Neurobiology of Disease

Mice with Genetically Altered Glucocorticoid Receptor Expression Show Altered Sensitivity for Stress-Induced Depressive Reactions

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Altered glucocorticoid receptor (GR) signaling is a postulated mechanism for the pathogenesis of major depression. To mimic the human situation of altered GR function claimed for depression, we generated mouse strains that underexpress or overexpress GR, but maintain the regulatory genetic context controlling the GR gene. To achieve this goal, we used the following: (1) GR-heterozygous mutant mice ($GR^{+/-}$) with a 50% GR gene dose reduction, and (2) mice overexpressing GR by a yeast artificial chromosome resulting in a twofold gene dose elevation. $GR^{+/-}$ mice exhibit normal baseline behaviors but demonstrate increased helplessness after stress exposure, a behavioral correlate of depression in mice. Similar to depressed patients, $GR^{+/-}$ mice have a disinhibited hypothalamic–pituitary–adrenal (HPA) system and a pathological dexamethasone/corticotropin-releasing hormone test. Thus, they represent a murine depression model with good face and construct validity. Overexpression of GR in mice evokes reduced helplessness after stress exposure, and an enhanced HPA system feedback regulation. Therefore, they may represent a model for a stress-resistant strain. These mouse models can now be used to study biological changes underlying the pathogenesis of depressive disorders. As a first potential molecular correlate for such changes, we identified a downregulation of BDNF protein content in the hippocampus of $GR^{+/-}$ mice, which is in agreement with the so-called neurotrophin hypothesis of depression.

Key words: **depression; stress; glucocorticoid receptor; helplessness; transgenic mice; behavior**

Introduction

Dysregulations and dysfunctions of corticosteroid receptors have been implicated in the pathogenesis of stress-related psychiatric disorders such as depression (De Kloet et al., 1998; Holsboer, 2000; Sapolsky, 2000; Nestler et al., 2002). Clinical studies have demonstrated a hyperactivity of the hypothalamic–pituitary–adrenal (HPA) system with elevated plasma cortisol levels in the majority of patients with major depression (Holsboer and Barden, 1996; Zobel et al., 1999; Holsboer, 2000). In these patients, diminished glucocorticosteroid receptor (GR) expression or function has been postulated as causative factor for a deficient feedback of cortisol that may explain their increased HPA activity and stress sensitivity. Because studies of the GR in the human brain are not feasible *in vivo*, such evidence derives mainly from pharmacological challenge tests, such as the dexamethasone (Dex) suppression test, which indirectly measures GR function in the pituitary and maybe in part also in the CNS (Heuser et al., 1994). Currently, the analysis of direct effects of GR dysfunction and its potential role for emotional behavior is restricted to animal models. For this purpose, targeted mutagenesis in mice seems to be particularly promising.

Several mouse strains with altered GR expression or function have been generated: (1) mice with a GR point mutation (GR^{dim}) that prevents GR dimerization and binding to its cognate element GRE (glucocorticoid response element) (Reichardt et al., 1998), (2) a strain with a brain-specific GR knock-out (GR^{NesCre}) (Tronche et al., 1999), and (3) a GR antisense model with reduced expression in brain and specific peripheral tissues (Pepin et al., 1992). Whereas GR^{dim} mice have not revealed changes in emotional behavior (Oitzl et al., 2001), brain-specific GR knock-out mice as well as GR antisense mice surprisingly exhibited reduced depression-like behavior (Montkowski et al., 1995; Tronche et al., 1999). However, with respect to human (psycho)pathology, the genetic defects generated in these mouse strains are unlikely to occur in humans.

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To test the "GR hypothesis of depression," we used mice that have a 50% reduced or a twofold increased GR gene dosage, which may mimic the situation of patients with affective disorders more accurately than a complete GR knock-out. Moreover, we tried to keep the genetic context controlling the GR gene as close as possible, to maintain the regulatory principles governing the normal gene. Such mice have been generated by homologous recombination [$GR^{+/-}$ mice (Tronche et al., 1999)] and by a transgenic approach [YGR mice (Reichardt et al., 2000)]. The latter strain contains two additional copies of the GR gene.

So far, $GR^{+/-}$ and YGR mice have not been characterized behaviorally. Therefore, we subjected $GR^{+/-}$ and YGR mice to a standardized battery of tests for emotional behavior, including tests for depression-like signs such as despair and helplessness. Furthermore, we analyzed the HPA system under baseline conditions, after stress, and after a Dex/corticotropin-releasing hormone (CRH) challenge. Moreover, we determined the content of brain-derived neurotrophic factor (BDNF) in the hippocampus, because BDNF has been postulated to play a critical role in pathogenesis and therapy of affective disorders (Duman et al., 1997; Altar, 1999; Nestler et al., 2002; Cryan and Mombereau, 2004).

Materials and Methods

Animals

The founders of GR-heterozygous mice ($GR^{+/-}$) were developed by using homologous recombination in embryonic stem cells as described previously (Tronche et al., 1999). Furthermore, so-called YGR mice were investigated that carry two additional copies of the GR, generated by a transgenic approach using a yeast artificial chromosome (Reichardt et al., 2000). For all experiments, male mice were used. Mice were bred as F_1 hybrids from two commonly used inbred strains, C57BL/6 and FVB/N. $GR^{+/-}$ mice were generated by crossing heterozygous C57BL/6N males (backcrossed for >10 generations) with wild-type FVB/N females. To obtain also an F_1 hybrid background in YGR mice, double-transgenic male YGR founders on a pure FVB/N background were paired with female C57BL/6N wild-type mice. Before all experiments, 3- to 6-month-old mice were single-housed for 2 weeks in a reversed dark/light cycle with lights on at 6:00 P.M. Animals were supplied with food and water *ad libitum*. German animal welfare authorities approved all experiments. The numbers of animals tested in each experiment were dependent on the availability of mice of approximately the same age.

