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Psychological Stress Increases Hippocampal Mineralocorticoid Receptor Levels: Involvement of Corticotropin-Releasing Hormone

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We investigated whether acute stressors regulate functional properties of the hippocampal mineralocorticoid receptor (MR), which acts inhibitory on hypothalamic-pituitary-adrenocortical activity. Exposure of rats to forced swimming or novelty evoked a significant rise in density of MR immunoreactivity in all hippocampal subfields after 24 hr, whereas exposure to a cold environment was ineffective. Time course analysis revealed that the effect of forced swimming on MR peaked at 24 hr and returned to control levels between 24 and 48 hr. In pyramidal neurons of CA2 and CA3, marked rises were already observed after 8 hr. Radioligand binding assays showed that corticotropin-releasing hormone (CRH) injected intracerebroventricularly into adrenalectomized rats also produced a rise in hippocampal MR levels; an effect for which the presence of corticosterone, but not dexamethasone, at the time of injection was a prerequisite. Moreover, pretreatment with the CRH receptor antagonist (D-Phe^{12},Nle^{21,38},α-Me-Leu^{37})-CRH$_{12–41}$ blocked the effect of forced swimming on hippocampal MR levels. To investigate whether the rise in MR levels had any functional consequences for HPA regulation, 24 hr after forced swimming, a challenge test with the MR antagonist RU 28318 was conducted. The forced swimming exposed rats showed an enhanced MR-mediated inhibition of HPA activity.

This study identifies CRH as an important regulator of MR, a pathway with marked consequence for HPA axis regulation. We conclude that the interaction between CRH and MR presents a novel mechanism involved in the adaptation of the brain to psychologically stressful events.

Key words: mineralocorticoid receptor; HPA axis; corticotropin-releasing hormone; ACTH; glucocorticoid hormone; hippocampus; stress

Glucocorticoid hormones represent the endproduct of the hypothalamic-pituitary-adrenocortical (HPA) axis. They play a principal role in energy metabolism, growth processes, immune function, neuroendocrine control, and brain function, including learning and memory processes underlying behavioral adaptation. Their regulation of the HPA axis has been classified as a negative feedback action and as a tonic inhibitory influence (De Kloet and Reul, 1987; De Kloet et al., 1998). Basically, these two modes of glucocorticoid action are mediated by a dual glucocorticoid-binding receptor system, i.e., the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (De Kloet and Reul, 1987; De Kloet et al., 1998), which act as ligand-dependent transcription factors (Evans and Arriza, 1989). The negative feedback is mediated by GRs at the hypothalamic and pituitary level of the HPA axis (Antoni, 1986; De Kloet and Reul, 1987; Dallman et al., 1987; De Kloet et al., 1998) and in suprahypothalamic structures (Meaney et al., 1996) to restrain circadian-driven and stress-induced elevations in HPA activity. The tonic inhibitory influence of these steroid hormones on HPA activity is exerted via MRs, which are mainly localized in pyramidal ($CA_{1–4}$) and granular (dentate gyrus) neurons of the hippocampus (Gerlach and McEwen, 1972; Herman et al., 1989). This limbic structure restrains HPA activity indirectly via stimulation of inhibitory GABAergic neurons located in the ventrolateral septal region and the bed nucleus of the stria terminalis (BNST), which project to corticotropin-releasing hormone (CRH)-containing parvocellular neurons of the hypothalamic paraventricular nucleus (Herman and Cullinan, 1997). Beside HPA regulation, MRs affect serotonergic neurotransmission (Joëls et al., 1991; De Kloet et al., 1998), electrophysiological events such as neuronal excitability (Joëls and De Kloet, 1990) and long-term potentiation (Pavlides et al., 1994), and behavioral responses (Oitzl et al., 1994; Smythe et al., 1997; Bitran et al., 1998).

The concept on the tonic inhibitory function of hippocampal MR on the activity of the HPA axis stems primarily from receptor occupancy studies. These studies showed that, because of the high affinity for endogenous glucocorticoids ($K_d \cong 0.1–0.5$ nM), MRs are >80% occupied already at the trough of the diurnal HPA cycle (Reul and De Kloet, 1985; Reul et al., 1987a, 1990; Spencer et al., 1990). This concept was further substantiated by the observation that intracerebroventricular and intrahippocampal injection of the synthetic MR antagonist RU 28318 resulted in an elevation of baseline corticosterone levels (Ratka et al., 1989; Oitzl et al., 1995; Van Haarst et al., 1997). However, the situation of a receptor (i.e., MR), which is always to a large extent occupied by hormone, prompts the question whether we are dealing with a static or a dynamic receptor system. A static system would be merely playing a cofactor function, whereas, in contrast, a dynamic receptor system would be responding, in terms of its capacity and function, rapidly to changing requirements. For GR this is much less relevant because this receptor becomes occupied in a graded manner by glucocorticoids during different physiological conditions (cf. circadian trough and peak, stress) (Reul and De Kloet, 1985; Reul et al., 1987a, 1990). Therefore, with regard

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to MR, we contemplated that, if the function of MR were to respond adequately to changing physiological conditions and needs, then an appropriate means would be to dynamically change its receptor capacity. Here, we show that an acute psychologically stressful experience raises hippocampal MR density, an event that is associated with an increased MR-mediated inhibition of HPA activity.

