

Effect of Chronic Administration of Selective Glucocorticoid Receptor Antagonists on the Rat Hypothalamic–Pituitary–Adrenocortical Axis

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The effects of the selective glucocorticoid receptor (GR) antagonists ORG 34850, ORG 34116, and ORG 34517 on the rat hypothalamic–pituitary–adrenocortical (HPA) system were investigated. To assess the potency of the compounds to occupy GR in the brain and pituitary, we applied a single acute subcutaneous (s.c.) injection (10 mg/kg). ORG 34517 was most potent to occupy GR in the anterior pituitary and distinct brain areas, whereas all compounds were unable to occupy mineralocorticoid receptor (MR). Chronic administration of ORG 34850, ORG 34116, and ORG 34517 (20 mg/kg/day) for 1, 3, and 5 weeks resulted only in minor changes in brain GR levels. However, profound increases of hippocampal MR were observed virtually at all time points. Treatment with ORG 34850 and ORG 34116 elicited episodic increases in HPA axis activity, whereas ORG 34517 did not cause any changes in HPA activity. Thus, the GR antagonists exert distinct effects on the HPA axis, which may be pertinent for the proposed antidepressant activity of these compounds.

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INTRODUCTION

Besides depressive psychopathology, patients suffering from major depression often have altered autonomic and neuroendocrine functioning. The system most commonly disturbed is the hypothalamic–pituitary–adrenocortical (HPA) axis (for a review see Holsboer, 2000). The control of HPA activity involves an autoregulatory feedback mechanism exerted by circulating corticosteroids via two different receptors (Reul and De Kloet, 1985; De Kloet and Reul, 1987): The mineralocorticoid receptor (MR) or Type I receptor shows a distinct distribution and is mainly expressed in the hippocampus (Gerlach and McEwen 1972; Reul and De Kloet, 1986; Van Eekelen *et al*, 1988; Herman *et al*, 1989; Reul *et al*, 2000) and pituitary gland (De Nicola *et al*, 1981; Moguilewsky and Raynaud, 1980; Reul and De Kloet, 1985, 1986; Reul *et al*, 1990). Glucocorticoid receptors (GRs) or Type II receptors are abundantly distributed in the brain and pituitary (De Kloet and McEwen, 1976; Rees *et al*, 1977; Fuxe *et al*, 1985; Reul

et al, 1990) with high levels in the hypothalamic PVN and in the hippocampal formation. MRs have a higher affinity for glucocorticoids ($K_d \sim 0.2$ nm), controlling basal HPA activity at low circulating glucocorticoid levels during the circadian trough of adrenocortical secretion (Reul and De Kloet, 1985; De Kloet and Reul, 1987; Reul *et al*, 2000). Elevated levels of glucocorticoids, present at the circadian peak or following stressful stimuli, exert their negative feedback action via lower affinity GRs ($K_d \sim 2$ –5 nm) (De Kloet and Reul, 1987; Dallman *et al*, 1987; Reul *et al*, 2000). Therefore, a coordinated functioning of this dual receptor system, which allows for a dynamic regulation of the HPA axis, is necessary for maintaining homeostasis.

Typical observations among depressed patients with a hyperactive HPA axis are elevated amplitudes of cortisol secretory episodes (Linkowski *et al*, 1986; Sachar *et al*, 1973), an increased frequency of ACTH secretory episodes (Mortola *et al*, 1987), and several other aberrations at the different levels of this neuroendocrine system. Several studies on long-term antidepressant treatment suggest that clinical remission of depressive psychopathology is often preceded by normalization of HPA axis functioning (Holsboer *et al*, 1982; Heuser *et al*, 1990; Holsboer-Trachsler *et al*, 1991). In line with these clinical findings are several preclinical studies on long-term antidepressant treatment in rats, which demonstrated that antidepressants, despite different primary neuropharmacological mechanisms, have the same restraining effect on HPA activity (Reul

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et al, 1993, 1994; Gesing and Reul, in preparation). Furthermore, these antidepressants increased the levels of hippocampal MR between 2 and 5 weeks of treatment, followed by moderate rises in GR level in the brain and pituitary. Other studies showed similar data (Kitayama *et al*, 1988; Brady *et al*, 1991, 1992). Reul *et al* (1993, 1994, 2000) have postulated that normalization of HPA disturbances might be a common denominator for the clinical efficiency of an antidepressive substance. Moreover, they hypothesized that an early increase in hippocampal MR levels might trigger a cascade of changes leading to normalization of HPA axis activity.

Since numerous clinical investigations point to hypercorticism as an important factor in the pathogenesis of depression, treating depression with GR antagonists or steroid synthesis inhibitors could be a promising strategy (Murphy *et al*, 1998; Wolkowitz *et al*, 1999; Belanoff *et al*, 2001; Reus and Wolkowitz, 2001). Administration of the GR antagonist RU 38486 clearly increased HPA axis drive in human subjects (Laue *et al*, 1989; Lamberts, 1991; Kawai *et al*, 1987) and rats (Lamberts *et al*, 1991; Van Haarst *et al*, 1996). However, application of RU 38486 as a GR antagonist is limited by its partial GR agonistic activity at the GR (Gruol and Altschmied, 1993; Havel *et al*, 1996) and its deficient receptor selectivity, as it also binds to progesterone receptors (PRs) (Gagne *et al*, 1985). Lately, several highly selective GR antagonists have been developed as derivatives of RU 38486 (Peeters *et al*, 1998) as a possible treatment to restore normal HPA axis activity in disorders associated with cortisol hypersecretion, such as major depression. Corresponding to our hypothesis of increased hippocampal MR capacity as the initial phenomenon of successive changes in HPA axis activity during the course of antidepressant treatment (Reul *et al*, 1993, 1994, 2000), we intended to investigate the effects of the newly developed GR antagonists on brain corticosteroid receptor capacity and HPA axis activity of rats using a similar approach as the previous antidepressant studies. Therefore, we chose for a long-term treatment at moderate dosages similar to the applied antidepressant administration rather than demonstrating their pronounced GR antagonism by application of high dosages.

MATERIALS AND METHODS

Animals

Male Wistar rats (140–160 g upon arrival; Charles River, Sulzfeld, Germany) were used for all experiments. They were housed in groups of three to six animals per cage under controlled conditions (constant temperature $22 \pm 1^\circ\text{C}$; lights on from 6.00–20.00 h; 40–60% humidity) with free access to food and water. Before the treatment was started, animals were allowed to adapt to the animal room for at least four days. All animal procedures were performed using protocols approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany.

Materials

GR antagonists were a gift from NV Organon (Oss, The Netherlands). Polyethylene glycol and dimethylsulfoxide

were purchased from Sigma (Deisenhofen, Germany), and aprotinine (Trasylol®) from Bayer (Leverkusen, Germany).

