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ORIGINAL ARTICLE

Intrahippocampal Corticosterone Response in Mice Selectively Bred for Extremes in Stress Reactivity: A Microdialysis Study

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The hypothalamic-pituitary-adrenocortical (HPA) axis is one of the major stress hormone systems, and glucocorticoids (GCs) play a pivotal role in homeostatic processes throughout the body and brain. A dysregulation of the HPA axis, leading to an aberrant secretion of GCs, is associated with affective disorders such as major depression. In the present study, three mouse lines selectively bred for high (HR), intermediate (IR) or low (LR) stress reactivity were used to elucidate the temporal dynamics of intrahippocampal corticosterone (CORT) in response to a standardised stressor. In particular, we addressed the question of whether the distinct differences in HPA axis reactivity between the three mouse lines, as determined by plasma CORT measurements, are present in the central nervous system as well, and if the respective endophenotype is brought about by alterations in blood–brain barrier (BBB) functionality. We applied *in vivo* microdialysis in the hippocampus, demonstrating that the concentrations of CORT released from the adrenals in response to restraint stress are not only distinctly different in the plasma, but can also be found in the central nervous system, although the differences between the three mouse lines were attenuated, particularly between IR and LR animals. Additionally, a time lag of approximately 60 min was observed in all three lines regarding intrahippocampal peak concentrations of CORT after the onset of the stressor. Furthermore, we showed that the penetration and clearance of CORT in the hippocampal tissue was not affected by differences in BBB functionality because the multidrug resistance 1 P-glycoprotein (Mdr1 Pgp) was equally expressed in HR, IR and LR mice. Furthermore, we could exclude surgical damage of the BBB because peripherally-injected dexamethasone, which is a high affinity substrate for the Mdr1 Pgp and therefore restricted from entering the brain, could only be detected in the plasma and was virtually absent in the brain.

Key words: corticosterone, microdialysis, blood–brain barrier, hippocampus, mouse model.

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Glucocorticoids (GCs) play a pivotal role in homeostatic processes throughout the body and brain (1–3). An aberrant secretion of GCs is associated with many diseases, such as diabetes, obesity and major depressive disorder (MDD) (3–5). The majority of patients suffering from MDD show profound neuroendocrine alterations with hyper- or hypocortisolism as the result of a dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis (1,4,6), often associated with a dysfunctional negative-feedback mechanism (7). Evidence has emerged that clinically relevant subtypes of MDD exhibit distinctively different pathophysiologies (8). Generally, melancholic and psychotic depression are associated with a hyperresponsive HPA system (4,9), whereas patients suffering from atypical depression exhibit a hypofunction of the HPA system (6,8). Interestingly, normalisation of HPA axis function was found to be a potent predictor for the successful treatment of MDD (9). Furthermore, it was shown that, despite an amelioration of psychological symptoms, an aberrant HPA axis function at the time of discharge was associated with a higher risk of relapse (10).

Focusing on the above mentioned findings, a new mouse model for affective disorders has recently been generated by selectively inbreeding CD-1 mice for extremes in HPA axis reactivity (11). This 'stress reactivity' (SR) mouse model consists of three independent

breeding lines, the high (HR), intermediate (IR) and low reactivity (LR) mouse line, which differ significantly with respect to their corticosterone (CORT) increase in response to stressors (i.e. modelling a key endophenotype of MDD). Previous studies characterising the SR mouse model revealed several alterations in neuroendocrine parameters, emotional behaviours, sleep architecture and circadian rhythms, as well as in cognitive abilities, associated with increased or decreased responsiveness of the HPA axis (11–14), thus, revealing further similarities to symptoms observed in depressed patients.

Psychiatric diseases such as MDD largely involve brain functions and, as noted above, an aberrant intracerebral GC signalling is critically involved in the development of such a disease (1,3–5,7). Because there is no clinically relevant way of directly monitoring brain GC concentrations in humans and GC responses are therefore normally monitored in the blood, the question remains as to whether GC levels in the blood truly reflect brain hormone concentrations. Previous studies in rats have shown that stress-induced changes in circulating plasma GC concentrations not necessarily reflect brain concentrations (15), raising the question of how these differences in stress-induced GC concentrations in plasma and brain may occur.

The multidrug resistance 1 P-glycoprotein (Mdr1 Pgp), which is located in the blood–brain barrier (BBB) (16) and restricts the access of many molecules to the brain (17), is potentially involved in the mechanism underlying the differential stress-induced GC response in plasma and brain. This Mdr1 Pgp not only functions as a drug efflux pump and protects the brain from xenobiotics (18), but also is potentially involved in the extrusion of endogenous steroids such as CORT (19,20), although an active transport of CORT by the Mdr1 Pgp is still a matter of debate (21–23). Because GCs exert important functions in the brain and are critically involved in the negative-feedback mechanisms of the HPA axis, BBB functionality can have a considerable impact on HPA axis regulation (24). Therefore, it is of special interest to investigate concentrations of free (i.e. bioavailable) CORT not only in the periphery, but also in the brain. There is a substantial body of evidence that *in vivo* microdialysis (MD) is a suited technique to continuously monitor neuroactive substances in the brain of rodents, including neurotransmitters, neuropeptides and endogenous steroids (15,25–34). Hence, in the present study, we used the MD technique to investigate the coherence of peripheral and intrahippocampal GC levels as well as BBB functionality in animals of the 'stress reactivity' mouse model.

Two experiments were performed. First, we determined the plasma CORT increase of HR, IR and LR males in response to a standardised psychological stressor and, in parallel, monitored the temporal dynamics of intrahippocampal CORT concentrations by means of *in vivo* MD. In a second experiment, we addressed the question whether the differential reactivity of the HPA axis between HR, IR and LR mice is brought about by alterations in BBB functionality because permeability differences in the BBB could account for differential intrahippocampal GC levels and, thus, an altered regulation of the HPA axis (24,35,36). The synthetic GC dexamethasone (Dex), which is a high affinity substrate for the Mdr1 Pgp and therefore restricted from entering the brain at the level of the BBB (37), was administered to all experimental animals by means of an i.p. injection. Subsequently, Dex concentrations were monitored in plasma samples and microdialysates from the hippocampus. Furthermore, the expression of Mdr1 Pgp, which is proposed to be involved in the transition of CORT across the BBB (19,20,38), was investigated in these animals by western blot analysis.

Materials and methods

Experimental design

In the present study, two experiments were performed (for a schematic overview, see Fig. 1).

In Experiment 1, we aimed to assess the peripheral GC response in animals of the three 'stress reactivity' mouse lines in response to a standardised stressor and, in parallel, monitor intrahippocampal CORT increase using *in vivo* MD.

Experiment 2 was conducted to investigate potential alterations in BBB functionality between the three mouse lines because permeability differences in the BBB could account for a differential responsiveness of the HPA axis.

Animals and housing conditions

Adult male mice (n = 11 for each breeding line) derived from the ninth breeding generation of the 'stress reactivity' (SR) mouse model (11) were used. Until the age of 11 weeks, the mice were housed in groups of four animals in transparent polycarbonate cages (standard Makrolon cages type III, Bayer MaterialScience, Leverkusen, Germany; $38 \times 22 \times 15$ cm^3) with wood chips as bedding and wood shavings as nesting material. Subsequently, they were transferred to smaller cages (standard Macrolon cages type II, Bayer MaterialScience; $23 \times 16 \times 14$ cm^3) and single housed at least for 2 weeks. Animals were aged 3–4 months during the experiment. Housing and experimental rooms were kept under standardised laboratory conditions (12/12 h light/dark cycle, lights on 08.00 h; temperature: 22 ± 1 °C; relative humidity: 55 ± 10%). Commercial mouse diet (Altromin No. 1324; Altromin GmbH, Lage, Germany) and tab water were available *ad lib.*

The presented work complies with the current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC). All experiments were announced to the appropriate local authority and were approved by the Animal Welfare Officer of the Max Planck Institute of Psychiatry (Az. 55.2-1-54-2531-64-07).

