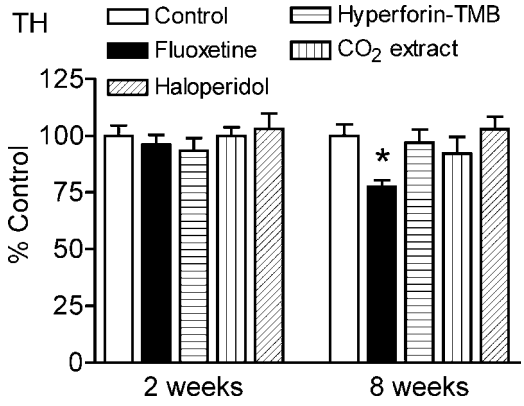

Figure 2 TH mRNA expression in the locus coeruleus after 2 and 8 weeks of daily administration.

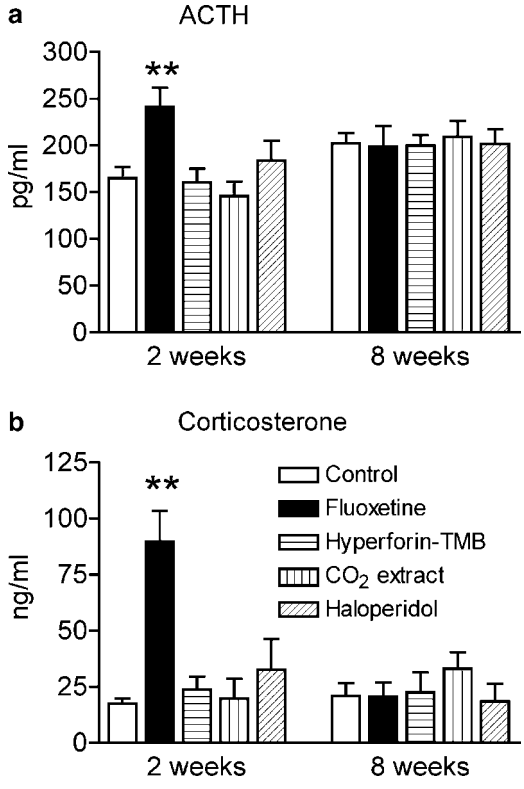

Figure 3 ACTH (a) and corticosterone (b) levels in plasma after 2 and 8 weeks of daily treatment.

We have previously reported that long-term administration of imipramine (15 mg/kg, p.o.), a methanolic SJW extract (500 mg/kg, p.o.), or hypericin (0.2 mg/kg, p.o.) all decreased CRH mRNA levels in the PVN (Butterweck *et al*, 2001b). The decrease in CRH mRNA levels in the PVN was accompanied by a decrease in POMC levels in the anterior lobe of the pituitary and in the plasma levels of ACTH and corticosterone. In light of the findings that CRH appears to be hypersecreted in depression, we suggested that the decrease in hypothalamic CRH expression induced by SJW extract and hypericin may be relevant to their therapeutic efficacy. The replication of the imipramine- (Brady *et al*, 1991) and fluoxetine-induced (Brady *et al*, 1992) delayed decreases in CRH mRNA levels in the PVN and the addition of significant data based on administration of plant substances (Butterweck *et al*, 2001b) further validate the short-term/long-term treatment paradigm for the assessment of efficacy of candidate antidepressant drugs. We hypothesize that if a downregulation of CRH mRNA in the PVN may be a common element relevant to the therapeutic efficacy of antidepressant drugs, the lack of effect of hyperforin and hyperforin derivatives on the regulation of HPA axis genes suggests that these substances are not necessarily involved in the beneficial effects of SJW after oral dosing.

In the present study, only fluoxetine altered mRNA levels of steroid hormone levels. In line with the finding of Brady *et al* (1992), the SSRI increased MR and GR mRNA levels in the hippocampus after 8 weeks, but not after 2 weeks, of drug treatment, indicating that differential regulatory mechanisms are operating to adjust limbic corticosteroid receptor number during antidepressant treatment. Similar antidepressant-induced increases of MR and GR mRNA expression have been reported in several *in vitro* and *in vivo*

