Central and Peripheral Aspects of Hypothalamic-Pituitary-Adrenal (HPA) Axis Dysfunction: Insights from Mice Selectively Bred for Extremes in Stress Reactivity

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Summary

An aberrant regulation of the hypothalamic-pituitary-adrenal (HPA) axis is closely associated with the pathophysiology of affective disorders such as major depression (MD). Accordingly, patients suffering from MD frequently show profound neuroendocrine alterations with hyper- or hypo-cortisolism as a result of a dysregulated stress hormone system. Focussing on this key endophenotype of MD, the 'stress reactivity' (SR) mouse model was recently established, consisting of three independent mouse lines, the high (HR), intermediate (IR) and low (LR) stress reactivity line, selectively bred for differences in their corticosterone (CORT) secretion in response to a psychological stressor. Previous studies revealed distinct differences between HR, IR and LR animals regarding sleep architecture, activity rhythms, emotional behaviour, cognition as well as neuroendocrine functions, resembling several endophenotypes observed in depressed patients.

In the series of studies presented in this work, we aimed to investigate whether the differences between HR, IR and LR mice were restricted to the peripheral phenomenon of adrenal CORT secretion, or whether these endophenotypes were brought about by an aberrant regulation of upstream control centres of the HPA axis. To this end, we performed experiments investigating all functional levels of HPA axis control, i.e. the adrenals, the pituitary and brain centres known to be involved in the neuroendocrine stress response. Moreover, we assessed the expression of corticosteroid-binding globulin (CBG), which contributes to the transport and delivery of CORT to its target tissues. Finally, we studied HPA axis regulatory mechanisms by means of the combined dexamethasone/corticotropin-releasing hormone (Dex/CRH) test.

At the level of the adrenal, we found that pharmacological inhibition of the biosynthesis and secretion of CORT using metyrapone had a significant impact on the stress-coping behaviour of HR, IR and LR animals as determined in the forced swim test (FST). As another peripheral factor influencing the secretion of CORT, we assessed the adrenal sensitivity of the animals to adrenocorticotropic hormone (ACTH) *in vivo*. After a Dex-mediated inhibition of endogenous ACTH release from the anterior pituitary, LR animals showed a markedly reduced CORT surge compared to HR mice in response to a stimulation of the adrenals with two doses of exogenous ACTH, indicating an enhanced adrenal sensitivity in HR mice and a blunted responsiveness to ACTH in LR mice. In addition, we found significant differences in plasma CBG levels between the three mouse lines (HR>IR>LR), concomitant with differences in free plasma CORT

both, basal and in response to 15 min restraint stress (HR>IR>LR). Since only free CORT is biologically active, these results indicate that CBG might play a role in the endophenotypes of the SR mouse lines.

At the pituitary level, we detected significantly altered ACTH protein levels (HR>IR≥LR) and proopiomelanocortin mRNA expression (HR>IR>LR), suggesting a differential activation of the anterior pituitary between the three lines, which is in line with the observed differences in stress reactivity.

In the brain, we assessed the neuronal activation induced by an acute stressor in regions known to be involved in HPA axis function such as the prefrontal cortex, the basolateral amygdala, the hippocampus and the paraventricular nucleus of the hypothalamus (PVN) using *c-fos in-situ* hybridisation. Acute stress exposure markedly increased neuronal activation in all investigated brain areas. However, significant differences in the neuronal excitation between the three lines were only detected in the PVN (HR>IR>LR), indicating an altered activation of the animals' HPA system orchestrated by this nucleus.

Finally, we assessed HPA axis regulatory mechanisms by means of the Dex/CRH test. Our results revealed considerable similarities to clinical studies, with HR mice showing signs of Dex non-suppression in addition to an overshooting CORT surge after CRH stimulation, mirroring the HPA axis hyper-active state of patients suffering from the psychotic or melancholic subtype of MD. In contrast, LR animals presented a strong Dex-induced CORT suppression and a blunted response to the CRH stimulation, resembling the situation observed in atypically depressed patients. Strikingly, chronic fluoxetine (Flx) treatment enhanced the negative feedback regulation of the HPA axis in all three lines of the SR mouse model. In particular, the Dex-mediated CORT suppression in HR mice was restored by Flx. Furthermore, Flx affected the stress-coping behaviour of the animals in the FST. Flx induced a reduction in active coping, indicating an attenuation of the hyper-aroused state, particularly in HR mice.

Taken together, the series of studies presented here demonstrated that the SR mouse model shows functional alterations on all levels of the HPA axis - peripheral, central and regarding the regulation – similar to the endophenotypes of MD patients, thus revealing a high level of face and construct validity of the model. Hence, the SR mouse model can serve as a valuable tool in the discovery and validation of new drug targets and improve already existing treatments of MD, particularly those targeting the HPA system.

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1 Introduction

Its not stress that kills us, it is our reaction to it
-Hans Selye (1907-1982)

1.1 Major depression

Stress-related disorders such as major depression (MD) are among the most prevalent and costly mental illnesses (Fava *et al.*, 2000; Nestler *et al.*, 2002) with about 120 million people worldwide being affected, accounting for an estimated lifetime prevalence of around 17 % to develop the disease (Fava *et al.*, 2000). According to the World Health Organisation (WHO), depression causes 6 % of the burden of all diseases in Europe in terms of disability adjusted life years (DALYs) (Sobocki *et al.*, 2006). Moreover, MD is projected to be the second most important cause of disability worldwide by the year 2020 (Davidson *et al.*, 1999).

MD occurs in persons of all genders, ages, and backgrounds, although women are affected twice as much as men (Nestler *et al.*, 2002; Levinson, 2006). The symptomatology is widespread and very heterogenic and includes psychological symptoms such as depressed mood, loss of interest or pleasure, feelings of worthlessness and guilt, low self-esteem, as well as psychosomatic alterations such as disturbed sleep, changes in appetite and weight, fatigue, and cognitive deficits (Lewinsohn *et al.*, 1998; Nestler *et al.*, 2002; Hasler *et al.*, 2004). Tragically, the personal suffering coming along with this symptomatology eventually peaks in about 850.000 suicides a year (http://www.who.int, accessed June 12th, 2012).

Apart from the tremendous personal afflictions of affected individuals and their relatives, MD also presents a considerable socioeconomic burden. The total annual cost of depression in Europe alone was estimated at 118 billion Euros in 2004. This makes depression the most costly brain disorder in Europe, accounting for 33 % of the total treatment costs of psychiatric diseases (Sobocki *et al.*, 2006). Several reasons help to explain why the economic burden of depression is so large. MD is among the most commonly occurring chronic diseases in both, the labour force and the general population. Moreover, MD is associated with substantial loss in productivity, much of

which results from the fact that MD has an earlier age of onset (typically late 20s) than other common diseases affecting individuals before or during their prime working years (Wang *et al.*, 2003).

Risk factors to develop MD are as heterogenic as the symptomatology. Epidemiologic studies showed that roughly 40-50 % of the risk for depression is genetic (Nestler *et al.*, 2002; Levinson, 2006), making MD a highly heritable disease. Moreover, non-genetic, 'environmental' factors such as the experience of a traumatic event, e.g. childhood abuse (Goldberg, 1994; Levitan *et al.*, 1998; Gibb *et al.*, 2003) or loss of a beloved person (Kendler *et al.*, 2003), drug abuse (Deykin *et al.*, 1987; Deykin *et al.*, 1992) as well as viral infections (Bode *et al.*, 1993) can increase the vulnerability to develop MD (Nestler *et al.*, 2002).

Thus, it becomes apparent that depressive disorders such as MD are multidimensional and heterogenic diseases, involving genetic (Levinson, 2006), epigenetic (Mill *et al.*, 2007), environmental (Kendler *et al.*, 1992) and psychosocial (Hirschfeld *et al.*, 1982) components, which are difficult to track down to one common mechanism of onset. Therefore, a major goal of psychiatric research is to understand the comprehensive network of factors which are involved in the aetiology of MD.

1.2 Subtypes of major depression

According to the 'diagnostic and statistical manual of affective disorders' (DSM-IV), MD is categorised in several subtypes with partly overlapping but characteristic (sometimes even contrasting) symptomatic features allowing diagnostic differentiation. The two probably most important subtypes of MD are the melancholic (also termed 'endogenous' or 'typical') and the 'atypical' subtype of depression. The melancholic subtype is commonly associated with increased anxiety, loss of responsiveness to environmental stimuli, insomnia as well as decreased appetite and weight. Furthermore, patients show diurnal variations in their depressive state which is worst in the morning. Interestingly, patients suffering from atypical depression virtually present the opposite symptomatology comprising lethargy and fatigue, hyperphagia and weight gain, hypersomnia, increased reactivity to the environment, social aggression as well as a diurnal variation in their emotional states that is at its best in the morning (Gold *et al.*, 2002; Nestler *et al.*, 2002; Hasler *et al.*, 2004; Antonijevic, 2006).

In addition, it has been reported that apart from psychosomatic dissimilarities, patients suffering from these two subtypes of MD show distinct differences regarding the organisation of their stress systems. Individuals with the psychotic and melancholic subtype of depression present high corticotropin-releasing hormone (CRH) as well as norepinephrine (NE) states, which are associated with high plasma cortisol (CORT) concentrations (hypercortisolism), while in atypically depressed patients, hypocortisolism was shown to be associated with low CRH and NE levels (Gold *et al.*, 1996; Gold *et al.*, 2002). Thus, in recent years evidence has emerged, that pathological alterations in the stress systems, i.e. the sympathetic nervous system and, in particular, the hypothalamic-pituitary-adrenal axis (HPA) axis, are prominently involved in the development of MD. Hence, a major sector of psychiatric research focuses on the understanding of HPA axis function both, under physiological as well as pathophysiological conditions (Plotsky *et al.*, 1998; Holsboer, 2000; Charmandari *et al.*, 2005; de Kloet *et al.*, 2005; Pariante *et al.*, 2008; Holsboer *et al.*, 2010).

1.3 The physiology of the hypothalamic-pituitary-adrenal axis

The HPA axis is a major neuroendocrine system in mammalian organisms and is involved in the regulation of a plethora of physiological processes implicating developmental, cardiovascular, immunological and metabolic aspects and in particular, the stress response (Tsigos *et al.*, 2002; Charmandari *et al.*, 2005; Melmed *et al.*, 2011). The key elements of the HPA axis are the paraventricular nucleus of the hypothalamus (PVN), the pituitary gland and the cortices of the adrenal glands (Tsigos *et al.*, 2002). The activity of the HPA axis underlies a diurnal as well as ultradian rhythmicity (Lightman *et al.*, 2008; Sarabdjitsingh *et al.*, 2012) which is under control of a pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Engeland *et al.*, 2012). This rhythmic activity of the HPA axis evokes a circadian release of CORT (cortisol in humans, corticosterone in murine rodents), that peaks at the beginning of the activity cycle, thus enabling the organism to cope with everyday physiological demands, and reaches trough levels at the start of the resting period (Melmed *et al.*, 2011). Furthermore, limbic brain structures, which are involved in the processing of environmental stimuli, project directly and/or via neuronal intermediaries to the PVN, thus interconnecting the

'emotional' brain centres and the neuroendocrine stress system (Herman *et al.*, 1997; Herman *et al.*, 2003).

In response to stress, the PVN receives stimulatory inputs from a multitude of brain areas, eventually culminating in the synergistical secretion of CRH and arginine-vasopressin (AVP) from parvocellular neurons of the PVN into the hypophyseal portal system of the median eminence (see Figure 1). Upon receptor binding at the anterior lobe of the pituitary, adrenocorticotropic hormone (ACTH) is released and transported via the systemic blood circulation to the adrenal glands where it binds to its receptor, the melanocortin-2-receptor (MC2R), which induces *de novo* steroidogenesis and release of glucocorticoids (GCs) from the *zona fasciculata* of the adrenal cortex (Simpson *et al.*, 1988; Abdel-Malek, 2001; Tsigos *et al.*, 2002; Charmandari *et al.*, 2005).

Adequate control of GC levels is accomplished by inducing a negative feedback mechanism that restricts the continuous release of HPA secretagogues. In the healthy organism, GCs bind to glucocorticoid (GR) and mineralocorticoid (MR) receptors in the pituitary, the hypothalamus (mainly in the PVN), and the hippocampus. By forming hetero- or homo-dimers, the cytoplasmic corticosteroid receptors act as a transcription factor and translocate to the nucleus to inhibit the expression of AVP, CRH, and ACTH via binding to GC response elements at the genomic DNA (Heitzer *et al.*, 2007; Prager *et al.*, 2009). In addition, non-genomic feedback mechanisms have been reported, involving fast (within minutes) GC-mediated signalling via membrane associated corticosteroid receptors (Tasker *et al.*, 2006; Joels *et al.*, 2008; Groeneweg *et al.*, 2011, 2012). Moreover, a significant impact of the endocannabinoid system in mediating these fast feedback mechanisms has been shown (Steiner *et al.*, 2008b; Tasker *et al.*, 2011).

Thus, it becomes clear, that the HPA axis is a fine-tuned orchestra involving a plethora of signalling molecules and receptors as well as many different regulatory circuits, assuring physiological homeostasis as well as an adequate stress response. Moreover, it is not surprising that an enduring disruption of this homeostatic balance, e.g. by repeated exposure to disproportional physical and/or psychological demands (stressors), can lead to pathophysiological conditions which can eventually culminate in the development of affective disorders such as MD.

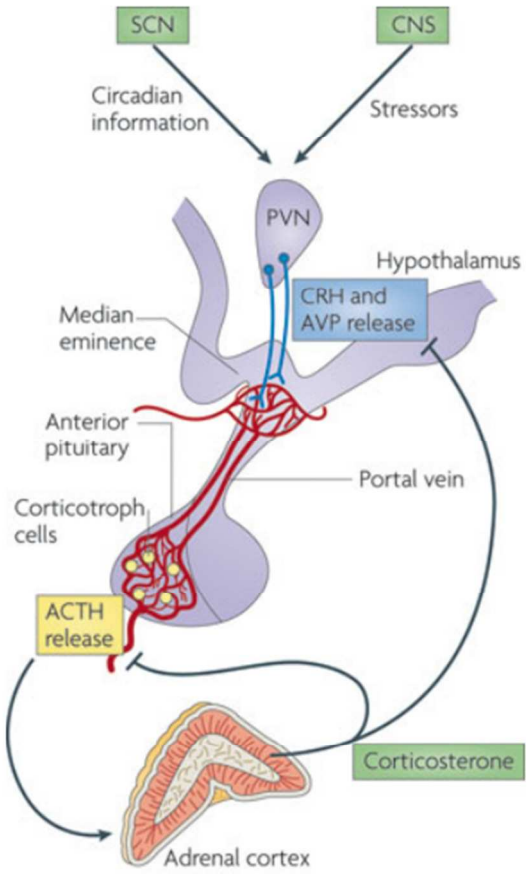

Figure 1: Schematic diagram illustrating the different levels of hypothalamic-pituitary-adrenal (HPA) axis control. In response to circadian stimuli or stress, the paraventricular nucleus of the hypothalamus (PVN) synergistically releases corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) into the portal system of the median eminence. Upon adrenal stimulation by adrenocorticotropic hormone (ACTH) released from the anterior pituitary, glucocorticoids such as corticosterone (CORT) are secreted from the adrenal cortex into the systemic circulation. Subsequently, negative feedback mechanisms at the pituitary and the PVN restrict the release of ACTH and CRH/AVP, respectively. SCN, suprachiasmatic nucleus; CNS, central nervous system. Figure adopted from (Lightman *et al.*, 2010).

1.4 The pathophysiology of the hypothalamic-pituitary-adrenal axis

In 1957, Board and colleagues were the first to report altered plasma CORT levels in depressed patients (Board *et al.*, 1957). Despite the very complex nature of MD, such neuroendocrine symptoms are observed in about 60% of depressed patients, namely hyper- or hypocortisolemia which is associated with a dysregulated HPA axis function (Ising *et al.*, 2005). Interestingly, pathological changes in HPA axis regulation could be found on all levels of the stress system, i.e. in the brain, the pituitary and the adrenals.

In the brain, post mortem studies on severely depressed suicide victims revealed high levels of CRH, concomitant with increased levels of CRH-expressing neurons and a

decreased amount of CRH receptors in brain regions such as the prefrontal cortex (Nemeroff *et al.*, 1988; Austin *et al.*, 2003; Merali *et al.*, 2004; Merali *et al.*, 2006). Furthermore, the number of AVP-containing PVN neurons was increased in the brain of depressed subjects (Merali *et al.*, 2006; Meynen *et al.*, 2006), suggesting an overstimulation of the pituitary by these neuropeptides. Accordingly, CRH levels were increased in the cerebrospinal fluid of MD patients (Nemeroff *et al.*, 1984; Banki *et al.*, 1987; Arato *et al.*, 1989). Moreover, a decreased function of the MR and the GR has been reported in depressed patients, which was associated with a decrease in the GC-mediated feedback inhibition of the PVN due to GC resistance (de Kloet *et al.*, 1998; Pariante *et al.*, 2001).

At the pituitary level, increased expression of ACTH and its precursor, proopiomelanocortin (POMC), have been reported (Charlton *et al.*, 1988). Consistently, increased numbers of ACTH-secretory episodes from corticotropes and subsequent exaggerated adrenal CORT secretion have been observed in depressed patients (Sher, 2004).

On the adrenal level, the increased stimulation by ACTH resulted in hyperplasia as well as hypertrophy of adrenal cells which was associated with an increased release of GCs from the adrenal cortex (Amsterdam *et al.*, 1987; Szigethy *et al.*, 1994; Plotsky *et al.*, 1998). The crucial role of GCs in the aetiology of MD is further supported by clinical observations of subjects suffering from pathophysiological adrenal GC secretion. Patients with Cushing's syndrome, who secrete high amounts of GC due to benign tumours in the pituitary or adrenocortical hyperplasia, show symptoms similar to melancholically depressed subjects. The treatment of Cushing's disease normalised GC levels and psychiatric symptoms were improved (Sonino *et al.*, 1993). In contrast, patients with a inherited or acquired pituitary hypofunction as seen in Addison's disease, resulting in adrenal insufficiency, display symptoms similar to those seen in atypically depressed patients (Melmed *et al.*, 2011).

Furthermore, MD patients commonly show profound alterations in the regulation of the HPA axis. In the combined dexamethasone/corticotropin-releasing hormone (Dex/CRH) test, patients suffering from MD show a decreased HPA axis suppression following an exogenously administered dose of the synthetic GR agonist Dex (Dex non-suppressors). A subsequent stimulation of the pituitary by CRH application, in turn, results in an overshooting CORT secretion, thus revealing a reduced function of the negative GC feedback, i.e. a decreased suppression of ACTH secretion. In contrast, it has been shown

that in patients suffering from post traumatic stress disorder (PTSD) or atypical depression (Kasckow *et al.*, 2001; Rydmark *et al.*, 2006), the GC response after CRH challenge was significantly attenuated compared to healthy controls, due to an increased negative feedback signal on the HPA axis. Therefore, the Dex/CRH test has been suggested to be a suitable surrogate marker for depression. Moreover, it has been shown that the outcome of the Dex/CRH test is a good indicator for the efficacy of antidepressant treatment (Ising *et al.*, 2005) as well as a measure for the risk of relapse at the time of discharge (Zobel *et al.*, 2001).

1.5 Treatment of major depression

Classical treatment approaches of MD focus on the monoaminergic system, since a depletion of monoamines has been reported as an important factor in the development of the disease (Delgado, 2000; Elhwuegi, 2004; Belmaker *et al.*, 2008).

Since the 1950s when monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs) were first discovered by serendipity (Pletscher, 1991), the development of psychopharmaca has steadily improved, especially gaining a better side-effect and safety profile of the drugs. Nowadays, the most common and first-line treatment of MD is a combination of psychotherapy and pharmacotherapy with selective serotonin (5-HT) reuptake inhibitors (SSRIs) such as fluoxetine (Flx) or combined 5-HT noradrenaline reuptake inhibitors (SNRIs) such as venlafaxine. All these classes of drugs have a common mechanism of action. They increase the availability of monoamines in the synaptic cleft by inhibiting the intracellular degradation of monoamines (MAOIs) or by blocking the reuptake of the monoaminergic neurotransmitters from the synaptic cleft back into the presynapse (TCAs, SSRIs, SNRIs), respectively (Nemeroff, 1998). However, as already mentioned the side-effects of pharmacotherapy are manifold and can comprise body weight changes, sexual dysfunction, nausea, constipation, insomnia or cardiovascular toxicity (Vanina *et al.*, 2002; Youdim *et al.*, 2006; Kennedy *et al.*, 2009) which, in turn, cause a high incidence of non-compliance in patients. In addition, approximately 50 % of patients do not respond to classical SSRI treatment. In those cases, therapy is switched to alternative substances (Rubinow, 2006; Maoz, 2007), which increases the risk of toxicity by drug-drug interactions through the inhibition of drug-

metabolising liver enzymes of the cytochrome P 450 family (Gillman, 2007). Hence, it is apparent that there is a need for better and more refined treatment strategies for MD.

However, the improvement of antidepressant drugs turned out to be highly challenging. There is still poor knowledge about how antidepressants exert their therapeutic effects since the biochemical function of these drugs are initiated shortly after their intake (minutes to hours), but an amelioration of psychic and physical symptoms in MD patients is delayed by approximately two to four weeks (Nestler *et al.*, 2002; Schloss *et al.*, 2004). This suggests, that not the accumulation of bioactive monoamines in the brain *per se* but rather functional and structural changes in neuronal networks, i.e. neuronal plasticity, are causative for the antidepressant effects (Schloss *et al.*, 2004).

Indeed, it has been shown that the SSRI Flx increased human hippocampal neurogenesis by activating the GR (Anacker *et al.*, 2011). Consistently, Surget and colleagues showed that Flx treatment initiated hippocampal neurogenesis in chronically stressed mice and thereby restored HPA axis function and negative feedback regulation (Surget *et al.*, 2008; Surget *et al.*, 2011). Furthermore, it has been shown that 5-HT transporter knockout (KO) mice presented an impaired HPA axis activity and negative feedback mechanism, providing evidence for the prominent role of 5-HT in the regulation of the HPA axis (Jiang *et al.*, 2009). In humans, a genetic variation resulting in a short 5-HT transporter gene promoter region was associated with an aberrant regulation of the HPA axis (Gotlib *et al.*, 2008), suggesting the involvement of genetic risk factors in the aetiology of MD (Caspi *et al.*, 2006). Considering the high heritability of MD (Fava *et al.*, 2000; Nestler *et al.*, 2002), it becomes apparent that psychiatric disorders are multidimensional diseases, developing from a combination of multiple factors such as a vulnerable genetic predisposition and environmental stimuli, leading to HPA axis dysregulation and alterations in the monoaminergic system. These factors interact in a highly complex manner to affect the risk of developing MD.

Hence, it becomes evident that, due to the complexity of the underlying biological mechanisms of the disorder, the research in humans is drastically limited and that the use of valid animal models is essential in psychiatric research.

1.6 Animal models in scientific research

Animal models have become an essential and powerful scientific tool in order to shed light on the complex molecular-genetic underpinnings as well as the behavioural endophenotypes of affective disorders such as MD. Mice and rats are frequently used model organisms in biomedical research as they can easily be kept in large numbers, are easy to handle and breed, have short generation times and cause relative low maintenance costs.

Additionally, the mouse genome is completely sequenced and mice can be genetically modified, which makes the mouse an interesting organism for e.g. gene association studies since approximately 99 % of the mouse genes have human counterparts (Tecott, 2003). Consistent with their genomic homologies, humans and mice share numerous features of brain organisation and behavioural responses to environmental stimuli as well as pharmacological agents despite their apparently different physiognomy (Tecott, 2003). Therefore, the mouse has become the favoured model organism for biomedical research in general and specifically in the field of psychiatric diseases.

A good animal model is characterised by its validity at different levels (Willner, 1984, 1995), i.e. the symptom profile (face validity) and the causality of the pathological condition (construct validity). Additionally, the amelioration or attenuation of symptoms by treatments (predicitive validity) should be similar between the model and clinical conditions. Moreover, events that potentially induce the pathology (aetiological validity) should match (Anisman *et al.*, 2005; Schmidt, 2011). Although it is apparent that depressive symptoms such as suicidal tendencies and recurrent thoughts of death cannot be modelled in mice, it is possible to study specific behavioural domains in relation to psychiatric endophenotypes such as anxiety, anhedonia, sleep disturbances, diminished cognitive function or hormonal dysregulations (Gould *et al.*, 2006). Accordingly, different approaches have been taken to induce these endophenotypes in mice thereby forming valid animal models, although none of these animal models is without shortcomings (Anisman *et al.*, 2005). Frequently, rodents are subjected to aversive, environmental stimuli such as chronic mild or chronic stress paradigms, or are exposed to early life stress (maternal or peer separation), which resembles cases of early neglect. However, these paradigms do not take into account the individual's genetic predisposition. Other models use genetically engineered mice with conventional (complete) or conditional (regionally and temporally controlled) gene KOs in order to

investigate the impact of a single gene on the behavioural and physiological endophenotype, thus disregarding the multi-genetic background of psychiatric diseases and the possibility of compensatory mechanisms resulting from the KO. Furthermore, many conventional KOs are lethal in early stages of development thereby prohibiting the manipulation of specific target genes (Cryan *et al.*, 2002; Cryan *et al.*, 2004; Cryan *et al.*, 2005). Therefore, another very promising approach is the use of selective, bidirectional inbreeding paradigms. In this approach, animals are selected for extremes of a specific behavioural phenotype such as anxiety (Krömer *et al.*, 2005; Landgraf *et al.*, 2007), aggressiveness (Veenema *et al.*, 2003), or avoidance behaviour (Steimer *et al.*, 2003). More recent approaches used a neuroendocrine phenotype such as the corticosterone secretion in response to stress as a selection criterion (Touma *et al.*, 2008). Consequently, the animals which show the most extreme phenotypes in either direction are mated, and hence, the shift from the average endophenotype is increased throughout the generations. This approach is of advantage since multiple genetic factors which are associated with the respective phenotype are taken into account and thus, the genetic heritability in the aetiology of psychiatric diseases is considered (Gershon *et al.*, 1976; Bienvenu *et al.*, 2011).

1.7 The 'stress reactivity' mouse model

Using a bidirectional selective inbreeding approach, a new mouse model for affective disorders was established in 2005, in order to model one key endophenotype of MD, a dysregulated HPA axis. This model consists of three mouse lines showing distinct differences in their adrenal CORT secretion in response to stressors, the high (HR), intermediate (IR) and low (LR) stress reactivity line (Touma *et al.*, 2008). Starting from a parental generation of outbred CD-1 mice, the animals were selected according to the outcome of a so-called 'stress reactivity test' (SRT), consisting of a 15-min restraint period and two blood samplings, one immediately before and one right after the restraint stressor (Touma *et al.*, 2008). The animals' CORT increase in response to the SRT served as the selection criterion.This test was repeated with every generation. Males and females of both extremes, i.e. with high or low CORT increase, were mated, creating the HR and LR line, respectively. Additionally, a third line was established showing intermediate stress reactivity, resembling the population mean phenotype. Already in the first

generation, animals of the three breeding lines which were subjected to the SRT displayed significant differences in CORT increase which was even more pronounced in the following generations (see Figure 2).

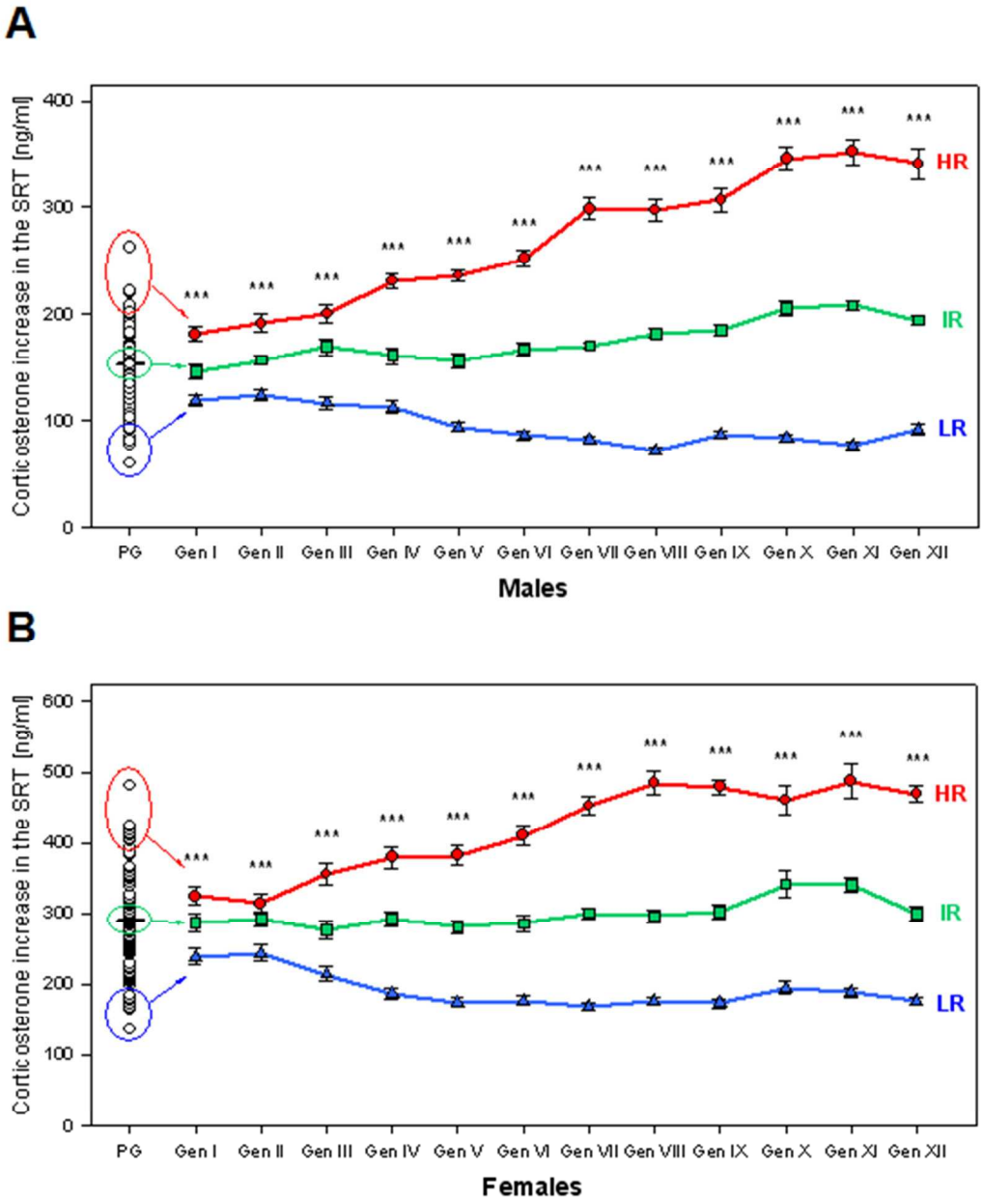

Figure 2: Corticosterone (CORT) increase in the stress reactivity test (SRT) of (A) male and (B) female mice of the CD-1 founder population (parental generation, PG) and the descendent generations (Gen I-XII) of high (HR), intermediate (IR) and low (LR) reactivity breeding line. Already in Gen I, significant differences in the stress-induced CORT increase between the three mouse lines could be observed (KWH-test, all $p \leq 0.001$*). This divergence was further increased in the subsequent generations (Figure adopted from Touma *et al.*, 2008; Touma, 2011).**

The stress reactivity (SR) mouse model has been established as a mouse model for affective disorders such as MD. In addition to the robust differences in plasma CORT concentrations in response to stressors between the three lines (Touma *et al.*, 2008; Touma *et al.*, 2009), elevated CORT levels were also found in the hippocampus (Heinzmann *et al.*, 2010) of HR mice which was associated with cognitive deficits, impaired learning abilities and reduced brain derived neurotrophic factor (BDNF) expression in these animals (Knapman *et al.*, 2010a). Interestingly, increased stress reactivity was also associated with reduced hippocampal activity and neuronal integrity in HR mice (Knapman *et al.*, 2012). Furthermore, HR mice presented a flattened diurnal rhythm of CORT secretion with elevated trough levels compared to IR and LR animals (Touma *et al.*, 2009). It was also shown that motor activity as well as sleep rhythmicity was different between the three lines, with HR mice presenting an elevated number of activity bouts and an increased sleep fragmentation in addition to increased rapid eye movement sleep and decreased slow wave activity compared to IR and LR animals, resembling symptoms of altered sleep architecture observed in melancholically depressed patients (Touma *et al.*, 2009).

Assessing the behavioural phenotype of HR, IR and LR mice, it was shown that animals of the HR line presented a hyper-active coping style in the forced swim test (FST), reflected by an increased struggling and decreased floating behaviour while LR mice displayed a more passive phenotype with decreased struggling and increased floating, which was interpreted as behavioural despair (Touma *et al.*, 2008; Knapman *et al.*, 2010a). Therefore, HR animals present a neuronendocrine and behavioural phenotype which resembles the melancholic or psychotic subtype of MD, while LR mice show features of atypical depession. In addition, animals of the LR line share other similarities to patients suffering from atypical depression, such as increased body weight and elevated social aggression (Touma *et al.*, 2008). Hence, HR and LR animals of the stress reactivity mouse model provide good face and construct validity as a model for melancholic/psychotic and atypical depression, respectively.