GR expression analyses

For Western blot analyses, whole hippocampi were homogenized in 400 μl of radioimmunoprecipitation assay buffer containing 0.4 M NaCl. Twenty micrograms of protein of the whole-cell extract were resolved on a denaturing SDS–7.5% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and stained with a GR-specific antibody (M-20; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized by enhanced chemiluminesence. ($GR^{+/-}$, $n = 4$; wild type, $n = 4$) (YGR, $n = 4$; wild type, $n = 4$).

For real-time quantitative reverse transcription (RT)-PCR, total hippocampal RNA was transcribed into cDNA with oligo-dT primers (Promega, Mannheim, Germany) and Superscript (Invitrogen, Karlsruhe, Germany). The transcripts of the housekeeping gene cyclophilin 1A (GenBank accession number NM_017101.1; bases 160–333) and of GR (GenBank accession number X_04435; bases 861–1186) were amplified from cDNA and negative controls without reverse transcription. A commercial master mix was used (Absolute SYBR Green Fluorescein; ABgene, Epson, UK) and primer concentrations of 400 nM (cyclophilin sense and antisense) or 200 nM (sense) and 800 nM (antisense of GR) at 60°C annealing temperature (iCycler; Bio-Rad, München, Germany). The cycle of amplification threshold (CT) was assessed, and the melting curve was used to verify the identity of the amplification products. We assessed the CT difference between the housekeeping gene and the gene of interest for every sample (Δ−CT) and compared the means of the groups of transgenes and their controls with the Student's *t* test ($n = 4$) to evaluate significantly different Δ−CT values (ΔΔ−CT) ($GR^{+/-}$, $n = 4$; wild type, $n = 4$) (YGR, $n = 4$; wild type, $n = 4$).

Behavioral experiments

All behavioral tests were conducted during the dark cycle (i.e., in the animals' active phase). Before each test, mice were acclimatized to the experimental room for at least 15 min. Test procedures were essentially performed as described previously (Fleischmann et al., 2003). The animals were subjected to several basal tests of locomotion, exploration, and anxiety, as well as depression-relevant paradigms such as the Porsolt forced-swim test, fear conditioning, and learned helplessness. The order of the tests followed previous recommendations ranking the tests from least stressful to more stressful (van Gaalen and Steckler, 2000; McIlwain et al., 2001). Between individual tests was a pause of at least 24 h. In all experiments, the investigator was blind to the genotype of the mice during behavioral testing.

Novel cage. The number of rearings in a novel home cage was analyzed for 5 min as an indicator for exploration ($GR^{+/-}$, $n = 14$; wild type, $n = 14$) (YGR, $n = 14$; wild type, $n = 14$).

Open field. Activity monitoring was conducted in a square-shaped, white open field, measuring 50×50 cm^2 and illuminated from above by 25 lux. Mice were placed individually into the arena and monitored for 15 min by a video camera (CCD IRIS; Sony, Tokyo, Japan). The resulting data were analyzed using the image-processing system EthoVision 2.3 (Noldus Information Technology, Wageningen, The Netherlands). For each sample, the system recorded position, object area, and the status of defined events. Parameters assessed for the present study were total distance moved, velocity, and time in center, which was defined as the area 10 cm distant from the walls ($GR^{+/-}$, $n = 14$; wild type, $n = 14$) (YGR, $n = 14$; wild type, $n = 14$).

Elevated O-maze. The maze consisted of a gray plastic annular runway (width, 6 cm; outer diameter, 46 cm; 50 cm above ground level), covered with black cardboard paper to prevent mice from slipping off the maze. Two opposing 90° sectors were protected by inner and outer walls of gray polyvinyl (height, 10 cm). Animals were placed in one of the protected sectors and observed for 5 min. The maze was illuminated by 25 lux. The following parameters were analyzed: (1) latency to first exit, (2) number of exits to and total time spent in the open compartments ($GR^{+/-}$, $n = 14$; wild type, $n = 14$) (YGR, $n = 14$; wild type, $n = 14$).

Dark–light box. The dark–light box consisted of two plastic chambers, connected by a small tunnel. The dark chamber measured 20×15 cm^2 and was covered by a lid. The other chamber, measuring 30×15 cm^2, was white and illuminated from above by an intensity of 600 lux. Mice were placed into the dark compartment, and latency to first exit, number of exits, and total time in the light compartment were recorded for 5 min ($GR^{+/-}$, $n = 14$; wild type, $n = 14$) (YGR, $n = 14$; wild type, $n = 14$).

Porsolt forced-swim test. Mice were placed into a glass cylinder (height, 23 cm; diameter, 13 cm), which was filled with water (22°C) up to a height of 8 cm, as described previously (Zörner et al., 2003). A testing period of 6 min was used to determine the onset and the percentage of time spent immobile. Immobility was defined as motionless floating in the water, only allowing movements necessary for the animal to keep its head above the water. In contrast, swimming was defined as time spent with active escape or struggling movements ($GR^{+/-}$, $n = 14$; wild type, $n = 14$) (YGR, $n = 14$; wild type, $n = 14$).

