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Mice selected for extremes in stress reactivity reveal key endophenotypes of major depression: A translational approach

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Summary Clear evidence has linked dysregulated hypothalamus—pituitary—adrenocortical (HPA) axis function to the aetiology and pathophysiology of major depression (MD), as observed in the majority of patients. Increased stress reactivity and hyperactivity of the HPA axis seem characteristic for psychotic/melancholic depression, while the atypical subtype of depression has been connected with the opposing phenotypes. However, the underlying molecular-genetic mechanisms are poorly understood.

In the present study, mouse lines selectively bred for extremes in stress reactivity (SR), i.e. presenting high (HR) or low (LR) corticosterone secretion in response to stressors, were used to characterise the molecular alterations on all levels of the HPA axis. Results were contrasted

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with clinical phenotypes of MD patients from the Munich Antidepressant Response Signature project, stratified according to their cortisol response in the Dex/CRH test.
Distinct differences between HR and LR mice were found in the expression of HPA axis-related genes in the adrenals, pituitary and selected brain areas. Moreover, HR animals presented an enhanced adrenal sensitivity, increased stress-induced neuronal activation in the PVN and an overshooting Dex/CRH test response, whereas LR animals showed a blunted response in these paradigms. Interestingly, analogous neuroendocrine, morphometric, psychopathological and behavioural differences were observed between the respective high and low HPA axis responder groups of MD patients.
Our findings suggests that (i) the SR mouse model can serve as a valuable tool to elucidate HPA axis-related mechanisms underlying affective disorders and (ii) a stratification of MD patients according to their HPA axis-related neuroendocrine function should be considered for clinical research and treatment.

1. Introduction

Clear evidence indicates that hypothalamus-pituitary-adrenocortical (HPA) axis dysregulation is closely associated with major depression (MD) (Raison and Miller, 2003; de Kloet et al., 2005; Thomson and Craighead, 2008; Holsboer and Ising, 2010; Stetler and Miller, 2011). Despite the heterogeneity of factors involved in the aetiology of this disease, neuroendocrine alterations in HPA axis activity can be observed in about 60—80% of depressed patients (Thomson and Craighead, 2008; Holsboer and Ising, 2010; Stetler and Miller, 2011). Moreover, it has been reported that normalisation of HPA axis function precedes the improvement of clinical symptoms and predicts the response to pharmacotherapy. Likewise the risk of relapse was found to be associated with continuous or recurrent HPA axis dysfunction (Zobel et al., 2001; Ising et al., 2007). This highlights a dysregulated HPA axis as one key endophenotype of MD and concurrently provides the opportunity to elucidate the underlying mechanisms of this disease.

Pathological changes in HPA axis function are found on many levels, i.e. in the brain, the pituitary and the adrenals of patients, leading to hyper- or hypo-cortisolism as a consequence of a dysregulated stress hormone system. This is of particular interest since these opposing neuroendocrine phenotypes coincide with distinct clinical symptoms of MD (Gold and Chrousos, 2002; Antonijevic, 2006; Wardenaar et al., 2011; Lamers et al., 2013). Accordingly, the outcome of the combined dexamethasone/corticotropin-releasing hormone (Dex/CRH) test, differs significantly between patients suffering from the psychotic/melancholic subtype and the atypical subtype of MD (Gold and Chrousos, 2002; Rydmark et al., 2006; Ising et al., 2007; Kloiber et al., 2007; Wahlberg et al., 2009). However, the molecular-genetic underpinnings of these aberrant HPA axis functions are still poorly understood, presumably due to the heterogeneity of mechanisms underlying this disorder and the lack of appropriate animal models.

Recently, we established a new genetic animal model of affective disorders by applying a bidirectional selective breeding approach, selecting mice for extremes in HPA axis reactivity (Touma et al., 2008). This 'stress reactivity' (SR) mouse model consists of three breeding lines showing distinct differences in their corticosterone (CORT) secretion in response to stressors: the high (HR), intermediate (IR) and low (LR) reactivity mouse line. Interestingly, previous studies revealed that in addition to the robust differences in HPA axis reactivity (Touma et al., 2008; Heinzmann et al., 2010), HR as well as LR animals share many similarities with symptoms observed in MD patients, particularly when the subtypes of melancholic and atypical depression are taken into account (Touma et al., 2008, 2009; Heinzmann et al., 2010; Knapman et al., 2010a,b; Fenzl et al., 2011; Knapman et al., 2012; Mattos et al., 2013). For instance, increased HPA axis activity was associated with cognitive deficits, reduced hippocampal activity and neuronal integrity in HR animals as well as in human patients (Knapman et al., 2010a,b, 2012). Furthermore, HR mice and MD patients presented a flattened diurnal rhythm of CORT secretion in addition to psychomotor alterations (increased motor activity/restlessness), increased REM sleep as well as disturbed sleep architecture (Touma et al., 2009; Fenzl et al., 2011). Investigating the behavioural phenotype of the three mouse lines, it has been shown that animals of the HR line present a hyperactive coping-style, while LR mice displayed more passive coping behaviours (Touma et al., 2008, 2009; Knapman et al., 2010a; Mattos et al., 2013), which can be interpreted as symptoms of agitation and lethargy, respectively, both observed in patients suffering from MD.

Considering the striking similarities between patients and animals of the SR mouse model, we sought to characterise the molecular underpinnings of the distinct differences in HPA axis function between HR, IR and LR mice in more detail. To this end, we investigated all functional levels of the HPA axis, in the periphery, the central nervous system and regulatory aspects. Translating these preclinical findings to humans, we analysed data from depressed inpatients, stratifying the patients according to their cortisol response in the Dex/CRH test and comparing their neuroendocrine, morphometric, psychopathological and behavioural symptoms in analogy to the phenotypes observed in the SR mouse lines.

2. Methods

A more detailed description of all methods and experimental procedures is provided in the Supplementary Material. Aliases and designation of investigated candidate genes can be found in Supplementary Table S1. All presented work is in

accord with accepted standards of humane care and use of experimental animals and was approved by the appropriate local authority.

2.1. Animals and housing conditions

In all experiments, adult male mice of the SR mouse model were used ((Touma et al., 2008) and Fig. S1). The animals were selected according to their plasma CORT increase in the 'stress reactivity test' (SRT) (Touma et al., 2008). The mice were 3—5 months of age and single housed at least ten days before the experiments. Animal husbandry was performed under standard laboratory conditions.

2.2. Experimental design

To characterise the molecular alterations underlying the differential HPA axis reactivity of HR, IR and LR mice, a series of experiments was performed, investigating all levels of the HPA axis, i.e. the adrenal, the pituitary and the brain as well as feedback regulatory functions.

For all candidate gene and protein expression analyses, animals were sacrificed under basal, unstressed conditions.

2.3. Functional characterisation at the adrenal level

Assessing differences in adrenal size, we determined the weight of both adrenal glands. Relative adrenal weights were obtained by dividing the weights by the respective body weight of the animal.

The expression of candidate genes involved in adrenocortical ACTH receptor function (*Mc2r*, *Mrap*), cholesterol trafficking (*Star*) as well as steroidogenesis (*Cyp11a1*, *Cyp11b1* and *Cyp11b2*) was analysed using quantitative real-time polymerase chain reaction (qPCR). Additionally, as marker of sympathetic-adrenomedullary activity, the expression of two enzymes (*Th*, *Pnmt*), controlling rate-liming steps in the catecholamine biosynthesis, was investigated.

As a functional readout for basal adrenocortical activity, animals of the SR mouse lines were sacrificed under unstressed conditions and circadian trough levels of plasma CORT as well as aldosterone were determined. Moreover, assessing steroid secretion in response to a moderate psychological stressor (15 min restraint), the increase of plasma CORT concentrations was measured in the SRT as described earlier (Touma et al., 2008).

The sensitivity of the adrenals to ACTH stimulation was assessed in vivo by determining the CORT release in response to different doses of administered ACTH, after suppression of endogenous ACTH levels with dexamethasone (Dex).

2.4. Functional characterisation at the pituitary level

The expression of candidate genes involved in pituitary activation (*Avpr1b*, *Crhr1*, *Pomc*) as well as negative feedback inhibition of pituitary function (*Nr3c1*, *Fkbp5*) was determined using qPCR. Moreover, protein abundance of the GR, the GR-associated protein FKBP5 and the pituitary secretagogue ACTH was assessed by Western blot.

2.5. Functional characterisation at the brain level

The expression of different genes involved in HPA axis activity and regulation was assessed in the following brain nuclei using qPCR: the paraventricular nucleus of the hypothalamus (*Nr3c1*, *Avp*, *Crh*, *Fkbp5*, *Fkbp4*), the basolateral amygdala (*Nr3c1*, *Crhr1*, *Fkbp5*, *Fkbp4*) as well as the dorsal and ventral hippocampus (*Nr3c2*, *Nr3c1*, *Crhr1*, *Hsd11b*, *Fkbp5*).

To assess whether animals of the three SR mouse lines show differences in their stress-induced neuronal activation, *c-fos* expression in response to a 15-min restraint stressor was determined by in situ hybridisation 45 min after termination of the stressor in brain structures involved in HPA axis function, including the prefrontal cortex (PFC), the paraventricular nucleus of the hypothalamus (PVN), the basolateral amygdala (BLA) and the hippocampus (Fig. S2).

2.6. Characterisation of regulatory functions

The combined dexamethasone/corticotropin-releasing hormone (Dex/CRH) test was used as previously described (Touma et al., 2011) to assess HPA axis activation and negative feedback regulation. Two independent Dex/CRH tests were performed with either a relatively high (2 mg/kg) or low (0.05 mg/kg) dose of Dex (CRH dose in both tests: 0.15 mg/kg). Additionally, using established techniques (Touma et al., 2003, 2004), the excretion profile of CORT metabolites (CM) was continuously monitored for 24 h in the faeces of HR, IR and LR mice subjected to the Dex/CRH test.

2.7. Molecular and behavioural analyses

A more detailed description of the performed molecular and behavioural analyses is provided in the Supplementary Material. Briefly, for the dissection of brain nuclei, the frozen brains of the animals were mounted in a cryostat and sectioned in 200 μm thick coronal slices. The dorsal and ventral hippocampus, the BLA and the PVN were acquired by micropuncture.

Gene expression analysis was performed by qPCR. To this end, total RNA was isolated from tissue punches, pituitaries and adrenals. Protein extraction and immunoblotting were performed as described previously. The expression of the immediate early gene *c-fos*, which is a CORT-independent marker of neuronal activation (Helmreich et al., 1996; Weiser et al., 2011), was monitored in the brain of HR/IR/LR mice using *c-fos* in situ hybridisation applying a previously described protocol (details in the Supplementary Material).

Plasma aldosterone, CORT and ACTH concentrations were measured by immunoassays.

The forced swim test (FST) was used to measure stress-coping behaviour. As described before (Touma et al., 2008; Knapman et al., 2010a), each mouse was placed for 6 min in an inescapable glass cylinder filled with 23 °C warm water. The sessions were video recorded and the amount of time

the animals spent struggling, swimming and floating was scored by a trained observer.

2.8. Studies involving depressed patients

In total, 657 inpatients with a current major depressive episode participating in the Munich Antidepressant Response Signature (MARS) project (54.9% female patients; mean age: 48.5 ± 13.4 years) were selected for the analysis. The MARS project is an open-label naturalistic longitudinal study with inpatients suffering from a depressive episode. Patients are recruited from referrals to the hospital of the Max Planck Institute of Psychiatry in Munich, Germany, and from collaborating hospitals in Southern Bavaria and Basel, Switzerland. Inclusion criteria were a first (33.9%, DSM-IV code 296.2) or a recurrent episode (66.1%, 296.3) of major depression with moderate to severe intensity (as indicated by a total score of larger than 14 in the 21-item Hamilton Depression Rating Scale, HDRS; mean HDRS score: 26.2 ± 6.1), and participation in the combined Dex/CRH test within the first ten days after admission to the hospital. Exclusion criteria were presence of a somatic disorder potentially interfering with the results of the Dex/CRH test, such as endocrine disorders or acute infectious diseases, and treatment with lithium or carbamazepine, as both mood stabilisers are known to invalidate the results of the Dex/CRH test (Zobel et al., 2001). Patients were included after study details were explained and written informed consent was obtained. The study was approved by the local ethics committee of the Ludwig Maximilians University (LMU), Munich. At the time of the Dex/CRH test, 20% of the patients were without medication, while the remaining 80% received different types of antidepressants according to the attending doctor's choice (16% tricyclic antidepressant, 25% selective serotonin reuptake inhibitors, 20% venlafaxine, 22% mirtazapine, 10% other antidepressants). 36% of the patients received benzodiazepines as add-on medication, 19.5% atypical antipsychotics, and 12% mood stabilisers (other than lithium and carbamazepine). In some patients comorbidity with anxiety disorders was observed: 7.6% of the participants were additionally suffering from generalised anxiety disorder, panic disorder and/or agoraphobia, or social phobia. The average number of previous depressive episodes was 2.5 (SD = 4.9), the mean age of disease onset was 37.3 (SD = 15.4) years, and the mean duration of the current episode was 39 (SD = 65) weeks.

Coping style was assessed using the German Stress-Coping Questionnaire (Ising et al., 2001), which was applied at the end of hospitalisation, when depression symptoms had substantially improved. The questionnaire asks for individual coping strategies in response to general stressful conditions and situations. The items were grouped into secondary scales of a predominantly active coping (distraction, substitute gratification, situational control, reaction control, positive self-instructions) and passive coping style (avoidance, escape).

To assess HPA axis function in these patients, fasting morning cortisol was collected the day before the combined Dex/CRH test as described previously (Heuser et al., 1994; Zobel et al., 2001; Ising et al., 2007). The Dex/CRH test was administered within 10 days (on average 6 d) after admission (for further details see Supplementary Material). The cortisol response in the Dex/CRH test (area under the time course curve, AUC) was used to stratify the patients into tertile groups according to their neuroendocrine phenotype in high (hHR, $N = 219$), intermediate (hIR, $N = 219$) and low (hLR, $N = 219$) HPA axis responders. Besides the AUC, basal morning cortisol levels (measured in a subset of 453 patients on the day before the Dex/CRH test), cortisol concentrations after Dex (but before CRH), as well as cortisol levels 30 min after CRH injection are additionally reported, the latter two matching the time points of the Dex/CRH test in mice. For the comparison of MD patients and HR/IR/LR mice, we subsequently analysed the data focussing on neuroendocrine, morphometric, psychopathological and behavioural characteristics in these three subgroups of patients.