MATERIALS AND METHODS

Animals and surgery

Male Wistar rats (weight at time of experiment 220–240 gm) were group-housed six per cage with *ad libitum* access to food and water in a light (lights on from 6.00 A.M. until 20.00 P.M.)-, temperature (22 ± 1°C)-, and humidity (55 ± 5%)-controlled animal room. Animals with intracerebroventricular cannulas were housed singly. All rats were handled at least 7 d (~3 min/rat per day) before the day of the experiment. All experimental protocols were approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany.

In some experiments, bilaterally adrenalectomized rats were used. Adrenalectomy (ADX) was performed aseptically under halothane anesthesia 1 d before application of stress or intracerebroventricular injection. After adrenalectomy, rats were given 0.9% saline in their drinking water and, in some experimental groups, corticosterone (15 μg/ml) or dexamethasone (5 μg/ml) was added as well. Steroids were first dissolved in ethanol before being added to the drinking solution (final concentration 0.5% ethanol). In some experiments, rats were equipped with an intracerebroventricular cannula. This operation was conducted 1 week before the experiment under halothane anesthesia using a stereotactic instrument (coordinates: lateral, −1.2 mm; anteroposterior, +0.5 mm).

Experimental procedures

Effect of forced swimming, cold exposure, and novelty on hippocampal MR immunoreactivity. All experiments were started between 7:00 and 9:00 A.M. To determine the effect of forced swimming on MR immunoreactivity, rats were placed in a glass beaker containing water (height, 20 cm) at 25°C for 15 min. Thereafter, they were dried and returned to their home cage. To induce novelty stress, rats were placed singly in a new cage for 30 min. Cold exposure consisted of placing the animals singly in a cage at 4°C for 4 hr without access to food and water. Thereafter rats were returned to their home cages. Control animals were kept undisturbed in their home cages. Twenty-four hours later rats were killed by decapitation under quiet conditions. Whole brains to be used for MR immunohistochemistry (see below) were snap-frozen in isopentane at −40°C and deep-frozen in dry-ice.

In a separate experiment, a time course was determined for the effect of forced swimming on MR immunoreactivity in the hippocampus. Therefore, rats were killed at 8, 24, or 48 hr, or at 7 d after forced swimming.

Effect of CRH on hippocampal MR and GR binding: involvement of glucocorticoids. To assess whether CRH could mimic the effects of stress on MR, 1 d ADX rats were injected intracerebroventricularly with CRH (3 μg in 10 μl saline) or saline only. To test for glucocorticoid involvement, separate groups of ADX rats were supplemented with corticosterone, dexamethasone, or no-steroid via the drinking solution from the time of surgery until the time of injection. Immediately after the intracerebroventricular injection, glucocorticoids were withdrawn from the animals' drinking solution because they would hamper the receptor binding assay (see below). Twenty-four hours after injection, rats were killed under quiet conditions to prevent acute unspecific stress. Next, various regions (to be used in the *in vitro* MR and GR binding assay) were dissected from the brain and frozen in liquid nitrogen. Plasma was prepared from trunk blood to check for the completeness of adrenalectomy.

Effect of forced swimming on MR: intermediary role of CRH. Corticosterone-substituted 1 d ADX rats were subjected to forced swimming (15 min at 25°C) or left untouched in their home cages (i.e., control). Ten minutes before the forced swimming procedure, rats received an intracerebroventricular injection with either CRH receptor antagonist (D-Phe^{12},Nle^{21,38},α-Me-Leu^{37})-CRH$_{12–41}$ (D-Phe-CRH$_{12–41}$; 5 μg in 10 μl saline) or vehicle. Directly after forced swimming, the corticosterone-containing drinking solution was replaced by saline. Rats were killed 24 hr later. The brain was dissected, and various parts (for *in vitro* MR and GR binding assay) were frozen in liquid nitrogen.

RU 28318 challenge test. To determine whether the changes in MR levels in the hippocampus resulted in an altered MR-mediated tonic inhibitory control of the HPA axis, a challenge test with the specific MR antagonist RU 28318 was conducted. Rats were subjected to forced swimming or left undisturbed. Twenty-four hours later, RU 28318 (100 ng in 0.5% ethanol/saline) or the vehicle was injected intracerebroventricularly, and rats were decapitated 30 min later. After decapitation, blood was collected in ice-chilled EDTA-coated Trasylol-containing tubes, and plasma was prepared to be used for radioimmunoassay for ACTH and corticosterone content (Reul et al., 1993).