Fear conditioning. Fear conditioning was done as described previously (Fleischmann et al., 2003). For both contextual and cued conditioning, mice were individually placed into the conditioning chamber ($58 \times 30 \times 27$ cm^3; TSE, Bad Homburg, Germany) and allowed to habituate for 2 min, before the onset of a discrete conditioned stimulus (2800 Hz tone; 85 dB) that lasted 30 s. At the end of the tone, animals were subjected to the unconditioned stimulus (2 s of continuous footshock of 0.8 mA). Twenty-four hours after training, context conditioning was assessed by measuring freezing, defined as a complete lack of movements apart from respiration. Context learning was tested in the same Plexiglas chamber that was used during the training. Freezing behavior was scored at intervals of 10 s for 5 min. Cued conditioning was analyzed in a visually and olfactorily novel context at 48 h after training by exposing the animals to

the tone for 3 min, during which freezing was scored as described above ($GR^{+/-}$, $n = 13$; wild type, $n = 14$) (YGR, $n = 14$; wild type, $n = 14$).

Learned helplessness. In the learned-helplessness paradigm, as described by Reif et al. (2004), the animals were exposed to a transparent Plexiglas shock chamber (18 × 18 × 30 cm), equipped with a stainless-steel grid floor (Coulborn precision regulated animal shocker; Coulborn Instruments, Düsseldorf, Germany), through which they received 360 footshocks (0.150 mA) on 2 consecutive days, respectively. The footshocks applied were unpredictable with varying shock episodes (1–3 s) and interval episodes (1–15 s), amounting to a total session duration of ~52 min. Twenty-four hours after the second shock procedure, learned helplessness was assessed by testing shuttle box performance (Graphic State Notation; Coulborn Instruments). The shuttle box consisted of two equal-sized compartments (18 × 18 × 30 cm) that were separated by a small gate (6 cm wide and 7 cm high). The shuttle box also contained a grid floor, through which current could be applied, and a signaling light at the top of both compartments. Spontaneous initial shuttles from one compartment to the other were counted during the first 2 min by red light beams at the bottom of each of the two divisions. Performance was analyzed according to the behavior during 30 shuttle escape trials. Each trial started with a light stimulus of 5 s, announcing a subsequent footshock of maximum 10 s duration. The intertrial interval was 30 s. The following behavioral reactions were defined as follows: "avoidance" as adequate reaction to the light stimulus by changing to the other compartment immediately, "escapes" as shuttling to the other section in reaction to the electric shock, and "failures" when no attempt to escape was made. For determination of the activity during the intervals, shuttles in between the trials were recorded. Total time of testing for helplessness was ~20 min, with the exact time period depending on the animal's ability to learn the paradigm. To exclude pain sensitivity as a confounding factor, all mice were tested on the hot plate (ATLab, Vendargues, France) at a temperature of 52°C for 45 s. Latency to first reaction (i.e., licking hind paws or jumping) was assessed ($GR^{+/-}$, $n = 28$; wild type, $n = 28$) (YGR, $n = 13$; wild type, $n = 11$).

Neuroendocrinological experiments

Basal corticosterone levels. To determine the circadian secretion of corticosterone, mice were decapitated at respective time points of the active (dark) and inactive (light) phase ($GR^{+/-}$, $n = 6$; wild type, $n = 6$) (YGR, $n = 6$; wild type, $n = 6$) (for each time point).

Corticosterone levels after restraint stress. Mice were restrained for a period of 30 min during the light phase in plastic tunnel. Forty and 60 min after termination of the immobilization stress, plasma corticosterone levels were assessed ($GR^{+/-}$, $n = 6$; wild type, $n = 6$) (YGR, $n = 6$; wild type, $n = 6$) (for each time point).

Dexamethasone suppression test. The mice received a intraperitoneal injection of dexamethasone (3 μg/100 g body weight; Sigma, Schnelldorf, Germany) 6 h before decapitation and determination of corticosterone suppression ($GR^{+/-}$, $n = 12$; wild type, $n = 11$) (YGR, $n = 11$; wild type, $n = 12$).

Dex/CRH test. Six hours after application of dexamethasone, the mice were injected intraperitoneally with 5 μg of CRH (Ferring, Kiel, Germany) and killed 30 min later ($GR^{+/-}$, $n = 8$; wild type, $n = 8$) (YGR, $n = 8$; wild type, $n = 8$). All corticosterone levels were analyzed by a commercial radioimmunoassay (ICN Biomedicals, Eschwege, Germany) as described previously (Zörner et al., 2003).

Determination of neurotrophin levels

After decapitation, the hippocampus was dissected and frozen on dry ice ($n = 8$–12 per genotype). Each specimen was homogenized by ultrasonication in 10–20 vol of lysing buffer containing 0.1 M Tris-HCl, pH 7.0, 0.4 M NaCl, 0.1% NaN_3, and a variety of protease inhibitors. BDNF protein levels were measured in the homogenates using commercial ELISA kits in principle according to the manufacturer's instructions (Promega) but adapted to the fluorometric technique also used for NGF determination as described in detail previously (Hellweg et al., 2003). The detection limit of the assay was 1 pg/ml. Similarly, NGF levels were determined by a fluorometric two-site ELISA, also described previously (Hellweg et al., 2003). The detection limit of this assay was 0.25 pg/ml.

