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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

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Summary
There is strong evidence for a pivotal interaction of corticosteroid signalling and behavioral adaptation to stress. To further elucidate this relation, we monitored the dynamics of free corticosterone in the murine hippocampus of two inbred mouse strains using *in vivo* microdialysis. C57BL/6JOlaHsd (C57BL/6) and DBA/2OlaHsd (DBA/2) inbred mouse strains have been shown to differ in their anxiety-related and depression-like behavior and provide, thus, an interesting animal model to study the stimulus-response profile of the hypothalamus–pituitary–adrenocortical (HPA) system as a function of emotional and physical load.
We, first, compared peripheral and intracerebral concentration patterns of corticosterone by simultaneous microdialysis of the jugular vein and the hippocampus in anesthetized mice and found strain differences in blood versus intracerebral free corticosterone concentrations. C57BL/6 showed almost the same steroid levels in either compartment, whereas DBA/2 mice displayed higher glucocorticoid levels in the circulation than in the hippocampus. This data suggest a strain difference in the tissue environment influencing the amount of biological active corticosterone at the receptor site.
Measurements of intrahippocampal corticosterone in freely moving mice revealed that DBA/2 display a prolonged glucocorticoid increase in response to a single forced swimming stress (FST), as compared to C57BL/6 mice indicating a reduced inhibitory HPA axis

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feedback. Exposure to a novel environment (NE) induced a desensitization of the HPA system in DBA/2 animals as they show an attenuated intracerebral corticosterone dynamics after a subsequent FST. Testing animals in an elevated plus-maze (EPM), however, did not significantly stimulate coriticosterone release in either strain. The analysis of the area under the curve revealed a high amount of corticosterone released through FST and a low glucocorticoid release after NE or EPM exposure that are independent of the strain. This data indicate a strong stimulus dependency of corticosterone secretion that is strain independent, whereas the dynamics and feedback of the HPA axis is different between both inbred strains.

Behavioral phenotyping of animals revealed a strong impact of microdialysis procedure on FST and EPM performance. Innate emotionality differences of both strains, however, were not affected.

Though descriptive in nature, the present results suggest an altered corticosteroid signalling in the DBA/2 strain compared to C57BL/6 mice. Whether this observation causally underlies the differences in anxiety-related and depression-like behavior has to be further experimentally validated. In addition, our study highlights the use of *in vivo* microdialysis to assess the neuroendocrine endophenotype of animal models via profiling of stimulus-response patterns of stress hormones.

1. Introduction

The hypothalamus–pituitary–adrenocortical (HPA) system plays a vital role in an organism's homeostatic function that is constantly challenged by intrinsic or extrinsic stress. Activation of the HPA system improves an individual's ability to restore homeostasis and facilitates adaptation via secretion of stress hormones (McEwen, 2000): Cortisol (in humans) and corticosterone (in rats and mice) act at multiple levels of homeostatic regulation. They are potent modulators of the function of neuronal cells and networks that control emotions and cognitive processes (de Kloet et al., 2002). In addition, corticosteroids play a crucial role regulating fear- and anxiety-related behavior (Korte, 2001). In close interaction with other components of the stress hormone system, glucocorticoids maintain basal activity of the HPA axis and control the threshold of HPA system's response to stress (Sapolsky et al., 2000). These hormones terminate the stress response by inhibitory feedback action at the level of the pituitary, hypothalamus and limbic brain areas, including the hippocampus, amygdala and septum (Herman et al., 2003). This essentially dynamic process is mediated by two corticosteroid receptor molecules, high-affinity mineralocorticoid receptors (MRs) and low-affinity glucocorticoid receptors (GRs), acting both by modifying gene transcription activity and by non-genomic mechanisms on cell signalling processes (de Kloet, 2004). The MR primarily mediates the effects of, and possibly controls, low basal circadian levels of circulating steroids, whereas the GR appears to act at stress-related levels of glucocorticoids and mediates inhibitory feedback effects on the HPA system (de Kloet et al., 1998). Within the HPA system, corticotropin-releasing factor (CRF), together with arginine vasopressin (AVP), appears to be the main hypothalamic regulator of basal and stress-induced release of pituitary corticotropin (ACTH), which, subsequently, mediates the synthesis and secretion of glucocorticoid hormones from the cortex of the adrenal glands (Antoni, 1993; Vale et al., 1981).

With the intent to gain more insight into HPA axis responsiveness, the present study was conducted to investigate several temporal and CNS-related aspects of the neuroendocrine system in an animal model comprising two different inbred mouse strains. C57BL/6 and DBA/2 inbred mice have previously been used as a genetic animal model for anxiety-related and depression-like behavior (Crawley et al., 1997; Griebel et al., 1997; Ohl et al., 2003; Jacobson and Cryan, 2007) as well as a model of distinct hippocampal functioning regarding learning and memory (Ammassari-Teule et al., 2000; Passino et al., 2002). Moreover, it has been reported that mice of both strains show differences in glucocorticoid hormone responses towards stress but not under basal conditions (Cabib et al., 1990; Jones et al., 1998; McNamara et al., 2003; Shanks et al., 1990). However, the estimation of basal hormone action and HPA system regulation is rather complicated in these two inbred strains as they differ in their plasma concentrations of corticosterone binding globuline (Jones et al., 1998). This protein binds circulating corticosterone in the blood with less than 5% remaining unbound, i.e. free and biological active (de Kloet et al., 1998).

Based on the published observations of different behavioral phenotypes, stress responsiveness and glucocorticoid status of these two inbred mouse strains, we performed *in vivo* microdialysis studies to determine the dynamics of biological active, free corticosterone. As this *in vivo* technique enables the continuous sampling of stress hormones at target receptor sites of laboratory animals, the present series of experiments were aimed at creating a stimulus-response profile of the HPA axis in both inbred mouse strains via determination of intrahippocampal free corticosterone. Importantly, the dynamics of the glucocorticoid hormone under basal conditions and in response to stressors with different emotional and physical load is thought to represent, at least phenomenologically, the feedback action of the HPA system. The hippocampus was selected as the site of probe insertion

because of its key role in the coordination of the adaptive neuroendocrine and behavioral response to stress and its postulated aberrant functioning in stress-related psychiatric diseases (Herman et al., 2003; Lupien and Lepage, 2001).

In the first experiment, however, simultaneous *in vivo* microdialysis in the jugular vein and the hippocampus was performed to determine basal and CRF-stimulated levels of peripheral versus intracerebral free corticosterone in both strains.

