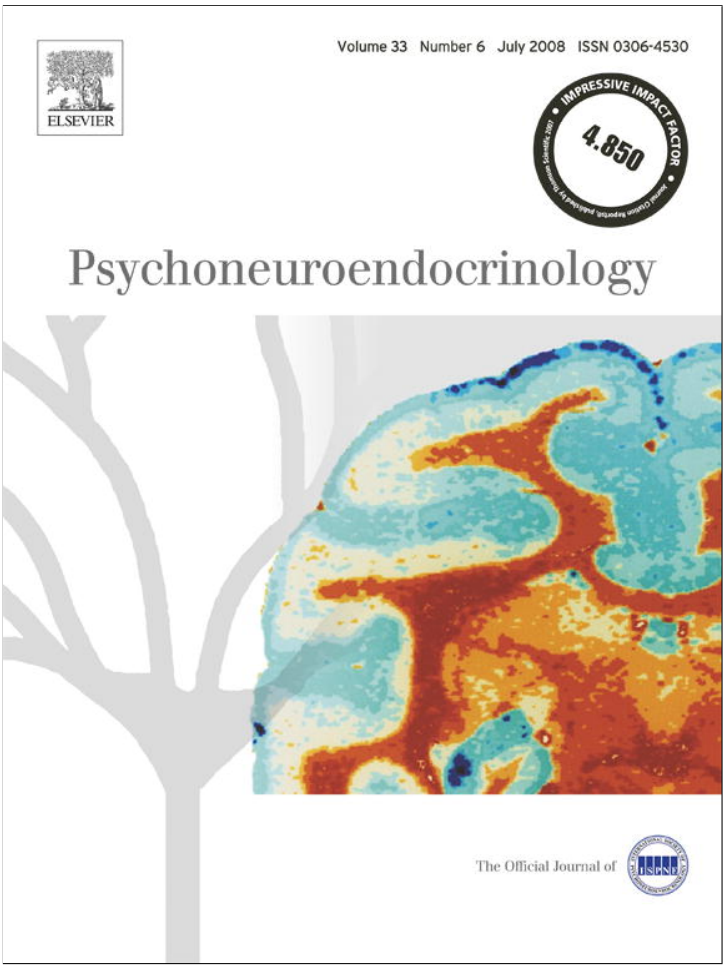

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Mice selected for high *versus* low stress reactivity: A new animal model for affective disorders

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Summary
Affective disorders such as major depression are among the most prevalent and costly diseases of the central nervous system, but the underlying mechanisms are still poorly understood. In recent years, it has become evident that alterations of the stress hormone system, in particular dysfunctions (hyper- or hypo-activity) of the hypothalamic–pituitary–adrenal (HPA) axis, play a prominent role in the development of major depressive disorders. Therefore, we aimed to generate a new animal model comprising these neuroendocrine core symptoms in order to unravel parameters underlying increased or decreased stress reactivity.

Starting from a population of outbred mice (parental generation: 100 males and 100 females of the CD-1 strain), two breeding lines were established according to the outcome of a 'stress reactivity test' (SRT), consisting of a 15-min restraint period and tail blood samplings immediately before and after exposure to the stressor. Mice showing a very high or a very low secretion of corticosterone in the SRT, i.e. animals expressing a hyper- or a hypo-reactivity of the HPA axis, were selected for the 'high reactivity' (HR) and the 'low reactivity' (LR) breeding line, respectively. Additionally, a third breeding line was established consisting of animals with an 'intermediate reactivity' (IR) in the SRT.

Already in the first generation, i.e. animals derived from breeding pairs selected from the parental generation, significant differences in the reactivity of the HPA axis between HR, IR, and LR mice were observed. Moreover, these differences were found across all subsequent generations and could be increased by selective breeding, which indicates a genetic basis of the respective phenotype. Repeated testing of individuals in the SRT furthermore proved that the observed differences in stress responsiveness are present already early in life and can be regarded as a robust genetic predisposition.

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Tests investigating the animal's emotionality including anxiety-related behavior, exploratory drive, locomotor activity, and depression-like behavior point to phenotypic similarities with behavioral changes observed in depressive patients. In general, HR males and females were 'hyperactive' in some behavioral paradigms, resembling symptoms of restlessness and agitation often seen in melancholic depression. LR mice, on the other hand, showed more passive-aggressive coping styles, corresponding to signs of retardation and retreat observed in atypical depression.
Several morphometric and neuroendocrine findings further support this view. For example, monitoring the circadian rhythm of glucocorticoid secretion revealed clearly increased trough levels in HR mice, resulting in a flattened diurnal rhythm, again adding to the neuroendocrine similarities to patients suffering from melancholic depression.
Taken together, our results suggest that distinct mechanisms influencing the function and regulation of the HPA axis are involved in the respective behavioral and neurobiological endophenotypes. Thus, the generated HR/IR/LR mouse lines can be a valuable model to elucidate molecular genetic, neuroendocrine, and behavioral parameters associated with altered stress reactivity, thereby improving our understanding of affective disorders, presumably including the symptomatology and pathophysiology of specific subtypes of major depression.

1. Introduction

Stress-related disorders such as major depression (MD) are among the most prevalent and costly diseases of the central nervous system. About 240 million people are affected worldwide, reflecting a high lifetime prevalence of about 17% (Wong and Licinio, 2001; Nestler et al., 2002; Kessler et al., 2003). In general, mood disorders are a major cause of morbidity as they show a high rate of recurrence ($>50\%$) and the duration of a depressive episode can be more than 2 years in about 30% of the patients, also involving a high risk for suicide (Wong and Licinio, 2001; Nestler et al., 2002; Kessler et al., 2003). Apart from the tremendous suffering of the affected individuals and their families, depressive disorders also cause a high economic burden (year 2000 estimated total costs of MD in the USA: 83.1 billion $ Greenberg et al., 2003). Furthermore, a considerable comorbidity with other psychiatric disorders (e.g. anxiety and substance abuse) is observed and MD has been identified as a risk factor for other clinical conditions such as obesity, cardiovascular, and neurodegenerative disorders (Kessler et al., 2003; Rumsfeld and Ho, 2005; Swaab et al., 2005; Bornstein et al., 2006).

Clinical features of MD encompass mood changes, vegetative dysfunctions, motor deficits, and cognitive impairments. According to current diagnostic algorithms (DSM-IV), patients attributed to major depression have depressed mood, diminished interest or pleasure in enjoyable activities/stimuli (e.g. sex, food, social interaction), disturbed sleep (insomnia or hypersomnia), decreased or increased appetite, significant changes in weight, psychomotor agitation or retardation, cognitive impairments, feelings of worthlessness and inappropriate guilt, and often recurrent suicidal ideation. Thus, the current diagnosis of MD is, in contrast to other diseases, not based on objectifiable signs or diagnostic markers and includes a highly variable, sometimes even contrasting set of symptoms. Therefore, attempts have been made to define 'subtypes' of depression with potentially distinct pathophysiology and response to treatment. Two quite common and clinically important subtypes of MD are the 'melancholic' (also termed 'endogenous' or 'typical') and the 'atypical' form of depression. Melancholia is associated with non-reactive mood, anxiety, insomnia (including early morning awakening), loss of appetite and weight, as well as marked psychomotor changes, involving hyperarousal and agitation (Gold and Chrousos, 2002; Nestler et al., 2002; Hasler et al., 2004a; Antonijevic, 2006). Atypical depression seems to be distinctly different, as it is characterized by reactive/labile mood, hypersomnia (oversleeping), increased appetite and weight gain, as well as lethargy and fatigue (Nierenberg et al., 1998; Angst et al., 2002; Gold and Chrousos, 2002; Nestler et al., 2002; Antonijevic, 2006).

In recent years, it has become evident that pathological alterations in the stress hormone systems play a major role in the development of depressive disorders. The activity of the sympathetic nervous system as well as the hypothalamic–pituitary–adrenal (HPA) axis is dysregulated in depressed patients and restored by successful antidepressant treatment during remission (findings of human and animal studies including discussions on potential mechanisms are reviewed in: Holsboer, 2000; Wong and Licinio, 2001; Gold and Chrousos, 2002; Nestler et al., 2002; Hasler et al., 2004a; Cryan and Holmes, 2005; de Kloet et al., 2005; Bale, 2006; Müller and Holsboer, 2006).

In particular, dysfunctions (hyper- or hypo-activity) of the HPA axis in patients suffering from mood disorders are a firmly established finding in biological psychiatry. About 50% of patients suffering from MD (mainly of the melancholic subtype) present a hyperactive HPA system resulting in hypercortisolism (see reviews cited above). The production and secretion of glucocorticoids (mainly cortisol in humans and corticosterone in murine rodents) from the adrenal cortex is regulated by the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which, in turn, is stimulated by corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) derived from parvocellular neurons of the paraventricular nucleus (PVN) of the

hypothalamus (Herman and Cullinan, 1997; Sapolsky et al., 2000, Engelmann et al., 2004; Aguilera et al., 2007). In these MD patients, the expression of CRH and AVP in the PVN is enhanced, increased CRH levels are found in the cerebrospinal fluid (CSF), and the ACTH secretory response is blunted, pointing to elevated endogenous release of CRH. As a consequence, the adrenals show hypertrophy, and basal corticosteroid and ACTH levels are elevated (see reviews cited above). In response to stress, their HPA axis is persistently activated and when challenged in different functional tests, it shows feedback resistance at the level of the PVN and pituitary (see reviews cited above). In contrast, patients suffering from atypical depression show virtually the opposite neuroendocrine phenotype. Their HPA axis activity is largely reduced, resulting in hypocortisolism presumably due to a central CRH deficiency mediated by an enhanced negative feedback of cortisol (Nierenberg et al., 1998; Gold and Chrousos, 2002; Antonijevic, 2006). A crucial involvement of HPA axis hyper- and hypo-function in affective disorders is also indicated by other clinical observations. For example, patients with Cushing's disease whose adrenals produce excessive cortisol frequently suffer from depression and overall psychopathology decreases significantly after correction of hypercortisolism (Fava et al., 1987; Sonino et al., 1998). Furthermore, patients with Addison's disease have adrenal glands that produce insufficient amounts of cortisol and depressive symptoms occur in this disorder as well (Fava et al., 1987; Thomsen et al., 2006). Thus, an inverted U-shaped function for the effects of glucocorticoid concentrations on mood is suggested.

There is also other compelling evidence for the involvement of HPA axis abnormalities in MD and these changes in HPA axis activity appear to be state-dependent; that is, they normalize prior to the elevation of mood and have been proposed as biomarkers predicting the relapse and remission of depressive symptoms (Zobel et al., 2001; de Kloet et al., 2005; Ising et al., 2007). Some studies also present evidence that feedback resistance and mild hypercortisolism are already present in healthy subjects at genetic risk for depression, suggesting an imbalance in drive and feedback inhibition of the HPA axis preceding the clinical manifestation of the disorder (Holsboer et al., 1995; Modell et al., 1998; Ising et al., 2005). Although some advances unraveling the genetic underpinnings and cellular neurobiology of MD could be made (Manji et al., 2001; Nestler et al., 2002; de Kloet et al., 2005; Levinson, 2006), the etiology, risk factors and underlying mechanisms (including genetic predisposition) of affective disorders are still poorly understood. Moreover, it is becoming increasingly clear that these disorders arise from a combination of environmental and genetic factors, which interact in a highly complex manner to affect the risk of developing MD (Holsboer, 2000; Lesch, 2004; Caspi and Moffitt, 2006; Kas et al., 2007; Mill and Petronis, 2007; Tsankova et al., 2007).

In order to understand the biological mechanisms underlying a complex disorder, valid animal models are indispensable and in the past there has been a surge in the use of mice in biomedical and neuropsychiatric research. Although it is impossible to recapitulate all aspects of a complex human disease such as MD in a mouse, mice genetically engineered or selectively bred to model-specific key symptoms prevalent in human depression can be successfully employed to discover neurobiological endophenotypes[1] bridging the gap between behavioral phenotype and genotype (Tecott, 2003; Hasler et al., 2004a; Cryan and Holmes, 2005; Urani et al., 2005; Müller and Holsboer, 2006; Jacobson and Cryan, 2007; Kas et al., 2007). Thus, focusing on individual endpoints of the disease, rather than the entire syndrome (endophenotype-based approach), can yield valuable insights into the genetic/epigenetic and neurobiological underpinnings of MD.