GR Antagonist Administration: Receptor Occupancy Study

Receptor occupancy experiments were essentially conducted as described previously (Reul and De Kloet, 1985; Reul *et al*, 1989). To determine the potency of the GR antagonists to occupy corticosteroid receptors *in vivo*, 2-day adrenalectomized rats received a single subcutaneous (s.c.) injection of GR antagonists (10 mg/kg body weight). This dose was declared as the threshold dose for the penetration of the blood–brain barrier by NV Organon (Peeters *et al*, 1998). All compounds were dissolved in 5% dimethylsulfoxide (DMSO)/polyethylene glycol-300 (PEG-300). Control animals received the vehicle. At 1 h after injection, animals were anesthetized quickly in a glass jar containing saturated halothane (Hoechst, Frankfurt am Main, Germany) and decapitated immediately. Brain and pituitary were rapidly removed from the skull and dissected quickly. Tissues were frozen in liquid nitrogen and stored at -80°C . Measurement of binding to corticosteroid receptors was determined in [^3H]steroid binding assays as described below. Receptor occupancy was determined by assessment of the residual MR and GR binding in tissues of GR antagonist treated rats as compared to receptor binding in vehicle-treated animals. The percentage difference between the receptor binding data was taken as the percentage occupancy.

GR Antagonist Administration: Chronic Drug Treatment

In choosing the route of drug administration, our objective was to save the animals from unspecific stress. Accordingly, similar to previous chronic antidepressant treatment experiments (Reul *et al*, 1993, 1994), the oral route was chosen for the chronic administration of the glucocorticoid antagonists. As all compounds showed a bad solubility in a drinkable liquid phase (eg ethanol 0.5%/water), application via the drinking water was impossible. Therefore, the drug was applied via the food (233 mg per 950 g food). The animals were treated for a period of maximum 5 weeks. Drug intake was monitored by weighing the food each time it was renewed (ie every third day). Based on the average food consumption per day, the drug concentration in the food and the average weight of the rats, a mean drug intake could be calculated (ie 20–25 mg/kg/day). Water intake was checked daily, body weight gain was determined once a week.

Surgery, Tissue Collection, and [^3H]Steroid Binding Assay

For corticosteroid binding measurement, treatment was terminated two days before decapitation and bilateral adrenalectomy was performed under halothane anesthesia to deplete their bodies of endogenous corticosteroids and GR antagonists. After surgery, the animals were maintained on 0.9% NaCl in drinking water and rat chow without drug. Immediately after removal from the body, adrenal glands

were placed in an Eppendorf tube (adrenals of one rat per tube) and kept on normal ice. The weight of the carefully cleaned adrenals was determined on the same day as the day of surgery to prevent unreliable adrenal weight data because of water loss from the frozen tissue during long-term storage. In addition, the rats were weighed at the time of adrenalectomy.

At 2 days after adrenalectomy, the rats were quickly (<20 s) anesthetized with halothane in a glass jar and killed by decapitation. Trunk blood was collected in ice-chilled EDTA-coated tubes, and the plasma was checked for the absence of any endogenous corticosterone by RIA (ICN Biomedicals, Costa Mesa, USA). Thymuses were removed, cleaned and weighed on the same day. Immediately after decapitation, the brain and the pituitary were rapidly removed from the skull. Subsequently, the anterior part of the pituitary was separated from the neurointermediate lobe, and the hippocampus, hypothalamus, amygdala, frontal cortex, and neocortex (consisting of parietal, cingular, and entorhinal cortex) were dissected from the brain. Dissected tissues were instantaneously frozen in liquid nitrogen and stored at -80°C until corticosteroid receptor binding assay.

The MR and GR binding assay was conducted as described (Reul *et al*, 1993; Gesing *et al*, 2001). Pooled tissues of six rats per group were homogenized (100 mg brain tissue/ml; 50 mg anterior pituitary tissue/ml; 10 strokes at 900 rpm) in ice-cold 5 mM Tris-HCl (pH 7.4) containing 5% glycerol, 10 mM sodium molybdate, 1 mM EDTA, and 2 mM β -mercaptoethanol using a glass homogenizer with a Teflon pestle milled at a clearance of 0.25 mm on the radius. The homogenate was centrifuged at 100 000 g for 60 min at $0-2^{\circ}\text{C}$ to obtain cytosol (ie supernatant fraction). All reagents used were of analytical grade. Aliquots of cytosol (100 μl) were incubated with [^3H]steroids over a concentration range of 0.1–10 nM (6–8 concentrations; total volume 150 μl). In some experiments, steroid receptors were measured with one saturating concentration of [^3H]steroid (10 nM, >90% saturation of binding) in five- to seven-fold. Total binding to soluble macromolecules was determined with [^3H]aldosterone (87–94 Ci/mmol NEN DuPont, Dreieich, Germany) or with [^3H]dexamethasone (85–106 Ci/mmol, Amersham, Braunschweig, Germany). For measurement of MR, total binding was assessed by incubating cytosol with [^3H]aldosterone in the presence of a 100-fold excess of the specific glucocorticoid agonist RU 28362 (11 β , 17 β -dihydroxy-6-methyl-17 α -(1-propionyl)androsta-1,4,6-triene-3-one). Unlabeled RU 28362 was included to prevent [^3H]aldosterone from binding to GR, so that only binding of this [^3H]ligand to MR was measured. Nonspecific binding was determined in parallel incubations containing a 1000-fold excess of corticosterone in addition to cytosol and [^3H]aldosterone. Total binding for the GR was determined by incubating cytosol with [^3H]dexamethasone. Since [^3H]dexamethasone also displays considerable affinity (at least *in vitro*) for MR (Reul *et al*, 2000), the fraction of [^3H]dexamethasone binding to MR was estimated by including a 100-fold excess of RU 28362 in parallel incubation tubes. Nonspecific binding was determined in parallel incubations containing a 1000-fold excess of dexamethasone in addition to cytosol and [^3H]dexamethasone.

After incubation for 20–24 h at 0°C , bound and free [^3H]steroid were separated by Sephadex LH-20 (Pharmacia, Uppsala, Sweden) gel filtration (100 μl of the cytosol-steroid mixture was applied to the LH-20 columns), and radioactivity was measured in a liquid scintillation counter. The protein concentration was determined by the method of Lowry *et al* (1951) with BSA as the standard. The binding data were expressed as femtomoles per milligram protein (fmol/mg) and nonspecific binding was subtracted from total binding to yield specific binding. In this manner, the MR concentration could be measured directly. However, GR binding was estimated by subtraction of the specific binding of [^3H]dexamethasone + 100 \times RU 28362 from the specific binding of [^3H]dexamethasone. [^3H]dexamethasone + 100 \times RU 28362 rather than [^3H]aldosterone + 100 \times RU 28362 binding data were used to estimate the amount of the specific [^3H]dexamethasone binding to MR, because [^3H]dexamethasone + 100 \times RU 28362 binding to MR was found to be about 30% less than [^3H]aldosterone + 100 \times RU 28362 binding to this receptor type (Reul *et al*, 2000).

