Chapter 4

Paradoxical decrease in basal and stress-induced corticosterone secretion after recurrent daily blockade of the glucocorticoid receptor

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Abstract

The present study tests the hypothesis that repeated daily administration of the glucocorticoid receptor antagonist mifepristone (MIF) would lead to chronic disinhibition of the hypothalamic-pituitary-adrenal axis. Male C57BL/6J mice were offered 200 mg/kg MIF *per os* in oats, either once (1xMIF) or daily for seven days (7xMIF), or vehicle. Plasma corticosterone levels were determined in blood samples obtained i) at various time points after MIF intake; ii),immediately following five min exploration of a circular hole board at 24h after the first and seventh administration. At that time the mice were sacrificed for quantification of mineralocorticoid (MR) and glucocorticoid receptor (GR) mRNA expression in the hippocampus and the paraventricular nucleus (PVN).

After 1xMIF corticosterone levels were elevated for about 16h, and then decreased towards vehicle control levels at 24h, while showing a much higher corticosterone response to circular hole board exposure. Following 7xMIF the basal and stress-induced corticosterone patterns were comparable to vehicle. The 1xMIF mice showed behavioral hyperactivity during exploration of the circular hole board, while the 7xMIF mice rather engaged in serial search patterns. MR mRNA was decreased in all hippocampal subregions of the 1xMIF group, and increased in the 7xMIF group only in the CA2 cell field. GR mRNA expression in hippocampus and PVN was not affected. Adrenal weights were increased in both MIF groups.

In conclusion, the data show that after recurrent blockade of GR the adrenal corticosterone secretion is downregulated rather than disinhibited because of intermittent glucocorticoid feedback, while MR-dependent characteristics become prominent in exploratory behavior.

Introduction

It has been reported that patients suffering from psychotic major depression benefit from a brief treatment with the glucocorticoid antagonist RU38486, also known as mifepristone (MIF; 600 – 1200mg/day, once a day for four to seven days). These high doses of the antiglucocorticoid improved emotional and cognitive abilities and restored aberrant levels of corticosteroids (Murphy et al. 1993; Belanoff et al. 2001a; DeBattista and Belanoff 2006; Flores et al. 2006; Blasey et al. 2009; Blasey et al. 2011). The fast amelioration of psychotic and depressive symptoms is thought to be at least in part due to restoration of glucocorticoid action to which untreated depressed patients are resistant, while the anti-progestin activity of MIF seems not implicated (Belanoff et al. 2001a; Thomson and Craighead 2008).

In the present study we asked how daily administration of MIF would affect the secretion of corticosterone. Recent studies have addressed this question but have provided different results. Wulsin et al. (Wulsin et al. 2010) using a daily dose of 10mg/kg of MIF in rats found attenuated basal and stress-induced Hypothalamic-Pituitary-Adrenal (HPA) axis activity and attenuated depression-like behavior. Bachman et al. (Bachmann et al. 2003) offered rats 20mg/kg/day of various GR antagonists added to food and observed episodic increases in HPA axis activity and a profound increase in hippocampal MR expression (see also Spencer et al. 1998; Oomen et al. 2007) gave twice a day MIF orally (50mg/kg rat) to chronically stressed rats and found that this treatment blocked the stress-induced reduction in neurogenesis. Revsin et al. (Revsin et al. 2009) also administered twice a day MIF orally (200mg/kg mouse) and observed elevated HPA axis activity. Finally, van Haarst et al. (van Haarst et al. 1996) infused MIF chronically 100ng/hr intracerebroventricularly in rats and observed after 3 days infusion enhanced stress-induced and circadian rises in corticosterone secretion under conditions in which spatial memory was improved (Oitzl et al. 1998). Common to these studies was that adrenal weight and responsiveness to ACTH were increased indicating that the changes were due to MIF's anti-glucocorticoid rather than anti-progestin activity. Based on these results we tested therefore the hypothesis that daily administration of a very high dose of MIF would disinhibit the HPA axis.