Immunohistochemistry

Twenty micrometer coronal brain cryosections were mounted on polylysine-coated slides and post-fixed for 30 min in 4% paraformaldehyde. Subsequent immunohistochemical staining was performed with the avidin–biotin–peroxidase system (Elite ABC goat kit; Vector Laboratories, Burlingame, CA) and diaminobenzidine/Ni^{+} as substrate according to company instructions. Detection of MR was achieved using a primary polyclonal goat anti-MR antibody (N-17; dilution 1:800; Santa Cruz Biotechnology, Santa Cruz, CA). As negative controls, purified IgGs from normal goat (Santa Cruz) serum were used. Specificity of primary antibodies was checked by both antigen preabsorption and Western analysis (data not shown). The immunohistochemical signal of MR-IR was quantified using a digital video image analyzer (Optimas System, Puchheim, Germany). The staining intensity (i.e., gray values) of nuclei of all neurons of the pyramidal and granular cell layers within an area of 320 × 410 μm (as shown in Fig. 1) was analyzed. From each animal, at least three sections of independent immunohistochemical assays were evaluated. The net immunohistochemical staining was determined by subtraction of the background signal from the nuclear staining signal. Data are presented as mean gray values ± SEM of six rats per group, except for the cold stress experiment (n = 4–5).

Receptor binding assay

The MR and GR binding assay was conducted as described (Reul et al., 1993). Briefly, pooled brain tissues were homogenized (100 mg tissue/ml; 10 strokes at 900 rpm) in ice-cold 5 mM Tris-HCl, pH 7.4, containing 5% glycerol, 1 mM EDTA, 10 mM sodium molybdate, 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°C to obtain cytosol (i.e., the supernatant fraction). Aliquots of cytosol were incubated for 20 hr at 4°C with [^{3}H]-aldosterone and [^{3}H]-dexamethasone at a concentration range of 0.05–10 nM to measure MR and GR, respectively. In [^{3}H]-aldosterone-containing incubations, a 100-fold excess of the specific GR ligand RU 28362 was included to block binding of [^{3}H]-aldosterone to GR. The binding of [^{3}H]-dexamethasone to MR was evaluated by adding a 100-fold excess of RU 28362. Nonspecific binding for MR and GR was determined by inclusion of a 1000-fold excess of corticosterone and dexamethasone, respectively. Bound and free [^{3}H]-steroid were separated by gel filtration on Sephadex LH-20 (Pharmacia, Uppsala, Sweden), and bound radioactivity was measured by liquid scintillation counting. Protein content was determined by the method of Lowry using BSA as a standard. The binding data were expressed as femtomoles per milligram of protein, and nonspecific binding was subtracted from total binding to yield specific binding. GR levels were calculated by subtraction of nonspecific binding as well as binding of [^{3}H]-dexamethasone to MR. Total binding (B_{max}) and binding affinity (K_d) were derived from Scatchard analysis.

Radioimmunoassay

Blood samples were centrifuged at 4°C for 10 min, and plasma aliquots were stored at −80°C for analysis by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA) as described previously (Reul et al., 1993). The interassay and intra-assay coefficients of variance for ACTH were 7 and 5%, respectively, with a detection limit of 2 pg/ml. For corticosterone, the interassay and intra-assay coefficients of variance were 7 and 4%, respectively, with a detection limit of 1.5 ng/ml.

Statistics

Data were analyzed with Student's t test, one-way ANOVA followed by a Dunnett's t test, or two-way ANOVA followed by a *post hoc* Duncan multiple range test. The experimental data were considered to be statistically different from control data when $p < 0.05$.