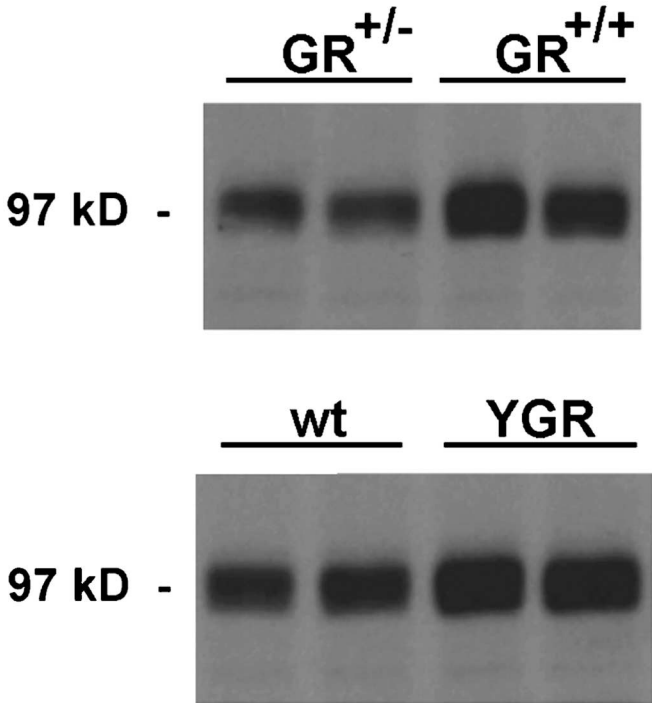

Figure 1. Expression levels of GR protein in $GR^{+/-}$ and YGR mice in the hippocampus. Western blot analyses show a marked downregulation of GR in $GR^{+/-}$ mice compared with wild-type littermates (top gel; each lane represents a hippocampal protein extract from a different animal). Conversely, YGR mice exhibit a distinct GR upregulation compared with their control littermates (bottom gel). wt, Wild type.

Statistics

Statistical analysis was performed in the XLStat statistics program (XLStat, version 7.5; Addinsoft, Brooklyn, NY). Intergroup comparisons were calculated by one-way ANOVAs. For analysis of the learned helplessness parameters, a Mann–Whitney *U* test was performed because samples are not normally distributed. Correlations were calculated with the Spearman method.

Results

GR expression levels in $GR^{+/-}$ and YGR mice

Loss of one functional GR allele in $GR^{+/-}$ mice led to a decrease in the 97 kDa GR band in Western blots of hippocampal protein extracts (Fig. 1). In contrast, doubling of the GR gene dosage by transfer of a yeast artificial chromosome containing two copies of the entire GR gene plus extensive flanking sequences caused the expected increase of GR (Fig. 1). Altered GR expression was also evident at the mRNA level. Real-time RT-PCR experiments demonstrated a downregulation in $GR^{+/-}$ mice to 33% of control littermates ($\Delta\Delta-CT$, +1.60; $p < 0.05$) and an upregulation in YGR mice to 219% ($\Delta\Delta-CT$, −1.13; $p < 0.05$).

$GR^{+/-}$ mice have normal basal but increased stress-induced corticosterone levels

To investigate the effects of reduced GR expression on the regulation of the HPA system, corticosterone levels of $GR^{+/-}$ mice and wild-type littermate controls were analyzed by a radioimmunoassay. Under basal, unstressed conditions, no differences were observed between $GR^{+/-}$ and control mice, neither during the dark phase nor during the light phase of the circadian rhythm (Fig. 2*a*). Both genotypes showed about three times higher levels of corticosterone in the dark than in the light phase (Fig. 2*a*). Because the HPA system is more active under stressful than under

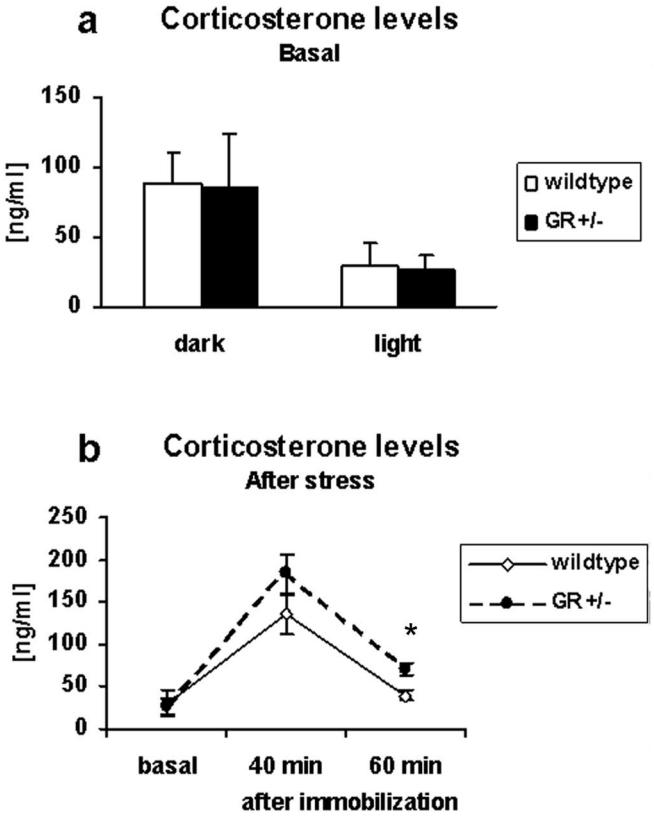

Figure 2. Corticosterone levels in GR $^{+/-}$ mice under basal and stressful conditions. ***a***, Plasma corticosterone levels are unchanged in GR $^{+/-}$ mice compared with wild-type mice, both during the animals' active (dark) and inactive (light) phase. ***b***, After stress exposure, however, corticosterone levels are higher in GR $^{+/-}$ mice at 40 and 60 min (*$p < 0.05$) after immobilization.