Experiment 1: monitoring the intrahippocampal and the peripheral glucocorticoid response

Implantation of the guide cannula

To investigate the intrahippocampal GC response, *in vivo* MD was used to monitor the increase of intrahippocampal CORT concentrations (15,25–28,30), including its temporal dynamics in response to a standardised psychological stressor (see below). Accordingly, a MD probe was implanted to the left hippocampus of all animals. The surgical procedure to implant the guide cannula (MAB 4.15.IC; Microbiotech/se, Stockholm, Sweden; length: 10.35 mm, outer diameter: 0.45 mm, inner diameter: 0.3 mm) was performed under isoflorane-oxygen anaesthesia using a custom-made vaporising device. The animals were positioned in a stereotactic device (TSE GmbH, Bad Homburg, Germany) and the guide cannula for the insertion of the MD probe was implanted. Surgery was performed as described previously (28,29), with slight modifications. Briefly, a 1-cm incision was placed rostrocaudally along the midline of the animal's head cutting skin and muscles. Bregma was used as a landmark to adjust the guide cannula to the position

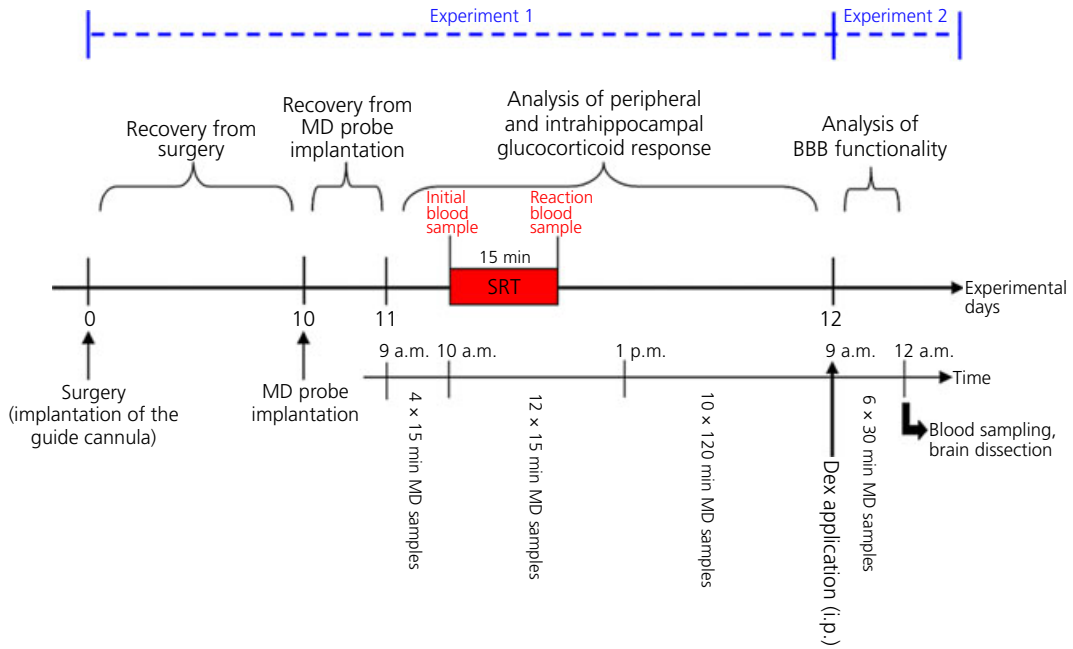

Fig. 1. Schematic overview of the experimental schedule with time and intervals of the collected microdialysis (MD) and blood samples. SRT, stress reactivity test; Dex, dexamethasone; BBB, blood–brain barrier.

above the hippocampus (rostrocaudally: −3.2 mm, laterally: +3.2 mm, relative to Bregma) and a small hole (approximately 500 μm in diameter) was drilled into the skull at this position. Coordinates were based on the atlas by Franklin and Paxinos (39). The guide cannula was inserted through the hole (ventrally: −2.6 mm, relative to Bregma; the ventral coordinate refers to the tip of the guide cannula) into the hippocampal tissue. Next to the guide cannula, two more holes (approximately 200 μm in diameter) were drilled into the left *os parietalis* of the mouse skull and two jeweller's screws (DIN 84, A1, AM 1.0 × 2, stainless steel; Paul Korth GmbH, Lüdenscheid, Germany) were mounted into these holes. The guide canulla and the screws were framed and glued together with dental cement (SPEIKO Kallocryl; Dr Speier GmbH, Münster, Germany). Additionally, a small metal peg was also embedded in the dental cement, which was eventually connected to a guidewire to prevent the MD probe from being removed accidentally by the animal's movements during the MD experiment (see below). After surgery, animals received a single dose (12.5 μg/kg body weight, injection volume 0.05 ml) of the analgesic 'metacam' (Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany) s.c. to reduce post-surgical pain.

Microdialysis probe implantation

After a 10-day post surgery recovery period (Fig. 1), the MD probe (MAB 4.15.2.Cu, membrane length: 2 mm, shaft outer diameter: 0.18 mm, membrane outer diameter: 0.2 mm, cut-off 6 kDa; Microbiotech/se) was inserted into the left hippocampus through the guide cannula under light isoflorane anaesthesia and tubes for the influx and efflux of Ringer's solution through the probe were connected. All tubes and the guide wire were attached to a swivel and counterbalancing system (Instech Laboratories, Plymouth Meeting, PA, USA), allowing the animals to move freely (28). MD tubes and probes were perfused with sterile Ringer's solution (147.2 mmol Na^+, 4 mmol K^+, 2.25 mmol Ca^{2+}, 155.7 mmol Cl^-; Berlin-Chemie AG, Berlin, Germany) at a constant flow rate of 2 μl/min using a microinfusion pump (Type No. 540220; TSE GmbH, Bad Homburg, Germany). Animals were allowed to recover for another 24 h subsequent to the probe implantation.

Monitoring the intrahippocampal glucocorticoid response

Sampling of microdialysates was started at 09.00 h on experimental day 11 (Fig. 1). Four baseline samples with a sampling interval of 15 min each were collected in the first hour of the experiment. Subsequently, the animals were subjected to a 15-min restraint stress (for details, see below). Simultaneously, MD samples were collected for the next 3 h to monitor the intrahippocampal GC response. The sampling interval was set to 15 min. After this period, the temporal resolution of the MD sampling was decreased to 2-h intervals until 09.00 h on the next day. All MD samples were collected in pre-cooled microvials using an automated sampling device (Microsampler 820; Univentor Ltd., Zejtun, Malta) and stored at −20 °C for later analysis of CORT concentrations.

Assessing the peripheral glucocorticoid response

At 10.00 h on the experimental day 11 (Fig. 1), all animals were subjected to a stress reactivity test (SRT). The SRT is described in detail elsewhere (11). Briefly, two blood samples were collected from small incisions made to the ventral tail vessel of the animal, one right before and one immediately after a 15-min restraint period. As a result of the MD-set up on the animals' heads, the restraint procedure had to be adjusted in this experiment. Instead of using tubes, the mice were restraint by fixing the tail and the body (by means of a hip-belt) with adhesive tape to the surface of the laboratory bench. The collected blood samples were centrifuged at 14 800 *g* for 5 min and the plasma was transferred into clean tubes and stored at −20 °C until further analysis.