Table 2 Adrenal and Body Weights in Short- and Long-term Administration Groups

	Control		Fluoxetine		Hyperforin-TMB		CO_2 extract		Haloperidol	
	2 weeks	8 weeks	2 weeks	8 weeks	2 weeks	8 weeks	2 weeks	8 weeks	2 weeks	8 weeks
Adrenal gland weight (mg)	56 ± 3	57 ± 3	50 ± 2	53 ± 3	53 ± 2	52 ± 3	59 ± 2	56 ± 3	58 ± 3	56 ± 3
Body weight (g)	388 ± 8	429 ± 15	359 ± 9*	386 ± 14*	375 ± 11	396 ± 13	392 ± 10	421 ± 19	388 ± 11	375 ± 10*

Values are expressed as mean ± SEM ($n = 8$ per group). Initial body weights were 160–180 g. *$P < 0.05$.

studies (Budziszewska *et al*, 1994; Pepin *et al*, 1989; Pfeiffer *et al*, 1991; Reul *et al*, 1993; Seckl and Fink, 1992). It has also been shown that chronic administration of lithium, which is known to augment clinical effects of medication in depressed patients, as well as prolonged application of electroconvulsive shock (ECS), regarded as a nondrug therapy of depression, elevate GR mRNA levels or the density of GR and MR in the rat hippocampus (Pfeiffer *et al*, 1991; Przegalinski *et al*, 1993). GR and MR biosynthesis appears to be under control of central monoaminergic systems (Maccari *et al*, 1990, 1992; Mitchell *et al*, 1990; Weidenfeld and Feldmann, 1991). *In vivo* studies with monoamine-depleting agents (eg reserpine) and neurotoxic substances that specifically destroy serotonergic, noradrenergic, and/or dopaminergic nerve terminals have provided evidence for a modulatory role of monoamines in brain corticosteroid receptor regulation (Lowy, 1990; Seckl *et al*, 1990; Siegel *et al*, 1983; Weidenfeld *et al*, 1983). After chronic fluoxetine treatment, *in vivo* microdialysis studies have shown that extracellular levels of serotonin are markedly elevated (Rutter *et al*, 1994). Thus, in the present study, the increase in GR and MR mRNA levels after long-term fluoxetine treatment may be connected with the actions of the SSRI on serotonergic neurotransmission.

Apart from a role of the monoamines, fluoxetine-induced changes in plasma levels of ACTH and corticosterone may also be a factor contributing to the regulation of the corticosteroid receptors. Support for a stimulatory role of fluoxetine on the HPA axis has been provided by a number of studies. For instance, acute administration of fluoxetine to rats stimulates the secretion of pituitary ACTH and adrenal corticosterone in a dose-dependent manner (Dinan, 1996; Li *et al*, 1993a, b). In the present study, ACTH as well corticosterone levels were significantly increased after 2 weeks of daily fluoxetine treatment, and they were back to baseline levels after 8 weeks. This result is in contrast to other reports of decreased or baseline levels of ACTH and corticosterone plasma levels after repeated treatment of rats with fluoxetine (Brady *et al*, 1991, 1992; Lopez *et al*, 1998; Raap *et al*, 1999). One reason for these discrepancies might be the large variability in daily fluoxetine doses used in the different studies. Many studies using fluoxetine in rats have used doses of 10–30 mg/kg/day (Gardier *et al*, 1994; Nestler *et al*, 1990; Trouvin *et al*, 1993); others have used lower doses of 2.5–5 mg/kg/day (Brady *et al*, 1992; Nibuya *et al*, 1996). In these studies, the substance was given i.p. or subcutaneously, whereas in the present study, 10 mg/kg of fluoxetine was given orally by gavage. However, the antidepressant doses used in the present study were selected from the doses shown in animal models (forced swim test) to correlate with antidepressant activity.

In our study, POMC mRNA levels in the anterior pituitary were slightly reduced after 2 weeks of fluoxetine treatment, but this effect was not statistically significant. CRH mRNA levels were significantly decreased after 8 weeks of daily treatment with fluoxetine. These apparent discrepancies between increased plasma ACTH and corticosterone levels slightly reduced mRNA POMC levels after 2 weeks, and reduced CRH mRNA levels after 8 weeks of fluoxetine treatment might be explained by the fact that substances like fluoxetine, which increase central 5-HT concentration, may increase plasma ACTH and corticosterone levels through mechanisms that are independent from serotonergic innervation of CRH neurons in the PVN. In fact, it has been reported that 5-HT causes a direct ACTH secretion from anterior pituitary cells *in vitro* (Calogero *et al*, 1995). On the other hand, corticosteroids have been shown to alter several elements of serotonergic neurotransmission. The removal of circulating corticosteroids by adrenalectomy resulted in anatomically specific decreases in the indices of 5-HT metabolism (Chalmers *et al*, 1993; De Kloet *et al*, 1982; Zhong and Ciaranello, 1995). It is therefore likely that the increased ACTH and corticosterone levels observed in the present study after 2 weeks of fluoxetine treatment do not result from changes in HPA activity, but rather reflect a direct effect on pituitary cells caused by increased levels of serotonin.