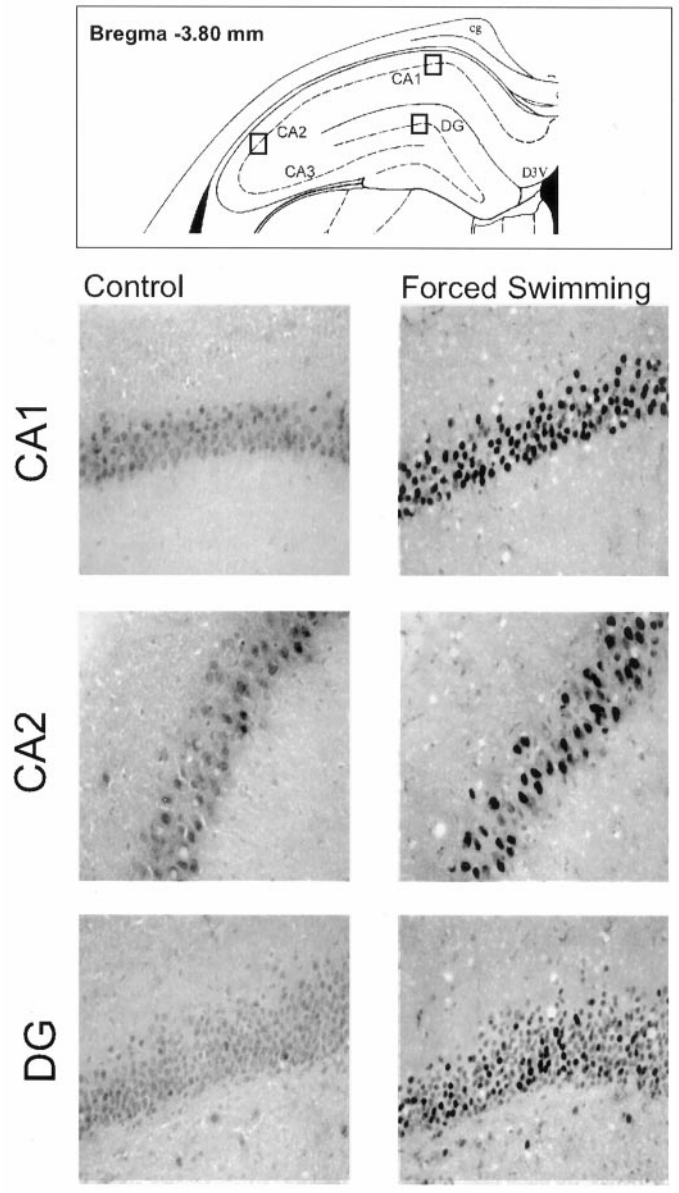

Figure 1. Increase in MR-IR in neuronal nuclei of CA1, CA2, and dentate gyrus (DG) 24 hr after a 15 min session of forced swimming (water temperature, 25°C). Representative immunohistochemical pictures are shown together with their localization in the hippocampus at level −3.8 mm of bregma. All images are equally magnified (200×). Please note the nuclear localization of MR in the pyramidal and granular layers. *cg*, Cingulum bundle; *D3V*, dorsal part of third ventricle. For quantitative analyses, see Figure 2.

RESULTS

Effect of forced swimming, cold exposure, and novelty on hippocampal MR immunoreactivity

Rats were subjected to a single forced swim session, and 24 hr later MR density in hippocampus was determined by semiquantitative immunohistochemistry. Analysis of MR immunoreactivity (MR-IR) in rat brain sections revealed a similar distribution, as previously reported using *in vitro* autoradiography and *in situ* hybridization (Reul and De Kloet, 1986; Herman et al., 1989). The hippocampus proved to be the richest source of MR-IR with highest levels in the pyramidal neurons. Within the different regions, a heterogeneous intensity of (nuclear) staining among neurons was observed (Fig. 1). Twenty-four hours after forced swimming, a rise in the average staining intensity of MR-IR was found in all cell layers of the hippocampus (Fig. 1), which was confirmed by semiquantitative image analysis (Fig. 2*A*). It was, however, evident that also after stress a marked heterogeneity in signal intensity among neurons was maintained. Beside forced swimming, we also found novelty stress, a mild psychological stressor (Fig. 2*B*), but not cold exposure (Fig. 2*C*), to increase hippocampal MR expression.

Time course analysis of the effect of forced swimming on MR levels in the cellular subfields of the hippocampus revealed significant rises at 24 hr, whereas in CA2 and CA3 also at 8 hr significant elevations could be observed (Fig. 3). At 48 hr, in all subfields MR levels had returned to baseline values.

Effect of CRH on hippocampal MR and GR binding: involvement of glucocorticoid hormones

Using a radioligand binding method, we investigated whether CRH would mimic the effects of forced swimming and novelty on hippocampal MR levels. With this method, we could also determine GR levels. Because corticosteroid receptor binding characteristics (i.e., B_{max}, K_d) can only be reliably determined in corticosteroid-free tissue, initial experiments were conducted in adrenalectomized rats. This approach also allowed us to control for CRH-induced (and stress-induced, see below) endogenous secretion of glucocorticoids, which are known to regulate brain MR and GR levels (Reul et al., 1987b; Spencer et al., 1991).

Intracerebroventricular injection of CRH produced no significant effect on hippocampal MR levels (Fig. 4*A*). We contemplated that endogenous corticosterone might be required to permit a CRH-evoked rise in receptor levels. Therefore, we supplemented the drinking solution of the ADX rats with a physiological dose of corticosterone (i.e., 15 μg/ml) until we gave the CRH–vehicle injection, after which a normal, steroid-free solution was given. Indeed, in corticosterone-substituted animals, CRH evoked a marked rise in hippocampal MR levels (Fig. 4*A*). In contrast, if rats were substituted with the selective GR agonist dexamethasone, CRH produced a profound decrease in MR levels (Fig. 4*A*). However, hippocampal tissue of dexamethasone-substituted control rats showed higher levels of MR than no-steroid and corticosterone-substituted animals; a well known, but still unclarified phenomenon (Reul et al., 1987b, 1989). At any rate, dexamethasone application obviously did not allow a CRH-induced rise of MR, suggesting that the effect of CRH on this receptor type involved a permissive glucocorticoid effect via MR and not GR. CRH application did not affect GR levels (Fig. 4*B*). In none of the experiments the ligand binding affinity of either MR or GR was affected (data not shown).

