



## Temporal and spatial dynamics of corticosteroid receptor down-regulation in rat brain following social defeat

Bauke Buwalda<sup>a,\*</sup>, Klára Felszeghy<sup>b</sup>, Katalin M. Horváth<sup>a</sup>, Csaba Nyakas<sup>b</sup>, Sietse F. de Boer<sup>a</sup>, Béla Bohus<sup>a</sup>, Jaap M. Koolhaas<sup>a</sup>

<sup>a</sup>Department of Animal Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, Netherlands

<sup>b</sup>Central Research Division, Haynal University of Health Sciences, Budapest, Hungary

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### Abstract

The experiments explored the nature and time course of changes in glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) binding in homogenates of various brain regions and pituitary of male Wistar rats following social defeat stress. One week after defeat, the binding capacity of GRs was decreased in the hippocampus and the hypothalamus while no changes were observed in the parietal cortex and the pituitary. The number of MRs remained at the same level as in undefeated rats. Three weeks postdefeat, the initially down-regulated GR returned to baseline level in the hippocampus and the hypothalamus. However, GR binding was now decreased in the parietal cortex. Severe down-regulation of MRs was detected in the hippocampal and septal tissue. The results show that brief but intense stress like social defeat induces a long-lasting down-regulation of corticosteroid receptors and that the temporal dynamics of these changes are not only differential for GRs and MRs but also for brain sites. © 2001 Elsevier Science Inc. All rights reserved.

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### 1. Introduction

The majority of basic stress research describing and analyzing behavioral and physiological responses to various stressors focuses either on acute responses that are short-lasting and return to baseline values within minutes or hours, or on changes at the end of a chronic stress period. However, in a number of animal models, it is shown that short-lasting stressors of sufficient intensity have long-term consequences in changing the neural or neuroendocrine control of physiology and behavior long after the actual stressor is gone [1–4]. One of the models in which this is exemplified uses the stress of social defeat in rats and is considered as a model with relatively high face validity in the study of risk factors involved in human psychopathology [5]. The temporal dynamics of a number of behavioral and physiological changes following a defeat experience have been summarized recently in a review of Koolhaas et al. [6]. The time course of these changes in various para-

meters after defeat is strikingly differential. For example, home cage locomotor activity is decreased for about 2 days, whereas body temperature during resting time is increased up to 7 days. Effects of defeat on behavioral reactivity in an orientation–attention test have a delay of about 1 week but from then on, the immobility response stays elevated for up to at least 2 months. A similar kind of delay in neural consequences of a brief stress experience has been described in a rat model applying footshock stress and immune challenges [7].

We recently described changes in the long-term regulation of the HPA axis in rats 1 and 3 weeks after a social defeat [8]. One week after defeat, rats were hyperresponsive in their ACTH release to intravenously administered corticotropin-releasing factor (CRF). This pituitary hyperresponse normalized within 3 weeks. We furthermore observed an escape from dexamethasone (DEX) suppression of a CRF challenge 1 week after defeat. This aberrant response to DEX/CRF challenge was persistent up to 3 weeks after being defeated [8]. These findings were confirmed in a study using rats of a wild-type strain [9]. This enduring escape from DEX suppression in the DEX/CRF challenge could be due to a decreased inhibition of HPA

\* Corresponding author. Tel.: +31-50-3632345; fax: +31-50-3635205.  
E-mail address: buwaldab@biol.rug.nl (B. Buwalda).

activity following social defeat. Since DEX poorly penetrates the brain [10], its inhibitory action most likely is exerted at the level of the pituitary. The failing DEX inhibition could also be indirect, through its negative effect on endogenous corticosterone (CORT) secretion, at the level of the brain. Actions of corticosteroids on the brain are mediated by glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) [11]. Differential temporal changes of GR and MR properties in the pituitary and/or brain may underlie the neuroendocrine changes observed in previously defeated rats. Since modulation of central nervous CORT receptors, especially in the hippocampus also affects behavioral performance in a wide array of tasks including stress-induced immobility [12,13], changes in these receptors might also be involved in some of the behavioral consequences of defeat.

The aim of the present study was, therefore, to determine the long-lasting effects of social defeat on GR and MR binding capacity in the pituitary and hypothalamus, hippocampus, septum and parietal cortex. Possible temporal dynamics in MR and GR binding were studied by measuring binding capacity of these receptors 1 and 3 weeks after defeat.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats (3 months old) were individually housed in a room with constant temperature ( $21 \pm 2^\circ\text{C}$ ) and fixed, 12 h light–dark regime (light on at 0800 h).