As an alternative to transgenic (overexpression or knockout) mice carrying specific mutations modulating for example the monoaminergic system or the HPA axis, selective breeding has proved to be a powerful strategy to unravel the genetic basis of disorders, providing unique information about pleiotropy,[2] epistasis,[3] and gene-by-environment interaction (Phillips et al., 2002; Swallow and Garland, 2005). The breeding program usually begins with evaluating the trait of interest in a genetically heterogeneous population, e.g. a commercially available outbred strain of mice. Individuals with responses at either extreme of the response curve are then selectively bred together for their opposing trait phenotypes over multiple generations. Thus, heritability characteristics of the trait can be evaluated and later generations of these inbred lines can be examined for underlying neurobiology and polygenetic or pleiotropic correlates of the trait of interest. Successfully established examples of this classical genetic approach include mice and rats selected for extremes in anxiety-related behavior (Krömer et al., 2005; Landgraf et al., 2007), helplessness/avoidance/depression-like behavior (Scott et al., 1996; Weiss et al., 1998; El Yacoubi et al., 2003; Steimer and Driscoll, 2003; Will et al., 2003; Henn and Vollmayer, 2005; Weiss et al., 2008), contextual fear conditioning (Ponder et al., 2007), aggressiveness (Lagerspetz, et al., 1968; Veenema et al., 2003; Gammie et al., 2006), novelty-seeking behavior (Stead et al., 2006), and nest building behavior (Lynch, 1980). Similarly, other studies selected animals for differences in glucocorticoid secretion in response to stressors or addressed the genetic linkage between behavioral and neuroendocrine stress response traits (domestic turkey: Brown and Nestor, 1973; domestic chicken: Edens and Siegel, 1975; Japanese quail: Satterlee and Johnson, 1988; Satterlee and Marin, 2006; rainbow trout: Pottinger and Carrick, 1999; zebra finch: Evans et al., 2006; domestic pig: Désautés et al., 2002; Kadarmideen and Janss, 2007; laboratory rat: Solberg et al., 2003). Most selection experiments involving rodents, however, focused on behavioral traits. As evidence from human and animal studies reveals a vital link between individual stress sensitivity and the predisposition towards mood disorders (Holsboer, 2000; de Kloet et al., 2005; Bale, 2006), applying selection for HPA axis reactivity might be a promising approach yielding insights into the genetic and mechanistic basis of complex traits underlying MD.

[1]Endophenotypes are defined as quantitative biological traits associated with a complex genetic disorder.

[2]Multiple effects of a single gene.

[3]Interaction of genes residing at different loci (non-allelic interaction).

Therefore, the aim of our study was to generate and validate a new genetic animal model comprising the neuroendocrine core symptom of altered stress reactivity by selectively breeding mice for increased or decreased HPA axis responsiveness. Here, we present findings regarding the activity of the HPA axis in the founder population, outbred CD-1 mice, and the response to selection for high, intermediate, or low stress reactivity. Furthermore, results of an extensive behavioral test battery applied to the selected mouse lines as well as neuroendocrine characterization experiments are presented.

2. Methods

2.1. Animals and general housing conditions

As mentioned above, a selective breeding approach (initiated in January 2005) was utilized to generate mice clearly differing in their reactivity of the HPA axis (for details see Section 2.2). Assuring genetic heterogeneity in the founder population, CD-1 outbred mice (purchased from Charles River Laboratories, Sulzfeld, Germany) were chosen as the background strain for establishing breeding lines with distinct differences in stress reactivity that are not brought about by environmental effects only, but relate to differences at the molecular genetic level, thereby mimicking the interaction of genetic susceptibility and environmental factors involved in the etiology of affective disorders in humans (Wong and Licinio, 2001; Nestler et al., 2002; Lesch, 2004, Levinson, 2006; Kas et al., 2007; Mill and Petronis, 2007).

Details about housing conditions, age, and the number of mice used in each experiment are given in the respective sections (see below). In general, from weaning at the age of about 4 weeks all animals were housed in same-sex groups of two to four mice in transparent polycarbonate cages (standard Macrolon cages type III, $38 \times 22 \times 15\,cm^3$) 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). The animal housing rooms as well as the experimental rooms were maintained under standard laboratory conditions (light–dark cycle: 12:12 h, lights on at 08:00 h; temperature: $22 \pm 1\,^{\circ}C$; relative humidity: $55 \pm 10\%$). Commercial mouse diet (Altromin no. 1324, Altromin GmbH, Lage, Germany) and bottled tap water were available *ad libitum*.

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

2.2. Selective breeding for differences in stress reactivity

Starting from a population of outbred CD-1 mice ('parental generation': 100 males and 100 females), two breeding lines were established by selective and bidirectional breeding according to the outcome of a 'stress reactivity test' (SRT, for details see Section 2.2.1). Mice showing a very high or a very low increase of corticosterone in the SRT, i.e. animals expressing a hyper- or a hypo-reactivity of the HPA axis, were selected for the 'high reactivity' (HR) and the 'low reactivity' (LR) breeding line, respectively (see Figure 1). Additionally, a third breeding line was established consisting of animals with an 'intermediate reactivity' (IR) in the SRT, resembling the mean phenotype of the founder population (see Figure 1).

From the parental generation (PG) eight males and eight females were selected as founder pairs for each breeding line (see Figure 1). Their offspring (generation I, Gen I) and all animals from each of the following generations (Gen II, Gen III, etc.) were also tested in the SRT and selected for breeding in the HR, IR, or LR line according to the criterion of high, intermediate, or low corticosterone increase, respectively. To minimize possible effects of genetic drift, for each line and generation eight to 16 breeding pairs were mated following a strict system of within family selection. This involved mating of more or less closely related individuals and did not exclude sib matings. Litter size was reduced to a maximum of 12 pups (eight males and four females when possible) on postnatal day one to three, ensuring that each family contributed equally to the next generation. Furthermore, additional measures were taken to assure the validity of our breeding approach. Besides establishing an independent 'control' line with an intermediate stress reactivity phenotype (IR mice), an internal replication was included for each of the three breeding lines. That is, every generation of HR, IR, and LR mice (from Gen II) consists of animals of two separate 'sub-lines' (A and B) within each breeding line, descending from two different breeding pairs of the PG, thus, conveying information about the impact of founder effects and genetic drift on our colonies (Henderson, 1997).

Despite increasing homozygosity, there was no evidence for inbreeding depression (i.e. reduction of fertility) in the selected lines. By the seventh generation of selection, no significant differences in breeding success rates and litter sizes were observed between the HR, IR, and LR lines as well as across generations (all $p > 0.05$). Furthermore, there was no indication of changes in general health or well-being of the animals.

2.2.1. Stress reactivity test (SRT)

For testing the reactivity of the HPA axis, we established a so-called SRT, applying a brief period of confinement as standardized, moderate stressor. The SRT is routinely performed at the age of 7–8 weeks and consists of a 15-min restraint period in a 50 ml plastic tube ($11.4 \times 2.8\,cm^2$) with holes for ventilation and an aperture in the cap for the tail. Immediately before and after exposure to the stressor, blood samples (30–70 μl) are collected from the ventral tail vessel (sampling technique according to Dürschlag et al., 1996; with slight modifications) using heparinized capillaries (Product code: 749311, Brand GmbH, Wertheim, Germany). After separation of cellular constituents by centrifugation (5 min, 14,800*g*), plasma was frozen at $-20\,^{\circ}C$ until analysis of corticosterone concentration (see Section 2.5.1).

All blood samplings and the restraint were performed in a separate room adjacent to the animal housing room within a maximum time of 2–3 min from disturbing the animal's cage (all mice in a cage were treated in parallel). In order to

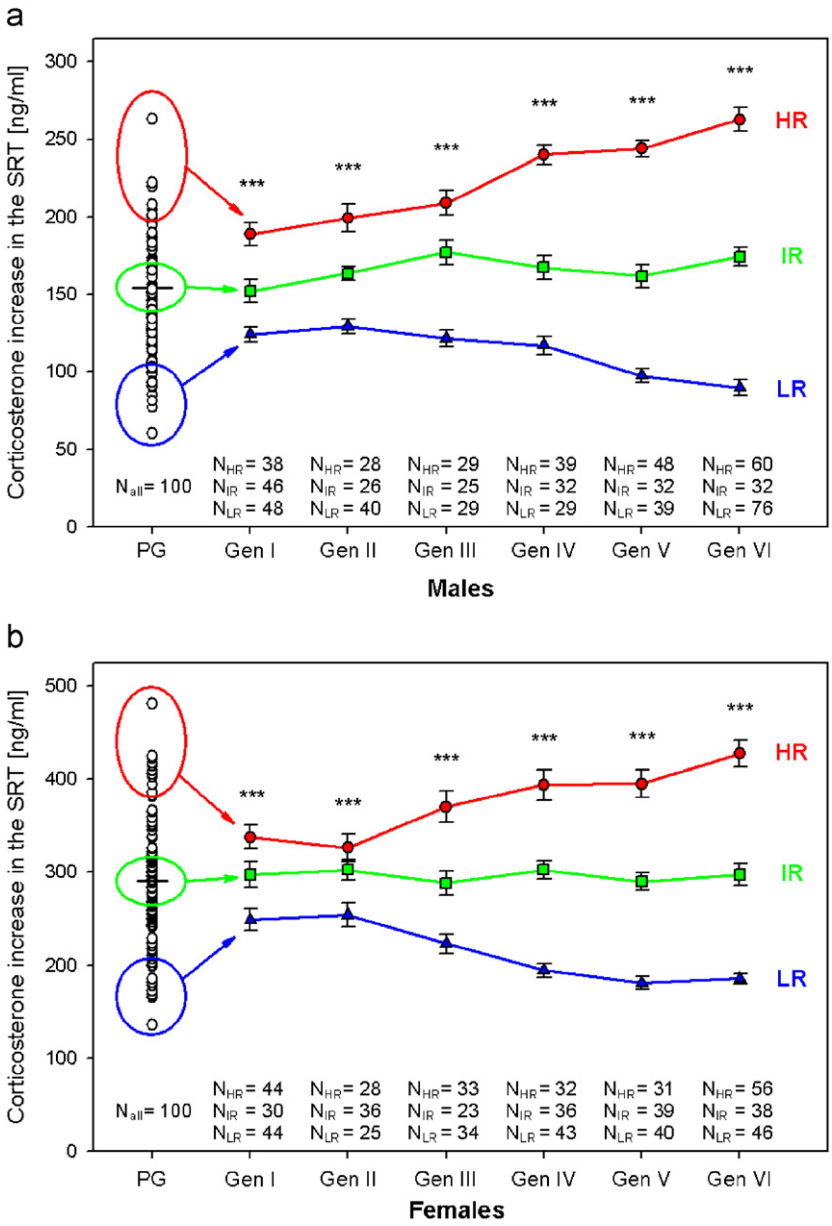

Figure 1 Corticosterone increase in the 'stress reactivity test' (SRT) of male (a) and female (b) CD-1 mice (parental generation, PG) and descendent generations (Gen I–VI) of the high (HR), intermediate (IR), and low (LR) reactivity breeding lines. Data are given as individual values (PG) and means ± SEM (Gen I–VI). Statistically significant differences between the three lines are indicated by asterisks (KWH-tests, all $p < 0.001$***; for details see text).

avoid confounding factors such as additional stress effects, e.g. from activities in the experimental room, mice in the tubes were covered with non-transparent boxes during the restraint period. Furthermore, the SRT was exclusively performed between 09:00h and 11:00h, i.e. in the trough of the circadian rhythm of glucocorticoid secretion (Halberg et al., 1959; Dallman et al., 1978; Kalsbeek et al., 1996; Buijs and Kalsbeek, 2001; Engeland and Arnhold, 2005).

2.3. Behavioral phenotyping

In order to investigate behavioral traits associated with differences in stress reactivity, a number of paradigms were used to characterize the animals' emotionality/coping style, including anxiety-related behavior, exploratory drive, locomotor activity, aggressiveness, and depression-like behavior (Cryan and Holmes, 2005).

At the age of 8–9 weeks, all animals of the PG (100 male and 100 female outbred CD-1 mice) were tested in the elevated plus-maze test (see Section 2.3.1) and 1 week later in the tail-suspension test (see Section 2.3.4), gaining information about the distribution of anxiety-related and depression-like behavior in the founder population.