2.9. Statistical analysis

Statistical analysis of the animal data was performed using non-parametric statistics. Two independent samples were compared using the Mann–Whitney *U*-test (MWU-test), while more than two independent samples were analysed using the Kruskal–Wallis *H*-test (KWH-test). Post hoc pairwise comparisons were calculated using the MWU-test. Consequently, significance levels were corrected according to the sequential Bonferroni technique.

In the patient sample, an analysis of covariance (ANCOVA) was used for group comparisons of quantitative variables, with age and gender as covariates in all analyses. Post hoc comparisons were performed using Fisher's Least Significant Difference (LSD) test and corrected for multiple comparisons with the Bonferroni-Holm (BfH) method. To analyse differences in psychopathology, we used five HDRS items (loss of weight; insomnia early, middle and late; loss of appetite) as ''non-atypical subscale'' (Kloiber et al., 2007), representing symptoms which are less prevalent or even absent in the subtype of atypical depression (Angst et al., 2002; Antonijevic, 2006). Additionally, we analysed HDRS item 9 ''agitation''. HDRS group analyses were controlled for the remaining HDRS items. All tests were applied two-tailed and were calculated using the software package SPSS. Statistical significance was accepted from $p \leq 0.05$, while $p < 0.1$ was considered as a trend.

3. Results

3.1. Functional differences at the adrenal level

Significant differences in the animals' body weight were observed, with HR mice being significantly lighter compared to animals of the IR and LR line (Fig. 1A). Relative to their body weight, HR mice showed significantly higher adrenal weights compared to IR animals, while LR mice had the lowest relative adrenal weight (Fig. 1B).

Our gene expression analysis in the adrenals did not reveal significant differences in the mRNA levels of adrenocortical *Mc2r*, *Star*, *Cyp11b1* and *Cyp11b2* nor adrenomedullar *Th* and *Pnmt* (Table 1A). However, the expression of *Mrap* and *Cyp11a1* was significantly increased in HR mice compared to the other two lines, while no differences were observed between IR and LR mice (Table 1A).

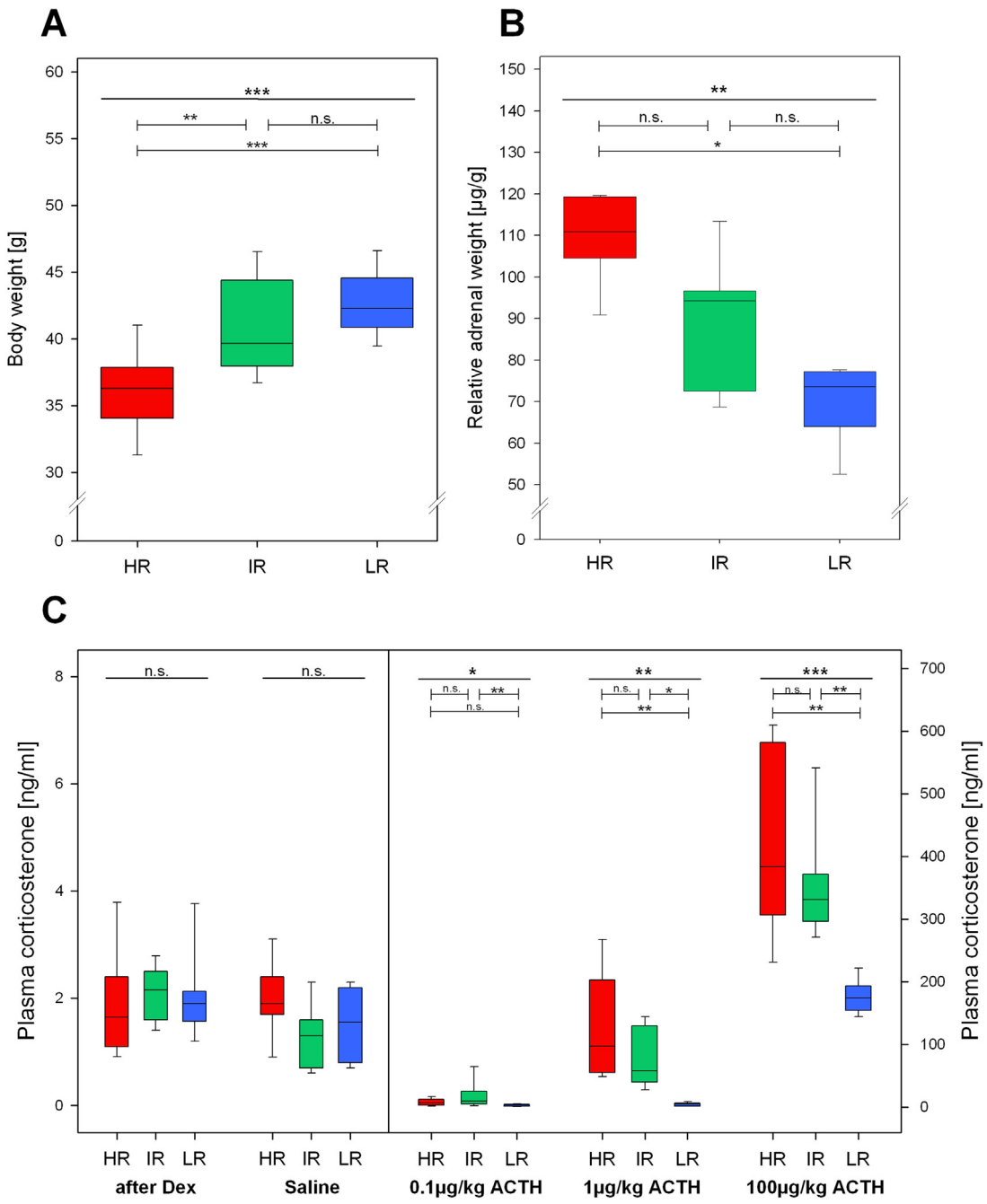

Figure 1 Body weight (A) and relative adrenal weight (B) of high (HR), intermediate (IR) and low (LR) reactivity mice ($N = 6$ for each line). (C) Plasma corticosterone response in the adrenal sensitivity test of HR, IR and LR mice 6 h after the injection of a relatively high dose of dexamethasone (Dex, 4 mg/kg) (after Dex value, $N = 30$ for each line) and 30 min after the subsequent treatment with either saline (Saline value, $N = 6$ for each line) or three different doses of ACTH (0.1 µg/kg, $N = 10$ for each line; 1 µg/kg, $N = 6$ for each line; or 100 µg/kg, $N = 8$ for each line). Note the different y-axis scales in the two panels. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes) as well as 10% and 90% percentiles (whiskers). Statistical differences between the three lines (KWH-test) are given at the top of each panel. Results of the pairwise group comparison (MWU-tests) are indicated below (Bonferroni-corrected p-values: $p > 0.1$ n.s., $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***). (A) HR vs. IR vs. LR, df = 2, $H = 22.6$, $p < 0.001$; HR vs. IR, $U = 36.5$, $p = 0.003$, HR vs. LR, $U = 12$, $p < 0.001$, IR vs. LR, $U = 84.5$, $p = 0.101$. B) HR vs. IR vs. LR, df = 2, $H = 9.9$, $p = 0.007$; HR vs. IR, $U = 6$, $p = 0.110$, HR vs. LR, $U = 0$, $p = 0.012$, IR vs. LR, $U = 8$, $p = 0.109$. C) *After Dex*: HR vs. IR vs. LR, df = 2, $H = 4.2$, $p = 0.124$; *Saline*: HR vs. IR vs. LR, df = 2, $H = 3.4$, $p = 0.180$; *0.1 µg/kg ACTH:* HR vs. IR vs. LR, df = 2, $H = 8.5$, $p = 0.014$, HR vs. IR, $U = 36.5$, $p = 0.307$, HR vs. LR, $U = 28.5$, $p = 0.208$, IR vs. LR, $U = 10.5$, $p = 0.009$; *1 µg/kg ACTH:* HR vs. IR vs. LR, df = 2, $H = 12.1$, $p = 0.002$, HR vs. IR, $U = 10$, $p = 0.200$, HR vs. LR, $U = 0$, $p = 0.008$, IR vs. LR, $U = 0$, $p = 0.012$; *100 µg/kg ACTH*: HR vs. IR vs. LR, df = 2, $H = 16.0$, $p < 0.001$, HR vs. IR, $U = 21$, $p = 0.248$, HR vs. LR, $U = 0$, $p = 0.002$, IR vs. LR, $U = 0$, $p = 0.003$.

Table 1 (A) Candidate gene expression in the adrenals of high (HR), intermediate (IR) and low (LR) reactivity mice ($N = 7–9$ for each line). (B) Candidate gene expression in the paraventricular nucleus of the hypothalamus (PVN), the basolateral amygdala (BLA) and the dorsal (dHC) and ventral (vHC) hippocampus of HR, IR and LR mice ($N = 11–16$ for each line). Expression (mean ± SEM) is given relative to two housekeeping genes (HPRT and TBP) and normalised to the mean of the IR line. Significant differences between the three mouse lines (KWH-test) are indicated, including the post hoc pairwise comparisons (Bonferroni-corrected MWU-tests). For more detailed information about abbreviations, aliases and function of the different candidate genes see Table S1 in the Supplementary Material.

Candidate gene	HR		IR		LR		Line comparison		Post hoc comparison (Bf corrected MWU-test)					
	Mean	SEM	Mean	SEM	Mean	SEM	(KWH-test, df = 2)		HR vs IR		HR vs LR		IR vs LR	
							H	*p*	*U*	*p*	*U*	*p*	*U*	*p*
A														
Mc2r	1.08	0.09	1.00	0.07	1.22	0.12	1.794	0.408 n.s.						
Mrap	1.75	0.26	1.00	0.08	1.02	0.23	**7.902**	**0.019***	7.5	0.018*	11	0.098 T	23.5	0.397
Star	1.46	0.15	1.00	0.08	1.50	0.21	5.897	0.050 T						
Cyp11a1	1.32	0.07	1.00	0.07	1.13	0.12	**6.334**	**0.042***	7.5	0.018*	20.5	0.452	29	0.500
Cyp11b1	1.03	0.10	1.00	0.06	0.88	0.09	1.979	0.372 n.s.						
Cyp11b2	1.08	0.32	1.00	0.21	1.11	0.07	0.461	0.794 n.s.						
Th	0.79	0.17	1.00	0.19	0.54	0.11	4.263	0.119 n.s.						
Pnmt	0.82	0.19	1.00	0.07	0.86	0.26	2.661	0.264 n.s.						

Brain region	Candidate gene	HR		IR		LR		Line comparison		Post hoc comparison (Bf corrected MWU-tests)					
		Mean	SEM	Mean	SEM	Mean	SEM	(KWH-test, df = 2)		HR vs IR		HR vs LR		IR vs LR	
								H	*p*	*U*	*p*	*U*	*p*	*U*	*p*
B															
paraventricular nucleus of the hypothalamus (PVN)	Nr3c1	0.96	0.04	1.00	0.03	0.92	0.02	3.837	0.147 n.s.						
	Avp	0.75	0.14	1.00	0.17	0.98	0.20	0.734	0.693 n.s.						
	Crh	0.97	0.10	1.00	0.09	1.00	0.09	0.087	0.957 n.s.						
	Fkbp5	1.30	0.18	1.00	0.21	1.26	0.10	3.267	0.195 n.s.						
	Fkbp4	0.81	0.08	1.00	0.13	0.92	0.18	1.984	0.371 n.s.						
Basolateral amygdala (BLA)	*Nr3c1*	0.94	0.03	1.00	0.05	0.85	0.04	**5.996**	**0.050***	106	0.580	70.5	0.100 T	59.5	0.084 T
	Crhr1	0.99	0.05	1.00	0.06	0.80	0.05	**8.273**	**0.016***	116	0.874	55	0.030*	55	0.034*
	Fkbp5	1.21	0.19	1.00	0.11	1.03	0.05	2.211	0.331 n.s.						
	Fkbp4	0.97	0.04	1.00	0.06	1.01	0.08	0.271	0.873 n.s.						
Dorsal hippocampus (dHC)	*Nr3c2*	0.86	0.04	1.00	0.05	0.87	0.04	5.368	0.068 T						
	Nr3c1	0.98	0.03	1.00	0.04	0.87	0.03	**8.982**	**0.011***	111	0.722	58	0.028*	47.5	0.021*
	Crhr1	0.98	0.04	1.00	0.08	0.93	0.07	0.702	0.704 n.s.						
	Hsd11b1	0.95	0.07	1.00	0.11	0.99	0.11	0.002	0.999 n.s.						
	Fkbp5	0.70	0.08	1.00	0.11	0.21	0.09	**19.735**	**0.001*****	69	0.044*	36	0.002**	20	0.001***
Ventral hippocampus (vHC)	*Nr3c2*	0.93	0.05	1.00	0.05	0.98	0.05	1.850	0.397 n.s.						
	Nr3c1	0.95	0.04	1.00	0.06	0.83	0.02	**6.612**	**0.037***	105	0.553	66	0.066 T	58.5	0.075 T
	Crhr1	1.02	0.24	1.00	0.19	0.76	0.16	1.544	0.462 n.s.						
	Hsd11b1	0.89	0.04	1.00	0.11	0.78	0.07	2.507	0.285 n.s.						
	Fkbp5	1.07	0.11	1.00	0.13	0.75	0.05	**6.447**	**0.040***	105	0.553	61	0.057 T	65.5	0.102

Note: $p > 0.1$ n.s., $0.05 < p \leq 0.1$ T, $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***.
Statistically significant differences are indicated in bold.

Concerning adrenal steroid secretion, morning levels of plasma CORT (mean ± SEM [ng/ml], HR: 10.6 ± 1.7, IR: 7.3 ± 1.1, LR: 7.9 ± 2.5) and aldosterone (mean ± SEM [pg/ml], HR: 197.0 ± 22.6, IR: 168.5 ± 25.7, LR: 117.3 ± 17.9) were slightly but significantly increased in HR mice compared to IR animals, while LR mice showed the lowest concentrations (Fig. S3).

Additionally, HPA axis reactivity, as reflected by the plasma CORT increase in response to 15-min restraint, differed significantly between HR, IR and LR animals. As expected, HR mice showed a much more pronounced increase of CORT compared to IR animals, while LR mice presented a clearly blunted response to the stressor (Fig. 3A).