1.8 Aims and scope of the thesis

The purpose of the studies presented in this thesis was to characterise the molecular-genetic underpinnings of HPA axis dysregulation in HR, IR and LR mice, mirrored by the differences in stress-induced adrenal CORT secretion. As outlined above, it has been reported, that MD patients show HPA regulatory malfunctions, affecting all levels of this stress hormone system. Hence, we were interested whether the robust neuroendocrine phenotypes of the animals of the SR mouse model are a peripheral phenomenon brought about by the distal branches of the HPA axis such as the pituitary and/or the adrenal. Additionally, we wanted to elucidate whether control centres in the brain, which are known to be involved in HPA axis function and negative feedback regulation such as the hippocampus, the PVN or the prefrontal cortex, are differentially activated in HR, IR and LR animals, thus suggesting a central origin of HPA dysfunction in the SR mouse lines. Since the physiological regulation of the HPA axis is critically dependent on negative feedback mechanisms, and dysregulations of this system in patients suffering from MD are assessed by the Dex/CRH test, we were also interested in adapting the clinical settings of the Dex/CRH test to our experiments and subjecting animals of the SR mouse model to this neuroendocrine test. Furthermore, we aimed to pharmacologically characterise the SR mouse model in order to assess its predictive validity and thus, highlighting the model as a good model for affective disorders.

To this end, we designed a series of experiments to investigate the activity and regulatory functions at the different levels of the HPA axis, i.e. in the brain, the pituitary and the adrenals.

At the adrenal level, we pharmacologically inhibited CORT synthesis and secretion using metyrapone in order to evaluate whether the differences in stress reactivity between the three lines are associated with the divergent coping strategies in behavioural tests. Furthermore, we determined the adrenal weight and assessed the adrenal sensitivity of HR, IR and LR animals towards ACTH. Gene expression analysis in the adrenals was performed to identify the expression pattern of candidate genes critically involved in CORT secretion and steroidogenesis.

At the pituitary level, we assessed ACTH protein abundance and release, both, basal and in response to stress. Furthermore, the gene expression pattern of candidate genes involved in the functional activity of the pituitary was studied.

In the blood, we investigated the role of corticosteroid-binding globulin (CBG), including its expression and release from the liver, since this protein is critically involved in the binding as well as the transport of CORT and regulate the amount of free, biologically active GCs.

Once peripheral aspects of HPA axis dysfunction in the SR mouse lines had been assessed, we addressed the question whether the different behavioural and neuroendocrine phenotypes of the SR mice have molecular correlates in the central nervous system. In the same experimental approach, we aimed to investigate whether these phenotypes are affected by antidepressant treatment.

In two experiments, we chronically treated mice of the three SR lines with the SSRI Flx, which is widely used as a first-line treatment in patients suffering from MD.

In the first experiment, we studied the effect of chronic Flx treatment on the emotional behaviour of the animals. Additionally, we assessed the stress-induced HPA axis reactivity in response to the FST as well as HPA axis regulation in the Dex/CRH test in response to Flx treatment. Moreover, gene expression studies of candidate genes relevant for HPA axis function in the hippocampus and the pituitary of the animals were determined. Furthermore, GR and FK506 binding protein (FKBP51) abundance in the hippocampus was assessed.

In the second experiment, we investigated whether Flx is able to exert beneficial effects on the cognitive performance of HR, IR and LR animals and is able to reverse the spatial learning deficits observed in HR mice in the Y-maze task. Subsequently, we wanted to elucidate whether the central perception and interpretation of stressful stimuli is different between HR, IR and LR animals. To this end, we assessed the stress-induced neuronal activitation in different limbic brain regions by means of *C-fos in-situ* hybridisation, i.e. monitoring the neuronal excitation in brain areas relevant for HPA axis activation and feedback regulation.

The overall aim of this thesis was to gain detailed insights into the molecular-genetic underpinnings of one key endophenotype of MD, a dysregulation of the HPA axis. In a translational experimental approach, we used the SR mouse lines to characterise the interplay of all functional levels of the multidimensional cascade of the HPA axis, thereby contributing to the understanding of the involvement of a dysfunctional stress hormone system in the aetiology of affective disorders such as MD.

2 Animals, material and methods

2.1 Animals

In all studies, young adult male mice derived from generation XVI to XX of the SR mouse model were used. After weaning, the mice were housed in groups of four animals in transparent polycarbonate cages (standard Makrolon cages type III, Bayer MaterialScience, Leverkusen, Germany; 38 x 22 x 15 cm) with wood chips as bedding and wood shavings as nesting material. At the age of about eight weeks, plasma CORT concentrations were assessed in all animals by means of the SRT (Touma *et al.*, 2008). At least ten days before performing the experiments of this study, animals were single housed by transferring them into smaller cages (standard Macrolon cages type II, Bayer MaterialScience; 23 x 16 x 14 cm). The mice were aged three to five months during all experiments. Housing and experimental rooms were kept under standardised laboratory conditions (12/12 h light/dark cycle, lights on 8:00 h; temperature: 22±1 °C; relative humidity: 55±10 %). Commercial mouse diet (Altromin No. 1324; Altromin GmbH, Lage, Germany) and tap water were available *ad libitum*. The presented work complies with the current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86 / 609 / EEC). All experiments were announced to the appropriate local authority and were approved by the Animal Welfare Officer of the Max Planck Institute of Psychiatry (Az. 55.2-1-54-2531-64-07).

2.2 Neuroendocrine tests

2.2.1 The 'stress reactivity test'

In order to test the reactivity of the HPA axis, the SRT was performed. This test is routinely carried out in all animals of the SR mouse model at the age of about eight weeks and consists of a 15-min restraint period, corresponding to an acute, moderate psychological stressor and two blood samplings drawn from the animals' ventral tail vessel by means of a small incision immediately before and right after the restraint period, with the first blood sampling not exceeding 2 min after touching the cage, i.e. to rule out acute effects of handling on the first, baseline blood sample. The animals were

restrained in a 50 ml Falcon tube (Sarstedt, Nürnbrecht, Germany) with a ventilation hole at the conical tip of the tube and a hole for the tail in the lid (see Figure 3). In order to shield the mice from external cues and additional stress other than the confinement, the tubes were covered with non-transparent aluminium boxes during the restraint period. Blood samples were collected in EDTA-coated tubes (Microvette, Sarstedt, Nürnbrecht, Germany), centrifuged at 4000 *x g* for 10 min and plasma CORT concentrations were determined by means of a radioimmunoassay (see 2.4.3.1). By substracting the plasma CORT concentrations of the first blood sample from the restraint stress-induced plasma CORT values of the second sample, the CORT increase in response to the stressor was calculated for each animal, which served as neuroendocrine marker for the selective breeding approach of the SR mouse model (Touma 2008).

Figure 3: Mouse being restraint during the 'stress reactivity' test (SRT).

2.2.2 The combined dexamethasone/corticotropin-releasing hormone test

In order to assess HPA axis activity and regulation in animals of the SR mouse model, we performed a combined Dex/CRH test. Therefore, we adapted the Dex/CRH test, which is widely used in clinical settings (Bardeleben *et al.*, 1989; Heuser *et al.*, 1994; Hatzinger *et al.*, 1996; Ising *et al.*, 2007) to mice. In humans, as well as in rats, blood samples are repeatedly drawn via a venous catheter to allow the determination of plasma ACTH and CORT concentrations over a particular period of time after CRH stimulation. Such an approach is not feasible in mice due to their small body size. Repeated blood sampling by means of incisions in the ventral tail vessel in mice of the SR mouse model are critical, since every sampling event would further activate the HPA axis, thus excluding unbiased neuroendocrine analysis. To avoid this, we performed the Dex/CRH test with slight modifications, i.e. only one blood sample was collected after Dex suppression and CRH challenge, respectively. A reference blood sample was collected by an incision in the ventral tail vessel at 15:00, three days prior to the test. On the experimental day, HR, IR

and LR mice were injected intraperitoneally (i.p.) with a low dose of Dex (0.05 µg/g BW; ratiopharm GmbH, Ulm, Germany) at 9:00, i.e. during the trough of the circadian CORT release, in order to suppress HPA axis activity. It should be noted that we chose a low dose of Dex to avoid (i) an overstimulation of the Dex-mediated negative feedback and (ii) a Dex surge in the brain, since this synthetic GC is restricted from entering the brain under physiological conditions (Schinkel, 1997). At 15:00, a second blood was drawn from the ventral tail vessel by means of a tail nick in order to analyse the CORT-suppressive effect of Dex in the plasma of the animals, followed by an immediate CRH injection (0.15 µg/g BW i.p.) to stimulate HPA axis activity. Thirty minutes later the mice were sacrificed and trunk blood was collected in EDTA-coated tubes (KABE Labortechnik GmbH, Nürnbrecht-Elsenroth) equipped with 10 µl of the protease inhibitor Trasylol (Bayer Vital GmbH, Leverkusen, Germany). All other blood samples were collected in EDTA-coated tubes (Sarstedt, Nürnbrecht, Germany), centrifuged for 10 min (4000 *x g* at 4 °C) and plasma CORT concentrations were determined (see 2.4.3.1).

2.2.3 The adrenal sensitivity test

To investigate whether the distinctly different neuroendocrine phenotypes, i.e. adrenal CORT secretion in response to stressors, of HR, IR and LR mice are brought about by differences in the sensitivity of the adrenals of the animals, an adrenal sensitivity test was performed. In this test, animals of all three lines received a high dose of Dex (4 µg/g BW) at 9:00 with the aim of suppressing the release of ACTH via GR-mediated negative feedback mechanism at the level of the pituitary, thus minimising masking effects of endogenous ACTH on adrenal CORT release. Six hours after the Dex injection (15:00), when maximal suppression of endogenous ACTH and CORT was reached (Keller-Wood 1984, Steimer 2007), a blood sample was collected by means of a tail nick in order to verify the ACTH- and CORT-suppressive effect of Dex. Subsequently, animals were injected with either vehicle (Ringer solution, Berlin-Chemie AG, Berlin, Germany) or one of two doses of ACTH (0.1 ng/g or 1 ng/g BW, Synacthen®, Defiante Farmacêutica, S.A., Funchal, Portugal) diluted in vehicle to stimulate CORT secretion from the adrenal cortex. Thirty minutes after the injection, animals were deeply anaesthetised (Isoflurane, Forene, Abbott GmbH, Wiesbaden, Germany), sacrificed by decapitation and trunk blood was collected in EDTA-coated tubes (KABE Labortechnik GmbH, Nürnbrecht-Elsenroth) equipped with 10 µl of the protease inhibitor Trasylol (Bayer Vital GmbH, Leverkusen,

Germany). The blood samples were centrifuged for 10 min (4000 *x g* at 4 °C) and plasma was transferred into fresh tubes and frozen at -20 °C. Plasma ACTH concentrations were determined from the blood sample taken 6 h after the Dex injection (see 2.4.3.2) to confirm the Dex-mediated ACTH suppression. Furthermore, plasma CORT concentrations were analysed in all collected blood samples (see 2.4.3.1).

2.3 Behavioural tests

2.3.1 Open field test

The Open Field (OF) test is based on evoking a conflict between the animal's exploratory drive and its evolutionary innate fear of novel and open areas. The OF apparatus consists of a black, open topped PVC cylinder (60 cm in diameter, 40 cm in height) mounted on a grey PVC floor plate (see Figure 4). The circular test area was divided into a more aversive, inner zone (30 cm in diameter) and a more protective, ring-shaped outer zone near the walls of the OF. The apparati were placed into test chambers surrounded on three sides by black PVC walls, the fourth side could be closed by a black curtain to prevent the experimental animals from visual or auditory cues. In all experiments, the test apparati were dimly and evenly illuminated with 15 lux, thereby reducing the averseness of the test situation since we were not interested in the assessment of anxiety-related behavioural parameters, but rather in the exploratory drive and locomotor activity of the mice. During the 10-min test period, the animals' total distance of travelling, the time within each zone, as well as the ratio between the path length in the inner and outer zone were automatically assessed utilising the ANY-Maze Video Tracking Software (version 4.72, Stoelting Co., Wood Dale, IL, USA). After testing an animal, the OF was thoroughly cleaned with soapy water and 70% ethanol.

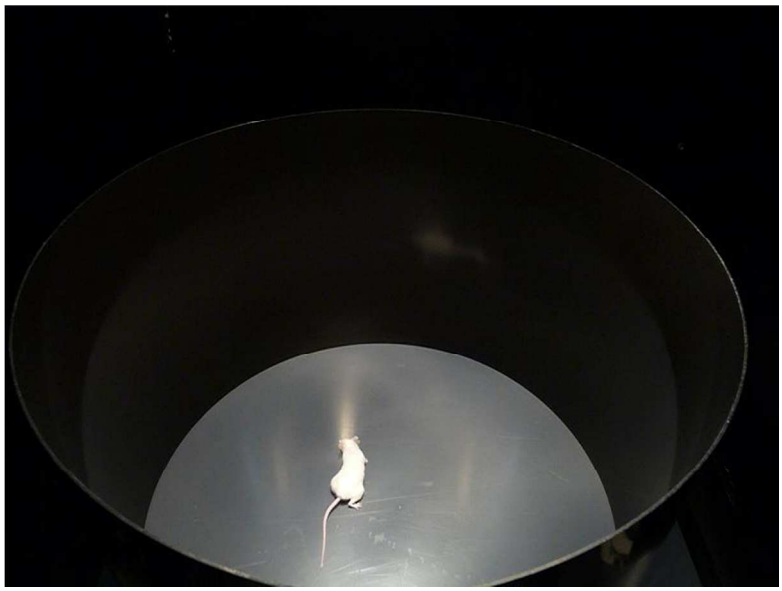

Figure 4: Experimental setup of the Open Field (OF) test.

2.3.2 Forced swim test

The Forced Swim Test (FST) is widely used in pharmacological research using mice and rats to screen pharmaceutical compounds for their antidepressant activity. In the FST, animals are subjected to a psychological (inescapable), as well as physiological (swimming), stressful situation in order to monitor their depression-like behaviour and individual stress-coping style, respectively. This paradigm is especially demanding for mice since they rarely swim in their natural environment. Being placed in a glass beaker (12 cm in diameter, 23.5 cm in height) filled two thirds with 23 °C warm water (see Figure 5) and unable to touch the bottom with their hind paws or tail, the mice actively try to escape from the situation by attempting to climb out of the tank (struggling), followed by periods of active swimming. Eventually, the animals adopt immobile postures, so called floating (defined as complete immobility of the animals extremities excluding small movements to keep balance in the water), with the amount of immobility representing depression-like behaviour or behavioural despair. In our studies, the duration of struggling, swimming and floating behaviour during a 6-min FST period was scored by a trained observer blind to line and treatment using EVENTLOG software (EVENTLOG Event Recorder, version 1.0, EMCO software).

Figure 5: Experimental setup of the Forced Swim Test (FST).

2.3.3 Y-Maze test

To assess the spatial, hippocampus-dependent learning and memory of the experimental animals, the Y-Maze test was used. The Y-Maze apparatus used in our tests consisted of three similarly shaped arms made of grey PVC, each 11 cm wide and 30 cm long, surrounded by 15 cm high walls and interconnected through a triangular central zone (see Figure 6). To facilitate the mice' orientation in the maze, the wall of each arm was tagged with a distinctive symbol in the shape of a bar, a triangle or a plus, respectively. During the 10-min acquisition phase, one arm was blocked by a grey PVC inlay, leaving two arms for the mice to explore. To avoid a preference for one arm during the acquisition phase, the animals were first placed into the central zone facing the partition wall. After returning the animals for 1 h to their home cages (inter-trial interval), they were placed back into the test apparatus (retrieval phase), this time with the opportunity to explore all three arms of the maze. The retrieval phase lasted for 5 min. The apparati were placed in test chambers surrounded by black PVC walls and a black curtain to avoid additional distal orientation cues apart from the proximal cues positioned on the Y-Maze walls. After each animal and each trial the Y-Maze was thoroughly cleaned with soapy water and 70 % ethanol to prevent mutual interference of the test animals.

The retrieval phase of each animal was analysed regarding the time spent in each of the three arms using ANY-Maze Video Tracking Software (version 4.72, Stoelting Co., Wood Dale, IL, USA). If the experimental animals were able to discriminate between the

familiar arms, i.e. the two arms open during the acquisition phase, and the novel arm, i.e. the newly opened arm during the retrieval phase, this was reflected by an extended amount of time spent in this new arm. In order to counterbalance differences in locomotion and therefore in total time spent in the respective arms, the mean percent time spent in the familiar arms was compared to the percent time spent in the novel arm.
Additionally, a 'discrimination ratio' (% time spent in the novel arm/familiar arms) was calculated reflecting the accuracy of the animals' memory performance. The higher the ratio, the more accurately the animals were assumed to remember the 'old', previously explored arms.

Figure 6: Experimental setup of the Y-maze test.

2.4 Molecular biology techniques

2.4.1 Western blot

In order to analyse hippocampal, pituitary and liver homogenates as well as plasma samples from the experimental animals regarding potential differences in protein expression, semi-quantitative protein analysis was performed using Western blotting.
Whole cell protein was extracted from the tissue (hippocampus and pituitary) using an automatic tissue homogeniser (VDI 12, VWR International GmbH, Darmstadt, Germany) in 150 µl homogenisation buffer (H-buffer). After homogenisation, 50 µl of extraction buffer (E-buffer) was added to the solution and the samples were sonified for

approximately 20 s (Branson Sonifier 250, Schwäbisch Gmünd, Germany). A detailed overview of all used buffers is outlined in the appendix (Table 1). The samples were subsequently incubated on ice for 15 min, centrifuged for 30 min (11350 *x g* at 4 °C) and the supernatant, holding the cell protein, was transferred into fresh tubes. The pellet was discarded. An aliquot of 4 µl was pipetted and further diluted 1:10 with distilled water for protein concentration analysis (see below).

Liver samples were processed as described above with slight modifications. Protein was extracted from liver samples using radioimmunoprecipitation assay (RIPA) buffer. After homogenisation and sonification, the samples were incubated for 30 min to accomplish cell lysis. After centrifuging 1 ml of the homogenate for 30 min (11350 *x g* at 4 °C), the supernatant was transferred into a new tube. An aliquot of 4 µl was pipetted and further diluted 1:100 with distilled water for protein concentration analysis.

Plasma samples were directly diluted 1:10 with distilled water in order to determine the plasma protein concentration.

The protein concentration of all tissue and plasma samples was detected using a BCA protein assay according to the manufacturer's protocol (Thermo Scientific, Rockford, USA). The samples were analysed in triple estimation by photometric measure (Dynatech MR 7000, Denkendorf, Germany) at a wavelength of 550 nm and the protein concentration was set to 0.5 µg/µl to 2 µg/µl with a final volume of 100 µl to 200 µl per sample, always containing protein loading buffer (PLB, see Table 1 in the appendix) in a 1/5 ratio of the final volume.

Ten microlitres (5 µg to 20 µg) of protein solution was loaded and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 10 % or 12 %, depending on the size of the protein of interest) for 90 min at 120 V and transferred (60 min at 400 mA) onto a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). To block unspecific binding sites, the membranes were incubated for 1-2 h in 5 % (w/v) fat free milk solution (Carl Roth GmbH, Karlsruhe, Germany) and incubated overnight at 4 °C in the respective first antibody in 2.5 % Tris-buffered saline and Tween 20 (TBST)/milk solution. After the membranes were rinsed with TBST on the next day, the incubation time with the respective horseradish peroxidase-conjugated secondary antibody was 1 h at room temperature, followed by washing with TBST. A detailed overview of the used primary and secondary antibodies is outlined in Table 1. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and Transferrin (TF) were used as standard proteins for hippocampal, pituitary and liver samples or plasma samples,

respectively. After being incubated in enhanced chemiluminescence (ECL) solution (see appendix, Table 2), the membranes were either (i) exposed to a ECL sensitive film (GE Healthcare, Amersham Hyperfilm™ ECL, Little Chalfont, UK), scanned and the signal intensity of the protein band (optical density) was assessed by Image J software (version 1.44p, National Institute of Health, USA, http://imagej.nih.gov/ij/) or (ii) the protein bands were detected by the chemoluminescence-sensitive camera of the ChemiDoc MP imaging system (Bio-Rad Laboratories GmbH, Munich, Germany) and analysed by Image Lab software (version 4.0.1 build 4, Bio-Rad Laboratories GmbH, Munich, Germany).

Table 1: List of antibodies with their respective specifications and dilutions used for Western blot analysis. kDa, kilodalton.

Target protein	Abbreviation	Protein size (kDa)	Primary antibody		Secondary antibody	
			specification	dilution	specification	dilution
Glucocorticoid Receptor	GR	95	GR (M-20) rabbit polyclonal antibody (Santa Cruz, sc 1004)	1:500	goat anti-rabbit horseradish peroxidase conjugated antibody (Sigma, A9169)	1:30000
FK506 binding protein 51	FKBP51	51	FKBP51 (F-14) goat polyclonal antibody (Santa Cruz, sc 11518)	1:500	donkey anti-goat horseradish peroxidase conjugated antibody (Santa Cruz, sc2056)	1:10000
Adrenocortico-tropic hormone	ACTH	29	ACTH rabbit polyclonal antibody (Abcam, ab 74976)	1:3000	goat anti-rabbit horseradish peroxidase conjugated antibody (Sigma, A9169)	1:30000
Glyceraldehyde-3-phosphate-dehydrogenase	GAPDH	37	GAPDH (A-3) mouse monoclonal antibody (Santa Cruz, sc 137179)	1:2000	goat anti-mouse horseradish peroxidase conjugated antibody (Sigma, A9917)	1:15000
Corticosteroid binding globulin	CBG	55	hCBG rabbit antibody (Affiland, CB017H)	1:2000	goat anti-rabbit horseradish peroxidase conjugated antibody (Sigma, A9169)	1:30000
Transferrin	TF	79	Transferrin (M-70) rabbit polyclonal antibody (Santa Cruz, sc 30159)	1:1000	goat anti-rabbit horseradish peroxidase conjugated antibody (Jackson, 111-035-003)	1:10000

2.4.2 Quantitative real-time PCR

2.4.2.1 RNA extraction and reverse transcription

RNA extraction from hippocampal, pituitary and adrenal tissue was performed using guanidinium thiocyanate-phenol-chloroform extraction (=TRIzol method). To this end, the tissue was homogenised in TRI reagent (1 ml TRI reagent per 50 mg tissue, Sigma-Aldrich, Steinheim, Germany) with a plastic pestle. After adding 200 µl chloroform (Carl Roth GmbH, Karlsruhe, Germany) per ml TRI reagent, the solution was incubated for 5 min at room temperature and subsequently centrifuged for 15 min (11350 *x g* at 4 °C). The supernatant was transferred into a fresh tube, 500 µl isopropanol (Carl Roth GmbH, Karlsruhe, Germany) per ml TRI reagent was added and the samples were incubated over night at -20 °C for RNA precipitation. The next day, samples were centrifuged for 10 min (11350 *x g* at 4 °C), the supernatant was discarded and the pellet was washed three times with 70 % ethanol, air dried and redissolved in approximately 50 µl of H_2O. Total RNA concentration was analysed using the NanoDrop photometer (Implen GmbH, Munich, Germany) and was set to 50 ng/µl. In order to control for RNA quality, the absorbance ratio of the RNA at a wavelength of 260 nm and 280 nm (A260/A280) was calculated. Values higher than 1.8 were accepted, indicating good RNA quality. A total amount of 500 ng of RNA per sample was reverse transcribed into complementary DNA (=cDNA) by means of a high-capacity cDNA reverse transcription kit (Applied Biosystems, Darmstadt, Germany) following the manufacturer's protocol (thermal cycler settings: 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C, ∞ at 4 °C) using a Peqlab thermal cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany). The cDNA concentration was analysed by the NanoDrop photometer (Implen GmbH, Munich, Germany) and set to 150 ng/µl.

2.4.2.2 Quantitative real-time PCR analysis

The quantitative real-time PCR (qPCR) is based on the simultaneous amplification and quantification of PCR products allowing quantitative conclusions regarding expression patterns of a gene of interest. cDNA gene transcripts were analysed using the Qiagen QuantiFast SYBR Green PCR kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Samples were prepared as follows: 5 µl QuantiFast SYBR Green, 1 µl forward primer (10 pM), 1 µl reverse complementary primer (10 pM), 2 µl cDNA and 1 µl nuclease-free water. Additionally, one control sample was prepared,

containing water instead of cDNA (negative control). Experiments were performed in duplicates using the Roche Lightcycler® 2.0 instrument (Roche Diagnostics, Mannheim, Germany) under following conditions: initial preincubation phase (95 °C 10 min), followed by 40 cycles of repetitive denaturation (95 °C 10 s) and combined annealing and extension (60 °C 30 s). After each cycle fluorescence was assessed. A melting curve was created at the end of each run in order to denature all PCR products (50-95 °C with 0.1 °C/s temperature increase) and control for their quality. Primer specificity was assured when an equal melting temperature of the amplicon was detected. TATA-binding protein (TBP) and Hypoxanthine-Guanine Phosphoribosyltransferase (HPRT) were used as housekeeping genes. A detailed list of all applied oligonucleotide primers is outlined in Table 2. Crossing points (CP) were calculated with the LightCycler® Software 4.05 (Roche Diagnostics GmbH, Mannheim, Germany) using the absolute quantification fit points method. Threshold and noise band were set to the same level in all compared runs. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ algorithm (Livak *et al.*, 2001). The efficiency of each run was used as calculated from an internal standard curve prepared from a dilution series (1/1, 1/5 and 1/10) containing an aliquot of cDNA from each sample. CPs were normalised to the mean of the two housekeeping genes TBP and HPRT and all values were calculated to the relative expression mean of IR animals.

Table 2: List of oligonucleotides (primers) used for qPCR analysis. For full gene names see 2.5.2.1.3. All primers were purchased from Sigma Aldrich. T_m, melting temperature; bp, base pairs

Target gene	Orientation (5' - 3')	Sequence	T_m [°C]	Product length [bp]
Nr3c1	forward	CAA GGG TCT GGA GAG GAC AA	64.2	220
	reverse	TAC AGC TTC CAC ACG TCA GC	64.1	
Nr3c2	forward	GTG TGT GGA GAT GAG GC	57.2	155
	reverse	GGA CAG TTC TTT CTC CGA AT	59.6	
Fkbp4	forward	CAA CGC CAC ACT TGT ATT TGA	63.5	143
	reverse	CTT CCA CCA TAG CAC CAT CAT	63.7	
Fkbp5	forward	AGA ATC AAA CGG AAA GGC GAG	66.3	103
	reverse	CTC GGC AAT CAA ATG TCC TTC	65.6	
Abcb1a	forward	GAA GAT CAA CTC GCA AAA GCA T	64.3	161
	reverse	GGC ACA GAA TAT ACC AAC CAC A	63.4	
Abcb1b	forward	GAA CAT TAC TTC CCC TCT TGA T	59.9	130
	reverse	CTG TTG CTG ATG ATC AGA GTA C	59.0	
Crhr1	forward	GGT CCT GCT GAT CAA CTT TA	59.2	152
	reverse	ACA TGT AGG TGA TGC CCA	59.9	
Cfl1	forward	ATG ATC TAT GCC AGC TCC AAG	63.0	127
	reverse	CGC TGC CAC CTA GTT TCT CT	63.3	
Gilz	forward	GTG GCC CTA GAC AAC AAG ATT	61.9	122

	reverse	GAG TTC TTC TCA AGC AGC TCA	61.5	
Pomc	forward	GAA GAT GCC GAG ATT CTG CT	63.4	222
	reverse	TTT TCA GTC AGG GGC TGT TC	64.1	
Star	forward	GCT GGA AGC TCC TAT AGA CAT	59.0	191
	reverse	GAC AGC TCC TGG TCA CTA TAG	58.0	
Mc2r	forward	CCA AGG AGA GGA GCA TTA TTG	62.5	141
	reverse	GTT TGC CGT TGA CTT ACA GAA	61.7	
Tbp	forward	CCC CCT TGT ACC CTT CAC C	65.4	285
	reverse	TGG ATT GTT CTT CAC TCT TGG	65.3	
Hprt	forward	GTT GGA TAC AGG CCA GAC TTT GT	65.1	225
	reverse	CCA CAG GAC TAG AAC ACC TGC TA	64.3	
Th	forward	CTG TGG AGT TTG GGC TGT GTA	65.0	160
	reverse	GTT TGA TCT TGG TAG GGC TGC	64.7	
Pnmt	forward	GCT GCA TGG CAC AAG TCT TTG	68.0	198
	reverse	CAC TCC AGT CAA AGG CTC CTG	66.2	

2.4.3 Radioimmunoassays

2.4.3.1 Corticosterone radioimmunoassay

Plasma CORT was measured using a commercially available CORT radioimmunoassay (RIA) kit (DRG Instruments GmbH, Marburg, Germany) following the manufacturer's protocol with slight modifications. In order to increase the applicable sample size of the RIA, half of the recommended volume of all reagents was used. Ten microlitres of plasma of each sample were applied to the assay and all samples were analysed in duplicate. Furthermore, the same "pool" samples were run in every assay as an internal standard to control for intra- and interassay variations. The CORT-RIA is based on the competitive binding of radiolabelled ^{125}Iodine (I)-CORT and non-radioactive CORT in the plasma samples for a limited number of accessible binding sites of a specific antibody. The higher the content of unlabelled antigen in the plasma samples, the less ^{125}I-CORT can be bound by the applied antibody. Hence, the amount of the ^{125}I-CORT is negatively proportional to the amount of unlabelled plasma CORT in the samples. To isolate the antibody-CORT-complex in the solution, a second antibody is applied and centrifuged, the unbound CORT is decanted and the radioactivity in the pellet is measured by a gamma counter (Wallac Wizard 1470 automatic gamma counter, Perkin Elmer life science, Rodgau, Germany). CORT concentrations were calculated by means of a standard curve. Double estimations were accepted if the coefficient of variation was below 10 %. The detection limit of the CORT-RIA was 1 ng/ml.

2.4.3.2 Adrenocorticotropic hormone radioimmunoassay

Plasma ACTH was measured using a commercially available ACTH-RIA kit (DRG Instruments GmbH, Marburg, Germany) following the manufacturer's protocol. 50 µl of plasma of each sample was analysed in duplicate if not otherwise indicated. Double estimations were accepted if the coefficient of variation was below 15 %. The ACTH-RIA is based on the same principle as the CORT-RIA (see 2.4.3.1) using the double antibody-antigen technique. The detection limit of the ACTH-RIA was 7 pg/ml.

2.4.4 *In-situ* hybridisation

The *in-situ* hybridisation (ISH) technique allows the detection of local expression patterns of a gene of interest in target tissues and therefore provides information of its distribution or involvement in particular brain functions and processes. In order to assess mRNA expression levels of *C-fos* as a marker for neuronal activity in HR, IR and LR mice, ISH was performed in different brain areas (see Figure 7) known to be critically involved in HPA axis regulation (prefrontal cortex, paraventricular nucleus of the hypothalamus, basolateral amygdala, dorsal and ventral hippocampus). An exon-spanning ribonucleotide probe for *C-fos* was designed using Primer3 software (http://primer3.sourceforge.net/releases.php), spanning from exon 1 to exon 4 of the murine *C-fos* gene, with a total length of 480 base pairs (forward primer 5'-3': ATGGGCTCTCCTGTCAACAC; reverse primer 5'-3': GGCTGCCAAAATAAACT CCA). Frozen brains were mounted in a cryostat (Cryo-Star HM 560 M, MICROM GmbH, Waldorf, Germany) and cut in 18 µm slices in the coronal section with a knife and sample temperature of -15 °C. Brain slices were adhered to superfrost plus slides (VWR, Darmstadt, Germany), shortly dried and stored at -80 °C until further analysis.
ISH using a ^{35}S-UTP-labelled riboprobe for *C-fos* was performed as described earlier (Schmidt *et al.*, 2007; Scharf *et al.*, 2011). Briefly, brain sections were fixed in 4 % paraformaldehyde, washed and acetylated in 0.25 % acetic anhydride. Subsequently, slides were dehydrated in ascending concentrations of ethanol, degreased with chloroform and air dried. After adding 100 µl of hybridisation buffer per slide, containing 0.65 to 1.7x10^6 counts per minute of ^{35}S-UTP-labelled riboprobe, the slides were coverslipped and incubated overnight at 55 °C. The next day, coverslips were removed, the slides were washed and incubated in RNase A solution. Finally, the sections were desalted and dehydrated and exposed to radiation-sensitive films (Kodak Biomax MR films, Eastman

Kodak Co., Rochester, NY, USA) for 5 days except for the ventral hippocampus, which was exposed for 12 days due to a lower signal intensity. The films were scanned and the *C-fos* signal intensity (optical density) was assessed by Image J software (version 1.44p, National Institute of Health, USA, http://imagej.nih.gov/ij/). For each animal, bilateral structures of one slice were calculated, substracting the background from the value. The background signal was measured in structures not expressing *C-fos*.