Experiment 2: functionality of the BBB

Dexamethasone analysis in microdialysates and plasma

After the monitoring of the peripheral and the intrahippocampal glucocorticoid response was finished (Experiment 1), the synthetic glucocorticoid Dex

(Dexa-ratiopharm 4 mg; Ratiopharm GmbH, Ulm, Germany) was injected i.p. (40 μg/kg body weight, injection volume 0.3 ml) to all animals at 09.00 h on experimental day 12 (Fig. 1). For the next 3 h, microdialysates were collected at 30-min intervals and frozen at −80 °C for the subsequent analysis of hippocampal Dex concentrations (see below). At 12.00 h, mice were deeply anaesthetised with isoflorane and rapidly decapitated. Blood was collected and centrifuged at 1500 *g* at 4 °C for 10 min. The plasma was transferred into clean tubes and stored at −20 °C for analysis of Dex concentrations (see below).

Brain dissection and verification of the microdialysis probe locus

After decapitation, the brains of the experimental animals were quickly removed from the skull. Approximately 4 mm of the frontal brain were cut and the frontal brain part was frozen at −80 °C until used for the quantification of Mdr1 Pgp protein expression by applying the western blot technique (see below).

Using a cryostat (HM 500 O; MICROM Laborgeräte GmbH, Walldorf, Germany), the brains (i.e. brain minus the frontal part) were cut into 20-μm slices at the site of the puncture channel of the guide cannula and were stained with cresyl violet. The brain slices were subsequently investigated under a binocular microscope (Olympus, Tokyo, Japan; magnification: × 20) for lesions in the hippocampal tissue. Only those animals that had a clearly visible puncture channel in the hippocampal tissue caused by the MD probe were included for further analysis (Fig. 2).

Neuroendocrine measures

Corticosterone and adrenocorticotrophic hormone (ACTH) measurements

Plasma CORT and ACTH concentrations were analysed in duplicate using a commercial radioimmunoassay (RIA; DRG Instruments GmbH, Marburg, Germany) in accordance with the manufacturers' instructions, with slight modifications (11). The detection limit for CORT and ACTH in the plasma was 1 ng/ml and 40 pg/ml, respectively. For both assays, intra- and inter-assay coefficients of variation were both below 10%. The collected microdialysates were analysed for immunoreactive CORT using an enzyme-immunoassay (EIA). Details of the characteristics and performance of this assay are provided elsewhere (40,41). In the EIA, the detection limit for CORT in the microdialysates was 0.1 ng/ml. Intra- and inter-assay coefficients of variation were 10% and 13.4%, respectively. Dialysate CORT concentrations were not corrected for probe recovery.

Dexamethasone measurements

The blood plasma and the dialysates collected over 3 h after the Dex application (Experiment 2) were analysed for Dex using the combined high-performance liquid chromatography/mass spectrometry technique. Analysis was performed using an Agilent 1100 Series liquid chromatograph (Agilent, Waldbronn, Germany), which was interfaced to the electrospray ionisation source of an Applied Biosystems API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany). All samples were prepared applying solid-phase extraction using a Waters OASIS HLB μElution Plate (Waters, Eschborn, Germany). The applied sample volume was 300 μl for the plasma and 100 μl for the microdialysates. Deuterated cortisol (Cort-D4) was used as internal standard. Chromatography was accomplished using an isocratic elution of 0.1% formic acid and 60% methanol in a Gemini 5 μ C18 column (2 × 50 mm; Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.2 ml/min. The retention times for Dex and Cort-D4 were 3.6 and 3.0 min, respectively. The ion source was operated in the positive mode at 350 °C, and multiple reaction monitoring collision-induced dissociation were performed using nitrogen gas as the collision gas. The collision energy was set to 17 V and 35 V for Dex and Cort-D4, respectively. The transitions monitored during analysis were *m/z* 393 to 355 for Dex and 367 to 121 for Cort-D4. The detection limit for Dex in the plasma samples and dialysates was 0.1 ng/ml.

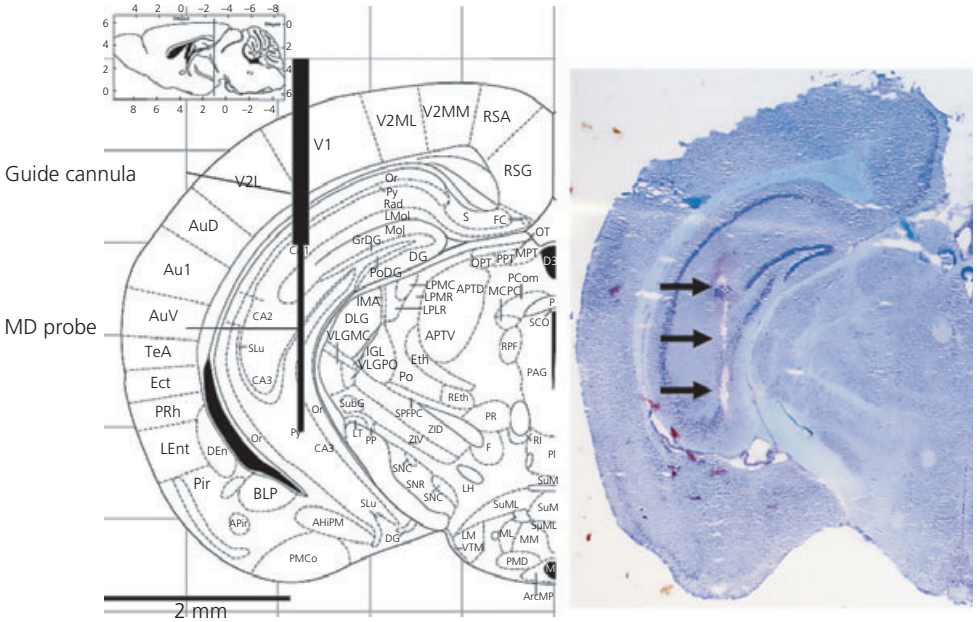

Fig. 2. Left: schematic overview of the microdialysis probe location in the left hippocampus; adapted from Franklin and Paxinos (39). Right: cresyl violet stained 20 μm coronal section of the microdialysis probe (MD) implantation site; the arrows indicate the probe's puncture channel in the hippocampal tissue.

Expression of the multidrug resistance 1 P-glycoprotein

The dissected frontal brain tissue (see above) was homogenised and protein expression levels of the Mdr1 Pgp were quantified using western blot analysis. The brain tissue was weighed and then homogenised in the five-fold volume of phosphate-buffered saline (PBS) tablets (Sigma-Aldrich, Taufkirchen, Germany), containing Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany) using a Dispomix Drive (Biolab Products, Gödenstorf, Germany). Subsequently, the samples were diluted with PBS to two different concentrations (dilutions of 1 : 30 and 1 : 60) and incubated for 30 min at room temperature with XT-loading buffer (XT Sample Buffer; Bio-Rad Laboratories GmbH, Munich, Germany; diluted 1 : 2 with aqua dest.). Fifteen microlitres of each sample was loaded on a 7% sodium-dodecyl-sulfate-polyacrylamide gel. After proteins had been separated by electrophoresis (1 h at 150 V) and transferred to a nitrocellulose membrane (30 min at 100 V), they were incubated with antibodies specific for Mdr1 Pgp (anti-mdr H-241 from rabbit, dilution 1 : 600; Santa Cruz, Heidelberg, Germany) and β-actin (anti-actin from rabbit, 1 : 40000; Sigma-Aldrich, Taufkirchen, Germany). The bands were detected by applying a horseradish peroxidase linked secondary antibody (anti-rabbit, peroxidase, dilution 1 : 2000; Amersham, Germany) and an enhancing chemiluminescence reagent (Lumilight; Roche Diagnostics GmbH, Mannheim, Germany). Semi-quantitative analysis of band signals was performed using a Kodak Image Station 440 CF with Kodak 1D 3.6 Software (Kodak, Rochester, NY, USA).