In the current study we mimicked in naive male C57BL/6J mice the high dose regimen of MIF common for the patient studies. For this purpose we applied a previously devised non-invasive stress-free method for steroid delivery via oats (Dalm et al. 2008). After the first (1xMIF) and the seventh administration (7xMIF) of the glucocorticoid antagonist or vehicle we assessed (i) in 2h-intervals the circadian corticosterone secretion

pattern; (ii) 24h post-treatment the behavioral and corticosterone response to novelty during five min exploration of a circular hole board. Immediately thereafter mice were sacrificed and hippocampal and hypothalamic MR, GR and CRH mRNA expressions were measured in brain sections with *in situ* hybridization. The data demonstrated contrary to our hypothesis that recurrent GR blockade with MIF downregulates HPA axis activity, while altering the behavioral response to novelty.

Materials and Methods

Animals

Male C57BL/6J mice, 8-10 weeks of age, were purchased from Janvier (France). Upon arrival at the animal facilities (Gorlaeus Laboratory, LACDR, University of Leiden, The Netherlands), mice were single housed in a temperature (21 ± 1˚C) and humidity (55 ± 5%) controlled room, with food and water *ad libitum;* for ten days before the start of the experiment (12-12h light-dark cycle; lights on 0700 to 1900h). During this period mice were weighed and handled every other day. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC).

Study design

The experiments were conducted with separate groups of mice. We measured: (1) the 24h circadian corticosterone secretion, following single and repeated administration of MIF (200mg/kg; 1x/day for seven days). In addition, we collected blood samples around the time of the circadian corticosterone peak 32h after the last administration of MIF; (2) corticosterone concentrations before (basal) and after five min of novelty exposure to the circular hole board during which behavior was recorded for further analysis; (3) following behavioral testing, mice were decapitated and brains were prepared for measuring the expression levels of MR, GR and CRH mRNA in the hippocampus and paraventricular nucleus of the hypothalamus (PVN).

Procedures

Familiarization of mice to oat administration and drug delivery procedures as described in (Dalm et al. 2008) are applicable to all experiments of this study.

Familiarization to oat administration

One week prior to the start of the experiment a feeding-cup (2.3cm diameter x 2.5cm high) was taped to the floor in a corner of the home cage, opposite the nest location. For familiarization, three flakes of oats (Speltvlokken, Biologische teelt, Graanpletterij de Halm, Netherlands; ± 140mg) were placed in the cup on days 1, 3 and 5 of week 1, 2 hours after lights on. The top of the home cage was lifted and the sawdust was removed from the cup using an air puff generated with a pipette. Next, the oats were placed into the cup using forceps to minimize human odor transfer. Thereafter, the home cage was closed and the mouse was allowed to eat the oats undisturbed. All the oats were consumed within 10 min.

Drug delivery

Preparation of drug delivery via oats: One day prior to the experiment three flakes of oats were placed in a glass vial and the solutions containing GR antagonist or dissolvent (VEH) were applied. The glass vials containing the oats were kept at room temperature over night. Within 16h, the solution was absorbed by the oats and they were dry when presented to the mice.

The GR antagonist mifepristone (MIF) (kindly provided by Corcept Pharmaceuticals, CA, U.S.A.) was dissolved in 1 ml 0.9% NaCl containing 0.25% carboxymethylcellulose and 0.2% Tween20 (VEH=dissolvent). From this solution 50µl was applied to the oats (mice received a dose of 200mg/kg MIF).

Hormone assays

The circadian corticosterone concentrations were measured in blood samples obtained via tail incision (Dalm et al. 2005). Briefly, a small incision with a razor blade at the base of the tail allowed collection of 50µl blood within 90s after opening of the animal's cage. Following decapitation, trunk blood was collected individually in capillaries coated with potassium-EDTA (Sarstedt, Germany), stored on ice, and centrifuged with 13000 rpm at 4°C for 10 min. Plasma was stored at –20°C. Corticosterone concentrations were measured using commercially available radio immunoassay kits ^{125}I-corticosterone (MP Biomedicals, Inc., NY, USA; sensitivity 3 ng/ml).