Moreover, in the third generation (Gen III), selected individuals from the HR/IR/LR breeding lines were subjected to a behavioral test battery assessing different ethological aspects relevant for affective disorders. These animals

(10 males and 10 females of each line) showed clear differences in the SRT (performed about 2 weeks earlier) and were later used to breed the next generation of HR, IR, and LR mice, respectively. The test battery consisted of the elevated plus-maze (EPM) test, the tail-suspension (TST) test, the elevated platform (EPF) test, the open-field test (OF), and the forced-swim test (FST) (see Sections 2.3.1–2.3.5).

All tests were performed in the order listed under standardized conditions (see Section 2.1) between 08:00 h and 12:00 h with an inter-test interval of 48 h, i.e. one day rest between the tests, as recommended for successive behavioral testing (Paylor et al., 2006; see also McIlwain et al., 2001 for effects of test history). The animals' behavior during the tests was videotaped and scored by a trained observer using 'Eventlog' (version 1.0, Emco Software Ltd.) or was automatically analyzed using the 'ANY-maze' video-tracking system (version 4.50, Stoelting Co., Wood Dale, IL, USA).

2.3.1. Elevated plus-maze test

Anxiety-related behavior was measured by means of the EPM test (Pellow et al., 1985; Lister, 1987). This paradigm is based on the 'approach-avoidance' conflict of mice to explore an unfamiliar environment versus avoiding unprotected and highly illuminated areas. The plus-shaped test apparatus was made of dark-gray plastic consisting of two opposing closed/shielded arms ($30 \times 5 \times 15\,cm^3$, dimly lit with about 50 lx) and two open/unprotected arms ($30 \times 5 \times 0.5\,cm^3$, brightly lit with about 300 lx) connected by a central platform ($5 \times 5\,cm^2$, illuminated with about 100 lx). The maze was elevated 40 cm above the floor and each mouse was tested for 5 min on the apparatus. At the beginning of each trial, the animals were placed on the central platform randomly facing one of the closed arms. Each entry into an open or closed arm of the apparatus was counted and the time spent in either type of arm was measured. Mice were considered to have entered an open or closed arm when both front paws and shoulders were on the arm. After each trial, the EPM was thoroughly cleaned with water containing detergents and dried with a tissue to avoid olfactory influences on subsequently tested animals.

2.3.2. Open-field test

The OF test was used to measure locomotor activity and explorative behavior, utilizing the animals' drive to explore unfamiliar environments (Walsh and Cummins, 1976). Each mouse was tested for 5 min in an evenly and dimly lit (about 50 lx) OF consisting of a circular arena (60 cm in diameter, surrounded by 40 cm high walls). The total distance traveled, the number of rearings, and the time the animal spent in the more aversive inner zone (30 cm diameter) or the more protective outer zone near the walls of the OF was monitored. After each test, the apparatus was thoroughly cleaned as described above, in particular in those areas where urine or feces have been voided.

2.3.3. Elevated platform test

Assessing the animals' explorative drive independent of locomotor activity, the EPF test was used. Here, the animals were exposed for 5 min to an inescapable circular platform (10 cm in diameter) that was elevated about 40 cm above the floor and the number of so-called 'head dippings' was counted. Head dippings are defined as exploratory head movements where the animal lowers its head over the edge of the platform at least to the eye level or below. The apparatus was brightly lit (with about 500 lx) and cleaned between consecutive tests as described above.

2.3.4. Tail-suspension test

The TST was used to assess the animals' depression-like behavior and coping styles (Steru et al., 1985). Similar to other tests in depression-related research, the paradigm is based on alterations in stress-induced coping strategies and has shown good predictive validity as an animal test for antidepressant activity (Steru et al., 1985; Cryan and Holmes, 2005; Jacobson and Cryan, 2007). During the TST, mice were suspended for 6 min by their tail (attached about 2 cm from the tail tip with adhesive tape) from a metal bar fixed about 50 cm above the floor and the total duration spent in an immobile body posture was measured ('total time immobility'). Animals that managed to climb up their tails, i.e. escaping the aversive situation of suspension, were excluded from the analysis.

2.3.5. Forced swim test

Similar to the TST, the FST is a frequently used paradigm for measuring depression-like behavior in rodents and its responsiveness to antidepressant treatment has repeatedly been validated (Porsolt et al., 1977a, b; Cryan and Holmes, 2005; Jacobson and Cryan, 2007). The test involves placing each mouse for 6 min in an inescapable 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, the animals intermittently adopt immobile postures interspersed with bouts of swimming. The total time spent immobile ('immobility' was 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 (Porsolt et al., 1977a, b) or an alteration in coping strategy from active to passive (Cryan and Holmes, 2005).

2.3.6. Attack-latency test

The attack-latency test (ALT) was performed with males of Gen V (12 of each breeding line) to investigate possible differences in social behavior and aggressiveness between HR, IR, and LR mice. In principle, the ALT is based on the 'resident–intruder' paradigm (Blanchard et al., 2001). Each test mouse (adult males, 14 weeks of age, single housed for at least 2 weeks prior to the experiment) was confronted in its home cage with a young adult male conspecific (a CD-1 mouse, about 8–9 weeks of age). The animal's behavior was directly observed and the time to the first attack of the resident mouse towards the intruder ('attack-latency') was measured. To assure that escalated fighting and injuries are prevented, the opponents were immediately separated after the first attack. If no aggressive interactions occurred within 5 min, the males were also separated and an attack-latency of 300 s was ascribed to the test mouse. Test animals that were first attacked by the intruder were excluded from the analysis.

2.4. Neuroendocrine investigations

In order to characterize the neuroendocrine phenotype of HR, IR, and LR mice in more detail, several experiments were performed, addressing the following questions:

- Are the observed differences in stress reactivity a stable individual trait or subject to variation over time, i.e. state dependent?
- How early during development do the differences in stress responsiveness appear?
- Are differences in the capacity of the adrenal cortex responsible for the differential secretion of corticosterone in response to stressors?
- Do HR, IR, and LR mice differ with regard to basal functions of the HPA axis, such as the corticosterone/ACTH concentration under resting conditions or the circadian rhythm of glucocorticoid secretion?
- Are differences in stress reactivity associated with differential expression patterns of relevant neuropeptides in the brain or morphometric parameters of the animals?

2.4.1. Trait stability

To address the question of trait stability, male HR/IR/LR mice of Gen III (10 of each breeding line) were subjected to two consecutive SRTs (see Section 2.2.1). The first SRT was performed at the age of about 7 weeks, while the second test took place 8 weeks later. The animals used in this experiment were the same males investigated in the behavioral test battery described above (see Section 2.3), but were left undisturbed (in single housing) for at least 3 weeks prior to the second SRT.

2.4.2. Development of the trait

In order to test whether the trait of differential reactivity of the HPA axis is already present relatively early in life, selected individuals of the HR/IR/LR mouse lines (Gen V, males: $N = 12/11/12$, females: $N = 8/12/8$) were subjected to the SRT shortly after weaning (SRT at around postnatal day 30).

2.4.3. ACTH challenge test

An 'ACTH challenge' test with 10 selected males of each breeding line (Gen II, about 4 months of age) was performed in order to assess potential differences in the maximal capacity of the adrenal cortex to produce and secrete glucocorticoids in HR, IR, and LR mice. This test involves a pharmacological stimulation of the adrenal glands with a relatively high dose of ACTH and measurement of the response in terms of glucocorticoid secretion (von Holst, 1998). In order to accurately follow the time course and detect the peak of the response without interfering activation of the HPA axis by repeated handling and blood sampling, a non-invasive technique to monitor adrenocortical activity by measuring corticosterone metabolites (CM) in the feces of mice was applied (Touma et al., 2004). This technique of glucocorticoid metabolite quantification in fecal samples has been established in an increasing number of species (for review see: Touma and Palme, 2005) and was extensively validated for laboratory mice (Touma et al., 2003, 2004).

At the beginning of the experiment, each mouse was injected at 9 a.m. intraperitoneally with 1 mg/kg body weight of synthetic ACTH (Synacthen, Ciba-Geigy, Basel, Switzerland) dissolved in 0.4 ml of sterile isotonic saline solution. The whole handling procedure of catching, fixation, injection, and returning the mouse into the cage lasted a maximum time of 3 min. For the following 24 h, all fecal samples were collected quantitatively and stored at −20 °C until analysis of CM (see Section 2.5.2). Sampling times were 0, 4, 6, 8, 10, 12, 14, 16, 20, 24 h postinjection.

To facilitate individual sampling and quantitative collection of all voided feces without handling the animal, the method described by Touma and colleagues (2003, 2004) was used. Briefly, the mice were housed individually in stainless steel wire cages ($38 \times 22 \times 15\,cm^3$), which were placed in standard Macrolon cages of the same size. All excreta dropped through the bars of the wire cage and could easily be collected from the floor of the lower cage, which was completely covered with filter paper that immediately adsorbed the urine. To habituate the mice to this sampling procedure and to the housing in wire cages the animals were already placed into this housing system 3 days prior to the injection and samples were collected in 12 h intervals during this time. Since mice are nocturnal animals and their steroid excretion pattern is known to be influenced by their activity (Touma et al., 2003), all sample collections performed during the dark phase of the light–dark cycle were conducted under dimmed light conditions (less than 5 lx) to avoid disturbing the animals' natural activity rhythms.

2.4.4. Basal activity of the HPA axis and diurnal rhythm of glucocorticoid secretion

As outlined in the introduction, several functional alterations of the HPA axis have been described in depressive patients, including basal concentrations of stress hormones and disturbances in the diurnal rhythm of glucocorticoid secretion.

In order to monitor this natural variation of adrenocortical activity over the 24-h cycle in the 'stress reactivity' mouse model, we applied the same non-invasive technique and experimental design that was used for the ACTH challenge experiment (see Section 2.4.3). Again, 10 adult males of the HR, IR, and LR breeding line (Gen II), respectively, were single housed in the wire cages and, after habituation, fecal samples were collected for 24 h in short sampling intervals as described above. In contrast to the ACTH challenge experiment, however, these animals were not injected and left undisturbed as much as possible. In order to confirm the findings of this 'diurnal variation study', the same experiment was repeated with males of Gen III (those used for behavioral testing, see Section 2.3).

After a recovery period of 2 weeks in standard housing conditions, the animals of the latter experiment were quickly sacrificed by decapitation under isoflurane anesthesia, and trunk blood was collected into prechilled, EDTA-coated tubes supplemented with 10 μl of the protease inhibitor 'aprotinin' (Trasylol 500000KIE, Bayer, Leverkusen, Germany). All blood samples were taken between 9 and 11 a.m., maintained on ice, and subsequently centrifuged (1500*g*) for 10 min at 4 °C, before transferring the plasma into clean tubes. Plasma was stored at −20 °C until

corticosterone and ACTH measurements were performed (see Section 2.5.1). To avoid stress effects of the sampling procedure on the investigated neuroendocrine parameters (Gärtner et al., 1980; Beynen, 1992), all blood and organ sampling was performed in a laboratory adjacent to the animal housing room within a few minutes from disturbing the animals' cages.

Within less than 3 min after decapitation, brains were removed, quickly frozen in dry ice-chilled *n*-methylbutane and stored at −80 °C until further processing for the measurement of expression profiles of AVP and CRH (see Section 2.6).

Both adrenals were also dissected immediately after decapitation (completed within 4 min), cleaned from fat and adherent tissue and frozen on dry ice in 0.2 ml of prechilled, 5 mM Tris–HCl buffer (pH 7.2). As indicator of the general activity of the HPA axis and the sympathetic-adrenomedullary (SAM) system (von Holst, 1998), the weight of the right adrenal gland was later determined (after thorough cleaning under a binocular microscope) to the nearest 10 μg.

Since morphometric parameters like body weight can largely interfere with behavioral as well as physiological characteristics, the weight of the animals was also regularly monitored. Body weight measurements were routinely performed in the afternoon on the day of SRT testing in all animals of each breeding generation.

2.5. Hormone analysis

All hormone measurements were performed in duplicate using commercial (see Section 2.5.1) or self-developed (see Section 2.5.2) immunoassay systems. In order to assure high quality standards of the analyses, assay results were only accepted if they were within the linear part of the standard curve and if the coefficient of variation between duplicates was below 5% and 10% for enzyme-immunoassays (EIA) and radioimmunoassays (RIA), respectively. Furthermore, the same two 'control' samples (with relatively high and low concentrations, respectively) were run in every assay in order to detect and adjust for shifts in measured hormone levels across assays.