### 2.2. Defeat procedure

One group of rats ( $n=20$ ) was subjected twice to social defeat (between 1000 and 1300 h) by placing them on two subsequent days in the cage of an aggressive male conspecific. The home cages of a group of control rats ( $n=20$ ) were placed for 1 h in a novel room. The resident rats were of a wild-type strain and at least 6 months of age. They were housed in large cages ( $80 \times 55 \times 40$  cm) with a female to stimulate territorial aggression. The residents were trained on a regular base by confronting them with naive male intruders. Before the start of the experiment, residents with attack latencies shorter than 2 min were selected. By using residents with equal readiness to and intensity of attack, variation in stress intensity was reduced. The experimental animals were transferred with their home cage to the room of the residents and immediately placed into the territory of the resident. One hour before the start of social interaction, females were removed from the residents' cage. On the first day, the intruders were attacked for a standard period of 15 min and subsequently put in a small wire-mesh cage ( $30 \times 15 \times 15$  cm), which was placed back into the cage of the resident for the rest of the hour.

Accordingly, during this remaining 45 min, the experimental animals were protected from repeated attacks and potential injury, but remained in auditory, olfactory, and visual contact with the resident. On the second day the intruders were attacked for a period of 5 min after which they were placed in the dominants cage in the wire-mesh cages for 55 min. Immediately after the 1-h defeat sessions, the experimental animals returned to their home cages and to their own room.

### 2.3. Corticosteroid receptor binding assay

To study the lasting effects of the defeats on MR and GR binding in brain regions and pituitary, 20 rats were sacrificed 1 week after the defeat ( $n=10$ ) or control ( $n=10$ ) procedure. The other 20 animals were studied 3 weeks after defeat or control treatment (decapitation was performed between 1000 and 1200 h). Rats were bilaterally adrenalectomized 24 h prior to sacrifice to leave enough time for elimination of endogenous CORT. Following decapitation, the brain was quickly removed and the hippocampus, hypothalamus, parietal cortex, septum, and pituitary were dissected on ice. Tissues were frozen in liquid nitrogen and kept at  $-80^\circ\text{C}$  until determination of receptor binding. For single-point assay to determine total CORT binding (MR+GR), 20 nM [ $^3\text{H}$ ]CORT was used ([ $^3\text{H}$ ]CORT 2.58 Tbq/mmol, Amersham) as a saturating concentration of the labeled ligand [14]. To determine separately the number of MR and GR sites, the incubation was carried out with 20 nM [ $^3\text{H}$ ]CORT either in the presence or in the absence of 10  $\mu\text{M}$  unlabeled RU 28362 (Roussel Uclaf, Romainville, France), a GR specific competitor [15] to displace [ $^3\text{H}$ ]CORT from GR sites. In the presence of unlabeled RU 28362, the remaining [ $^3\text{H}$ ]CORT activity represented the binding to MR, while GR could be calculated by subtraction of MR from total [ $^3\text{H}$ ]CORT binding. The nonspecific binding was determined by the inclusion of 20  $\mu\text{M}$  unlabeled CORT. The receptor assay was performed as described earlier [16]. Briefly, for homogenization and incubation, a buffer containing 5 mM Tris, 1 mM EDTA, 10 mM sodium molybdate, 5% (vol/vol) glycerin and 1 mM  $\beta$ -mercaptoethanol (TEMG buffer, pH 7.4) was used. To obtain cytosol fractions, the homogenates were centrifuged for 30 min with  $100,000 \times g$  at  $4^\circ\text{C}$ . The cytosol was collected and kept on ice. The labeled and unlabeled steroids were dried in the test tubes and 200  $\mu\text{l}$  cytosol was then added. After 1 h shaking of the mixture in an ice bath, the incubation proceeded for 6 h at  $2^\circ\text{C}$ . For separation of receptor bound and free fractions of [ $^3\text{H}$ ]CORT, a filtration method was used as described by van Haarst and Szuran [17]. Briefly, glass fibre filters (Whatman GF/B, Whatman, Clifton, NJ, USA) were immersed in a 0.3% polyethylenimine solution for 60–90 min at  $4^\circ\text{C}$ . The incubation mixture was rapidly filtered under vacuum by these filters. Subsequently, the filters were washed twice with 5 ml TEM buffer (homogenization buffer without

glycerol). Filters were dried for 1 h at 80°C and counted in 5 ml of scintillation liquid.