Statistical analysis

Because a normal distribution and variance homogeneity of the data could not always be assumed, analyses were exclusively performed using nonparametric statistics (42). All tests were applied two-tailed and were calculated using the software package SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). Group comparisons between more than two independent samples were performed using the Kruskal–Wallis H-test. In the case of a significant variation verified by the Kruskal–Wallis H-test, post-hoc pairwise comparisons were performed using the the Mann–Whitney U-test. Consequently, significance levels were corrected according to the sequential Bonferroni technique. Group comparisons between two dependent samples were performed using the Wilcoxon-test for repeated measures. For all tests, $P < 0.05$ was considered statistically significant.

Results

Experiment 1: monitoring the peripheral and the intrahippocampal glucocorticoid response

Plasma corticosterone concentrations

The concentrations of CORT in the blood samples collected immediately before the restraint (initial values) were not significantly different between the experimental animals selected from generation IX of the HR, IR and LR breeding lines (mean $\pm$ SEM, HR: 38.3 $\pm$ 8.1, IR: 17.5 $\pm$ 5.4, LR: 19.6 $\pm$ 8.6; Kruskal–Wallis H-test: n = 11/11/11, H = 5.54, d.f. = 2; $P > 0.05$; Fig. 3A). In the reaction blood samples collected immediately after the 15-min restraint stressor, elevated CORT concentrations were observed in all animals, manifesting a significant difference between initial and reaction values in all three mouse lines (Wilcoxon-test: n = 11/11/11, all $Z = -2.93$; $P < 0.01$; Fig. 3A). Comparing the magnitude of the CORT increase, as expected, HR, IR and LR animals differed significantly (Kruskal–Wallis H-test: n = 11/11/11, H = 26.3, d.f. = 2;

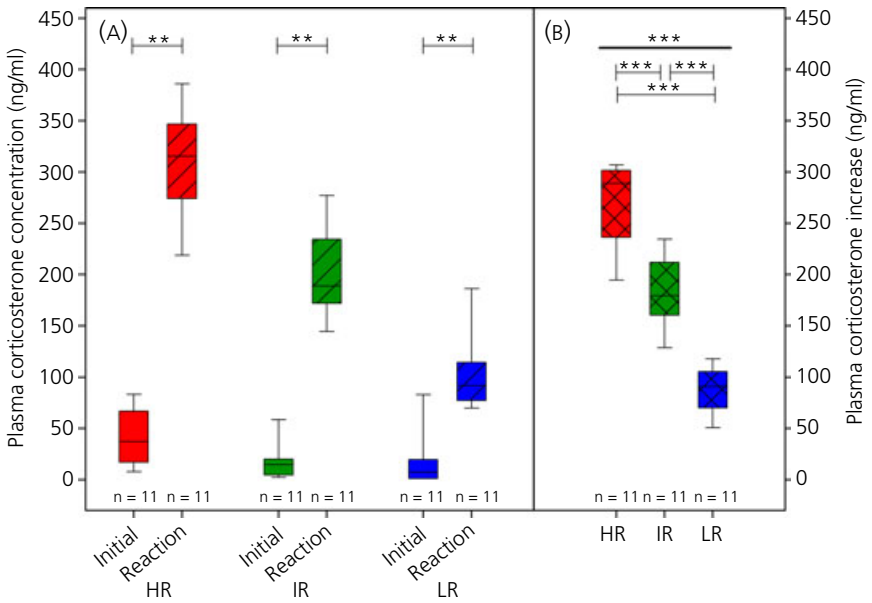

Fig. 3. (A) Plasma corticosterone concentrations immediately before (initial) and after (reaction) the 15-min restraint in male mice selectively bred for high (HR), intermediate (IR) and low (LR) stress reactivity, respectively. The corticosterone concentrations differed significantly between initial and reaction samples in all three lines (Wilcoxon-test for repeated measures, all $P < 0.01$**). (B) Increase of plasma corticosterone in response to the 15-min restraint in male mice selectively bred for HR, IR and LR stress reactivity, respectively. The corticosterone increase differed significantly between the three lines (Kruskal–Wallis H-test followed by *post hoc* Mann–Whitney U-tests, all $P < 0.001$***). Data are given as box plots showing medians (lines in the boxes) and 25% to 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers.

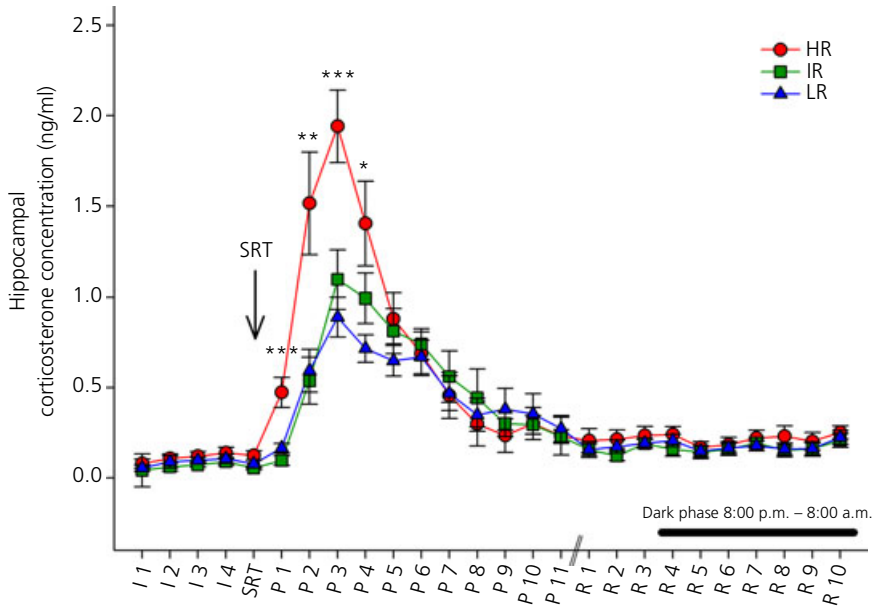

Fig. 4. Time course of hippocampal corticosterone concentrations in high (HR), intermediate (IR) and low (LR) stress reactivity mice (n = 11/10/10). HR mice showed a greatly pronounced increase of corticosterone in the hippocampus in response to the stress reactivity test (SRT) indicated by the arrow (Kruskal–Wallis H-test: P1, P3 $P < 0.001$***; P2, $P < 0.01$**; P4, $P < 0.05$*). Length of the sampling intervals: I (initial): 15 min, P (post SRT): 15 min, R (recovery): 120 min. The beginning of the dark phase is indicated by the black bar in the lower part. Data are given as line plots showing the mean ± SEM.

$P < 0.001$; Fig. 3B). HR mice showed a clearly higher CORT increase compared to the IR line, whereas LR mice showed the lowest increase of CORT (post-hoc Mann–Whitney U-test: U = 0–9, HR versus IR, $P < 0.001$; HR versus LR, $P < 0.001$; IR versus LR, $P < 0.001$; Fig. 3B). This is in line with findings from previous breeding generations where the secretion of ACTH in response to the 15-min restraint stressor also was found to be significantly different between the three mouse lines (mean ± SEM, HR: 1120.6 ± 56.4, IR: 998.6 ± 61.2, LR: 604.3 ± 64.9; Kruskal–Wallis H-test: n = 8/6/8, H = 14.4, d.f. = 2; $P < 0.001$) (i.e. further indicating a clearly increased or decreased reactivity of the HPA axis in HR and LR mice, respectively).

Hippocampal corticosterone concentrations

As a result of technical problems, the microdialysates of one IR and one LR mouse were lost. Thus, the final number of experimental animals was reduced to eleven HR, ten IR and ten LR mice in this experiment.