2.5.1. Plasma corticosterone and ACTH

Plasma concentrations of corticosterone and ACTH were determined by RIA kits (MP Biomedicals, Solon, Ohio, USA) according to the manufacturers' instructions with a slight modification (half of the recommended volume was used for all components, i.e. doubling the number of samples analyzed per kit). For the corticosterone analysis, 10 μl of plasma were used (diluted 1:13.5 for 'initial' samples, 1:100 for 'reaction' samples), while 25 μl of plasma were directly used for a single estimation in the ACTH RIA, yielding a sensitivity of 1 ng/ml and 40 pg/ml, respectively (values below the detection limit were raised to these levels). Intra- and inter-assay coefficients of variation were both below 10% for the low and the high control sample ($N > 50$).

2.5.2. Fecal corticosterone metabolites

The collected fecal samples were analyzed for immunoreactive CM using a 5α-pregnane-3β,11β,21-triol-20-one EIA. Details regarding development, biochemical characteristics, and biological validation of this assay are described by Touma and colleagues (2003, 2004). Before EIA analysis the fecal samples were homogenized and aliquots of 0.05 g were extracted with 1 ml of 80% methanol. A detailed description of the assay performance has been published elsewhere (Touma et al., 2003). Briefly, the EIA used a double-antibody technique and was performed on anti-rabbit-IgG-coated microtiter plates. After overnight incubation (at 4 °C) of standards (range: 0.8–200 pg/well) and samples with steroid antibody and biotinylated label, the plates were emptied, washed and blotted dry, before a streptavidin horseradish peroxidase conjugate was added. After 45 min incubation time, plates were emptied, washed, and blotted dry. The substrate (tetramethylbenzidine) was added and incubated for another 45 min at 4 °C before the enzymatic reaction was stopped with 1 mol/l sulfuric acid. Then, the optical density (at 450 nm) was recorded with an automatic plate reader and the hormone concentrations were calculated. The intra- and inter-assay coefficients of variation were 9.1% and 14.0%, respectively.

2.6. Expression profiling

The expression levels of AVP and CRH mRNA, two neuropeptides largely involved in HPA axis function, were assessed in the PVN using *in situ* hybridization histochemistry. The frozen brains (see Section 2.4.4) were sectioned into 14 μm thick slices using a cryostat (HM 500, Microm Laborgeräte, Walldorf, Germany). Several sections at PVN level were taken for each individual (5 sections per slide). These were processed according to published protocols (Müller et al., 2003; Wigger et al., 2004) comparing mRNA expression levels between HR, IR, and LR mice.

2.6.1. AVP mRNA *in situ* hybridization

For each individual, a set of sections was dehydrated in increasing concentrations of ethanol, degreased with chloroform, rinsed in ethanol, and subsequently air-dried. A highly specific 48-base-long oligonucleotide directed against the last 16 amino acids of the glycoprotein that AVP does not share with oxytocin (OXT) (5′gggcttggcagaatccacggactcttgtgtcccagccagctgtaccag3′; Ivell and Richter, 1984; Villar et al., 1994) was applied for hybridization. The oligonucleotides were labeled with ^{35}S by using ^{35}S-ATP (NEN Chemicals, Dreieich, Germany) and terminal transferase (Tdt, Roche Molecular Biochemicals, Mannheim, Germany) including purification by tRNA precipitation (Sigma-Aldrich, Munich, Germany). Tissue sections were saturated with 100 μl of hybridization buffer containing 10^6 dpm ^{35}S-labeled oligoprobe (for details see Wigger et al., 2004). Coverslipped sections were incubated in humid chambers for 18–22 h at 45 °C. After several washes in 1 × SSC (standard saline citrate), slides were dehydrated and air-dried before they were exposed to the film (see Section 2.6.3).

2.6.2. CRH mRNA *in situ* hybridization

A second set of sections was used for *in situ* hybridization with a ^{35}S-UTP-labeled ribonucleotide CRH probe. The cRNA riboprobe was produced from a 356 bp 5′ fragment (nucleotides 1308–1664) of the mouse CRH cDNA in the pCR II TOPO

vector (Invitrogen, Karlsfeld, Germany) using SP6 (antisense probe) transcription systems in a standard labeling reaction mixture consisting of 1.5 μg of linearized plasmid, transcription buffer, 0.12 mCi of ^{35}S-UTP, 1 mM NTPs, 16.7 mM DTT (DL-Dithiothreitol), 40 U of RNAse inhibitor, and 20 U of the appropriate polymerase. The reaction mix was incubated at 37 °C for 90 min and the labeled probe was subsequently separated from free nucleotides via Qiagen spin columns (for details see Müller et al., 2003). As described elsewhere (Schmidt et al., 2002), for the riboprobe *in situ* hybridization, sections were fixed in 4% paraformaldehyde and were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Slides were dehydrated via increasing ethanol concentrations, degreased with chloroform, rinsed in ethanol, and subsequently air-dried. Tissue sections were saturated with 100 μl of hybridization buffer containing approximately 10^6 dpm ^{35}S-labeled riboprobe. Slides were coverslipped and incubated in humid chambers over night at 55 °C. On the following day, slides were rinsed in 2 × SSC, treated with RNAse A (20 mg/l), washed by decreasingly concentrated SSC solutions, dehydrated by increasing ethanol concentrations, and air-dried before exposure to the film (see Section 2.6.3).

2.6.3. *In situ* hybridization data analysis

For all *in situ* hybridizations, sections were exposed to radiation-sensitive films (Kodak BioMax, Eastman Kodak Co., Rochester, New York, USA) for 1–14 days. The radiation-induced blackening of the film was digitized and quantified by means of image analysis using the software Optimas (version 5.22, Optimas Corp., Silver Spring, Maryland, USA). Autoradiograms from *in situ* hybridizations were analyzed using the OD readings (grey intensity). For each individual, three to five brain sections were analyzed (by an observer blind to the assignment of animals to treatment or breeding line), but only the highest expression (hybridization signal minus background consisting of a nearby region without specific labeling on the same brain slice) was used for calculation of AVP and CRH mRNA expression per nucleus, respectively.

2.7. Statistical analysis

Since a normal distribution of the data could not always be assumed, analyses were exclusively performed using non-parametric statistics (Siegel and Castellan, 1988). All tests were applied two-tailed and were calculated using the software package SPSS (version 12.0).

ANOVA on ranks (Friedman-test) was used to evaluate differences between more than two dependent (related) samples. Two independent samples were compared using the Mann–Whitney *U*-test (MWU-test), while differences between more than two independent samples were calculated with the Kruskal–Wallis *H*-test (KWH-test). In the case of significant variation proved by the KWH-test, post-hoc comparisons between the groups were done using multiple MWU-tests. Here, significance levels were corrected according to the sequential Bonferroni technique (Rice, 1989). For all tests, differences were considered significant if their probability of occurring by chance was less than 5% ($p < 0.05$).

3. Results

3.1. Reactivity of the HPA axis and response to selection

As expected for an outbred strain of mice, a large variation was observed between individuals of the PG regarding the corticosterone increase in the SRT (range males: 60–263 ng/ml, range females: 136–481 ng/ml; see Figure 1). In general, females had corticosterone levels about twice as high as males and are therefore presented separately (see Figure 1a and b). Those male and female individuals showing a very high, intermediate, or low reactivity of the HPA axis, respectively, were selected for founding the HR/IR/LR breeding lines (see Section 2.2 and Figure 1).

Interestingly, already from the first generation of offspring, highly significant differences in stress reactivity were found between the three lines (KWH-tests for Gen I–VI, males: $H = 41.6$–98.2, $df = 2$, all $p < 0.001$; females: $H = 13.2$–101.9, $df = 2$, all $p < 0.001$; post-hoc MWU-tests, males: $U = 0$–670, all group comparisons $p < 0.001$; females: $U = 8$–480, all group comparisons $p < 0.001$; see Figure 1). For both sexes, the restraint-induced corticosterone increase in the SRT was highest in HR, intermediate in IR, and lowest in LR mice (see Figure 1a and b). Moreover, these differences also remained stable in the subsequent generations and could be further increased by assortative breeding, confirming the rapid response to selection for the HR and LR trait, respectively. The same picture emerges, when the two sub-lines (A and B) within each breeding line are considered separately (see Figure S1a and b). By generation IV, the average SRT corticosterone response in HR males was well above 200 ng/ml, around 150 ng/ml in IR males, and below 100 ng/ml in LR males, respectively (see Figure 1a). HR females reached levels above 400 ng/ml, compared to about 300 ng/ml in IR females, and below 200 ng/ml in LR females (see Figure 1b).

To estimate the heritability of the trait, we calculated the realized heritability (h^2) defined as the response to selection[4] (R) relative to the strength of selection[5] (S). The mean heritability of the selection trait (corticosterone increase in the SRT) turned out to be about 0.4 (range: 0.28–0.48) for the HR and LR breeding line, including both sexes separately.

In addition to differences in the corticosterone increase in the SRT, mice of the three breeding lines also differed significantly with regard to other characteristics of the HPA axis. Not only in response to stressors, but also under basal conditions (undisturbed sampling in the first hours of the light phase), elevated corticosterone and ACTH concentrations were observed in HR males and females of generation III, while LR animals showed the lowest concentrations (see Table 1). This difference between the three lines was also reflected in the relative weight of the adrenal glands

[4] R = difference in offspring and whole parental generation mean trait.

[5] S = difference in mean trait between the population as a whole and the selected parents of the next generation; also called selection differential.

Table 1 HPA axis parameters measured under basal conditions in high (HR), intermediate (IR), and low (LR) reactivity males and females of generation III ($N = 10$ for each line and gender).

Gender	Parameter measured	HR		IR		LR		Group comparison[a]		
		Median	Ranges	Median	Ranges	Median	Ranges	H	p	
Males	Plasma corticosterone (ng/ml)	13.0	1.1–31.3	2.3	1.0–6.4	1.5	1.0–4.4	10.2	0.006	**
	Plasma ACTH (pg/ml)	125.7	90.8–211.3	111.0	87.9–163.9	88.8	50.8–164.9	5.6	0.061	T
	Relative adrenal weight (mg/g BW)	0.072	0.060–0.082	0.066	0.055–0.071	0.061	0.041–0.071	14.9	0.001	***
Females	Plasma corticosterone (ng/ml)	26.9	11.5–58.6	10.4	2.0–44.1	7.2	2.5–28.1	7.2	0.027	*
	Plasma ACTH (pg/ml)	88.8	56.6–134.8	72.7	46.6–90.4	59.0	41.0–81.2	8.1	0.017	*
	Relative adrenal weight (mg/g BW)	0.153	0.146–0.196	0.147	0.113–0.168	0.134	0.095–0.159	10.5	0.005	**

[a]Group comparisons between the three breeding lines were calculated using the Kruskal–Wallis H-test, df = 2.

(HR > IR > LR, see Table 1). Interestingly, both corticosterone levels and relative adrenal weights were clearly higher in female mice, while the opposite was true for ACTH concentrations (see Table 1).

3.2. Behavioral emotionality

As mentioned above (see Section 2.3), all animals of the PG were tested in the EPM test and the TST. Similar to the results of the SRT, a considerable variation was observed between individuals regarding for example the proportion of time spent on the open arms of the EPM (see Figure 2) and the total time spent immobile in the TST (see Figure 3). However, the emotionality of the animals selected as founders of the HR, IR, and LR breeding lines, respectively, did not differ significantly in either test (KWH-tests: $N = 8$ for each line and sex, $H = 0.3$–4.3, df = 2, all $p > 0.1$; see symbols in Figures 2 and 3, and S2 and S3).

Searching for alterations in emotional responsiveness associated with the observed differences in stress reactivity, male and female HR/IR/LR mice of generation III were subjected to a series of behavioral tests (see Section 2.3).

No distinct effects on anxiety-related behavior and locomotor activity could be found. In the EPM test, males and females of the three breeding lines did not differ significantly in the proportion of time they spent exploring the unprotected open arms of the apparatus or the latency to enter this compartment (males: see Table 2; females: see Table 3). Furthermore, HR, IR, and LR mice spent about the same time in the more aversive/anxiogenic inner zone of the OF and also did not differ significantly regarding the total distance traveled in the circular arena (see Tables 2 and 3).

However, significant differences were observed for both sexes in the number of rearings in the OF test (see Figure 4) and the number of head dippings in the EPF test (see Figure 5). In both tests, HR animals clearly performed more of these explorative activities, while LR mice showed less exploratory drive, compared to IR males and females (see also Tables 2 and 3).