Pituitaries from three animals per group were pooled to obtain sufficient cytosol samples. Instead of CORT, which binds to transcortine-like globulins in the pituitary, [<sup>3</sup>H]DEX (1.52 Tbq/mmol, Amersham) was used for labeling receptor sites [18]. GR binding was determined by the amount of total [<sup>3</sup>H]DEX (20 nM) binding displaced by RU 28362 (10 μM). MR binding was determined by the amount of [<sup>3</sup>H]DEX binding in the presence of RU 28362 displaced by CORT (20 μM) [18]. Following 3 h incubation, the bound steroid was separated from the unbound one by gel filtration [14]. GR and MR binding capacity were expressed as fmol/mg protein.

### 2.3.1. Control experiments for the corticosteroid receptor binding assay

In the experiments, the rats were not perfused at sacrifice with saline. Therefore, since CORT binds strongly to CBG, residual CBG in remaining blood might contribute to the reported MR signal, which is not suppressed by RU 28362 but is suppressed by cold CORT. In a control experiment, rats were saline perfused and data analysis showed that there was no significant difference in MR and GR binding between perfused and nonperfused brain regions. In these control experiments, we also compared the  $B_{\max}$  value with the [<sup>3</sup>H]CORT binding on 20 nM saturation concentration. These values did not differ significantly ([<sup>3</sup>H]CORT maximal binding capacity was  $315 \pm 25.6$  fmol/mg prot and total [<sup>3</sup>H]CORT binding on 20 nM saturation concentration  $341 \pm 28.4$  fmol/mg prot ( $df=10$ ,  $t=0.65$ ,  $P=.5317$ ; compared by Student's  $t$  test)).

### 2.4. Western blot analysis of pituitary GRs

The necessary pooling of three to four pituitaries in the binding assay reduced the number of measurements performed to a statistically unreliable number ( $n=3$ ). Since the glucocorticoid feedback regulation of the HPA axis at this level is crucial, we decided to repeat the defeat procedure for another cohort of rats ( $n=36$ ) to measure changes in GR properties in individual pituitaries by using a sensitive Western blot technique following a modified protocol of Spencer et al. [19]. For whole-cell preparations, frozen pituitaries from individual animals were individually sonicated in 1.2 ml homogenization buffer/100 mg tissue on ice. The buffer consisted of 25 mM Tris buffer (pH 7.6; 4°C) containing 250 mM glucose, 150 mM NaCl, 10 mM EGTA, 2 mM EDTA, 5 mM MgCl<sub>2</sub>, 1 mM orthovanadate, 5mM dithiothreitol, 2 tbl/25 ml protease inhibitor cocktail (Complete, Boehringer Mannheim, Almere, The Netherlands). Sonication was followed by an additional 30 min incubation period adding Triton X-100 and Tween-20 to the homogenization buffer (in 1–1% end-concentration). After incubation, the homogenate was ultracentrifuged for 20 min at 4°C (20,000 ×  $g$ ). Supernatant was collected, aliquoted, and

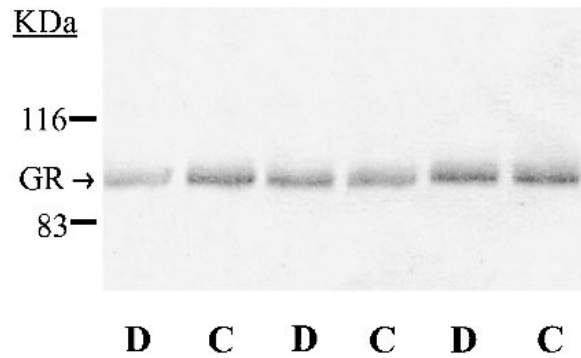


Fig. 1. Representative blot illustrating immunoreactive GR (arrow) levels in pituitary homogenates of rats 7 days after prior defeat (D) and control rats (C).

used as whole-cell extract. Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Samples were mixed with sample buffer, denaturated at 93–95°C for 4 min, loaded into 8% bis-acrylamide gel, and separated by SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred from gel onto nitrocellulose membrane (NY 12N, Nytran; Schleicher & Schuell, Germany). Membranes were blocked for 30 min at room temperature with 5% nonfat dry milk (Bio-Rad) in Tris-buffered saline (TBS) solution. Immunoblot analysis was carried out with polyclonal rabbit anti-GR IgG (0.25 μg/ml TBS; M-20, Santa Cruz Biotech., CA) incubating overnight at room temperature. The epitope of the antibody has been mapped onto the amino terminus of glucocorticoid receptor  $\alpha$  of mouse origin. The next day blots were exposed to goat anti-rabbit-HRP (1:600, Bio-Rad). Finally, immunolabelling was visualised by reacting with 0.4% diaminobenzidine and 0.016% H<sub>2</sub>O<sub>2</sub> (see Fig. 1 for representative blot illustrating immunoreactive GR levels rats 7 days after defeat and control rats). Leica Quantimet 500 image analysis system was used for the measurement of optical density of GR reactive bands on the immunoblots. Density was quantified with Sigmagel (Jandel Scientific Software). Samples were run in duplicate and treatment groups were counterbalanced across gels. All resulting blots were exposed to the chromogen for the same time period.