Effect of forced swimming on MR: intermediary role of CRH

We next investigated whether the effect of forced swimming would be evoked by a CRH receptor-mediated action. Given the necessity of the presence of corticosterone in the previous experiment, ADX rats were substituted with corticosterone until the forced swim session. Forced swimming evoked a marked rise in MR levels (Fig. 5), confirming the data obtained with immunohistochemistry (Figs. 1, 2). No effects were apparent on GR (data not shown). The same results were found using *in vitro* autoradiography of MR binding in brain sections (data not shown). Pretreatment of rats intracerebroventricularly with D-Phe-CRH_{12-41} 10 min before forced swimming blocked the effect of

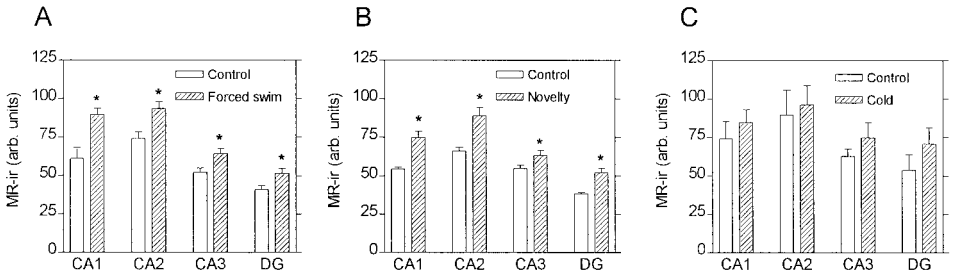

Figure 2. Effect of forced swimming (15 min at 25°C; *A*), novelty (30 min in new cage; *B*), and cold exposure (4 hr at 4°C; *C*) on MR-IR in neuronal nuclei of CA1, CA2, CA3, and DG after 24 hr. Optical densities of neuronal nuclei were determined by an image analysis program. MR-IR data are expressed as arbitrary units (*net gray level*). Data in *A* ($n = 6$ rats), *B* ($n = 6$), and *C* ($n = 4$–5) are presented as mean ± SEM. $^*p < 0.05$, if compared with respective control (Student's *t* test).

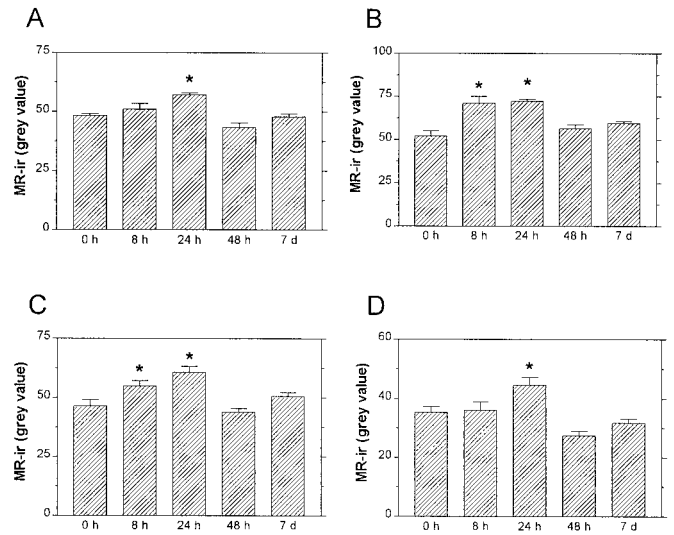

Figure 3. Time course analysis of the effect of forced swimming on MR-IR in neuronal nuclei of CA1 (*A*), CA2 (*B*), CA3 (*C*), and DG (*D*). Rats ($n = 6$ per group) were killed under early morning baseline conditions (*0 h*) or 8 hr, 24 hr, 48 hr, or 7 d after a single forced swimming session (15 min at 25°C). $^*p < 0.05$ if compared with 0 hr controls, *post hoc* Dunnett's test.

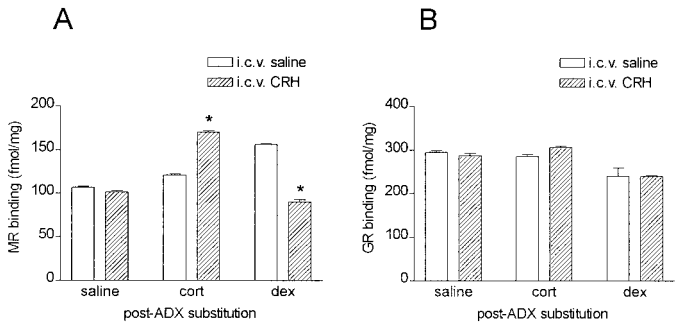

Figure 4. Effect of intracerebroventricular injection of saline or 3 μg of CRH on MR (*A*) and GR (*B*) binding in hippocampus of corticosterone, dexamethasone, and nonsubstituted ADX rats. After the CRH injection procedure, rats received no-steroid-containing 0.9% NaCl in tap water to drink. All animals were killed 24 hr later, and hippocampal MR and GR binding was determined by a radioligand binding assay (see Materials and Methods). Data (femtomoles per milligram of protein) are expressed as mean ± SEM of five independent experiments. $^*p < 0.05$, if compared with the respective intracerebroventricular saline group (*post hoc* Duncan multiple range test).