Experiment 1: Effect of GR antagonism on corticosterone secretion

Animals

Mice (n = 54) were randomly assigned to three treatment groups (n = 18/group): (1) single mifepristone (1xMIF); (2) MIF once a day on seven consecutive days (7xMIF) or (3) VEH on seven consecutive days (VEH). Oats+MIF or Oats+VEH were placed in the feeding cup at 0900h, and consumed within 10 min.

Experimental design

The circadian corticosterone secretion was determined in blood samples collected via tail incision every two hour over a period of 24h. The first blood sample was taken at 1100h, i.e., two hours after MIF or VEH was administrated, and the last at 0900h the next day. Subsequent blood samples were collected starting 32h after the last administration around the circadian corticosterone peak at 1700, 1900, 2100 and 2300h.

The three treatment groups were divided in three subgroups each, consisting of six mice. Thus, from each mouse, one blood sample was taken every six hours and each time point consisted of six mice per group. During the dark period, blood sampling took place under red light conditions.

Experiment 2: Corticosterone and behavioral responses to the circular hole board

Animals

Mice (n = 24) were randomly assigned to three treatment groups (n = 8/group): (1) single mifepristone (1xMIF); (2) mifepristone once a day on seven consecutive days (7xMIF) or (3) VEH on seven consecutive days (VEH). Oats+MIF or Oats+VEH were placed in the feeding cup at 0900h, and consumed within 10 min.

Experimental design

Twenty-four-hours after the last administration of MIF or VEH we took a blood sample via tail incision, and placed the mouse for 5 min on the circular hole board; the behavioral response was analyzed. Immediately following behavioral testing, mice were decapitated. Corticosterone concentrations were determined in trunk blood. Brains were snap frozen in isopentane, pre-cooled on dry ice/ethanol and stored at –80°C until further use, i.e. to determine MR, GR and CRH mRNA expression levels in brain tissue. Thymus and adrenals were removed and weighed.

Circular hole board

Apparatus: A grey round plate (Plexiglass; 110cm diameter) with 12 holes (5cm diameter, 5cm deep) at equal distances from each other, and at a distance of 10cm from the rim of the hole to the rim of the plate, was situated one meter above the floor in a different experimental room then the housing room. Light conditions on the surface of the board were 120lux. To minimize, and distribute odour cues, the surface was cleaned with 1%HAc and the board was turned (randomly clock- and anticlockwise) before a mouse was tested. Behavior was recorded on videotape and analyzed with an automated tracking system (Ethovision 3.1, Noldus Information Technology, Wageningen, The Netherlands). The position of the mouse was sampled five times per second. To calculate the distance walked, we set the minimal distance between samples to 3cm. The following parameters related to general activity, exploratory strategies and possible anxiety-related behaviors were analyzed: distance walked (m) on the board (=total arena) and in specified zones defined as: start center = circle of 30cm diameter, rim zone = a ring of 4.5cm at the outer perimeter of the plate. Parameters: velocity (cm/s), number of holes visited; sequence of hole visits (*serial*: more than two holes in sequence; *perseveration*: repeatedly visiting the same hole or alternately visiting two neighbouring holes); latency (s) to leave the center; latency (s) to and time spent (s) in rim zone.

In situ hybridization for MR, GR and CRH mRNA

Brains were sectioned at –20°C in a cryostat microtome at 10µm in the coronal plane through the level of the hypothalamic paraventricular nucleus (PVN) and dorsal hippocampus. Sections were thaw-mounted on poly-L-lysine coated slides (0.001%), air dried and kept at –80°C until further use.

In situ hybridizations using ^{35}S-labeled ribonucleotide probes (MR, GR, CRH) were performed as described previously (Schmidt et al. 2003). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense RNA probes were transcribed from linearised plasmids containing exon-2 of mouse MR and GR, and the full length coding regions of CRH (rat). Tissue sections (3–4/slide) were saturated with 100µl hybridization buffer containing 20mM Tris-HCl (pH 7.4), 50% formamide, 300mM NaCl, 1mM EDTA (pH 8.0), 1x Denhardt's, 250 µg/ml yeast transfer RNA, 250 µl/ml total RNA, 10mg/ml salmon sperm DNA, 10% dextran sulfate, 100mM dithiothreitol, 0.1% SDS, 0.1% sodium thiosulfate and supplemented with approximately 1.5 x 10^6cpm ^{35}S-labeled riboprobe. Brain sections

were cover slipped and incubated overnight at 55°C. The next day sections were rinsed in 2xSSC, treated with RNaseA (20mg/ml) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1xSSC at 65°C for 30 min and dehydrated through increasing concentrations of ethanol. All age groups were assayed together. Films were opposed to Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY) and developed.