As the hippocampus is also suggested to be a key component in the mediation of depression (Duman *et al*, 1997), the expression level of 5-HT_{1A} mRNA was measured in this structure. Several studies provide evidence that the activity and levels of 5-HT_{1A} receptors are modulated by glucocorticoid levels. It has been shown that removal of circulating corticosteroids acts to induce 5-HT_{1A} receptor expression. Autoradiographic studies first identified increased 5-HT_{1A} receptor binding in the rat hippocampus after adrenalectomy (Biegon *et al*, 1985). Subsequent investigations have confirmed the sensitivity of 5-HT_{1A} receptors to circulating corticosteroid levels (Chalmers *et al*, 1993, 1994; Zhong and Ciaranello, 1995). However, the fact that in the present study, plasma ACTH and corticosterone levels were found to be elevated after 2 weeks whereas hippocampal 5-HT_{1A} mRNA was upregulated after 8 weeks indicates that differential or delayed regulatory mechanisms are operating to adjust limbic 5-HT_{1A} receptor number during fluoxetine treatment.

Long-term oral administration of fluoxetine was associated with a decrease in mRNA expression in the LC, whereas haloperidol (1 mg/kg), the CO_2 extract (27 mg/kg), and hyperforin-TMB (8 mg/kg) had no effect on TH message levels. Data from previous studies suggest that the common action of antidepressants could be related to an effect on the regulation of TH in the LC, but there is controversy in the literature in this regard. Whereas Brady *et al* (1992) report an upregulation of TH mRNA levels in the LC after 8 weeks of fluoxetine treatment (5 mg/kg, i.p.), Nestler *et al* (1990) found decreased levels of TH after 2 weeks of fluoxetine administration (15 mg/kg, i.p.). The apparent downregulation of TH by fluoxetine is particularly interesting and supports the view that the serotonergic system exerts an influence on the functional state of noradrenergic neurons (Valentino *et al*, 1990).

In summary, as a positive control, we replicated the effects of long-term fluoxetine administration on gene transcription in selected brain areas that are thought to be involved in HPA axis control (Brady *et al*, 1992). In contrast, a hyperforin-rich lipophilic CO_2 extract as well as hyperforin-TMB failed to affect gene transcription associated with HPA axis control. Although some authors emphasize hyperforin as the major active principle of SJW, its efficacy could not be demonstrated in the present paradigm. In the present study, hyperforin was used as TMB, a hyperforin prodrug. The application of hyperforin as an ester appeared to be necessary because hyperforin is

extremely sensitive to light and oxidation processes and is decomposed in aqueous solutions. In the present study, we tested the pharmacological activity of hyperforin-TMB, a new derivative synthesized with the aim of obtaining a more stable compound. Although hyperforin-TMB was inactive in the present study, it can be excluded that this effect is due to lack of its bioavailability. The release of hyperforin from the ester was demonstrated in pharmacokinetic studies. Furthermore, the dosage used in the present study proved to be active when tested in the forced swimming test (see Materials and methods). However, recently Cervo *et al* (2002) showed that hyperforin—used as a hyperforin-dicyclohexylammonium salt—was active in several behavioral models, but that the ester as well as the free drug could not be detected in the brain probably because of its poor passage through the blood–brain barrier. The authors speculate that the antidepressant-like effect of hyperforin might be mediated by a still unknown metabolite of this compound.

In our previous study, we showed that a methanolic SJW extract as well as hypericin reduced measures of HPA axis activity with delayed onset (after 8 weeks), indicating that the delayed changes may be important for their therapeutic efficacy. Based on our present study, we conclude that hyperforin and hyperforin derivatives might belong to the active compounds that contribute to the beneficial effects of SJW after oral dosing, but that these lipophilic compounds are not involved in the regulation of HPA axis control. However, other mechanisms relevant to antidepressant activity of these compounds are not excluded and need to be investigated in further *in vivo* studies.

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