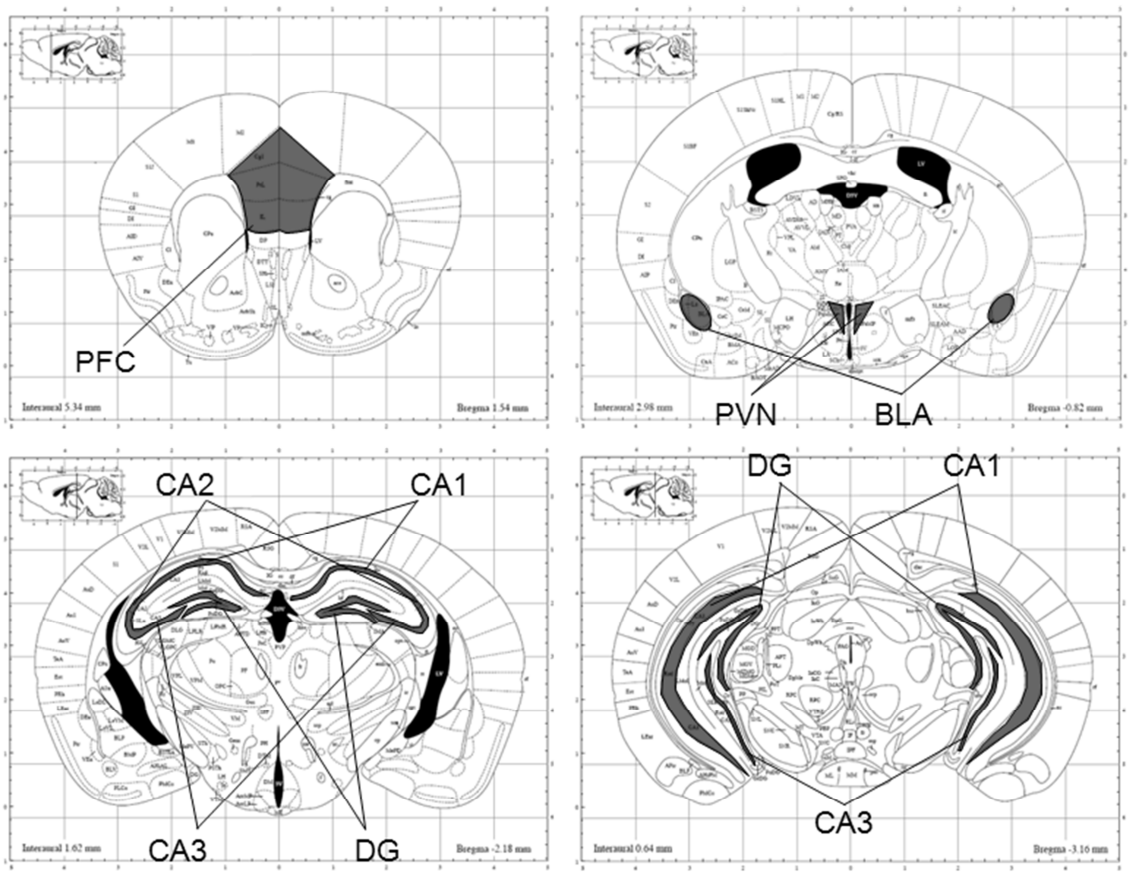

Figure 7: Schematic overview of the anatomical location of the brain regions investigated for *C-fos* expression by *in–situ* hybridisation. Upper left: prefrontal cortex (PFC). Upper right: basolateral amygdala (BLA) and paraventricular nucleus of the hypothalamus (PVN). Lower left: dorsal hippocampus including the cornu ammonis 1-3 subfields (CA1, CA2, CA3) and the dentate gyrus (DG). Lower right: ventral hippocampus. Figures adopted from (Franklin *et al.*, 2001).

2.5 Experimental design

2.5.1 Peripheral regulation of the hypothalamic-pituitary-adrenal axis

2.5.1.1 Experiment 1: Inhibition of corticosterone secretion by the 11β-hydroxylase inhibitor metyrapone

2.5.1.1.1 Introduction

Adrenal cortex steroid biosynthesis is stimulated by ACTH released from the anterior pituitary and performed by multiple enzymatic steps, converting cholesterol into different steroid hormones, among them CORT (Jefcoate *et al.*, 1992; Miller *et al.*, 2011). It has been repeatedly reported that CORT, either endogenously secreted in response to stress or exogenously administered, has the ability to shape behavioural phenotypes, e.g. in memory tasks (de Quervain *et al.*, 1998; Akirav *et al.*, 2004), stress-coping paradigms (Baez *et al.*, 1994) or anxiety-related tests (Calvo *et al.*, 1998). In the same studies, it was shown that metyrapone was able to reverse the CORT-induced behavioural effects in the experimental animals, suggesting antidepressant properties of this pharmaceutical compound. Metyrapone was originally synthesised by Allen and Rencze in 1958 (Jenkins *et al.*, 1958). It inhibits the enzymatic activity of cytochrome P450 11β-hydroxylase, the final enzymatic step of CORT production (Yanagibashi *et al.*, 1988), leading to a dose-dependent decrease in CORT secretion from the adrenal cortex (Jenkins *et al.*, 1958). However, the induction and depletion of CORT synthesis or secretion, respectively, is not an intrinsic mechanism but is functionally interwoven with the biosynthetic pathway of catecholamines in the adrenal medulla (Axelrod *et al.*, 1984). It has been reported that glucocorticoids can modulate the enzymatic production of the catecholamines adrenaline and noradrenaline (Axelrod *et al.*, 1984) and *vice versa* (Bornstein *et al.*, 1999), which are originally released from the adrenal medulla in response to sympathetic nervous system activity via the splanchnic nerve (Engeland, 1998). The rate-limiting step of catecholamine biosynthesis in the adrenal medulla is the conversion of tyrosine into Dopa by tyrosine hydroxylase (TH), whereas the enzyme phenylethanolamine-N-methyltransferase (PNMT) synthesises adrenaline from noradrenaline. Collectively, glucocorticoids and catecholamines have complementary actions throughout the body, including energy metabolism and maintenance of blood pressure (Ulrich-Lai *et al.*, 2009), respectively, thus playing a pivotal role in the physiological stress response.

2.5.1.1.2 Objective of the experiment

In this experiment, we were interested in investigating the behavioural and physiological effects of CORT in animals of the SR mouse model. It has been shown that in these animals, which are selectively bred for extremes in stress reactivity, the HR line has a significantly increased secretion of CORT from the adrenal cortex compared to LR animals in response to stress, concomitantly showing differences in stress-coping strategies, e.g. in the FST. HR mice display a hyperactive coping, i.e. more struggling, while LR animals show more passive behaviour, i.e. reduced mobility in this test (Touma *et al.*, 2008). Based on this observation, we investigated (i) whether pharmacological inhibition of CORT synthesis and secretion by metyrapone affects the behavioural phenotype of the animals of the SR mouse model and (ii) whether the three mouse lines of the SR mouse model show differences in the gene expression patterns of TH and PNMT in the adrenals according to their distinctly different CORT release in response to stress. Furthermore, we assessed adrenal mRNA expression of the ACTH receptor (melanocortin 2 receptor, *Mc2r*) as well as the steroid acute regulatory protein (*Star*), which are both critically involved in the release of CORT from the adrenals and in the induction of steroidogenesis in the adrenal cortex, respectively (Lefkowitz *et al.*, 1971; Xia *et al.*, 1996; Stocco, 2001; Miller, 2007).

2.5.1.1.3 Experimental setup

Adult males of the HR, IR and LR line derived from Gen XVI of the SR mouse model were acutely injected intraperitoneally (i.p.) with a dose of 50 µg/g BW metyrapone dissolved in vehicle (Ringer solution containing 5 % ethanol). Control animals received just the vehicle solution. At this dose metyrapone produces a greater than 50 % reduction in plasma CORT within the first hour after injection, which gradually returns to baseline within 20-24 h (Jenkins *et al.*, 1958; Roberts *et al.*, 1984; Piazza *et al.*, 1994). Moreover, we performed dose-finding studies, which confirmed the effectiveness of this particular dose on stress-induced CORT levels in a separate batch of HR, IR and LR mice. One hour after the injection, two behavioural tests were performed consisting of a 10-min OF test, directly followed by a 6-min FST in order to assess locomotor activity and stress-coping behaviour, respectively. Additionally, plasma CORT concentrations were determined. To this end, a blood sample of the yet untreated animals (reference) was obtained three days before the start of the experiment. To assess the stress-induced rise in plasma CORT, a second blood sample was collected immediately after the FST (trunk blood sample).

Animals were sacrificed by decapitation, trunk blood was collected and both adrenals were dissected, cleared from fat tissue and quickly frozen on dry ice until further analysis. RNA was isolated from the adrenals and gene expression analysis was performed using qPCR (see 2.4.2). The blood samples were centrifuged for 10 min (4000 *x g* at 4 °C) and plasma was transferred into fresh tubes and frozen at -20 °C until CORT concentration was analysed (see 2.4.3.1). An overview of the experimental schedule is depicted in Figure 8.

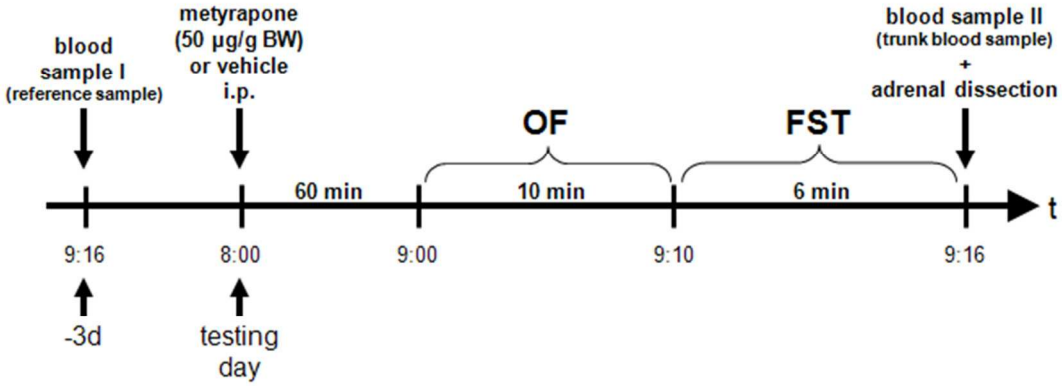

Figure 8: Schematic overview of the experimental schedule with time and intervals of the collected blood samples, metyrapone injection and behavioural tests. OF, open field test; FST, forced swim test; i.p., intraperitoneal; t, time; d, day.

2.5.1.2 Experiment 2: Adrenal sensitivity towards adrenocorticotropic hormone

2.5.1.2.1 Introduction

It has been shown in a number of studies that adrenal sensitivity, i.e. GC release from the adrenal cortex upon binding of ACTH, is largely dependent on ligand binding of the MC2R. The MC2R is a G-protein coupled receptor with seven transmembrane domains (Abdel-Malek, 2001), which is located mainly in the zona fasciculata and zona glomerulosa of the adrenal cortex, the site of glucocorticoid and aldosterone synthesis, respectively (Mountjoy *et al.*, 1992; Xia *et al.*, 1996). Upon binding of ACTH to this receptor, a signalling cascade is activated, stimulating the production of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase, which, in turn, activates catalytic subunits of the protein kinase A (PKA). The PKA then triggers the phosphorylation of proteins and enzymes involved in the conversion of cholesterol to corticosteroids, i.e. activating *de novo* steroidogenesis (Lefkowitz *et al.*, 1971; Abdel-Malek, 2001; Kirschner, 2002). However, changes in MC2R sensitivity towards its ligand and/or alterations in the subsequent signalling cascade can lead to pathological conditions such as adrenal

insufficiency, e.g. familial glucocorticoid deficiency (FGD) or Addison's disease. It has been reported that in FGD mutations in the MC2R gene result in an impaired cAMP response due to decreased ligand binding affinity of the receptor (Elias *et al.*, 1999; Elias *et al.*, 2000). Furthermore, it was shown that the MC2R accessory protein (MRAP) is required for processing, trafficking and proper insertion of functional MC2R in the plasma membrane of adrenal cells and that disruption of this protein can lead to an insufficient response of the adrenal to ACTH (Metherell *et al.*, 2005). On the other hand, Swords and colleagues showed that two missense mutations in the MC2R gene lead to adrenal hypersensitivity towards ACTH resulting in increased plasma CORT concentrations (Swords *et al.*, 2004). Additionally, it has been reported that repeated adrenal stimulation with ACTH induces an enhanced expression of the MC2R, which results in an increased ACTH binding and cAMP response in adrenal cells (Penhoat *et al.*, 1989; Mountjoy *et al.*, 1994; Abdel-Malek, 2001). Furthermore, it was shown that stress exposure leads to adrenal hypertrophy associated with elevated concentrations of plasma CORT (Gamallo *et al.*, 1986; Moraska *et al.*, 2000; Ulrich-Lai *et al.*, 2006). Both, adrenal insufficiency, as well as adrenal hyperactivity, are pathological states involving the MC2R and are associated with a dysregulation of the HPA axis under basal conditions or in response to stressors, a condition which is mirrored in the animals of the SR mouse model.

2.5.1.2.2 Objective of the experiment

The purpose of this experiment was to address the question whether the distinct differences in stress-induced plasma CORT concentrations observed in HR, IR and LR mice arise from differences in adrenal sensitivity. Previous studies by Touma and colleagues showed that an ACTH challenge of these animals with a supraphysiological dose (1 µg/g BW) of exogenously administered ACTH caused a similar increase in plasma CORT concentrations in all three lines, suggesting that the capacity of the adrenals to produce and secrete CORT was not different between the lines (Touma *et al.*, 2008). Hence, we hypothesised that the significant differences in CORT secretion in response to stressors could potentially be based on differences in adrenal sensitivity to ACTH.

To test this hypothesis, we performed an adrenal sensitivity test *in vivo* with HR, IR and LR mice (see 2.2.3). Two different doses of ACTH were chosen with the lower dose resembling the physiological ACTH response to a relatively strong stressor, e.g. FST

(Müller *et al.*, 2003), and the higher dose corresponding to a supraphysiological level of ACTH, to induce ACTH-mediated CORT release from the adrenals. Furthermore, we assessed the adrenal weight of HR, IR and LR animals since it has been shown that repeated stress exposure can lead to adrenal hypertrophy (Gamallo *et al.*, 1986; Moraska *et al.*, 2000; Ulrich-Lai *et al.*, 2006).

2.5.1.2.3 Experimental schedule

An overview of the experimental schedule is depicted in Figure 9. A detailed description of the adrenal sensitivity test is given in section 2.2.3. Briefly, at 9:00 on the experimental day, HR, IR and LR males derived from Gen XX of the SR mouse model were acutely injected intraperitoneally (i.p.) with a high dose (4 µg/g BW) of dexamethasone in order to suppress endogenous ACTH and CORT secretion. Six hours later, blood was collected from the ventral tail vessel for the verification of the ACTH- and CORT-suppressive effect of Dex, immediately followed by an ACTH (or vehicle) injection to stimulate adrenal CORT secretion. Thirty minutes later, i.e. at 15:30, the animals were sacrificed and trunk blood was collected to determine plasma CORT concentrations. In a final step, the adrenals were dissected, cleared from fat tissue and immediately weighed.

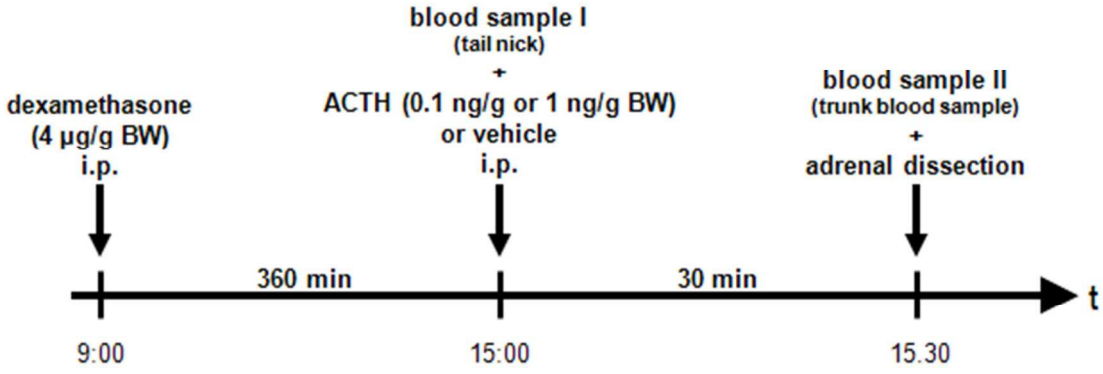

Figure 9: Schematic overview of the experimental schedule with time and intervals of the collected blood samples and dexamethasone or adrenocorticotropic hormone (ACTH) injections. i.p., intraperitoneal; t, time.

2.5.1.3 Experiment 3: Expression and release of corticosteroid-binding globulin in response to stress

2.5.1.3.1 Introduction

Once released into the blood stream, steroid hormones such as oestrogens, progesterone and testosterone, but also CORT, bind to albumin (Slaunwhite *et al.*, 1959), which is the

most abundant plasma protein synthesised in the liver, comprising as much as 50 per cent of the productive effort (Rothschild *et al.*, 1972a,b). Albumin binds CORT with high capacity but low affinity. In contrast, transcortin, also called corticosteroid binding globulin (CBG), binds CORT with high affinity but low capacity (Sandberg *et al.*, 1964; Breuner *et al.*, 2002; Moisan, 2010). Like albumin, CBG is synthesised in the liver and secreted from hepatocytes (Weiser *et al.*, 1979; Kuhn *et al.*, 1986; Henley *et al.*, 2011) into the plasma, binding 80-90 % of circulating CORT while 10-15 % of total CORT is bound to albumin, leaving a fraction of about 5 % free CORT to be biologically active (Lewis *et al.*, 2005; Henley *et al.*, 2011; Cizza *et al.*, 2012) according to the free hormone hypothesis by Mendel (Mendel, 1989). This equilibrium between CBG-bound CORT, albumin-bound CORT and a minor fraction of free CORT in the plasma is challenged as soon as an organism is exposed to stressful stimuli and CORT secretion from the adrenal cortex is elevated. In a study by Qian and colleagues it was shown in rats that a 15-min swim stress evoked a fast and profound rise in plasma CBG with a concomitant rise in plasma total CORT. Interestingly, in compartments such as the brain, plasma and subcutaneous tissue, the rise in free CORT was delayed for 20-30 min as measured by microdialysis. Hence, it was concluded that the increase in circulating CBG levels after stress restrains the rise in free CORT concentrations for approximately 20 min in face of mounting total hormone levels in the circulation, suggesting that CBG may serve as a buffer allowing the organism to respond to stress in an adequate way (Qian *et al.*, 2011). Moreover, it was reported that CBG deficient mice display an increased activity of the HPA axis associated with a 10-fold increase in free CORT levels. Furthermore, the half-life of CORT was significantly reduced in CBG deficient mice and drug metabolising enzymes in the liver were elevated (Petersen *et al.*, 2006). In a recent study, CBG deficient mice were studied with the aim of unravelling the effect of CORT-binding to CBG on HPA axis function and behaviours linked to anxiety and depression traits. These mice showed a dysregulated HPA axis function with reduced total CORT levels, basally and in response to stress, while free CORT was normal at rest but presented a reduced surge after restraint stress (Richard *et al.*, 2010). In addition to this, CBG deficient mice showed a depression-like behavioural phenotype with increased immobility in the FST as well as enhanced learned helplessness after prolonged uncontrollable stress. However, there were no alterations in paradigms measuring anxiety-related behavioural (Richard *et al.*, 2010).

In summary, these findings highlight the critical role of corticosteroid binding proteins in providing an adequate neuroendocrine and behavioural response to stress, and underline the eminent role of CBG in the bioavailability, local delivery and cellular signal transduction of CORT, thereby exceeding its role as a mere steroid transporter (Petersen *et al.*, 2006; Moisan, 2010; Richard *et al.*, 2010; Henley *et al.*, 2011).

2.5.1.3.2 Objective of the experiment

Considering the essential role of CBG in the physiological stress response, we investigated whether HR, IR and LR mice, with their distinctly different neuroendocrine phenotypes regarding their stress-induced secretion of CORT, would differ in the expression and release of CBG in the liver and in plasma, both, basally and in response to restraint stress. Additionally, we addressed the question (i) whether the increased CORT release in response to stressors was reflected by elevated levels of ACTH in the pituitary of the animals and (ii) whether stress-induced plasma ACTH concentrations parallel the rise in plasma CORT levels, implicating a decrease in the amount of vesicularly stored pituitary ACTH.

2.5.1.3.3 Experimental setup

HR, IR and LR animals derived from Gen XX of the SR mouse model were subjected to a SRT (see 2.2.1) on the experimental day at 9:00. Blood was sampled immediately before (t=0) and straight after (t=15) the 15-min restraint period. Subsequently, animals were transferred back to their homecages. Thirty minutes after the beginning of the restraint period, when animals were assumed to have reached maximal concentrations of CBG in the plasma (according to the experiment of Qian *et al.*, 2011), animals were sacrificed and trunk blood was collected (t=30). The brain, pituitary and liver were dissected, immediately frozen on dry ice and stored at -80 °C until further analysis. Animals of the control group were not subjected to the SRT but killed under unstressed conditions. Trunk blood was collected and organs were dissected, stored and processed as described above. All blood samples were centrifuged for 10 min (4000 *x g* at 4 °C) and plasma CORT concentrations were determined (see 2.4.3.1). Plasma ACTH concentrations were determined in the unstressed control group, as well as in the samples collected straight after (t=15) and 15 min after the cessation of the stressor (t=30) (see 2.4.3.2). Due to the lower amount of plasma available, ACTH was determined in single estimation in the sample collected straight after the end of the stressor (t=15). CBG in the liver and in the

plasma, as well as ACTH in the pituitary, was quantified by Western blot (see 2.4.1). An overview of the experimental schedule is depicted in Figure 10.

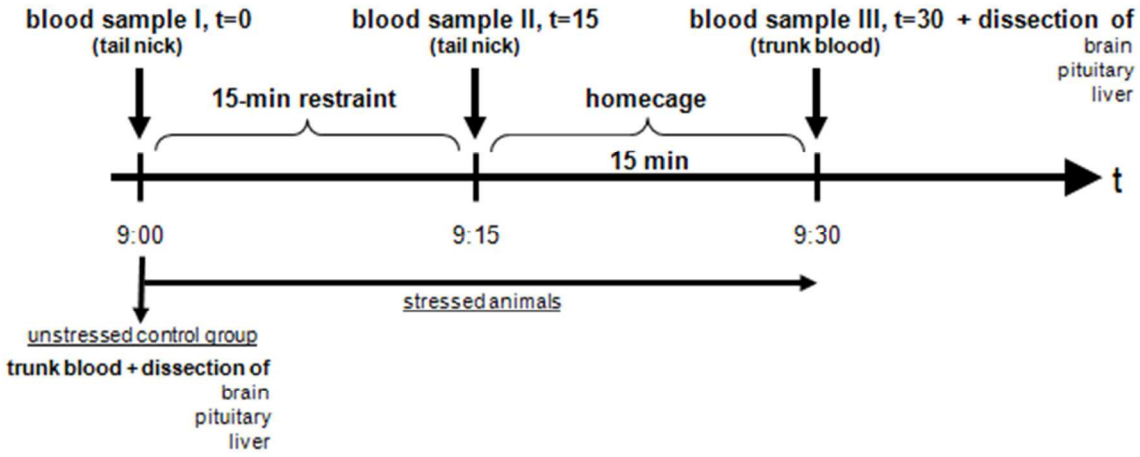

Figure 10: Schematic overview of the experimental schedule with time and intervals of the collected blood samples. Stressed animals were subjected to 15 min of restraint stress and returned back to their homecages for 15 min before they were sacrificed. The unstressed control group was killed under basal conditions. t, time.

2.5.2 Central regulation of the hypothalamic-pituitary-adrenocortical axis

In the experiments presented in this work so far, we mainly focused on the peripheral aspects of HPA axis function in HR, IR and LR animals, such as regulatory mechanisms at the pituitary level, GC secretion from the adrenals and corticosteroid-binding globulin in the plasma. However, since alterations or maladaptions of the HPA system, both peripherally and centrally, contribute to the pathogenesis of affective disorders such as MD (Holsboer, 2000; Juruena *et al.*, 2004; Charmandari *et al.*, 2005; Pariante *et al.*, 2008) and psychiatric diseases such as MD originate from the brain, we further investigated central aspects of HPA axis dysfunction in the animals of the SR mouse model in order to establish a more comprehensive picture. Simultaneously, we addressed the question whether treatment with the SSRI Flx, which is widely used in clinical settings as a first-line treatment in patients suffering from MD, would affect the neuroendocrine and behavioural phenotype of HR, IR and LR mice. Although its mechanism of action is still poorly understood, it has been shown in preclinical and clinical studies that Flx influences GC receptor expression (Budziszewska *et al.*, 2000; Heydendael *et al.*, 2010; Elakovic *et al.*, 2011), emotional behaviour (Dulawa *et al.*, 2004; Norcross *et al.*, 2008) and cognition (Grote *et al.*, 2005). Furthermore, the impact of Flx treatment on the regulation of the HPA axis has been shown (Szymanska *et al.*,

2009; Surget *et al.*, 2011). This is of interest since normalisation of HPA axis function was found to be a potent predictor for the successful treatment of MD (Ising *et al.*, 2005; Ising *et al.*, 2007) and can significantly reduce the risk of relapse (Zobel *et al.*, 2001), emphasising the importance of a balanced HPA system.

Therefore, in two translational experimental approaches, we investigated the impact of chronic Flx treatment on the neuroendocrine and behavioural phenotype of HR, IR and LR mice. In the first experiment, we focused on the effects of chronic Flx treatment on the emotional behaviour as well as HPA axis regulation. In the second experiment, we were interested whether animals of the SR mouse model were affected by chronic Flx treatment regarding their cognitive performance as well as HPA axis reactivity and neuronal activation in response to stress.

2.5.2.1 Experiment 1: Effects of chronic fluoxetine treatment on emotional behaviour and hypothalamic-pituitary-adrenal axis regulation in HR, IR and LR mice

HR, IR and LR mice derived from Gen XVII of the SR mouse model were chronically administered 10 µg/g BW Flx or vehicle by means of a daily intraperitoneal (i.p.) injection over a time period of 34 days. The injection was given once daily at 16:00. Flx was dissolved in Ringer solution. Control animals (Veh) only received Ringer solution. A detailed outline of the experimental schedule is depicted in Figure 11. After 25 days of treatment, a reference blood sample was collected at 9:00 from all animals to assess basal CORT conditions. In order to exclude interfering effects of the blood sampling procedure with the behavioural testing, the animals were allowed to recover for 3 days before the start of the behavioural test battery. However, the daily injection was also maintained on these days.

2.5.2.1.1 Assessment of emotional behaviour and hypothalamic-pituitary-adrenal axis activity in response to stress

On day 28, a behavioural test battery was performed to assess the animals' locomotor activity and exploratory drive in a 10-min OF test (see 2.3.1). Subsequently, depression-like behavioural parameters were analysed by means of a 6-min FST (see 2.3.2). Right after the FST, a blood sample was collected in order to assess stress-induced plasma CORT concentrations.

2.5.2.1.2 Assessment of hypothalamic-pituitary-adrenal axis regulation by means of a combined dexamethasone/corticotropin-releasing hormone test

On day 32, a reference blood sample was taken at 15:00, resembling basal/untreated conditions. After allowing the animals to recover for 3 days, a combined Dex/CRH test was performed (see 2.2.2). Subsequently, the animals were sacrificed and the hippocampus and the pituitary were dissected and immediately frozen on dry ice. One hippocampal hemisphere was used for protein expression analysis using Western blot (see 2.4.1). From the other hippocampal hemisphere, as well as from the pituitary, RNA was isolated and gene expression analysis was performed (see 2.4.2). All blood samples were centrifuged for 10 min (4000 *x g* at 4 °C) and CORT concentrations were determined from the plasma (see 2.4.3.1).

2.5.2.1.3 Gene expression analysis

After the Dex/CRH test, animals were sacrificed and the hippocampus and the pituitary were dissected for qPCR-based gene expression analysis. In the hippocampus, the following candidate genes were investigated known to be involved in HPA axis function as well as negative feedback regulation: the glucocorticoid receptor (nuclear receptor subfamily 3, group C, member 1, *Nr3c1*), the mineralocorticoid receptor (nuclear receptor subfamily 3, group C, member 2, *Nr3c2*), the glucocorticoid receptor co-chaperones FK506 binding protein 4 (*Fkbp4*) and FK506 binding protein 5 (*Fkbp5*), the multidrug resistance transporters ATP-binding cassette, sub-family B, member 1A (*Abcb1a*) and 1B (*Abcb1b*), the corticotropin-releasing hormone receptor 1 (*Crhr1*), the actin modulating protein cofilin-1 (*Cfl1*) and the glucocorticoid induced leucine zipper (*Gilz*).
Additionally, the following candidate genes were analysed in the pituitary of the animals: *Nr3c1*, proopiomelanocortin (*Pomc*), *Fkbp5*, *Abcb1a*, *Abcb1b*, *Crhr1* and *Gilz* (for abbreviations see above).

2.5.2.1.4 Protein expression in the hippocampus

In addition to the hippocampal gene expression analysis, GR and FKBP51 protein abundance was assessed in Veh- and Flx-treated HR, IR and LR animals in the hippocampal hemisphere which was not used for qPCR analysis.

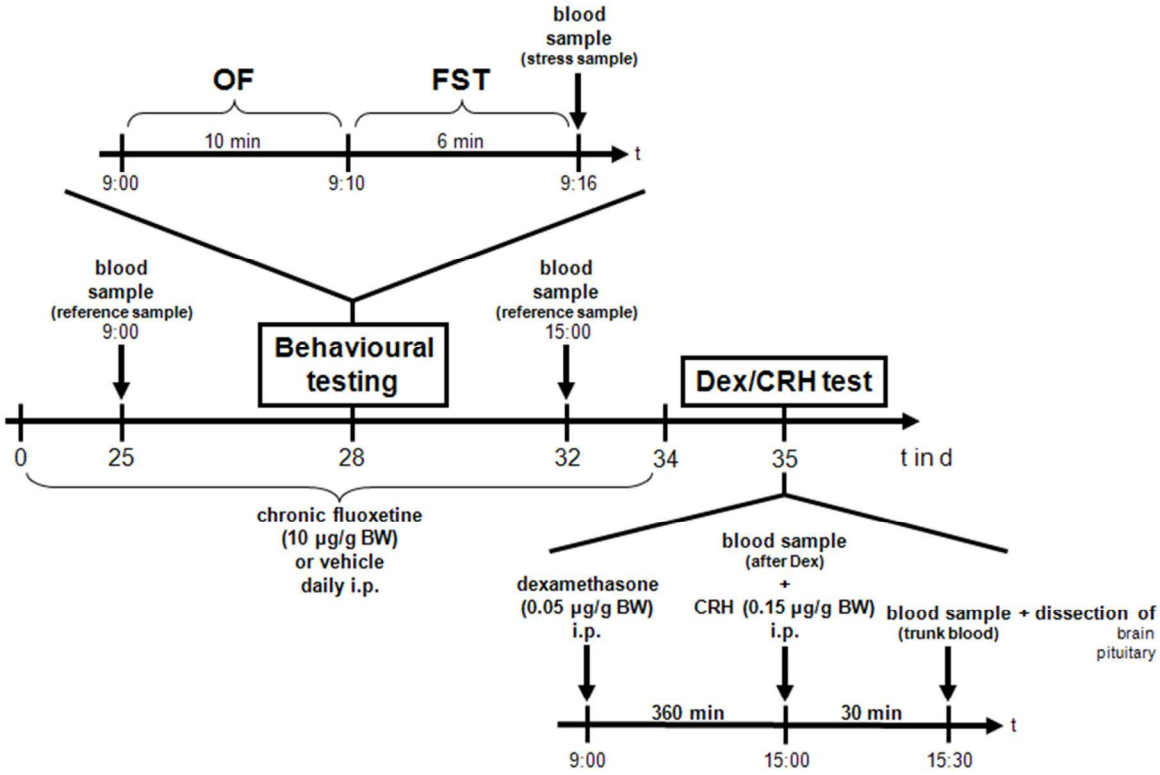

Figure 11: Schematic overview of the experimental schedule showing the day of behavioural testing (day 28) and the Dex/CRH test (day 35). The detailed experimental schedules on the respective days are given in the timelines on top or below. OF, open field test; FST, forced swim test; Dex, dexamethasone; CRH, corticotropin-releasing hormone; i.p., intraperitoneal; t, time; d, day; BW, body weight.

2.5.2.2 Experiment 2: Effects of chronic fluoxetine treatment on spatial reference memory, hypothalamic-pituitary-adrenal axis function and neuronal activation in HR, IR and LR mice

In the second Flx experiment, HR, IR and LR mice derived from Gen XIX of the SR mouse model were used. Similarly to the first experiment, animals were chronically administered 10 µg/g BW of Flx by means of a daily intraperitoneal (i.p.) injection over a time period of 34 days. The injection was given once daily at 16:00. Flx was dissolved in Ringer solution. Control animals (Veh) only received Ringer solution. In this experiment, two control groups consisting of animals of the HR, IR and LR line, were used (control I and control II, see Figure 12). Similar to the experimental animals, mice of control I received a daily injection of Flx or Veh over a period of 34 days and were subjected to the Y-maze test. This group served to control for effects of the daily injection procedure on (i) CORT levels and (ii) *C-fos* expression, while control II consisted of entirely untreated, naïve HR, IR and LR mice reflecting basal plasma CORT levels and brain *C-fos* expression.

2.5.2.2.1 Assessment of spatial reference memory by means of the Y-maze test

After 28 days of chronic Flx treatment, mice of the experimental and control I group were subjected to the Y-maze test to assess their hippocampus-dependent, spatial reference memory (see 2.3.3). The Y-maze test has been validated previously as a hippocampus-dependent spatial task (Conrad *et al.*, 1996; Conrad *et al.*, 1997) and previous experiments in HR, IR and LR mice showed that animals of the HR line display cognitive deficits regarding their hippocampus-dependent memory performance (Knapman *et al.*, 2010a; Knapman *et al.*, 2012). Thus, we were interested whether the cognitive deficits in HR mice could be rescued by Flx treatment.

2.5.2.2.2 Assessment of stress-induced hypothalamic-pituitary-adrenal axis function and neuronal activation in the central nervous system

After allowing the animals to recover for seven days to avoid confounding influences of the Y-maze test on plasma CORT concentrations and brain *C-fos* expression, they were subjected to a SRT starting at 9:00. A blood sample was collected immediately before and after the 15-min restraint period in order to assess unstressed and stress-induced CORT levels, respectively. Subsequently to the SRT, animals were transferred back to their homecages for 45 min. Sixty minutes after the onset of the stressor, the mice were sacrificed, trunk blood was collected and the brain was dissected. The brain was immediately shock-frozen in dry-ice cooled methylbutane (Carl Roth GmbH, Karlsruhe, Germany), wrapped in aluminium foil, and stored at -80 °C until analysis of *C-fos* expression by means of *in-situ* hybridisation (see 2.4.4). All blood samples were centrifuged for 10 min (4000 *x g* at 4 °C) and CORT concentrations were determined from the plasma (see 2.4.3.1). An overview of the experimental schedule is depicted in Figure 12.