The behavior of HR, IR, and LR mice also differed significantly in the TST and the FST, both assessing depression-like behavior/coping style in a stressful situation. LR males and females adopted immobile body postures more quickly and spent more time immobile/floating compared to HR and IR animals (see Figure 6 and Tables 2 and 3). HR mice showed the shortest durations of immobility, i.e. spent more time struggling and swimming in the TST and FST, respectively (see Figure 6 and Tables 2 and 3).

The ALT revealed further significant differences between HR, IR, and LR mice (KWH-test: $N = 12/10/12$, $H = 6.5$, df = 2, $p < 0.05$; see Figure 7). LR males showed a much shorter latency to attack the intruder compared to HR and IR males. Seven of the twelve HR males did not even initiate any aggressive interaction within the 5-min test; while only three and one animal of the IR and LR line, respectively, were scored an attack latency of 300 s (see Figure 7).

3.3. Neuroendocrine characterization

Regarding the stability of the trait of differential reactivity to stressors, repeated testing of HR/IR/LR mice of

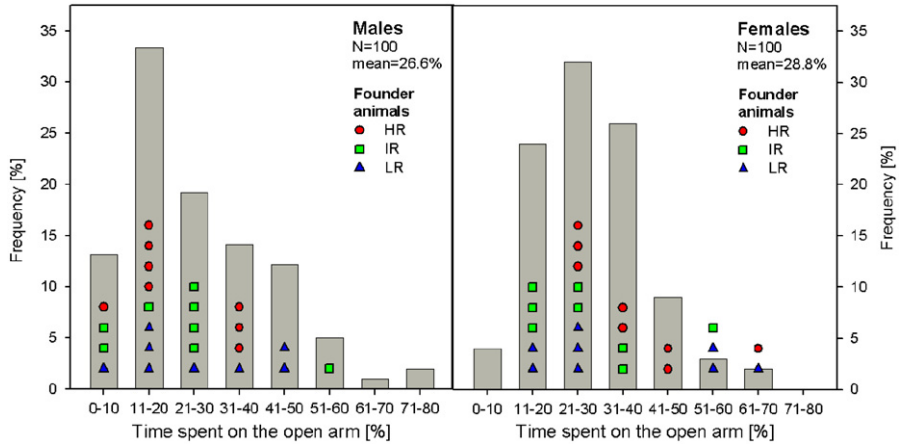

Figure 2 Frequency distribution of anxiety-related behavior (time spent on the open arms in the elevated plus-maze test) in the parental generation of CD-1 mice (bars), including those individuals selected as founder animals for the high (HR), intermediate (IR), and low (LR) reactivity breeding lines, respectively (symbols).

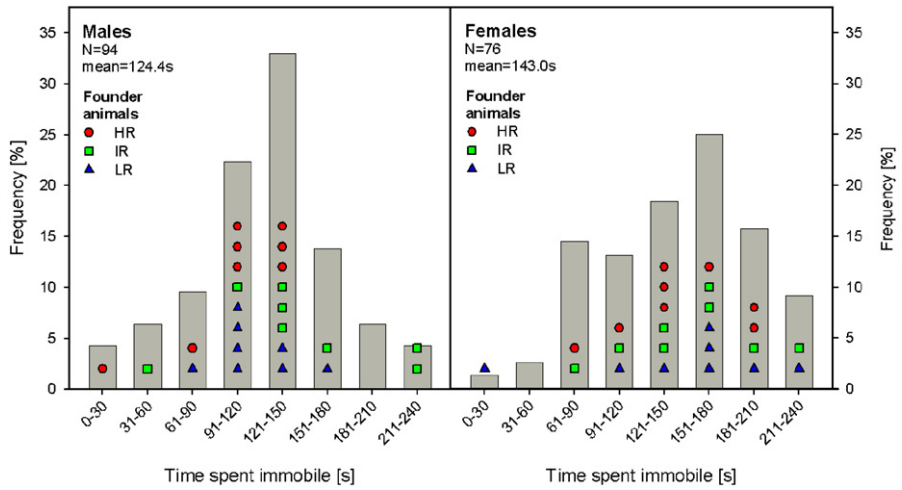

Figure 3 Frequency distribution of depression-like behavior (time spent immobile in the tail-suspension test) in the parental generation of CD-1 mice (bars), including those individuals selected as founder animals for the high (HR), intermediate (IR), and low (LR) reactivity breeding lines, respectively (symbols).

generation III in the SRT revealed very consistent results. In the first as well as in the second SRT, HR animals showed a much stronger increase of corticosterone concentration than IR and LR males, the latter displaying the smallest rise in corticosteroids (KWH-tests: $N = 10$ for each line and test, $H = 25.6$ and 18.9, df $= 2$, both $p < 0.001$; see Figure 8).

These differences in stress reactivity proved to be present already quite early in life (KWH-tests: $N_{males} = 10/11/12$, $N_{females} = 8/12/8$, $H = 24.1$ and 16.7, df $= 2$, both $p < 0.001$; see Figure 9). Male and female HR/IR/LR mice of generation V tested in the SRT shortly after weaning (SRT at around 30 days of age) displayed corticosterone increases very similar to those observed in adult animals, including the significant differences between the lines (see Figure 9). Interestingly, however, the juvenile males of all three breeding lines had corticosterone values in the range usually observed in females, i.e. showed distinctly higher concentrations than during adulthood (cp. Figures 8 and 9).

In order to address potential differences in the capacity of the adrenal cortex to produce and secrete glucocorticoids, HR, IR, and LR males of generation II were subjected to an ACTH challenge test (see Section 2.4.3). As expected, the injection of ACTH caused a sharp rise of circulating corticosterone levels in all three breeding lines, which was reflected in clearly increased CM concentrations in the feces about 10–12 h postinjection, rapidly decreasing to background levels within the next hours (see Figure 10). Both, the time course and magnitude of the response to ACTH were very similar between HR, IR, and LR animals, although a trend was evident towards a higher adrenocortical activation in HR mice (KWH-tests, $N = 10$ for each line, df $= 2$, maximum response: $H = 5.6$, $p = 0.061$; area under the curve (AUC): $H = 4.8$, $p = 0.089$; mean location (ML): $H = 5.1$, $p = 0.076$; see Figure 10). Significant differences in the amount of excreted CM were only detected 4 and 6 h after injection, i.e. before the lag time

Table 2 Results obtained for high (HR), intermediate (IR), and low (LR) reactivity males of generation III in the tests assessing anxiety-related behavior, locomotor activity, exploratory drive, and depression-like behavior.

Behavioral test, sample size	Parameter measured	HR		IR		LR		Group comparison[a]		
		Median	Ranges	Median	Ranges	Median	Ranges	*H*	*p*	
Elevated plus-maze test, $N_{HR/IR/LR} = 10/9/10$	Proportion of time spent on the open arms (%)	36.8	23.8–65.7	37.3	26.1–53.6	41.4	18.9–62.4	0.1	0.933	n.s.
	Latency to the first open arm entry (s)	29.2	7.1–51.4	20.9	1.3–62.9	17.7	5.0–46.9	2.0	0.364	n.s.
Open-field test, $N_{HR/IR/LR} = 9/9/10$	Total distance traveled (m)	24.6	17.0–52.6	19.3	10.5–30.0	18.9	8.0–33.1	3.1	0.213	n.s.
	Time spent in the inner zone (s)	19.1	1.7–46.9	22.5	0.0–113.2	22.0	0.1–79.8	0.6	0.757	n.s.
	Number of rearings (#)	43.0	27–88	32.0	11–65	25.0	11–49	5.9	0.050	*
Elevated platform test, $N_{HR/IR/LR} = 10/8/10$	Total number of head dippings (#)	40.5	22–103	29.0	10–82	24.0	3–44	6.0	0.049	*
Tail-suspension test, $N_{HR/IR/LR} = 10/9/9$	Total time spent in an immobile body posture (s)	90.5	53.3–152.9	82.2	15.1–124.3	134.8	14.5–195.5	5.5	0.065	T
	Latency to the first immobility bout (s)	84.4	54.0–111.5	161.1	54.5–238.8	57.2	41.0–208.4	6.5	0.039	*
Forced swim test, $N_{HR/IR/LR} = 9/9/10$	Total time spent immobile/floating (s)	55.8	4.7–182.9	132.0	24.6–208.3	173.3	72.8–280.7	7.0	0.031	*
	Latency to the first immobility bout (s)	87.7	19.5–279.3	70.9	34.8–123.1	69.9	12.8–186.2	1.4	0.486	n.s.

[a]Group comparisons between the three breeding lines were calculated using the Kruskal–Wallis *H*-test, df = 2.

Table 3 Results obtained for high (HR), intermediate (IR), and low (LR) reactivity females of generation III in the tests assessing anxiety-related behavior, locomotor activity, exploratory drive, and depression-like behavior.

Behavioral test, sample size	Parameter measured	HR		IR		LR		Group comparison[a]		
		Median	Ranges	Median	Ranges	Median	Ranges	*H*	*p*	
Elevated plus-maze test, $N_{HR/IR/LR} = 10/10/10$	Proportion of time spent on the open arms (%)	43.6	24.9–75.5	37.4	18.9–53.2	27.9	14.7–47.5	4.1	0.130	n.s.
	Latency to the first open arm entry (s)	26.0	1.0–57.2	24.4	1.0–61.7	19.7	1.0–52.9	0.1	0.947	n.s.
Open-field test, $N_{HR/IR/LR} = 9/10/10$	Total distance traveled (m)	25.3	17.8–33.3	18.7	4.8–35.7	16.1	7.4–33.9	5.2	0.073	T
	Time spent in the inner zone (s)	22.2	12.5–38.3	21.6	1.1–43.3	16.8	5.9–33.9	0.3	0.867	n.s.
	Number of rearings (#)	38.0	14–71	35.0	2–52	19.5	2–40	7.6	0.022	*
Elevated platform test, $N_{HR/IR/LR} = 9/10/10$	Total number of head dippings (#)	39.0	17–64	28.5	5–73	13.5	3–22	10.2	0.006	**
Tail-suspension test, $N_{HR/IR/LR} = 9/9/10$	Total time spent in an immobile body posture (s)	97.8	0.0–144.1	116.6	0.0–160.3	144.2	71.8–194.5	4.6	0.098	T
	Latency to the first immobility bout (s)	108.9	33.8–360.0	78.4	48.3–360.0	54.9	22.3–115.9	5.7	0.059	T
Forced swim test, $N_{HR/IR/LR} = 10/10/10$	Total time spent immobile/floating (s)	48.8	8.5–164.1	136.5	18.8–203.6	185.6	56.7–286.5	8.8	0.012	*
	Latency to the first immobility bout (s)	91.3	52.6–133.2	72.5	41.7–186.2	56.4	35.9–103.1	4.6	0.099	T

[a]Group comparisons between the three breeding lines were calculated using the Kruskal–Wallis *H*-test, df = 2.

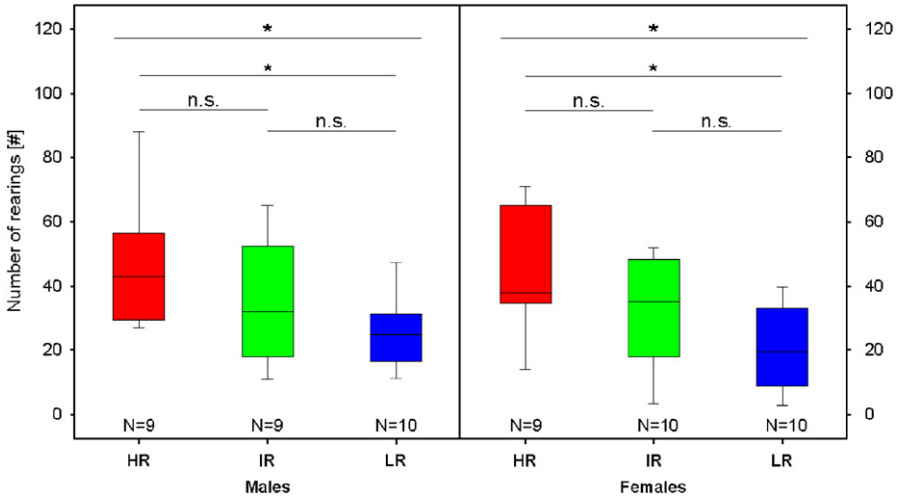

Figure 4 Exploratory behavior (total number of rearings) of high (HR), intermediate (IR), and low (LR) reactivity mice from generation III in the open-field test. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes) as well as 10% and 90% percentiles (whiskers). Statistical differences between the three lines (KWH-test, for details see Tables 2 and 3) are given at the top of each panel and results of the pairwise group comparisons (post-hoc MWU-tests) are indicated below (Bonferroni corrected $p > 0.1$ n.s., $p < 0.05$*).