The maximal number of binding sites (B_{max}) and relative binding affinity (K_d) were determined by Scatchard analysis.

Neuroendocrine Experiments

All experiments and surgical procedures were performed between 7.00 and 11.00 am. Great effort was undertaken to keep the rats undisturbed during the day before the experiment. On the day of the experiment, rats were killed either under basal early morning conditions or 15 min after onset of forced swim stress. The forced swimming procedure was carried out similar to the Porsolt protocol (Porsolt *et al*, 1978). Briefly, the rats were placed for 15 min in glass cylinders (height 35 cm, diameter 24 cm) containing water to a height of 20 cm and at 25°C . Immediately thereafter rats were rapidly anesthetized in a glass jar containing saturated halothane (<20 s; Hoechst, Frankfurt am, Germany) and decapitated immediately. Brain and pituitary were rapidly removed from the skull and frozen in isopentane (-40°C) and on dry ice. Trunk blood was collected in prechilled EDTA-coated tubes containing Trasylol (Bayer, Leverkusen, Germany). Plasma was prepared by centrifugation (3000 rpm, 4°C , 20 min) and stored at -80°C . Adrenal glands and thymuses were removed from the body and treated as described.

Measurement of Plasma ACTH and Corticosterone

Plasma ACTH and corticosterone concentrations were measured using commercially available radioimmunoassay kits (ICN Biomedicals, Costa Mesa, USA). The inter- and intra-assay coefficients of variability for ACTH were 7 and 5% with a detection limit of 2 pg/ml. For corticosterone, the inter- and intra-assay coefficients were 7 and 4%, respectively, with a detection limit of 1.5 ng/ml.

Statistical Analysis

Results are presented as means \pm SEM. Assessment of statistically significant differences between the groups of

time course experiments on physical parameters (body weight, adrenal weight, and thymus weight), receptor binding measurements, and plasma hormone concentrations was performed with two-way analysis of variance (ANOVA). Since RU 38486 treatment was only conducted for 3 weeks, these data were omitted from the two-way ANOVA. However, to assess the statistical significance of the effect of RU 38486 treatment, the data were included in the one-way ANOVA analysis of the 3-week time-point data. Data obtained from acute antagonist treatment experiments were tested with one-way ANOVA. In appropriate cases, ANOVA was followed by a *post hoc* Dunnett's test. Values of $p < 0.05$ were considered to indicate significant difference if compared with the control group.

RESULTS

Chemical Structures

Figure 1 shows the chemical structures of the compounds Org 34517, Org 34850, Org 34116 and RU 38486. These graphs illustrate the chemical relation between the different molecules.

Occupancy of Brain and Anterior Pituitary Corticosteroid Receptors by GR Antagonists

Occupancy of corticosteroid receptors by GR antagonists was determined in various brain areas and in the pituitary. Figure 2 shows that there were marked differences between the GR antagonists in their efficacy to occupy GRs, not only among the compounds but also among the tested tissues. ORG 34517 clearly showed the highest potency in binding to brain and anterior pituitary GRs, because binding of [3 H]dexamethasone to GR was most substantially reduced in animals treated with this antagonist. As compared to the binding of ORG 34517 to GRs, that of ORG 34850 and ORG 34116 showed much less potency and was very much

dependent of the tissue. ORG 34850, at least at the used dose, was unable to occupy GRs in the hippocampus, amygdala, and neocortex, whereas GR occupancy in the hypothalamus, frontal cortex, and anterior pituitary was moderate to high (Figure 2). ORG 34116 did not occupy GRs in the hippocampus and showed low to moderate occupancy levels in all other tissues (Figure 2). Overall, for all ligands the anterior pituitary GRs presented the highest occupancy levels of all tissues (Figure 2). No occupancy of MR by GR antagonists was observed (data not shown).

Time Course of Physical Parameters

Over the 5 weeks course of treatment an overall significant increase in body weight and adrenal weight (two-way ANOVA) was observed, whereas thymus weight significantly decreased (two-way ANOVA). Oral administration of the GR antagonists did not result in any significant differences in body weight at the respective time points, except for the RU 38486 treatment of 3 weeks that resulted in a small, but significant, reduction in body weight (Figure 3a). Adrenal weight (= weight per 2 adrenals) of animals treated with ORG 34850 and ORG 34116 was significantly increased after 1, 3, and 5 weeks of treatment (Figure 3b). Application of ORG 34517 and RU 38486 yielded no significant changes in adrenal weight. The thymus weight was markedly elevated after 1 and 3 weeks of treatment with ORG 34116, as it was after a 3-week administration of ORG 34850 in comparison with the respective control groups (Figure 3c). Administration of ORG 34517 did not change thymus weight at any time. RU 38486 treatment for 3 weeks resulted in a significant reduction of thymus weight.

Effect of Chronic Treatment with GR Antagonists on Hippocampal Corticosteroid Receptors

Measurement of hippocampal MR levels revealed marked time-dependent and GR-antagonist-specific increases at all

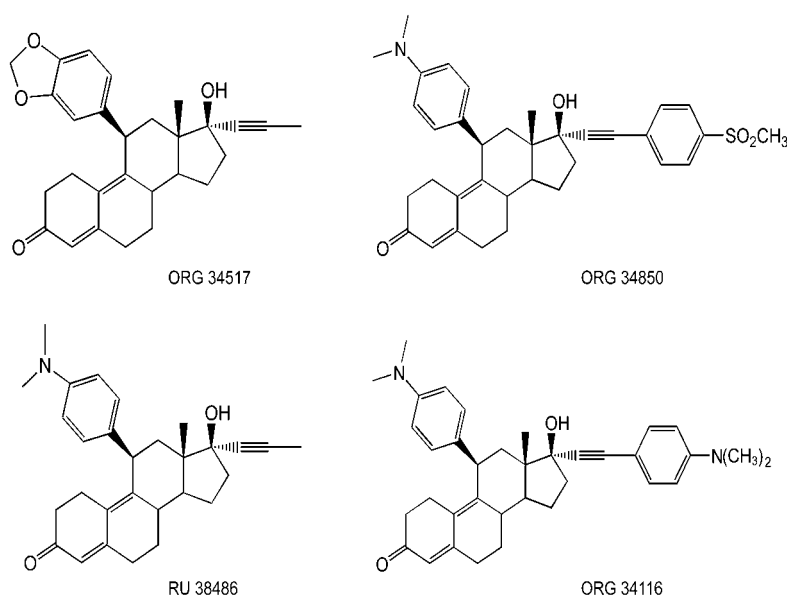


Figure 1 Chemical structures of the compounds Org 34517, Org 34850, Org 34116, and RU 38486.