Autoradiographs were digitized, and optical density of the areas of interest was quantified using image analysis computer software (analySIS 3.1, Soft Imaging System GmbH). The average density of six measurements for each animal was calculated.

Statistical analysis

The circadian profile of corticosterone was analyzed by analysis of variance (ANOVA - factor: treatment) with repeated measurements, followed by LSD *post-hoc* test. Total corticosterone (AUC: area under the curve) over 24h was calculated for light and dark periods of 12h, subjected to ANOVA, with treatment and time of the day as fixed factors. Body-, adrenal-, and thymus weights were analysed using one-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test. Data are presented as mean ± S.E.M. Statistical significance was accepted at $p < 0.05$.

Results

Experiment 1: Effect of GR antagonism on corticosterone secretion

Circadian pattern of plasma corticosterone level

Mice of all groups showed a circadian corticosterone rhythm (Figure 1A; time $F_{(11,165)}=35.051$, $p < 0.001$) as previously described (Dalm et al. 2005). The corticosterone secretion of control mice increased from 1500h onwards, with peak levels (± 100ng/ml) at the end of the light phase and the beginning of the dark phase (between 1700 and 2100h). Interestingly, the frequency of MIF administration affected the course of the circadian rhythm (time*group: $F_{(22,165)}=15.992$, $p < 0.001$). Corticosterone concentrations in 1xMIF-mice were significantly higher from 1100 until 0100h ($p < 0.01$), reaching and maintaining peak levels from 1300 until 2300h (± 300ng/ml). Around 2300h, concentrations readily declined until there was no difference in corticosterone concentration at 0300h vs. control and 7xMIF-administrated mice. There was a sudden significant increase vs. controls ($p = 0.001$) and 7xMIF mice ($p = 0.013$), at 0500h. In contrast, repeated MIF

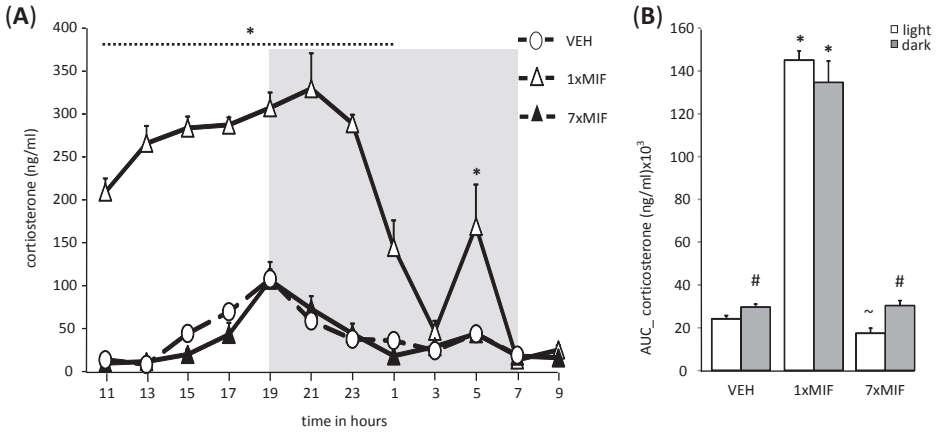

Figure 1

(A) Circadian secretion of corticosterone in ng/ml measured every 2 hours in blood plasma of male mice C57BL/6J that received RU38486 (MIF) once (1xMIF) or for seven days (7xMIF). Mice were entrained in a 12-12h light-dark cycle (dark phase from 1900 to 0700h represented by the gray shaded area). **(B)** Total corticosterone secretion in ng/ml during the light and dark period of the day, determined as Area Under the Curve (AUC); ng/ml. Data are presented as mean ± S.E.M; $p < 0.05$ * vs. other groups, # within groups, ˜ 7xMIF vs. VEH.

administration did not boost the concentrations of corticosterone as was observed for 1xMIF administrated mice; the time course was similar to VEH mice. Overall, there was a main effect of treatment due to the high corticosterone concentrations in the 1xMIF mice ($F_{(2,15)}$=550.923, $p < 0.001$).