Regarding the sensitivity of the adrenals to ACTH, significant differences between HR, IR and LR mice could also be detected. After pharmacological suppression of pituitary function with a high dose of Dex, plasma ACTH concentrations were very low in all three lines (mean ± SEM [pg/ml], HR: 34.4 ± 4.4, IR: 45.2 ± 8.2, LR: 52.2 ± 11.3; KWH-test: HR vs. IR vs. LR, df = 2, $H = 0.6$, $p = 0.741$). Similarly, plasma CORT levels were readily suppressed after Dex treatment (Fig. 1C, after Dex value) and remained low in response to saline injection (Fig. 1C, Saline value). However, adrenal stimulation with ACTH resulted in a dose-dependent increase in plasma CORT levels in HR and IR mice, which was clearly attenuated in LR animals (Fig. 1C, right panel).

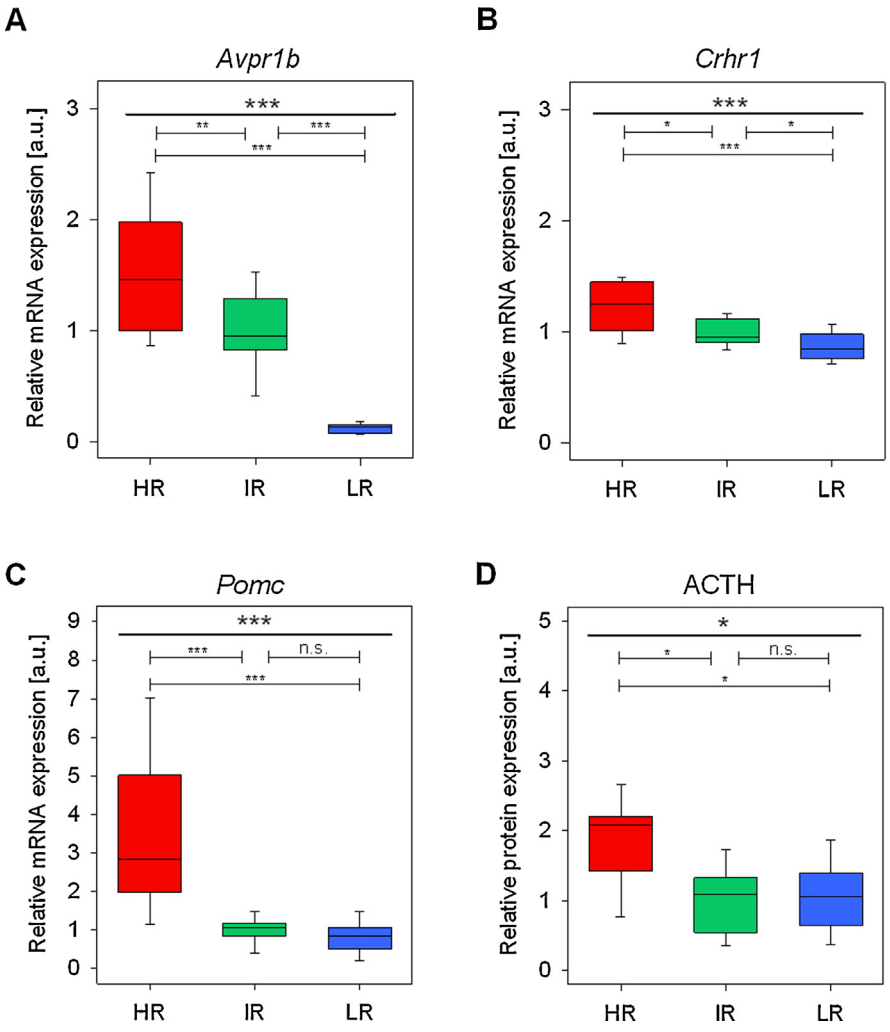

Figure 2 Expression of *Avpr1b* (A), *Crhr1* (B) and *Pomc* (C) mRNA in the pituitary of high (HR), intermediate (IR) and low (LR) reactivity mice ($N = 10–16$ for each line), relative to two housekeeping genes (HPRT and TBP) and normalised to the mean of the IR line. (D) ACTH protein expression ($N = 8$ for each line) in the pituitary of HR, IR and LR mice relative to the internal standard protein (GAPDH) and normalised to the mean of the IR line. Data are given as box plots (for description see legend of Fig. 1). Statistical differences between the three lines (KWH-test) are given at the top of each panel. Results of the pairwise group comparison (MWU-tests) are indicated below (Bonferroni-corrected p-values: $p > 0.1$ n.s., $0.05 < p \leq 0.1$ T, $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***). (A) *Avpr1b:* HR vs. IR vs. LR, df = 2, $H = 32.7$, $p < 0.001$, HR vs. IR, $U = 47$, $p = 0.007$, HR vs. LR, $U = 0$, $p < 0.001$, IR vs. LR, $U = 0$, $p < 0.001$. (B) *Crhr1:* HR vs. IR vs. LR, df = 2, $H = 17.5$, $p < 0.001$, HR vs. IR, $U = 33$, $p = 0.026$, HR vs. LR, $U = 24$, $p < 0.001$, IR vs. LR, $U = 35$, $p = 0.027$. (C) *Pomc:* HR vs. IR vs. LR, df = 2, $H = 25.7$, $p < 0.001$, HR vs. IR, $U = 10$, $p < 0.001$, HR vs. LR, $U = 4$, $p < 0.001$, IR vs. LR, $U = 51$, $p = 0.142$. (D) ACTH: HR vs. IR vs. LR, df = 2, $H = 8.7$, $p = 0.013$, HR vs. IR, $U = 7$, $p = 0.027$, HR vs. LR, $U = 9$, $p = 0.032$, IR vs. LR, $U = 31$, $p = 0.916$.

3.2. Functional differences at the pituitary level

Analysing the expression of candidate genes involved in the activation of the anterior pituitary, we found significantly increased *Avpr1b* and *Crhr1* mRNA levels in HR mice compared to IR animals, while the expression of both receptors was significantly decreased in the pituitaries of LR mice (Fig. 2A and B). Moreover, *Pomc* mRNA levels were significantly higher in HR mice compared to animals of the other two lines (Fig. 2C). Similarly, HR mice showed a higher ACTH protein abundance in the pituitary compared to IR and LR animals (Fig. 2D). However, no statistical differences were observed between HR, IR and LR mice regarding the mRNA and protein expression of the GR and FKBP5 (Figs. S4 and S5).

3.3. Functional differences at the brain level

The results of our expression analysis of several candidate genes in brain nuclei relevant for HPA axis function are summarised in Table 1B. No significant differences between HR, IR and LR animals were found in the PVN. In the BLA, however, the expression of *Nr3c1* and *Crhr1* was significantly decreased in LR mice. Decreased expression of *Nr3c1* and *Fkbp5* was also observed in the hippocampus (dorsal and ventral) of these animals.

Investigating the neuronal activation of brain nuclei involved in HPA axis function in response to an acute psychological stressor, *c-fos* expression was assessed 45 min after the end of a 15-min restraint period. Unstressed animals from the three mouse lines served as controls for basal *c-fos* expression. In these animals the intensity of the *c-fos* signal did hardly differ from background values. Moreover, there were no statistical differences between the three lines in any of the investigated brain regions (Table S2A). After the stressor, as expected, considerably increased *c-fos* mRNA expression was observed, although there were no significant differences between HR, IR and LR mice in most of the investigated brain areas (Table S2B). Interestingly, however, line-specific differences in *c-fos* signal intensity could be detected in the PVN, with HR mice showing an increased and LR mice a decreased activation in this nucleus (Table S2B and Fig. 3B). Furthermore, significant but less prominent differences were found in the CA3 region of the dorsal hippocampus as well as in the DG of the ventral hippocampus. Here, HR mice showed a reduced *c-fos* expression, whereas LR mice presented a stronger *c-fos* signal compared to animals of the IR line (Table S2B).

3.4. Differences in HPA axis regulation

To assess differences in regulatory functions of the HPA axis between HR, IR and LR mice, we performed independent Dex/CRH tests, applying either a relatively high or a relatively low dose of Dex. The high dose of Dex strongly suppressed adrenocortical activity in all three mouse lines (Fig. 4A). Accordingly, CORT levels were close to the detection limit and did not differ significantly between the three lines. Similarly, the low dose of Dex readily suppressed plasma CORT concentrations in IR and LR mice. However,

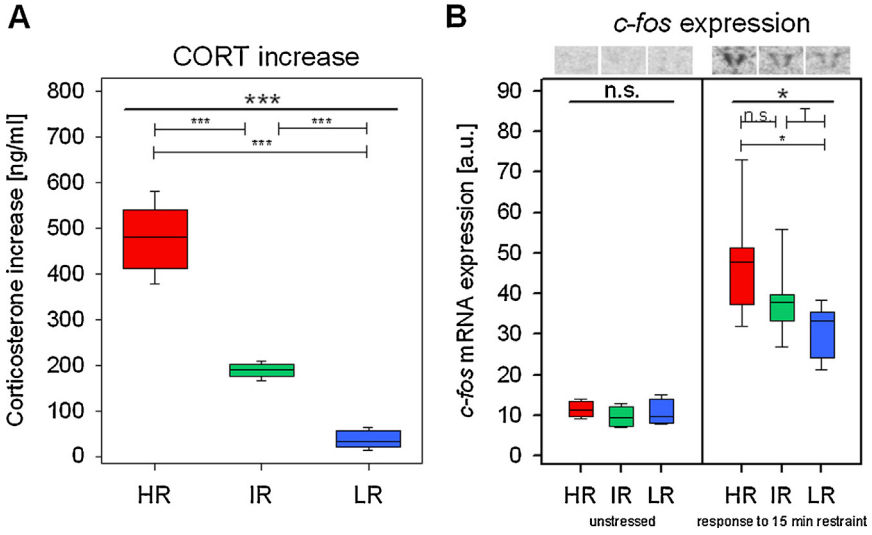

Figure 3 (A) Plasma corticosterone increase of high (HR), intermediate (IR) and low (LR) reactivity mice ($N = 16$ for each line) in response to a 15-min restraint stressor. (B) Expression of *c-fos* mRNA in the paraventricular nucleus of the hypothalamus (PVN) of HR, IR and LR mice under basal, unstressed conditions (left panel, $N = 4$ for each line) and in response to a 15-min restraint stressor (right panel, $N = 9–10$ for each line). The animals were sacrificed 45 min after the termination of the stressor and *c-fos* mRNA expression was quantified by in situ hybridisation. Representative in situ hybridisation images are shown. Data are given as box plots (for description see legend of Fig. 1). Statistical differences between the three lines (KWH-test) are given at the top of each panel. Results of the pairwise group comparison (MWU-tests) are indicated below (Bonferroni-corrected p-values: $p > 0.1$ n.s., $0.05 < p \leq 0.1$ T, $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***). (A) Corticosterone increase: HR vs. IR vs. LR, df = 2, $H = 41.8$, $p < 0.001$; HR vs. IR, $U = 0$, $p < 0.001$, HR vs. LR, $U = 0$, $p < 0.001$, IR vs. LR, $U = 0$, $p < 0.001$. (B) *c-fos* expression unstressed: HR vs. IR vs. LR, df = 2, $H = 1.6$, $p = 0.437$; *c-fos* expression after 15 min restraint: HR vs. IR vs. LR, df = 2, $H = 9.0$, $p = 0.011$, HR vs. IR, $U = 32$, $p = 0.288$, HR vs. LR, $U = 8$, $p = 0.012$, IR vs. LR, $U = 20$, $p = 0.082$.