basal conditions, we also analyzed corticosterone levels after 30 min of restraint stress. Immobilization caused a strong increase of corticosterone levels in both genotypes, but levels were higher in GR $^{+/-}$ mice when measured at 40 and 60 min ($p < 0.05$) after restraint stress (Fig. 2*b*). These data indicate a disturbed feedback control of the HPA system in GR $^{+/-}$ mice under stress.

GR $^{+/-}$ mice display normal behavior under basal conditions but increased helplessness after stress

To analyze whether the reduced GR expression also influences the animals' behavior, a series of behavioral tests was performed. GR $^{+/-}$ mice exhibited normal behavior under basal conditions and were indistinguishable from their wild-type littermates in the open-field test and the novel-cage test (data not shown). These two tests evaluate various aspects of locomotor and explorative behavior. GR $^{+/-}$ mice also showed unaltered anxiety-related behavior in two tests based on an approach–avoidance conflict (i.e., the elevated O-maze and the dark–light box paradigm) (data not shown). In both tests, GR-heterozygous and wild-type mice exhibited similar scores in visiting the aversive, anxiety-related compartments of the maze, respectively. In addition, no differences between the two genotypes were observed in fear-conditioning experiments that tested both context- and cue-dependent fear-associated learning (data not shown). GR $^{+/-}$ mice also demonstrated normal scores in the Porsolt forced-swim test (data not shown). In this assay, the latency to start floating and the total time spent immobile are regarded as correlate of "despair-like behavior" (Cryan et al., 2002).

The learned-helplessness paradigm evaluates the coping capabilities of mice in an aversive test situation after 2 d of intense stress evoked by exposure to a series of unpredictable and uncontrollable foot shocks. When tested for helpless behavior, GR $^{+/-}$ mice displayed significantly increased escape latencies ($p < 0.05$) (Fig. 3*a*) and a higher number of escape failures ($p < 0.05$) (Fig. 3*b*). When mice were scored individually (Fig. 3*c*), the most helpless individuals were exclusively GR $^{+/-}$ (GR $^{+/-}$, $r = 0.984$; wild type, $r = 0.929$, $\alpha = 0.05$). These results reflect true coping deficits because they were not caused by an altered pain sensitivity in a hot-plate test (data not shown) or general changes in activity.

GR $^{+/-}$ mice exhibit a depression-like Dex/CRH test

Because coping deficits in the learned-helplessness paradigm have been postulated to reflect depression-like behavior, we subjected the animals to two clinically established neuroendocrinological tests for a depressive state, the dexamethasone suppression test and the combined Dex/CRH test. Six hours after injection of dexamethasone, GR $^{+/-}$ mice exhibited significantly higher corticosterone levels than wild-type controls ($p < 0.01$) (Fig. 4*a*). In the combined Dex/CRH test, GR $^{+/-}$ mice showed also significantly higher corticosterone levels in response to the CRH challenge than the control littermates ($p < 0.001$) (Fig. 4*b*). Similar changes are typical in patients with severe depressive episodes.

YGR mice have a stress-resistant HPA axis

After the observation that loss of one copy of the GR leads to a "depression-like syndrome," we tried to confirm the specificity of these findings by analyzing mice that overexpress GR. These YGR mice possess and express two additional copies of the GR gene. We therefore asked whether these mice accordingly have a lower sensitivity to stress-induced behavioral and endocrinological alterations. Similar to GR $^{+/-}$ mice, YGR mice had unaltered corticosterone levels under basal conditions (Fig. 5*a*). After 30 min of restraint stress, YGR mice exhibited significantly decreased peak levels of corticosterone at 40 min after stress exposure ($p < 0.01$), whereas at 60 min, no difference was observed any longer (Fig. 5*a*). In the dexamethasone suppression test, corticosterone levels in YGR mice were significantly more suppressed than in wild-type controls ($p < 0.01$) (Fig. 5*b*). In the combined Dex/CRH test, corticosterone levels were lower in YGR mice than in control littermates, but this difference did not reach statistical significance ($p = 0.07$) (Fig. 5*c*). In summary, YGR mice demonstrated exactly the opposite changes in HPA system (dys)regulation as GR $^{+/-}$ mice.

YGR mice are more resistant to develop helplessness

Finally, we intended to investigate whether increased levels of GR would also lead to altered behavior. Similar to GR $^{+/-}$ mice, YGR mice did not show any phenotype in the test battery for baseline behavior (open-field, novel-cage, O-maze, dark–light box, fear-conditioning, Porsolt test) (data not shown). Again, significant differences were restricted to the learned-helplessness paradigm. In this test, YGR mice displayed significantly lower escape latencies ($p < 0.05$) (Fig. 6*a*) and, correlating to that, lower numbers of escape failures (YGR, $r = 0.898$; wild type, $r = 0.997$, $\alpha = 0.05$) (Fig. 6*c*) compared with their littermate controls ($p < 0.05$) (Fig. 6*b*). Again, altered pain sensitivity could be excluded to account for these differences, because both groups showed similar results in the hot-plate test (data not shown). Thus, whereas GR $^{+/-}$ mice were more prone to develop depression-like coping deficits, YGR mice were more resistant to develop helplessness.