No significant differences in CORT concentrations between the lines were found in the samples collected before the SRT (baseline: I1–I4, Kruskal–Wallis H-test: n = 11/10/10, H = 1.3–2.4, d.f. = 2; all $P > 0.05$; Fig. 4). Similarly, the levels of hippocampal CORT did not differ significantly with respect to the microdialysates collected during the 15-min restraint (SRT, Kruskal–Wallis H-test: n = 11/10/10, H = 4.1, d.f. = 2; $P > 0.05$; Fig. 4).

However, in the first MD sample collected after the SRT (P1; Fig. 4), animals differed significantly with respect to the CORT concentrations measured in the hippocampus (Kruskal–Wallis H-test: n = 11/10/10, H = 15.5, d.f. = 2; $P < 0.001$), with HR mice showing clearly increased CORT levels compared to IR and LR animals. These differences were also found in the following three post-SRT intervals (P2–P4; Kruskal–Wallis H-test: n = 11/10/10, H = 8.5–14.8, d.f. = 2; all $0.001 < P < 0.05$; Fig. 4). Calculations of the AUC (SRT-P6) revealed significantly higher CORT levels in the hippocampus of HR mice than in animals from the IR and LR line. However, there were no significant differences in the AUC between the IR and the LR line (mean ± SEM, HR: 1.65 ± 0.21, IR: 0.98 ± 0.12, LR: 0.85 ± 0.09; Kruskal–Wallis H-test: n = 11/10/10, H = 12.2, d.f. = 2; $P < 0.01$; post-hoc Mann–Whitney U-test: U = 11–35, HR versus IR, $P < 0.05$; HR versus LR, $P < 0.01$; IR versus LR, $P > 0.05$; Fig. 4). Peak concentrations were reached approximately 60 min after the onset of the stressor (P3; Fig. 4), with HR mice showing the largest increase in hippocampal CORT concentrations (P3, Kruskal–Wallis H-test: n = 11/10/10, H = 14.8, d.f. = 2; $P < 0.001$; post-hoc Mann–Whitney U-test: U = 4–35, HR versus IR, $P < 0.05$; HR versus LR, $P < 0.001$; IR versus LR, $P > 0.05$; Fig. 4). At P5 (i.e. 90 min after the onset of the restraint), no significant difference could be found between the three lines. This holds true also for the rest of the experimental time period (P5-R10, Kruskal–Wallis H-test: n = 11/10/10, H = 0.1–3.2, d.f. = 2; all $P > 0.05$; Fig. 4).

Experiment 2: functionality of the BBB

Plasma and hippocampal Dexamethasone concentrations

As a result of technical problems, the microdialysates of one HR, IR and LR mouse were lost, respectively, reducing the sample size to ten individuals per line in this experiment.

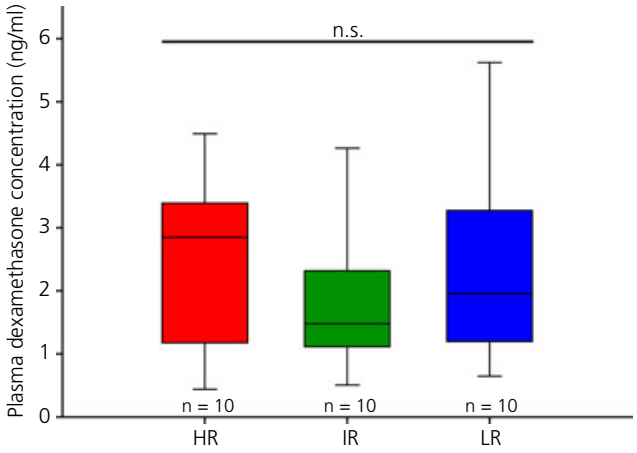

Fig. 5. Concentration of dexamethasone (Dex) in plasma samples of high (HR), intermediate (IR) and low (LR) stress reactivity mice collected 3 h after i.p. injection of Dex (40 μg/kg body weight). No significant differences could be observed between the lines (Kruskal–Wallis H-test: $P > 0.1$, n.s.). Data are given as box plots showing medians (lines in the boxes) and 25% to 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers.

The synthetic GC Dex was administered peripherally to the experimental animals to investigate potential alterations in BBB functionality between the three mouse lines. Three hours after i.p. injection, mean plasma Dex levels were approximately 1.8–2.5 ng/ml in the animals of all three breeding lines, with no significant differences between the lines (Kruskal–Wallis H-test: n = 10/10/10, H = 1.1, d.f. = 2; $P > 0.05$; Fig. 5). However, in all microdialysates collected after the injection of Dex, no concentration above the detection limit of 0.1 ng/ml could be found. That is, Dex was not detectable in the brains of the animals regardless of breeding line, indicating an intact BBB even in the dialysed area.

Expression of the multidrug resistance 1 P-glycoprotein

Western blot analysis performed in the frontal brain tissue of HR, IR and LR mice revealed no significant differences concerning the expression of the Mdr1 Pgp between the three lines (Kruskal–Wallis H-test: n = 11/10/10, H = 2.7; d.f. = 2; $P > 0.05$; Fig. 6). That is, all animals expressed approximately the same level of this efflux pump.

Discussion

In the present study, three mouse lines selectively bred for HR, IR or LR stress reactivity were used to elucidate the temporal dynamics of HPA axis reactivity in the periphery, as well as in the brain of these animals. In the first MD experiment, we demonstrated that the concentrations of CORT released from the adrenal cortex of the three breeding lines in response to restraint stress are not only distinctly different in the plasma, but also in the hippocampus, although the differences between the three mouse lines were attenuated. Significant differences in intrahippocampal CORT increase were only found when comparing HR mice to IR and LR mice but not between IR and LR animals. Furthermore, a time lag of approximately 60 min was observed regarding intrahippocampal peak concentrations of CORT after the onset of the stressor in all three lines. In the second experiment, we showed that the differential stress-induced increase of intrahippocampal CORT in the three mouse lines is neither caused by a different functionality, nor by surgical damage of the BBB.

The aim of the first experiment was to assess the peripheral GC response of the animals of the three mouse lines to restraint stress and, in parallel, monitor the intrahippocampal CORT increase using *in vivo* MD. The results obtained revealed that there is an association between plasma and intrahippocampal CORT dynamics in response to stress in all three mouse lines, although the CORT differences between the lines were not as pronounced in the hippocampus as in the plasma; in particular, the difference between IR and LR animals was diminished. There were no significant differences in hippocampal CORT levels between HR, IR and LR mice before the onset of the SRT (Fig. 4, I1–I4). Additionally, in these samples, hippocampal CORT concentrations were in a very low range, close to the detection limit of the assay (0.1 ng/ml). Thus, the surgical procedure of the MD probe implantation itself had a similar and rather minor impact on basal hippocampal CORT levels in animals of all three breeding lines. Similarly, the CORT concentrations in the initial plasma samples (i.e. immediately before restraint) were relatively low and not significantly different between the three lines (Fig. 3A). After the 15-min restraint period, however, the HR mice showed a significantly higher plasma CORT response than mice of the IR line and LR mice presented the lowest increase (Fig. 3). However, these significant differences in CORT increase were not observed in the respective dialysate samples collected during this time interval (Fig. 4, SRT). It was only in the first post-SRT (Fig. 4, P1) sample interval (i.e. within 30 min after the onset of the stressor) that significant differences in CORT levels were detected in the hippocampus. These differences were also found in the after two sampling intervals (Fig. 4, P2 and P3). Intrahippocampal peak concentrations of CORT were measured approximately 60 min after the onset of the stressor (Fig. 4, P3). These results are in accordance with data obtained from other studies (15,30), where peak concentrations of CORT in the hippocampus of rats were measured approximately 50 min after the beginning of a 15-min forced swim test. In another study, Thoeringer et al. (28) compared intra-hippocampal CORT dynamics of two inbred mouse strains in response to a 5-min forced swim test. The latency to reach intrahippocampal peak levels was approximately 45 min for C57BL/6J and approximately 75 min for DBA/2 mice, with CORT concentrations returning back to basal values after approximately 60 min for C57BL/6J and about approximately min for DBA/2 mice, indicating strain differences in intrahippocampal CORT dynamics as well as negative-feedback regulation. In the cited studies, the distinct delay between plasma and intrahippocampal peak levels of CORT was explained by the potential involvement of certain proteins (for a more detailed discussion, see below) that regulate the bioavailability of CORT, and thus have an impact on the local abundance of CORT in the brain. In the present study, the animals of the three breeding lines [all derived from a CD-1 founder population; for details, see (11)] not