In order to confirm strain differences in depression-like and anxiety-related behavior under our laboratory conditions, we performed a forced swimming test (FST) (Porsolt et al., 1977) and the elevated plus-maze test (EPM) (Pellow and File, 1986) in naïve mice of both strains. In addition, animals subjected to microdialysis were also phenotyped to determine the influence of this procedure *per se* on behavior.

2. Material and methods

2.1. Animals

All experiments were performed with male C57BL/6JOlaHsd (C57BL/6; $n = 62$) and DBA/2OlaHsd (DBA/2; $n = 67$) mice purchased from Harlan Winkelmann Laboratories (Borchen, Germany) at least 2 weeks before surgery. All animals were housed individually in polycarbonate cages ($30 \times 20 \times 14$ cm) under standard laboratory conditions in the animal facility of the Max Planck Institute (temperature 22–23 °C, relative humidity 60%) with lights on between 06:00 and 18:00 h. Food pellets and water were available *ad libitum*. At the time of the experiments, mice were 11–13 weeks old, with a body weight of 23–28 g.

The animal studies were conducted in accordance with the guidelines set by the National Institutes of Health, USA, and the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany.

2.2. Behavioral phenotyping

2.2.1. Forced swimming test

The FST was used to induce an emotional stress in order to analyze the HPA axis responsiveness and to assess stress coping behavior (Porsolt et al., 1977). Microdialyzed animals were forced to swim in a glass beaker (diameter 12.5 cm) containing water (25 °C) for 5 min. The water depth was 11.5 cm, preventing the mice to touch the bottom of the glass with their paws or tail. After completion of the swim procedure mice were returned to their home cages. Behavioral activity was recorded on a video tape during the entire experiment and scored by a trained observer by means of the Observer software (Noldus, Wageningen, NL). Parameters were classified according to the following three categories (Cryan et al., 2002): time spent on climbing, swimming, and immobility, with the latter reflecting a passive stress coping behavior. No diving was observed. Additionally, naïve mice of both strains that did not undergo microdialysis were tested to obtain baseline behavioral data (control group). All experiments were performed between 06:00 and 12:00 h.

2.2.2. Elevated plus-maze

For the assessment of anxiety-related behavior, mice were exposed to the EPM for 5 min (Pellow and File, 1986). The maze was elevated to a height of 50 cm and comprised two opposing open arms ($30 \times 5 \times 0.5$ cm) and two opposing enclosed arms ($30 \times 5 \times 15$ cm) connected by a central platform (5×5 cm), forming the shape of a plus sign (Rodgers and Dalvi, 1997). All parts of the apparatus were made of dark gray polyvinyl plastic. The lightening in the experimental room was adapted to illuminate the open arms with an intensity of 150 lx. To enable the microdialyzed mice to move freely within all compartments of the EPM, a special device of flexible arms was designed to which the microdialysis swivel and counterbalancing apparatus were connected.

At the beginning of the experiment, a mouse was placed in the center of the maze facing one of the open arms. The anxiety-related parameters comprised the time spent in, or the entries made into, the open and closed arms and the central platform, respectively. Results were also calculated as ratio of time spent and entries into the open arms. Additionally, grooming behavior was scored. The behavioral performance was recorded on a video tape during the entire experiment and later scored by a trained observer.

2.3. Surgical procedures

2.3.1. Surgery for hippocampal microdialysis

Surgery and microdialysis were performed as described previously (Linthorst et al., 2000; Sillaber et al., 1998). Briefly, mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf, Tujunga, CA, USA) with adapted components. After exposure of the skull a hole was drilled to implant a sterile, stainless-steel guide cannula (CMA/7; CMA/Microdialysis AB, Stockholm, Sweden) entering the dorsal left hippocampus. Coordinates were determined according to a stereotaxic mouse brain atlas (Franklin and Paxinos, 2001): +1.4 mm lateral, −2.0 mm posterior, and −1.4 mm ventral, with the bregma as an overall zero. The implanted guide cannula was fixed with epoxy glue and dental cement. To connect a liquid swivel and counterbalancing arm during the microdialysis experiments, a small peg was mounted to the skull.

After surgery, mice were moved to the experimental room with similar housing conditions as in the animal facility and housed singly in special plexiglas cages ($25 \times 25 \times 35$ cm) with free access to water and food. Animals were kept there for 7 days for recovery. Until testing, mice were handled daily to reduce non-specific stress during the experiments.

2.3.2. Jugular vein preparation

Animals were anesthetized by i.p.-injection of sodium pentobarbital (see above) and were kept anesthetized during the whole experiment. Additionally, they were placed under a heating lamp to prevent hypothermia. Then, the jugular vein was dissected and a microdialysis probe (CMA/7; CMA/Microdialysis AB; for details see later) was carefully inserted and surgically fixed. In order to prevent blood coagulation around the inserted probe mice were treated with heparin (30 IU, s.c.).

Additionally, 0.3 ml of saline was administered subcutaneously to avoid dehydration.

2.4. *In vivo* microdialysis procedures

Seven days after intrahippocampal guide cannula implantation, a microdialysis probe (CMA/7; CMA/Microdialysis AB; membrane: cuprophane with a molecular cut-off of 6000 DA; outer diameter: 0.24 mm; length: 1 mm) was inserted under light and short-lasting isoflurane anesthesia. Probe insertion was regularly performed in the evening, the day before the actual experiment. In addition, mice were connected to a liquid swivel and counterbalancing system (Instech Laboratories, Plymouth Meeting, PA, USA). Probes were perfused with sterile, pyrogen-free Ringer solution (Fresenius, Bad Homburg, Germany; 147 mM NaCl, 4 mM KCl, 2.25 mM $CaCl_2$) at a flow rate of 2.0 μl/min by use of a microinfusion pump. Microdialysis samples were collected in a plastic vial on top of the swivel, stored at −20 °C until measurement of free corticosterone.

2.5. Experimental protocols

2.5.1. Experiment 1: simultaneous measurement of free corticosterone in the hippocampus and the jugular vein

In the first *in vivo* microdialysis study, free corticosterone was measured simultaneously both in the murine hippocampus and the jugular vein in order to be able to (i) compare the levels of circulating free corticosterone in the peripheral blood with intracerebral concentrations and (ii) to determine the dynamics of corticosterone release within the blood and the hippocampus triggered by intraperitoneally administered CRF. First, three consecutive 10 min samples were collected to obtain basal levels of intrahippocampal corticosterone. Then, during continuous 10 min sampling from the cerebral probe, mice were anesthetized and a second microdialysis probe was implanted in the jugular vein as described above. Subsequently, three consecutive venous and cerebral microdialysates were collected simultaneously every 10 min. During the fourth dialysis period, a CRF challenge (10 ng/kg, i.p.) was performed. To assess the effects of HPA axis stimulation on peripheral and intracerebral corticosterone concentrations, seven additional dialysates were collected. Importantly, animals were kept under general anesthesia (see above) from jugular vein surgery on for the remaining experimental procedure.