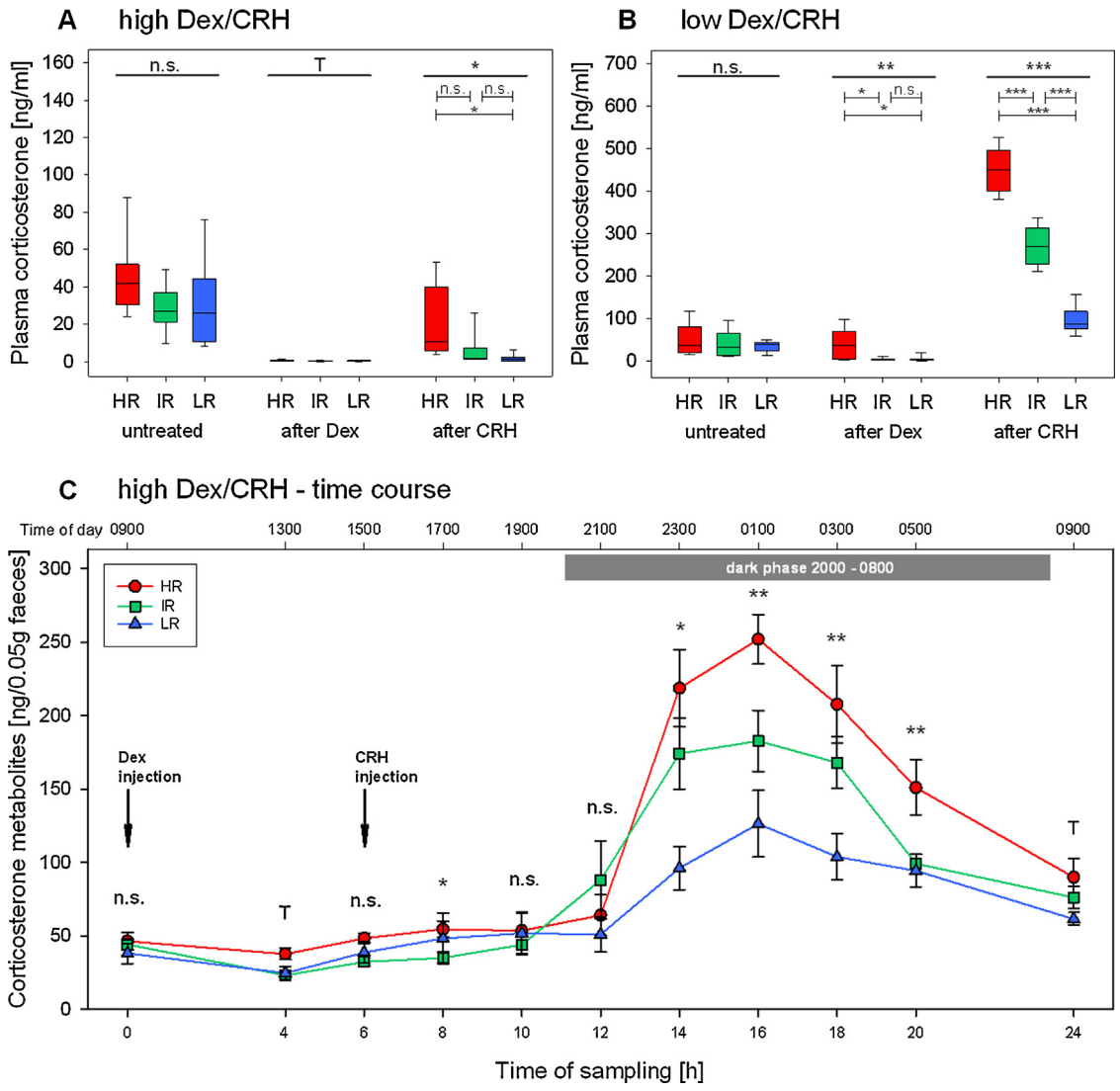

Figure 4 Plasma corticosterone (CORT) concentrations of high (HR), intermediate (IR) and low (LR) reactivity mice in the combined dexamethasone/corticotropin-releasing hormone (Dex/CRH) test; response to a pharmacological suppression of adrenocortical activity with either a relatively high (2 mg/kg, panel A) or a relatively low (0.05 mg/kg, panel B) dose of Dex and subsequent stimulation with CRH (0.15 mg/kg). Plasma CORT concentrations were measured three days before (untreated) and 6 h after Dex treatment (after Dex) as well as 30 min after CRH injection (after CRH). Data are given as box plots (for description see legend of Fig. 1). Statistical differences between the three lines (KWH-test) are given at the top of each panel. Results of the pairwise group comparison (MWU-tests) are indicated below (Bonferroni-corrected p-values: $p > 0.1$ n.s., $0.05 < p < 0.1$ T, $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***). (A) high-dose Dex ($N = 6$ for each line): untreated, HR vs. IR vs. LR, df = 2, $H = 2.5$, $p = 0.281$; after Dex, HR vs. IR vs. LR, df = 2, $H = 5.8$, $p = 0.056$; after CRH, HR vs. IR vs. LR, df = 2, $H = 7.5$, $p = 0.023$, HR vs. IR, $U = 6$, $p = 0.110$, HR vs. LR, $U = 2$, $p = 0.030$, IR vs. LR, $U = 12.5$, $p = 0.378$. (B) low-dose Dex ($N = 9$ for each line): untreated, HR vs. IR vs. LR, df = 2, $H = 0.7$, $p = 0.721$; after Dex, HR vs. IR vs. LR, df = 2, $H = 9.6$, $p = 0.008$, HR vs. IR, $U = 11.5$, $p = 0.020$, HR vs. LR, $U = 10$, $p = 0.021$, IR vs. LR, $U = 33.5$, $p = 0.536$; after CRH, HR vs. IR vs. LR, df = 2, $H = 23.1$, $p < 0.001$, HR vs. IR, $U = 0$, $p < 0.001$, HR vs. LR, $U = 0$, $p < 0.001$, IR vs. LR, $U = 0$, $p < 0.001$. (C) Excretion profile of immunoreactive faecal corticosterone metabolites (CM) in the Dex/CRH test (Dex dose 2 mg/kg, CRH dose 0.15 mg/kg). The time of the day and the dark phase (horizontal bar) are indicated at the top of the panel. The arrows mark the time of injection. Note that the delayed responses to the injection of Dex and CRH are due to the gut passage time. Data are given as means ± SEM for each line. Statistical differences between the three lines are indicated by asterisks. HR vs. IR vs. LR, $N = 10$ for each line, df = 2, $H = 0.5$—13.7, $p > 0.1$ n.s., $0.05 < p < 0.1$ T, $p \leq 0.05$*, $p \leq 0.01$**. Calculations of the area under the curve (AUC, time of sampling 0—24) revealed significantly higher CM levels in HR mice compared to animals of the IR and LR line (mean ± SEM, HR: 2643.4 ± 178.9, IR: 2039.8 ± 164.0, LR: 1513.0 ± 221.7; HR vs. IR vs. LR, KWH-test: df = 2, $H = 12.6$, $p = 0.002$, post hoc MWU-tests: HR vs. IR, $U = 17$, $p = 0.026$, HR vs. LR, $U = 8$, $p = 0.003$, IR vs. LR, $U = 29$, $p = 0.112$).

HR mice presented a clearly less pronounced suppression of plasma CORT levels in response to this lower dose of Dex, when compared to the other two lines (Fig. 4B).

In response to the stimulation with CRH, all mice showed increased CORT levels (Fig. 4A and 4B). In the high-dose Dex/CRH test, CORT concentrations were moderately increased in all three lines, but were significantly higher in HR compared to LR mice (Fig. 4A). In the low-dose Dex/CRH test, the CRH injection induced a robust increase of CORT concentrations in all three lines, with HR mice showing a clearly overshooting CORT surge, while LR mice presented a blunted CORT response (Fig. 4B).

The 24 h excretion profile of immunoreactive CORT metabolites (CM) in the Dex/CRH test is depicted in Fig. 4C. In response to the CRH stimulation, all three lines showed a clear increase in faecal CM compared to their undisturbed diurnal CM excretion pattern (Fig. S6). Similar to the results presented above, HR mice presented an overshooting CM excretion compared to the IR line, while LR mice displayed a clearly blunted response. Calculations of the area under the curve revealed significantly higher values in HR mice compared to animals of the IR and LR line (Fig. 4C).

3.5. Comparison of phenotypes in mice and men

Following the three-group approach of the HR/IR/LR mouse lines, depressed patients were stratified according to their cortisol response in the Dex/CRH test in high (hHR), intermediate (hIR) and low (hLR) HPA axis responders (Fig. S7). This stratification revealed considerable similarities regarding the neuroendocrine, morphometric, psychopathological and behavioural phenotypes observed in the three patient groups and the SR mouse lines. The findings are summarised in Table 2.

MD patients of the hHR group as well as HR mice showed a significantly less pronounced Dex-mediated CORT suppression, followed by an overshooting CORT surge in response to CRH. In contrast, hLR patients and LR mice readily suppressed CORT after Dex application and only showed a blunted CRH-triggered CORT response. Interestingly, hLR patients and LR mice also had a significantly higher body weight/BMI compared to the respective high responders. Regarding psychopathological characteristics, patients in the hLR group showed less sleep disturbance, less appetite loss and less weight loss, which have been grouped into the HDRS subscale of non-atypical depression symptoms (Table 2A). Moreover, hHR patients showed a strong trend towards higher 'agitation' scores and increased active stress-coping behaviour compared to hLR patients. Similarly, as has also been described earlier (Touma et al., 2008), HR mice showed clearly hyper-active stress-coping behaviours in several behavioural tests, e.g. increased struggling and decreased floating in the forced swim test (FST) (Table 2B). In contrast, LR mice reacted in a much more passive way, i.e. presenting decreased struggling and increased floating behaviour in the FST (Table 2B).

4. Discussion

Our study included a detailed analysis of peripheral, central and feedback regulatory aspects of HPA axis function in the HR/IR/LR mouse lines, a recently established animal model of affective disorders. The model was generated by selective breeding for extremes in stress reactivity, matching with the view that depression is a stress-related disorder. We could show that HR as well as LR animals have functional alterations on all levels of the HPA axis, providing insights into the complex molecular mechanisms orchestrating neuroendocrine functions under physiological and pathological conditions.

HR mice showed increased adrenal weights (Fig. 1B), an enhanced adrenocortical sensitivity to ACTH (Fig. 1C) and an increased expression of genes involved in steroidogenesis (Mrap and Cyp11a1, Table 1A), resulting in a differential activity of the adrenal cortex (Figs. 3A, S1 and S3) in the three mouse lines. In humans, adrenal hyper-activity and resulting elevated levels of circulating cortisol, as observed in Cushing's disease, are closely associated with symptoms of MD (Starkman et al., 1986; Sonino et al., 1998). Interestingly, adrenocortical hypo-activity with low cortisol levels has also been connected to pathologies such as familial glucocorticoid deficiency and Addison's disease, which are likewise associated with depression and anxiety (Hunt et al., 2000; Metherell et al., 2005; Thomsen et al., 2006). Thus, both adrenal hyperactivity as well as adrenal insufficiency are HPA axis dysfunctions mirrored in our SR mouse model. It is noteworthy, that steroidogenesis as well as adrenal activity are under control of both, the neuroendocrine HPA axis and the nervous sympathetic-adrenomedullary (SAM) system, two functionally interwoven stress response systems (Axelrod and Reisine, 1984; Ulrich-Lai and Herman, 2009). However, we could show that the expression of *Th* and *Pnmt* did not differ significantly between HR, IR and LR mice (Table 1A), indicating no or only a minor influence of the SAM system on the distinct differences in adrenocortical function between our mouse lines. This is further supported by previous findings showing no significant differences between the SR mouse lines in the stress-induced hyperthermia test (Touma et al., 2008).

At the pituitary level, the differences in *Avpr1b* and *Crhr1* expression between HR, IR and LR animals (Fig. 2A and B) are in accord with the differential activation of the HPA axis. It has been shown that CRHR1 activity induces pituitary *Pomc* expression (Bale and Vale, 2004; Karalis et al., 2004). Moreover, activation of the AVPR1b by the CRH-cosecretagogue AVP is known to potentiate *Pomc* expression and ACTH synthesis/release (DeBold et al., 1984). Hence, our results suggest that the increased expression of *Pomc* and ACTH in the pituitary (Fig. 2C and D) as well as the elevated plasma ACTH levels (Touma et al., 2008) of HR mice compared to IR and LR animals are caused by an enhanced activation of the CRHR1 and/or AVPR1b (Axelrod and Reisine, 1984; Bale and Vale, 2004; de Kloet et al., 2005; Holsboer and Ising, 2010). Interestingly, very similar results were found in patients suffering from affective disorders. MD patients showed an increased *POMC* expression and augmented ACTH levels in the pituitary, in addition to an increased number of ACTH secretory episodes, which were closely associated with alterations in adrenal cortisol secretion (Thomson and Craighead, 2008; Holsboer and Ising, 2010; Stetler and Miller, 2011). Moreover, in-depth studies on the CRH/CRHR1 system revealed that genetic polymorphisms are largely implicated in the pathophysiological changes of HPA axis function in MD

Table 2 Summary of demographic characteristics, neuroendocrine, morphometric, psychopathological and behavioural phenotypes observed in high (hHR, $N = 219$), intermediate (hIR, $N = 219$) and low (hLR, $N = 219$) HPA axis responder patients (A) and high (HR, $N = 9$), intermediate (IR, $N = 9$) and low (LR, $N = 9$) reactivity mice (B). Data are given as means ± SEM. Significant differences between the three groups are indicated (humans: ANCOVA, mice: KWH-test), including the appropriate post hoc pairwise comparisons (humans: Bonferroni-Holm-corrected LSD-tests, mice: Bonferroni-corrected MWU-tests).

	Variable	hHR		hIR		hLR		Group comparison		Post hoc comparison (BfH corrected LSD-tests)					
		Mean	SEM	Mean	SEM	Mean	SEM	(ANCOVA)		hHR vs hIR		hHR vs hLR		hIR vs hLR	
								F	*p*	*d*	*p*	*d*	*p*	*d*	*p*
A															
Demographic and clinical characteristics	Age [years]	49.81	0.91	48.43	0.91	47.26	0.92	2.0[1]	0.139						
	Previous depressive episodes [n]	2.90	0.36	2.58	0.36	2.16	0.36	1.0[1]	0.357						
	Age at disease onset [years]	38.06	1.04	37.55	1.04	36.26	1.05	0.8[1]	0.455						
	Duration current episode [weeks]	43.17	4.59	37.92	4.48	36.60	4.60	0.6[1]	0.564						
Dex/CRH test	Basal morning cortisol [ng/ml]	200	5.89	176	5.96	164	5.92	9.93[2]	<0.001***	24.7	0.006**	36.6	0.001***	11.9	0.161
	Cortisol after Dex [ng/ml]	34.25	1.28	15.15	1.28	9.23	1.29	**103.2**[3]	**<0.001*** **	19.1	0.001***	25.0	0.001***	5.92	0.001***
	Cortisol after CRH [ng/ml)	87.4	1.93	23.4	1.93	9.5	1.95	**459.0**[3]	**<0.001*** **	64.0	0.001***	77.9	0.001***	14.0	0.001***
	Cortisol AUC after CRH	6447	129	1908	129	733	130	**540.1**[3]	**<0.001*** **	4540	0.001***	5714	0.001***	1175	0.001***
BMI (body mass index)	BMI: onset of treatment	24.52	0.29	24.75	0.29	26.01	0.30	**7.2**[4]	**0.001*** **	−0.22	0.589	−1.49	0.001**	−1.26	0.006**
	BMI: end of treatment	24.83	0.29	25.21	0.28	26.58	0.29	**10.1**[4]	**<0.001*** **	−0.38	0.351	−1.75	0.001***	−1.37	0.002**
Depression symptoms	Total HDRS-21	26.85	0.41	25.77	0.41	25.99	0.41	1.9[3]	0.146						
	HDRS: non-atypical subscale (5 items)	5.85	0.18	5.33	0.18	4.94	0.18	**6.3**[3]	**0.002** **	0.52	0.084 T	0.91	0.001**	0.39	0.126
	HDRS item 9: agitation	1.07	0.07	1.08	0.07	0.86	0.07	**3.0**[3]	**0.050***	−0.01	0.942	0.22	0.074 T	0.22	0.093 T
	HDRS: all other items (15 items)	20.24	0.33	19.70	0.33	20.25	0.33	0.9[3]	0.397						
Coping behaviour	Active stress-coping	13.45	0.27	12.68	0.28	12.58	0.27	**3.1**[5]	**0.048***	0.77	0.094 T	0.88	0.075 T	0.10	0.795
	Passive stress-coping	13.21	0.45	13.91	0.46	13.12	0.46	0.9[5]	0.419						

[1]F(2,654), [2]F(2,448), [3]F(2,652), [4]F(2,610), [5]F(2,287)

Table 2 (*Continued*)

	Variable	HR		IR		LR		Line comparison		Post-hoc comparison (Bf corrected MWU-tests)					
										HR vs IR		HR vs LR		IR vs LR	
		Mean	SEM	Mean	SEM	Mean	SEM	*H*	*p*	*U*	*p*	*U*	*p*	*U*	*p*
B															
Dex/CRH test	Corticosterone after Dex [ng/ml]	36.9	11.8	3.6	1.0	4.1	1.9	**9.6**	**0.008****	11.5	0.020*	10.0	0.021*	33.5	0.536
	Corticosterone after CRH [ng/ml]	452.3	17.1	269.6	15.0	95.1	10.3	**23.1**	**<0.001*****	0.0	0.001***	0.0	0.001***	0.0	0.001***
Body weight	Five months of age [g]	35.5	0.5	38.6	0.9	42.2	0.8	17.6	<0.001***	17.0	0.026*	0.0	0.001***	18.0	0.016*
Stress-coping behaviour in the FST	Struggling time [s]	165.9	14.3	118.9	15.7	60.2	7.3	18.6	<0.001***	19.0	0.029*	1.0	0.001***	10.0	0.004**
	Swimming time [s]	174.0	17.2	155.7	16.1	110.9	18.2	**5.5**	**0.063 T**						
	Floating time [s]	19.4	9.2	84.0	17.5	187.6	19.0	**20.7**	**<0.001*****	11.0	0.003**	0.0	0.001***	10.0	0.004**
	Latency to 1st floating [s]	299.0	24.6	114.5	20.9	65.2	6.7	**20.2**	**<0.001*****	3.0	0.002**	0.0	0.001***	24.0	0.049*

Note: $p > 0.1$ n.s., $0.05 < p \leq 0.1$ T, $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***.
Statistically significant differences are indicated in bold.

patients, which could also be used as new drug targets (Bale and Vale, 2004; Thomson and Craighead, 2008; Holsboer and Ising, 2010).