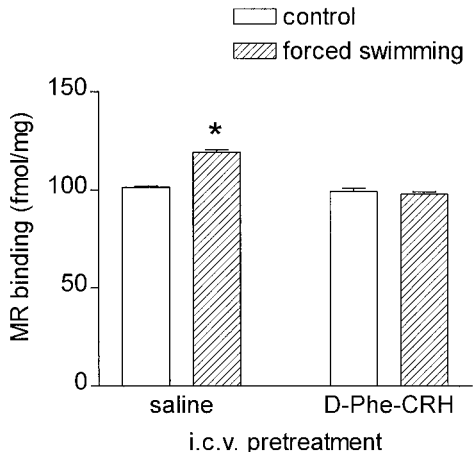

Figure 5. Effect of forced swimming on hippocampal MR is mediated by the CRH receptor. Ten minutes before a 15 min forced swimming session (water temperature 25°C), corticosterone-substituted ADX rats were intracerebroventricularly injected with 5 μg of D-Phe-CRH_{12-41} or vehicle. After the forced swimming procedure, rats received no-steroid-containing 0.9% NaCl in tap water to drink. All animals were killed 24 hr later, and MR binding was determined by a radioligand binding assay. Data (femtomoles per milligram of protein) are expressed as mean ± SEM of five independent experiments. $^*p < 0.05$, if compared with the respective intracerebroventricular saline group (*post hoc* Duncan multiple range test).

the stressor on MR levels (Fig. 5), strongly suggesting an important intermediary role of CRH receptors. In addition to hippocampus, forced swimming evoked increases in MR concentrations in neocortex, frontal cortex, and amygdala (but not hypothalamus) that were completely abolished by D-Phe-CRH_{12-41} (Table 1). Thus, the effect of forced swim stress on MR levels is confined to extrahypothalamic limbic and neocortical brain regions and is mediated by an action via CRH receptors within the brain.

Forced swimming-induced rise in hippocampal MR levels: implications for HPA axis activity

The hippocampal MR mediates a tonic inhibitory influence of low levels of corticosterone on the HPA axis, as was shown in neuroendocrine challenge tests in rats and humans using MR antagonists such as RU 28318 and spironolactone (Ratka et al., 1989; Deuschle et al., 1998). Pharmacologically, the action of the antagonists is based on the high occupancy of MRs by endogenous glucocorticoids already at the trough of HPA activity (Reul and De Kloet, 1985; De Kloet and Reul, 1987; Reul et al., 1987a, 2000; Spencer et al., 1990). Hence, application of MR antagonists evolves in transient rises in plasma ACTH and glucocorticoid hormone levels (Ratka et al., 1989). We applied an RU 28318 challenge to explore the functional significance of stress-induced elevations in hippocampal MR. In control rats, RU 28318 injection led to increases in plasma ACTH and corticosterone, but they did not reach statistical significance (Fig. 6). However, in rats stressed by forced swimming 24 hr before, the MR antagonist produced marked rises in plasma corticosterone ($p < 0.05$) and

Table 1. Blockade of forced swimming-induced rise in MR levels by D-Phe-CRH$_{12-41}$

	Saline/control (i.c.v.)	Saline/stress (i.c.v.)	D-Phe-CRH$_{12-41}$/control (i.c.v.)	D-Phe-CRH$_{12-41}$/stress (i.c.v.)
Hippocampus	101.3 ± 0.6	119.1 ± 1.2*	99.2 ± 1.6	97.8 ± 1.2
Neocortex	21.3 ± 1.6	26.9 ± 0.3*	21.8 ± 0.7	21.8 ± 0.2
Amygdala	27.8 ± 0.7	33.1 ± 0.4*	29.2 ± 1.4	31.0 ± 0.9
Frontal cortex	14.7 ± 0.4	23.8 ± 0.3*	14.7 ± 0.2	15.7 ± 1.3
Hypothalamus	16.8 ± 0.7	16.2 ± 0.3	14.2 ± 0.2	14.7 ± 0.5

Corticosterone-substituted adrenalectomized rats (n = 6/group) were intracerebroventricularly injected with either 5 μg of D-Phe-CRH$_{12-41}$ or saline 10 min before a 15 min forced swim session. No-swim controls were left undistributed in their cages after the injection. After the injection/stress session, all animals received steroid-free saline as drinking solution. We killed all animals 24 hr later. MR levels in indicated brain tissues were measured by radioligand binding. Results (B_{max} values) are shown of five independent experiments and are expressed as femtomoles per milligram of protein (means ± SEM). *$p < 0.05$, if compared with the intracerebroventricular saline group (*post hoc* Duncan multiple range test).

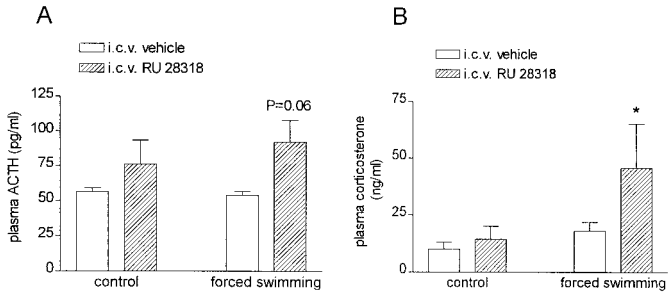

Figure 6. Increased MR-mediated HPA axis inhibition 24 hr after forced swimming. Twenty-four hours after a 15 min forced swim period, rats were intracerebroventricularly injected with the MR antagonist RU 28318 (100 ng in 10 μl of 0.5% ethanol/saline) or vehicle. Animals were decapitated 30 min later, and trunk blood was collected. Data (n = 5–7) on ACTH (in picograms per milliliter) (*A*) and corticosterone (in nanograms per milliliter) (*B*) plasma concentrations are presented as mean ± SEM. *$p < 0.05$, if compared with respective intracerebroventricular vehicle (*post hoc* Duncan multiple range test).