In addition, naïve animals of both strains were decapitated under basal conditions and trunk blood was taken to assay total amounts (free and protein bound) of peripheral corticosterone.

All experiments were performed on day 1 after the insertion of the microdialysis probe between 06:00 and 12:00 h to avoid interference with rodents' physiologically rising corticosterone levels in the afternoon (Oshima et al., 2003).

2.5.2. Experiment 2: analysis of a single forced swimming stress on intrahippocampal corticosterone levels

To study the effects of a pronounced emotional and physical stress on HPA system activity, intrahippocampal corticosterone levels were determined in response to a single forced swimming exposure. To monitor basal concentrations, five consecutive 10 min dialysates were collected. Then, during the sixth sampling period, animals were subjected to a 5 min forced swimming stress. To assess post-stress levels, microdialysis samples were collected every 10 min for the next 2 h. Simultaneously, the behavioral activity during FST was videotaped for behavioral assessment.

In addition, a group of naïve mice was tested in a FST to assess the effects of microdialysis on the behavioral performance.

2.5.3. Experiment 3: effects of exposure to a novel environment as a "primer" on FST-induced HPA system activation

In a further experiment, we wanted to investigate whether a first exposure to a novel environment (NE), representing a rather mild stimulus, exerts a modulating influence on HPA system dynamics in these two inbred mouse strains when further subjected to a more severe stressor (i.e. FST).

Therefore, C57BL/6 and DBA/2 mice were dialysed under basal conditions (four consecutive 10 min dialysates) and, during the fifth dialysis period, animals were subjected to a NE for 10 min, i.e. the ground plates of their home cages were replaced by fresh and clean plates that did not contain any bedding. After 10 min, the ground plates were re-exchanged. Assessing post-stress levels, 12 consecutive 10 min samples were collected until mice were exposed to a 5 min forced swimming stress. Subsequently, 12 additional 10 min microdialysates were sampled to determine post-stress corticosterone dynamics. Behavioral performance in the FST was videotaped.

2.5.4. Experiment 4: determination of corticosterone release in response to an elevated plus-maze exposure

Intrahippocampal corticosterone levels were analyzed under basal conditions and in response to an EPM exposure. As in experiment 2, five consecutive 10 min basal dialysates were sampled and, during the sixth sampling period, mice were placed on the EPM for 5 min. Then, mice were returned to their home cages and a 2 h follow-up sampling was performed every 10 min. Behavior was videotaped during the exposure period for further characterization. As in experiment 2, a group of naïve mice was tested to assess the effects of microdialysis on EPM behavioral performance.

2.6. Measurement of free corticosterone

Microdialysis samples were assayed for free corticosterone by means of a radioimmunoassay (ICN Biomedicals, Costa Mesa, CA, USA). An aliquot of 10 μl was taken from each sample and assayed without prior dilution. To increase the detection limit of the assay (approximately 0.02 ng/ml), the standard curve was modified with additional calibrators in the low-concentration range. Analyzing total plasma corticosterone levels was performed according to the manufacturer's guide. The intra-assay coefficient of variation for corticosterone was approximately 5.0%.

The *in vitro* recovery of the microdialysis probes for corticosterone were determined as 19.8±2.3% (mean± S.E.M.; $n = 3$).

2.7. Histology

At the end of microdialysis experiments, animals were sacrificed by an overdose of isoflurane. Brains were removed, frozen in prechilled methylbutane on dry ice and stored at −80 °C. For the histological verification of the probes' neuroanatomical localization, brains were sectioned in a cryostat and 18 μm-sections were stained with cresyl violet. Only data from mice with correctly placed microdialysis probes were included in the analysis.

2.8. Data analysis and statistics

All results are reported as means±S.E.M. Behavioral data of the EPM and the FST were analyzed by means of multivariate and univariate analyses of variance (MANOVA/ANOVAs) with factors comprising strain, microdialysis and exposure to a NE, respectively. Separate analyses were calculated by means of *t*-tests.

For microdialysis data, corticosterone levels are expressed as percentage of baseline, with the exception of the experiments dealing with simultaneous blood–brain microdialysis (data expressed as absolute values). Analyses of variance with repeated measures were performed to estimate the effects of strain and stress-related stimuli on levels of free corticosterone. When appropriate, Newman–Keuls *post-hoc* tests were calculated. For further analyses, we used Mann–Whitney *U*-Tests, *t*-tests and parametric correlation statistics (see Section 3). The total area under the curve (AUC) of absolute corticosterone levels was calculated according to the trapezoid rule. Statistical significance was accepted if $p < 0.05$.

3. Results

3.1. Behavioral phenotyping: forced swimming test

In the first analysis, we compared FST data from mice undergoing microdialysis (experiment 2) with data obtained from control animals (Table 1). Multivariate analysis revealed a significant effect for the factor strain (Wilks' Lambda: $F_{3.32} = 3.798$, $p = 0.019$) and microdialysis (Wilks' Lambda: $F_{3.32} = 22.621$, $p = 0.000$); no interaction effects were found (Wilks' Lambda: $F_{3.32} = 0.832$, $p = 0.486$). Univariate analysis revealed that the microdialysis procedure significantly influenced swimming behavior ($F_{1.34} = 51.603$, $p = 0.000$), the time the animals spent climbing ($F_{1.34} = 55.334$, $p = 0.000$) and the immobility time ($F_{1.34} = 13.606$, $p = 0.001$). In addition, we found significant strain differences for swimming ($F_{1.34} = 6.245$, $p = 0.017$), climbing ($F_{1.34} = 9.424$, $p = 0.004$) and immobility ($F_{1.34} = 4.661$, $p = 0.038$). In general, C57BL/6 mice spent less time immobile and swimming but more time climbing as compared to DBA/2 animals.

In a separate analysis, we compared the behavioral performance of naïve mice versus animals subjected to microdialysis for each mouse strain. We observed a significant decrease of swimming behavior and an increase of climbing behavior in mice of both strains undergoing microdialysis. In addition, immobility times were significantly decreased in the C57BL/6 strain but only nominally in DBA/2 animals.