Total amount corticosterone

The total amount of corticosterone calculated as area under the curve (AUC) over 24h showed a main effect of treatment (Figure 1B AUC: $F_{(2,17)}$=392.094, $p < 0.001$). AUC corticosterone during the dark period (1900 to 0700h) was higher than during the light period (0700 to 1900h) in VEH and 7xMIF mice (paired t-test; both $p < 0.01$). 1xMIF mice had similar high AUC corticosterone levels during the light and dark periods, both significantly higher than VEH and 7xMIF mice. Interestingly, AUC corticosterone was lowest during the light period of 7xMIF mice ($p < 0.039$ vs. VEH) due to the low corticosterone concentrations measured from 1500 till 1700h.

Corticosterone around the circadian peak, 32h after mifepristone administration

Treatment effects were found around the time of the circadian peak (Figure 2, 1700 to 2300h; $F_{(2,15)}$=6.308, p =0.01). Thirty-two hours after the last administration, 1xMIF mice secreted less corticosterone than VEH (p = 0.007) and 7xMIF mice (p = 0.008). No statistical difference was found for corticosterone secretion patterns of VEH and 7xMIF groups.

Experiment 2: Corticosterone and behavioral responses to the circular hole board

Basal and novelty induced corticosterone secretion

Basal resting as well as novelty induced corticosterone were affected 24h after the last treatment (Figure 3; treatment $F_{(2,44)}$=17.175, p < 0.0001; time $F_{(1,44)}$=45.980, p < 0.0001; treatment*time $F_{(2,44)}$=17.626, p < 0.0001). Basal resting corticosterone differed

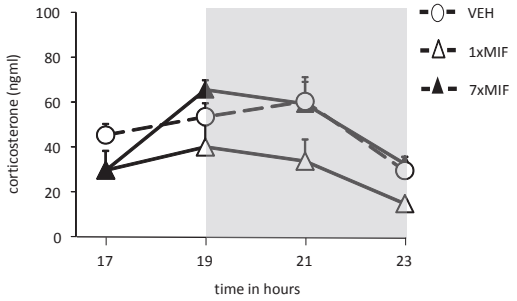

Figure 2
Corticosterone (ng/ml) secretion during the circadian peak in mice, 32h after last administration of RU38486 (MIF), 1xMIF, 7xMIF or VEH (dark phase from 1900 to 2300h represented by the gray shaded area). Data are presented as mean ± S.E.M.

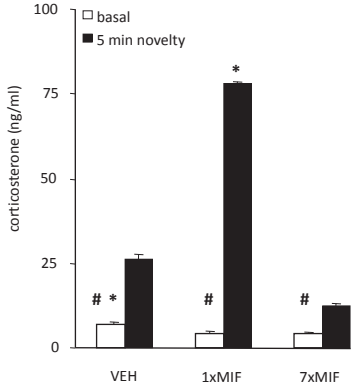

Figure 3
Basal and novelty (5 min exposure to the circular hole board) induced corticosterone (ng/ml) were determined in mice, 24h after last administration of VEH, 1xMIF or 7xMIF. Data are presented as mean ± S.E.M.; p < 0.05 * vs. other groups, # within groups.

significantly between the groups ($F_{(2,23)}$=14.656, p < 0.001) and was lower in both MIF treated groups than in VEH mice (p < 0.001). Basal corticosterone of 1xMIF and 7xMIF mice was comparable. After 5 min on the circular hole board, corticosterone was increased in all groups compared to baseline, however to a different degree ($F_{(2,23)}$=19.074, p < 0.0001). Corticosterone levels in 1xMIF where 300% of the VEH group and 700% of the 7xMIF group (both p < 0.0001); corticosterone of the VEH group was about twice as much as in the 7xMIF group (p < 0.05).