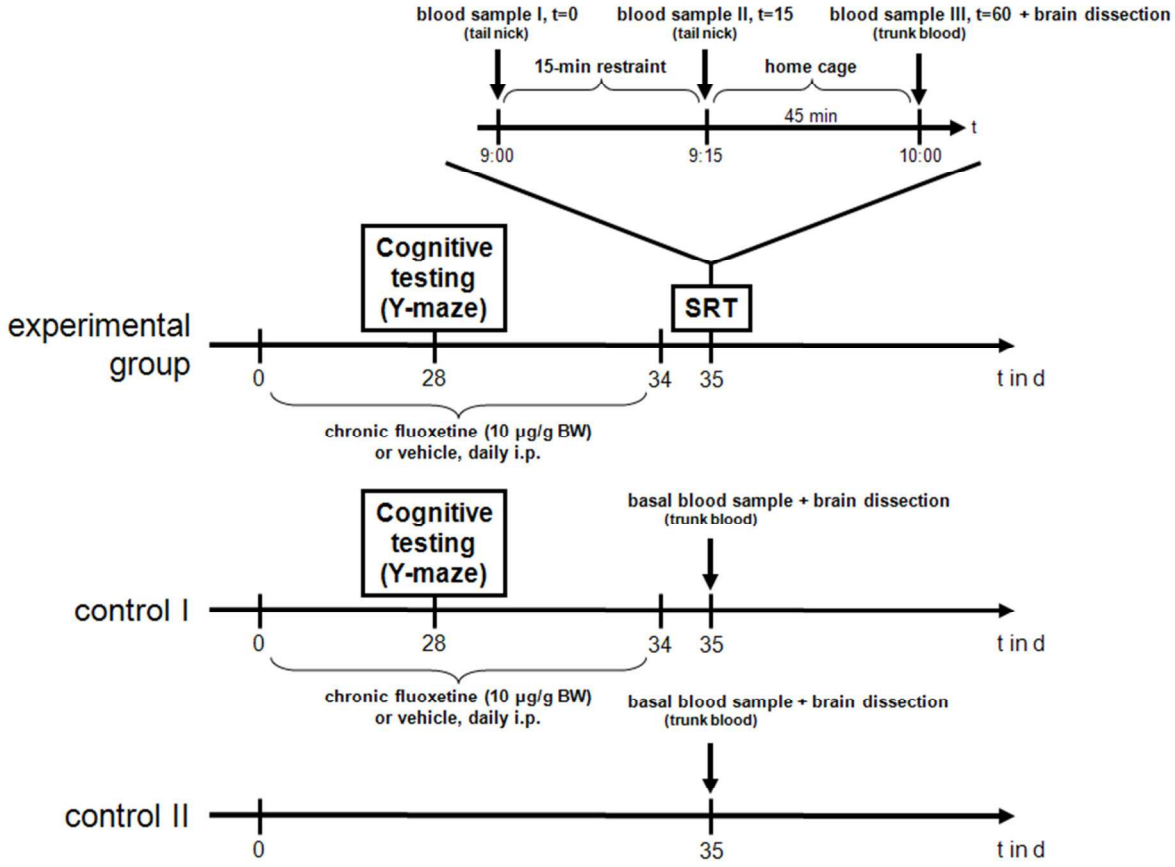

Figure 12: Schematic overview of the experimental schedule of the experimental group, control I and control II, respectively. The day of Y-maze testing (day 28) and the SRT (day 35) are given. The detailed experimental schedule of the SRT is outlined in the timeline on top. i.p., intraperitoneal; t, time; d, day; BW, body weight.

2.6 Statistical analysis

Since normal distribution and variance homogeneity of the data could not always be assumed, statistical analysis was performed using exclusively non-parametric statistics. Two independent samples were compared using the Mann-Whitney U-tests (MWU-test), while group comparisons of more than two independent samples were analysed using the Kruskal-Wallis H-Test (KWH-test). In case of a significant difference revealed by the KWH-test, post-hoc pairwise comparisons were applied using the MWU-test. Consequently, significance levels were corrected according to the sequential Bonferroni technique for multiple testing.

In order to evaluate differences between two dependent samples the Wilcoxon test (W-test) was applied. More than two dependent samples were analysed using the Friedman test (F-test). Post-hoc pairwise comparisons applying the W-test were calculated if the F-test yielded significant results. Consequently, significance levels were corrected according to the sequential Bonferroni technique for multiple testing.

All tests were applied two-tailed and were calculated using the software package SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was accepted from $p<0.05$, while $p<0.1$ was considered as a trend.

2.7 Graphic illustration

Graphs were created using SigmaPlot software package version 9.01 (Systat Software Inc., San Jose, USA). Data are given as box plots showing medians (lines in the boxes) and 25 % to 75 % percentiles (boxes). The 10 % percentile and 90 % percentile are indicated by the whiskers.

3 Results

3.1 Peripheral regulation of the hypothalamic-pituitary-adrenal axis

3.1.1 Experiment 1: Inhibition of corticosterone secretion by the 11β-hydroxylase inhibitor metyrapone

In this experiment, animals derived from Gen XVI of the SR mouse model were used. The animals of the HR, IR and LR line were subdivided into two groups receiving vehicle solution (Veh; HR/IR/LR, N=8/9/9) or metyrapone (Met; HR/IR/LR, N=8/8/8), respectively.

3.1.1.1 Plasma corticosterone concentrations

In the reference blood sample (-3d) collected three days prior to the testing day, animals of the Veh and Met group did not differ regarding their plasma CORT concentrations in all three lines. Only in the Veh group the between-line comparison revealed significant differences between HR, IR and LR mice (KWH-test: N=8/9/9, H=10.2, df=2, $p<0.01$) with HR and IR mice showing higher basal CORT concentrations than LR mice (post-hoc MWU-test: HR vs IR, U=27.5, $p>0.1$; HR vs LR, U=10.5, $p<0.05$; IR vs LR, U=8, $p<0.05$). However, all groups showed low CORT concentrations with a mean range of 3 ng/ml to 10 ng/ml (mean ± SEM, Veh, HR: 6.9 ± 2.1, IR: 9.5 ± 3.3, LR: 2.2 ± 0.4; Met, HR: 3.2 ± 0.4, IR: 4.9 ± 1.8, LR: 2.3 ± 0.3), which is in line with initial CORT levels detected prior to the restraint period in the SRT (Touma *et al.*, 2008; Heinzmann *et al.*, 2010; Knapman *et al.*, 2010a).

There was a significant increase in plasma CORT concentrations in the Veh group (W-test: HR/IR/LR, N=8/9/9, Z=-2.5/-2.6/-2.6, all $p<0.05$), as well as in the Met group (W-test: HR/IR/LR, N=8/8/8, all Z=-2.5, all $p<0.05$) of all three lines compared to the reference sample in response to the OF and FST. However, the swim stress-induced increase in CORT was significantly lower in Met-treated animals compared to Veh animals (MWU-test: Veh vs Met; HR: N=8/8, U=0, $p\leq0.001$; IR: N=9/8, U=0, $p\leq0.001$; LR: N=9/8, U=0, $p\leq0.001$, see Figure 13), indicating a drastically decreased secretion of CORT from the adrenals following Met treatment. Comparing the magnitude of the CORT increase in the Veh group, HR, IR and LR mice differed significantly (KWH-test:

N=8/9/9, H=12.5, df=2, p<0.01), with HR mice showing a clearly higher CORT increase compared to the IR line, while LR animals showed the lowest increase in CORT (post-hoc MWU-test: HR vs IR, U=8, p<0.05; HR vs LR, U=5, p<0.01; IR vs LR, U=20, p<0.1). Interestingly, similar significant differences between the lines could be observed in Met-treated animals (HR>IR>LR; KWH-test: N=8/8/8, H=14.2, df=2, p≤0.001; post-hoc MWU-test: HR vs IR, U=15, p<0.05; HR vs LR, U=0, p<0.01; IR vs LR, U=10, p<0.05). However, the absolute CORT levels were much lower in all Met-treated animals. In summary, animals of all three lines showed a significant increase in plasma CORT concentrations in response to the OF and FST, which was dramatically attenuated by Met treatment. However, the line specific differences in plasma CORT increase in response to stress (HR>IR>LR) were conserved also after Met treatment.

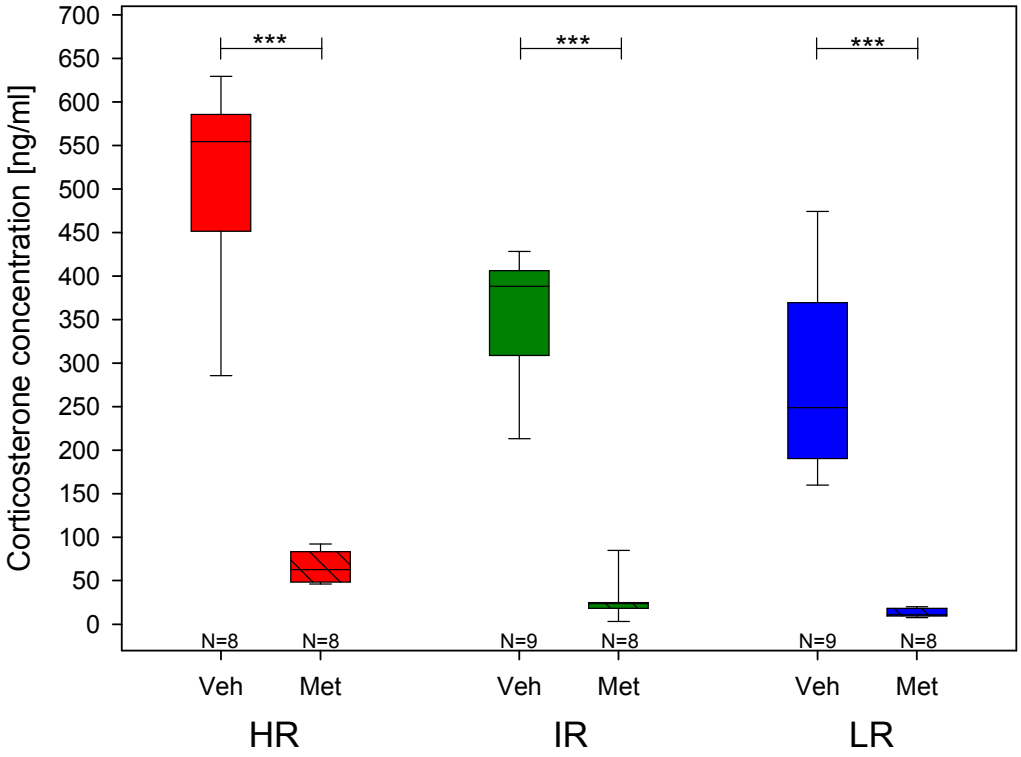

Figure 13: Plasma corticosterone increase in response to the forced swim test in high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVI. For statistical differences between the three lines see text. Statistical differences between the Veh and Met group are given on top of the panel (MWU-test: p≤0.001*).**

3.1.1.2 Assessment of emotional behaviour

3.1.1.2.1 Locomotor activity and exploratory drive in the open field test

One hour after the Met or Veh injection, the animals were subjected to a 10-min OF test in order to assess their locomotor activity and exploratory drive. Due to technical problems of the ANY maze tracking software, one LR animal of the Veh group had to be excluded from analysis, thereby reducing the number of LR animals in this group from nine to eight.

Independent of treatment, the animals of all three lines neither showed significant differences in their total distance travelled, time spent in the inner or outer zone of the OF apparatus, nor in the ratio between inner and outer zone path length (see Table 3). Thus, Met treatment did not induce changes in the locomotion or exploratory drive in the tested animals.

Table 3: Behavioural readout of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVI determined in the open field (OF) test. Independent of treatment, the animals of all three lines did not show significant differences in their locomotor activity or exploratory drive.

Parameter measured	$N_{HR/IR/LR}$	treatment	HR		IR		LR		between-line comparison		
			mean	SEM	mean	SEM	mean	SEM	H	p	
total distance travelled [m]	8/9/8	Veh	39.70	4.27	42.11	4.98	42.48	3.68	0.1	0.906	n.s.
	8/8/8	Met	43.13	6.20	44.80	5.49	45.71	2.86	0.1	0.924	n.s.
time inner zone [s]	8/9/8	Veh	75.14	16.77	59.06	15.76	73.38	11.18	1.9	0.383	n.s.
	8/8/8	Met	74.59	13.17	44.46	5.95	64.95	8.96	2.4	0.296	n.s.
time outer zone [s]	8/9/8	Veh	524.86	16.77	540.94	15.76	526.61	11.18	1.9	0.383	n.s.
	8/8/8	Met	525.40	13.17	555.54	5.95	535.05	8.96	2.4	0.296	n.s.
ratio inner/ outer path length	8/9/8	Veh	0.19	0.02	0.20	0.05	0.20	0.02	0.7	0.691	n.s.
	8/8/8	Met	0.20	0.02	0.14	0.02	0.15	0.02	3.5	0.168	n.s.

3.1.1.2.2 Assessment of stress-coping behaviour in the forced swim test

In the FST performed immediately after the OF test, a line comparison between HR, IR and LR animals of the Met-treated group revealed significant differences in the stress-coping behaviour of these animals regarding their floating time (KWH-test: N=8/8/8, H=16.6, df=2, $p<0.001$). Met-treated HR mice showed a significantly reduced floating time compared to IR animals, while LR mice displayed the highest immobility (post-hoc

MWU-test: HR vs IR, U=0, $p<0.01$; HR vs LR, U=2, $p<0.01$; IR vs LR, U=10, $p<0.05$). In the Veh-treated group, similar significant differences between the three lines could be observed (KWH-test: N=8/9/9, H=6.6, df=2, $p<0.05$), although they were less pronounced in this group (post-hoc MWU-test: HR vs IR, U=7, $p<0.05$; HR vs LR, U=20.5, $p>0.1$; IR vs LR, U=35, $p>0.1$, see Figure 14 A). Interestingly, the within-line comparison showed that Met induced a significant increase in active stress-coping behaviour, i.e. struggling, in the HR and IR line compared to the Veh-treated group of the respective line (MWU-test: Veh vs Met; HR: N=8/8, U=13, $p\leq0.05$; IR: N=9/8, U=13.5, $p\leq0.05$). However, Met treatment did not affect the stress-coping behaviour of LR animals (MWU-test: Veh vs Met; LR: N=9/8, U=33, $p>0.1$, see Fig. Figure 14 B).

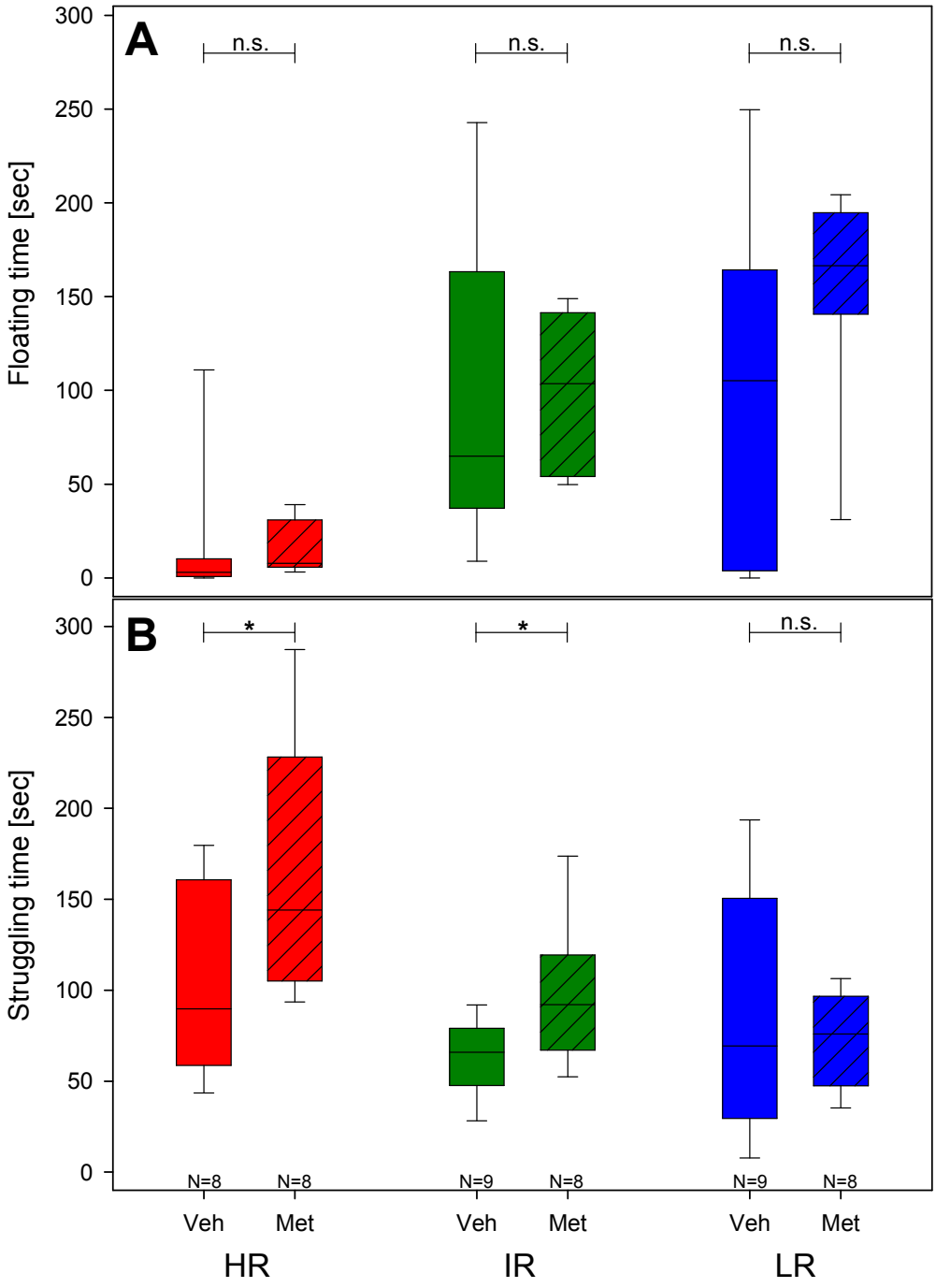

Figure 14: Stress-coping behaviour of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVI during the 6-min forced swim test (FST) period. (A) time spent floating (B) time spent struggling. For statistical differences between the three lines (KWH-test) see text. Statistical differences between the Veh and Met group are given on top of the panel (MWU-test: p>0.1 n.s., p≤0.05*).

In summary, this data shows that Met efficiently inhibited adrenal CORT secretion in response to stressors in HR, IR and LR animals. Additionally, the pharmacological inhibition of CORT secretion induced behavioural changes regarding the stress-coping style in the FST of HR and IR animals in the absence of changes in general locomotion or exploratory drive as shown in the OF.

3.1.1.3 Gene expression analysis

To investigate whether the distinct differences in CORT increase between the three lines in response to stress have persistent effects on adrenal gene expression patterns, total RNA was isolated from the adrenals of the Veh animals and relative gene expression analysis was performed using quantitative real-time PCR. One LR animal had to be excluded due to low RNA quality. Hence, the final number of samples was eight for HR, nine for IR and eight for LR animals.

There were no significant differences between HR, IR and LR mice regarding the relative gene expression of tyrosine hydroxylase (*Th*, see Figure 15 A), phenylethanolamine-N-methyltransferase (*Pnmt*, see Figure 15 B) and the melanocortin 2 receptor (*Mc2r*, see Figure 15 C) (KWH-test: N=8/9/8, H=1.1-2.2, df=2, all $p>0.1$). However, significant differences were detected between HR, IR and LR animals in the relative gene expression of steroid acute regulatory protein (*StAR*, see Figure 15 D) (KWH-test: N=8/9/8, H=9, df=2, $p<0.05$). Interestingly, *StAR* mRNA was increased in HR and LR animals compared to IRs (post-hoc MWU-test: HR vs IR, U=6, $p<0.05$; IR vs LR, U=13, $p<0.1$), whereas there was no significant difference between HR and LR animals (post-hoc MWU-test: HR vs LR, U=31.5, $p>0.1$).

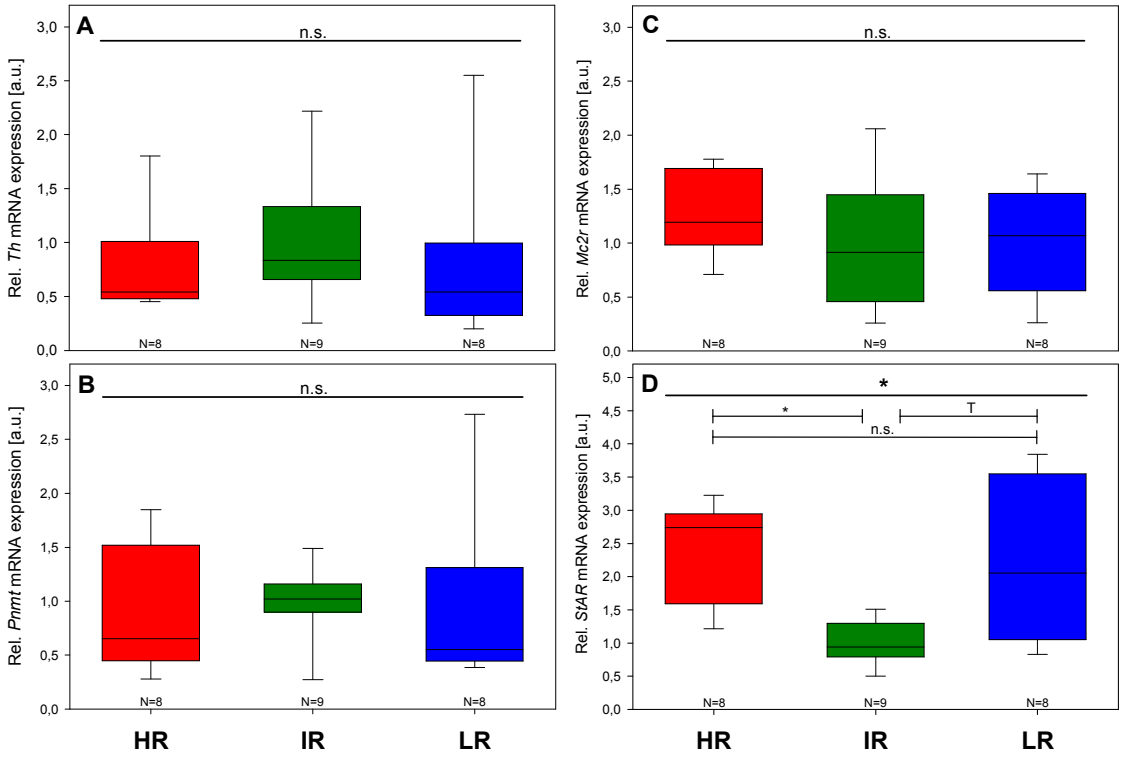

Figure 15: Gene expression in the adrenals of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVI (Veh group) relative to two housekeeping genes (*Hprt* and *Tbp*), normalised to the IR group. (A) tyrosine hydroxylase (*Th*) mRNA expression. (B) phenylethanolamine-N-methyltransferase (*Pnmt*) mRNA expression. (C) melanocortin 2 receptor (*Mc2r*) mRNA expression. (D) steroid acute regulatory protein (*StAR*) mRNA expression. Statistical differences between the three lines are given on top of each panel (KWH-test), pairwise group comparisons (post-hoc MWU-test) are indicated below (p≤0.05*, p<0.1 T, p>0.1 n.s.).

3.1.2 Experiment 2: Adrenal sensitivity towards adrenocorticotropic hormone

3.1.2.1 Neuroendocrine response

Whether the distinct differences in stress-induced plasma CORT concentrations of HR, IR and LR mice were brought about by differences in adrenal sensitivity towards ACTH, was investigated by means of an adrenal sensitivity test. To this end, mice were injected with a high dose of Dex, suppressing endogenous ACTH release from the pituitary. Subsequently, defined doses of ACTH were applied and the CORT release from the adrenals in response to this stimulation with exogenous ACTH was determined.

For the assessment of the ACTH-suppressive effect of Dex in the plasma of the animals, one HR animal had to be excluded due to the low amount of blood obtained, reducing the number to 15 HR, 16 IR and 16 LR animals.

In the blood sample collected six hours after the Dex injection, ACTH concentrations were determined with a median range between 29 pg/ml and 33 pg/ml in the HR, IR and

LR group. There were no significant differences in plasma ACTH concentration between the three lines (KWH-test: N=15/16/16, H=0.6, df=2, p>0.1), suggesting a similar suppression of ACTH by Dex in HR, IR and LR animals (see Figure 16). It should be noted, that similarly to CORT, ACTH follows a circadian rhythm with trough levels in the morning and peak levels in the afternoon. Therefore, the determined plasma ACTH levels (at 15:00) were clearly below the expected levels for this time of day, supporting the effectiveness of Dex in clearly suppressing ACTH release from the pituitary.

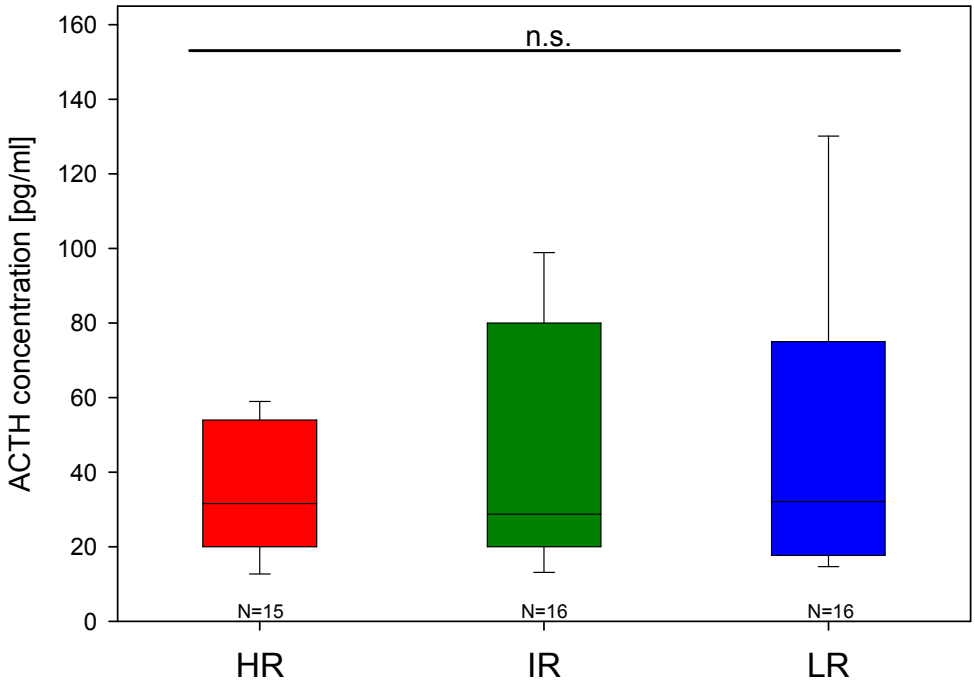

Figure 16: Plasma adrenocorticotropic hormone (ACTH) concentration after dexamethasone (Dex) suppression in high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XX. Statistical differences between the three lines are given on top of the panel (MWU-test: p>0.1 n.s.).

Similar to ACTH, plasma CORT concentrations of HR, IR and LR mice were markedly decreased after the Dex treatment (mean ± SEM, HR: 2.1 ± 0.1, IR: 2.2 ± 0.2 LR: 2.3 ± 0.3) with no significant difference between the lines (KWH-test: N=22/22/22, H=1.8, df=2, p>0.1). Furthermore, after saline injection, the CORT concentrations remained very low (mean ± SEM, HR: 1.9 ± 0.3, IR: 1.3 ± 0.3 LR: 1.5 ± 0.3), close to the detection limit of the radioimmunoassay, showing no significant differences between HR, IR and LR animals (KWH-test: N=6/6/6, H=3.4, df=2, p>0.1). This indicates (i) that the injection procedure *per se* did not induce a release of CORT from the adrenals and (ii) that the low concentrations of endogenous ACTH detected in the plasma after Dex injection (see above) did not stimulate the release of meaningful amounts of CORT from the adrenal

cortex (see Figure 17 A). However, after stimulation with the lower dose of ACTH (0.1 ng/g BW), HR and IR animals showed a significant increase in plasma CORT concentrations (mean ± SEM, HR: 7.5 ± 1.8, IR: 17.0 ± 6.4 LR: 2.9 ± 0.5), whereas LR animals remained unaffected (KWH-test: N=10/10/10, H=8.5, df=2, $p<0.05$; post-hoc MWU-test: HR vs IR, U=36.5, $p>0.1$; HR vs LR, U=28.5, $p>0.1$; IR vs LR, U=10.5, $p<0.01$). This rise in ACTH-induced CORT secretion was also observed in the group of HR and IR animals receiving the high dose (1 ng/g BW) of ACTH (mean ± SEM, HR: 127.9 ± 37.4, IR: 76.1 ± 20.0 LR: 4.5 ± 1.2) compared to animals of the LR line (KWH-test: N=6/6/6, H=12.1, df=2, $p<0.01$; post-hoc MWU-test: HR vs IR, U=10, $p>0.1$; HR vs LR, U=0, $p<0.01$; IR vs LR, U=0, $p<0.05$), although the effect was much more pronounced. Hence, LR animals did not respond with a marked increase in their plasma CORT concentrations to either dose of ACTH (see Figure 19 B).

In summary, this experiment revealed that high doses of Dex efficiently suppressed plasma CORT and ACTH concentrations in all three lines. Furthermore, we could show that the adrenal responsiveness towards defined, exogenously administered doses of ACTH is markedly decreased in LR mice compared to animals of the HR and IR line, suggesting a decreased adrenal sensitivity in LR animals.

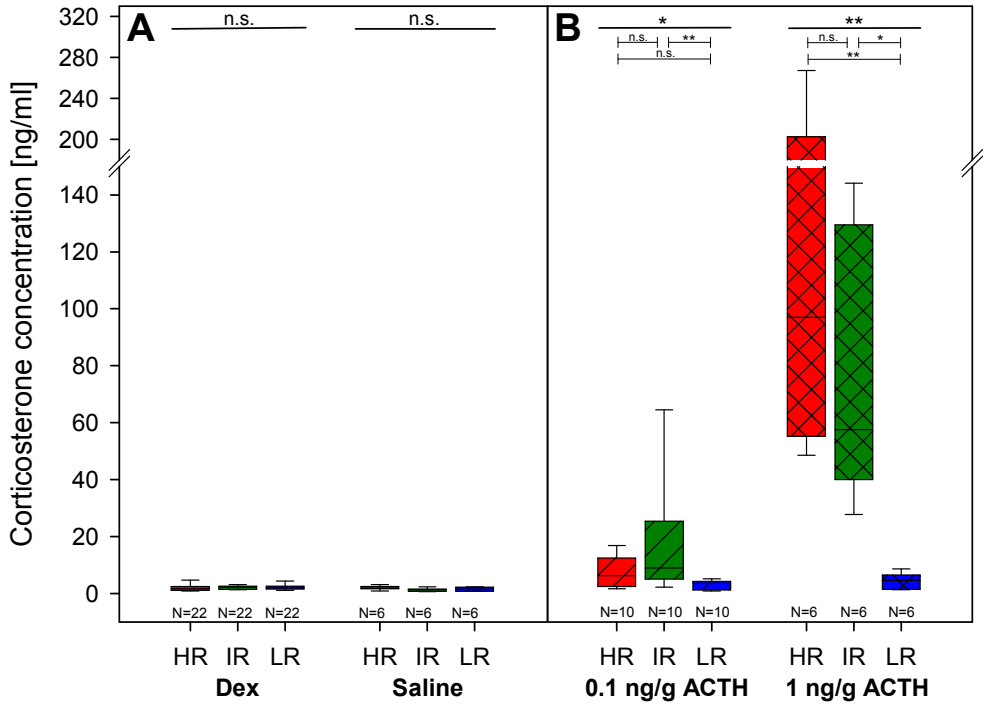

Figure 17: Plasma corticosterone (CORT) concentrations in high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XX. (A) left: plasma CORT concentrations 6h after dexamethasone injection, right: plasma CORT concentrations 6 h after dexamethasone injection followed by a saline injection. (B) plasma CORT concentrations after stimulation with 0.1ng/g BW ACTH (left) and 1ng/g BW ACTH (right). Statistical differences between the three lines are given on top of the panel (KWH-test), pairwise group comparisons (post-hoc MWU-test) are indicated below ($p\leq0.01$, $p\leq0.05$*, $p>0.1$ n.s.).**

3.1.2.2 Determination of the adrenal weight

The adrenal weight was assessed using six animals from each line of the SR mouse model and was calculated as a ratio of the body weight of the respective animal.

As it has been shown before (Touma *et al.*, 2008), there was a significant difference in the relative adrenal weight between HR, IR and LR animals (KWH-test: N=6/6/6, H=9.8, df=2, $p<0.01$, see Figure 18 A) with HR animals showing an increased relative adrenal weight compared to LR animals (post-hoc MWU-test: HR vs IR, U=6, $p>0.1$; HR vs LR, U=0, $p<0.05$; IR vs LR, U=8, $p>0.1$). When weighed separately, a trend towards a heavier left adrenal was observed in HR and LR animals (MWU-test: left vs right; HR: N=6/6, U=7, $p<0.1$; LR: N=6/6, U=7, $p<0.1$, see Figure 18 B).