To investigate whether or not a prior exposure to a NE influences the animals' performance in the FST, we further compared the behavioral data of both microdialysis groups (Table 2). We were able to detect multivariate effects for the factors strain (Wilks' Lambda: $F_{3.31} = 0.906$, $p = 0.018$) and NE exposure (Wilks' Lambda: $F_{3.31} = 25.950$, $p = 0.000$), while the interaction of both factors was not significant (Wilks' Lambda: $F_{3.31} = 0.292$, $p = 0.831$). With respect to the prior exposure to a NE, univariate effects

Table 1 Behavioral performance of C57BL/6 and DBA/2 mice during microdialysis (EPM- and single FST-exposure) as compared to naive animals.

Behavioral parameter	C57BL/6		DBA/2	
	Naive ($n = 10$)	MD ($n = 8$)	Naive ($n = 10$)	MD ($n = 10$)
Forced swimming				
Time (%) swimming	28.45±6.12	9.57±1.24*	38.61±5.96[1,2]	13.44±2.07*
Time (%) climbing	25.96±5.78	69.31±5.95*	11.75±1.89[1,2]	48.35±3.66*
Time (%) immobility	45.58±4.98	21.12±4.18*	49.29±3.20[1,2]	38.21±6.70
Elevated plus-maze	Naive ($n = 10$)	MD ($n = 10$)	Naive ($n = 10$)	MD ($n = 10$)
Entries (no.) into open arms	18.20±0.87	14.78±0.95*	13.60±1.34[1,2]	12.33±0.76
Time (%) in open arms	49.37±2.88	44.33±5.80	21.45±2.72[1]	18.34±1.90
Entries (no.) into closed arms	10.80±1.62	8.56±1.63	13.01± 1.91[1]	14.78±1.33
Time (%) in closed arms	28.81±1.88	32.33±5.97	53.64±3.29[1]	49.39±4.91
Entries (no.) into the center	29.60±1.51	19.11±2.02*	25.90±2.29[2]	26.44±1.97
Time (%) in the center	17.07±1.26	16.47±1.26	19.26±2.09[1]	24.91±3.22
Ratio time/entry in open arms	2.77±0.21	2.93±0.50	1.68±0.21[1]	1.53±1.18

Data presented as means±S.E.M. ANOVAs revealed [(1)]significant main effects for the factor strain ($p < 0.05$), [(2)]significant main effects for microdialysis (MD) ($p < 0.05$).
*$p < 0.05$ as observed in a separate *t*-test calculation for naive versus microdialysed animals.

Table 2 Behavior of C57BL/6 and DBA/2 mice in the FST during microdialysis without (no exposure) versus prior priming through novel environment exposure (NE exposure).

Behavioral parameter	C57BL/6		DBA/2	
	No exposure ($n = 8$)	NE exposure ($n = 9$)	No exposure ($n = 10$)	NE exposure ($n = 10$)
Time (%) swimming	9.57 ± 1.24	4.60 ± 1.43	13.44 ± 2.07[1,2]	7.80 ± 1.36*
Time (%) climbing	69.31 ± 5.95	73.39 ± 6.80	48.35 ± 3.66[1]	60.23 ± 6.45
Time (%) immobility	21.12 ± 4.18	21.97 ± 6.16	38.21 ± 6.70[1]	31.59 ± 5.85

Data presented as means ± S.E.M. ANOVAs revealed [(1)]significant main effects for strain ($p < 0.05$), [(2)]significant main effects for the factor novel environment exposure ($p < 0.05$).
*$p < 0.05$ as observed in a separate *t*-test calculation for non-exposed versus NE-exposed animals.

were found for swimming behavior only ($F_{1.33} = 9.053$, $p = 0.005$). Additional statistical comparisons for each strain revealed a significant reduction of swimming levels in DBA/2 mice that were subjected to a NE, whereas in the C57BL/6 strain swimming levels were nominally reduced.

3.2. Elevated plus-maze test

MANOVA revealed a significant strain effect (Wilks' Lambda: $F_{7.28} = 8.254$, $p = 0.000$), a significant effect for microdialysis procedure (Wilks' Lambda: $F_{7.28} = 2.832$, $p = 0.023$) and a significant interaction of both factors (Wilks' Lambda: $F_{7.28} = 3.248$, $p = 0.012$). At the univariate level (Table 1), we observed significant strain effects for all behavioral parameters, except entries made into the center ($F_{1.34} = 0.847$, $p = 0.364$). This seems to be due to a disordinal interaction with the microdialysis procedure ($F_{1.34} = 7.815$, $p = 0.008$), as we found significant effects for the factor microdialysis on entries made into the center ($F_{1.34} = 6.348$, $p = 0.017$), which was, however, also observed for entries into the open arms ($F_{1.34} = 5.245$, $p = 0.028$). No significant effects were observed regarding grooming behavior (data not shown).

In detail, C57BL/6 animals displayed more entries and more time in the open arms and less entries and time in the closed compartment of the maze than DBA/2 mice.

In an additional analysis using *t*-tests, the behavioral performance of naïve versus mice subjected to microdialysis for each mouse strain was compared. We observed significant fewer entries into the open arms and in the center of the maze in C57BL/6 mice of the microdialysis group as compared to naïve animals. No significant differences in the parameters investigated were found between naïve and dialysed DBA/2 mice.

Importantly, these data indicate that the microdialysis procedure *per se* leads to a general reduction in locomotion, but it does not affect the differences in emotionality between both inbred mouse strains.

3.3. Neuroendocrine stress responses

3.3.1. Experiment 1: simultaneous measurement of free corticosterone in the hippocampus and the peripheral blood

In the first experiment, we assessed total (i.e. free and protein bound) basal plasma glucocorticoid levels in naïve C57BL/6 and DBA/2 mice and observed nominally higher corticosterone concentrations in DBA/2 as compared to C57BL/6 animals (Fig. 1A; *t*-test: $p = 0.38$).

With respect to *in vivo* microdialysis, ANOVAs with repeated measures for each strain were calculated to compare free corticosterone levels in the jugular vein and the hippocampus under basal conditions (the first three simultaneously measured concentrations) and in response to CRF. In C57BL/6 mice, we found significant effects for the factors time (Huynh–Feldt: $F_{9.126} = 3.309$, $p = 0.013$), no effects for blood versus brain microdialysis (Huynh–Feldt: $F_{1.14} = 2.965$, $p = 0.107$) and no interaction (Huynh–Feldt: $F_{9.126} = 1.187$, $p = 0.326$). As presented in Fig. 1B, CRF administration led to a single significant corticosterone peak as determined by *post-hoc* testing for the factor time. Furthermore, there appears to be a time lag between blood and brain elevations of the stress hormone (see corticosterone levels measured 40 until 60 min after CRF administration).