It has been shown that stimulation of the anterior pituitary by hypothalamic neuropeptides is indispensable for triggering *Pomc* expression and ACTH release (Dallman et al., 1985). In line with this, HR mice presented an increased stress-induced neuronal activation in the PVN compared to LR animals (Fig. 3B and Table S2B). Thus, the differences in stress reactivity between HR, IR and LR animals are not only a peripheral phenomenon but have a neuronal underpinning in the central nervous system. In depressed patients too, increased expression of CRH and AVP in the PVN, concomitant with an increased number of AVP-containing neurons and enhanced CRH levels in the cerebrospinal fluid, have been observed (Nemeroff et al., 1984; Heuser et al., 1998; Wang et al., 2008). Although basal *Crh* and *Avp* expression in the PVN was not different between the three SR mouse lines (Table 1B and (Touma et al., 2008)), our results suggest that the CORT-independent (Helmreich et al., 1996; Weiser et al., 2011) differences in activation of PVN neurons evoked a differential neurotransmitter release, which would eventually culminate in the observed differences in HPA axis reactivity (HR > IR > LR). It would also be conceivable that despite a similar release of CRH and AVP from the PVN, an increased or decreased activation of the anterior pituitary is triggered by the increased or decreased expression of CRHR1 and AVPR1b in HR and LR mice, respectively. However, the PVN is thought to be the 'executing' nucleus in the hypothalamus, suggesting that its stress-induced activation is evoked by superior limbic brain structures such as the hippocampus, the BLA and the PFC, known to project multisynaptic neuronal efferents to the PVN (de Kloet et al., 2005; Ulrich-Lai and Herman, 2009). Accordingly, we found significant differences in the stress-induced neuronal activation in the CA3 subregion of the dorsal hippocampus and in the dentate gyrus of the ventral hippocampus (HR < IR < LR, Table S2B). This is of interest, as the hippocampus exerts inhibitory activity on the HPA axis (de Kloet et al., 2005; Ulrich-Lai and Herman, 2009) and reduced hippocampal activity/neuronal integrity has been implicated in cognitive dysfunctions as well as increased stress reactivity observed in HR mice (Knapman et al., 2010a,b, 2012) and psychiatric patients (Hinkelmann et al., 2009; Lupien et al., 2009; Holsboer and Ising, 2010).

When comparing the findings in our mouse model and the clinical condition in humans, we found strong resemblances. The corresponding stratification of MD patients in high, intermediate and low HPA axis responder groups revealed similar neuroendocrine, morphometric, psychopathological and behavioural phenotypes in mice and men (Table 2). In the Dex/CRH test of the high responder groups (HR mice and hHR patients), Dex-nonsuppression and an overshooting CRH-induced CORT response (Fig. 4 and S7) was associated with a more active/hyperactive stress-coping behaviour, decreased body weight/BMI as well as higher scores in non-atypical symptoms of depression compared to the low responders (LR mice and hLR patients, Table 2). Thus, our results emphasise that the Dex/CRH test could be of considerable use to distinguish between distinct biological subtypes of MD. This is of critical importance for clinical research, diagnosis and treatment, as HPA axis

hyper-activity, psychomotor agitation and weight loss are associated with the subtypes of psychotic and melancholic depression, whereas hypo-activity of the HPA axis, lethargy and weight gain (as seen in LR mice and hLR patients) are frequently observed in atypical depression (Gold and Chrousos, 2002; Antonijevic, 2006; Lamers et al., 2013). Interestingly, clinical as well as preclinical research has mainly focussed on HPA axis hyper-activity and only few studies investigated the role of an insufficient adrenocortical response in MD (Gold and Chrousos, 2002; Raison and Miller, 2003; Fries et al., 2005; Antonijevic, 2006; Henkel et al., 2006). This, however, might have severe consequences for choosing the appropriate treatment for the disease, since pharmacotherapy designed for psychotic or melancholically depressed patients could fail or even have adverse effects in patients suffering from atypical depression and vice versa (Holsboer and Ising, 2010).

Thus, we suggest that a stratification of patients according to their HPA axis-related neuroendocrine phenotype should be taken into consideration in order to determine the most successful pharmacotherapy, in particular because HPA axis dysregulation is considered to play an important role in MD (Angst et al., 2002; Raison and Miller, 2003; Henkel et al., 2006; Thomson and Craighead, 2008; Holsboer and Ising, 2010; Stetler and Miller, 2011). Several studies also presented evidence that feedback resistance and mild hyper-cortisolism are already present in healthy subjects at risk for depression, indicating that an imbalanced HPA axis might precede the clinical manifestation of MD (Holsboer et al., 1995; Modell et al., 1998; Wahlberg et al., 2009; Walker et al., 2013). Hence, the SR mouse model could yield important insights into the mechanisms by which functional alterations of the HPA axis are linked to the increased risk of developing affective disorders. Furthermore, the selective inbreeding approach used to establish the HR, IR and LR mouse lines has enriched the respective genetic factors associated with the mentioned neuroendocrine, morphometric and behavioural endophenotypes. Thus, our model also considers the multigenetic background of depression, a key issue in psychiatric genetics, which is not reflected in single gene manipulation approaches e.g. applied in current transgenic mice, as well as in chronic stress models using wild-type animals.

In conclusion, the prominent analogies between our SR mouse lines and MD patients reveal a high degree of face and construct validity of this model for human depression. Thus, our animal model has the potential to become a valuable tool that allows for detailed insights into the molecular-genetic mechanisms leading to the observed pathophysiological changes in HPA axis function and related endophenotypes. Furthermore, it can serve in the search for novel drug targets and help to improve already existing treatments of MD. In particular, different subtypes of MD may benefit from different treatments.

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Conflict of interest

Florian Holsboer is cofounder of the biotech company HMNC which develops personalised treatments for depression. The current study is not involved in any of this co-authors' entrepreneurial endeavours. All other authors report no financial interests or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2014.07.008.

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SUPPLEMENTAL INFORMATION

Heinzmann JM, Kloiber S, Ebling-Mattos G, Bielohuby M, Schmidt MV, Palme R, Holsboer F, Uhr M, Ising M, Touma C (2014): Mice selected for extremes in stress reactivity reveal endophenotypes of major depression: a translational approach. *Psychoneuroendocrinology.*

1. Methods

The animal studies presented in this manuscript were conducted at the Max Planck Institute of Psychiatry and are in accord with accepted standards of humane animal care, as outlined in the National Institutes of Health Guide for the Care and Use Experimental Animals. All conducted experiments are in accord with the current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC). The experiments were announced to and approved by the appropriate local authority and the Animal Welfare Officer of the Max Planck Institute of Psychiatry.

1.1. Experimental animals and housing conditions

In all experiments, adult male mice derived from generation XVI to XX of the stress reactivity (SR) mouse model were used (for details see (Touma et al., 2008)). In this mouse model, males and females of both extremes, i.e. with high or low corticosterone (CORT) increase in the 'stress reactivity test' (SRT, see 1.3.1.), were mated, generating the high reactivity (HR) and low reactivity (LR) breeding line, respectively. In addition, a third breeding line was established showing intermediate stress reactivity (IR), resembling the mean phenotype of the CD-1 strain founder population. Already in the first generation, animals of the three breeding lines displayed significant differences in their CORT increase in the SRT, which could be further increased by selective breeding in the following generations (Figure S1).

After weaning, the mice were housed in groups of four animals in transparent polycarbonate cages (standard Macrolon cages type III, Bayer MaterialScience, Leverkusen, Germany; 38 x 22 x 15 cm) with wood chips as bedding and wood shavings as nesting material (Product codes: LTE E-001 and NBF E-011, ABEDD – LAB and VET Service GmbH, Vienna, Austria). At the age of about eight weeks, HPA axis responsiveness to a psychological

stressor was assessed in all animals by means of the SRT (see 1.3.1.). At least ten days before performing the experiments presented in this study, animals were single housed (standard Macrolon cages type II, Bayer MaterialScience, Leverkusen, Germany; 23 x 16 x 14 cm) to avoid potential dominance hierarchy effects. The mice were 3 – 5 months of age in all experiments. Housing and experimental rooms were kept under standard laboratory conditions (12/12 h light/dark cycle, lights on 8:00 h; temperature: 22±1 °C; relative humidity: 55±10 %). Commercial mouse diet (Altromin No. 1324; Altromin GmbH, Lage, Germany) and tap water were available *ad libitum*.

1.2. Blood sampling

Blood sampling was performed as described previously (1). Blood samples obtained from the animals' ventral tail vessel were collected in EDTA-coated tubes (Microvette, Sarstedt, Nürnbrecht, Germany). Trunk blood was collected in EDTA-coated tubes (KABE Labortechnik GmbH, Nürnbrecht-Elsenroth, Germany) equipped with 10 µl of the protease inhibitor Tryasylol (Bayer Vital GmbH, Leverkusen, Germany). All blood samples were immediately cooled on ice and centrifuged at 4000 *g* for 10 min at 4 °C. The plasma was transferred into fresh tubes (SafeSeal, Sarstedt, Nürnbrecht, Germany) and stored at -20 °C until further analysis.

1.3. Behavioural testing

All tests were performed between 9:00 and 12:00. The animals' behaviour during the tests was videotaped and scored by a trained observer.

1.3.1. The forced swim test (FST)

The FST was used to measure stress-coping behaviour. It involves putting each mouse for 6 min in an inescapable and highly aversive situation by placing them in a glass cylinder (12 cm diameter, 24 cm height) filled two-thirds with 23°C warm water. After an initial period of vigorous activity ('swimming' and 'struggling') in an attempt to escape (swimming is characterized by relatively strong movements of the limbs and the tail, while 'struggling' also includes breaking the water surface with the front paws or trying to climb up the walls of the beaker), the animals intermittently adopt immobile postures ('floating') interspersed with bouts of swimming. The total time spent floating (defined as ceasing to move altogether, making only those movements necessary to keep the head above water) during the test was

scored and has been proposed to reflect a state of despair or an alteration in coping strategy from active to passive.

1.4. Paradigms assessing hypothalamic-pituitary-adrenal axis activity and regulation

1.4.1. The stress reactivity test (SRT)

The SRT is described in detail elsewhere (Touma et al., 2008). Briefly, the test consists of a 15-min restraint period and two tail blood samplings, one immediately before and one right after the restraint stressor. The animals' plasma CORT increase in response to the SRT served as criterion for selecting the animals for the respective experimental groups of the three mouse lines.

1.4.2. The adrenal sensitivity test

To determine the adrenal responsiveness of HR, IR and LR mice to ACTH, an adrenal sensitivity test was performed. In this test, the animals received a high dose of dexamethasone (Dex, 4 mg/kg BW) at 0900 to suppress endogenous ACTH secretion. Six hours later (1500), a blood sample was collected by means of a tail nick to confirm the Dex-mediated suppression of ACTH and CORT, which was immediately followed by an i.p. injection of either vehicle (Saline solution, Berlin-Chemie AG, Berlin, Germany) or one of three doses of ACTH (0.1 µg/kg, 1 µg/kg or 100 µg/kg, Synacthen®, Defiante Farmacêutica, S.A., Funchal, Portugal) dissolved in vehicle (0.3 ml) to stimulate CORT secretion from the adrenal cortex. Thirty minutes after this injection, the animals were sacrificed by decapitation after brief isoflurane anaesthesia (Isoflurane, Forene, Abbott GmbH, Wiesbaden, Germany) and trunk blood was collected. Plasma ACTH concentrations were determined (see 1.4.3.) from the first blood sample taken 6 h after the Dex injection to confirm the Dex-mediated ACTH suppression. Plasma CORT concentrations were analysed in all collected blood samples (see 1.4.2.).

1.4.3. The combined Dex/CRH test in mice

In order to assess HPA axis activity and regulation in animals of the SR mouse model, the combined Dex/CRH test was performed as described previously (1). Briefly, a reference blood sample was collected by an incision in the ventral tail vessel at 1500, three days prior to the actual test ('untreated' value). On the experimental day, at 0900, HR, IR and LR mice were injected intraperitoneally (i.p.) with either a relatively high (2 mg/kg) or a relatively low

(0.05 mg/kg) dose of Dex (ratiopharm GmbH, Ulm, Germany). At 1500, a second blood sample was drawn from the tail vessel ('after Dex' value), immediately followed by an injection of CRH (0.15 mg/kg, i.p.). Thirty minutes later, the mice were sacrificed and trunk blood was collected ('after CRH' value). All blood samples were stored frozen at -20 °C until plasma CORT concentrations were analysed (see 1.4.2.).

1.5. Hormone analysis by immunoassays

1.5.1. Aldosterone enzyme-linked immunosorbent assay

Aldosterone was measured using a recently described method (Manolopoulou et al., 2008). Briefly, 50 µl of the plasma sample collected from each animal was extracted using 2 ml of dichloromethane (DCM)/ polyethylene glycol 10000 (PEG; 50 mg/l), reconstituted and pipeted in duplicate directly onto microtiter-plates previously coated with the monoclonal mouse anti-aldosterone capture antibody, along with aldosterone calibrators and controls. Subsequently, 100 µl (5 pg/well) of biotinylated aldosterone tracer were pipetted into each well and incubated overnight at 4 °C. The following day, the incubation was terminated and 200 µl per well of a Streptavidin-Europium conjugate (PerkinElmer/Wallac, Turku, Finland) were added followed by addition of an in-house "enhancement solution" used to transfer the Europium conjugate into a fluorescent complex. The amount of biotinylated aldosterone bound was then measured using a fluorometer (VICTOR^3, PerkinElmer). Intra-assay coefficients of variation were determined by 20-fold measurements of pooled plasma samples with aldosterone concentrations of 18, 34 and 139 pg/ml which were extracted, reconstituted, pooled again and then added as samples on the same plate and found to be 7.3, 6.3, 4.4 %, respectively. Inter-assay coefficients of variation were determined by 20-fold measurements (on consecutive day) of plasma samples of 14, 37 and 161 pg/ml and were found to be 15.2, 15.1 and 8.0 %, respectively.