Expression of MR, GR, CRH mRNA in hippocampus and PVN

Hippocampal MR mRNA expression was differentially affected by treatment, 24h post-administration, across all subfields (Figure 4; treatment – DG: $F_{(2,23),}$=11.005, p = 0.001; CA1: $F_{(2,23)}$=12.887, p = 0.001; CA2: $F_{(2,23)}$=14.267, p = 0.001; CA3: $F_{(2,23)}$=11.550, p = 0.001). MR mRNA expression was reduced across all subfields in 1xMIF-mice compared to VEH and 7xMIF-mice (p < 0.05). Repeated MIF administration increased MR mRNA expression in the CA2 specifically vs. VEH and 1xMIF-mice (p = 0.016 and p = 0.001, respectively).

Neither GR nor CRH mRNA expression in hippocampus and PVN were affected by treatment (data not shown).

Exploration on the circular hole board

Twenty-four hours after administration the behavioral response differed during five min exploration on the circular hole board (Table 1: MANOVA: $F_{(20,26)}$=3.772, p = 0.001). Following initial slower movement out of the central start position, 1xMIF mice showed hyperactivity: they walked longer distances, with a faster speed of moving, visited more

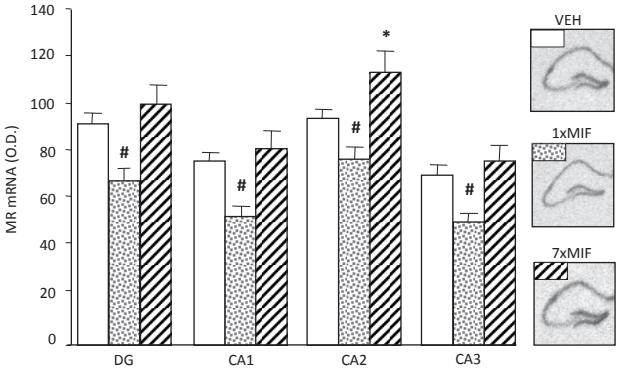

Figure 4
Expression of MR mRNA, measured as optical density (O.D.) in the hippocampal subfields dentate gyrus (DG), CA1, CA2 and CA3, 24h after last administration of VEH, 1xMIF or 7xMIF. Data are presented as mean ± S.E.M.; p < 0.05 * vs. other groups, # within groups.

Table 1: The behavioral response during five min circular hole board exposure, 24h after the last administration with RU38486 (MIF).

	VEH	1xMIF	7xMIF
General activity			
distance walked (m)	7.9 ± 0.7	***15.0 ± 1.9****	7.5 ± 0.9
speed of moving (cm/s)	8.6 ± 0.4	***11.6 ± 0.8****	9.9 ± 0.3
total hole visits	14.8 ± 2.1	***24.8 ± 1.8****	17.0 ± 2.4
Search strategy			
latency (s) from center	8.4 ± 1.4	***14.0 ± 1.4**$^{\#}$*	11.8 ± 2.1
latency (s) first hole visit	13.9 ± 0.8	16.0 ± 2.6	***18.4 ± 0.7**$^{\#}$*
%serial	16.5 ± 5.2	28.2 ± 3.6	**36.6 ± 10.2**$^{\#}$
%perseveration	48.6 ± 5.8	39.3 ± 5.2	52.1 ± 5.1
Anxiety-related			
latency (s) to rim	63.0 ± 13.1	55.1 ± 12.4	69.9 ± 8.5
number of rim dips	12.8 ± 1.4	***18.4 ± 2.0****	11.1 ± 1.2
number of boli	1.1 ± 0.7	0.8 ± 0.4	1.3 ± 0.8

Data are presented as mean ± S.E.M.; $p < .05$ * vs. other groups; # vs. VEH.
Bold italic indicates significant differences.

holes and made more rim dips (vs. VEH and 7xMIF-mice: $p < 0.05$). Interestingly, 7xMIF-mice made more use of a serial search strategy (vs. VEH-mice: $p = 0.05$).