ACTH (p = 0.06) (Fig. 6). Thus, stress-induced rises in MR density are associated with profoundly higher hormonal responses to an MR antagonist challenge. This observation indicates that within 24 hr after a stressful experience an enhancement occurs in the MR-mediated tonic inhibitory control of the HPA axis.

DISCUSSION

Here, we showed that psychologically stressful events such as novelty and forced swimming induced a rise in hippocampal MR levels that was associated with changes in the regulatory control of the HPA axis. Elevations in MR density were evident in nuclei of pyramidal and granular neurons in all hippocampal subfields. The stress-induced rises were transient and peaked at 24 hr, whereas in CA2 and CA3, significant elevations were already seen at 8 hr. Notably, CRH mediated the effect of forced swim stress on MR for which the presence of corticosterone was a prerequisite. In addition to the hippocampus, forced swimming-induced, CRH receptor-mediated rises in MR were observed in the amygdala and neocortical regions, including the frontal cortex. The effects were specific with regard to MR, because no effects on GR levels were found. The rise in MR was associated with an increased, MR-mediated tonic inhibition of HPA activity. Thus, the capacity and function of MR is under a dynamic control participating in HPA and, most likely, other changes in the brain after a stressful challenge.

Apart from neuroanatomical specificity, the response in MR was stressor-specific. Forced swimming and novelty stress evoked an increase in MR density, whereas cold exposure was ineffective. This stressor specificity may reflect the involvement of limbic and neocortical forebrain structures required for appropriate interpretation of the situation, which is in line with the concept on the differential circuitry involved in the processing in the brain of psychological (cf. novel environment, forced swimming) versus physical (cf. cold exposure) stress (Herman and Cullinan, 1997). This notion is underlined by the observation that stress-evoked rises were only observed in extrahypothalamic limbic and neocortical brain regions. Previously, restraint stress was shown to reduce MR heteronuclear RNA, but not MR mRNA, in DG and CA1 within 1–2 hr (Herman and Watson, 1995). Our preliminary data show that forced swimming did not affect MR mRNA levels in the rat hippocampus (data not shown). In tree shrews subjected to chronic psychosocial stress, within the hippocampus elevations as well as reductions in MR mRNA levels were observed (Meyer et al., 2000). In conjunction with our data, this suggests that psychological stress differentially affects MR gene transcription and translation.

We found that CRH plays an important role in the effect of forced swimming on the density of MR in the hippocampus and other extrahypothalamic parts of the brain. CRH might have evoked this effect on MR directly or indirectly. A direct action of CRH may have evolved via CRH receptors that are known to be present in the hippocampus, neocortex, and amygdala (Chalmers et al., 1995). Indirectly, CRH activates several neurotransmitter systems, including the serotonergic (Linthorst et al., 1999) and noradrenergic systems (Curtis et al., 1997), which exert positive effects on corticosteroid receptor expression (Mitchell et al., 1990; Seckl et al., 1990; Maccari et al., 1992; Vedder et al., 1993). Our study identifies CRH as an important regulator of MR expression in certain brain areas. Conceptually, this interaction presents a novel element in the function of CRH in the stress response. Until now, CRH is regarded as the key mediator of neuroendocrine, autonomic, and behavioral responses to stress (Owens and Nemeroff, 1991; Holsboer, 1999). Evidently, by its effects on MR expression, CRH with regard to its time point acts beyond the acute phase of the stress response and participates in the regulation of a primary control instrument of the HPA axis, i.e., MR. This interaction adds a new component to the regulation of the HPA axis: the notion that a proactively acting HPA neuropeptide (i.e., CRH) strengthens an HPA axis controlling instrument (i.e., MR). In addition to HPA regulation, MR regulates autonomic output and stress-related behavioral performance (Korte et al., 1993; Oitzl et al., 1994). On the cellular level, hippocampal MR reduces serotonergic signal transduction (Joëls et al., 1991; Meijer and De Kloet, 1998), potentiates electrical activity of pyramidal neurons (Joëls and De Kloet, 1990), extends long-term potentia-

tion (Pavlides et al., 1994), and has anti-apoptotic properties in the dentate gyrus (Sloviter et al., 1989; Hassan et al., 1997). This underlines that the effect of CRH on MR function comprises a general organizational change in the stress response, possibly as part of adaptive processes. Also urocortin could play a role in this mechanism because this CRH-like neuropeptide binds with high affinity to both CRH-R1 and CRH-R2 (Vaughan et al., 1995). Therefore, future investigations should reveal whether CRH or urocortin is recruited by the effect of psychological challenges on MR and whether this evolves via CRH-R1 or CRH-R2.