1.5.2. Corticosterone radioimmunoassay

Plasma corticosterone (CORT) was measured as described before (1), using a commercially available CORT radioimmunoassay (RIA) kit (DRG Instruments GmbH, Marburg, Germany) following the manufacturer's protocol with slight modifications, i.e. using half of the recommended volume of all reagents. Ten microliters of plasma of each sample were applied to the assay and all samples were analysed in duplicate. Furthermore, the same pool samples (one in the upper and one in the lower concentration range) were run in every assay as an internal standard to control for intra- and inter-assay variations, which were both below 10 %

in this study. Radio-labelled samples were measured by a gamma counter (Wallac Wizard 1470 automatic gamma counter, Perkin Elmer life science, Rodgau, Germany). CORT concentrations were calculated by means of a seven point standard curve. Double estimations were accepted if the coefficient of variation was below 10 %. The detection limit of the CORT RIA was 1 ng/ml plasma.

1.5.3. Adrenocorticotropic hormone radioimmunoassay

Plasma ACTH was measured as described before (1), using a commercially available ACTH RIA kit (DRG Instruments GmbH, Marburg, Germany) following the manufacturer's protocol. 50 µl of plasma of each sample was analysed in duplicate. Double estimations were accepted if the coefficient of variation was below 15 %. The detection limit of the ACTH RIA was 7 pg/ml plasma.

1.5.4. Faecal corticosterone metabolites enzyme-immunoassay

Faecal corticosterone metabolites (CM) were extracted and analysed according to the method described previously (Touma et al., 2003; Touma et al., 2004). Briefly, each faecal sample was dried, homogenized and an aliquot of 0.05 g was shaken with 1 ml of 80 % methanol for 30 min on a multi-vortex. After centrifugation (10 min at 2500 *g*) an aliquot of the supernatant was diluted (1:10) with assay buffer (Tris/HCl 20 mM, pH 7.5) and frozen at -20 °C until analysis. To determine the amount of faecal CM, a 5α-pregnane-3β,11β,21-triol-20-one enzyme-immunoassay (EIA) was used, which proved well suited to assess CM in faecal samples of mice (Touma et al., 2003; Touma et al., 2004). This EIA utilizes a group-specific antibody measuring steroids with a 5α-3β,11β-diol structure and shows very little cross-reaction with Dex (<0.01 %) or other relevant steroids (progesterone, androstenedione and dehydroepiandrosterone, <1 %). The intra- and inter-assay coefficients of variation were 9.1 % and 14.0 %, respectively.

1.6. Dissection of brain nuclei for mRNA extraction

For the dissection of brain nuclei, the mouse brains were quickly removed from the skull and frozen at -80 °C. The frozen brains were mounted in a cryostat (Cryo-Star HM 560 M, MICROM GmbH, Waldorf, Germany) and sectioned in 200 µm thick coronal slices. The dorsal and ventral hippocampus, the basolateral amygdala and the paraventricular nucleus of

the hypothalamus were acquired from these slices by micropuncture as described previously (Palkovits, 1973).

1.7. RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted as described previously (Bettscheider et al., 2011). Briefly, the tissue (adrenals, pituitaries and brain nuclei) was homogenized in 200 µl of guanidium thiocyanate buffer (4.5 M guanidium thiocyanate, 2 % N-lauroylsarcosine, 50 mM EDTA pH 8, 25 mM Tris-HCl pH 7.5, 0.1 M betamercaptoethanol, 0.2 % antifoam A). Subsequently, 20 µl NaOAc, 200 µl acidic phenol and 100 µl of chloroform:isoamyl alcohol (24:1) was added. The solution was incubated on ice (10 min), centrifuged (20 min, 11300 g at 4 °C) and the aqueous phase was transferred to a new tube and mixed with 70 % ethanol. Finally, the precipitate was transferred to a RNA spin column (RNeasy Micro Kit, Qiagen, Hilden, Germany) and further processed according to the manufacturer's protocol. Approximately 50 ng of total RNA was reverse transcribed into cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA gene transcripts were analysed in 384 well-plates using the Qiagen QuantiFast SYBR Green PCR kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. All samples were analysed in duplicates using the Roche Lightcycler® 480 instrument (Roche Diagnostics, Mannheim, Germany). TATA-binding protein (TBP) and Hypoxanthine-Guanine Phosphoribosyltransferase (HPRT) were used as housekeeping genes. A detailed list of all applied oligonucleotide primers is provided in Supplementary Table S1. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ algorithm (Livak and Schmittgen, 2001). Crossing Points were normalised to the mean of the two housekeeping genes TBP and HPRT and all values were calculated to the relative expression mean of the IR group.

1.8. Protein extraction and immunoblotting

For protein extraction, frozen hippocampal and pituitary tissue was homogenised in 150 µl homogenisation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 3.33 mM EDTA, 10 % Complete Mini Protease Inhibitor, Roche Diagnostics, Mannheim, Germany). Subsequently, 50 µl of extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 % NP-40 (Igepal), 2 % sodium deoxycholate, 4 % SDS) was added to the solution and the samples were sonified. Whole cell protein concentration was assessed using a BCA protein assay according to the manufacturer's protocol (Thermo Scientific, Rockford, USA). Ten microlitres (20 µg) of protein solution was loaded and separated by sodium dodecyl sulphate polyacrylamide gel

electrophoresis (SDS-PAGE, 12 %) and transferred onto a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). To block unspecific binding sites, the membranes were incubated for 1h in 5 % (w/v) fat free milk solution (Carl Roth GmbH, Karlsruhe, Germany) and incubated overnight at 4 °C with the respective first antibody in 2.5 % Tris-buffered saline and Tween 20 (TBST)/milk solution. After the membranes were rinsed with TBST on the next day, the incubation time with the respective horseradish peroxidase-conjugated secondary antibody was 1 h at room temperature, followed by washing with TBST. A detailed overview of the used primary and secondary antibodies is provided in Supplementary Table S3. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as standard protein. After being incubated in enhanced chemiluminescence (ECL) solution (100 mM Tris-HCl pH 6.8, 10 % DMSO, 0.0025 % (w/v) luminol, 0.001 % (w/v) p-coumaric acid, 0.003 % (v/v) 30 % H_2O_2), the protein bands were detected by the chemoluminescence-sensitive camera of the ChemiDoc MP imaging system (Bio-Rad Laboratories GmbH, Munich, Germany) and analysed by Image Lab software (version 4.0.1 build 4, Bio-Rad Laboratories GmbH, Munich, Germany).

1.9. *In-situ* hybridisation (ISH) for *c-fos* expression

For the quantification of *c-fos* expression in the brains of HR, IR and LR mice, an exon-spanning ribonucleotide probe for *c-fos* was designed using Primer3 software (http://primer3.sourceforge.net/releases.php), spanning from exon 1 to exon 4 of the murine *c-fos* gene, with a total length of 480 base pairs (forward primer 5'-3': ATGGGCTCTCCTGTCAACAC; reverse primer 5'-3': GGCTGCCAAAATAAACTCCA). The frozen brains were mounted in a cryostat (Cryo-Star HM 560 M, MICROM GmbH, Waldorf, Germany) and cut in 18 µm thick slices in the coronal section with a knife and sample temperature of -15 °C. Brain slices/sections were adhered to superfrost plus slides (VWR, Darmstadt, Germany), shortly dried and stored at -80 °C until further analysis. When slicing the brain, every 10th section is sampled on the same glass slide, with a total of four sections per slide. This results in a representative representation of the rostral to caudal extent of the respective brain structure.

ISH using a ^{35}S-UTP-labelled riboprobe for *c-fos* was performed as previously described (Scharf et al., 2011). Briefly, brain sections were fixed in 4 % paraformaldehyde, washed and acetylated in 0.25 % acetic anhydride. Subsequently, slides were dehydrated in ascending concentrations of ethanol, degreased with chloroform and air dried. After adding 100 µl of hybridisation buffer per slide, containing 0.65 to 1.7x10^6 counts per minute of ^{35}S-UTP-

labelled riboprobe, the slides were coverslipped and incubated overnight at 55 °C. The next day, coverslips were removed, the slides were washed and incubated in RNase A solution. Finally, the sections were desalted, dehydrated and exposed to radiation-sensitive films (Kodak Biomax MR films, Eastman Kodak Co., Rochester, NY, USA) for 5 days, except for the ventral hippocampus which was exposed for 12 days due to a lower signal intensity. The films were scanned and the *c-fos* signal intensity (optical density) was assessed by Image J software (version 1.44p, National Institute of Health, USA, http://imagej.nih.gov/ij/). For each animal, the expression in four (at least two) separate sections in both hemispheres on one slide was measured, including subtraction of the background value. These expression values were averaged to obtain a representative value for each brain region. The background signal was measured in adjacent structures not expressing *c-fos*. *C-fos* signal intensity was assessed in the prefrontal cortex (PFC), the paraventricular nucleus of the hypothalamus (PVN), the basolateral amygdala (BLA) as well as the dorsal and ventral hippocampus (HC).

1.10. Studies involving depressed patients

1.10.1. Patients

In total, 657 inpatients with a current major depressive episode participating in the Munich Antidepressant Response Signature (MARS) project (54.9 % female patients; mean age: 48.5 ± 13.4 years) were selected for the analysis. The MARS project is an open-label naturalistic longitudinal study with inpatients suffering from a depressive episode. Patients are recruited from referrals to the hospital of the Max Planck Institute of Psychiatry in Munich, Germany, and from collaborating hospitals in Southern Bavaria and Basel, Switzerland (see also (Hennings et al., 2009). Inclusion criteria were a first (33.9%, DSM-IV code 296.2) or a recurrent episode (64.1%, 296.3) of a major depression with moderate to severe intensity (as indicated by a total score of larger than 14 in the 21-item Hamilton Depression Rating Scale, HDRS, (Overall and Rhoades, 1982); mean HDRS score: 26.2 ± 6.1), and participation in the combined Dex/CRH within the first ten days after admission to the hospital. Exclusion criteria were presence of a somatic disorder potentially interfering with the results Dex/CRH test, such as endocrine disorders or acute infectious diseases, and treatment with the lithium or carbamazepine, as both mood stabilizers are known to invalidate the results of the Dex/CRH test (Zobel et al., 2001; Watson et al., 2007). Patients were included after study details were explained and written informed consent was obtained. The study was approved by the local ethics committee of the Ludwig Maximilians University (LMU), Munich. At the time of the Dex/CRH test, 20% of the patients were without medication, while the remaining 80%

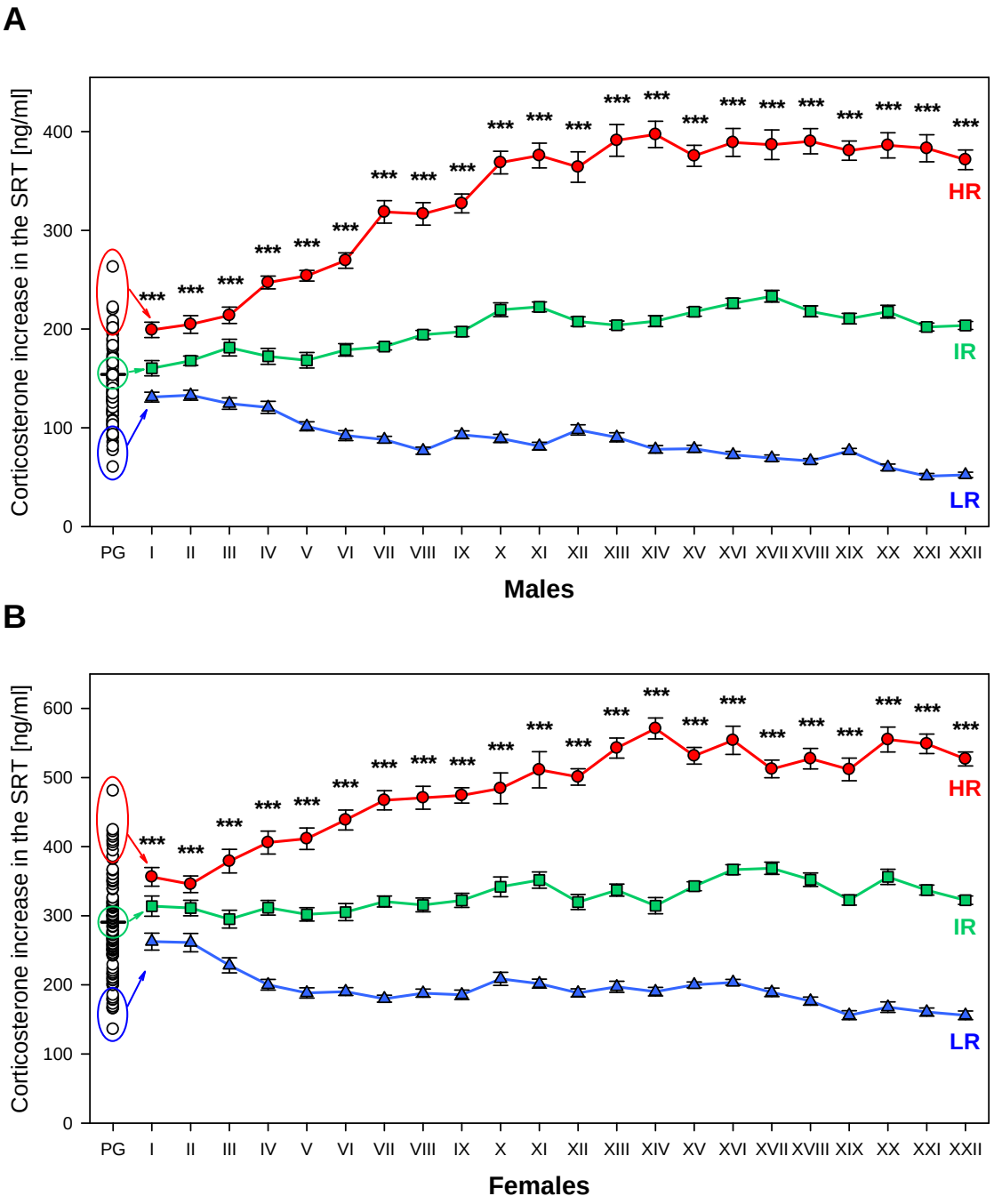
received different types of antidepressants according to the attending doctor's choice (16% tricyclic antidepressant, 25% selective serotonin reuptake inhibitors, 20% venlafaxine, 22% mirtazapine, 10% other antidepressants). 36% of the patients received benzodiazepines as add-on medication, 19.5% atypical antipsychotics, and 12% mood stabilizers (other than lithium and carbamazepine). In some patients comorbidity with anxiety disorders was observed: 7.6% of the participants were additionally suffering from generalized anxiety disorder, panic disorder and/or agoraphobia, or social phobia. The average number of previous depressive episodes was 2.5 (SD = 4.9), the mean age of disease onset was 37.3 (SD = 15.4) years, and the mean duration of the current episode was 39 (SD = 65) weeks.