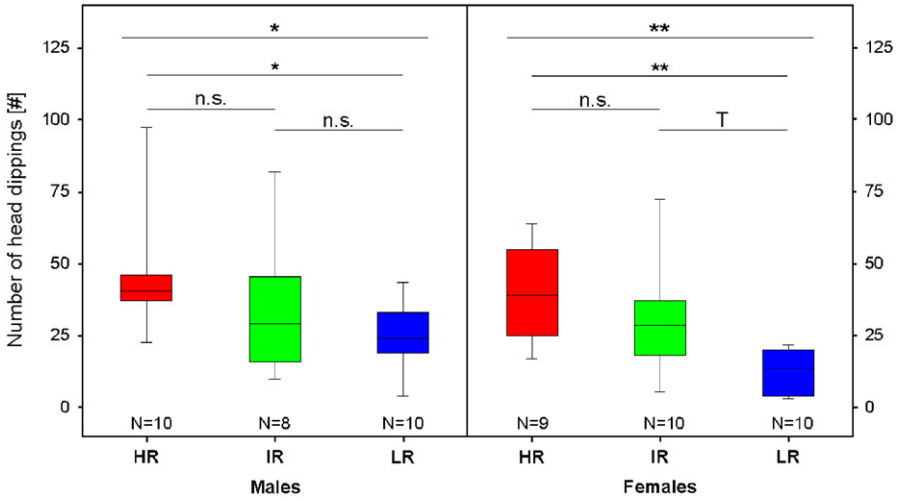

Figure 5 Exploratory behavior (total number of head dippings) of high (HR), intermediate (IR), and low (LR) reactivity mice from generation III in the elevated platform test. Data are given as box plots (for a description, see legend of Figure 4). Statistical differences between the three lines (KWH-test, for details see Tables 2 and 3) are given at the top of each panel and results of the pairwise group comparisons (post-hoc MWU-tests) are indicated below (Bonferroni corrected $p > 0.1$n.s., $p < 0.1$T, $p < 0.05$*, $p < 0.01$**).

of about 8–10 h between plasma and fecal measures (Touma et al., 2003).

Regarding the natural circadian rhythm of glucocorticoid secretion (investigated in animals of Gen II and Gen III), all three lines showed a significant variation of CM concentrations over the 24-h cycle (Friedman-tests, HR/IR/LR males, Gen II and Gen III, $N = 10$ for each line, $Chi_r^2 = 34.9–66.2$, $df = 9$, all $p < 0.001$; see Figure 11). Highest concentrations were measured during the dark phase (around midnight), while relatively low CM levels were observed during the light phase. Comparing the concentrations of excreted CM between HR, IR, and LR animals across the day revealed no significant differences during the night or during the end of the light period. However, at several sampling time points at the beginning of the light phase (between 9 a.m. and 5 p.m.), HR mice showed distinctly and significantly higher CM concentrations than IR and LR animals (see Figure 11). Furthermore, the difference between the maximum and minimum CM concentration across the 24-h cycle was significantly smaller in HR animals compared to IR and LR mice (KWH-tests, HR/IR/LR males, Gen II and Gen III, $N = 10$ for each line, $H = 6.9$ and 7.7, $df = 2$, both $p < 0.05$).

From generation IV on, the differences in stress reactivity were also associated with morphometric parameters such as body weight. In both sexes, adult LR mice were significantly heavier than IR and HR mice, with the latter presenting the

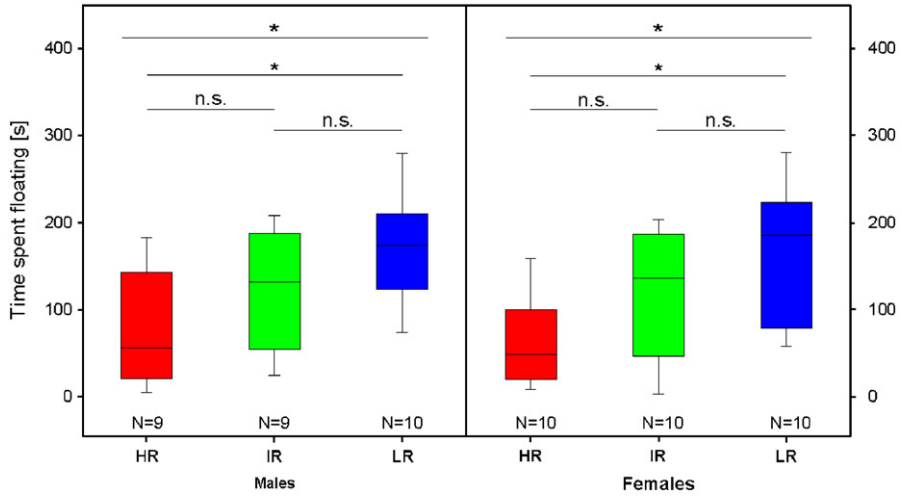

Figure 6 Depression-like behavior (total time spent floating) of high (HR), intermediate (IR), and low (LR) reactivity mice from generation III in the forced swim test. Data are given as box plots (for a description, see legend of Figure 4). Statistical differences between the three lines (KWH-test, for details see Tables 2 and 3) are given at the top of each panel and results of the pairwise group comparisons (post-hoc MWU-tests) are indicated below (Bonferroni corrected $p > 0.1$ n.s., $p < 0.05$*).

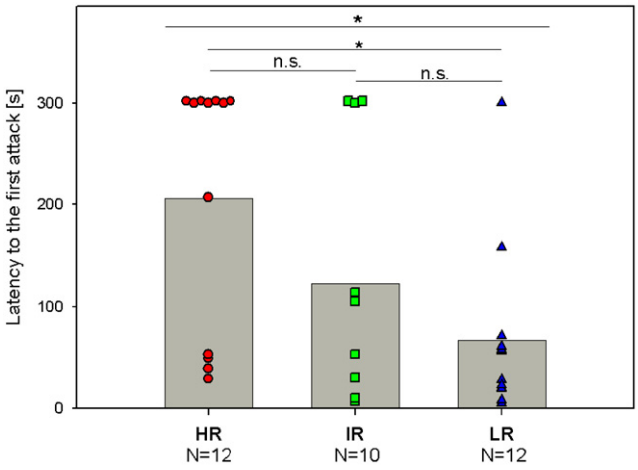

Figure 7 Latency to attack the intruder mouse in the attack latency test. Data are given as means (bars) and individual values (symbols) for each male from generation V of the high (HR), intermediate (IR), and low (LR) reactivity breeding lines. If no aggressive interactions occurred within 5 min, an attack latency of 300 s was ascribed to the test mouse. Statistical differences between the three lines (KWH-test, for details see text) are given at the top of the panel and pairwise group comparisons (post-hoc MWU-tests) are indicated below (Bonferroni corrected $p > 0.1$n.s., $p < 0.05$*).

lowest body weights (Gen V, body weight at about 2 months of age, mean $\pm$ SEM; males: HR = 34.2 $\pm$ 0.4 g, IR = 36.2 $\pm$ 0.4 g, LR = 37.2 $\pm$ 0.4 g; females: HR = 26.3 $\pm$ 0.6 g, IR = 28.4 $\pm$ 0.5 g, LR = 29.7 $\pm$ 0.3 g; see Figure S4). These differences in body weight also proved to be stable across the following generations, but the absolute difference between the lines did not increase. These effects on body weight, however, could not be attributed to differential caloric intake, as HR, IR, and LR mice of both sexes consumed about the same amount of food (Gen V, mean relative food intake measured daily in adult mice over a period of 6 days [g/g BW/d]; males: HR = 0.15, IR = 0.14, LR = 0.14; females: HR = 0.17, IR = 0.16, LR = 0.15; see Figure S5), which has also been confirmed repeatedly in later generations.

Regarding the expression of AVP and CRH mRNA in the hypothalamic PVN, the *in situ* hybridization histochemistry revealed no significant differences between the three breeding lines (see Table S1). The signal intensity as well as the size of the labeled area were very similar in HR, IR, and LR animals (males of Gen III, $N = 10$ for each line, KWH-tests: $H = 0.14$–1.54, df = 2, all $p > 0.1$; representative autoradiographs are shown in Figure S6).

4. Discussion

Affective disorders such as MD provide a challenging degree of complexity with respect to genetic and environmental factors and their interactions. As chronic stress or the propensity to a hypersensitive stress response contributes to the development of anxiety-related disorders and depression, animal models are indispensable tools to discover underlying mechanisms and to identify potential drug targets (Holsboer, 2000; Nestler et al., 2002; de Kloet et al., 2005; Bale, 2006; Kas et al., 2007).

Focusing on the endophenotype of altered stress reactivity frequently observed in patients with MD, the overall aim of our study was to develop a new mouse model comprising this neuroendocrine core symptom by selectively breeding CD-1 mice for high, intermediate, or low reactivity of the HPA axis. Using this approach, we succeeded in establishing three mouse lines (HR, IR, and LR) showing strong and robust differences in the selected trait as well as other functions of the HPA axis, including basal activity and circadian rhythm of glucocorticoid secretion. Furthermore, the bidirectional phenotypic divergence of HPA axis reactivity was associated with alterations in behavioral emotionality, revealing considerable similarities with changes observed in depressive

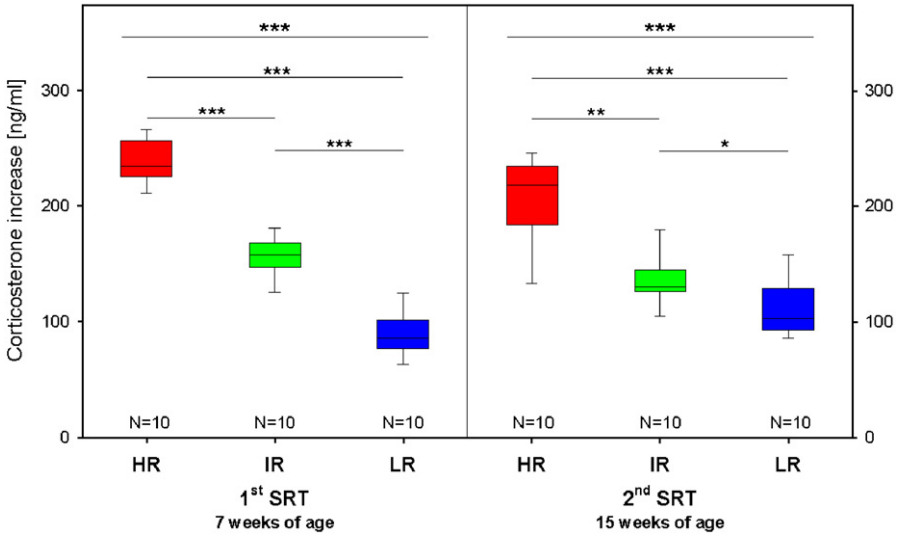

Figure 8 Corticosterone increase in the first and second stress reactivity test (SRT) of high (HR), intermediate (IR), and low (LR) reactivity males from generation III (SRT performed at about 7 and 15 weeks of age, respectively). Data are given as box plots (for a description, see legend of Figure 4). Statistical differences between the three lines (KWH-tests, for details see text) are given at the top of each panel and results of the pairwise group comparisons (post-hoc MWU-tests) are indicated below (Bonferroni corrected $p<0.05$*, $p<0.01$**, $p<0.001$***).