The presence of corticosterone was a prerequisite for the effects of CRH on MR, because no effects were seen in nonsubstituted ADX rats. High glucocorticoid levels are known to affect corticosteroid receptor expression (Reul et al., 1987b, 1989; De Kloet et al., 1998) and, therefore, the use of ADX rats and steroid substitution allowed to control for indirect effects of stress and CRH on MR via elevations in corticosterone because of HPA axis activation. The plasma levels of corticosterone in ADX rats attained after substitution of this steroid were in the same range as found during the early morning hours in intact rats, i.e., <10 ng/ml. Thus, these low levels of corticosterone sufficed to allow CRH-induced upregulation of MR levels. However, substitution of ADX rats with dexamethasone was ineffective in allowing MR upregulation. In contrast, as shown before (Reul et al., 1987b, 1989, 2000), dexamethasone treatment itself induced an upregulation of MR, the mechanism of which is still unknown. Under these conditions, CRH injection caused a reduction in MR levels. Presently, the interaction between dexamethasone and CRH is unclear, but it is clearly distinct from the stimulatory effects of stress and CRH on MR in corticosterone-substituted and intact animals. Nevertheless, given the differential *in vivo* receptor binding profile of dexamethasone (GR) versus low corticosterone (MR), it appears that MR occupancy by corticosterone is required for the stimulatory effects of CRH on MR levels.

The RU 28318 challenge test showed that the forced swimming-evoked increase of hippocampal MR levels was accompanied by an enhanced MR-mediated inhibitory tonus on the activity of the HPA axis. This observation underscores that the changes in MR density are of physiological significance. However, in view of the increased MR-mediated inhibition of HPA activity 24 hr after forced swimming or novelty stress, reduced ACTH and corticosterone plasma were to be expected, but did not occur (data not shown). This observation points to the involvement of compensatory mechanisms aimed to balance the enhanced tonic inhibition and, thus, to maintain "normal" baseline HPA output. Such mechanisms might act at different levels of the HPA axis: intrahypothalamic circuits modulating the corticotrophic secretory system, corticotrophic sensitivity in the anterior pituitary, and adrenal mechanisms. Alternatively, afferent pathways within the CNS might convey stimulatory influences to the HPA axis (Whitnall, 1993), such as those originating in the central nucleus of the amygdala and the thalamic paraventricular nucleus (Bhatnagar and Dallman, 1998). Thus, an overall adjustment in the afferent control of the HPA axis occurs after emotional and psychological stress.

The newly acquired insight into the interaction between CRH and MR after stressful events is of importance for the elucidation of the pathophysiology of stress-related disorders such as major depression. Depressed patients show elevated cerebrospinal fluid levels of CRH (Nemeroff et al., 1984), increased numbers of CRH and CRH/vasopressin-expressing neurons in their paraventricular nucleus (PVN) (Raadsheer et al., 1994), and elevated CRH mRNA levels in this nucleus (Raadsheer et al., 1995). Reduced levels of CRH-binding sites have been measured in brains of suicide victims having suffered from depression (Nemeroff et al., 1988), and also neuroendocrine function tests favor elevated CRH secretion in depressed patients (Holsboer et al., 1984; Gold et al., 1986). Thus, a hyperactivity of CRH very likely exists in depressed patients, which is at least partly responsible for the elevated HPA activity, the vegetative disturbances, and psychopathology observed in these patients (Holsboer, 2000). The HPA aberrations seen in depression seem to involve disturbed MR function (De Kloet et al., 1998; Lopez et al., 1998; Reul et al., 2000), whereas their role in autonomic and psychological aspects of the disease is still unknown. Successful antidepressant drug treatment, besides amelioration of psychopathology, results in normalization of HPA function (Holsboer and Barden, 1996; De Kloet et al., 1998). Animal studies have revealed that chronic antidepressant treatment primarily raises hippocampal MR density, which is thought to be instrumental in the observed attenuation of parvocellular paraventricular CRH expression and HPA axis activity (Brady et al., 1991; Reul et al., 1993, 1994). Decreased hippocampal MR density and increased CRH expression, often associated with HPA hyperactivity, have also been observed in aging animals and man (Meaney et al., 1988, 1992; Reul et al., 1988; Swaab et al., 1994; Raadsheer et al., 1995). We postulate that hypersecretion of CRH in brain resulting from chronic stress or during aging leads, via desensitization of CRH receptors and post-receptor systems, to a decline in the CRH-mediated regulation of MR levels resulting in a growing loss of control of the HPA axis and other MR-sensitive systems. Whether it comes in individuals to this aberrant CRH–MR interaction seems to depend on the person's genetic make-up, frequency, and gravity of major life events, age, and early life experiences (Levine, 1967; Meaney et al., 1988, 1989; Sutanto et al., 1996; Vallée et al., 1997; De Kloet et al., 1998; Holsboer, 2000).

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