Coping style was assessed using the German Stress-Coping Questionnaire (Ising et al., 2001), which was applied at the end of hospitalisation, when depression symptoms had substantially improved. The questionnaire asks for individual coping strategies in response to general stressful conditions and situations. The items were grouped into secondary scales of a predominantly active coping (distraction, substitute gratification, situational control, reaction control, positive self-instructions) and passive coping style (avoidance, escape).

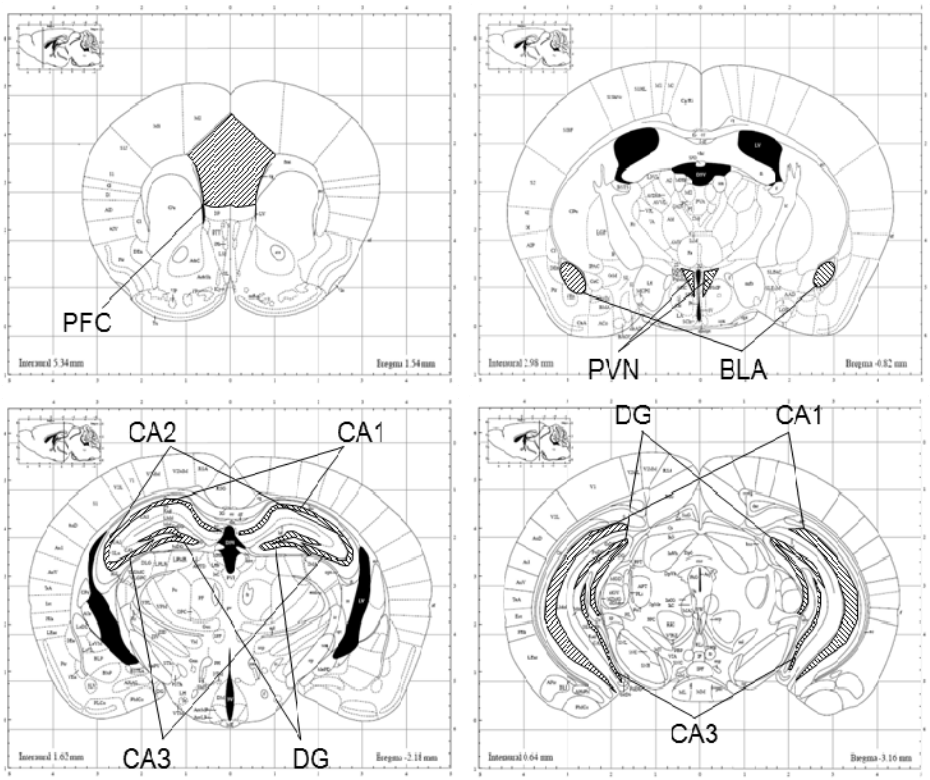
1.10.2. The combined Dex/CRH test in humans

The combined Dex/CRH test was administered within 10 days (on average 6 d) after admission to the clinic. Dexamethasone (Dex, 1.5 mg) was orally administered in the evening (2300) the day before stimulation with 100 µg human CRH at 1500. Blood samples were drawn at 1500, 1530, 1545, 1600, and 1615. CRH was injected within 30 seconds, just after the first sample was collected. Plasma cortisol concentrations were determined by radioimmunoassay (ICN Biomedicals, Carson, CA; detection limit 0.3 ng/ml). The subjects rested supine throughout the test. Hormone concentrations of the first specimen collected at 1500 (baseline) reflect the suppressive effects of the Dex application the day before, whereas the other four samples depict the response to the CRH injection, which was assessed as the total area under the curve (AUC) of the five cortisol concentrations.

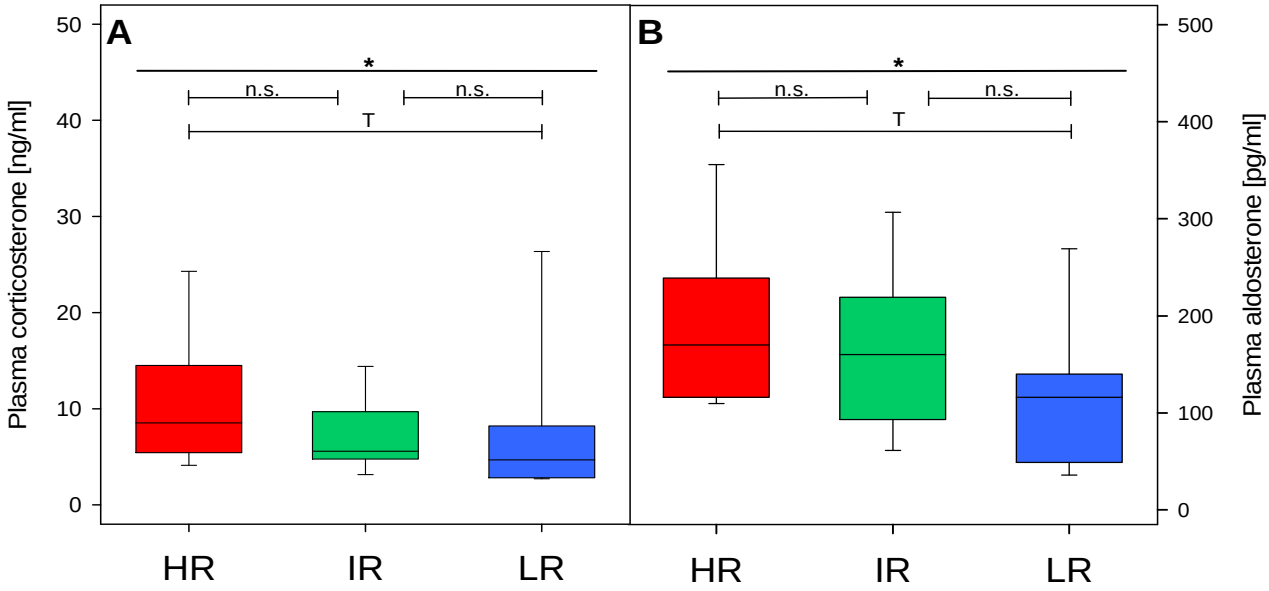
2. Supplementary Figures and Tables



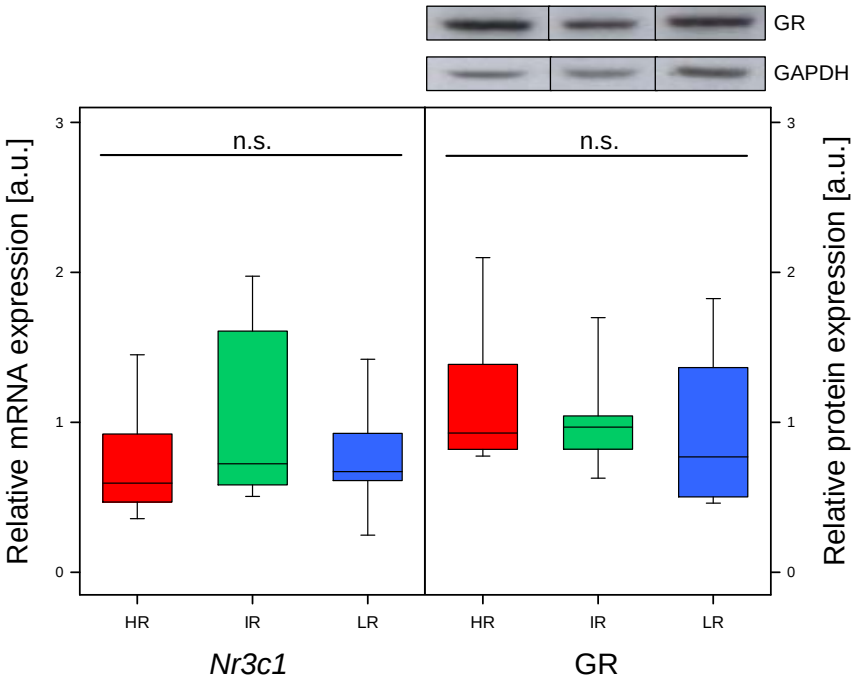
Supplementary Figure S1: Corticosterone (CORT) increase in the stress reactivity test (SRT) of male (**A**) and female (**B**) mice of the CD-1 founder population (parental generation, PG) and the descendent generations (Gen I – XXII) of the high (HR), intermediate (IR) and low (LR) reactivity breeding line. Data are given as individual values (PG) and means ± SEM (Gen I – XXII). Already from generation I, significant differences in the stress-induced CORT increase between the three mouse lines could be observed (HR vs. IR vs. LR, KWH-test, df=2, Gen I – XXII, males N=25-83 for each line, H=41.6-175.4, females N=23-77 for each line H=13.8-153.7, all $p \leq 0.001$***). This divergence was further increased by selective breeding in the subsequent generations. Figure adopted and extended from Touma et al., 2008 (1).



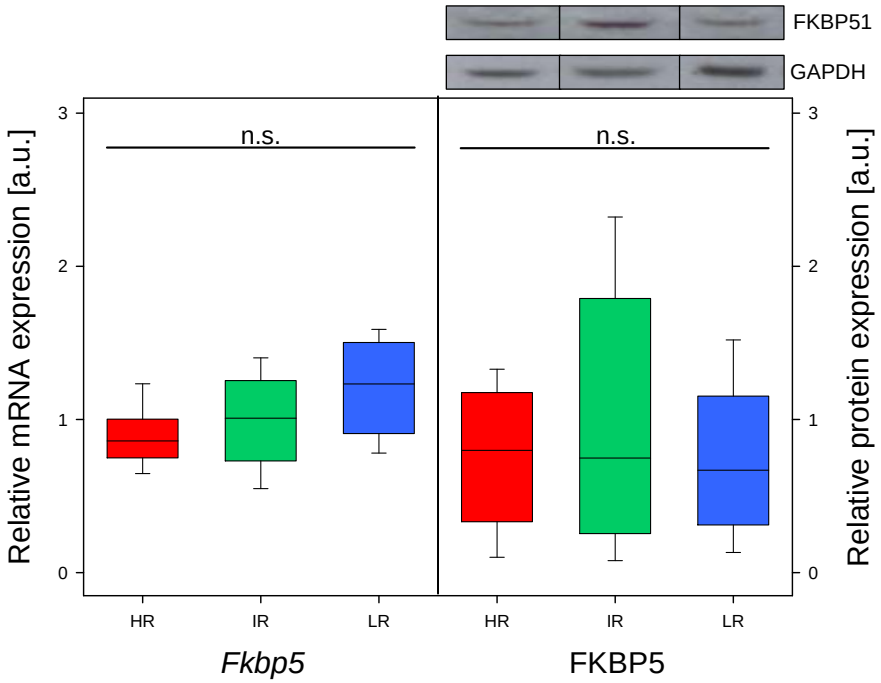
Supplementary Figure S2: Schematic overview of the anatomical location of the brain regions investigated for *c-fos* mRNA expression by *in-situ* hybridisation. Upper left: prefrontal cortex (PFC). Upper right: basolateral amygdala (BLA) and paraventricular nucleus of the hypothalamus (PVN). Lower left: dorsal hippocampus including the cornu ammonis subfields (CA1, CA2, CA3) and the dentate gyrus (DG). Lower right: ventral hippocampus including subfields and the DG. Images adopted from the mouse brain atlas by Franklin and Paxinos 2001.



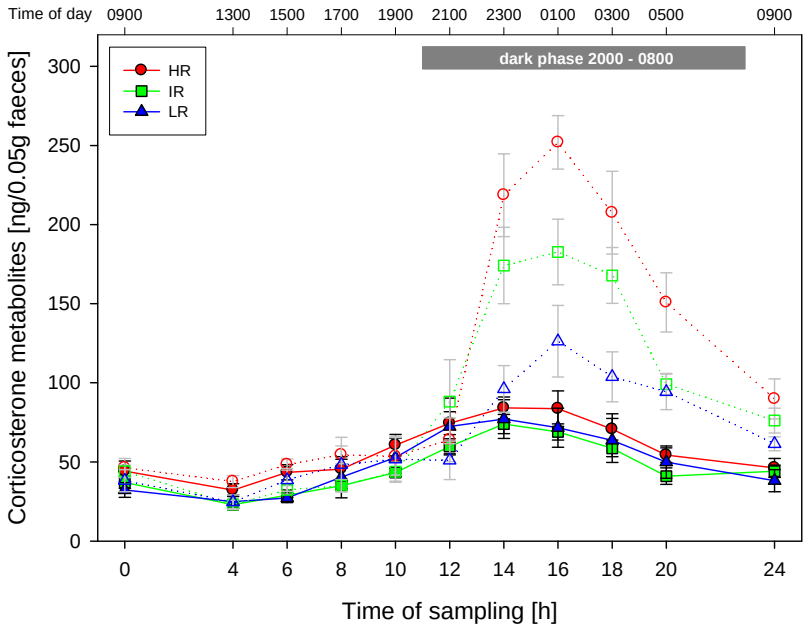
Supplementary Figure S3: Morning plasma corticosterone (**A**) and aldosterone (**B**) concentrations of high (HR), intermediate (IR) and low (LR) reactivity mice (N=16 for each line) under basal, unstressed conditions. Data are given as box plots (for description see legend of Fig. 1). Statistical differences between the three lines (KWH-test) are given at the top of each panel. Results of the pairwise group comparison (MWU-tests) are indicated below (Bonferroni corrected p-values: $p>0.1$ n.s., $0.05<p\leq0.1$ T, $p\leq0.05$*). **A**) HR vs. IR vs. LR, df=2, H=6.7, p= 0.035; HR vs. IR, U=87.5, p=0.254, HR vs. LR, U=65, p=0.054, IR vs. LR, U=89.5, p=0.146. **B**) HR vs. IR vs. LR, df=2, H=6.3, p= 0.043; HR vs. IR, U=98, p=0.258, HR vs. LR, U=65, p=0.051, IR vs. LR, U=86.5, p=0.234.