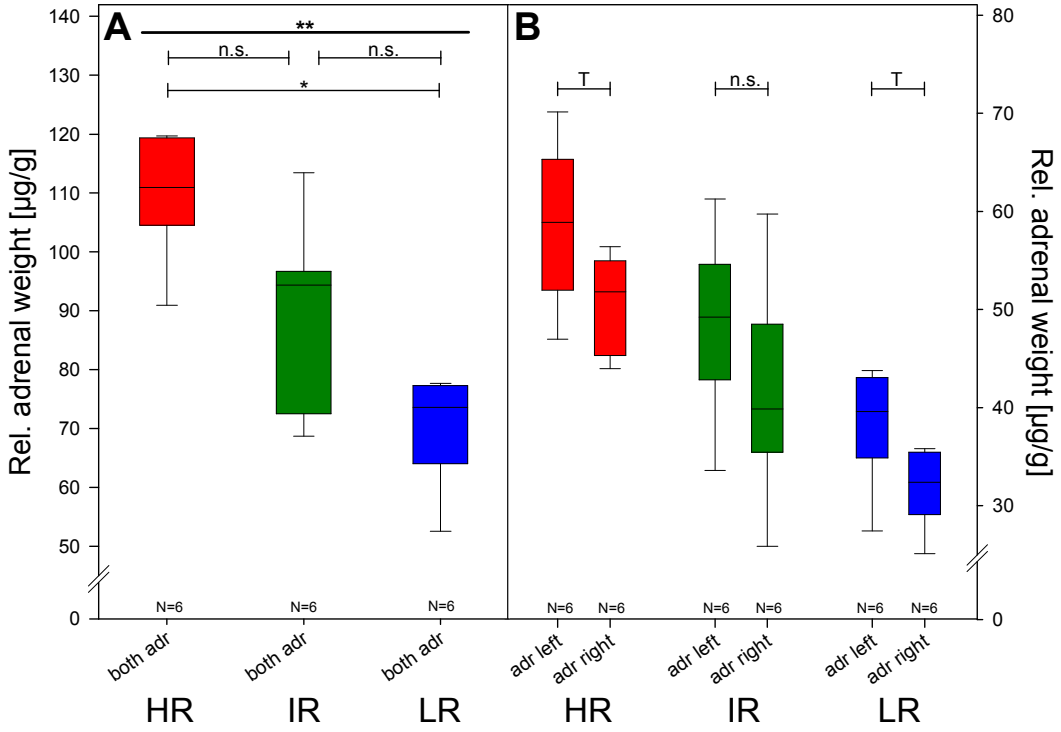

Figure 18: Relative adrenal weight (with respect to body weight) of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XX. (A) both adrenals pooled. (B) adrenals weighed separately. Statistical differences between the three lines are given on top of the panel (KWH-test), pairwise group comparisons (MWU-tests) are indicated below ($p\leq0.01$, $p\leq0.05$*, $p\leq0.1$ T, $p>0.1$ n.s.).**

3.1.3 Experiment 3: Expression and release of corticosteroid-binding globulin in response to stress

In this experiment, animals derived from Gen XX of the SR mouse model were used. The unstressed control group consisted of eight animals per line (HR/IR/LR, N=8/8/8), while ten animals of each line were subjected to the SRT (HR/IR/LR, N=10/10/10).

3.1.3.1 Plasma corticosterone concentrations

As expected, in the blood sample collected immediately before the 15-min restraint period (t=0), plasma CORT concentrations were low in HR, IR and LR animals (mean ± SEM [ng/ml], HR: 8.7 ± 2.9, IR: 16.5 ± 11.2, LR: 4.6 ± 0.8) with no significant differences between the lines (KWH-test: N=10/10/10, H=3.0, df=2, $p>0.1$, see Figure 19). Similarly, plasma CORT concentrations were low in all three lines of the control group, (mean ± SEM [ng/ml], HR: 21.6 ± 6.9, IR: 11.0 ± 3.8, LR: 4.7 ± 0.7) although a line comparison showed that HR animals had significantly increased plasma CORT concentrations compared to mice of the LR line (KWH-test: N=8/8/8, H=6.8, df=2, $p<0.05$; post-hoc MWU-test: HR vs IR, U=19, $p>0.1$; HR vs LR, U=9, $p<0.05$; IR vs LR, U=17, $p>0.1$). However, a within-line comparison revealed no significant differences in plasma CORT concentrations between control animals and samples of the stressed group (t=0) immediately before the restraint (MWU-test: unstressed vs t=0; HR: N=8/10, U=20, $p<0.1$; IR: N=8/10, U=32.5, $p>0.1$; LR: N=8/10, U=39.5, $p>0.1$), indicating basal plasma CORT levels in these two groups.

Right after the 15-min restraint period (t=15), plasma CORT concentrations were markedly increased in all three lines and were even further elevated 30 min (t=30) after the onset of the restraint stressor (F-test: HR/IR/LR, N=10/10/10, all $Chi^2=20$, all df=2, all $p<0.001$; post-hoc W-test: t=0 vs t=15, all Z=-2.8, all $p\leq0.01$; t=0 vs t=30, all Z=-2.8, all $p<0.01$; t=15 vs t=30, all Z=-2.8, all $p<0.05$). Furthermore, there was a significant difference in the magnitude of the CORT increase between the three lines, both at t=15 and t=30 (HR>IR>LR; KWH-test: N=10/10/10, H=24.0-24.3, df=2, all $p\leq0.001$), with HR animals displaying higher plasma CORT concentrations than IR mice, while LR animals had the lowest increase (post-hoc MWU-test: HR vs IR, U=4-5, all $p\leq0.001$; HR vs LR, all U=0, $p<0.01$; IR vs LR, U=1-3, $p<0.05$).

In summary, controls and unstressed animals showed low plasma CORT concentrations at t=0, while there was a significant rise in plasma CORT levels after the restraint (t=15), which was further elevated 30 min (t=30) after the onset of the stressor (see Figure 19).

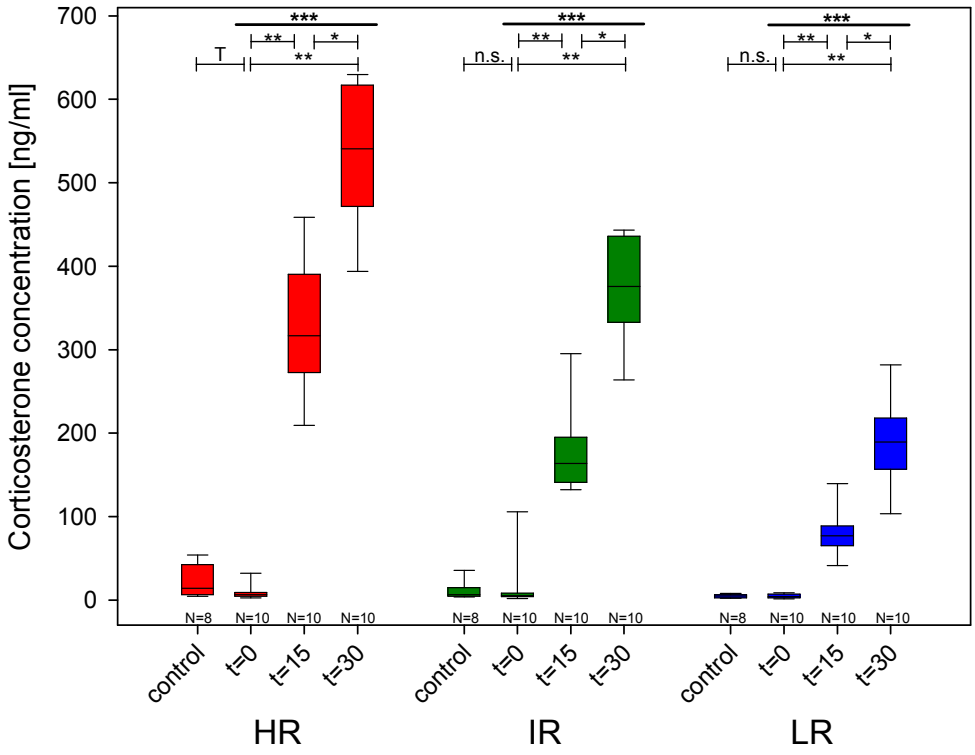

Figure 19: Plasma corticosterone (CORT) concentrations in high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XX. Control animals were killed under basal conditions. Plasma CORT concentrations of HR, IR and LR animals which were subjected to a 15-min restraint period were determined right before (t=0) and immediately after (t=15) the stress as well as 30 min (t=30) after the onset of the stressor. Statistical differences between the three lines are given on top of the panel (F-test), pairwise group comparisons (W-tests) are indicated below (p≤0.01, p≤0.05*, p≤0.1 T, p>0.1 n.s.).**

3.1.3.2 Corticosteroid-binding globulin expression

In order to quantify corticosteroid binding globulin (CBG) protein abundance and dynamics in response to stress in the liver and in the plasma of the experimental animals, Western blot analysis was performed.

3.1.3.2.1 Liver corticosteroid-binding globulin

CBG abundance in the liver of HR, IR and LR animals was analysed, in eight animals per line and treatment (HR/IR/LR, $N_{unstressed}=N_{stressed}=8/8/8$).

The between-line comparison revealed no significant differences between HR, IR and LR mice of both, the unstressed and stressed group (KWH-tests: HR/IR/LR, $N_{unstressed}=N_{stressed}=8/8/8$, H=0.1-2.7, df=2, all p>0.1). Furthermore, there was no significant effect of stress within IR and LR animals (MWU-test: unstressed vs stressed; IR: N=8/8, U=20, p>0.1; LR: N=8/8, U=19, p>0.1). In contrast, stressed HR animals showed a significantly increased CBG expression in the liver after stress (MWU-test: unstressed vs stressed; HR: N=8/8, U=11, p<0.05, see Figure 20).

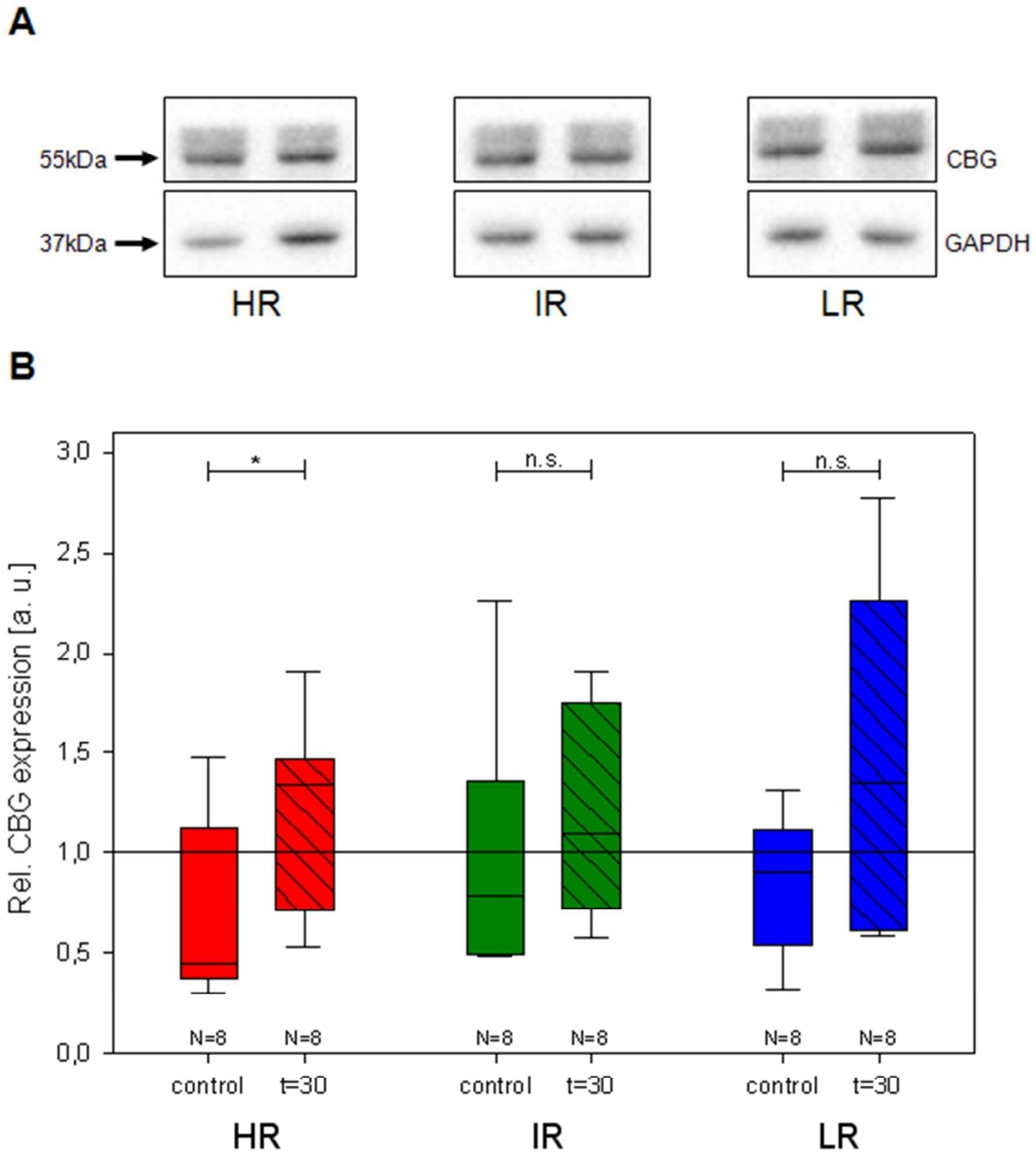

Figure 20: (A) Representative Western blot pictures of corticosteroid binding globulin (CBG) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). (B) CBG expression in the liver of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XX relative to GAPDH, normalised to the IR control group. Statistical differences between the three lines (KWH-test) are given in the text, within-line comparisons (MWU-test) are indicated on top of the panel ($p \leq 0.05$*, $p > 0.1$ n.s.).

3.1.3.2.2 Plasma corticosteroid-binding globulin

Due to technical problems, one IR animal of the unstressed control group had to be excluded, reducing the number of samples to seven in this group (HR/IR/LR, N=8/7/8). The number of samples in the stressed group was ten per line (HR/IR/LR, N=10/10/10).

Figure 21 shows the relative plasma CBG protein amount of control and stressed (t=30) HR, IR and LR animals normalised to the IR control group. In contrast to CBG protein abundance in the liver, there was a significant difference between HR, IR and LR animals regarding the CBG protein amount in the plasma in both, the control group and the stressed animals (KWH-tests: $HR/IR/LR_{control}$, N=8/7/8, $HR/IR/LR_{stressed}$, N=10/10/10, H=13.6-18.9, df=2, all $p \leq 0.001$). HR mice showed a clearly increased plasma CBG content compared to IR animals, while animals of the LR line had the lowest amount of CBG (post-hoc MWU-tests: control, HR vs IR, U=5, $p<0.05$; HR vs LR, U=3, $p<0.01$; IR vs LR, U=9, $p<0.05$; stressed, HR vs IR, U=11, $p<0.01$; HR vs LR, U=0, $p<0.01$; IR vs LR, U=18, $p<0.05$). However, a within-line comparison did not reveal significant differences between control and stressed animals of the respective line (MWU-test: control vs stressed; HR: N=8/10, U=23, $p>0.1$; IR: N=7/10, U=32, $p>0.1$; LR: N=8/10, U=40, $p>0.1$).

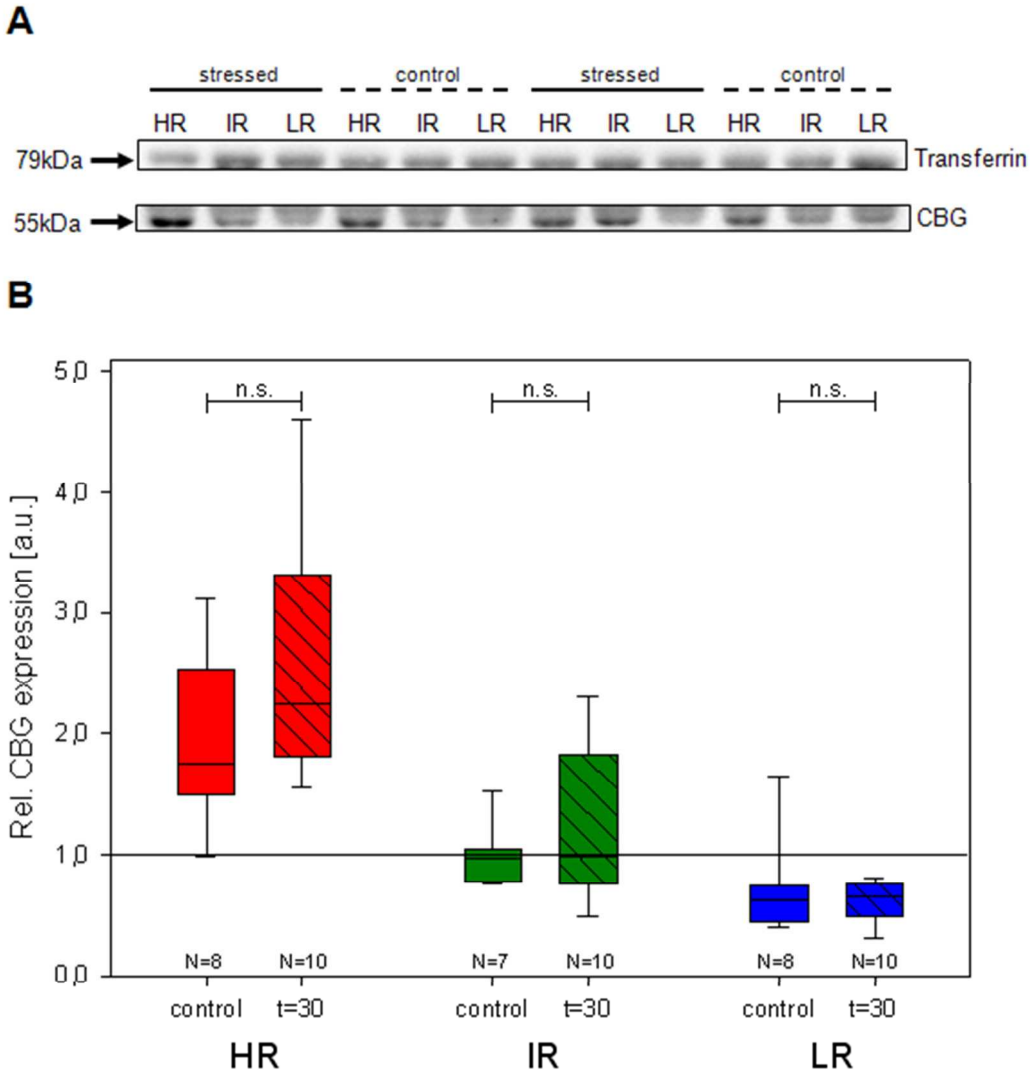

Figure 21: (A) Representative Western blot pictures of corticosteroid binding globulin (CBG) and transferrin. (B) CBG expression in the plasma of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XX relative to transferrin, normalised to the IR control group. Statistical differences between the three lines (KWH-test) are given in the text, within-line comparisons (MWU-test) are indicated on top of the panel (p>0.1 n.s.).

In summary, this data revealed significant differences in plasma concentrations of CBG between HR, IR and LR animals (HR>IR>LR), which were not affected by stress 30 min after the onset of the stressor. Furthermore, a within-line comparison showed that an acute stress exposure did not induce a significant change in liver CBG abundance in animals of the IR and LR line, while there was a slight increase in liver CBG after stress (t=30) in HR animals.

3.1.3.3 Plasma adrenocorticotropic hormone concentrations

Due to technical problems during the ACTH RIA, the plasma sample of one LR animal collected immediately after the stressor (t=15), as well as one HR plasma sample

collected 30 min after the onset of the stressor (t=30) were lost. Thus, the final sample size was HR/IR/$LR_{control}$ N=8/8/8, HR/IR/$LR_{t=15}$ N=10/10/9 and HR/IR/$LR_{t=30}$ N=9/10/10. The plasma ACTH concentrations in the animals of the control group did not show significant differences between the three lines (KWH-test: HR/IR/LR, N=8/8/8, H=2.9, df=2, p>0.1; see Figure 22). In HR animals, however, a higher individual variation in ACTH concentrations with markedly increased mean levels compared to the other two lines (mean ± SEM [pg/ml], HR: 327.3 ± 103.8, IR: 135.3 ± 34.4, LR: 222.0 ± 47.1) was observed. However, median plasma ACTH levels in the animals of all three lines were similarly low, ranging between 106 pg/ml and 233 pg/ml. After 15 min of restraint stress, only the IR line showed a significant surge of ACTH concentrations (MWU-test: control vs t=15, U=4, p≤0.001), whereas there was no statistical detectable stress-induced rise in animals of the HR and LR line (MWU-test: control vs t=15, U=23-31, all p>0.1). However, a between-line comparison showed that immediately after the restraint period (t=15), LR animals had lower plasma ACTH levels compared to HR and IR mice (KWH-test: HR/IR/LR, N=10/10/9, H=12.1, df=2, p<0.01; post-hoc MWU-test: HR vs IR, U=29, p>0.1; HR vs LR, U=13, p<0.05; IR vs LR, U=8, p<0.01). It was not until 30 min after the onset of the stressor that significantly increased plasma ACTH levels could be detected in the animals of all three lines (MWU-test: control vs t=30; HR: N=8/9, U=8, p<0.01; IR: N=8/10, U=0, p<0.001; LR: N=8/10, U=1, p≤0.001). Interestingly, there was no difference in the magnitude of the ACTH rise between HR, IR and LR animals at this time point (KWH-test: HR/IR/LR, N=9/10/10, H=1.2, df=2, p>0.1).

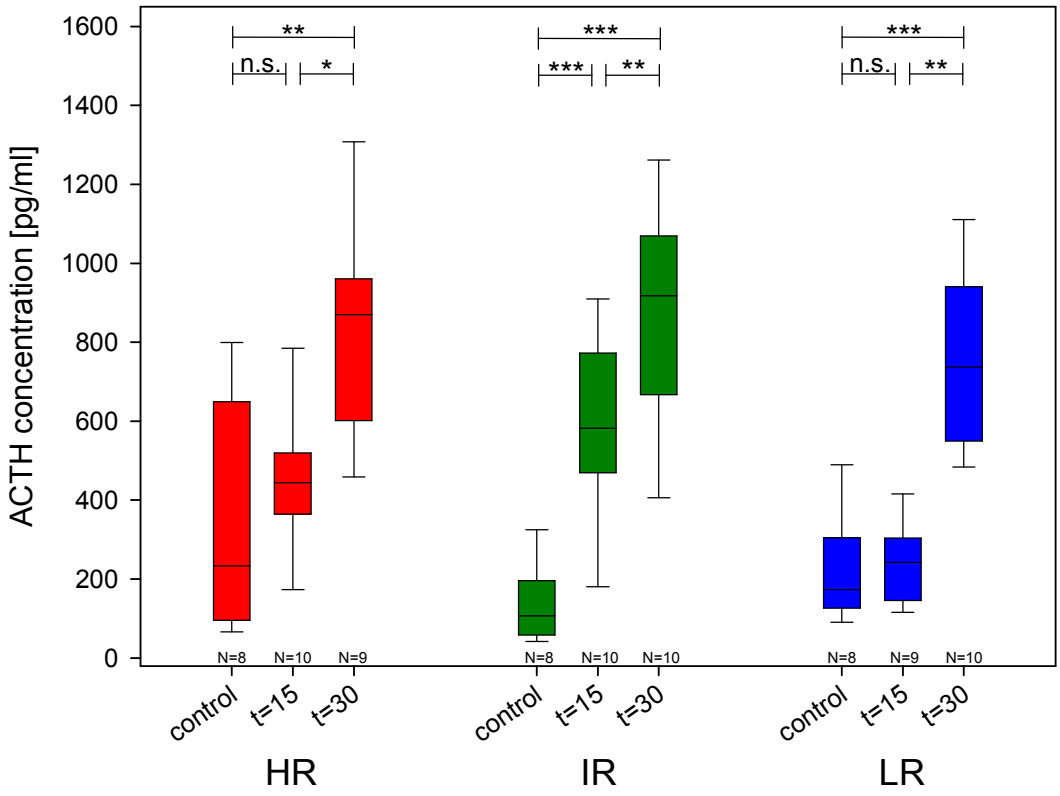

Figure 22: Plasma adrenocorticotropic hormone (ACTH) concentrations in high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XX. Control animals were killed under basal conditions. Plasma CORT concentrations of HR, IR and LR animals which were subjected to a 15-min restraint period were determined immediately after (t=15) the stress as well as 30 min (t=30) after the onset of the stressor. Statistical differences between independent samples (MWU-test: control vs t=15, control vs t=30) and dependent samples (W-test: t=15 vs t=30) are given on top of the panel (p≤0.001*, p≤0.01**, p≤0.05*, p>0.1 n.s.).**

3.1.3.4 Adrenocorticotropic hormone expression in the pituitary

In order to determine the amount of pituitary ACTH protein, Western blot analysis was performed. Due to technical problems during the protein extraction, one IR sample of the stressed group was lost, reducing the number of samples in this group to nine (HR/IR/LR, N=10/9/10). The number of samples in the unstressed control group was eight per line (HR/IR/LR, N=8/8/8).

Figure 23 shows the relative ACTH protein amount of control and stressed (t=30) HR, IR and LR animals standardised to GAPDH and the IR control group. HR animals showed a significantly higher ACTH protein amount in the pituitary compared to IR and LR animals in both the control (KWH-test: HR/IR/LR, N=8/8/8, H=8.6, df=2, $p<0.05$; post-hoc MWU-test: HR vs IR, U=7, $p\leq0.05$; HR vs LR, U=9, $p<0.05$; IR vs LR, U=31, $p>0.1$) and the stressed group (KWH-test: HR/IR/LR, N=10/9/10, H=10.6, df=2, $p<0.01$; post-hoc MWU-test: HR vs IR, U=13, $p<0.05$; HR vs LR, U=11, $p<0.01$; IR vs LR,

U=44, p>0.1). Interestingly, a within-line comparison revealed no significant differences in pituitary ACTH protein abundance between control and restraint-stressed animals of the HR, IR and LR line (MWU-test: control vs stressed; HR: N=8/10, U=40, p>0.1; IR: N=8/9, U=29, p>0.1; LR: N=8/10, U=33, p>0.1).

Thus, despite the prominent release of CORT from the adrenals in response to stress in all three lines, the restraint stress had only a minor impact regarding the depletion of the available ACTH pool in the pituitary. Furthermore, animals of the IR and LR line showed a similar ACTH abundance in the pituitary despite significant differences in their stress-induced adrenal CORT release. In addition, the stress-induced increase in plasma ACTH levels did not parallel the rise in plasma CORT concentrations in response to stress, particularly in HR and LR animals. This suggests that further mechanisms in addition to ACTH expression, abundance and release are involved in the distinctly different stress response of HR, IR and LR animals.

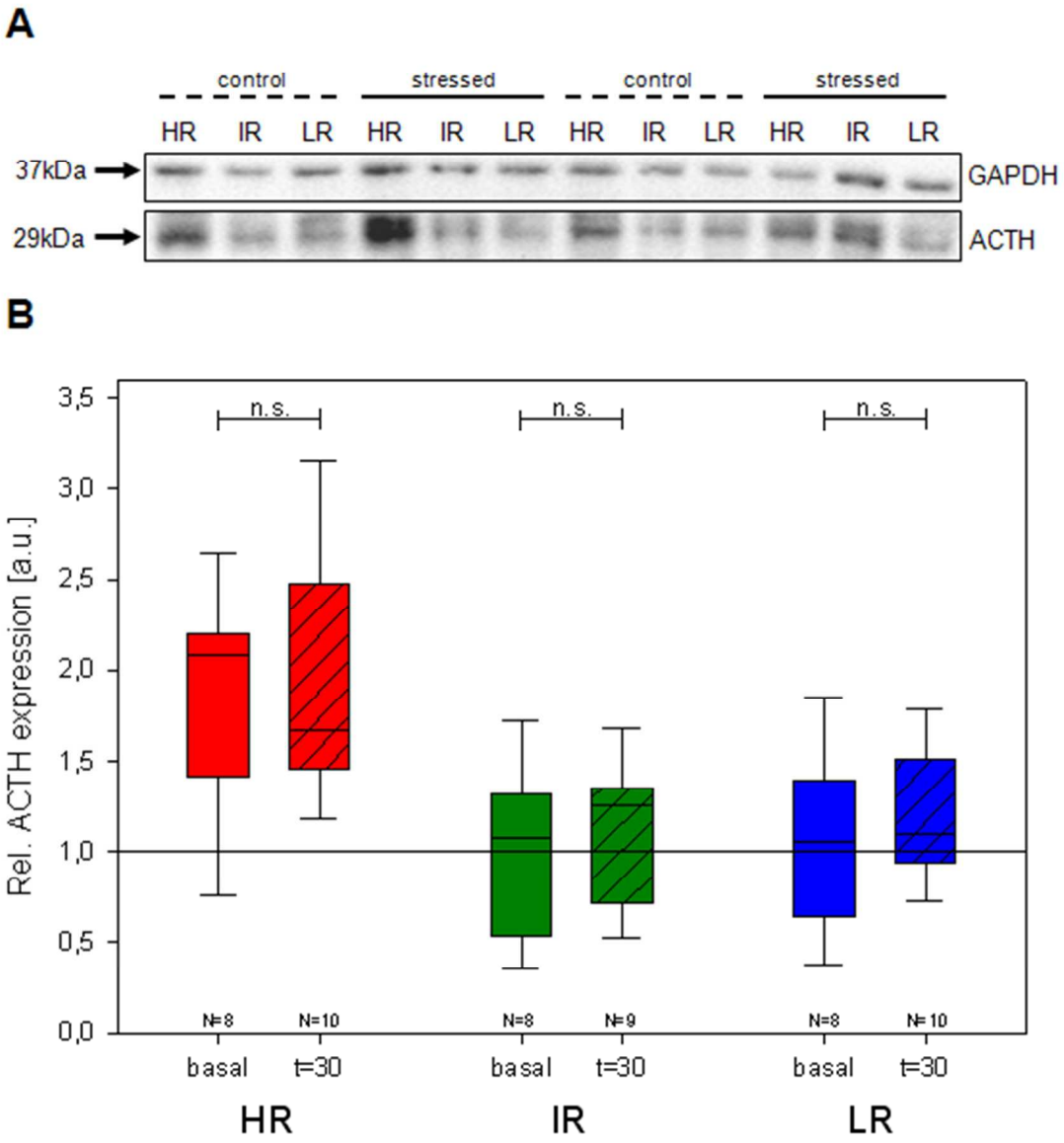

Figure 23: (A) Representative Western blot pictures of adrenocorticotropic hormone (ACTH) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). (B) ACTH expression in the pituitary of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XX relative to GAPDH, normalised to the IR control group. Statistical differences between the three lines (KWH-test) are given in the text, within-line comparisons (MWU-test) are indicated on top of the panel (p>0.1 n.s.).

3.2 Central regulation of hypothalamic-pituitary-adrenal axis activity

3.2.1 Experiment 1: Effects of chronic fluoxetine treatment on emotional behaviour and hypothalamic-pituitary-adrenal axis regulation in HR, IR and LR mice

3.2.1.1 Assessment of emotional behaviour

3.2.1.1.1 Locomotor activity and exploratory drive in the open field test

After 28 days of chronic Flx treatment, the animals were subjected to a 10-min OF test in order to assess their locomotor activity and exploratory drive. Due to technical problems of the ANY-maze software, one Flx-treated LR animal had to be excluded from the analysis, thereby reducing the number of LR animals in this group from nine to eight.

Independent of treatment, the animals of all three lines neither showed significant differences in the total distance travelled, time spent in the inner or outer zone of the OF apparatus, nor in the ratio between inner and outer zone path length (see Table 4). Thus, Flx treatment did not induce changes in locomotion or exploratory drive of the tested animals.

Table 4: Behavioural readout of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII determined in the open field (OF) test. Independent of treatment, the animals of all three lines did not show significant differences in their locomotor activity or exploratory drive.

Parameter measured	$N_{HR/IR/LR}$	treatment	HR		IR		LR		between-line comparison		
			mean	SEM	mean	SEM	mean	SEM	H	p	
total distance travelled [m]	8/8/8	Veh	48.41	6.18	37.96	4.98	34.75	2.89	4.3	0.113	n.s.
	8/9/8	Flx	39.84	2.95	37.66	2.37	33.48	1.34	3.1	0.206	n.s.
time inner zone [s]	8/8/8	Veh	47.28	9.96	39.38	9.26	34.54	9.2	1.8	0.403	n.s.
	8/9/8	Flx	32.79	5.23	40.03	6.77	32.58	6.88	0.5	0.776	n.s.
time outer zone [s]	8/8/8	Veh	552.73	9.96	560.63	9.26	565.46	9.2	1.8	0.403	n.s.
	8/9/8	Flx	567.21	5.23	559.96	6.77	567.43	6.88	0.5	0.776	n.s.
ratio inner outer path length	8/8/8	Veh	0.18	0.03	0.16	0.02	0.16	0.04	0.7	0.682	n.s.
	8/9/8	Flx	0.14	0.02	0.17	0.02	0.13	0.03	2.1	0.342	n.s.

3.2.1.1.2 Assessment of stress-coping behaviour in the forced swim test

Immediately after the OF test, a 6-min FST was performed. A line comparison between HR, IR and LR animals of the Veh-treated group revealed significant differences in stress-coping behaviour regarding their struggling time (KWH-test: N=8/8/8, H=8.8, df=2, $p<0.05$, see Figure 24 B). Veh-treated HR mice showed a significantly increased time spent struggling compared to IR and LR animals (post-hoc MWU-test: HR vs IR, U=10, $p<0.05$; HR vs LR, U=6, $p<0.05$). There was no difference between Veh-treated IR and LR animals (post-hoc MWU-test: HR vs IR, U=27.5, $p>0.1$). Furthermore, a trend in the same direction was observed in Flx-treated HR, IR and LR animals (KWH-test: N=8/9/9, H=4.6, df=2, $p<0.1$). In line with this, HR, IR and LR animals of both treatment groups displayed a significantly different passive stress-coping behaviour, i.e. floating (KWH-test: Veh, N=8/8/8, H=11.6, df=2, $p<0.01$; Flx, N=8/9/9, H=9.3, df=2, $p<0.01$, see Figure 24 A), with HR mice of both treatment groups showing less immobility compared to IR and LR animals (post-hoc MWU-test: Veh, HR vs IR, U=8, $p<0.05$; HR vs LR, U=4, $p<0.01$; Flx, HR vs IR, U=8, $p<0.05$; HR vs LR, U=10, $p<0.05$). Strikingly, a within-line comparison revealed that Flx-treated HR and IR animals had a significantly increased floating time compared to the Veh-treated animals of the respective line (MWU-test: HR and IR, Veh vs Flx, all U=11, all $p<0.05$).