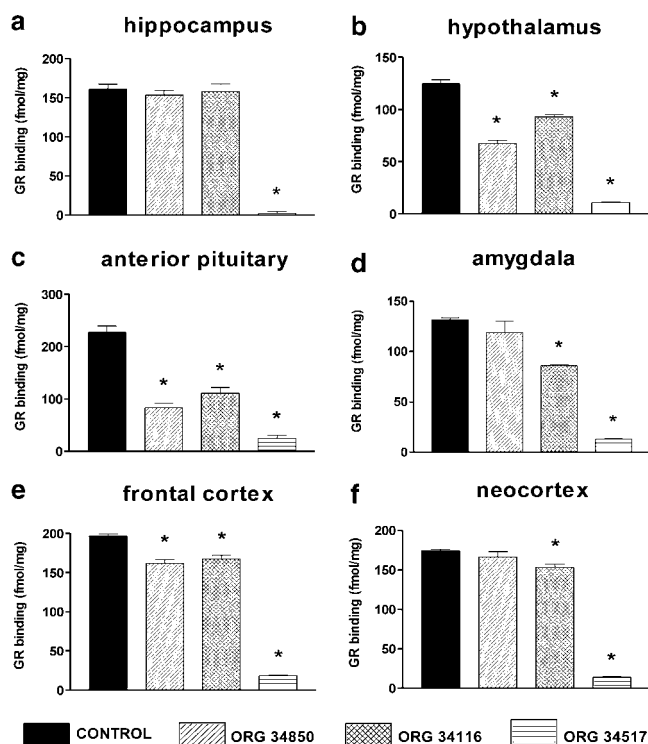


Figure 2 Occupancy of corticosteroid receptors by GR antagonists. Effect of acute administration of antagonists on binding of [3 H]dexamethasone to GR in the anterior pituitary and distinct brain regions. Rats (six per group) received a single s.c. injection of either GR antagonists (10 mg/kg) or 5% DMSO/PEG (control) and were decapitated 1 h thereafter. Measurements of GR and MR concentrations, expressed as femtomoles per mg protein (mean \pm SEM), were performed at a single concentration of [3 H]steroid (10 nM, >90% saturation). No effect on binding of [3 H]aldosterone to MR was obtained (data not shown). One-way ANOVA: hippocampus-GR (effect of treatment), $F(3,16)=130.708$, $p<0.0005$; anterior pituitary-GR: effect of treatment: $F(3,16)=75.926$, $p<0.0005$; hypothalamus-GR (effect of treatment), $F(3,16)=346.205$, $p<0.0005$; amygdala-GR (effect of treatment), $F(3,16)=85.723$, $p<0.0005$; neocortex-GR (effect of treatment), $F(3,16)=337.714$, $p<0.0005$; frontal cortex-GR (effect of treatment), $F(3,16)=480.004$, $p<0.0005$; * $p<0.05$ (vs the control group, *post hoc* Dunnett's *t*-test).

times during the course of the treatment (Figure 4a). As compared to the controls, the MR concentration in ORG 34116-treated rats was elevated after 1 (+26%), 3 (+52%), and 5 weeks (+24%). Administration of ORG 34850 resulted in statistically significant rises in hippocampal MR levels after 1 (+50%) and 5 (+16%) weeks, whereas hippocampal MR levels determined in animals treated with ORG 34517 were only elevated after 3 weeks (+41%). A pronounced increase (+61%) in hippocampal MR levels was observed in animals treated with RU 38486 for 3 weeks (Figure 4a).

Regarding the effects on hippocampal GRs, significant changes (ie decreases) were only observed after the administration of ORG 34116 (Figure 4b). Data obtained from Scatchard analyses yielded no changes in K_d values of MR and GR binding during the course of the treatment (K_d values for [3 H]aldosterone binding to MR and [3 H]dexamethasone binding to GR were 0.09–0.15 and 1.2–1.5 nM, respectively).

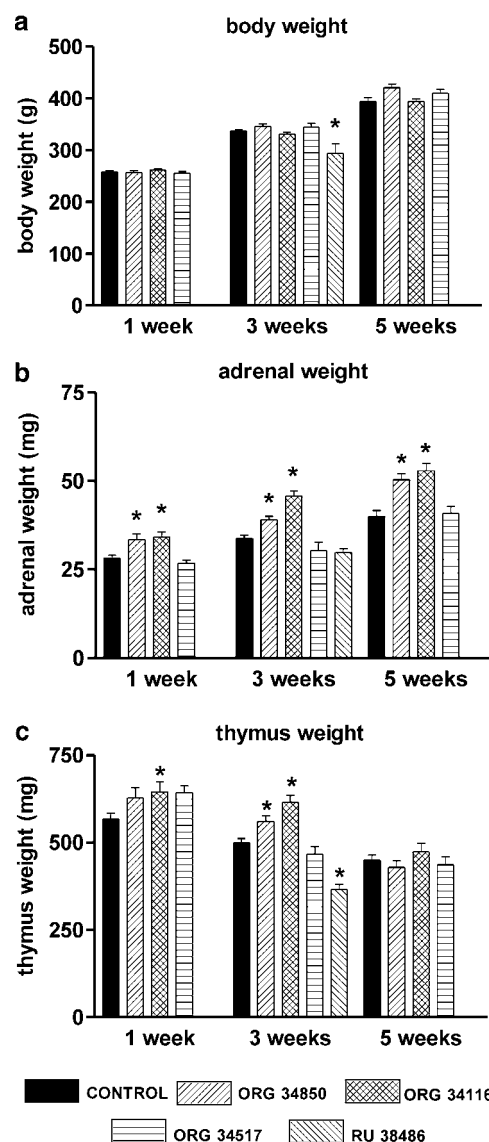


Figure 3 Time course of the effect of chronic GR antagonist administration on body weight (a), adrenal weight (b), and thymus weight (c). The data on the adrenal weights were expressed as the weight per 2 adrenals in milligram (mean \pm SEM). Rats (15–60 per group) received either GR antagonists (233 mg per 950 g food: 25 mg/kg/day) or food (control, black bar). The animals were killed after 1, 3, and 5 weeks of treatment. Adrenalectomy was conducted 2 days before decapitation, and treatment was stopped at the same time. Two-way ANOVA: body weight: effect of duration, $F(2,333)=673.19$, $p<0.0005$; effect of treatment, $F(3,333)=4.80$, $p<0.01$; effect by interaction of duration and treatment, $F(6,333)=2.59$, $p<0.02$; adrenal weight: effect of duration, $F(2,333)=91.890$, $p<0.0005$; effect of treatment, $F(3,333)=39.716$, $p<0.0005$; effect by interaction of duration and treatment, $F(6,333)=1.914$, $p>0.05$; thymus weight: effect of duration, $F(2,333)=63.686$, $p<0.0005$; effect of treatment, $F(3,333)=7.815$, $p<0.0005$; effect by interaction of duration and treatment, $F(6,333)=3.343$, $p<0.01$; *post hoc* Dunnett's *t*-test; * $p<0.05$ (vs the control group).