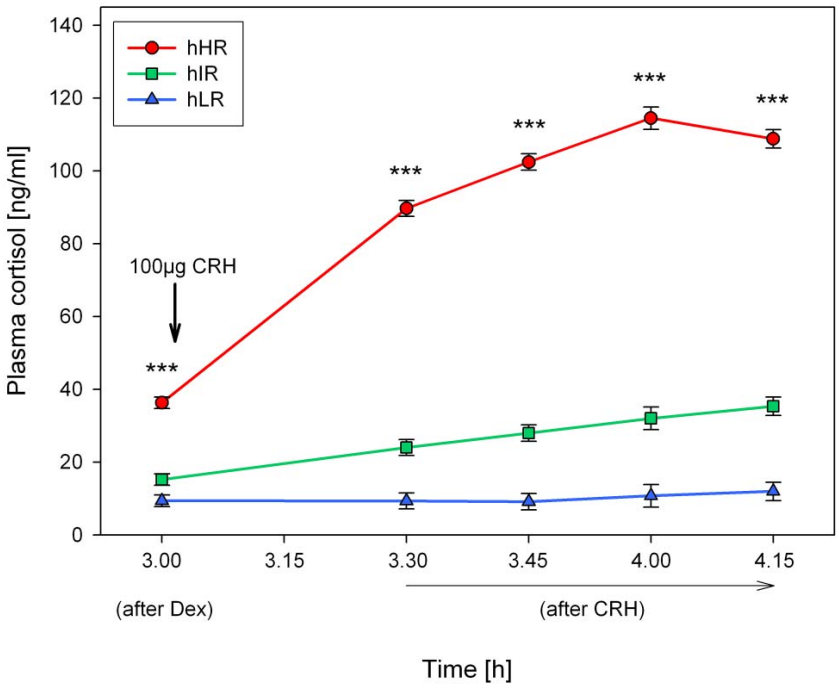
Supplementary Figure S4: Expression of *Nr3c1* mRNA in the pituitary of high (HR), intermediate (IR) and low (LR) reactivity mice (N=8 for each line), relative to two housekeeping genes (HPRT and TBP) and normalised to the mean of the IR line (left panel). GR protein expression (N=8 for each line) in the pituitary of HR, IR and LR mice relative to the internal standard protein (GAPDH) and normalised to the mean of the IR line (right panel). Representative western blot images of the internal standard (GAPDH) and the target (GR) protein are presented (top right panel). Data are given as box plots (for description see legend of Fig. 1). Statistical differences between the three lines (KWH-test) are given at the top of each panel (p>0.1 n.s.). *Nr3c1:* HR vs. IR vs. LR, df=2, H=1.5, p=0.464; GR: HR vs. IR vs. LR, df=2, H=1.4, p= 0.505.



Supplementary Figure S5: Expression of *Fkbp5* mRNA in the pituitary of high (HR), intermediate (IR) and low (LR) reactivity mice (N=8 for each line), relative to two housekeeping genes (HPRT and TBP) and normalised to the mean of the IR line (left panel). FKBP5 protein expression (N=8 for each line) in the pituitary of HR, IR and LR mice relative to the internal standard protein (GAPDH) and normalised to the mean of the IR line (right panel). Representative western blot images of the internal standard (GAPDH) and the target (FKBP5) protein are presented (top right panel). Data are given as box plots (for description see legend of Fig. 1). Statistical differences between the three lines (KWH-test) are given at the top of each panel (p>0.1 n.s.). *Fkbp5:* HR vs. IR vs. LR, df=2, H=4.6, p=0.102; FKBP5: HR vs. IR vs. LR, df=2, H=0.2, p= 0.898.



Supplementary Figure S6: Diurnal variation of immunoreactive corticosterone metabolites (CM) in the faeces of high (HR), intermediate (IR) and low (LR) reactivity mice (solid lines). The excretion profile of CM in response to pharmacological suppression with Dex and subsequent stimulation with CRH (Dex/CRH test, c.f. Figure 3C) is indicated (dashed lines). Note the distinct increase of faecal CM concentrations after CRH stimulation in all three mouse lines compared to the respective circadian rhythm of CM secretion. The time of day and the dark phase (horizontal bar) are indicated at the top of the panel. Data are given as means ± SEM (N=10 for each line).



Supplementary Figure S7: Plasma cortisol concentrations of human high (hHR), intermediate (hIR) and low (hLR) responder patients in the combined dexamethasone/corticotropin-releasing hormone (Dex/CRH) test; response to a pharmacological suppression of adrenocortical activity with Dex (1.5 mg) and subsequent stimulation with CRH (100 µg). Plasma cortisol concentrations were measured after the Dex treatment (after Dex), 30 minutes following the CRH injection (after CRH) and then in 15-min intervals. The arrow marks the time of CRH injection. Data are given as means ± SEM for each group. Statistical differences between the three patient groups (ANCOVA) are indicated by asterisks. hHR vs. hIR vs. hLR, N=219 for each group, F(2,652)=103.2-574.9, all $p \leq 0.001$***.

Supplementary Table S1: List of candidate genes including designation, aliases, oligonucleotide primer sequences, melting temperature (T_m) and amplicon length in base pairs (bp) assessed in high (HR), intermediate (IR) and low (LR) reactivity mice using quantitative real-time polymerase chain reaction (qPCR).

Candidate gene	Designation	Aliases	Direction	Sequence	T_m	Amplicon length (Binder et al.)
Avp	Arginine Vasopressin	Vp	forward	TCGCCAGGATGCTCAACAC	67.6	174
			reverse	TTGGTCCGAAGCAGCGTC	67.7	
Avpr1b	arginine vasopressin receptor 1B	AVPR3, V3/V1b	forward	CCTTTCTTCAGTGTCCAGATG	61.3	141
			reverse	GTTGAAGCCCATATAGATCCA	60.3	
Crh	corticotropin releasing hormone	CRF	forward	GCATCCTGAGAGAAGTCCCTCTG	67.5	135
			reverse	GCAGGACGACAGAGCCA	64.2	
Crhr1	corticotropin releasing hormone receptor 1	CRFR1	forward	GGTCCTGCTGATCAACTTTA	59.2	152
			reverse	ACATGTAGGTGATGCCCA	59.9	
Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1; cholesterol desmolase; cytochrome P450 side-chain cleavage enzyme	P450scc	forward	GATGCGTCGATACTCTTCTCA	61.9	222
			reverse	GATGAAGTCCTGAGCTACACCT	61.3	
Cyp11b1	cytochrome P450, family 11, subfamily b, polypeptide 1; steroid-11-beta-hydroxylase		forward	CATGGTAGCTTCTCTATGGACT	58.9	181
			reverse	GCAAGTACAAGAGCTGTGTG	58.6	
Cyp11b2	cytochrome P450, family 11, subfamily b, polypeptide 2; aldosterone synthase; steroid 11-beta-hydroxylase		forward	CCTGGGATGTCATCTCTGAG	62.3	152
			reverse	CATAGAGTTCGCTTTGATGGC	63.5	
Fkbp4	FK506-binding protein 4	FKBP-4, FKBP52	forward	CAACGCCACACTTGTATTTGA	63.5	143
			reverse	CTTCCACCATAGCACCATCAT	63.7	
Fkbp5	FK506-binding protein 5	FKBP-5, FKBP51	forward	AGAATCAAACGGAAAGGCGAG	66.3	103
			reverse	CTCGGCAATCAAATGTCCTTC	65.6	
Hprt	hypoxanthine guanine phosphoribosyl transferase	Hprt1	forward	GTTGGATACAGGCCAGACTTTGT	65.1	225
			reverse	CCACAGGACTAGAACACCTGCTA	64.3	
Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1		forward	GCACTATGGAAGACATGACAT	59.0	159
			reverse	AGTTGACCTCCATGACTCTTC	59.7	
Mc2r	melanocortin 2 receptor	ACTHR	forward	CCAAGGAGAGGAGCATTATTG	62.5	141
			reverse	GTTTGCCGTTGACTTACAGAA	61.7	
Mrap	melanocortin 2 receptor accessory protein		forward	CTGTGGACGAGAAGAAGCTGA	64.6	169
			reverse	GTCCATGAACATATTGGCTGG	64.0	
Nr3c1	glucocorticoid receptor	GR	forward	CAAGGGTCTGGAGAGGACAA	64.2	220
			reverse	TACAGCTTCCACACGTCAGC	64.1	
Nr3c2	aldosterone receptor; mineralocorticoid receptor	MR	forward	GTGTGTGGAGATGAGGC	57.2	155
			reverse	GGACAGTTCTTTCTCCGAAT	59.6	
Pnmt	phenylethanolamine-N-methyltransferase		forward	GCTGCATGGCACAAGTCTTTG	68.0	198
			reverse	CACTCCAGTCAAAGGCTCCTG	66.2	
Pomc	pro-opiomelanocortin-alpha	Pomc1, alpha-MSH, alphaMSH, beta-MSH, gamma-MSH	forward	GAAGATGCCGAGATTCTGCT	63.4	222
			reverse	TTTTCAGTCAGGGGCTGTTC	64.1	
Star	steroidogenic acute regulatory protein		forward	GCTGGAAGCTCCTATAGACAT	59.0	191
			reverse	GACAGCTCCTGGTCACTATAG	58.0	
Tbp	TATA box binding protein	TFIID	forward	CCCCCTTGTACCCTTCACC	65.4	285
			reverse	TGGATTGTTCTTCACTCTTGG	65.3	
Th	tyrosine hydroxylase		forward	CTGTGGAGTTTGGGCTGTGTA	65.0	160
			reverse	GTTTGATCTTGGTAGGGCTGC	64.7	
Tsc22d3	glucocorticoid-induced leucine zipper protein	Gilz	forward	GTGGCCCTAGACAACAAGATT	61.9	122
			reverse	GAGTTCTTCTCAAGCAGCTCA	61.5	

Supplementary Table S2: Expression of *c-fos* mRNA (**A**) under basal, unstressed conditions (N=4 for each line) and (**B**) in response to a 15-min restraint stressor (N=11-16 for each line) in the prefrontal cortex (PFC), the paraventricular nucleus of the hypothalamus (PVN), the basolateral amygdala (BLA) as well as the dorsal (dHC) and ventral (vHC) hippocampus of high (HR), intermediate (IR) and low (LR) reactivity mice. The brains of the stressed animals were dissected 45 min after the termination of the stressor. All brains were analysed by *in-situ* hybridisation. *C-fos* signal intensities (optical density) are given as means ± SEM. Significant differences between the three mouse lines (KWH-test) are indicated, including the post-hoc pairwise comparisons (Bonferroni-corrected MWU-tests).

A

brain region		HR		IR		LR		line comparison (KWH-test, df=2)			post-hoc comparison (Bf corrected MWU-tests) HR vs IR		HR vs LR		IR vs LR	
		mean	SEM	mean	SEM	mean	SEM	H	p		U	p	U	p	U	p
PFC		9.74	3.13	13.09	3.49	9.45	2.34	0.5	0.779	n.s.						
PVN		11.47	1.03	9.62	1.26	10.49	1.60	1.6	0.437	n.s.						
BLA		4.10	0.89	7.42	2.29	5.18	2.49	0.7	0.694	n.s.						
dHC	CA1	12.92	3.64	16.54	4.40	14.64	4.38	0.2	0.874	n.s.						
	CA2	9.84	2.25	13.73	3.47	12.96	3.04	0.5	0.779	n.s.						
	CA3	13.56	3.09	15.84	3.20	15.05	2.94	0.4	0.794	n.s.						
	DG	18.54	2.04	19.26	1.35	22.81	2.52	1.8	0.397	n.s.						
vHC	CA1	9.51	0.84	8.71	1.28	10.72	1.67	0.4	0.794	n.s.						
	CA3	8.34	0.70	7.26	0.73	8.25	0.90	1.4	0.491	n.s.						
	DG	10.56	1.29	8.08	0.86	11.90	1.39	5.1	0.077	T						

B

brain region		HR		IR		LR		line comparison (KWH-test df=2)			post-hoc comparison (Bf corrected MWU-tests) HR vs IR		HR vs LR		IR vs LR	
		mean	SEM	mean	SEM	mean	SEM	H	p		U	p	U	p	U	p
PFC		47.33	2.37	45.46	1.47	51.38	2.29	3.2	0.200	n.s.						
PVN		46.47	4.14	38.92	2.89	30.32	2.13	**9.0**	**0.011**	*	32	0.288	8	0.012 *	20	0.082 T
BLA		35.41	3.23	29.90	1.53	28.43	1.86	2.4	0.288	n.s.						
dHC	CA1	29.09	2.81	28.34	1.62	33.10	2.76	1.8	0.397	n.s.						
	CA2	23.25	3.04	29.89	2.01	29.23	2.39	3.1	0.208	n.s.						
	CA3	21.20	1.70	27.03	1.80	28.14	2.48	**6.4**	**0.040**	*	18	0.081 T	16	0.062 T	45	1.000
	DG	25.63	1.68	22.46	0.92	27.15	1.86	3.8	0.148	n.s.						
vHC	CA1	20.15	2.22	23.86	1.96	26.99	2.42	2.9	0.225	n.s.						
	CA3	12.14	1.13	12.24	0.84	16.05	1.36	4.7	0.093	n.s.						
	DG	11.45	1.17	12.40	0.93	16.15	1.30	**6.1**	**0.046**	*	35	0.414	14.5	0.076 T	16	0.099 T

Supplementary Table S3: List of antibodies including abbreviations, size (kilodalton, kDa), specifications and the dilution used for the western blot analysis of protein samples from high (HR), intermediate (IR) and low (LR) reactivity mice.

target protein	abbreviation	protein size (kDa)	primary antibody		secondary antibody	
			specification	dilution	specification	dilution
Adrenocorticotropic hormone	ACTH	29	ACTH rabbit polyclonal antibody (Abcam, ab 74976)	1:3000	goat anti-rabbit horseradish peroxidase conjugated antibody (Sigma, A9169)	1:30000
FK506 binding protein 51	FKBP51	51	FKBP51 (F-14) goat polyclonal antibody (Santa Cruz, sc 11518)	1:500	donkey anti-goat horseradish peroxidase conjugated antibody (Santa Cruz, sc2056)	1:10000
Glucocorticoid Receptor	GR	95	GR (M-20) rabbit polyclonal antibody (Santa Cruz, sc 1004)	1:500	goat anti-rabbit horseradish peroxidase conjugated antibody (Sigma, A9169)	1:30000
Glyceraldehyde-3-phosphate-dehydrogenase	GAPDH	37	GAPDH (A-3) mouse monoclonal antibody (Santa Cruz, sc 137179)	1:2000	goat anti-mouse horseradish peroxidase conjugated antibody (Sigma A9917)	1:30000

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