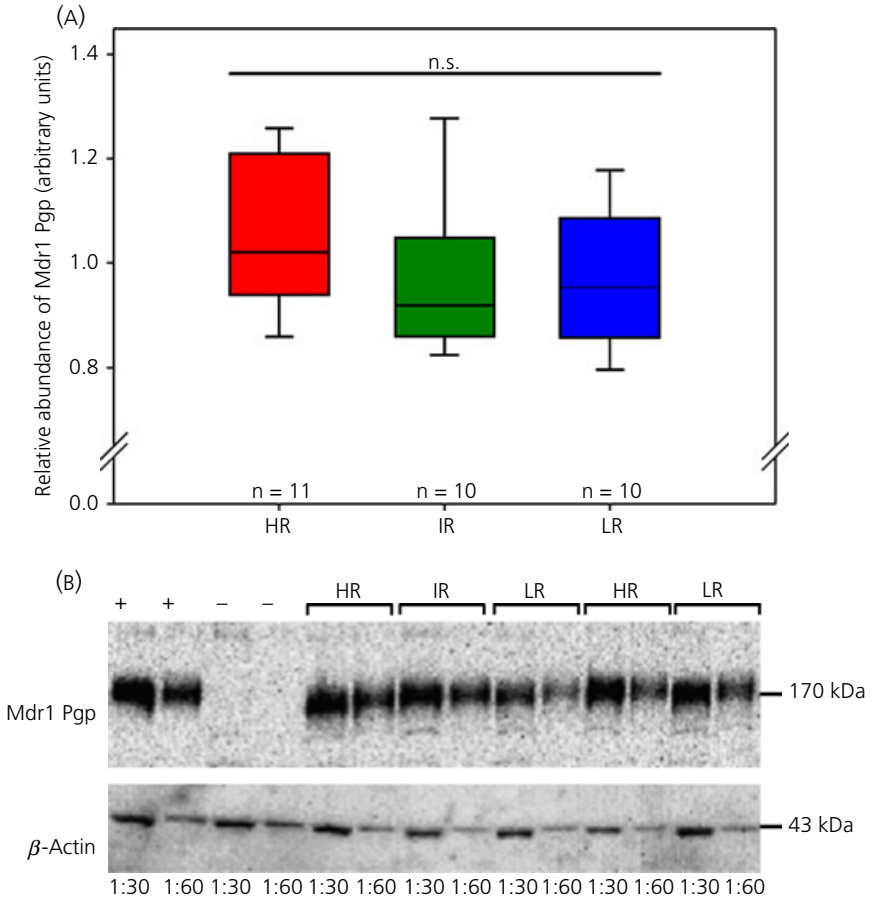

Fig. 6. (A) Relative (to β-actin) abundance of the multi-drug resistance 1 P-glycoprotein (Mdr1 Pgp) in the brains of high (HR), intermediate (IR) and low (LR) stress reactivity mice. No significant differences were found between the lines (Kruskal–Wallis H-test: $P > 0.1$, n.s.). Data are given as box plots showing medians (lines in the boxes) and 25% to 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. (B) Above: representative western blot image of the Mdr1 Pgp expression in the brains of HR, IR and LR stress reactivity mice. Below: western blot image of the β-actin expression in the respective samples of HR, IR and LR stress reactivity mice. *Abcb1 a*/b double knockout mice (FVB-abcb1a/b –/–) were used as negative controls (–). The respective wild-type animals (FVB-abcb1a/b +/+) were used as positive controls (+). Each sample was applied in duplicate at two different concentrations (dilutions of 1 : 30 and 1 : 60).

only showed peak concentrations of CORT approximately 60 min after the onset of the stressor, but also the intrahippocampal CORT concentrations differed significantly between the lines. HR animals showed clearly higher peak concentrations compared to IR and LR animals (Fig. 4), which is in accordance with the elevated stress-induced plasma CORT concentrations in these animals (Fig. 3B). Interestingly, the difference in the stress-induced hippocampal CORT increase between IR and LR mice was attenuated (Fig. 4), implicating a potential contribution of multiple mechanisms involved in the transport, binding, metabolism and clearance of CORT in the brain of these animals (for a more detailed discussion, see below). CORT concentrations no longer differed between the lines after approximately 90 min (Fig. 4, P5) and returned back to basal values after approximately 180 min (Fig. 4, P11). That is, despite the distinctively different HPA axis reactivity in response to stressors, animals of all three lines showed a similar clearance of CORT in the hippocampus, indicating functional negative-feedback mechanisms in HR, IR and LR animals. Additionally, no differences in intrahippocampal CORT levels were found between basal (Fig. 4, I1–I4) and recovery (Fig. 4, R1–R10) samples, suggesting that there were no long-term effects of the SRT on HPA axis activity. However, calculating the AUC of intrahippocampal CORT concentrations (Fig. 4, SRT-P6) revealed a clearly increased activation of the HPA axis in animals of the HR line relative to IR and LR mice after exposure to the stressor, suggesting that the brains of HR mice are subjected to higher amounts of CORT during each stress response.

Chronic or repeated exposure to excessive amounts of stress hormones, particularly CORT, contributes to an impairment of cognitive functions and promotes damage to important brain structures such as the hippocampus (1,5,43). Indeed, in a recent study, we showed that HR animals have cognitive deficits in hippocampus-dependent memory tasks compared to IR and LR mice, thereby indicating a possible contribution of exposure to higher levels of CORT to a decreased hippocampal functioning (13).

As outlined in the Introduction, the Mdr1 Pgp, which is located at the apical membrane of endothelial cells of the BBB (16,17),

functions not only as a drug efflux pump and protects the brain from xenobiotics (18), but also is considered to be involved in the extrusion of endogenous steroids such as CORT (19,20). The bioavailability of CORT in the brain, which also triggers the negative-feedback mechanism, could therefore be dependent on the expression and activity of the Mdr1 Pgp which, in turn, could account for a differential regulation of the HPA axis (23,44).

Therefore, in the second experiment, we investigated the potential influence of a differential BBB functionality on the observed stress reactivity phenotype in HR, IR and LR mice. With respect to the BBB, the strong increase of intrahippocampal CORT concentrations in HR mice as well as the attenuated differences in intrahippocampal CORT increase between IR and LR animals suggests two main possibilities. First, the MD probe implantation caused damage to the BBB, leading to an unrestricted 'leaking' of CORT into the brain according to the differential CORT response in the plasma of the respective mouse line. Second, the Mdr1 Pgp is differentially expressed in the BBB of HR, IR and LR mice, leading to an altered transport dynamic of CORT by this efflux pump. Therefore, mice expressing more Mdr1 Pgp would be able to extrude more steroid hormone out of the brain in the same time interval than mice with a lower expression of Mdr1 Pgp. That is, the peripheral stress-induced CORT increase would not necessarily reflect the intrahippocampal CORT response in these animals.

To test the possibility that the BBB was damaged by the surgical implantation of the MD probe, we applied Dex via i.p. injections to all experimental animals. We found that the synthetic steroid hormone Dex, which is a high affinity substrate for the Mdr1 Pgp and thus restricted from entering the brain (37,44), could exclusively be detected in the plasma (Fig. 5), whereas it was virtually absent in the brain (i.e. below the detection limit in all MD samples). This is in accordance with data from other studies, where it has been shown that, after surgical intervention (i.e. implantation of a MD probe), the BBB had fully recovered at the latest 24 h after surgery (31–33).