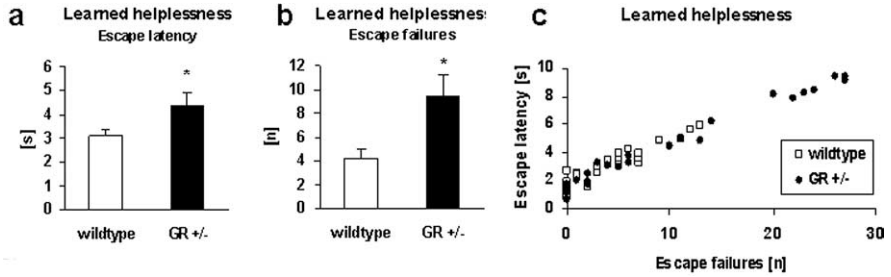

Figure 3. GR $^{+/-}$ mice display increased helplessness in a shuttle box test after exposure to inescapable footshocks on the 2 d preceding the test. ***a***, GR $^{+/-}$ mice exhibit significantly increased escape latencies compared with their wild-type littermates (*$p < 0.05$). ***b***, GR $^{+/-}$ mice also show a higher number of escape failures (*$p < 0.05$). ***c***, Latencies and numbers of failures are highly correlated when plotted for individual mutant and wild-type mice, demonstrating that the subjects with the worst coping (in the top right area of the graph) are all GR $^{+/-}$ mice.

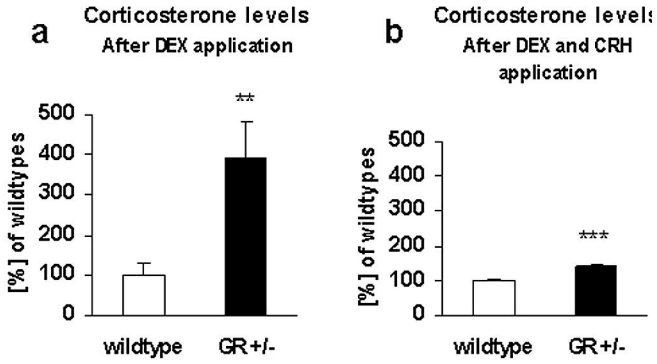

Figure 4. GR $^{+/-}$ mice are nonsuppressors in the Dex/CRH test. ***a***, After administration of dexamethasone, wild-type mice show decreased corticosterone levels, whereas GR $^{+/-}$ mice exhibit nonsuppression (**$p < 0.01$). ***b***, In the combined Dex/CRH test, corticosterone levels are significantly elevated in GR $^{+/-}$ mice compared with the levels of wild-type mice (***$p < 0.001$).

GR $^{+/-}$ and YGR mice show altered levels of hippocampal BDNF expression

Because downregulation of BDNF has been postulated to play a critical role in the pathogenesis of depression disorders (Duman et al., 1997; Altar, 1999; Wong and Licinio, 2001; Nestler et al., 2002; Cryan and Mombereau, 2004), we analyzed the levels of BDNF protein in the hippocampus by ELISA. GR $^{+/-}$ mice exhibited a significant downregulation of BDNF to 56.9% of controls, whereas YGR mice revealed a significant upregulation of 51.7% (Fig. 7). In contrast, the expression levels of NGF protein, another key member of the neurotrophin family, were unaltered in both GR mutated strains, indicating the specificity of the results obtained for BDNF (Fig. 7).

Discussion

The present study analyzed the effects of reduced and increased expression of GR on behavioral responses and the HPA system in mice. On both levels, alterations became evident only after a stressful challenge, whereas none of the strains showed a phenotype under basal conditions in a test battery for locomotor, exploratory, anxiety-related, and emotional behavior. GR $^{+/-}$ mice exhibited a depression-like phenotype (i.e., increased helplessness after challenge by uncontrollable footshocks). GR-overexpressing mice, conversely, revealed a resistance to develop helpless behavior. Whereas GR $^{+/-}$ mice demonstrated a disinhibited HPA axis after stress exposure and a depression-like Dex/CRH test (as the consequence of altered feedback regulation attributable to a reduced GR gene dosage at the level of the paraventricular nucleus and the anterior pituitary), YGR mice had a stress- and challenge-resistant HPA system.

Mice underexpressing GR have a predisposition for depression

The learned-helplessness model represents a rodent depression model with good face and construct validity (Vollmayr and Henn, 2001; Shirayama et al., 2002). It is based on the concept that depressed individuals show a loss of coping strategies in aversive environmental situations (Shumake and Gonzalez-Lima, 2003). In the present study, GR $^{+/-}$ mice demonstrated significantly prolonged escape latencies and increased failures in the active avoidance task of the learned helplessness paradigm after two sessions of footshock exposure. The impaired coping behavior strongly indicates the development of depression-like behavior in GR $^{+/-}$ mice.