In DBA/2 mice (Fig. 1C), the factors time (Huynh–Feldt: $F_{9.144} = 8.712$, $p = 0.000$), blood versus brain microdialysis (Huynh–Feldt: $F_{1.16} = 568.755$, $p = 0.000$) and the interaction (Huynh–Feldt: $F_{9.144} = 2.435$, $p = 0.032$) achieved significance. By means of subsequent *post-hoc* analyses, we found significantly higher stress hormone levels in the jugular vein compared to intracerebral concentrations. CRF, however, did not significantly raise glucocorticoid release.

Importantly, the observed strain differences in hippocampal versus jugular vein free corticosterone obtain special attention in the context of higher plasma corticosterone binding globuline (CBG) levels in DBA/2 than in C57BL/6 mice (Jones et al., 1998).

In an additional analysis, we investigated whether the dynamics of hippocampal and blood corticosterone levels are closely correlated and performed parametric correlation analyses of central versus peripheral means of each time point. We observed a non-significant correlation of cerebral versus jugular vein corticosterone for C57BL/6 mice (Pearson: $r = 0.48$, $p = 0.160$) but an almost significant correlation for DBA/2 animals (Pearson: $r = 0.62$, $p = 0.054$). Though correlation coefficients are not significant, which is probably due to a small sample size, they suggest a medium ($r \geqslant 0.30$) and a strong ($r \geqslant 0.50$) effect size, respectively, for these associations (Cohen, 1988).

3.3.2. Experiment 2: effects of forced swimming stress on intrahippocampal corticosterone dynamics

We, first, assessed basal hippocampal corticosterone concentrations and observed no significant differences between

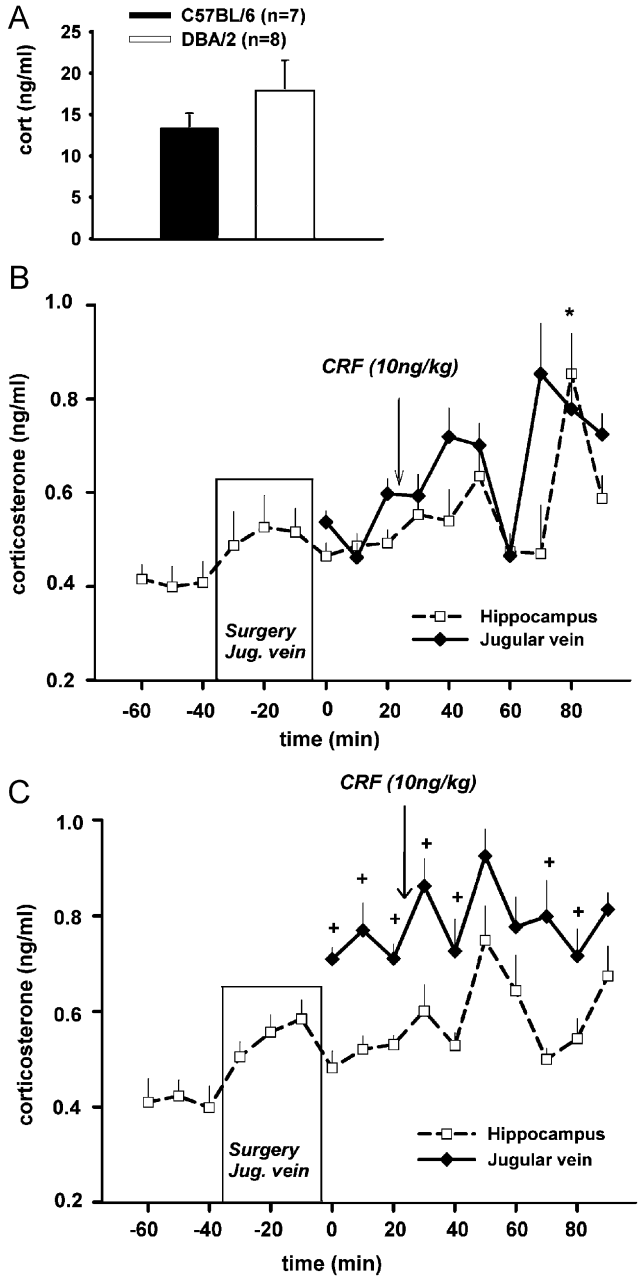

Figure 1 (A) Total (i.e. free and protein bound) basal plasma corticosterone in naïve mice of both inbred strains. (B) Simultaneous microdialysis of free corticosterone (ng/ml) in the jugular vein and the hippocampus of C57BL/6 mice ($n = 8$) under basal conditions and in response to corticotropin-releasing factor (CRF). (C) Blood versus brain microdialysis in DBA/2 mice ($n = 9$). Animals were anesthetized from the beginning of jugular vein surgery on until the end of the experiment (sampling time −30 to 90 min). Data are expressed as means+S.E.M. Newman–Keuls *post-hoc* testing: $^{*}p<0.05$ versus CRF pre-injection corticosterone levels, $^{+}p<0.05$ versus intrahippocampal corticosterone levels.

C57BL/6 and DBA/2 mice (basal mean: C57BL/6, 0.39 ± 0.01 ng/ml; DBA/2, 0.42 ± 0.08 ng/ml; *t*-test: $p = 0.55$).

Evaluating the neuroendocrine stress response to a forced swimming stress, we detected a significant effect for the factor time (Huynh–Feldt: $F_{17.272} = 6.132$, $p = 0.000$) only. As presented in Fig. 2, the stress-related stimuli induced a significant increase in glucocorticoid levels. The strains, however, were statistically not distinguishable (Huynh–-Feldt: $F_{1.16} = 159.229$, $p = 0.983$) and the interaction was not significant (Huynh–Feldt: $F_{17.272} = 1.459$, $p = 0.156$). *Post-hoc* analysis for the time effect revealed a significant elevation of hippocampal corticosterone in a time-frame of 30–90 min after FST.

In addition, we assessed the time for each strain to reach hormonal peaks (i.e. the dialysate that showed the highest corticosterone concentration) by counting the 10 min-intervals of each animal (starting at time point 0 (i.e. FST-sample)) and further calculating the mean for both inbred mouse strains. We observed a significant difference in the latency of the corticosterone peak concentrations (C57BL/6: 39.25 ± 6.39 min; DBA/2: 67.00 ± 7.37 min; Mann–Whitney *U*-Test: $p = 0.021$). Furthermore, DBA/2 animals showed a more prolonged period of time to return to baseline concentrations than C57BL/6 mice.