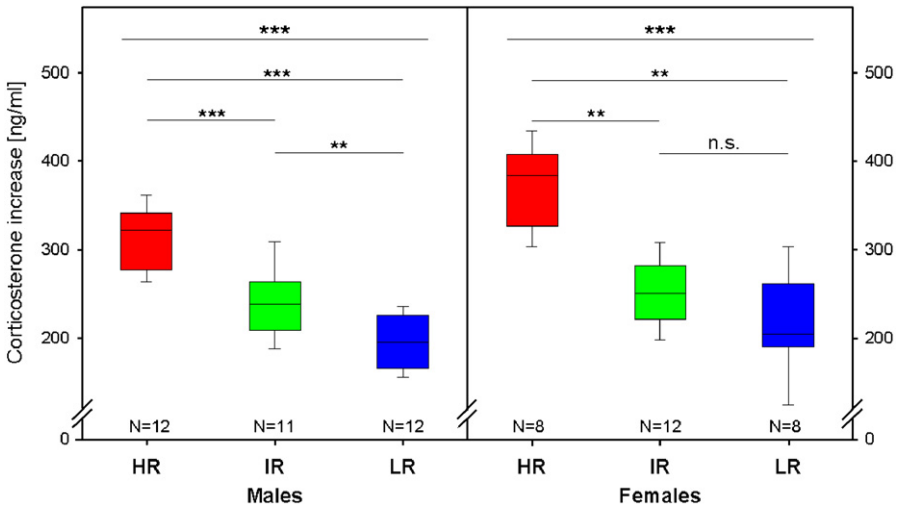

Figure 9 Corticosterone increase in an 'early' stress reactivity test (SRT) of high (HR), intermediate (IR), and low (LR) reactivity males and females from generation V (SRT performed at about 30 days of age). Data are given as box plots (for a description, see legend of Figure 4). Statistical differences between the three lines (KWH-tests, for details see text) are given at the top of each panel and results of the pairwise group comparisons (post-hoc MWU-tests) are indicated below (Bonferroni corrected $p>0.1$n.s., $p<0.01$**, $p<0.001$***).

patients, in particular, when the two opposing subtypes of melancholic and atypical depression are considered.

Selective breeding approaches represent a powerful tool for integrative neuroscience research (Phillips et al., 2002; Swallow and Garland, 2005). Interestingly, however, most investigators in the field of biological psychiatry have only applied selective breeding to shape the behavior of mice or rats to reflect specific features or symptoms of affective disorders (e.g. anxiety-related behavior: Krömer et al., 2005; Stead et al., 2006; Landgraf et al., 2007; depression-like behavior: Scott et al., 1996; Weiss et al., 1998, 2008; El Yacoubi et al., 2003; Steimer and Driscoll, 2003; Will et al., 2003; Henn and Vollmayer, 2005; Weiss et al., 2008) but did not use physiological responses as selection criterion. To our knowledge, we are the first to report results of a selective breeding experiment involving laboratory mice and applying increased or decreased HPA axis reactivity as selected trait.

The response to selection for high, intermediate or low corticosterone increase in the SRT, as depicted in Figure 1, turned out to be quite strong for males and females and was present in both sub-lines within the HR, IR, and LR breeding line, respectively (see Figure S1). Already in the first

generation, i.e. offspring derived from breeding pairs selected from the founder population of outbred CD-1 mice, significant differences in stress reactivity were found. These differences could be increased further from generation to generation by assortative breeding (see Figures 1 and S1 for breakdown into sub-lines). By generation VI, the mean corticosterone increase of males and females in the HR and LR lines was well within the range or even exceeded the responses observed in the respective founder pairs. This rather rapid and stable response to selection for extremes in stress reactivity strongly indicates a genetic basis of the respective phenotype. Furthermore, our heritability estimates indicate that about 40% of the phenotypic variance within the offspring of the founding population of CD-1 mice was determined by heritable, thus potentially genetic, factors. These results are also in line with findings of other studies selecting animals for glucocorticoid secretion in response to stressors (domestic turkey: Brown and Nestor, 1973; domestic chicken: Edens and Siegel, 1975; Japanese quail: Satterlee and Johnson, 1988; rainbow trout: Pottinger and Carrick, 1999; zebra finch: Evans et al., 2006; domestic pig: Kadarmideen and Janss, 2007). In these studies, the heritability of the trait was described to range between 0.2–0.3 in Japanese quail and zebra finches, around 0.4 in the rainbow trout, and 0.4–0.7 in the domestic pig (see references cited above). A segregation study in rats also revealed (at least for the males) a narrow sense heritability of 0.26 for restraint stress-induced corticosterone concentrations (Solberg et al., 2003). Together with the considerable individual variation found in the PG of mice (see Figure 1), this suggests that HPA axis reactivity is a highly heritable trait probably determined by a set of major impact genes that presumably have been conserved during evolution (see also Overli et al., 2007). Given this relatively high degree of heritability, future studies will be possible using a candidate gene approach to identify genetic variants

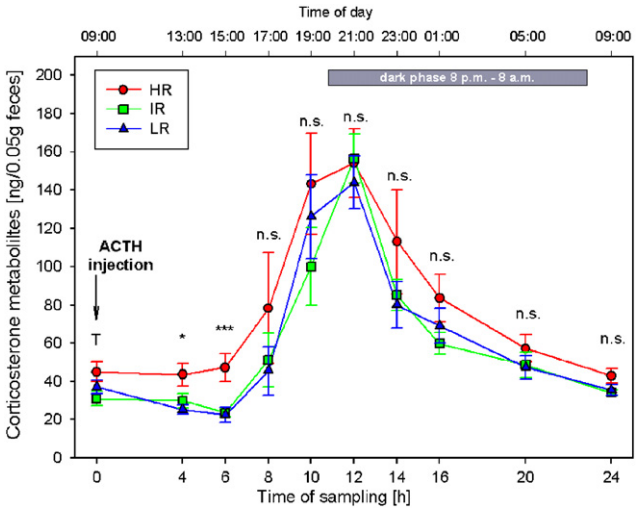

Figure 10 Excretion profile of immunoreactive corticosterone metabolites (CM) in fecal samples of high (HR), intermediate (IR), and low (LR) reactivity males from generation II after stimulation with ACTH. Data are given as means ± SEM for each line. Statistical differences between the three lines are indicated by asterisks (KWH-tests, $N = 10$ for each line, df = 2, $H = 0.1$–13.8, $p > 0.1$n.s., $p < 0.1$T, $p < 0.05$*, $p < 0.001$***). The time of day and the dark phase (horizontal bar) are indicated at the top of the panel. The arrow marks the time of injection.

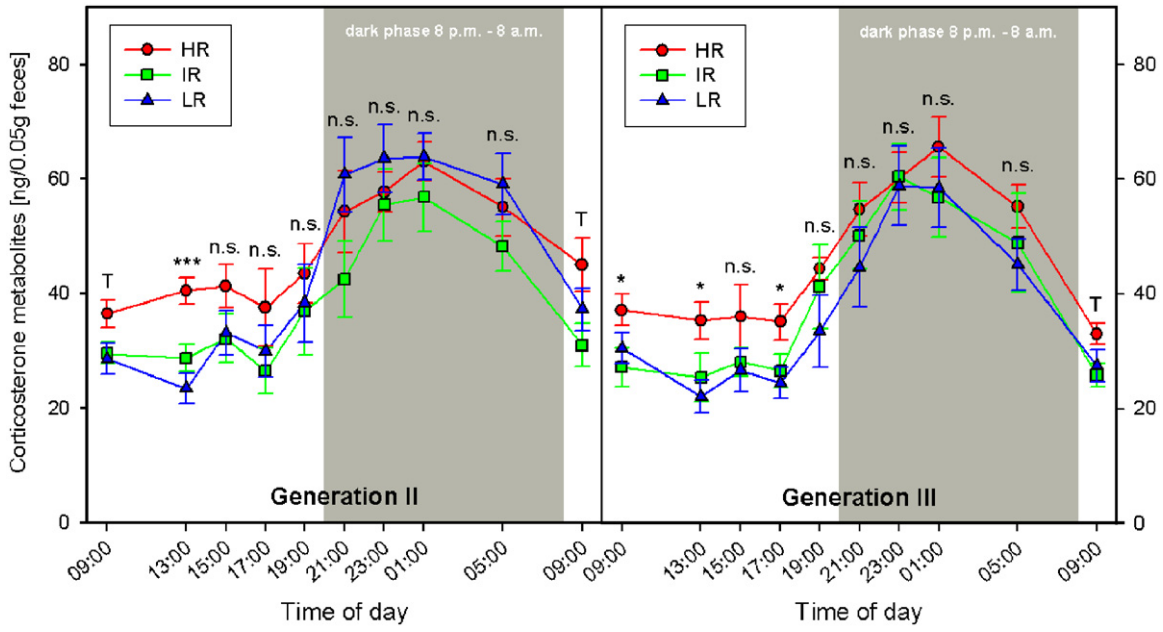

Figure 11 Diurnal variation of immunoreactive corticosterone metabolites (CM) in fecal samples of high (HR), intermediate (IR), and low (LR) reactivity males from generations II and III over the 24-h cycle. Data are given as means ± SEM for each line. Statistical differences between the three lines are indicated by asterisks (KWH-tests, $N = 10$ for each line, df = 2, $H = 0.7$–14.4, $p > 0.1$n.s., $p < 0.1$T, $p < 0.05$*, $p < 0.001$***). The dark phase of the light–dark cycle is indicated by the shaded area.

that underlie the observed differences in stress reactivity between the HR, IR, and LR lines (see also Kas et al., 2007).

Although these findings strongly point into the direction of a genetically driven trait, many studies on rodents and primates (including humans) have also shown strong effects of developmental determinants such as the early life environment (which is essentially determined by maternal behavior in murine rodents) on emotionality and HPA axis reactivity in the offspring (Meaney, 2001; Schmidt et al., 2002; Champagne et al., 2003; Levine, 2005; Szyf et al., 2005; Macri and Würbel, 2006; Coutellier et al., 2008). Thus, it is possible that variation in maternal care between HR, IR, and LR mice brings about the observed differences in stress reactivity. Addressing this question, all pup-related behaviors (including nursing as well as licking and grooming) of dams from the three breeding lines (Gen IV) were continuously monitored over the entire 24-h cycle on postnatal day (PND) 2, 4, and 8. The analysis, however, did not reveal significant differences in maternal care between HR and LR mothers, indicating that the trait of differential reactivity of the HPA axis is unlikely to be strongly influenced by epigenetic programming by maternal behavior.

Regarding the observed sex differences in HPA axis activity and reactivity (c.p. Figure 1a and b; and see Table 1) our findings are also in accordance with published results. In general, female mice and rats are reported to show higher circulating corticosterone concentrations under resting conditions as well as a greater magnitude and duration of HPA axis response to stressors (Handa et al., 1994; Armario et al., 1995; Timpl et al., 1998; Rhodes and Rubin, 1999). Human studies also revealed higher stress vulnerability in women, although the neuroendocrine evidence is much more equivocal compared to the animal data (Kudielka and Kirschbaum, 2005). Nevertheless, numerous significant sex influences on brain anatomy, physiology, and function are apparent and sex differences should seriously be taken into account, in particular, as many psychiatric disorders affect males and females differently (Seeman, 1997; Altemus, 2006; Cahill, 2006). Interestingly, several syndromes that have been associated with alterations in HPA axis function and regulation, including MD, anxiety disorders, and Alzheimer's disease, disproportionally affect women; for example, females suffer from depression about twice as often as males (Seeman, 1997; Swaab et al., 2005; Altemus, 2006). However, this gender bias in major depression seems to be restricted mainly to atypical depression, as men present more often with melancholic features than women (Angst et al., 2002; Khan et al., 2006). A straightforward relationship between higher HPA axis activity and melancholic depression is obviously too simplistic. In our model, we therefore focussed on HPA axis reactivity, which might be a more appropriate indicator of susceptibility.

In addition to a thorough stress-physiological characterization of the unselected founder population, we also tested all CD-1 mice of the PG regarding anxiety-related and depression-like behaviors. As expected for an outbred strain of mice (see also: Vaugeois et al., 1997; Jacobson and Cryan, 2007), in the EPM test as well as in the TST a considerable individual variation was observed with a more or less normal distribution (see Figures 2 and 3). However, the behavioral emotionality of the founder animals of the HR, IR, and LR breeding lines, respectively, did not differ significantly (see Figures S2 and S3). This indicates that the behavioral as well as the neuroendocrine phenotype are brought about by a complex interaction of genetic and environmental factors, making predictions from one to the other extremely difficult. Nevertheless, a genetic predisposition for high, intermediate, or low stress reactivity is associated with changes in behavioral emotionality, as we have shown by our selective breeding experiment.