Effect of Chronic Treatment with GR Antagonists on Corticosteroid Receptors: Neuroanatomical Specificity

Chronic administration of GR antagonists generated time-dependent changes in corticosteroid receptor concentrations in distinct brain and pituitary tissues. Oral application

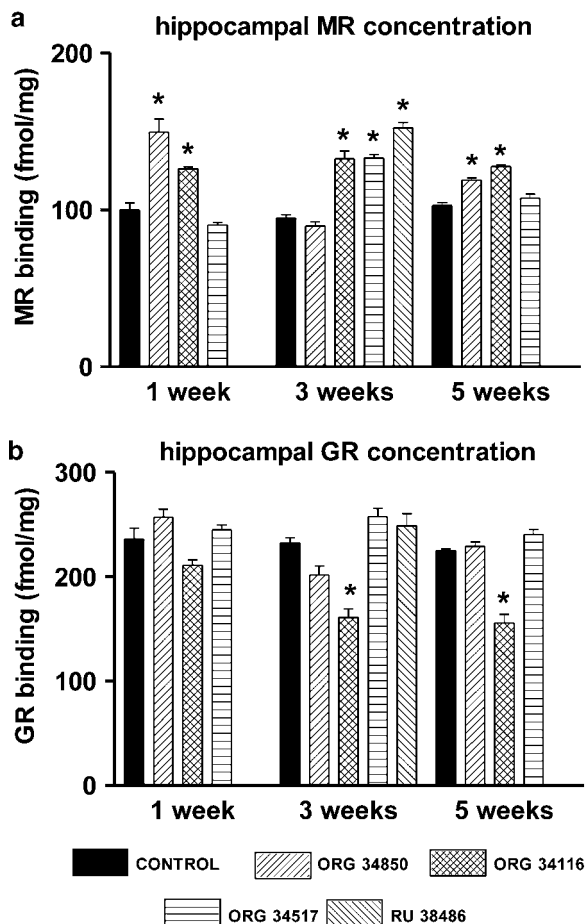


Figure 4 Time course of the effect of GR antagonist treatment on MR (a) and GR (b) concentrations in the rat hippocampus. Rats (5–7 per group) were treated with GR antagonists, including RU 38486, adrenalectomized and decapitated as outlined in the legend to Figure 3. Hippocampal MR and GR, expressed as femtomoles per mg protein (mean \pm SEM), were measured by radioligand binding assay (see *Materials and methods*). Two-way ANOVA: MR: effect of duration, $F(2,52) = 0.465$, $p > 0.05$ (nonsignificant); effect of treatment, $F(4,52) = 56.056$, $p < 0.0005$, effect by interaction of duration and treatment, $F(6,52) = 37.787$, $p < 0.0005$; GR: effect of duration, $F(2,52) = 14.334$, $p < 0.0005$; effect of treatment, $F(4,52) = 44.797$, $p < 0.0005$; effect by interaction of duration and treatment, $F(6,52) = 6.229$, $p < 0.0005$; *post hoc* Dunnett's *t*-test; * $p < 0.05$ (vs the control group).

of ORG 34850 caused only small, mostly statistically insignificant changes of MR and GR levels in all tested regions (Figure 5), except for a marked MR and GR downregulation in the neocortex after 1 week of treatment (Figure 5e, f).

In ORG 34116-treated rats, anterior hypophyseal MR concentrations were profoundly downregulated only at 1 week, while overall brain MR levels were found to be elevated, reaching statistical significance at 1 and 3 weeks in the amygdala and neocortex, and at 3 weeks in the frontal cortex. Brain and pituitary GR binding levels were profoundly reduced in animals treated with ORG 34116 (Figure 5), albeit different time courses were observed. GR levels in pituitary and hypothalamus were already downregulated after 1 week of treatment, whereas for the other brain structures including hippocampus (see Figure 4b) this took at least 3 weeks of treatment.

In the pituitary, neocortex, and frontal cortex transient rises in MR levels were observed in animals treated with ORG 34517 with a statistically significant MR upregulation at 3 weeks. At 1 week, a marked downregulation in amygdaloid and neocortical MR concentration was found. Only minor effects were measured in the brain and pituitary GR levels after chronic administration of ORG 34517 (Figure 5).

RU 38486 was administered for 3 weeks and caused a marked increase in MR concentration in the amygdala and frontal cortex. GR numbers were increased in the amygdala and frontal cortex, but significantly reduced in the hypothalamus. Determination of hypophyseal GR concentration after chronic application of RU 38486 was impossible because of loss of tissue.

Data obtained from Scatchard analyses yielded no changes in K_d values of MR and GR binding during the course of the treatment in any of the measured tissues (K_d values for [3 H]aldosterone binding to MR and [3 H]dexamethasone binding to GR were 0.09–0.15 and 1.2–1.5 nM, respectively).

Effect of Chronic Treatment with GR Antagonists on HPA Hormones

Since the changes observed in the brain and pituitary MR and GR concentrations and adrenal weight might be related to alterations in HPA functioning, basal and stress-induced ACTH and corticosterone plasma levels were measured (Figure 6). Basal corticosterone levels were below or slightly over the detection limit. Baseline plasma ACTH levels were unchanged except for a significant rise at 5 weeks in ORG 34850-treated rats (Figure 6a). Chronic treatment with ORG 34850 for 5 weeks resulted in elevated basal plasma ACTH levels. The stress-induced plasma corticosterone response to forced swim stress was enhanced in 5 weeks ORG 34850 and ORG 34116 treated rats. The poststress plasma ACTH response was attenuated after 3 weeks in ORG 34850-treated rats.

DISCUSSION

The present report shows that administration of the selective GR antagonists ORG 34850, ORG 34116, and ORG 34517 causes significant changes in the various components of the HPA axis, involving corticosteroid receptors in distinct regions of the brain and pituitary, the adrenal gland, and secretion of ACTH and corticosterone. In particular, this study indicates that chronic oral application of the selective GR antagonists to rats results in a time-dependent enhancement of hippocampal MR capacity, which corresponds with our previous observations, showing an enhanced hippocampal MR capacity during chronic application of different antidepressants to rats (Reul *et al*, 1993, 1994; Gesing and Reul, in preparation).