3.3.3. Experiment 3: effects of exposure to a novel environment as a modulator of FST-induced HPA system activation

Increases in intrahippocampal free corticosterone concentrations in mice of both strains in response to a NE as a modulating factor of HPA system activation followed by a forced swimming stress are shown in Fig. 3. An ANOVA for all time points with repeated measures revealed significant effects for the factors time (Huynh–Feldt: $F_{29.435} = 5.985$, $p = 0.000$), strain (Huynh–Feldt: $F_{1.15} = 5.349$, $p = 0.035$) and the interaction of both (Huynh–Feldt: $F_{29.435} = 1.949$, $p = 0.036$). We further calculated an ANOVA for NE-induced corticosterone elevations versus basal levels and found a significant effect for time (Huynh–Feldt: $F_{12.175} = 1.937$, $p = 0.034$) but not for strain (Huynh–Feldt: $F_{1.415} = 0.486$,

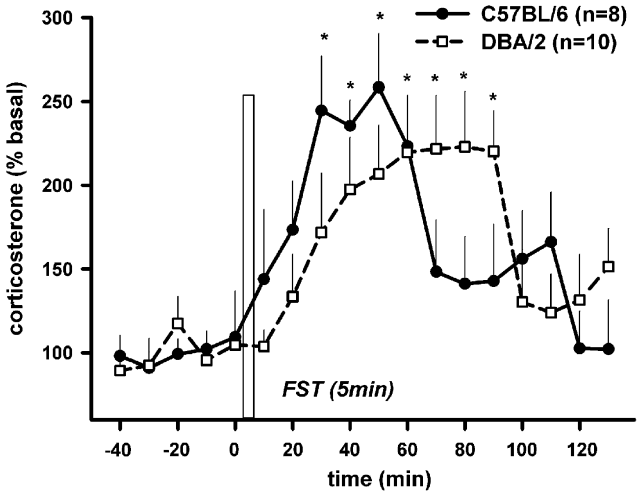

Figure 2 Effects of a 5 min forced swimming stress (FST) on intrahippocampal corticosterone dynamics. Data are expressed as means+S.E.M., representing percentage of baseline. $^{*}p<0.05$ versus basal (0 until −40 min), determined by *post-hoc* tests (Newman–Keuls) for the factor time.

$p = 0.497$) or the factorial interaction (Huynh–Feldt: $F_{12.175} = 0.357$, $p = 0.975$). Subsequent *post-hoc* analyses revealed a significant increase in corticosterone concentration after NE exposure (independent of strain). With respect to a subsequent forced swimming stress, this stressor triggered a strong elevation of hippocampal corticosterone in C57BL/6 mice, whereas in DBA/2 animals only a weak (but significant) release of the steroid hormone was observed.

Basal hormonal concentration was indistinguishable between the two strains (basal mean: C57BL/6, 0.30 ± 0.05 ng/ml; DBA/2, 0.38 ± 0.05 ng/ml).

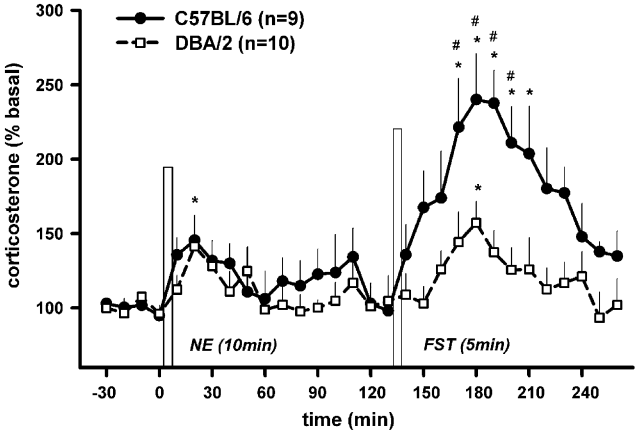

Figure 3 Effects of a 10 min exposure to a novel environment (NE) and a 5 min forced swimming stress (FST) on intrahippocampal corticosterone concentrations illustrated as percentage of baseline. Data are expressed as means+S.E.M. Newman–Keuls *post-hoc* testing: $^{*}p<0.05$ versus basal (0 until −30 min), $^{\#}p<0.05$ versus DBA/2.

3.3.4. Experiment 4: corticosterone release in response to an elevated plus-maze exposure

Intrahippocampal corticosterone release in response to an EPM exposure (Fig. 4) showed no significant effects for the factors time (Huynh–Feldt: $F_{17.396} = 0.956$, $p = 0.485$), strain (Huynh–Feldt: $F_{1.18} = 2.624$, $p = 0.123$) or the interaction of time and strain (Huynh–Feldt: $F_{17.396} = 0.698$, $p = 0.733$). As in the experiments before, basal hormonal concentration were indistinguishable between the two strains (basal mean: C57BL/6, 0.45 ± 0.07 ng/ml; DBA/2, 0.43 ± 0.05 ng/ml).

3.4. Area under the curve (AUC) calculations

As presented in Fig. 5, we determined the total AUC for each experimental stimulus, i.e. FST (single FST exposure), NE and EPM. We observed a significant effect for the factor stimulus ($F_{2.49} = 31.814$, $p = 0.000$) with FST inducing the largest corticosterone release. No effects were detected for the factor strain ($F_{1.49} = 1.088$, $p = 0.302$) and the factorial interaction ($F_{2.49} = 0.016$, $p = 0.984$). Intriguingly, this result is in large contrast to the observed differences in the temporal profile of HPA axis activation (e.g., Fig. 2).

4. Discussion

In the ambition to gain insight into human affective disorders, numerous attempts have been performed to establish animal models of psychiatric diseases or at least of core features. In addition to genetic or environmental manipulations and pharmacological interventions, the analysis and comparison of inbred mouse strains has been proven useful in the determination of biological variants