This altered behavioral reactivity to stress is accompanied by depression-like alterations of the HPA system. Similar to the behavioral level, changes of the HPA axis are not present under basal conditions in GR $^{+/-}$ mice, but a significant disinhibition occurs after stress. Furthermore, GR $^{+/-}$ mice exhibit a pathological Dex/CRH test, currently the most relevant biological marker in patients for both florid depression and the risk to develop a depressive episode. This test combines the suppressive effect of dexamethasone with the stimulatory potency of CRH (Heuser et al., 1994). In 70–80% of severely depressed patients, the CRH-elicited ACTH and cortisol response is blunted, and dexamethasone pretreatment does not elicit a suppressive effect and paradoxically induces enhanced cortisol levels after a CRH challenge. In healthy individuals, in contrast, the Dex/CRH test creates the situation of a pharmacological (partial and transient) adrenalectomy in which hypothalamic CRH expression is increased because of a decrease in plasma cortisol (Holsboer, 2000). Similar to depressed patients, GR $^{+/-}$ mice display a characteristic nonsuppression after dexamethasone treatment and the paradoxical increase of corticosterone levels after combined Dex/CHR application.

While preparing this manuscript, a depression-like phenotype similar to that of GR $^{+/-}$ mice was described in mice with a forebrain-specific GR knock-out (Boyle et al., 2005). These animals also exhibit impaired negative-feedback regulation of the HPA axis as well as increased depression-like behavior. These findings indicate that crucial brain regions responsible for the phenotype of GR $^{+/-}$ mice may be located in the forebrain.

Normal baseline behavior and neuroendocrinology of GR mutant mice may reflect developmental compensation or genetic background effects

Mice with a forebrain-specific complete GR knock-out, induced by the calcium-calmodulin-dependent protein kinase II (CaMKII) promoter, exhibit at 3 weeks of age a depression-like phenotype already under basal (unstressed) conditions (Boyle et al., 2005). Our results show that GR underexpression and overexpression from early development on does not lead to an overt phenotype under baseline conditions with low levels of stress. This could be attributable to a mere gene dosage effect. Furthermore, a complete GR knock-out in some brain areas with preserved GR levels in other regions may result in a different pheno-

type than a more subtle and homogenous GR reduction throughout the brain. However, the lack of a specific phenotype might also be caused by early ontogenetic compensatory or adaptive processes, possibly reflecting the condition of patients with a high genetic (familial) risk for depression who are initially inconspicuous but develop depressive episodes after stressful life events. The lack of a phenotype under baseline conditions in the GR mutant strains examined here may be also attributable to the distinct genetic background of the animals. In accordance with the recommendations of the Banbury Conference (1997), all mice used in our experiments were F_1 hybrids. This results in a so-called hybrid vigor that can reduce the impact of genetic alterations compared with a mixed or inbred genetic background (Wolfer et al., 2002). However, the detection of phenotypic alterations or pathologies in mice on a hybrid background (in our GR mutant mice, altered stress sensitivity) implies that the consequences of the underlying mutations are robust and reliable. These considerations regarding the behavioral findings can also be used with physiological systems such as the HPA axis. Despite their altered GR expression, GR mutant mice show normal baseline corticosterone levels during circadian cycle. After immobilization stress, however, significant changes in the response of the HPA system are present in GR $^{+/-}$ as well as in YGR mice.

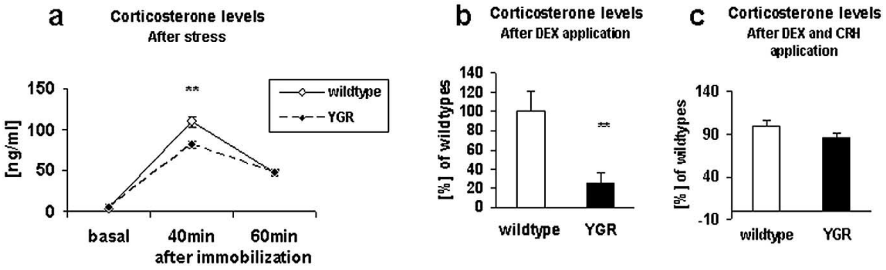

Figure 5. YGR mice have a stress-resistant HPA system and are oversuppressors in the Dex test. ***a***, After stress exposure, corticosterone levels are significantly lower in YGR mice at 40 min after immobilization than in wild-type controls (**$p < 0.05$). ***b***, After administration of dexamethasone, YGR mice show a significant oversuppression of corticosterone levels compared with wild-type mice (**$p < 0.01$). ***c***, In the combined Dex/CRH test, corticosterone levels are lower in YGR mice compared with the levels of wild-type mice ($p = 0.07$).

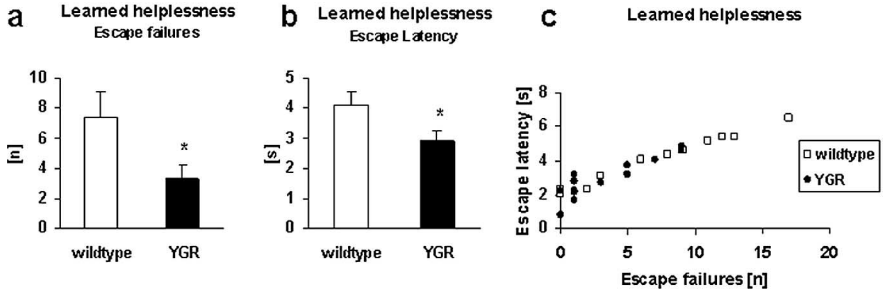

Figure 6. YGR mice are more resistant in the learned-helplessness paradigm. ***a***, YGR mice exhibit significantly decreased escape failures compared with their wild-type littermates (*$p < 0.05$). ***b***, YGR mice also show a reduced number of escape latencies (*$p < 0.05$). ***c***, Latencies and numbers of failures are highly correlated when plotted for individual mutant and wild-type mice, demonstrating worse coping (in the top right area of the graph) in wild-type mice, whereas YGR mice cluster in the bottom left area of the graph.