Our present data corroborate that the substances have a distinct potency to occupy brain and pituitary GR. All compounds were synthesized as derivatives of RU 38486 and display a higher GR selectivity because of a significant reduction in PR binding (Peeters *et al*, 1998). A single s.c. injection of the three GR antagonists (10 mg/kg body

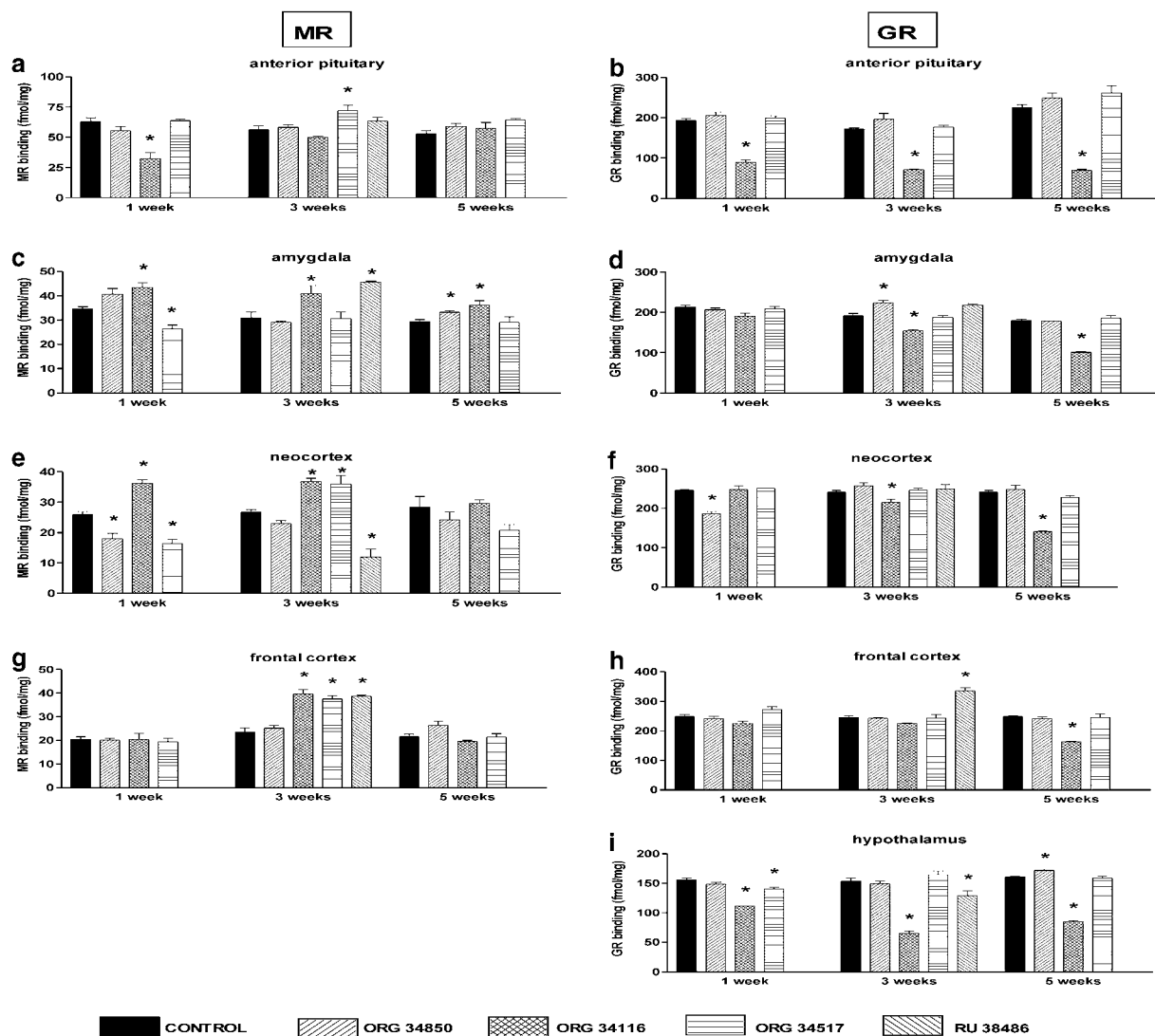


Figure 5 Effect of chronic administration on MR and GR concentration in the anterior pituitary and distinct brain regions. Rats (5–7 per group) received either ORGANON GR antagonists (233 mg substance per 950 g food) or food only. The rats were killed after 3 weeks of treatment. Adrenalectomy and dissection of brain and pituitary tissues was conducted as described in *Materials and methods*. Measurements of MR and GR concentrations, expressed as femtomoles per mg protein (mean \pm SEM), were measured by radioligand binding assay (see *Materials and methods*). Two-way ANOVA: anterior pituitary-MR: effect of duration, $F(2.61) = 3.778$, $p < 0.05$; effect of treatment, $F(4.61) = 17.279$, $p < 0.0005$; effect by interaction of duration and treatment, $F(6.61) = 6.358$, $p < 0.0005$; GR: effect of duration, $F(2.42) = 26.431$, $p < 0.0005$; effect of treatment, $F(3.42) = 142.433$, $p < 0.0005$; effect by interaction of duration and treatment, $F(6.42) = 4.816$, $p < 0.01$; hypothalamus-GR: effect of duration, $F(2.52) = 7.533$, $p < 0.001$; effect of treatment, $F(4.52) = 164.815$, $p < 0.0005$; effect by interaction of duration and treatment, $F(6.52) = 15.645$, $p < 0.0005$, *post hoc* Dunnett's *t*-test; * $p < 0.05$ (vs the control group).

weight) into adrenalectomized rats showed that ORG 34517 was more potent than the others in occupying GR in the pituitary and various brain areas, while all were unable to occupy MR, clearly underscoring the specificity of the GR antagonists. Furthermore, our findings confirmed results from earlier studies indicating that the 11,21-bisaryl steroids displayed a three-fold lower (ORG 34116) and two-fold lower (ORG 34850) binding affinity to human GR as compared to the 11-monoaryl steroid ORG 34517 (Peeters *et al*, 1998). Binding of [3 H]dexamethasone to GR was most significantly reduced in the anterior pituitary, suggesting that penetration of the highly lipophilic GR antagonists was limited by the blood–brain barrier. Hence, the substances may be substrates for the *mdr1a* P-glycoprotein transporter (Cordon-Cardo *et al*, 1989). In

addition, imaging studies have provided evidence for distinct regional differences in cerebral blood flow in rats (Lin *et al*, 1999) and humans (Schneider *et al*, 1996), possibly contributing to a region-dependent availability of the substances.

In spite of their strong potency to occupy brain and pituitary GR, chronic oral administration of ORG 34517 and ORG 34850 resulted only in modest changes of GR levels. In contrast, a significantly reduced binding of [3 H]dexamethasone to brain and pituitary GR of animals chronically treated with ORG 34116 was observed. Different pharmacokinetics of the compounds might account for these different findings, as rats chronically treated with ORG 34116 still had considerably high circulating levels of the compound even 48 h after administration of the drug had