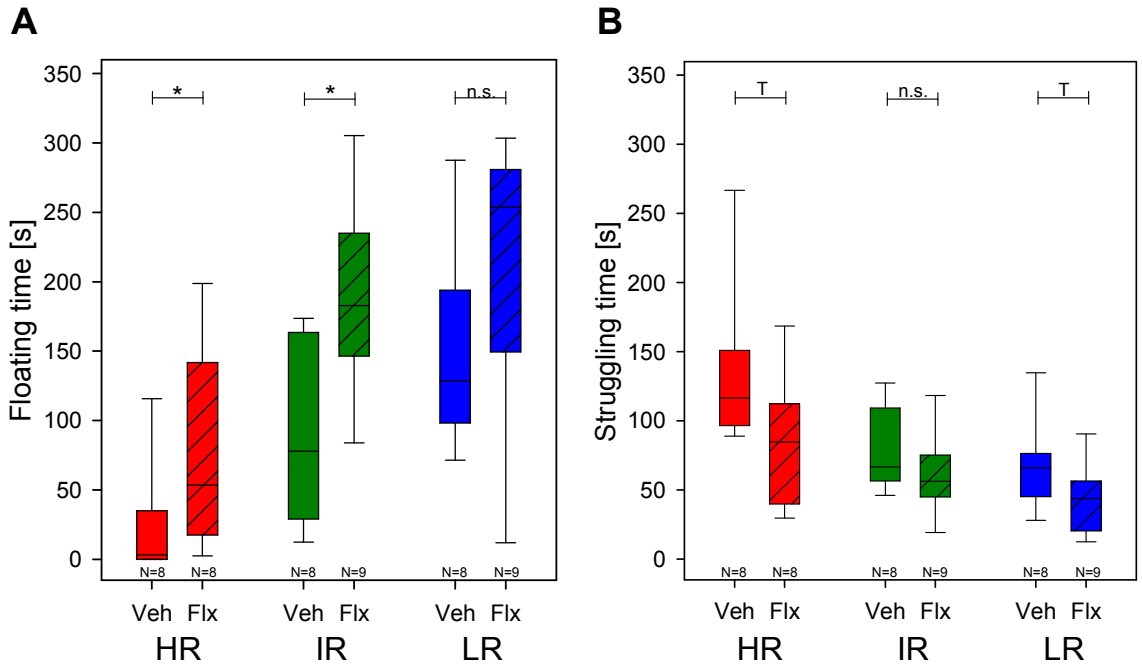

Figure 24 Stress-coping behaviour of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII during the 6-min forced swim test (FST) period. A: time spent floating. B: time spent struggling. For statistical differences between the three lines (KWH-test) see text. Statistical differences between the Veh and Flx group are given on top of the panel (MWU-test: $p\leq0.05$*, $p\leq0.1$ T, $p>0.1$ n.s.).

In addition, the latency until the onset of the first floating episode was reduced in Flx-treated animals of all lines compared to Veh-treated animals (MWU-test: Veh vs Flx; HR, U=8, p<0.05; IR, U=7, p<0.01; LR, U=18, p<0.1, see Figure 25), while HR mice of both treatment groups had a significantly higher latency until their first floating episode compared to the other two lines (KWH-test: Veh, N=8/8/8, H=15, df=2, p<0.001; Flx, N=8/9/9, H=8.3, df=2, p<0.05; post-hoc MWU-test: Veh, HR vs IR, U=3, p<0.01; HR vs LR, U=0, p<0.01; Flx, HR vs IR, U=8, p<0.05; HR vs LR, U=12, p<0.05).

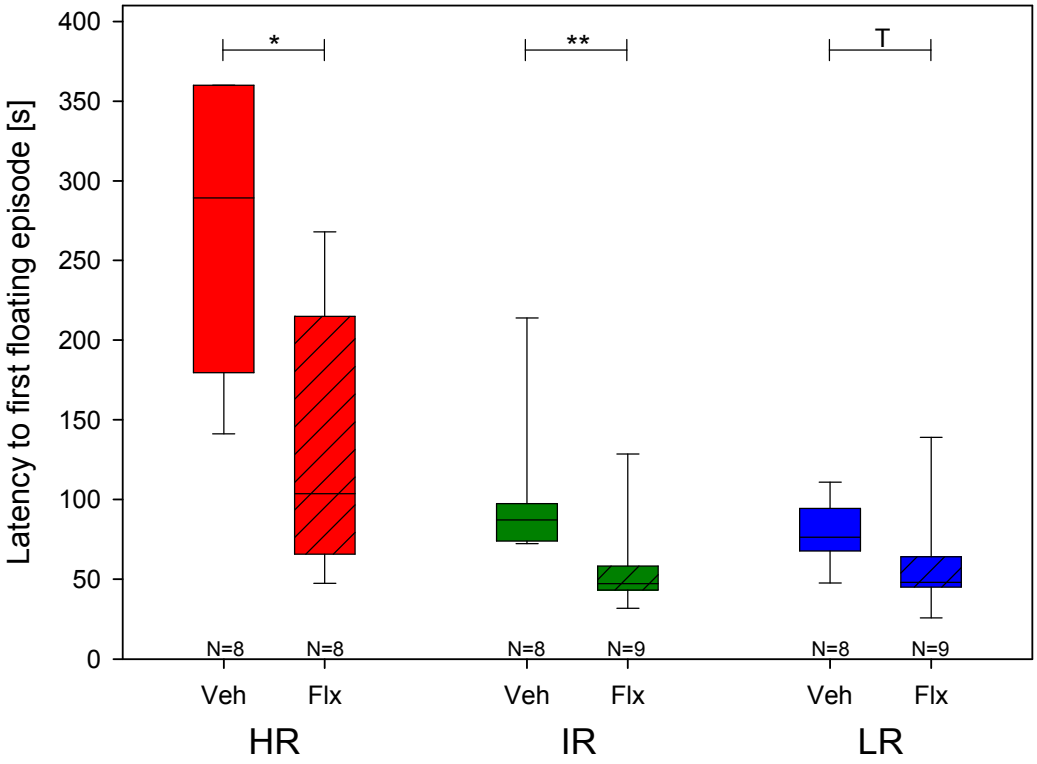

Figure 25: Latency to the first floating episode of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII during the 6-min forced swim test (FST) period. For statistical differences between the three lines (KWH-test) see text. Statistical differences between the Veh and Flx group are given on top of the panel (MWU-test: p≤0.01, p≤0.05*, p≤0.1 T).**

In summary, these results show that HR, IR and LR mice use a distinctly different strategy to cope with the physiologically as well as psychologically demanding situation of the FST, with LR mice resembling a phenotype of behavioural despair, while HR mice display clearly more agitated behaviour. Furthermore, the results suggest that Flx treatment increases the passive stress-coping behaviour of HR and IR animals in the FST compared to their Veh-treated littermates, while LR mice are less affected by the Flx treatment.

3.2.1.2 Hypothalamic-pituitary-adrenal axis reactivity in response to swim stress

In the reference blood sample collected three days prior to the behavioural testing, CORT concentrations were low in HR, IR and LR animals, ranging between mean values of 5 ng/ml and 26 ng/ml (mean ± SEM, Veh, HR: 25.8 ± 8.6, IR: 11.5 ± 5.4, LR: 5.1 ± 1.2; Flx, HR: 14.3 ± 4.0, IR: 13.0 ± 2.9, LR: 5.1 ± 1.6), with no significant differences between Veh and Flx-treated animals (see Figure 26). However, HR animals of the Veh group showed slightly elevated plasma CORT concentrations compared to LR animals (KWH-test: Veh, N=8/8/8, H=6.1, df=2, $p<0.05$; post-hoc MWU-test: HR vs IR, U=16, $p>0.1$; HR vs LR, U=9.5, $p<0.1$; IR vs LR, U=24, $p>0.1$). In the Flx-treated group, HR and IR animals displayed higher plasma CORT concentrations in the reference sample compared to the LR group (KWH-test: Flx, N=8/9/9, H=8.1, df=2, $p\leq0.05$; post-hoc MWU-test: HR vs IR, U=35, $p>0.1$, HR vs LR, U=9.5, $p<0.05$; IR vs LR, U=14, $p<0.05$). Regarding the plasma CORT concentrations determined right after the behavioural test battery (OF + FST), a between-line comparison showed significant differences in the swim stress-induced plasma CORT concentrations between HR, IR and LR animals of both the Veh and Flx group (KWH-test: Veh, N=8/8/8, H=19.2, df=2, $p<0.001$; Flx, N=8/9/9, H=21.0, df=2, $p<0.001$), with HR mice showing clearly higher plasma CORT concentrations compared to the IR line, while LR animals had the lowest increase (post-hoc MWU-test: Veh, HR vs IR, U=3, $p<0.01$; HR vs LR, U=0, $p<0.01$; IR vs LR, U=1, $p<0.01$; Flx, HR vs IR, U=2, $p<0.01$; HR vs LR, U=0, $p\leq0.001$; IR vs LR, U=2, $p<0.01$). However, there was no significant effect of Flx-treatment within the three lines (MWU-test: Veh vs Flx; HR, U=18, $p>0.1$; IR, U=20, $p>0.1$; LR, U=30, $p>0.1$), i.e. chronic Flx treatment did not affect the acute, swim stress-induced CORT secretion from the adrenals.

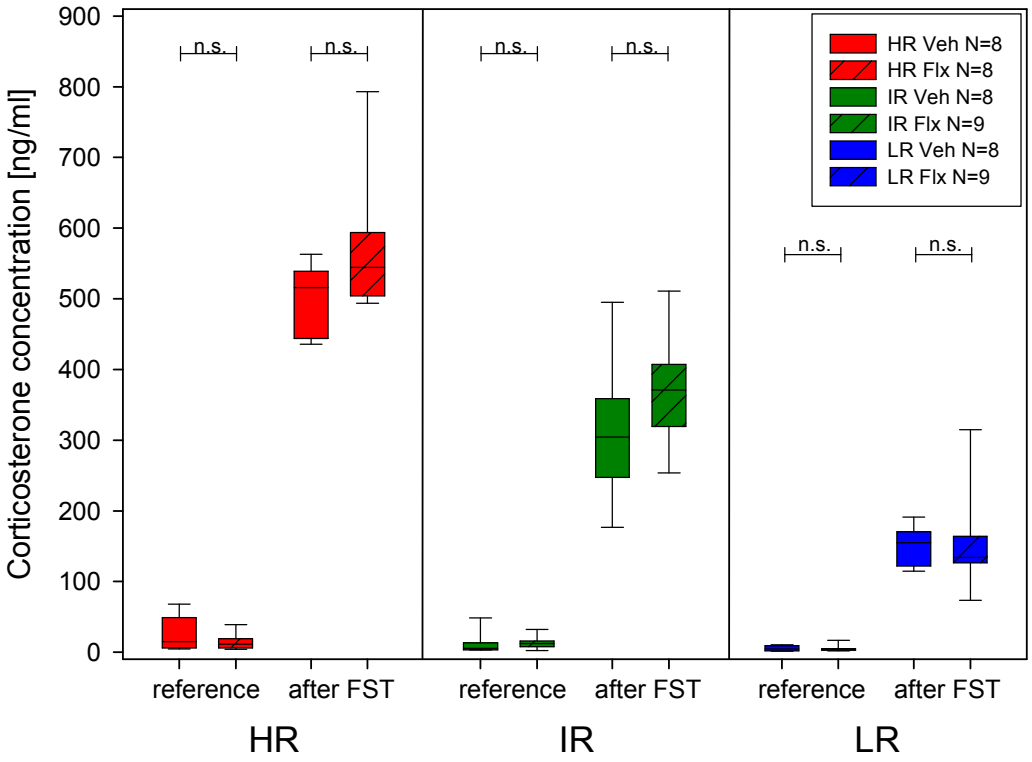

Figure 26: Plasma corticosterone (CORT) concentrations in high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII three days before (reference) and immediately after the behavioural testing (after FST). Statistical differences between the three lines (KWH-test) are given in the text, pairwise group comparisons (MWU-tests) are indicated on top of the panel (p>0.1 n.s.).

3.2.1.3 Hypothalamic-pituitary-adrenal axis activity in the combined dexamethasone/corticotropin-releasing hormone test

Not only to assess HPA axis reactivity in response to an acute stressor (e.g. FST, see above) but to further investigate regulatory mechanisms involved in HPA axis activity, we performed a combined Dex/CRH test with the same animals of the HR, IR and LR line. Due to technical problems, one IR animal of the Flx-treated group had to be excluded, reducing the number of experimental animals to eight in this group (Veh, HR/IR/LR, N=8/8/8; Flx, HR/IR/LR, N=8/8/9).

On day 32, three days prior to the Dex/CRH test, a reference blood sample was collected at 15:00 and plasma CORT concentrations were determined. There were no significant differences between HR, IR and LR mice of both, the Veh and Flx-treated group (KWH-test: Veh, HR/IR/LR, N=8/8/8, H=0.4, df=2, p>0.1; Flx, HR/IR/LR N=8/8/9, H=3.4, df=2, p>0.1). Additionally, a within-line comparison revealed no significant differences between the Veh- and Flx-treated animals (see Figure 27). It should be noted that in general, the plasma CORT levels in these samples (afternoon) were higher compared to

morning samples due to the circadian rhythm of CORT secretion (Touma *et al.*, 2004; Touma *et al.*, 2008; Touma *et al.*, 2009).

On day 35, all animals received an i.p. injection with a low dose of Dex. Six hours after the injection, blood was sampled and plasma CORT levels were assessed. Independently of treatment, Dex induced a suppression of plasma CORT concentrations in all animals (Wilcoxon-test: Veh, HR/IR/LR, N=8/8/8, Z=-2.1/-2.1/-2.5, all $p<0.05$; Flx, HR/IR/LR, N=8/8/9, Z=-2.5/-2.6/-2.6, all $p<0.05$). In Veh-treated animals, the between-line comparison revealed a significantly enhanced Dex-mediated CORT suppression in LR mice compared to HR and IR animals (KWH-test: N=8/8/8, H=10.7, df=2, $p<0.01$; post-hoc MWU-test: HR vs IR, U=25, $p>0.1$; HR vs LR, U=2, $p<0.01$; IR vs LR, U=10, $p<0.05$), while Dex induced a prominent CORT suppression in all Flx-treated animals, which was not different between the lines (KWH-test: N=8/8/9, H=2, df=2, $p>0.1$). Interestingly, however, the CORT-suppressive effect of Dex was significantly higher in HR and IR animals of the Flx-treated group (MWU-test: Veh vs Flx; HR: N=8/8, U=1, $p\leq0.001$; IR: N=8/8, U=9.5, $p<0.05$, see Figure 27), indicating that Flx treatment enhanced the Dex-mediated negative feedback signal on the HPA axis. In animals of the LR line both, Veh and Flx-treated mice showed a highly effective CORT suppression by Dex, which was not significantly different between the two treatment groups.

In response to the injection of CRH, significant differences in plasma CORT concentrations between HR, IR and LR mice of the Veh and Flx group were observed. Independent of treatment, HR mice showed the most pronounced rise in CORT, while LR animals had a clearly attenuated CORT release in response to CRH (HR>IR>LR; Veh, KWH-test: N=8/8/8, H=19.2, df=2, $p\leq0.001$; post-hoc MWU-test: HR vs IR, U=3, $p<0.01$; HR vs LR, U=0, $p<0.01$; IR vs LR, U=1, $p<0.01$; Flx, KWH-test: N=8/8/9, H=20.4, df=2, $p\leq0.001$; post-hoc MWU-test: HR vs IR, U=3, $p<0.01$; HR vs LR, U=0, $p<0.01$; IR vs LR, U=0, $p<0.01$). There was no significant effect of Flx treatment in animals of the HR and IR line. In LR mice, however, the Flx treatment produced a markedly decreased CORT secretion in response to the CRH stimulation compared to Veh-treated LR animals (MWU-test: Veh vs Flx, U=4, $p<0.01$).

Taken together, Flx induced a more pronounced suppression of CORT after Dex treatment as seen in animals of the HR and IR line, which is indicative of a higher Dex-induced negative feedback signal in these animals. In LR animals, albeit their already prominent Dex-induced CORT suppression (as observed in the Veh-treated group), Flx further enhanced the Dex-mediated negative feedback, resulting in a significantly

decreased CORT surge after CRH stimulation. Thus, the results revealed that chronic Flx treatment amplified the negative feedback signal on the HPA axis in all three lines of the SR mouse model, most considerable LR mice.

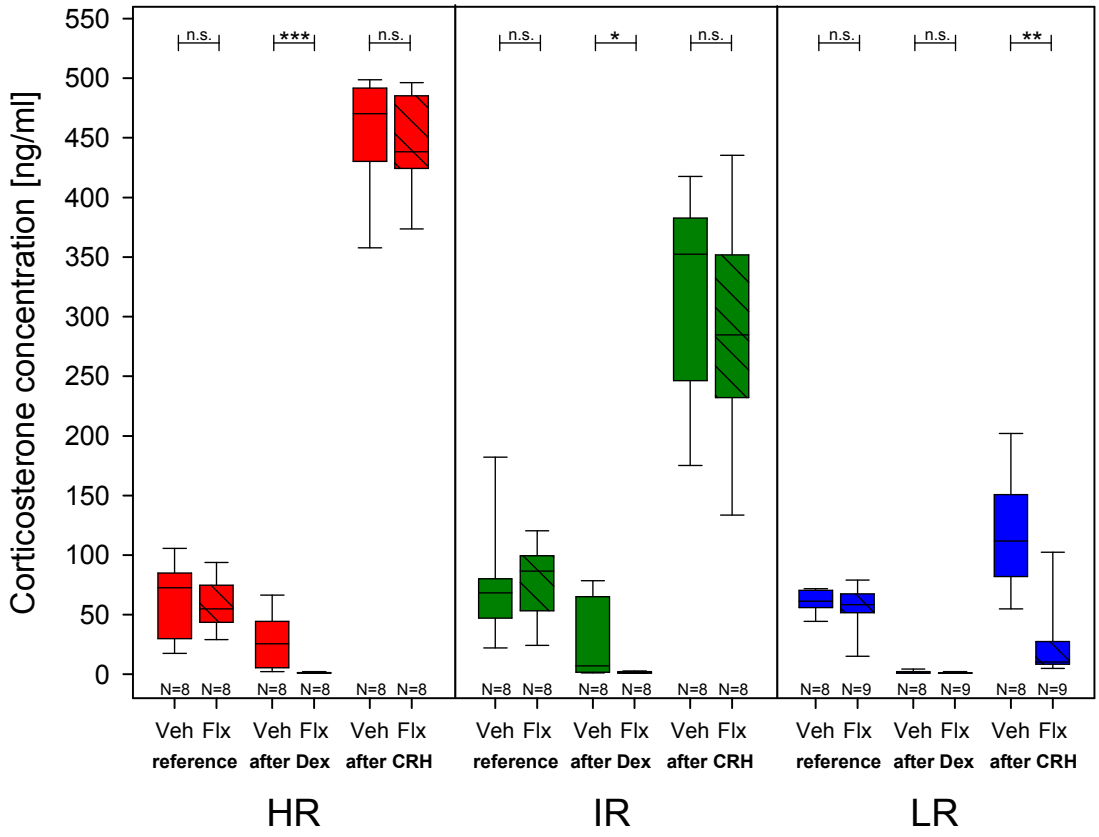

Figure 27: Plasma corticosterone (CORT) concentrations of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII in the Dex/CRH test. Blood samples were collected three days before the test (reference), six hours after Dex application (after Dex) and 30 min after CRH stimulation (after CRH). Statistical differences between the three lines (KWH-test) are given in the text, pairwise group comparisons (MWU-tests) are indicated on top of the panel (p≤0.001*, p≤0.01**, p≤0.05*, p>0.1 n.s.).**

3.2.1.4 Gene expression analysis

3.2.1.4.1 Gene expression in the hippocampus

Directly after the Dex/CRH test, animals were sacrificed and their hippocampi were dissected. From one hippocampal hemisphere RNA was isolated and gene expression analysis was performed.

In the hippocampus of Flx-treated animals, a significant difference between the lines was found in the gene expression profile of *Fkbp4*, *Abcb1b* and *Crhr1*. In Veh-treated animals, only *Abcb1b* was differentially expressed between HR, IR and LR animals.

Solely in LR animals, a within-line comparison (Veh vs Flx-treated animals) revealed significant differences regarding *Fkbp5*, *Abcb1a* and *Gilz*. The results of the qPCR analysis are summarised in Table 5.

Table 5: Relative gene expression in the hippocampus of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII. Values are given as 'fold regulation' relative to the Veh-treated IR group. Post-hoc MWU-tests were performed if the between-line comparison (KWH-test) yielded significant results. Exact p values are given, asterisks represent the significance levels ($p \leq 0.05$*, $p \leq 0.01$). Significant differences between Veh and Flx-treated HR, IR or LR animals are indicated in bold numbers; the corresponding statistical details (MWU-test) are given in Table 3 of the appendix.**

		HR		IR		LR		between line comparison (KWH-test)		post-hoc MWU-test					
										HR vs IR		HR vs LR		IR vs LR	
candidate	treatment	mean	SEM	mean	SEM	mean	SEM	H	p	U	p	U	p	U	p
Nr3c1	Veh	0.98	0.08	1.00	0.07	1.01	0.12	0.1	0.945						
	Flx	1.14	0.11	0.90	0.09	1.01	0.06	2.0	0.362						
Nr3c2	Veh	1.08	0.12	1.00	0.16	0.92	0.09	1.0	0.581						
	Flx	1.17	0.30	0.87	0.10	1.19	0.16	2.0	0.352						
Fkbp4	Veh	1.04	0.15	1.00	0.16	0.88	0.14	0.5	0.756						
	Flx	0.97	0.15	0.64	0.06	1.20	0.16	8.3	0.016*	15	0.086	27	0.386	10	0.021*
Fkbp5	Veh	0.86	0.11	1.00	0.06	**0.76**	**0.04**	5.8	0.054T						
	Flx	0.95	0.07	0.98	0.08	**0.95****	**0.05**	0.0	0.979						
Abcb1a	Veh	1.04	0.09	1.00	0.11	**0.80**	**0.05**	5.6	0.059T						
	Flx	1.07	0.09	0.90	0.10	**1.09***	**0.10**	1.4	0.478						
Abcb1b	Veh	1.91	0.13	1.00	0.27	0.42	0.06	12.4	0.002**	11	0.054	0	0.003**	18	0.141
	Flx	1.94	0.17	1.00	0.29	0.70	0.27	7.7	0.020*	14	0.068	8	0.021*	39	0.895
Crhr1	Veh	1.18	0.16	1.00	0.10	1.17	0.14	0.9	0.636						
	Flx	1.28	0.14	0.90	0.07	1.29	0.13	6.2	0.043*	16	0.108	36	1.000	14	0.057
Cfl1	Veh	1.03	0.08	1.00	0.07	1.00	0.10	0.2	0.888						
	Flx	1.25	0.11	1.00	0.13	1.16	0.08	2.1	0.341						
Gilz	Veh	1.12	0.08	1.00	0.07	**0.95**	**0.08**	3.7	0.153						
	Flx	1.22	0.10	1.10	0.10	**1.27****	**0.08**	1.0	0.596						

3.2.1.4.2 Gene expression in the pituitary

Similarly to the hippocampus, RNA was isolated from the pituitary and gene expression analysis was performed.

In the pituitary of Flx-treated animals, a significant difference between the lines was found in the gene expression profile *Pomc* and *Abcb1b*. In Veh-treated animals, solely *Abcb1a* was differentially expressed between HR, IR and LR animals.

A within-line comparison (Veh vs Flx-treated animals) revealed significant differences regarding *Fkbp5*, *Abcb1a* and *Gilz* in IR animals. Additionally, *Abcb1b* was significantly different in Veh vs Flx-treated animals of the LR line. The results of the qPCR analysis are summarised in Table 6.

Table 6: Relative gene expression in the pituitary of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII. Values are given as 'fold regulation' relative to the Veh-treated IR group. Post-hoc MWU-tests were performed if the between-line comparison (KWH-test) yielded significant results. Exact p values are given, asterisks represent the significance levels (p≤0.05*, p≤0.01, p≤0.001***). Significant differences between Veh and Flx-treated HR, IR or LR animals are indicated in bold numbers, the corresponding statistical details (MWU-test) are given in Table 4 of the appendix.**

		HR		IR		LR		between line comparison (KWH-test)		post-hoc MWU-test					
										HR vs IR		HR vs LR		IR vs LR	
candidate	treatment	mean	SEM	mean	SEM	mean	SEM	H	p	U	p	U	p	U	p
Nr3c1	Veh	0.72	0.13	1.00	0.20	0.75	0.12	1.5	0.464						
	Flx	1.00	0.16	0.80	0.04	1.13	0.19	1.8	0.389						
Pomc	Veh	1.98	0.51	1.00	0.19	0.79	0.15	4.4	0.108						
	Flx	2.63	0.44	1,31	0.23	0.87	0.13	15.0	0.001***	9	0.018*	0	0.003**	18	0.047*
Fkbp5	Veh	0.89	0.06	**1.00**	**0.10**	1.23	0.11	4.5	0.102						
	Flx	1.13	0.14	**1.68 ****	**0.16**	1.41	0.12	4.9	0.084T						
Abcb1a	Veh	1.78	0.22	**1.00**	**0.13**	1.44	0.20	8.0	0.018*	7	0.027*	19	0.172	15	0.148
	Flx	1.99	0.48	**1.89 ***	**0.24**	1.62	0.19	0.4	0.784						
Abcb1b	Veh	0.91	0.18	1.00	0.18	**0.57**	**0.06**	4.8	0.090T						
	Flx	1.32	0.22	0.71	0.09	**0.91 ***	**0.12**	7.1	0.028*	11	0.048*	17	0.136	26	0.200
Crhr1	Veh	1.03	0.12	1.00	0.12	0.65	0.13	3.6	0.160						
	Flx	1.01	0.15	0.73	0.09	0.81	0.12	2.0	0.363						
Gilz	Veh	1.06	0.10	**1.00**	**0.05**	1.09	0.08	0.7	0.692						
	Flx	1.29	0.16	**1.41 ***	**0.12**	1.08	0.11	3.4	0.174						

3.2.1.5 Protein expression in the hippocampus

Protein expression analysis of GR and FKBP51 in Veh- and Flx-treated HR, IR and LR animals was performed in the contralateral hippocampal hemisphere, which was not used for qPCR analysis.

A between-line comparison showed no significant differences in the expression of GR between HR, IR and LR animals of both the Veh and Flx group (KWH-test: Veh, N=8/8/8, H=1.3, df=2, $p>0.1$; Flx, N=8/9/9, H=2.0, df=2, $p>0.1$). In addition, there were no significant differences comparing Veh and Flx-treated animals within the breeding lines (MWU-test: Veh vs Flx; HR: N=8/8, U=28, $p>0.1$; IR: N=8/9, U=25, $p>0.1$, LR: N=8/9, U=22, $p>0.1$, see Figure 28). Similarly, no significant differences in the expression of FKBP51 between HR, IR and LR mice of the Veh group could be detected (KWH-test: Veh, N=8/8/8, H=0.2, df=2, $p>0.1$, see Figure 28). However, Flx treatment increased FKBP51 in animals of the LR line compared to HR and IR mice (KWH-test: Flx, N=8/9/9, H=10.1, df=2, $p<0.01$; post-hoc MWU-test: HR vs IR, U=24, $p>0.1$; HR vs LR, U=7, $p<0.05$; IR vs LR, U=13, $p<0.05$). A within-line comparison, i.e. Veh vs Flx treatment, revealed no significant differences in HR and IR animals. However, a trend could be observed in the LR line (MWU-test: Veh vs Flx; HR: N=8/8, U=27, $p>0.1$; IR: N=8/9, U=34, $p>0.1$, LR: N=8/9, U=16, $p<0.1$).

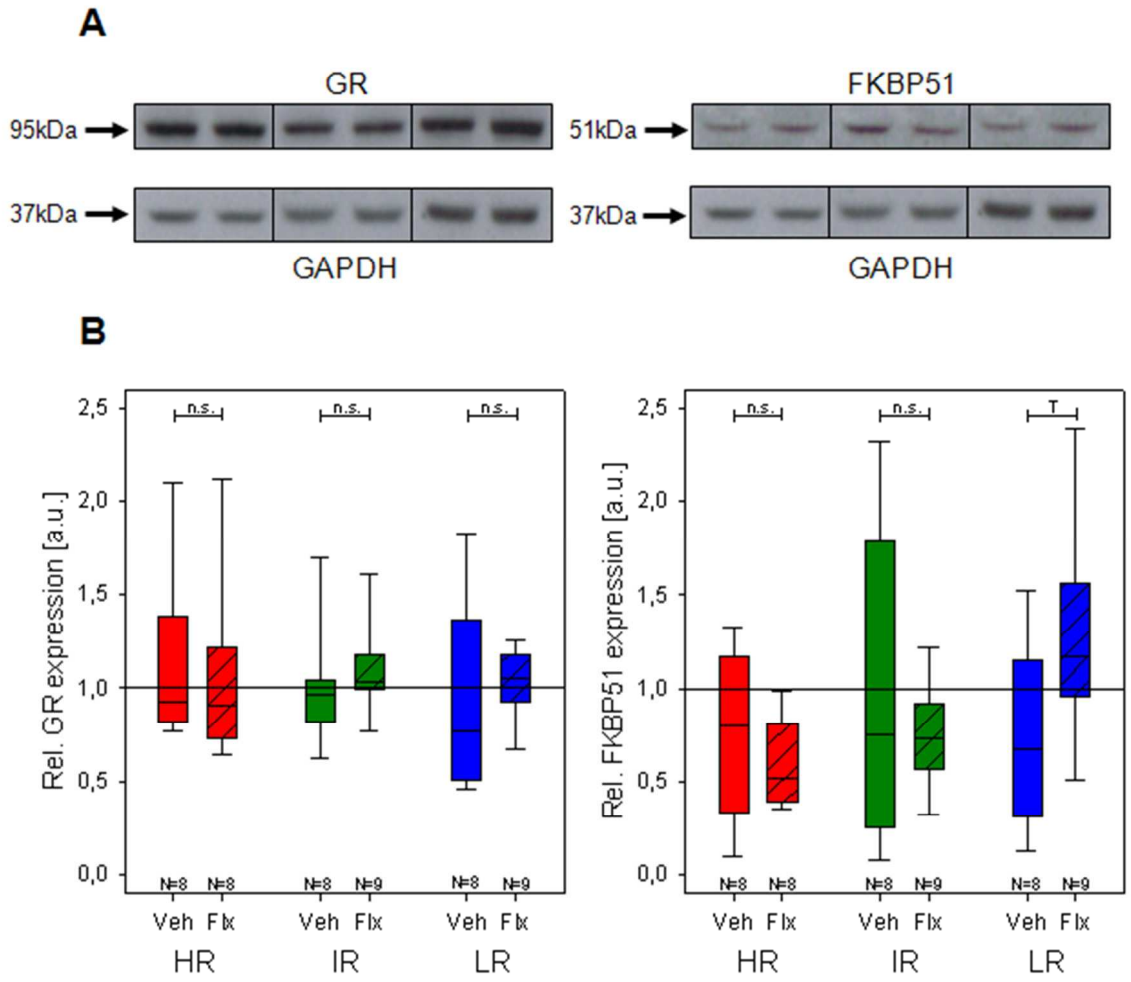

Figure 28: (A) Representative Western blot pictures of the glucocorticoid receptor (GR), FK506 binding protein 51 (FKBP51) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). (B) GR expression (left) and FKBP51 expression (right) in the hippocampus of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII relative to GAPDH, normalised to the IR Veh group. Statistical differences between the three lines (KWH-test) are given in the text, within-line comparisons (MWU-test) are indicated on top of the panel (p≤0.1 T, p>0.1 n.s.).

3.2.2 Experiment 2: Effects of chronic fluoxetine treatment on the spatial reference memory, hypothalamic-pituitary-adrenal axis function and neuronal activation in response to stress in HR, IR and LR mice

3.2.2.1 Assessment of spatial reference memory

After 28 days of chronic fluoxetine treatment, the animals were subjected to a Y-maze test in order to assess their hippocampus-dependent, spatial reference memory abilities. In the retrieval phase, i.e. after a 60-min period in their homecages between acquisition and retrieval phase, animals of all three lines independent of treatment did not show significant differences in the percentage of time exploring exploration the novel arm versus the familiar arms of the apparatus, indicating that neither HR, nor IR, nor LR mice

differentiated between the newly opened arm and the previously explored ones (see Table 7).

Table 7: High (HR), intermediate (IR) and low (LR) reactivity mice of Gen XIX during the retrieval phase of the Y-maze test; per cent time spent in the novel and familiar arms.

			novel arm		familiar arms		Wilcoxon-test		
line	N	treatment	mean	SEM	mean	SEM	Z	p	
HR	12	Veh	28.58	3.01	35.71	1.50	-1.4	0.136	n.s.
	11	Flx	40.34	3.82	29.83	1.91	-1.6	0.110	n.s.
IR	13	Veh	34.01	3.05	33.00	1.53	-0.2	0.807	n.s.
	12	Flx	31.63	2.70	34.19	1.35	-0.9	0.347	n.s.
LR	12	Veh	38.11	4.14	30.94	2.07	-1.0	0.308	n.s.
	7	Flx	34.66	3.90	32.67	1.95	-0.0	1.000	n.s.