Furthermore, addressing the second possibility mentioned above, our western blot analysis did not reveal significant differences in Mdr1 Pgp expression between the three mouse lines (Fig. 6). However, the absence of a difference in immuno-reactivity for the Mdr1 Pgp pump does not necessarily mean that there is no difference in the activity of the pump. Uhr and colleagues showed in a clinical study that single nucleotide polymorphisms in the human drug transporter gene *ABCB1* predicted the treatment response to antidepressants (35). That is, despite the equal abundance of the drug transporter protein, polymorphisms in the genotype of this protein could account for differences in its functionality and therefore in its pharmacokinetics. Hence, it is possible that the differences in intrahippocampal CORT increase and clearance between the three breeding lines were brought about by alterations in the genotype of the Mdr1 Pgp of the experimental animals. In this respect, it is of note that murine rodents express two isoforms of the Mdr1 Pgp (45), Mdr1a and Mdr1b Pgp (also called Abcb1a and Abcb1b), which are coded by two separate genes, *abcb1a* and *abcb1b*. Although *abcb1a* and *abcb1b* are not always expressed in the same organs, the overall distribution of their gene products coincides approximately with that of the single *ABCB1* gene in humans, suggesting that Mdr1a and Mdr1b together function in the same manner as human MDR1 (46). Unfortunately, it is not possible to distinguish between the two highly homologous isoforms of Mdr1 Pgp on the protein level as a result of a lack of specific antibodies.

Hence, our findings demonstrate that the significant difference in stress-induced intrahippocampal CORT increase of HR mice compared to IR and LR animals, as well as the similar clearance of CORT between the three mouse lines, can neither be explained by an altered expression of the Mdr1 Pgp in the BBB, nor by damages to the BBB.

In addition to the Mdr1 Pgp, other proteins contribute to the bioavailability of CORT in the brain and thus to the activation of the negative-feedback mechanisms of the HPA axis. For example, corticosteroid binding globulin is known to restrict the amount of bioavailable CORT, with more than 90% of circulating corticosteroid molecules being bound in the blood by this carrier protein (47). Corticosteroid binding globulin was also detected in the rat hypothalamus, suggesting that it might exceed its role as a mere steroid transporter and actively participate in the neuroendocrine regulation of the central nervous system, including the stress response (48). A particular role in the negative-feedback mechanism of the HPA axis is also exerted by the CORT metabolising enzyme 11-beta-hydroxysteroid-dehydrogenase type 1 (11β-HSD1). 11β-HSD1 converts inactive dehydrocorticosterone to active corticosterone and is very abundant in the CA3 regions of the hippocampus and the granule cell layer of the dentate gyrus (49). Interestingly, it was shown that mice deficient for 11β-HSD1 responded with an exaggerated release of ACTH and CORT to a 10-min restraint stress, indicating a diminished activation of the negative-feedback mechanisms in the brain of these animals (50). If and how these factors are associated with the observed endophenotypes in our SR mouse model requires investigation and will be the subject of future studies.

Taken together, in the present MD study, we demonstrated that the concentrations of CORT released from the adrenal cortex of HR, IR and LR mice in response to restraint stress are distinctly different in the plasma and can also be observed in the hippocampus, although the differences are markedly attenuated in this tissue, particularly between IR and LR animals. Additionally, a certain time delay between increased levels of CORT in the periphery and the central nervous system was found. The data obtained suggest that the differences in the intrahippocampal CORT response are not brought about by differences in BBB functionality because Mdr1 Pgp was equally expressed in HR, IR and LR mice. Furthermore, we could exclude surgical damage of the BBB because Dex could exclusively be detected in the plasma and was virtually absent in the brain. Regarding the scientific approach to model the aetiology and molecular–genetic mechanism of affective disorders, the SR mouse model asserts a clear advantage compared to other animal models in that the mice are not genetically manipulated to express a morbid phenotype but rather reflect the situation in MD patients (i.e. being genetically predisposed and therefore more susceptible or resilient to develop the disease). This allows an investigation of the progression of the disease as well as predisposing genetic and

environmental factors in more detail. Therefore, knowledge gained from studies on the SR mouse model might contribute to the development of new treatment strategies for MDD, in particular regarding HPA axis dysregulation as an important neuroendocrine endophenotype of the disease (3–7).

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Conflict of Interest

J-M Heinzmann, C-K Thoeringer, A Knapman, R Palme, F Holsboer, M Uhr, R Landgraf and C Touma report no conflict of interest associated with the content of this paper and have no financial interest regarding any of its content.

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References

1 de Kloet ER, Vreugdenhil E, Oitzl MS, Joels M. Brain corticosteroid receptor balance in health and disease. *Endocr Rev* 1998; **19**: 269–301.
2 Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions *Endocr Rev* 2000; **21**: 55–89.
3 Holsboer F, Ising M. Stress hormone regulation: biological role and translation into therapy. *Annu Rev Psychol* 2010; **61**: 81–109, C101–111.
4 Holsboer F. The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* 2000; **23**: 477–501.
5 de Kloet ER, Joels M, Holsboer F. Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 2005; **6**: 463–475.
6 Gold PW, Chrousos GP. Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states. *Mol Psychiatry* 2002; **7**: 254–275.
7 Thomson F, Craighead M. Innovative approaches for the treatment of depression: targeting the HPA axis. *Neurochem Res* 2008; **33**: 691–707.
8 Antonijevic IA. Depressive disorders – is it time to endorse different pathophysiologies? *Psychoneuroendocrinology* 2006; **31**: 1–15.
9 Ising M, Kunzel HE, Binder EB, Nickel T, Modell S, Holsboer F. The combined dexamethasone/CRH test as a potential surrogate marker in depression. *Prog Neuropsychopharmacol Biol Psychiatry* 2005; **29**: 1085–1093.
10 Zobel AW, Nickel T, Sonntag A, Uhr M, Holsboer F, Ising M. Cortisol response in the combined dexamethasone/CRH test as predictor of relapse in patients with remitted depression. a prospective study. *J Psychiatr Res* 2001; **35**: 83–94.
11 Touma C, Bunck M, Glasl L, Nussbaumer M, Palme R, Stein H, Wolferstatter M, Zeh R, Zimbelmann M, Holsboer F, Landgraf R. Mice selected for high versus low stress reactivity: a new animal model for affective disorders. *Psychoneuroendocrinology* 2008; **33**: 839–862.
12 Touma C, Fenzl T, Ruschel J, Palme R, Holsboer F, Kimura M, Landgraf R. Rhythmicity in mice selected for extremes in stress reactivity: behavioural, endocrine and sleep changes resembling endophenotypes of major depression. *PLoS ONE* 2009; **4**: e4325.
13 Knapman A, Heinzmann JM, Hellweg R, Holsboer F, Landgraf R, Touma C. Increased stress reactivity is associated with cognitive deficits and decreased hippocampal brain-derived neurotrophic factor in a mouse model of affective disorders. *J Psychiatr Res* 2010; **44**: 566–575.
14 Knapman A, Heinzmann JM, Holsboer F, Landgraf R, Touma C. Modeling psychotic and cognitive symptoms of affective disorders: disrupted latent inhibition and reversal learning deficits in highly stress reactive mice. *Neurobiol Learn Mem* 2010; **94**: 145–152.
15 Droste SK, Collins A, Lightman SL, Linthorst AC, Reul JM. Distinct, time-dependent effects of voluntary exercise on circadian and ultradian rhythms and stress responses of free corticosterone in the rat hippocampus. *Endocrinology* 2009; **150**: 4170–4179.
16 Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR, Bertino JR. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci USA* 1989; **86**: 695–698.
17 Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* 2003; **55**: 3–29.
18 Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CA, van der Valk MA, Robanus-Maandag EC, te Riele HP, Berns AJM, Borst P. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* 1994; **77**: 491–502.
19 Uhr M, Holsboer F, Muller MB. Penetration of endogenous steroid hormones corticosterone, cortisol, aldosterone and progesterone into the brain is enhanced in mice deficient for both mdr1a and mdr1b P-glycoproteins. *J Neuroendocrinol* 2002; **14**: 753–759.
20 Wolf DC, Horwitz SB. P-glycoprotein transports corticosterone and is photoaffinity-labeled by the steroid. *Int J Cancer* 1992; **52**: 141–146.
21 Mason BL, Pariante CM, Thomas SA. A revised role for P-glycoprotein in the brain distribution of dexamethasone, cortisol, and corticosterone in wild-type and ABCB1A/B-deficient mice. *Endocrinology* 2008; **149**: 5244–5253.
22 Karssen AM, Meijer OC, van der Sandt IC, Lucassen PJ, de Lange EC, de Boer AG, de Kloet ER. Multidrug resistance P-glycoprotein hampers the access of cortisol but not of corticosterone to mouse and human brain. *Endocrinology* 2001; **142**: 2686–2694.
23 Pariante CM, Makoff A, Lovestone S, Feroli S, Heyden A, Miller AH, Kerwin RW. Antidepressants enhance glucocorticoid receptor function in vitro by modulating the membrane steroid transporters. *Br J Pharmacol* 2001; **134**: 1335–1343.
24 Thoeringer CK, Wultsch T, Shahbazian A, Painsipp E, Holzer P. Multidrug-resistance gene 1-type p-glycoprotein (MDR1 p-gp) inhibition by tariquidar impacts on neuroendocrine and behavioral processing of stress. *Psychoneuroendocrinology* 2007; **32**: 1028–1040.
25 Linthorst AC, Flachskamm C, Holsboer F, Reul JM. Local administration of recombinant human interleukin-1 beta in the rat hippocampus increases serotonergic neurotransmission, hypothalamic-pituitary-adrenocortical axis activity, and body temperature. *Endocrinology* 1994; **135**: 520–532.
26 Linthorst AC, Flachskamm C, Muller-Preuss P, Holsboer F, Reul JM. Effect of bacterial endotoxin and interleukin-1 beta on hippocampal serotonergic neurotransmission, behavioral activity, and free corticosterone levels: an in vivo microdialysis study. *J Neurosci* 1995; **15**: 2920–2934.
27 Linthorst AC, Reul JM. Stress and the brain: solving the puzzle using microdialysis. *Pharmacol Biochem Behav* 2008; **90**: 163–173.
28 Thoeringer CK, Sillaber I, Roedel A, Erhardt A, Mueller MB, Ohl F, Holsboer F, Keck ME. The temporal dynamics of intrahippocampal