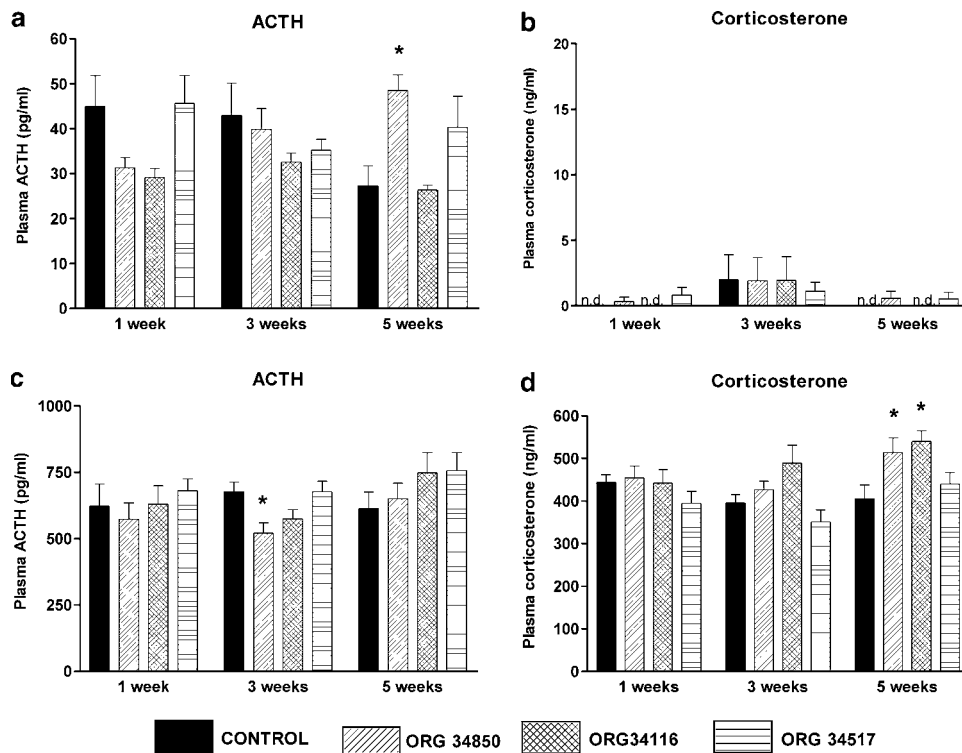


Figure 6 Time course of the effect of GR antagonists on HPA axis activity. Rats (9–15 per group) were treated with GR antagonists (20–25 mg/kg/day) for 1, 3, and 5 weeks. The animals were decapitated either under basal morning conditions or after 15 min forced swim stress, as described in *Materials and Methods*. (a and b) Basal ACTH and corticosterone levels (mean \pm SEM), respectively; (c and d) stress-induced ACTH and corticosterone concentrations (mean \pm SEM), respectively. In some cases, basal corticosterone levels were too low to be detected (ND = not detectable). Two-way ANOVA: ACTH-basal: effect of duration, $F(2,60) = 0.268$, $p > 0.05$; effect of treatment, $F(3,60) = 3.707$, $p < 0.02$; effect by interaction of duration and treatment, $F(6,60) = 3.057$, $p < 0.02$; ACTH-poststress: effect of duration, $F(2,96) = 3.067$, $p = 0.051$; effect of treatment, $F(3,96) = 1.464$, $p > 0.05$; effect by interaction of duration and treatment, $F(6,96) = 0.925$, $p > 0.05$; CORT-poststress: effect of duration, $F(2,95) = 4.491$, $p < 0.02$; effect of treatment, $F(3,95) = 7.024$, $p < 0.0005$; effect by interaction of duration and treatment, $F(6,95) = 1.357$, $p > 0.05$; *post hoc* Dunnett's *t*-test; * $p < 0.05$ (vs the control group).

been terminated (Dr O Epemolu, NV Organon, Oss, The Netherlands). This in contrast to any of the other compounds. Consequently, GR might still have been occupied by ORG 34116 and therefore unavailable for binding of [3 H]dexamethasone. The pharmacokinetics of RU 38486 have been extensively studied (Deraedt *et al*, 1985; Lähteenmäki *et al*, 1987): The substance displays extensive binding to α 1-acid glycoprotein in humans and to albumin (for a review see Bamberger and Chrousos, 1995; Cadepond *et al*, 1997), accounting for its long plasma half-life of 20 h. The pharmacokinetics of ORG 34850 and ORG 34116 have not been investigated by the manufacturer. Moreover, no reports describing the pharmacokinetic properties of the selective GR antagonists have been published so far. However, ORG 34517 does not bind to CBG and its $t_{1/2}$ in humans is 13–20 h (BWMM Peeters, personal communication). However, a rapid metabolization and a pronounced first pass effect of ORG 34517 and ORG 34850 could result in only transiently increased GR antagonist levels, but apparently such episodic antagonism seems not sufficient to cause a significant upregulation of GR capacity. Therefore, our results demonstrated that application of GR antagonists does not induce conditions observed after adrenalectomy, which has been shown to upregulate GR levels (Reul *et al*, 1987).

Even though no significant effects on hippocampal GR capacity could be observed, chronic treatment with ORG

34517 for 3 weeks and bisarylsteroids at almost all time points resulted in significantly increased MR levels in the hippocampus and various other brain areas (cortex, amygdala) via a yet unknown mechanism. Herman and Spencer (1998) demonstrated that the hippocampal GR is subject to heterologous regulation by MR. Reul *et al* (1987) presented data pointing to a vice versa regulation, as administration of the GR agonist dexamethasone resulted in increased hippocampal MR levels. However, regulation of MR expression in the hippocampus is very complex, involving monoaminergic and serotonergic fibers from various brain regions and growth factors. Gesing *et al* (2001) showed that CRH increases hippocampal MR expression. In line with our findings, Spencer *et al* (1998) reported an increase in hippocampal MR levels after administration of the highly selective GR antagonist RU 40555. Furthermore, RU 38486 has been shown to control MR expression via human PR (McDonnell *et al*, 1994). Previously, Reul *et al* (unpublished) observed that MR upregulation was triggered by the MR antagonist spironolactone via an intrinsic mechanism, because the effect was seen in adrenalectomized rats. In contrast, the fact that we did not observe any GR upregulation upon GR antagonist binding suggests the absence of such an intrinsic mechanism in GR regulation. Thus, it appears that MR and GR antagonists exert different interactions with their respective receptors. The data of Trapp and Holsboer (1995) indicate

that binding of an agonist *vs* an antagonist to MR induces distinct conformational changes, as proteolysis of the antagonist-bound MR resulted in smaller fragments than proteolysis of the agonist-occupied MR. Correspondingly, Brzozowski *et al* (1998) and Shiau *et al* (1998) postulated that the estrogen receptor (ER) antagonists raloxifene and tamoxifen exert their antagonistic properties by inducing a different conformation of the ER ligand binding domain (LBD), and, by that, blocking coactivator binding, resulting in a reduced transactivation capacity. No studies on the molecular effects of specific GR antagonists have been published. Nevertheless, induction of distinct changes in the conformation of GR after binding of the respective GR antagonists could lead to distinct differences in interactions at the genomic level.