Interestingly, Flx-treated HR mice revealed a significantly higher discrimination ratio regarding the exploration time in the novel arm compared to Veh-treated HR animals (MWU-test: Veh vs Flx; HR: N=12/11, U=29.5, $p<0.05$, see Figure 29). This indicates that the accuracy to visit and explore the novel arm was increased in Flx-treated HR mice. However, Flx did not affect the memory performance of IR and LR animals (MWU-test: Veh vs Flx; IR: N=13/12, U=68, $p>0.1$; LR: N=12/7, U=35, $p>0.1$). Furthermore, a between-line comparison did not show significant differences independent of treatment regarding the spatial memory performance in HR, IR and LR animals (KWH-test: HR/IR/LR; Veh, N=12/13/12, H=3.0, df=2, $p>0.1$; Flx, N=11/12/7, H=4.3, df=2, $p>0.1$).

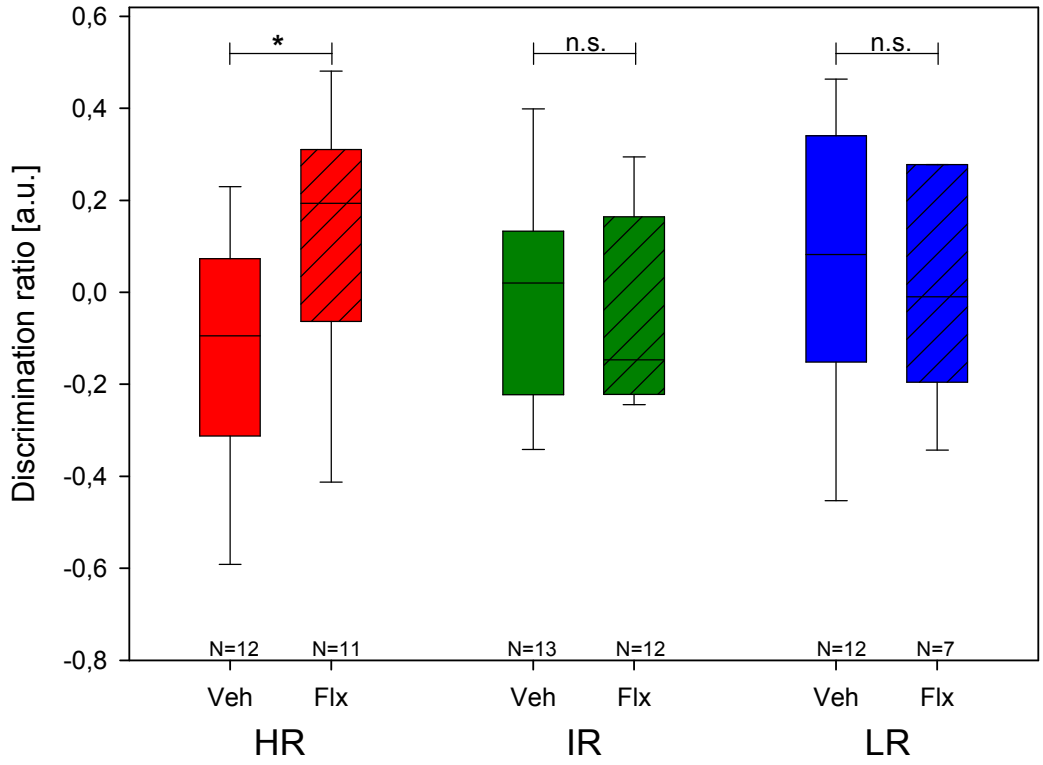

Figure 29: Discrimination ratio (% time spent in novel arm/familiar arms) of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XIX during the retrieval phase of the Y maze test. For statistical differences between the three lines (KWH-test) see text. Statistical differences between the Veh and Flx group are given on top of the panel (MWU-test: p≤0.05*, p>0.1 n.s.).

3.2.2.2 Assessment of stress-induced hypothalamic-pituitary-adrenal axis function and neuronal activation in the central nervous system

On day 35 of chronic Flx treatment, after allowing the animals to recover from the Y-maze testing for seven days, the experimental animals were subjected to a SRT in order to assess their HPA axis response to an acute stressor. The SRT also served as a standardised stressor to induce *C-fos* mRNA expression in the brain as a marker for neuronal activation. Animals of the two control groups (control I and II, for details see methods 2.5.2.2) were euthanised under basal conditions and plasma CORT concentrations as well as basal *C-fos* expression were assessed.

3.2.2.3 Assessment of stress-induced hypothalamic-pituitary-adrenal axis activation

As expected, the plasma CORT concentrations of Veh and Flx-treated HR, IR and LR animals of control I (mean ± SEM [ng/ml], Veh, HR: 14.0 ± 4.6, IR: 9.2 ± 3.1, LR: 12.6 ± 5.8; Flx, HR: 9.8 ± 2.6, IR: 13.9 ± 5.5, LR: 10.6) were similar to the CORT values of the experimental group, which were obtained immediately before the restraint (see below). The plasma CORT levels of the naïve control II animals were slightly lower (mean ±

SEM [ng/ml], HR: 3.9 ± 0.8, IR: 6.2 ± 2.4, LR: 5.9 ± 2.4) than the values of the experimental animals and control I. This suggests that the chronic daily injection procedure induced a moderate rise in basal CORT levels, which were, however, still in the range of unstressed CORT values (mean maximum of approx. 14 ng/ml).

In the experimental animals that were subjected to the SRT, plasma CORT concentrations did not differ significantly between the three lines of the Veh and Flx-treated group immediately before the SRT (KWH-test: Veh, N=9/10/9, H=3.8, df=2, $p>0.1$; Flx, N=9/8/7, H=5.0, $p<0.1$), with a range between 3 ng/ml and 14 ng/ml (mean ± SEM, Veh, HR: 9.9 ± 2.8, IR: 11.0 ± 2.5, LR: 5.2 ± 0.8; Flx, HR: 11.5 ± 3.4, IR: 13.7 ± 4.9, LR: 3.8 ± 0.8). Furthermore, there were no significant differences between Veh and Flx-treated animals in any of the three breeding lines (MWU-test: Veh vs Flx; HR: N=9/9, U=37, $p>0.1$; IR: N=10/8, U=38, $p>0.1$; LR: N=9/7, U=22.5, $p>0.1$). After 15 min of restraint, plasma CORT levels in HR, IR and LR animals were elevated independently of treatment, with HR animals displaying markedly increased CORT concentrations compared to IR animals, while LR mice had the lowest stress-induced plasma CORT levels (HR>IR>LR; Veh, KWH-test: N=9/10/9, H=22.4, df=2, $p\leq0.001$; post-hoc MWU-test: HR vs IR, U=6, $p\leq0.001$; HR vs LR, U=0, $p<0.01$; IR vs LR, U=0, $p<0.01$; Flx, KWH-test: N=9/8/7, H=20.4, df=2, $p\leq0.001$; post-hoc MWU-test: HR vs IR, U=0, $p<0.01$; HR vs LR, U=0, $p\leq0.001$; IR vs LR, U=0, $p<0.01$). However, Flx-treated LR animals revealed a tendency towards lower stress-induced plasma CORT levels compared to Veh-treated LR mice. Interestingly, 60 min after the onset of the stressor, Flx-treated LR animals showed significantly reduced plasma CORT concentrations compared to Veh-treated LR mice (MWU-test: Veh vs Flx; N=9/7, U=12, $p<0.05$). This indicates that Flx treatment increased the negative feedback inhibition of the adrenal CORT secretion in animals of the LR line. However, Flx treatment did not affect the plasma CORT concentrations 60 min after the onset of the SRT in HR and IR animals (MWU-test: Veh vs Flx; HR: N=9/9, U=36, $p>0.1$; IR: N=10/8, U=32, $p>0.1$). Moreover, 60 min after the onset of the stressor, plasma CORT concentrations were significantly reduced compared to the CORT levels immediately after the SRT only in HR animals (W-test: Veh/Flx, N=9/9, Z=-2.66, all $p<0.01$, see Figure 30), while there was no decrease in the respective samples for IR and LR animals of the Veh and Flx-treated group (W-test: Veh/Flx, IR: N=10/8, Z=-1.27/-0.56, all $p>0.1$; LR: N=9/7, Z=-1.00/0.00, all $p>0.1$).

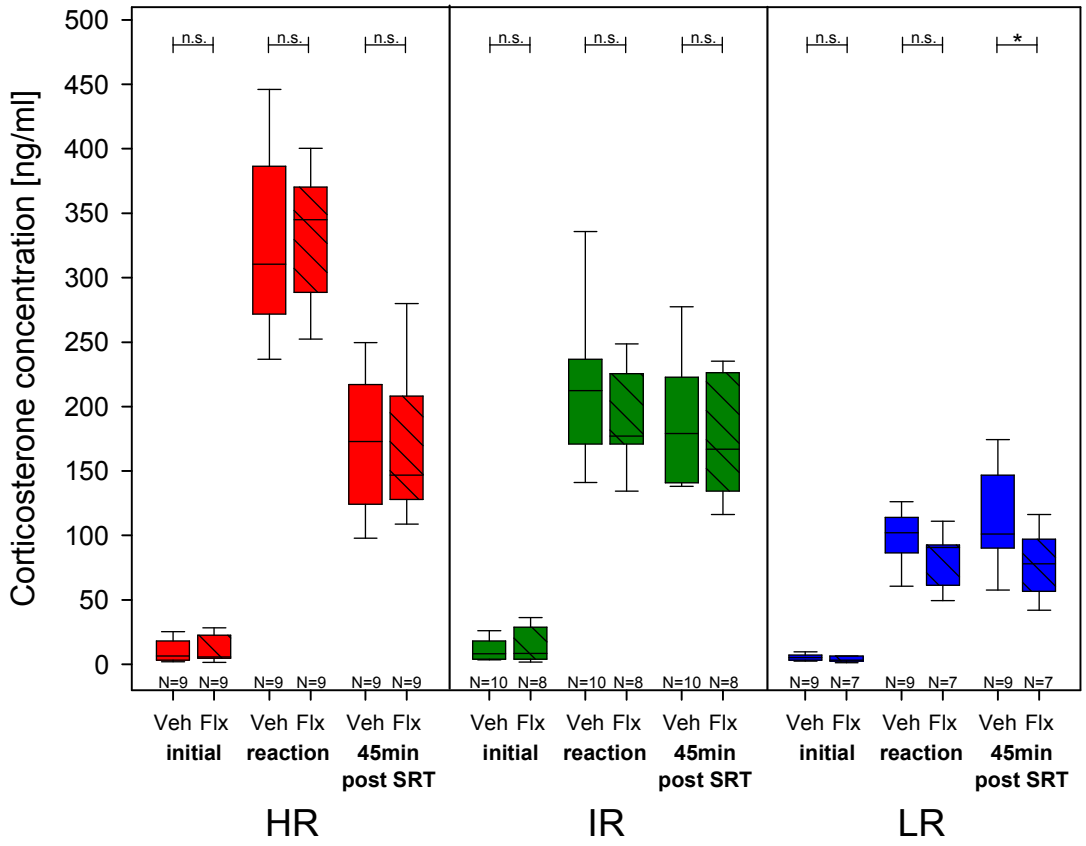

Figure 30: Plasma corticosterone concentrations of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XIX. Blood samples were collected immediately before (initial) and right after (reaction) the 15-min restraint period as well as 45min after the termination of the restraint (45min post SRT). Statistical differences between the three lines (KWH-test) and within treatment groups (W-test) are given in the text, pairwise group comparisons (MWU-tests) are indicated on top of the panel (p≤0.05*, p>0.1 n.s.).

3.2.2.4 Assessment of stress-induced neuronal activation in the brain

As expected, there was only low *C-fos* expression (as a marker for neuronal activation) in control I and control II animals (reflected by the very low grey values of the *in-situ* signal hardly deviating from the background) compared to HR, IR and LR animals subjected to the SRT (see Figure 31).

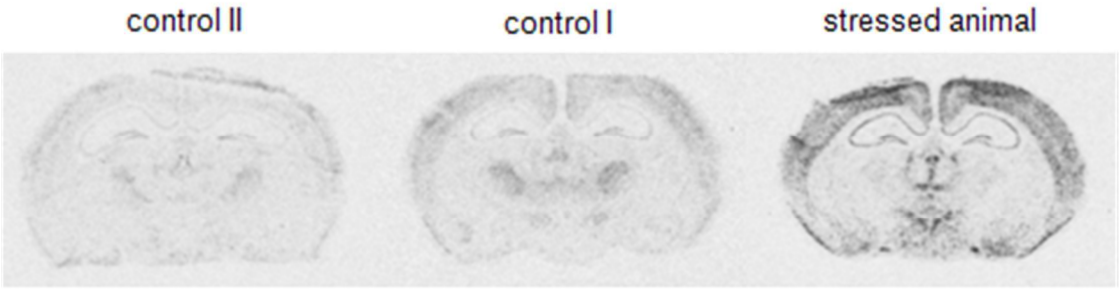

Figure 31: Representative pictures of *C-fos* expression in the brain of the experimental mice. Left: naïve animal (control II). Middle: animal that received chronic injections but was not subjected to the SRT (control I). Right: animal that was subjected to the SRT (stressed animal).

Additionally, no differences could be observed between animals of the control I and control II group regarding the *C-fos* expression in the investigated brain areas.

However, Flx-treated HR, IR and LR animals of control I showed significant differences in *C-fos* expression in the BLA, CA3 of the dorsal hippocampus and CA1, CA3 and DG of the ventral hippocampus (see Table 5 in the appendix). In this respect, however, it should be emphasised that these statistical differences were potentially brought about by the small sample size of control I (Veh: $N_{HR/IR/LR}$=4/4/4, Flx: $N_{HR/IR/LR}$=4/4/2) as well as by the limited sensitivity (close to the detection limit) of the method. Thus, reliable statistical conclusions cannot be drawn from this data.

Table 8 shows the *C-fos* mRNA expression obtained from HR, IR and LR which were subjected to the 15-min SRT. As expected, the 15-min restraint stress considerably increased *C-fos* mRNA expression 60 min after the onset of the stressor in all analysed brain areas with the exception of the dentate gyrus of the dorsal and ventral hippocampus, where the increase in *C-fos* was less prominent and already the animals of both control groups showed a considerable *C-fos* expression. In most of the analysed brain regions, Flx treatment had no major effect on the neuronal activation, except for the CA2 of the dorsal hippocampus and CA3 of the ventral hippocampus in LR, as well as the CA3 of the dorsal hippocampus in HR animals. Here, significant differences between Veh and Flx-treated animals were detected.

Table 8: *C-fos* mRNA expression (optical density) in the brain of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XIX which were subjected to the SRT. Post-hoc MWU-tests were performed if the between-line comparison (KWH-test) yielded significant results. Exact p values are given, asterisks represent the significance levels (p≤0.05*). Significant differences within animals of the HR, IR or LR line are indicated by bold numbers, the corresponding statistical details (MWU-test) are given in Table 7 of the appendix.

				HR		IR		LR		between line comparison (KWH-test)		post-hoc MWU-test					
												HR vs IR		HR vs LR		IR vs LR	
brain region	subregion	$N=_{HR/IR/LR}$	treatment	mean	SEM	mean	SEM	mean	SEM	H	p	U	p	U	p	U	p
PFC		9/10/9	VEH	47.33	2.37	45.46	1.47	51.38	2.29	3.2	0.200						
		8/8/6	Flx	47.54	1.81	44.43	2.48	50.98	3.30	1.0	0.599						
PVN		9/10/9	VEH	46.47	4.14	38.92	2.89	30.32	2.13	9.0	0.011*	32	0.288	8	0.012*	20	0.082T
		8/8/6	Flx	47.00	2.58	36.56	3.51	28.49	4.43	8.6	0.013*	13	0.092T	5	0.042*	10	0.071T
BLA		9/10/9	VEH	35.41	3.23	29.90	1.53	28.43	1.86	2.4	0.288						
		8/8/6	Flx	30.95	2.13	30.95	2.56	22.33	2.14	7.8	0.020*	31	0.916	5	0.028*	5	0.042*
dHC	CA1	9/10/9	VEH	29.09	2.81	28.34	1.62	33.10	2.76	1.8	0.397						
		8/8/6	Flx	28.95	2.06	27.44	3.13	28.32	2.42	1.0	0.581						
	CA2	9/10/9	VEH	23.25	3.04	29.89	2.01	**29.23**	**2.39**	3.1	0.208						
		8/8/6	Flx	22.30	1.99	25.38	1.79	**22.76***	**0.58**	1.5	0.450						
	CA3	9/10/9	VEH	**21.20**	**1.70**	27.03	1.80	28.14	2.48	6.4	0.040*	18	0.081T	16	0.062T	45	1.000
		8/8/6	Flx	**25.32***	**1.25**	27.17	2.74	24.28	1.36	1.2	0.545						
	DG	9/10/9	VEH	25.63	1.68	22.46	0.92	27.15	1.86	3.8	0.148						
		8/8/6	Flx	23.98	0.78	20.86	1.96	25.37	2.18	2.0	0.353						
vHC	CA1	9/10/8	VEH	20.15	2.22	23.86	1.96	26.99	2.42	2.9	0.225						
		8/8/6	Flx	19.59	1.45	21.03	1.48	22.13	2.01	1.0	0.587						
	CA3	9/10/8	VEH	12.14	1.13	12.24	0.84	**16.05**	**1.36**	4.7	0.093						
		8/8/6	Flx	12.64	1.38	12.69	1.18	**11.84***	**1.69**	0.6	0.715						
	DG	9/10/8	VEH	11.45	1.17	12.40	0.93	16.15	1.30	6.1	0.046*	35	0.414	14.5	0.076T	16	0.099T
		8/8/6	Flx	13.18	1.17	11.48	0.88	12.91	1.19	1.6	0.429						

More strikingly, independent of treatment, HR animals showed a significantly higher *C-fos* expression in the PVN compared to LR animals. Additionally, a trend was observed between both, Veh and Flx-treated IR and LR animals (see Table 8 and Figure 32). This is of interest, since the PVN is highly involved in the control of HPA axis function, integrating neuronal inputs from a multitude of brain regions and consequently initiating HPA axis activity which, eventually, results in CORT secretion from the adrenal cortex. These results show that the distinctly different CORT increase between HR, IR and LR animals in response to stressors is not only a peripheral phenomenon (e.g. ACTH release from the pituitary or differential adrenal sensitivity) but also has a CORT-independent neuronal correlate in the central nervous system.

In summary, *C-fos* expression analysis in HR, IR and LR animals showed that (i) acute restraint stress prominently increased *C-fos* expression in all investigated brain regions, (ii) strikingly, in the PVN of HR animals, this increase was significantly higher compared to LR animals and (iii) Flx treatment had no major effect on neuronal activation in the investigated brain regions.

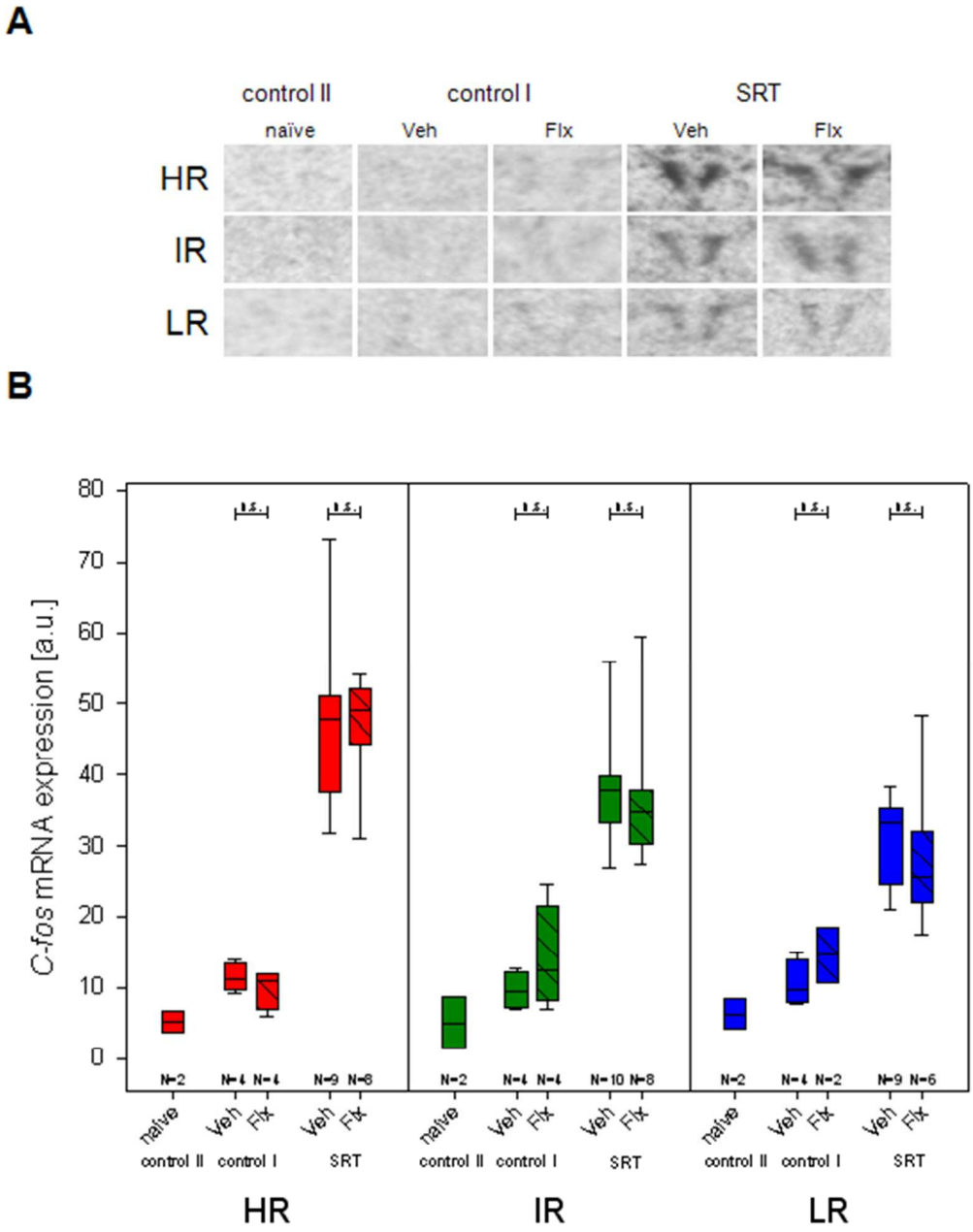

Figure 32: (A) Representative pictures of C-fos mRNA expression in the paraventricular nucleus of the hypothalamus (PVN) detected by in-situ hybridisation (B) C-fos mRNA expression in the PVN of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XIX. Statistical differences between the three lines (KWH-test) are given in the text, within-line comparisons (MWU-test) are indicated on top of the panel (p>0.1 n.s.).

4 Discussion

The body of work presented here aimed to characterise the molecular-genetic underpinnings of the distinctly different neuroendocrine and associated behavioural phenotypes of the HR, IR and LR line of the SR mouse model in more detail. Therefore, not only the distal branches of the HPA axis such as the adrenals, the pituitary as well as corticosteroid-binding globulin were investigated in the three lines, but we furthermore aimed to find molecular correlates of HPA axis dysfunction in the central nervous system of the animals. The HPA axis is a complex and highly dynamic system with regulatory elements on many different levels, peripheral as well as in the CNS. Thus, we wanted to elucidate whether in HR, IR and LR animals, albeit these mice are selectively bred for differences in CORT secretion in response to stressors, preceeding levels in the chain of HPA axis regulatory elements would be affected.

Indeed, through our studies, we gained insight into the mechanisms of HPA axis dysfunction beyond the selection criterion of stress-induced CORT secretion, thus revealing differences between HR, IR and LR mice on all levels of HPA axis function, i.e. the hypothalamus, the pituitary as well as the adrenals.

4.1 Peripheral regulation of the hypothalamic-pituitary-adrenal axis

Metyrapone inhibits corticosterone secretion in response to stress

At the adrenal level, we pharmacologically inhibited CORT synthesis and secretion using metyrapone (Met) in order to evaluate whether the differences in stress reactivity between the three lines were associated with the divergent coping strategies in behavioural tests.

After Met treatment, there was a significant decline in plasma CORT concentrations in animals of all three lines compared to Veh-treated animals in response to the FST (see Figure 13). This was expected, since at this dose Met, which suppresses *de novo* steroidogenesis by inhibiting cytochrome P450 11β-hydroxylase (CYP450c11), produces a greater than 50% reduction in plasma CORT within the first hour in rats (Jenkins *et al.*, 1958; Roberts *et al.*, 1984; Piazza *et al.*, 1994). Interestingly, the significant differences in stress-induced plasma CORT concentrations between the three lines persisted in Met-treated animals. This indicates that (i) the enzymatic activity of CYP450c11 was not

completely suppressed by Met at a dose of 50 µg/g BW or (ii) other mechanisms than *de novo* synthesis were involved in the secretion of CORT from the adrenals in HR, IR and LR animals. In a pilot study, validating the CORT-suppressive effect of Met, we injected animals of all three lines with two doses of Met, either 50 µg/g BW or 100 µg/g BW and subjected them to a FST. We observed that neither the lower dose, nor the high dose of Met completely suppressed the CORT secretion of HR, IR and LR animals. Moreover, the differences between the three lines (HR>IR>LR) in plasma CORT levels still persisted. Therefore, we suggest that the differences in plasma CORT concentration between Met-treated animals of the three lines are brought about by the stress-induced secretion of CORT vesicles stored in the adrenal cortex. This is supported by studies demonstrating the involvement of coated vesicles in the secretion of CORT by the *zona fasciculata* of the rat adrenal cortex (Bassett *et al.*, 1980; Mohn *et al.*, 2005).

Inhibition of corticosterone secretion alters the emotional behaviour of stress reactive mice

In the FST, HR, IR and LR animals showed significant differences in their stress-coping behaviour with HR mice displaying an hyper-active coping style, while LR animals showed signs of behavioural despair reflected by high immobility (see Figure 14). This was expected since previous studies showed that already in generation III of the SR model, animals of the three lines showed robust differences in their stress-coping behaviour which persisted in the following generations (Touma *et al.*, 2008; Knapman *et al.*, 2010a). Interestingly, Met treatment induced a further increase in active stress-coping behaviour in HR and IR mice, i.e. increased struggling. It remains to be clarified whether these effects were brought about by the inhibition of CORT synthesis and secretion *per se*. In a study performed in our group with a similar experimental setup, we could show that exogenously administered CORT did not affect the behavioural phenotype of HR, IR and LR animals in the FST (data not shown). Therefore, we hypothesised that the differences in the stress-coping behaviour between HR, IR and LR animals were brought about by other mechanisms shaping the behavioural endophenotypes of the animals of the three lines in the FST. The neuropeptide CRH plays a considerable role in the modulation of emotional behaviour. It has been shown that rats, which received i.c.v. injections of CRH, presented increased locomotion (Sutton *et al.*, 1982) as well as an increased active coping behaviour in the FST (Dunn *et al.*, 1990). Accordingly, mice overexpressing CRH

in the central nervous system displayed a more active stress-coping behaviour in the FST (Lu *et al.*, 2008; Dedic *et al.*, 2011). This increase in activity/hyperarousal was associated with a CRH-induced increase in activation of monoaminergic neurotransmission in brain structures such as the ventral tegmental area (Summers *et al.*, 2003), the dorsal raphe nucleus (Linthorst *et al.*, 2002) and the locus coeruleus (Valentino *et al.*, 1983; Curtis *et al.*, 1997). Interestingly, co-expression of glucocorticoid receptors was detected in many of these areas (Maras *et al.*, 2012). Additionally, it was shown that glucocorticoids have suppressive effects on CRH signalling, decreasing the impact of the CRH system in these areas, for instance locus coeruleus (LC) activity (Lechner *et al.*, 1999). Since the LC is prominently involved in the modulation of behavioural states such as anxiety and arousal (Berridge *et al.*, 2003), we suggest that the pharmacological inhibition of endogenous CORT synthesis and secretion by Met induced an amplifying effect on the CRH-induced activity of the LC noradrenergic system, thus, further increasing the active stress-coping behaviour in HR and IR mice. On the other hand, we propose that LR animals were less affected by Met treatment regarding their stress coping style in the FST, since these animals show a less active brain CRH system, e.g. reflected by a decreased stress-induced excitation of the PVN (see discussion below) which, in turn, might have had a less prominent influence on the central monoaminergic system.

In line with this, *in-situ* hybridisation studies in HR, IR and LR mice revealed a significantly increased CRH mRNA expression in the CeA of HR animals, while LR mice had the lowest expression (Touma *et al.*, in preparation). This is of interest, since it was shown that repeated or chronic exposure to elevated levels of CORT (reflecting the condition in HR mice) induced an upregulation of CRH mRNA in the CeA (Makino *et al.*, 1994a,b), a brain region critically involved in modulating the behavioural response to stress (Regev *et al.*, 2011; Regev *et al.*, 2012). Furthermore, CRH-antagonism in the CeA decreased anxiety-related behaviour (Liebsch *et al.*, 1995) and attenuated foot-shock-induced freezing behaviour (Swiergiel *et al.*, 1993), supporting the prominent role of the CeA CRH system in behavioural emotionality. Furthermore, Lechner and colleagues showed that CRH immunoreactive neurons in the CeA colocalised with GR, suggesting the GC-sensitivity of this brain region (Lechner *et al.*, 1999). Therefore, we suggest that (i) the hyper-active stress-coping behaviour of HR mice in the FST is associated with elevated levels of CRH mRNA in the CeA which is in contrast to LR mice showing more passive coping strategies and decreased levels of CRH mRNA in the CeA and (ii) that due to the GC-sensitivity of CRH neurons in the CeA, pharmacological inhibition of

endogenous CORT synthesis and secretion by Met potentially disinhibited the activity of CRH neurons, thus, further increasing the active stress-coping behaviour in HR and IR mice. Following this line of reasoning, the question remains why the exposure to the 10 min open field test did not induce differential effects regarding locomotion or exploration between the three lines (see Table 3). However, it was shown that the intensity of a stressor markedly affects the adrenal response in terms of CORT secretion (Djordjevic *et al.*, 2003) and that, in contrast to mild stressors such as exposure to a novel environment, a severe and life threatening stressor such as the FST induces a much higher secretion of CORT from the adrenals (Briski, 1996; Qian *et al.*, 2011). Therefore, we speculate that during the 10 min open field test (resembling a relatively mild novel environment stressor), the level of secreted CORT was not high enough to affect GC-sensitive brain structures involved in behavioural emotionality.

Adrenal gene expression in HR, IR and LR mice

It is known that the dominant mechanism of CORT secretion in response to stressors is *de novo* biosynthesis of CORT upon ACTH stimulation (Garren *et al.*, 1965; Boyd *et al.*, 1973), although CORT biosynthesis is not solely induced by ACTH (see discussion below). Thus, we were interested in whether the observed differences in CORT secretion in response to stressors between HR, IR and LR mice were brought about by mechanisms beyond the ACTH-induced adrenal stimulation. It has been shown that, in addition to the neuroendocrine signalling cascade of the HPA system, the sympathetic adrenomedullary system (SAM) is prominently involved in the stress response. Neuronal efferents, descending from the hypothalamic PVN (Harris, 1950; Yoshimatsu *et al.*, 1987) innervate the adrenal cortex as well as the adrenal medulla (Bornstein *et al.*, 1999; Engeland *et al.*, 2005) and that stimulation of splanchnic nerve fibres result in CORT release from the adrenals (Bornstein *et al.*, 2008). Moreover, a mutual interaction of the adrenal medulla and the adrenal cortex has been suggested, since catecholamines released from chromaffin cells of the adrenal medulla stimulate steroidogenesis (Bornstein *et al.*, 1999), while phenylethanolamine-N-methyltransferase (PNMT) expression in the adrenal medulla is dependent on high concentrations of glucocorticoids (Wurtman *et al.*, 1965), for instance after stress. Kvetnansky and colleagues revealed a virtual absence of stress-induced adrenomedullary PNMT expression in CRH knockout mice with low adrenal glucocorticoid concentrations (Kvetnansky *et al.*, 2006) while in rats immobilisation stress increased PNMT (Axelrod *et al.*, 1984) and tyrosine hydroxylase (TH) (Axelrod *et*

al., 1984; McMahon *et al.*, 1992) expression in the adrenal medulla. This supports the glucocorticoid-dependency of adrenal PNMT and TH expression. Interestingly, PNMT as well as TH mRNA expression were not different between HR, IR and LR animals (see Figure 15), albeit significant differences in stress-induced plasma CORT concentrations. However, it is of note that the animals of the SR mouse model are selected based on their HPA axis reactivity in response to stress, while they do not show prominent differences in plasma CORT concentrations under basal conditions (for examples see Touma *et al.*, 2008 or Figure 19). Considering the CORT-sensitivity of PNMT and TH mRNA expression, this might explain the similar mRNA expression levels of these two enzymes. Furthermore, the gene expression analysis was performed in adrenals of mice which were sacrificed immediately after the termination of the stressor, therefore probably leaving insufficient time for the induction of transcriptional processes of adrenal PNMT as well as TH mRNA.