### 2.5. Statistical analysis

Results are presented as means  $\pm$  S.E.M. One-way ANOVA was used to analyze the data.  $P$  values less than .05 were considered to be statistically significant.

## 3. Results

After 7 days, thymus weight was significantly lower in defeated rats [ $127.9 \pm 5.6$  mg/100 g body weight (bw)] than that in controls ( $155.6$  mg/100 g bw;  $P<.01$ ). Also, seminal vesicles were smaller after defeat ( $341 \pm 5.6$  mg/100 g bw

vs.  $389 \pm 5.6$  mg/100 g bw;  $P < .05$ ). There was no significant increase in adrenal weights. Three weeks after defeat, organ weights were similar in controls and defeated rats.

Changes in binding characteristics of GR and MR 1 week following social defeat are presented in the left panel of Fig. 2. The number of GRs in defeated rats decreased in the hippocampus and hypothalamus to  $73 \pm 6.3\%$  and  $76.3 \pm 7.3\%$ , respectively, of the control level ( $P < .05$ ). There was no change in binding capacity to MRs in any of the regions being measured. In the group of rats that were sacrificed 3 weeks following defeat (right panel of Fig. 2), the reduced GR binding was restored to baseline level in the hippocampus and hypothalamus. Interestingly, there was a significant reduction ( $65.7 \pm 11\%$ ) in GR number in the parietal cortex compared to controls. After 3 weeks, the number of MRs decreased in the hippocampus to  $56.4 \pm 4.1\%$  ( $P < .01$ ) and in the septum to  $54 \pm 9.9\%$

( $P < .01$ ) of control levels. The results in the pooled pituitaries indicated no change in DEX binding. This was confirmed in the Western blot study using an antibody against the GR in individual pituitaries (see Fig. 1 for a representative blot illustrating immunoreactive GR levels in pituitary homogenates of rats 7 days after defeat and control rats). Optical density readings of the 90-kDa band 7 days after defeat were  $105 \pm 16.1\%$  of control values and at 21 days,  $106 \pm 6.6\%$ .

#### 4. Discussion

The present study shows that besides site- and subtype-specific effects of stress on corticosteroid receptors in the brain [4,20,21], time-specific receptor alterations exist for weeks after the cessation of a brief but intense social stressor like defeat. These temporal dynamic changes in receptor binding capacity in various brain sites may be involved not only in the abnormal regulation of the HPA axis [8,9], but also in other long-lasting effects of defeat in a number of behavioral and physiological parameters [2,5,6,9].

Temporal and spatial dynamics in corticosteroid receptor down-regulation also occurs for at least 2 weeks after the end of a chronic treatment with high CORT levels [22]. Changes in hippocampal MRs appeared to be more persistent than down-regulation of GRs. In animals provided with permanent blood sampling catheters, plasma CORT levels are elevated for about 5 h following defeat [23]. In former studies, we did not find a change in basal CORT secretion beyond this period [8,9]. The involution of the thymus 1 week after defeat, however, reflects an increased plasma level of the free fraction of CORT. Therefore, a transient increase in plasma CORT levels following defeat may play a role in the differential down-regulation of GRs and MRs. Changes in steroid receptor binding without concomitant changes in plasma total steroid concentrations have been reported before [4,20,24]. Corticosteroid receptors can also be affected by an altered neuronal control as suggested in studies of Maccari et al. [25] showing that central noradrenergic systems exert an inhibitory influence on the number of hippocampal MRs and hypothalamic GRs. The long-lasting effects of defeat on central nervous noradrenergic functioning are, however, not known at present.