Other physiological measures

Treatment did not influence body weight. Adrenal weight ($F_{(3,34)}$=3.733, $p = 0.035$) was highest in both MIF-groups, but significantly higher in 7xMIF than in VEH ($p = 0.005$): adrenals in mg, mean ± SEM: VEH 23.5 ± 2.8; 1xMIF 31.3 ± 4.3; 7xMIF 39.3 ± 2.9. Thymus weight was lower in both MIF groups, but passed statistical significance ($F_{(3,34)}$=3.100, $p = 0.059$): thymus in mg, mean ± SEM: VEH 411.0 ± 38.9; 1xMIF 371.5 ± 15.1; 7xMIF 321.1 ± 23.6.

Discussion

The present study demonstrated that single delivery of a very high dose of MIF caused a profound increase in circulating corticosterone levels starting 2h first after ingestion and lasting 16h before reaching vehicle control levels at 24h. At that time exposure to a novelty stressor still triggered a profound corticosterone response. This disinhibitory

effect exerted by the antagonist was in line with our expectation. We did not expect, however, that after one week of daily consumption of the same high dose of MIF the basal and stress-induced corticosterone levels were back to baseline concentrations not different from vehicle controls. Accordingly, the current data reject the hypothesis that daily repeated MIF administration would produce a state of hypercorticism. At the same time the weight of the adrenals was further increased, while the thymus weight progressively decreased towards significance. The increased adrenal weight suggests that in spite of the progressive downregulation of the HPA axis apparently still sufficient ACTH is released over the week to exert its corticotrophic action.

How does the slow downregulatory adaptation of adrenal corticosterone secretion come about? One obvious explanation is related to the recurrent nature of daily GR blockade with the antagonist. MIF is rapidly cleared because the antagonist is not bound to albumin, a high capacity low affinity binder and not α1-acid glycoprotein, which is a low capacity high affinity binder, and rapidly metabolized (Heikinheimo and Kekkonen 1993). We found in another study that after a 50mg/kg rat dose orally, MIF is already depleted from the circulation in 90 min, while low amounts of the antagonist and its metabolites are retained in the brain for at least 3 hours (Karssen, Belanoff and de Kloet, unpublished observation). Corticosterone remained elevated though for 16 hours, while the genomic effects will persist even longer. Our experimental design thus allowed us to study a recurrent pattern of GR-mediated actions including negative feedback which are transiently interrupted by daily application of the GR antagonist. We propose therefore that the HPA axis progressively adapts to this daily cycle of GR blockade and subsequent GR activation. Hence, during the seventh day of GR antagonist administration, the circadian and stress-induced corticosterone pattern had become similar to that observed in control mice.

This slow adaptation of the HPA axis to MIF has been observed before. The elegant study by Wulsin et al (Wulsin et al. 2010) revealed that a twenty fold lower dose of MIF *i.p.* (10mg/kg rat) produced an attenuated HPA axis response to a forced swim stressor, after one week. Interestingly, this course of MIF treatment also evoked a differential pattern of activation and inhibition of central inputs to the PVN. The ventral subiculum of the hippocampus and all regions of the medial frontal cortex showed enhanced stress-induced c-Fos activity after daily GR blockade, while the c-Fos response was reduced, however, in other subregions of the hippocampus and in the amygdala. These data suggest that MIF enhanced inhibitory and suppressed excitatory inputs to the PVN that collectively may contribute to downregulation of HPA axis activity.

Also the mode of MIF application is important. A previous study showed that if MIF was chronically infused in the cerebral ventricles using an Alzet minipump a constant blockade of all brain GR sites was achieved. After four days of infusion the amplitude in the circadian and stress-induced corticosterone patterns were gradually enhanced because the peak levels of corticosterone had become higher, while the troughs remained low and did not alter (van Haarst et al. 1996). In the present study using recurrent daily rather than chronic blockade of GR the opposite adaptation occurred: initially a large surge in circadian and stress-induced corticosterone secretion was observed which then subsided over the next days of GR antagonist oral administration. This finding is reminiscent to the behavioral dichotomy between repeated and chronic infusions of MIF. It was found that continuous blockade of brain GR facilitated spatial learning and memory of rats, while phasic blockade caused a deficit (Oitzl et al. 1998). It is conceivable that besides blocking feedback suppression in pituitary and PVN, the effect of MIF on limbic circuitry noted by Wulsin et al (Wulsin et al. 2010) underlies this *opposite* change in stress sensitivity and behavioral performance after phasic vs. continuous blockade of the GR.