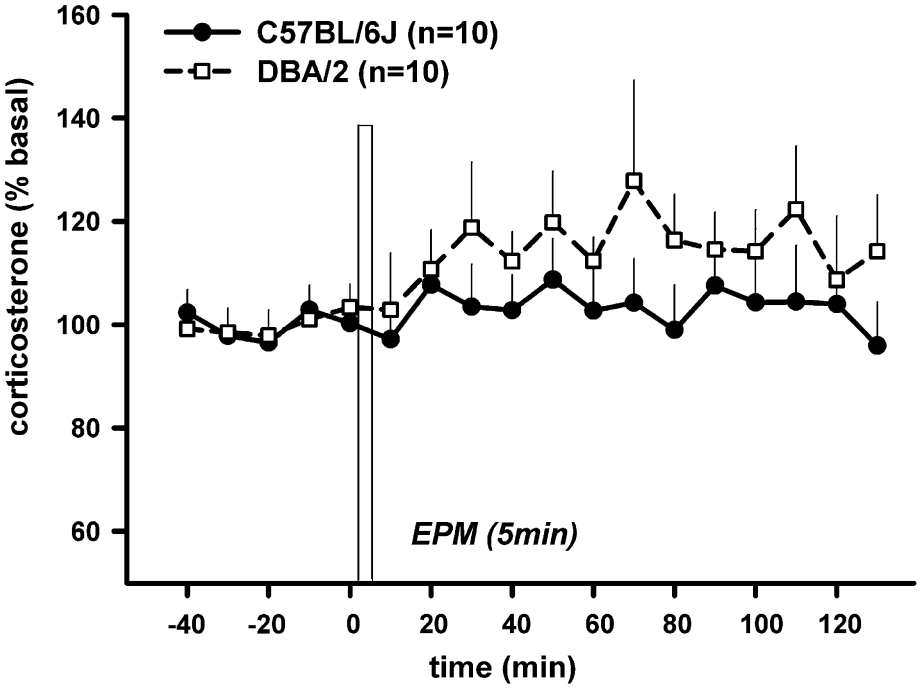

Figure 4 Effects of a 5 min exposure to the elevated plus-maze (EPM) on intrahippocampal corticosterone. Data are expressed as means+S.E.M., representing percentage of baseline.

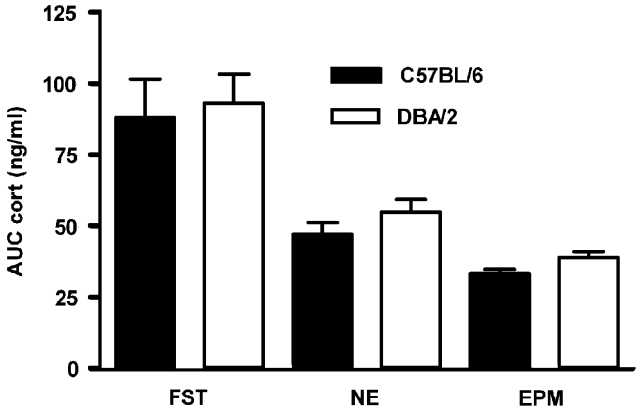

Figure 5 Total area under the curve (AUC) for single FST exposure, NE and EPM. Absolute corticosterone concentrations are presented as means+S.E.M.

underlying anxiety-related and depression-like behavior or stress responsiveness (Jacobson and Cryan, 2007).

Based on such an approach, the present study was aimed at creating a stimulus-response profile of the HPA axis in C57BL/6 and DBA/2 inbred mouse strains via *in vivo* microdialysis of basal versus stimulus-related intrahippocampal corticosterone. Dynamic profiling of an animal's stress response is traditionally performed by measuring glucocorticoid hormones in plasma. However, blood sampling in small laboratory animals requires distressing methods like cutting a tail vein, orbital sinus puncturing or decapitation of the animal and extracting blood directly from the aorta. Thus, profiling of stress-related corticosterone dynamics is not (easily) feasible as the number of blood samples is limited and biased due to inter-individual differences. In contrast, *in vivo* microdialysis enables continuous sampling of glucocorticoids at their respective receptors within the brain, and only the free and biological active fraction of the hormone is determined. However, this approach has only been used scarcely for the assessment of HPA axis activity in mutant mice or in response to pharmacological manipulations (e.g., Sillaber et al., 1998; Linthorst et al., 2000; Oshima et al., 2003; Keeney et al., 2006).

In our first series of experiments, we compared blood and intrahippocampal glucocorticoid hormone concentrations by simultaneous blood versus brain microdialysis in anesthetized animals under basal conditions and after a CRF challenge. To the best of our knowledge, this is the first study that describes the relationship of peripheral versus cerebral free corticosterone dynamics in mice. With respect to C57BL/6 mice, free corticosterone concentrations in the periphery reflected the concentrations in the brain. CRF administration induced a significant increase of the stress hormone in both compartments, although a certain lag of time in intrahippacampal concentrations was observed. Injecting CRF in DBA/2 animals, however, did not increase corticosterone levels in either compartment. This non-responding may be caused by a desensitization through prior surgery which stimulated glucocorticoid release (Fig. 1C). Interestingly, mice of the DBA/2 strains displayed significantly higher glucocorticoid levels in blood as compared to hippocampal dialysates. We further assessed basal plasma glucocorticoid levels in naïve mice of both strains determined as the total amount (free and protein bound) of the steroid hormone. Nominally, higher corticosterone concentrations were found in DBA/2 as compared to C57BL/6 animals, which, in turn, might reflect higher levels of free corticosterone in the jugular vein and previously reported higher levels of CBG in the DBA/2 strain (Jones et al., 1998).

In general, several factors control local tissue concentrations of glucocorticoids that are able to modify steroid hormone effects on cerebral neurons and networks. 11ß-Hydroxysteroid dehydrogenases (11ß-HSDs), for instance, are enzymes that metabolize glucocorticoids and, thus, tune the intracellular levels of these hormones that are available for activation of the nuclear corticosteroid receptors (Tomlinson et al., 2004). Mice homozygous for a target disruption of the 11ß-HSD type 1 gene display an impaired regulation of the stress hormone system (Holmes et al., 2003). A particular role in tissue steroid bioavailability is exerted by the multidrug-resistance gene 1-type *p*-glycoprotein (MDR1 *p*-gp) that actively transports corticosteroid hormones across the blood–brain barrier out of the brain (Uhr et al., 2002). In this context, Müller et al. (2003) have recently provided first evidence for a sustained impact of MDR1 *p*-gp function on the HPA axis. They found that genetic disruption of both the murine *mdr*1a and *mdr*1b gene leads to a profound suppression of the HPA axis at the hypothalamic level under basal conditions and following stress. Given the physiologically relevant issue of the local and cellular environment controlling the HPA system, it is important to assess the biological active hormone at cerebral target sites when profiling HPA axis feedback.

The major aim of the present study was to create a stimulus-response profile of the HPA axis via *in vivo* microdialysis of basal versus stimulus-related intrahippocampal corticosterone. If mice of both strains were subjected to a FST, a strong increase in intrahippocampal corticosterone release was detected in both strains. However, different latencies to reach hormonal peaks and to regain basal values were observed with DBA/2 mice displaying a more prolonged elevation of the steroid hormone. Importantly, this temporal profile suggests a reduced inhibitory HPA axis feedback regulation in the DBA/2 strain as compared to C57BL/6 animals.