The data demonstrate that the studied antagonists had substantial effects on the physical parameters: ORG 34116 and ORG 34850 exerted protective effects on thymus involution, induced by endogenous glucocorticoids, after a treatment period of 1 week and 3 weeks. Interestingly, also long-term treatment with the tricyclic antidepressant amitriptyline has been shown to act protective on thymus involution (Steckler *et al*, 1999), possibly as a result of its dampening effects on glucocorticoid secretion (Reul *et al*, 1993). In animals treated with the 11,21-bisaryl steroids ORG 34850 and ORG 34116, adrenal weight was enhanced at all time points. This observation points to an enhanced corticotrophic influence during chronic administration, suggesting disinhibition of HPA axis activity in the 11,21-bisarylsteroid-treated groups. In contrast, treatment with ORG 34517 did not cause any changes in HPA axis activity, as ORG 34517-treated animals showed no alterations in physical parameters and hormone levels, suggesting a mild antagonistic potency of this compound. Their different GR antagonistic properties of the compounds may be a reflection of the different pharmacological characteristics, that is, metabolism, half-life, and tissue penetration. As mentioned, the pharmacokinetics of ORG 34116 and ORG 34517 have not been investigated, and very limited data have been published on the newly developed compounds so far. Chronic administration of RU 38486 for 3 weeks induced thymus involution, corresponding with its catabolic effect on body weight; both findings are most likely because of the compound's partial GR agonistic properties (Gruol and Altschmied, 1993; Havel *et al*, 1996). However, no evidence for partial agonism could be found after chronic treatment with the selective GR antagonists.

Our hormone measurements, demonstrating unchanged baseline early morning levels, do not support a disinhibition of the HPA axis during the circadian trough. They correspond with several studies reporting no effect of acute or chronic treatment with RU 38486, administered icv or locally, on basal morning trough ACTH and corticosterone levels (De Kloet *et al*, 1988; Ratka *et al*, 1989; Van Haarst *et al*, 1997), whereas basal corticosterone concentrations during the diurnal peak and after novelty stress were significantly increased (Van Haarst *et al*, 1996, 1997). These results are in line with the notion that MRs mediate the effects of circadian trough basal levels of corticosteroids, whereas GRs mediate the effects of high glucocorticoid levels during the circadian peak and stress (for a review see De Kloet *et al*, 1998; Reul *et al*, 2000). This concept could

also elucidate why we did not observe any effects of the specific GR antagonists on basal morning corticosterone levels: During the circadian trough GRs are only poorly occupied by low circulating corticosterone levels (Reul and De Kloet, 1985), and, thus, GR antagonists do not generate 'antagonism', therefore not leading to any alterations in plasma hormone concentrations. Enhanced hippocampal MR concentrations during ongoing GR antagonist treatment could additionally account for a dampening of ACTH and corticosterone secretion during the circadian trough. Moreover, rats consumed most of the antagonists during the night, presumably resulting in higher nocturnal GR antagonist plasma concentrations. Thus, our data suggest that GR antagonism occurred episodically, that is, during the nocturnal peak and stress. These preclinical observations correspond with a clinical study showing HPA axis disinhibition after oral administration of RU 38486 during the morning peak of HPA axis activity in humans (Gaillard *et al*, 1984). However, several investigations on chronic oral administration of RU 38486 (200 mg/day or 10 mg/kg-day, respectively) to volunteers demonstrated an overall enhanced activity of the HPA axis, reflected by increased circulating plasma ACTH and cortisol concentrations, and enhanced urinary cortisol secretion (Laue *et al*, 1989; Lamberts *et al*, 1991).

Furthermore, adrenocortical sensitivity to ACTH shows a characteristic circadian rhythm with a decreased sensitivity to ACTH during the morning trough in rats (Nicholson *et al*, 1985; Dallman *et al*, 1987). As we measured the basal hormone levels only in the morning, the obtained low corticosterone levels may also reflect decreased adrenocortical sensitivity to ACTH. If we had measured basal ACTH and corticosterone levels during the secretion peak, we might have obtained increased corticosterone levels despite low ACTH, reflecting increased adrenocortical sensitivity (Van Haarst *et al*, 1996). However, our findings of increased adrenal weight, corresponding with increased stress-induced corticosterone plasma concentrations after 3 and 5 weeks of chronic ORG 34850 and ORG 34116 administration despite reduced poststress ACTH levels after 3 weeks, suggest that the zona fasciculata of the adrenal gland displayed a gradually enhanced sensitivity to circulating ACTH plasma levels with ongoing chronic treatment in those groups, but not in animals treated with ORG 34517. This notion is consistent with a previous study (Van Haarst *et al*, 1996) pointing to a shift towards elevated adrenal responsiveness to ACTH after chronic icv infusion of RU 38486 (100 ng/h). Moreover, several studies have provided strong evidence that adrenocortical sensitivity is modulated by the autonomic nervous system: Jasper and Engeland (1994) obtained increased ultradian and stress-induced corticosterone plasma concentrations in splachniectomized animals. Furthermore, Korte *et al* (1993) demonstrated that blockade of brain MR and GR after icv application of RU 28318 (a selective MR antagonist) and RU 38486, respectively, resulted in an attenuated CRH-induced increase in heart rate and adrenaline plasma concentrations.

We examined the effects of the highly selective GR antagonists ORG 34850, ORG 34116, and ORG 34517 on the rat HPA axis. Several clinical studies point to HPA axis hyperactivity as an important component in the etiology of

major depression. In this light, the studied selective GR antagonists were developed as a potential therapy for disorders associated with cortisol hypersecretion including major depression. Depressed patients typically show increased HPA activity with enhanced CRH drive and a reduced GR feedback (for a review see Holsboer, 2000). Therefore, GR antagonist administration at moderate dosages was preferred to high-dose administration, which could have led to HPA axis disinhibition. A dose, at which disinhibition would occur, would also be unfavorable to be applied in patients. Overall, chronic oral application of any compound to rats caused a time-dependent enhancement of hippocampal MR capacity, corresponding with our previous observations, which showed an enhanced hippocampal MR capacity during chronic application of different antidepressants to rats (Reul *et al*, 1993, 1994; Gesing and Reul, in preparation). Moreover, our present finding of significantly increased hippocampal MR capacity after treatment with RU 38486 may correspond with several clinical reports suggesting that treatment with RU 38486 may be effective in psychotic major depression (Van der Lely *et al*, 1991; Belanoff *et al*, 2001). Increased hippocampal MR capacity was postulated to be the initial phenomenon in a cascade of successive changes in the HPA axis during the course of a successful antidepressant therapy. However, animals treated with ORG 34116 and ORG 34850 showed increased adrenal weight and elevated poststress corticosterone plasma levels, pointing to—at least episodically—enhanced HPA activity. In contrast, treatment with ORG 34517 did not cause any enhancement in HPA axis activity. In addition to enhancing hippocampal MR capacity, ORG 34517 showed the strongest potency to occupy GR and did not cause any significant changes in HPA activity. Therefore, ORG 34517 might be a promising therapeutic strategy in the treatment of major depression. Indeed, in preliminary clinical trials, this compound has shown antidepressive effects in depressed patients (Peeters, personal communication 2001).

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