corticosterone in response to stress-related stimuli with different emotional and physical load: an in vivo microdialysis study in C57BL/6 and DBA/2 inbred mice. *Psychoneuroendocrinology* 2007; **32**: 746–757.
29 Beekman M, Flachskamm C, Linthorst AC. Effects of exposure to a predator on behaviour and serotonergic neurotransmission in different brain regions of C57bl/6N mice. *Eur J Neurosci* 2005; **21**: 2825–2836.
30 Droste SK, de Groote L, Atkinson HC, Lightman SL, Reul JM, Linthorst AC. Corticosterone levels in the brain show a distinct ultradian rhythm but a delayed response to forced swim stress. *Endocrinology* 2008; **149**: 3244–3253.
31 Benveniste H, Huttemeier PC. Microdialysis – theory and application. *Prog Neurobiol* 1990; **35**: 195–215.
32 Plock N, Kloft C. Microdialysis – theoretical background and recent implementation in applied life-sciences. *Eur J Pharm Sci* 2005; **25**: 1–24.
33 Watson CJ, Venton BJ, Kennedy RT. In vivo measurements of neurotransmitters by microdialysis sampling. *Anal Chem* 2006; **78**: 1391–1399.
34 Engelmann M, Ebner K, Landgraf R, Wotjak CT. Effects of Morris water maze testing on the neuroendocrine stress response and intrahypothalamic release of vasopressin and oxytocin in the rat. *Horm Behav* 2006; **50**: 496–501.
35 Uhr M, Tontsch A, Namendorf C, Ripke S, Lucae S, Ising M, Dose T, Ebinger M, Rosenhagen M, Kohli M, Kloiber S, Salyakina D, Bettecken T, Specht M, Putz B, Binder EB, Muller-Myhsok B, Holsboer F. Polymorphisms in the drug transporter gene ABCB1 predict antidepressant treatment response in depression. *Neuron* 2008; **57**: 203–209.
36 Schinkel AH. The physiological function of drug-transporting P-glycoproteins. *Semin Cancer Biol* 1997; **8**: 161–170.
37 de Kloet ER. Why dexamethasone poorly penetrates in brain. *Stress* 1997; **2**: 13–20.
38 Altuvia S, Stein WD, Goldenberg S, Kane SE, Pastan I, Gottesman MM. Targeted disruption of the mouse mdr1b gene reveals that steroid hormones enhance mdr gene expression. *J Biol Chem* 1993; **268**: 27127–27132.
39 Franklin KBJ, Paxinos G. The Mouse Brain in Stereotaxic Coordinates. San Diego, CA: Academic Press, 2001.
40 Touma C, Sachser N, Mostl E, Palme R. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *Gen Comp Endocrinol* 2003; **130**: 267–278.
41 Palme R, Möstl E. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *Z Säugetierkd – Int J Mamm Biol* 1997; **62**(Suppl 2): 192–197.
42 Siegel S, Castellan NJ. *Nonparametric Statistics for the Behavioral Science*. New York, NY: McGraw-Hill Book Company, 1988.
43 McEwen BS. The neurobiology of stress: from serendipity to clinical relevance. *Brain Res* 2000; **886**: 172–189.
44 Müller MB, Keck ME, Binder EB, Kresse AE, Hagemeyer TP, Landgraf R, Holsboer F, Uhr M. ABCB1 (MDR1)-type P-glycoproteins at the blood-brain barrier modulate the activity of the hypothalamic-pituitary-adrenocortical system: implications for affective disorder. *Neuropsychopharmacology* 2003; **28**: 1991–1999.
45 Devault A, Gros P. Two members of the mouse mdr gene family confer multidrug resistance with overlapping but distinct drug specificities. *Mol Cell Biol* 1990; **10**: 1652–1663.
46 Uhr M, Grauer MT, Holsboer F. Differential enhancement of antidepressant penetration into the brain in mice with abcb1ab (mdr1ab) P-glycoprotein gene disruption. *Biol Psychiatry* 2003; **54**: 840–846.
47 Breuner CW, Orchinik M. Plasma binding proteins as mediators of corticosteroid action in vertebrates. *J Endocrinol* 2002; **175**: 99–112.
48 Mopert B, Herbert Z, Caldwell JD, Jirikowski GF. Expression of corticosterone-binding globulin in the rat hypothalamus. *Horm Metab Res* 2006; **38**: 246–252.
49 Pelletier G, Luu-The V, Li S, Bujold G, Labrie F. Localization and glucocorticoid regulation of 11beta-hydroxysteroid dehydrogenase type 1 mRNA in the male mouse forebrain. *Neuroscience* 2007; **145**: 110–115.
50 Harris HJ, Kotelevtsev Y, Mullins JJ, Seckl JR, Holmes MC. Intracellular regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase (11beta-HSD)-1 plays a key role in regulation of the hypothalamic-pituitary-adrenal axis: analysis of 11beta-HSD-1-deficient mice. *Endocrinology* 2001; **142**: 114–120.