In a further approach, we exposed the animals to a mild stressor, i.e. a NE, which induced a slight increase of the steroid hormone in both strains and, 2 h afterwards, subjected them to the FST. In contrast to C57BL/6, DBA/2 mice displayed a rather attenuated corticosterone dynamics after the FST. This is an intriguing observation indicating that an exposure to a mild emotional stimulus induces a desensitization of this organism's HPA system. In contrast to a single FST exposure, in the context of prior stimulation the stress hormone system partly loses its capability to fully react to a subsequent, pronounced stressor. A similar effect has been observed in the blood versus brain microdialysis experiment, as surgery *per se* activates the HPA axis in DBA/2 mice and possibly renders the system non-responsive to the otherwise stimulant effects of CRF.

Determining the AUC of each experimental stimulus revealed that the amount of corticosterone released depends on the severity or aversiveness of the stressor. Importantly, no

AUC differences were observed between C57BL/6 and DBA/2 animals. However, as shown for the single forced swimming stress, the dynamics of the intrahippocampal corticosterone is different between the two strains.

In general, our data extend previous findings of HPA axis responsiveness in C57BL/6 and DBA/2 mice determined via plasma glucocorticoid concentrations. It has been reported that C57BL/6 and DBA/2 mice display similar plasma corticosterone concentrations under basal conditions (Jones et al., 1998; McNamara et al., 2003), but marked differences under stress-related conditions. Cabib et al. (1990) reported that C57BL/6 show a larger increase in plasma corticosterone levels than DBA/2 mice after a 30min exposure to an unfamiliar environment, with glucocorticoid concentrations returning back to basal values after 60 min in mice of the C57BL/6 strain and after 90 min in DBA/2 mice. In addition, it has been observed that DBA/2 mice exhibit a more prolonged elevation of corticosterone levels following inescapable footshocks (Shanks et al., 1990).

In view of the marked differences in the stress-related dynamics of the HPA axis in both strains, it is to note that Cabib et al. (1996) reported a higher number of GR and MR receptors (i.e. B_{max}) in un-stressed DBA/2 mice as compared to C57BL/6. At this point of the study, however, we are not able to provide any further evidence regarding the molecular basis (e.g., a different strain-related regulation of corticosteroid receptors) of the desensitization effect and neuroendocrine feedback mechanism in mice of both strains.

Investigating behavioral phenotypes of C57BL/6 and DBA/2 mice, we performed FST and EPM tests. The FST is the most frequently used rodent test of depression-related behavior due to its sensitive prediction of antidepressant treatment efficacy (Porsolt et al., 1997). The time spent immobile as the major behavioral parameter reflects adaptation to persistent forced swimming stress, i.e. passive stress coping, and alternates with active escape (Thierry et al., 1984). Our analysis of the stress coping behavior in the FST revealed that naïve (i.e. control) DBA/2 animals displayed only slightly higher levels of immobility. When exposed to the microdialysis procedure, immobility times are reduced and climbing levels are increased in both strains. This effect is less pronounced in DBA/2 mice. However, they display a significant greater immobility behavior and less active stress coping than C57BL/6 animals. Prior exposure to the NE had only a minor influence on stress coping behavior as it mainly decreased swimming behavior in both strains. It should be noted that there are discrepancies with previous reports that (un-stressed) C57BL/6 mice are more immobile in the FST than DBA/2 mice (Hwang et al., 1999; Ventura et al., 2002). Such differences in the outcome of behavioral studies can be partly explained by the influence of laboratory environment and experimental procedure (Chesler et al., 2002; Crabbe et al., 1999) and, importantly, by the use of different strain subtypes (Siegmund et al., 2005; Wotjak, 2003).

With respect to anxiety-related behavior in the C57BL/6 and DBA/2 strains, however, we could show that naïve (i.e. control) DBA/2 mice displayed greater avoidance of open arms and, thus, higher levels of anxiety than C57BL/6 mice, which is in accordance with previous observations (Crawley et al., 1997; Griebel et al., 1997; Ohl et al., 2003; Rodgers et al., 1999). On the neuroendocrine level, previous studies reported a significant increase in plasma corticosterone after EPM exposure (File et al., 1994; Holmes et al., 1998). Intriguingly, exposure of our animals to the EPM induced a small, insignificant increase of intrahippocampal corticosterone in both strains, although the EPM represented a "novel environment". An interpretation of the lack of effect could be that the stimulus was largely controllable for the mice. For instance, DBA/2 mice preferred to stay in the low aversive compartment of the maze, i.e. the closed arms, and, thus, did not display a neuroendocrine stress response. However, the microdialysis procedure *per se* influenced the behavioral performance of mice in terms of a reduced locomotion (i.e. reduced entries in the center and the open arms). This effect is more pronounced in the C57BL/6 strain, which is generally more active. In contrast, the anxiety-related behavior, i.e. the strain-specific, innate behavioral profile, remained unaffected in both strains.

4.1. Implications for anxiety disorders and depression: C57BL/6 and DBA/2 mice as an animal model?

The corticosteroid receptor hypothesis of depression relates to the finding that signs and symptoms characteristic for anxiety disorders and major depression are causally related to alterations in the HPA system and its limbic pathways (Holsboer, 2000). Based on the concept of "biological endophenotypes" (Hasler et al., 2004), it implies that intracellular corticosteroid signalling via MR and GR receptors is defunct in specific brain areas and, thus, HPA axis feedback is impaired, subsequently affecting anxiety-related and depression-like behavior via dysregulated CRF and AVP neuropeptides (Keck et al., 2004).

In the present series of experiments, we used a phenomenological approach aimed to characterize the dynamics of the HPA system in an animal model of C57BL/6 and DBA/2 inbred mice that differ in their anxiety-related and stress coping behavior. By means of profiling of corticosterone's stimulus-response patterns in the murine hippocampus, we observed strain differences in the (temporal) feedback of the stress hormone system and a desensitizing effect of emotional and stress-related stimuli on HPA axis activity. As these results resemble, at least in part, the neuroendocrine endophenotype observed in affective disorder patients C57BL/6 and DBA/2 inbred mouse strains are an interesting animal model for studying the neuroendocrine basis underlying anxiety and depression.

However, it has to be noted that the present study is descriptive in nature. The causal inference about the effects of corticosterone and HPA system regulation on anxiety-related and depression-like behavior in these two inbred mouse strains, thus, needs to be further tested and experimentally validated in future experiments, for instance by means of metyrapone challenges or receptor-targeted approaches using MR and GR antagonists.

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Conflict of interest

The authors declare that they do not have any conflicts of interest.

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