The above noted dichotomy in behavior may develop because after phasic (one time per day) GR blockade by MIF the rebound corticosterone response exerts its effect including the feedback suppression of the HPA axis, while during continuous blockade such agonist corticosterone actions are excluded. It also seems that if the phasic blockade is stepped up to two times per day with a high dose of MIF the condition of continuous blockade is approached (Oomen et al. 2007; Revsin et al. 2009). However, irrespective of phasic or continuous GR blockade, corticosterone binding to the MR will always occur (Reul and de Kloet 1985; Wodarz et al. 1992; Calfa et al. 2003). MR is known to mediate control over appraisal processes, behavioral reactivity to novel experiences and the onset of HPA axis activity (De Kloet et al. 1998). The changes in corticosterone action via MR activation were reflected by the lower expression of hippocampal MR mRNA for the 1xMIF-group, whereas expression was increased in the CA2 region of the hippocampus for 7xMIF-group. Interestingly, GR blockade after MIF treatment *per os* for three weeks, increased total hippocampal MR mRNA expression by 1.5 compared to controls (Bachmann et al. 2003). Therefore, it would be of interest to determine longitudinal effects of repeated GR blockade on MR function, particularly since previous studies have clearly shown that MR and GR interact in the control of HPA axis activity (Herman and Spencer 1998; Spencer et al. 1998).

In the studies by Bachmann et al (Bachmann et al. 2003) and Wulsin et al. (Wulsin et al. 2010) chronic MIF affected forced swimming behavior in a similar manner to antidepressants. In another study by Oitzl et al (Oitzl et al. 1998) chronic MIF leaving

MR active improved memory performance. Studies with mouse mutants overexpressing MR in limbic forebrain revealed enhancement of memory (Ferguson and Sapolsky 2007; Lai et al. 2007), perseveration of learned behavior (Harris et al. 2012 under review) and reduction of anxiety (Korte et al. 1996). Previously, also the pharmacological blockade of MR had shown altered appraisal processes and selection of the appropriate behavioral response, i.e., search strategy (Oitzl and de Kloet 1992; Oitzl et al. 1994; Oitzl et al. 1997a). In the current study twenty-four-hours following repeated GR antagonism, mice used the serial search strategy more often, compared to controls. This strategy increases the likelihood that the animals will visit all possible escape routes that the circular hole board provides (during the exploration trial all holes were closed). Indeed, the choice of applied strategy does affect performance in spatial learning trials (Dalm et al. 2000; Grootendorst et al. 2001a). In the current study following 1xMIF mice were initially slower to move away from the start center, and subsequently hyperactive on the circular hole board. This could indicate a change in the level of anxiety induced by previous GR antagonism. If so, then the effect is transient as repeated GR antagonism did not induce any of the above described features.

In conclusion, based on the current findings the efficacy of GR antagonism in clinical studies could be due to the following factors: (1) The detrimental effects of high corticosteroid levels via GR activation are prevented by GR antagonism. This possibility is prominent during continuous or high frequency blockade of the GR. (2) If during daily cycles of MIF application, the blockade by the GR antagonist wanes, GR becomes activated by the high circulating corticosterone levels and shuts off its own secretion (Wodarz et al. 1992; De Kloet et al. 1998; Calfa et al. 2003). (3) As a result of GR blockade and the subsequent rise in corticosterone levels, the MR becomes strongly activated irrespective of phasic or continuous GR antagonism.

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Conflict of interest

MSO and SD report no conflict of interest. ERdK is on the Scientific Advisory Board & owns stock of Corcept Therapeutics.

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