Already in the third generation, HR and LR animals of both sexes differed significantly in their coping styles.[6] Although no distinct effects on anxiety-related behavior could be observed and locomotor activity did also not differ significantly between the three breeding lines, clear differences were found regarding exploratory drive, depression-like behavior, and coping with aversiveness in general (see Tables 2 and 3 and Figures 4–6). The number of rearings in the OF test, the number of head dippings in the EPF test, as well as the time spent immobile in the TST and FST clearly indicate a passive coping style in LR mice compared to IR animals, while HR males and females showed a more active or even hyperactive/agitated phenotype in these tests (see also: Cryan and Holmes, 2005). These behavioral differences, in particular regarding the FST results, have also been repeatedly found in later generations of the HR, IR, and LR breeding lines (see Figure S8), which further underlines the significance of our findings in this relatively early generation III. Furthermore, the differences in coping behavior were present in each of the two independent sub-lines (A and B) within the HR, IR, and LR mouse line, respectively, indicating a causal link between HPA axis reactivity and behavioral emotionality (see Figure S8).

According to the concept of 'proactive' *versus* 'reactive' coping styles (reviewed in: Koolhaas et al., 1999), it would be expected that animals showing more passive behavioral responses to aversive situations present a high reactivity of the HPA axis (see for example: Veenema et al., 2003; Satterlee and Marin, 2006). This, however, is obviously not the case in our LR mouse line, where passive coping behavior is observed along with decreased stress reactivity. Interestingly, similar findings have been published for Wistar Kyoto (WKY) rats, another rodent model for depression (Pare, 1994; Solberg et al., 2001, 2003; Will et al., 2003). Compared to Fischer 344 rats, WKY rats show clearly increased immobility in the FST and also respond to restraint stress with a less pronounced secretion of corticosterone (Armario et al., 1995; Solberg et al., 2003), which might be linked to alterations in pituitary corticotroph responsiveness to CRH and AVP (Hauger et al., 2002).

In light of the neuroendocrine and behavioral changes observed in MD, these differences in coping between HR and LR mice can be interpreted as resembling the symptoms of melancholic and atypically depressed patients, respectively. As outlined in the Introduction, melancholia is often associated with HPA axis hyper-activity and psychomotor changes such as hyper-arousal and restlessness, while in atypical depression HPA axis hypo-reactivity coupled with

[6]Coping styles can be defined as a coherent set of behavioral and physiological stress responses, consistent over time and characteristic to certain individuals (Koolhaas et al., 1999).

lethargy and fatigue predominate (Holsboer, 2000; Gold and Chrousos, 2002; Nestler et al., 2002; de Kloet et al., 2005; Antonijevic, 2006). The opposing symptoms of decreased *versus* increased appetite and weight gain observed in patients with melancholic and atypical depression, respectively, also support this interpretation, as the significant body weight differences between HR and LR animals of both sexes (consistently present from Gen IV; see Section 3.3 and Figure S4) point in the same direction. The food intake, however, did not differ significantly between the breeding lines (see Section 3.3 and Figure S5), making this effect on body weight likely to be attributed to differences in body composition, which is probably brought about by the metabolic functions of glucocorticoids (Sapolsky et al., 2000; Bornstein et al., 2006) Furthermore, the ALT revealed significant differences in social behavior and aggressiveness between the three breeding lines, with LR males showing a much shorter latency to attack the intruder mouse than IR and HR males (see Figure 7). This higher level of aggression is also reflected by more frequent incidents of escalated fighting between LR males under normal group housing conditions (Touma, personal observation). Hence, our results further support data from human and other animal studies, strongly indicating a causal link between chronic glucocorticoid deficiency and antisocial aggressiveness (for review see: Haller and Kruk, 2006; Kim and Haller, 2007). Additionally, clinical observations associate atypical depression with being overweight and aggressive personality traits or sociopathy (Nierenberg et al., 1998; Hasler et al., 2004b), further underlining the validity of our interpretation that LR mice can be a model for this subtype of MD.

Regarding the neuroendocrine endophenotype, repeated testing in the SRT confirmed that the differential HPA axis reactivity is a stable individual trait and not, or only to a minor extent, state-dependent (see Figure 8). Furthermore, subjecting HR, IR, and LR mice of both sexes to the SRT shortly after weaning revealed that the differences in stress responsiveness are present already relatively early in life (see Figure 9). Together, these findings further support the assumption of genetic factors playing a major role in shaping the neuroendocrine and behavioral phenotypes observed in the HR/IR/LR mouse model. Concerning the increased corticosterone concentrations of juvenile/periadolescent males (but not females) compared to adults (c.p. Figures 8 and 9), very similar findings have been reported by Laviola and colleagues (2002). As testosterone has inhibiting effects on HPA axis function (Handa et al., 1994), and prepubertal androgen concentrations are low, this might be a possible explanation for this phenomenon.

In order to investigate whether differences in the maximal capacity of the adrenal cortex to produce and secrete glucocorticoids are responsible for the differential corticosterone response of HR, IR, and LR mice to stressors, an ACTH challenge test was performed (von Holst, 1998). The excretion pattern of fecal CM, reflecting circulating corticosterone concentrations with a lag time of about 10 h (Touma et al., 2003), showed no major differences between the three lines (see Figure 10), and the response to stimulation with a high dose of ACTH was very similar to findings of previous studies that applied the same test to C57BL/6J mice (Touma et al., 2004). In IR and LR males, the ACTH injection induced virtually identical activation of the adrenal cortex, indicating that the reduced stress response of LR animals in the SRT can not be attributed to functional impairments of their adrenals. HR animals, on the other hand, tended to show a stronger adrenocortical activation, which is in line with the increased adrenal weight observed in males and females of generation III (see Table 1). This suggests in HR mice an elevated secretion of ACTH, which is a trophic hormone, resulting in adrenocortical hyperplasia secondary to a hyper-reactive HPA axis (von Holst, 1998; Sapolsky et al., 2000; Ulrich-Lai et al., 2006). Additionally, differential sensitivity of the adrenals to ACTH (potentially mediated by adrenal neural activity) might contribute to the phenotypic differences between HR, IR, and LR mice (Dallman et al., 1978; Carsia et al., 1988; Engeland and Arnhold, 2005). Addressing this potential mechanism underling the variation in stress reactivity, future experiments should for example use a lower, sub-maximal stimulation with ACTH and block endogenous ACTH release by dexamethasone (for example see: Ulrich-Lai et al., 2006). It should also be noted that the animals used in the ACTH challenge test derived from generation II, i.e. relatively early during the selective breeding process, when the segregation of genetic loci contributing to the neuroendocrine phenotype was obviously not complete (see Figure 1). Conclusions about the underlying mechanisms must therefore be drawn with care. Nevertheless, obviously some stable characteristics of the animal model appeared very early; for example (similar to the behavioral effects mentioned above), the significantly increased adrenal weight found in HR mice of generation III was also repeatedly confirmed in later breeding generations.

Differences in adrenal weight can be brought about by enlargement of the adrenal cortex as well as the adrenal medulla, driven by an increased tone of the HPA axis and the sympathetic adrenomedullary (SAM) system, respectively (Engeland and Arnhold, 2005; Ulrich-Lai et al., 2006). In order to address the question, whether HR, IR, and LR mice do not only differentially activate their HPA axis, but might also respond differently to stressors with an increased or decreased secretion of epinephrine and norepinephrine from the adrenal medulla, we subjected all males and females of generation V to the 'stress-induced hyperthermia' (SIH) test. Hyperthermia (an increase in body temperature) is an integral part of an individual's response to situations perceived as stressful and this phenomenon is mediated mainly by the SAM system (Spooren et al., 2002; Olivier et al., 2003). Therefore, the SIH test, consisting of two rectal temperature measurements, the first under basal conditions ($T = 0$) and the second 15 min later ($T = 15$), can be used to assess the stress response of the second stress axis with relatively minor interference from the HPA axis (Spooren et al., 2002; Olivier et al., 2003). The results of the SIH test revealed no significant differences between HR, IR, and LR mice regarding basal body temperature as well as stress-induced temperature increase (see Figure S7). Thus, a differential activation of the SAM system is unlikely to contribute to the differences in stress reactivity observed between the three lines.

Our studies monitoring the circadian rhythm of glucocorticoid secretion in HR/IR/LR males of generation II and III revealed further significant differences between the breeding lines. Although the typical pattern of increased CM

concentrations during the dark phase and relatively low concentrations during the light phase (cf. Touma et al., 2004; Dallmann et al., 2006; Voigtländer et al., 2006) are found in all three mouse lines, HR animals had clearly elevated trough levels, resulting in a flattened diurnal variation of glucocorticoids (see Figure 11 and the first three sampling time points in Figure 10). Remarkably, disturbances of circadian functions and sleep architecture, including corticosteroid dysrhythmia, are among the main symptoms of MD (Deuschle et al., 1997; Wong and Licinio, 2001; Steiger, 2002; Antonijevic, 2006). Particularly in patients with melancholic or psychotic features, alterations in the circadian rhythm of cortisol secretion have been described, with elevated trough levels leading to a flattened diurnal profile (Deuschle et al., 1997; Wong et al., 2000; Keller et al., 2006). Thus, together with the significantly higher plasma corticosterone and ACTH concentrations (see Table 1) measured in samples from HR males and females collected during the first hours of the light phase, i.e. when nadir values are expected, this further underlines the similarity between the neuroendocrine phenotypes observed in melancholic depression and the HR mouse line.

Addressing potential hypothalamic correlates of the differential stress responsiveness between HR, IR, and LR mice, we focused on CRH and AVP, two neuropeptides largely involved in HPA axis function and which have been associated in clinical as well as preclinical studies with the behavioral and neurobiological endophenotypes of affective disorders (Holsboer, 2000; Nestler et al., 2002; Engelmann et al., 2004; de Kloet et al., 2005; Bale, 2006; Müller and Holsboer, 2006). The expression of both ACTH secretagogues, CRH and AVP, assessed by *in situ* hybridization histochemistry, however, did not differ significantly between the three breeding lines (see Table S1 and Figure S6). Thus, at least under basal conditions, there is no indication for up- or down-regulation of these neuropeptides in the PVN of either line (this was also confirmed by similar findings in animals from Gen V). However, the absence of a difference in basal AVP and CRH mRNA expression does not necessarily rule out differences at the protein level or the release pattern of these neuropeptides in response to stressors (see for example: Veenema et al., 2003).

As outlined in the Introduction, one of the most common findings in affective disorder research is a dysregulation of the stress hormone system, and impaired glucocorticoid signaling mediated by mineralocorticoid (MR) and glucocorticoid receptors (GR) has been proposed to be a key mechanism in the pathogenesis of depression (for review see: Holsboer, 2000; de Kloet et al., 2005; Ising et al., 2007). Therefore, we think that promising candidates for elucidating the mechanistic underpinnings of the differential stress responsiveness in the HR/IR/LR mouse model are the feedback mechanisms of the HPA axis, acting at different sites in the brain and the periphery, and involving corticosteroid receptors (MR and GR) triggering fast/short-term effects on neuronal activity and plasticity, as well as slower/long-term genetic effects on various brain functions (for reviews see: de Kloet et al., 1998, 2005; Holsboer, 2000; Sapolsky et al., 2000; Makara and Haller, 2001; Dallman, 2005). However, other central or peripheral mechanisms might also be involved, including differences in hormone bioavailability and adrenal sensitivity to ACTH.

Taken together, already in the first generation of our selection experiment, clear-cut differences in the reactivity of the HPA axis between HR, IR, and LR mice were observed. These differences were also found across subsequent generations and could even be increased by selective breeding, strongly indicating a genetic basis of this phenotype. We could further show that the neuroendocrine stress responsiveness is an individually stable and highly heritable trait, which also correlates with other functional alterations of the HPA axis. Additionally, the results of the behavioral tests applied to the selected mouse lines revealed distinct differences in emotionality associated with the differential reactivity of the HPA axis. As our selective breeding approach included an independent 'control' line (IR mice) as well as internal replications (two independent sub-lines within each breeding line), we believe that the generated HR/IR/LR mouse lines are a valid animal model for altered stress reactivity and show considerable similarities to the symptomatology of specific subtypes of MD.

Thus, given its unique strengths as an experimental system for studying genetics, neurobiology, and behavior, the HR/IR/LR mouse model can be central to meeting the challenge for cross-species translational research in biological psychiatry, improving our understanding of affective disorders and thereby opening new perspectives for the development of new, targeted treatment strategies.

Role of the funding sources

Funding for this study was provided by the Max Planck Society (MPS). The MPS had no further role in study design, in the collection, analysis and interpretation of data, in writing of the report, and in the decision to submit the paper for publication.

Conflict of interest

All authors declare that they have no 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 doi:10.1016/j.psyneuen.2008.03.013.

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