Mice overexpressing GR are stress resistant

An indirect genetic approach to prove the specificity of the depression-like alterations of GR $^{+/-}$ mice seemed to be the behavioral and neuroendocrinological examination of mice overexpressing GR. In these mice, one would expect the opposite phenotype of GR $^{+/-}$ mice. Indeed, YGR mice carrying four copies of the GR gene demonstrate a stress resistance on both the behavioral and the hormonal levels. These animals exhibit less helplessness, lower corticosterone levels after immobilization, and oversuppression after dexamethasone treatment. This is in agreement with the predictions of our hypothesis.

Recently, it was reported that mice overexpressing GR specifically in the forebrain display increased emotional lability in the Porsolt forced-swim test indicated by increased baseline immobility (Wei et al., 2004). Furthermore, these animals exhibited increased anxiety-like behavior in the elevated plus-maze and in the dark–light box (Wei et al., 2004). This was not observed in our YGR mice that express GR under control of its own regulatory sequences. These contradictory results could be explained by the late onset and forebrain-restricted mutation induced by the CaMKII promoter used in the former study (Wei et al., 2004). Conflicting results also come from the human field (van Rossum and Lamberts, 2004; van Rossum et al., 2004). Although enhanced glucocorticoid feedback has always been considered positive for better limiting the stress response, it is important to remember that overactive GR may transduce a stronger glucocorticoid signal (i.e., may produce effects of glucocorticoid excess). In a large elderly Dutch (Rotterdam) population cohort, two different polymorphisms in GR have been found, one that enhances sensitivity to dexamethasone and another that confers resistance to dexamethasone (van Rossum and Lamberts, 2004; van Rossum et al., 2004). However, the group with increased sensitivity to dexamethasone (and lower basal cortisol) shows a glucocorticoid excess phenotype with greater incidence of dementia and reduced overall survival (van Rossum and Lamberts, 2004; van Rossum et al., 2004). Thus, our understanding of these animal models needs to examine additional possibilities of what reduced or increased glucocorticoid signaling entails beyond HPA feedback or learned-helplessness models.

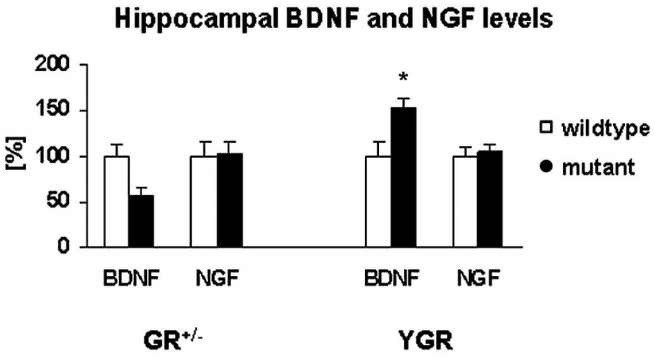

Figure 7. BDNF protein levels in the hippocampus are significantly diminished in GR $^{+/-}$ mice ($p < 0.05$) and significantly increased in YGR mice (*$p < 0.05$). In contrast, the expression levels of NGF are unaltered in both mutant strains.

GR mutant mice may represent a tool to study molecular correlates of depression-like behavior

The results in $GR^{+/-}$ mice resemble the human situation in depressive disorders, in which individuals at risk are predisposed to develop depressive episodes after stress. In this respect, $GR^{+/-}$ mice differ from mice with a forebrain-specific complete GR knock-out (Boyle et al., 2005), which already demonstrate depression-like behavioral and neurochemical alterations under baseline conditions. However, the findings in both strains indicate that compromised GR function constitutes a crucial molecular risk factor in the pathophysiology of depression. Therefore, we suggest that the mouse lines with altered GR expression are suitable tools for additional physiological, biochemical, and pharmacological investigations of GR function with regard to depressive disorders. These analyses are also of clinical relevance, because in preliminary studies, GR antagonists have been of value in the treatment of severe depressive episodes with psychotic features (Belanoff et al., 2002).

Recently, the so-called neurotrophin hypothesis of depression postulated that a downregulation of BDNF is crucial for the pathogenesis of depression in humans and rodents (Smith et al., 1995; Duman et al., 1997; Wong and Licinio, 2001; Nestler et al., 2002; Cryan and Mombereau, 2004; Lang et al., 2004). A stress- or corticosterone-induced BDNF downregulation in the hippocampus can be partially blocked by adrenalectomy, or can be reversed by antidepressant therapy (Nibuya et al., 1995; Smith et al., 1995; Schaaf et al., 1997; Chao et al., 1998). In accordance with this hypothesis, $GR^{+/-}$ mice, which have a predisposition to stress-induced depression-like behavior, show a significant downregulation of BDNF in the hippocampus, whereas YGR mice exhibit a significant BDNF upregulation. Indeed, this is the first experimental evidence that compromised GR function concurrently evokes a BDNF dysregulation and a predisposition to depressive behavior. In future experiments, we will investigate in these strains additional mechanisms of neural plasticity thought to be involved in the molecular and cellular pathogenesis of depression (e.g., monoaminergic systems, arginine vasopressin, and neurogenesis). In addition to the potential involvement of neurotrophins, respective experiments could also reveal a link between these systems, GR function, and depression-like behavior.

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