Long-term changes in corticosteroid receptor binding capacity after the termination of a stressor have been reported before. Sutanto et al. [26] reported that 3 weeks following defeat the number of hippocampal GRs and MRs were significantly reduced. In that study, Tryon Maze Dull S3 rat intruders were exposed to dominants for a period of 2 h, which may have contributed to a reduction in both GRs and MRs. Short inescapable footshock stress enhanced hippocampal GR and MR binding 2 weeks later without affecting binding in the hypothalamus and the pituitary [4]. Receptor subtype-specific effects were reported in social stress conditions [20] and after an immune challenge [21],

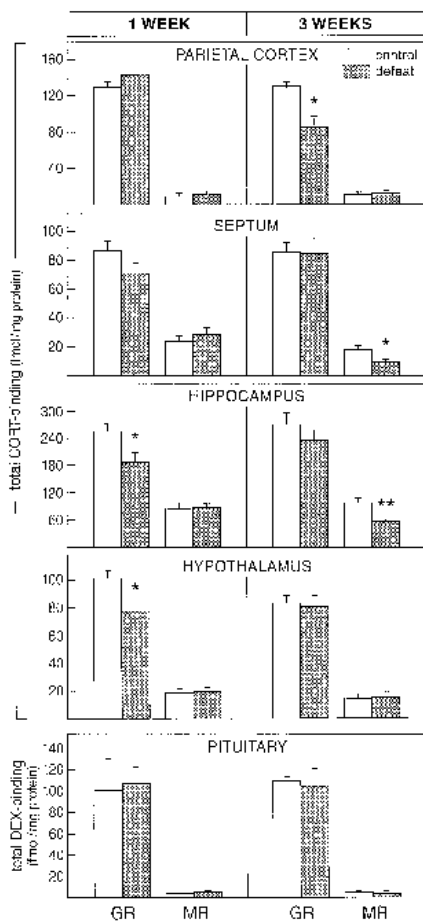


Fig. 2. Changes in corticosteroid binding to GRs and MRs 1 week (left panel) and 3 weeks (right panel) after a double social defeat in homogenates of various brain sites of defeated rats (gray bars) and controls (white bars). In the pituitary tissue DEX binding was calculated. Binding in defeated rats was compared with binding in proper controls. One week after defeat, hippocampal and hypothalamic GR binding was reduced, whereas 3 weeks after defeat, GRs in those regions normalized but decreased in the parietal cortex. MR binding was severely down-regulated 3 weeks after defeat in the septum and the hippocampus. \* $P < .05$ ; \*\* $P < .01$ .

both decreasing hippocampal MRs without affecting GRs. Very recently, Liberzon et al. [24] demonstrated that a single prolonged stress period (about 4 h) initially down-regulates hippocampal GR mRNA and, to a lesser extent, MR mRNA. At 1 and 2 weeks after stress, GR mRNA was up-regulated whereas MR mRNA remained below control levels. Since de Kloet [12] proposed that deviations of an idiosyncratic MR/GR balance alter individual-specific susceptibility to stress and perhaps to stress-related brain diseases, the authors also presented the MR/GR ratio. The results indicated a clear shift in hippocampal corticosteroid receptor balance. MR/GR ratio increased 24 h after stress. Following this initial increase, the ratio decreased for at least 2 weeks. Although the time course of the shift in hippocampal receptor balance is different in the present study, an initially increased hippocampal MR/GR ratio after 1 week is followed by a significant decrease 3 weeks following defeat in the hippocampus and the septum. Liberzon et al. [24] relate the shift in receptor ratios following single prolonged stress to earlier published findings of this group showing a fast feedback hypersensitivity of the HPA axis in this stress paradigm to cortisol [27]. It is surprising to notice that hippocampal MR/GR ratios change in a similar temporal pattern in these two stress paradigms, whereas long-term feedback sensitivity of the HPA axis appears to be opposite. Whether these functionally opponent findings are related to differences in transportation of cortisol and DEX over the blood–brain barrier is not known. The synthetic glucocorticoid mainly inhibits HPA activity at the level of the pituitary and the present findings indicate that 1 and 3 weeks following defeat, the number of pituitary GRs is not affected. Consequently, the long-term escape from DEX inhibition in the DEX/CRF challenge in defeated rats [8,9] is not caused by a failing inhibition in the pituitary. The severe reduction of hippocampal MRs 3 weeks following defeat, resulting in a decreased MR/GR balance, might be causally involved in the abnormal neuroendocrine response. Dorsal hippocampus lesions significantly reduced the inhibitory effect of DEX on basal and stress-induced adrenocortical responses [28].

It is not known whether the down-regulation of corticosteroid receptors as described above play a role in the long-lasting enhanced behavioral response to a novel environmental challenge as observed in the orientation–attention test [5]. Results of Korte et al. [13] and Oitzl et al. [29], however, suggested a critical role of hippocampal MRs in the behavioral reactivity to stress. Further studies are needed to elucidate the role of corticosteroid receptor changes in the brain following defeat in the previously described behavioral changes.

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