StAR, which is critically involved in cholesterol trafficking (the precursor of all steroid hormones) from the cytoplasm to the inner mitochondrial membrane, catalyses the rate-limiting step in a long enzymatic cascade of steroidogenesis (Jefcoate *et al.*, 1992; Kim *et al.*, 1997; Stocco, 2002). Moreover, the StAR system enables steroidogenic cells of the *zona fasciculata* and *zona glomerulosa* of the adrenal cortex to initiate and terminate steroidogenesis within a few minutes, permitting the rapid regulation of serum steroid hormone concentrations, e.g. in response to stress (Bose *et al.*, 2002). Interestingly, differences in StAR mRNA expression were observed between the three lines, with HR animals showing a significantly increased expression of StAR compared to IR animals (see Figure 15 D). It has been shown that increased StAR expression and activity paralleled increases in plasma pregnonolone, progesterone and corticosterone levels (Khisti *et al.*, 2003). Therefore, the overexpression of StAR in HR mice is in line with the high CORT secretion from the adrenals in response to stressors in these animals. The question remains, however, why animals of the LR line also showed high levels of StAR mRNA expression, albeit their low stress-induced adrenal CORT release. The following possibilities are conceivable. First, a plethora of enzymatic steps is involved in the conversion of cholesterol which eventually leads to CORT synthesis (Miller *et al.*, 2011). Therefore, we cannot exclude that dysfunctional enzymatic activities downstream of the rate-limiting step of cholesterol trafficking to the inner mitochondrial membrane are involved in bringing about the decreased plasma CORT concentrations in response to stress in LR mice. Second, it has been shown that steroidogenesis is dependent on ACTH

and its mediator cAMP by regulating the mitochondrial precursor pool of cholesterol (Mahaffee *et al.*, 1974) and that cholesterol is depleted from adrenal lipid droplets in response to stress inducing steroidogenesis (Trzeciak *et al.*, 1973). Therefore, a diminished availability of adrenal cholesterol in LR animals could induce compensatory mechanisms, i.e. increasing StAR expression, to assure a functional steroidogenesis despite low concentrations of adrenal cholesterol, although this hypothesis has to be tested in further experiments in mice of the SR mouse model assessing adrenal cholesterol content. Since it has been shown that steroidogenesis is critically dependent on ACTH signalling (Garren *et al.*, 1965) and that ACTH binding to its receptor, the MC2R, stimulates cholesterol transfer by increasing the susceptibility of mitochondrial outer membranes to cholesterol (Jefcoate *et al.*, 1992), we investigated the expression of the ACTH receptor in HR, IR and LR mice and its function. Interestingly, we did not observe differences in MC2R mRNA expression levels in HR, IR and LR mice, indicating that the distinct differences in CORT secretion were not brought about by the abundance of the ACTH receptor.

Assessment of the adrenal sensitivity in HR, IR and LR mice

In the second experiment addressing peripheral mechanisms of HPA axis function, we showed that after pharmacological hypophysectomy by a high dose of Dex, which resulted in a prominently reduced baseline CORT secretion, HR, IR and LR animals revealed distinct differences in their adrenal CORT secretion in response to exogenously administered doses of ACTH (see Figure 17). HR mice showed a pronounced CORT secretion, whereas LR animals did virtually not respond to the same dose of ACTH, indicating differences in adrenal sensitivity towards ACTH between the three lines. Moreover, it is likely that these differences were brought about by changes in adrenal MC2R sensitivity towards its ligand ACTH, since the expression of the MC2R was not different between the three lines (see Figure 15 C).

Studies by Ulrich-Lai and colleagues showed that chronic variable stress (CVS) in rats induced adrenal enlargement with hyperplasia in the outer *zona fasciculata* as well as hypertrophy in the inner *zona fasciculata* and medulla (Ulrich-Lai *et al.*, 2006). Furthermore, adrenal enlargement has been observed after several types of stressors in rats (Gamallo *et al.*, 1986; Ulrich-Lai *et al.*, 2002) and mice (Reber *et al.*, 2007; Uschold-Schmidt *et al.*, 2012) as well as after physical exercise (Moraska *et al.*, 2000; Droste *et al.*, 2003). This is in line with our findings since HR mice present a significantly higher

adrenal weight compared to LR animals (see Figure 18) according to their differences in HPA axis reactivity. Interestingly, CVS induced an enhanced plasma corticosterone response to exogenous ACTH in dexamethasone-blocked rats, specifically by augmenting the adrenal maximal response without affecting sensitivity to ACTH. It is argued, that *in vivo* tests of adrenal responses commonly used one to two doses of ACTH, thereby precluding definitive differentiation between maximal adrenocortical responses vs. sensitivity to submaximal doses of ACTH (Ulrich-Lai *et al.*, 2006). However, since in our mouse model, the capacity of the adrenal, i.e. adrenal CORT secretion in response to a very high dose of ACTH (1 µg/g BW) was not significantly different between the three lines (Touma *et al.*, 2008), we demonstrated that LR animals, irrespective of their decreased adrenal size, are able to adequately respond to ACTH stimulation, resulting in plasma CORT concentrations similar to HR and IR animals. This further supports the current finding that differences in adrenal sensitivity towards ACTH, at least partly, account for the distinct differences in stress reactivity between the three lines, although future studies should address whether the differences in adrenal size between HR, IR and LR animals are related to morphological changes, i.e. adrenal hypertrophy or hyperplasia in HRs and atrophy in LRs, respectively, in the different zones of the adrenal cortex and medulla.

The expression and release of corticosteroid-binding globulin in HR, IR and LR mice

Once released into the blood stream, CORT binds mainly to two types of carrier proteins, CBG and albumin (Slaunwhite *et al.*, 1959; Burton *et al.*, 1972) which are exclusively synthesised and stored in the liver (Rothschild *et al.*, 1972b, a; Weiser *et al.*, 1979). However, evidence for an intracellular location of CBG in the CNS and the pituitary is emerging (de Kloet *et al.*, 1984; Möpert *et al.*, 2006), thus exceeding its role as a mere steroid transporter (Henley *et al.*, 2011). In the blood, approximately 80-90% of the secreted CORT is bound to CBG and 10-15% to albumin, while the remaining 5% is thus unbound and biologically active (Lewis *et al.*, 2005; Henley *et al.*, 2011), according to the free hormone hypothesis by Mendel (Mendel, 1989). Furthermore, a stress-induced, time-dependent release of CBG from the liver has recently been shown in rats (Qian *et al.*, 2011), while following certain types of stressors or chronic stress, CBG expression was downregulated, increasing free glucocorticoid levels (Neufeld *et al.*, 1994; Fleshner *et al.*,

1995; Spencer *et al.*, 1996), attributing CBG a highly dynamic role in the physiological stress response.

In HR, IR and LR mice, we could show significant differences in plasma CBG levels already under unstressed conditions, with HR mice showing increased amounts of CBG, while LR animals presented the lowest plasma CBG protein abundance (see Figure 21). Furthermore, there were no major differences in plasma CORT concentrations between the three lines, which were equally low in the naïve control animals as well as immediately before the stressor (t=0, see Figure 19) in animals of all three lines. This is in accordance with studies by Richard and colleagues who revealed that partial or total deficiency of plasma CBG in hetero- and homozygous CBG KO mice, respectively, did not affect HPA axis function in resting conditions compared to wild-type controls (Richard *et al.*, 2010). Thus, the significant differences in plasma CBG between HR, IR and LR animals might not necessarily affect plasma CORT concentrations under resting conditions. The question remains, why the low amounts of CORT which are secreted from the adrenals under non-stressed conditions are not fully bound to CBG despite its high affinity. This is of interest, since it was shown that total and also free (i.e. not CBG bound) CORT follows a distinct circadian as well as an ultradian cycle, interacting with MRs and GRs to regulate the basic HPA axis tone (Lightman *et al.*, 2008), indicating that even under non-stressed conditions, free CORT is available to steroid receptors. Evidence comes from studies by Gayrard and colleagues who reported, that in resting conditions 68% of circulating CBG is not occupied by CORT in many species (Gayrard *et al.*, 1996), thus providing a pool of biologically active free CORT to exert its physiological functions at target tissues even under resting conditions.

In response to 15 min of restraint stress, we observed significant differences between the three lines regarding their stress-induced total plasma CORT levels (HR>IR>LR, see Figure 19) which was similarly reflected by the increase in free plasma CORT concentrations (HR>IR>LR; Moisan *et al.*, in preparation). However, plasma CBG levels remained unaffected by restraint stress, showing no further increase 30 min after the onset of the stressor (see Figure 21). These results are indicative of the following physiological properties of CBG. First, at least in animals of the SR mouse model, plasma CBG concentrations are unaffected by stress, albeit significant differences in plasma CBG abundance between the three lines exist. This is supported by our data providing no evidence for a depletion of liver-stored CBG 30 min after the onset of the 15 min restraint stressor (see Figure 20). Furthermore, we did not observe differences between HR, IR and

LR animals in liver-stored CBG, although CBG mRNA levels in the liver were significantly different between the three lines, with LR mice having the lowest expression (Moisan *et al.*, in preparation). Given that CBG mRNA is consistently translated into CBG protein at the hepatocytic proteome and only a minor moiety is stored in the liver, we suggest that CBG directly tranlocates through liver sinusoidal endothelial cell fenestrae into the capillary lumen (Braet *et al.*, 2002), resulting in the observed differences in plasma CBG between the three lines. Second, CBG is quickly saturated in animals of all three lines by CORT secreted from the adrenals in response to stress. Hence, the stress-induced surge of CORT overshoots the binding capacity of CBG in HR, IR and LR animals resulting in significantly increased but different free plasma CORT concentrations (HR>IR>LR, data not shown), thus paralleling the line-specific differences in total plasma CORT concentrations after stress. Accordingly, these differences in free CORT concentrations were also present in CORT target tissues such as the hippocampus (Heinzmann *et al.*, 2010), as measured by microdialysis, although the rise of free CORT in the CNS was delayed by about 30 min. This is in line with other studies showing a delayed increase in free CORT levels in the brain and other GC target tissues in rats (Droste *et al.*, 2008; Droste *et al.*, 2009; Qian *et al.*, 2011) and mice (Thoeringer *et al.*, 2007a; Tronche *et al.*, 2010). Qian and colleagues showed that this delay was associated with a concomitant increase in plasma CBG in response to stress. Thus, the authors concluded that the increase in circulating CBG levels after stress restrains the rise in free CORT concentrations for approximately 20 min in the face of mounting total hormone levels in the circulation, suggesting a highly dynamic role of CBG in CORT binding and transport (Qian *et al.*, 2011). However, in our experiment, applying a similar experimental setup as the study by Qian and colleagues, we already observed elevated free plasma CORT concentrations after the end of the 15-min restraint stress without changes in plasma CBG concentrations. Therefore, it remains to be clarified whether the regulatory interactions of CBG and CORT are different in mice and rats or whether the timing of these physiological processes follows different patterns in these two rodent species. In addition, the binding of CORT to CBG could be stressor-dependent since in our experiment, the mice were subjected to a more psychological, 15-min restraint stressor, whereas the rats in the study by Qian and colleagues experienced a clearly stronger physical and psychological stressor, being exposed to 15 min of forced swimming. However, preliminary data from our group indicate, that the stress-induced differences in free plasma CORT levels between HR, IR and LR animals persist

(HR>IR>LR) even after a 6-min forced swim period (Moisan *et al.*, in preparation). Furthermore, it needs to be clarified in additional studies whether the same physiological mechanisms in mice and rats underlie the delayed transition of plasma CORT into GC target tissues such as the brain.

It should be noted that the neuroendocrine and behavioural endophenotypes of HR, IR and LR animals of the SR mouse model significantly parallel those of hetero- and homozygous CBG knockout mice. In studies by Richard *et al*. it was shown that mice, which were fully or partly deficient of CBG, presented a reduced secretion of CORT from the adrenals in response to restraint stress (Richard *et al.*, 2010). Additionally, these mice displayed an increased passive stress-coping strategy in the FST. This parallels the conditions observed in LR mice, which show a decreased stress response as well as higher immobility in the FST compared to HR and IR animals, concomitant with decreased plasma CBG concentrations. This suggests that the distinct differences in plasma CBG levels between HR, IR and LR animals could play a pivotal role in the neuroendocrine and behavioural endophenotypes of these mice, although CBG release from the liver is not affected by stress. Furthermore, it was reported that CBG-deficient mice showed a very fast (within 5 min) and 4-fold higher CORT clearance compared to wild-type controls, which was paralleled by the upregulation of several drug-metabolising enzymes in the liver (Petersen *et al.*, 2006). Thus, it can be speculated whether the reduced stress-induced CORT concentrations in LR mice were partly brought about by an increased CORT clearance in the liver of these animals, beside the observed reduction in adrenal sensitivity (see discussion above). Future studies should address this possibility, e.g. by determining the amount and the time course of excreted radiolabelled CORT metabolites in the urine and in the feces of HR, IR and LR mice, which previously have been injected with ^{3}H-CORT (cf. Touma *et al.*, 2003). Moreover, functional alterations in liver enzymes due to alterations in GC signalling are frequently associated with obesity (Livingstone *et al.*, 2000; Seckl *et al.*, 2004; Bornstein *et al.*, 2006). This is of interest, since LR animals present an increased body-weight associated with larger fat storages compared to HR and IR animals in the absence of increased food intake (Touma *et al.*, 2008).

Moreover, it is of note that CBG acts as a protein thermocouple (Burton *et al.*, 1972). Studies by Cameron and colleagues showed that the affinity of glucocorticoids for CBG drops approximately 16-fold as the temperature increases from 35 °C to 42 °C, while the binding of GCs to albumin remained unaffected by temperature changes (Cameron *et al.*,

2010). According to this study, a rise in body temperature from 36.5 °C to 38 °C, as seen in HR, IR and LR animals subjected to the stress-induced hyperthermia test (Touma *et al.*, 2008), would increase the dissociation constant of CBG towards GCs from 25 nM to 40 nM (Cameron *et al.*, 2010). This accounts for an almost 50 % reduction in binding affinity of CBG at a temperature increase of 1.5 °C. Since a 15-min restraint period would induce similar changes in body temperature in animals of the SR mouse model, this would result in a decreased binding affinity of CBG towards CORT.

Therefore, it can be speculated, by which mechanism the temperature-sensitive decrease in CBG binding affinity towards CORT is counterbalanced. Although CORT-binding to albumin in temperature-insensitive (Cameron *et al.*, 2010), it is unlikely that this second major CORT binding protein 'adopts' the function of CBG. Preliminary data from our group show that albumin is not differentially expressed in the liver and in the plasma of HR, IR and LR animals. Moreover, the release of this protein is apparently not modulated by stress (data not shown).

Taken together, we conclude that CBG is important for the transport of GCs, such as CORT and it largely contributes to the bioavailability of free CORT at target tissues. We further suggest that, according to the presented results that the significant differences in plasma CBG concentrations might contribute to the neuroendocrine and behavioural phenotypes of HR, IR and LR animals. However, our data could not confirm the highly dynamic role of CBG proposed by other studies (Qian *et al.*, 2011). Further studies are necessary to provide a more comprehensive picture on the role of corticosteroid-binding globulin in HR, IR and LR mice, particularly considering the intracellular localisation of CBG in the CNS and in the pituitary (de Kloet *et al.*, 1984; Möpert *et al.*, 2006).

Adrenocorticotropic hormone synthesis and secretion

In the pituitary of HR, IR and LR animals, significant differences in ACTH abundance were observed between the three lines (see Figure 23), with HR mice showing a significantly higher amount of ACTH compared to animals of the IR and LR line. This is in accordance with an increased POMC mRNA expression in the pituitary of the animals (see Table 6), indicating alterations in HPA axis regulatory mechanisms at levels preceding the adrenocortical (dys)function of HR and LR animals. Interestingly, we did not observe a decrease in pituitary ACTH abundance 30 min after the onset of a 15 min of restraint stressor (see Figure 23). This indicates that (i) in response to this moderate psychological stressor, only a minor part of the available pool was secreted from the

pituitary into the blood stream to trigger the release of CORT from the adrenal cortex and/or (ii) the chosen method for detecting pituitary ACTH, i.e. Western blot, was too insensitive to uncover differences between stressed animals and their unstressed littermates. However, there was a significant increase in plasma ACTH levels in response to stress in all three lines. Unexpectedly, the stress-induced rise in plasma ACTH levels did not parallel the increase in plasma CORT concentrations (see Figure 22 and Figure 19). In response to the 15-min of restraint stress, plasma CORT levels significantly increased in animals of all three lines (HR>IR>LR) with a further increase 30 min after the onset of the stressor. Only in IR animals, plasma ACTH was significantly increased after 15 min of restraint stress with a further significant rise 30 min after the onset of the stressor (as expected), whereas there were no differences in HR and LR animals at t=15 compared to the unstressed control group. Moreover, a significant increase in stress-induced plasma ACTH concentrations in HR and LR animals was not reached until 30 min after the onset of the stressor. Interestingly, the significant surge in plasma ACTH concentrations at t=30 was not different between the three lines, albeit the observed line-specific differences in plasma CORT concentrations (see Figure 19). However, dissociations between plasma ACTH and CORT levels have been frequently reported (Bornstein *et al.*, 2008) and the existence of a temporal lag between stimulus-induced changes in ACTH and CORT levels are well documented (Engeland *et al.*, 1977). Both, ACTH and CORT are secreted in a pulsatile or episodic fashion under basal (Lightman *et al.*, 2008) and stress conditions (Windle *et al.*, 1998), which might well occur in non-overlapping pulses (Bornstein *et al.*, 2008). Moreover, as discussed previously, neuronal efferents, descending from the hypothalamic PVN (Harris, 1950; Yoshimatsu *et al.*, 1987) innervate the adrenal cortex as well as the adrenal medulla through splanchnic nerve fibres (Bornstein *et al.*, 1999; Engeland *et al.*, 2005), which, in turn, stimulate CORT release from the adrenals (Engeland, 1998; Bornstein *et al.*, 2008). Thus, it can be speculated that starting from the PVN of the hypothalamus, two independent but synergistically acting mechanisms, the SAM system and the endocrine pituitary-adrenal signalling cascade, are involved in bringing about the significant, line-specific differences in stress-induced adrenal CORT secretion between HR, IR and LR animals. This hypothesis is supported by data presented in this study. First, after 15 min of restraint stress there was a significant increase in plasma CORT concentrations of HR and LR animals in absence of rising plasma ACTH levels. However, we could show, that in response to 15 min restraint stress, *C-fos* mRNA expression, a CORT-independent marker

for neuronal activation (Melia *et al.*, 1994; Helmreich *et al.*, 1996; Weiser *et al.*, 2011), was markedly enhanced in the PVN of HR, IR and LR animals (see Figure 32). Strikingly, HR mice showed a significantly increased *C-fos* activity compared to IR animals, while animals of the LR line presented the lowest neuronal activation. Given that excitatory nerve fibers descending from the PVN directly stimulate the adrenals (Katafuchi *et al.*, 1988; Coote *et al.*, 1998), an ACTH-independent stimulation of the adrenal cortex through these nerve fibres must be considered, which would act faster than the endocrine signalling pathway mediated by ACTH. Thus, rising plasma CORT concentrations in response to stress in absence of increasing plasma ACTH levels are plausible. Second, 30 min after the onset of the stressor, plasma ACTH levels were significantly increased between HR, IR and LR animals without showing differences between the three lines. However, at this time point of sampling, plasma CORT levels showed the distinct line-specific differences in HR, IR and LR mice. Interestingly, this very well reflects the animals' response in the adrenal sensitivity test to a standardised dose of exogenous ACTH (see discussion above). In this test, HR animals presented a significantly increased CORT secretion 30 min after the administration of ACTH compared to LR animals which virtually did not respond to the same dose (cf. Figure 17). Thus, HR, IR and LR animals show significant differences in their ACTH-induced CORT release, irrespective of an exogenously or endogenously transmitted ACTH stimulation.

Taken together, our data indicates that in addition to alterations in functional mechanism on the adrenal level, preceding levels of HPA axis function such as the pituitary and/or the PVN are involved in bringing about the neuroendocrine phenotype of the HR and LR mouse line. We could show an upregulation of POMC mRNA and ACTH protein in pituitary corticotrophs of HR mice, presumably due an increased activation of the PVN. In contrast, LR animals presented a decreased POMC mRNA expression compared to IRs while the ACTH protein content was similar in both lines. Furthermore, we observed that the available pool of pituitary ACTH was not decreased after restraint stress in all three lines. However, ACTH was released from the pituitary in a time-dependent manner, not strictly paralleling stress-induced plasma CORT levels. Taking into account the significant differences in adrenal sensitivity between the three lines, we suggest that the endocrine pituitary-adrenal signalling cascade as well as the SAM system is considerably involved in bringing about the distinct differences in stress reactivity between HR, IR and LR animals.

4.2 Central regulation of the hypothalamic-pituitary-adrenal axis

In our final experiments, we aimed to investigate whether in addition to the differences between HR, IR and LR animals in the peripheral functions of the HPA axis (as discussed above), molecular correlates in the CNS of these mice could be found, which would support the assumption of a central origin of these line-specific differences in stress reactivity. Moreover, we addressed the question, whether pharmacological treatment with fluoxetine (Flx), one of the most frequently applied antidepressants, would impact the behavioural and neuroendocrine phenotype of HR, IR and LR animals.

The impact of fluoxetine on stress-coping behaviour

Our results show that Veh-treated as well as Flx-treated animals presented the robust, line-specific differences regarding their coping-strategies in the FST, which were already observed in earlier generations of the SR mouse model (Touma *et al.*, 2008; Knapman *et al.*, 2010a). Interestingly, after Flx treatment, animals of the SR mouse model showed an increase in passive stress-coping behaviour. In particular, an increased floating behaviour and, accordingly, less time spent struggling could be observed in HR and IR mice (see Figure 24).

As discussed previously, it has been shown that mice overexpressing CRH in the central nervous system displayed a more active stress-coping behaviour in the FST (Lu *et al.*, 2008; Dedic *et al.*, 2011), similar to the behavioural phenotype observed in HR mice. This increase in activity/hyperarousal was associated with a CRH-induced increase in activation of monoaminergic neurotransmission in brain structures such as the locus coeruleus. Interestingly, in this study, inhibition of NE synthesis by the tyrosine hydroxylase inhibitor α-methyl-*para*-tyrosine methyl ester (AMPT) resulted in a significant increase in floating behaviour of the animals in the FST, supporting the pivotal role of NE in active stress-coping strategies (Lu *et al.*, 2008).

Since the LC is innervated by serotonergic (5-HT) afferents of the dorsal raphe nucleus (Pickel *et al.*, 1977; Morgane *et al.*, 1979; Kim *et al.*, 2004) and these 5-HT neurons have been shown to attenuate the activation of NE-neurons in the LC (Segal, 1979; Aston-Jones *et al.*, 1991), an increase in 5-HT in the dorsal raphe nucleus, induced by the Flx-mediated inhibition of 5-HT reuptake into the pre-synapse, might suppress the activity of the LC-NE system in animals of the SR mouse model. Thus, the increase in passive stress-coping behaviour after Flx treatment in HR, IR and LR animals could be brought

about by augmenting levels of 5-HT in the dorsal raphe nucleus resulting in a suppression of the LC and hence, NE activity. So far, it can only be speculated about the molecular origin of the different stress-coping strategies observed between HR, IR and LR animals. Future studies also need to address the question to what extent the sympathetic nervous system is involved in bringing about the behavioural phenotype of the animals. Our results revealed no significant differences in adrenal PNMT and TH mRNA expression between the SR lines. However, future studies should investigate basal and stress-induced plasma NE levels. Moreover, brain areas known to be critically involved in catecholaminergic and serotonergic neurotransmission such as the raphe nucleus or the locus coeruleus need to be studied. Subsequently, the functional implications of adrenergic receptor antagonists should be tested, since stress is associated with alterations in catecholamine synthesis as well as changes in the noradrenergic receptor system, such as reduced β-adrenoceptor or increased α1- and α2-adrenoceptor expression (Brunello *et al.*, 2002).

Impact of fluoxetine on hypothalamic-pituitary-adrenal axis reactivity

We were interested whether Flx impacts the key endophenotype of HR, IR and LR animals, i.e. the distinctly different CORT secretion in response to stressors, since it has been reported that antidepressants such as Flx can modify the negative feedback mechanisms of the HPA axis (Holsboer, 2000; Pariante *et al.*, 2004). To this end, two different experimental approaches were conducted. First, after four weeks chronic Flx treatment, we subjected the animals to an acute stressor (10-min OF + 6-min FST) in order to evaluate the impact of Flx on rapid, non-genomic effects of corticosteroids on HPA axis activation (de Kloet *et al.*, 2008; Evanson *et al.*, 2010; Tasker *et al.*, 2011). Second, we aimed to investigate the effects of chronic Flx treatment on delayed, genetically-driven negative feedback mechanisms by means of the Dex/CRH test.

In response to the FST, HR, IR and LR animals showed the expected line-specific differences in stress-induced plasma CORT secretion (HR>IR>LR, see Figure 26). However, Flx had no significant effect on stress-induced plasma CORT levels in any of the three lines. Thus, our data indicates that Flx did not modulate the fast feedback mechanisms of the HPA axis in HR, IR and LR mice (see Figure 26). Consistently, chronic Flx treatment did also not affect plasma CORT concentrations in response to a 15-min restraint stressor (see Figure 30). In recent years, evidence has emerged that fast feedback mechanisms, involving corticosteroid binding to the MR as well as the

activation of the endocannabinoid system in the PVN, are implicated in a rapid downregulation of HPA axis activity in response to stressors (Atkinson *et al.*, 2008; Joels *et al.*, 2008; Groeneweg *et al.*, 2012). It has been shown that fast feedback mechanisms are sensitive to blocking or knocking out the MR, suggesting a role for this receptor in the rapid GC signalling. Moreover, this fast feedback was mediated by membrane receptors which involved the activation of G-protein coupled receptors (GPCR) and intracellular signalling cascades downstream from GPCRs, e.g. by modulating membrane potentials through inhibiting high-voltage gated Ca^{2+} channels (Tasker *et al.*, 2006). The endocannabinoid system has likewise been implicated in the regulation of the HPA axis (Tasker *et al.*, 2011). For instance, endocannabinoid receptor 1 (CB1) knockout mice showed an enhanced response to stress, indicating an endocannabinoid-mediated inhibitory tone on HPA axis activity (Aso *et al.*, 2008; Steiner *et al.*, 2008a;b). In addition, it has been shown that acute restraint stress induced a rapid (within 10 min) rise in 2-arachidonoylglycerol (2-AG) levels in the PVN, resulting in an immediate downregulation of HPA axis activity. This suggests a fast, non-genomic glucocorticoid suppression by the endocannabinoid system (Evanson *et al.*, 2010). Until now, it can only be speculated, to what extent these fast feedback mechanisms might contribute to the neuroendocrine phenotype of the animals of the SR mouse model. Future studies should address this question, for instance by applying selective MR or CB1 receptor antagonists. So far, our data demonstrate that these fast feedback mechanisms are obviously not modulated by chronic Flx treatment in HR, IR and LR mice.

Impact of fluoxetine on hypothalamic-pituitary-adrenal axis regulation in the combined dexamethasone/corticotropin-releasing hormone test

In contrast to the fast feedback mechanisms, Flx treatment significantly affected the delayed, genetically-driven negative feedback mechanism of the HPA axis. Furthermore, HR, IR and LR animals showed a differential response in the Dex/CRH test already under untreated conditions. This is of interest since it has been shown that the re-establishment of a balanced HPA system is an important prerequisite for the recovery from affective disorders such as MD (Zobel *et al.*, 2001). The outcome of the Dex/CRH test was also used to evaluate the effectiveness of antidepressant therapy in clinical settings (Holsboer-Trachsler *et al.*, 1991; Heuser *et al.*, 1994; Ising *et al.*, 2005).

Veh-treated animals of all three lines showed significant differences in the suppression of their plasma CORT concentrations after Dex treatment. In particular, HR mice presented

a reduced CORT-suppression, while the plasma CORT levels of IR and LR mice were readily suppressed (see Figure 27). After the stimulation of the HPA axis with CRH, in turn, HR, IR and LR mice showed the line-specific, significant rise in plasma CORT levels (HR>IR>LR). This is consistent with clinical findings. It has been shown that patients suffering from MD frequently show an insufficient suppression of CORT after Dex treatment (Dex non-suppressors), whereas they present an overshooting CORT response after CRH stimulation (Bardeleben *et al.*, 1989; Heuser *et al.*, 1994; Ising *et al.*, 2005). This neuroendocrine response pattern is reflected by the hyper-reactive HPA axis phenotype of HR animals. In contrast, a hypo-reactive HPA axis has been associated with a blunted response in the Dex/CRH test (Rydmark *et al.*, 2006), similar to the situiation seen in LR animals. This finding underlines the clinical relevance of the three mouse lines of the SR mouse model to gain more insight into the mechanisms responsible for HPA axis dysfunction. Moreover, the model could serve as a valuable tool to investigate the effect of antidepressant drugs on HPA axis function. Indeed, our experiment demonstrated that Flx treatment affected HPA axis function in HR, IR and LR mice. Strikingly, the CORT non-suppression observed in HR animals was restored after Flx treatment, which was reflected by markedly decreased plasma CORT levels after Dex administration in these mice. Similarly, Dex administration after chronic Flx treatment induced a decrease in plasma CORT concentrations in IR animals (see Figure 27). However, comparing the low median levels of the Veh- and Flx-treated group, the CORT-suppressive effect of Dex was less prominent though statistically significant in this line. An impact of Flx treatment on plasma CORT levels of LR mice could not be observed, presumably due to floor effects.

The CRH stimulation induced an increase in plasma CORT concentrations in all three lines. Flx treatment did not affect the CRH-mediated CORT increase in HR mice. However, in animals of the IR line, Flx-treated animals showed a reduced CORT surge after CRH stimulation mirrored by the lower median levels in this group (see Figure 27). Finally, Flx-treated LR mice presented a significantly blunted CORT response after CRH compared to their Veh-treated littermates.

Taken together, the data demonstrate that (i) Flx restored Dex-mediated CORT suppression in HR animals, (ii) after Flx treatment, Dex clearly suppressed CORT in all three lines and (iii) this Dex-induced suppression was weakest in HR mice since it could be overridden by CRH stimulation resulting in similar plasma CORT levels after CRH challenge, while in Flx-treated LR animals, CRH stimulation resulted in a blunted CORT

response. Thus, we conclude that chronic Flx treatment had a significant impact on the delayed negative feedback mechanism in animals of the SR mouse model.

Gene expression in the hippocampus and pituitary of HR, IR and LR mice

The synthetic GC Dex is a selective GR agonist (Rousseau *et al.*, 1972; de Kloet *et al.*, 1999). Hence, it is likely that the Dex-mediated feedback suppression of the HPA axis in HR, IR and LR mice was brought about by activation of the GR, which was even more enhanced after chronic Flx treatment. Surprisingly, we could not detect differences in corticosteroid receptor mRNA levels in the pituitary (GR) as well as in the hippocampus (GR and MR) between the three lines, both under control conditions or after chronic Flx treatment (see Table 5 and Table 6). Moreover, GR and FKBP51 protein levels in the hippocampus did not differ between the three lines and were not affected by chronic Flx treatment (see Figure 28). This indicates that neither the differences in the Dex-mediated feedback suppression of the HPA axis between HR, IR and LR mice, nor its enhancement after Flx treatment were associated with an increase in receptor abundance in the pituitary or in the hippocampus. However, other mechanisms in addition to receptor expression have been reported to be involved in the activation of GR signalling.

The access of GCs into the brain is tightly regulated by the multidrug resistance 1 P-glycoprotein (MDR1 Pgp) (Schinkel, 1997; Schinkel *et al.*, 2003). In contrast to humans, two isoforms of this efflux pump (Mdr1a Pgp and Mdr1b Pgp) are expressed in rodents, which are encoded by two different genes (*Abcb1a* and *Abcb1b*). The Mdr1a isoform is mainly located in the endothelial cells of microvessels forming the blood-brain barrier (BBB) (Regina *et al.*, 1998), whereas the Mdr1b isoform is particularly expressed in glia cells and neurons (Volk *et al.*, 2004; Löscher *et al.*, 2005) probably regulating the access of GCs on a cellular level. Both isoforms function as drug efflux pumps and protect the brain from xenobiotics (Schinkel, 1997) but are also involved in the extrusion of synthetic steroids such as Dex (Meijer *et al.*, 1998) as well as endogenous steroids such as CORT (Wolf *et al.*, 1992; Uhr *et al.*, 2002), implicating an involvement of Mdr1 Pgp on HPA axis function and regulation.

In a study by Uhr and colleagues, it has been shown that the endogenous steroid CORT is transported by the Mdr1 Pgp since *Abcb1ab* double-knockout resulted in an increased accumulation of CORT in the brain of these animals (Uhr *et al.*, 2002). Moreover, Thoeringer and colleagues reported that Mdr1 Pgp inhibition by tariquidar in mice resulted in decreased stress-induced plasma CORT levels, which was attributed to an

enhanced negative feedback suppression of HPA axis activity in these animals (Thoeringer *et al.*, 2007b). In another study, *Abcb1ab* Pgp knockout mice presented decreased stress-induced plasma CORT levels compared with their wildtype littermates, while plasma CORT concentrations in these mice did not differ under basal conditions (Müller *et al.*, 2003). Moreover, in this study the *Abcb1ab* knockout mice showed increased Dex-mediated CORT suppression compared to wildtype animals, indicating a prominent role of Mdr1 Pgp in HPA axis activity and negative feedback regulation.

Interestingly, clear differences in the expression levels of *Abcb1b* were detected in animals of the SR mouse model with HR mice showing a significantly increased *Abcb1b* expression in the hippocampus, whereas LR animals presented markedly reduced *Abcb1b* mRNA levels (see Table 5). Thus, the findings of the above mentioned studies are consistent with the neuroendocrine phenotype of animals of the SR mouse model. HR animals with an increased expression of *Abcb1b* in the hippocampus showed a reduced negative feedback and, in turn an increased stress-induced CORT secretion. This might be due to an increased extrusion of GCs from brain cells by Mdr1b Pgp resulting in a restricted access of GCs to the cytosolic GR. In contrast, LR animals with a low expression of Mdr1b Pgp presented an enhanced negative feedback suppression due to an increased GC receptor binding, resulting in a reduced CORT surge in response to stress. Taken together, a contribution of Mdr1b Pgp to the neuroendocrine phenotype of HR, IR and LR mice as well as the outcome of the Dex/CRH test must be considered. However, the question remains by which molecular mechanism chronic Flx treatment enhanced the negative feedback suppression of plasma CORT levels in the animals of all three lines in the Dex/CRH test. A potential mechanism is provided by studies from Peer and colleagues who showed that Flx significantly increased the accumulation of anticancer drugs within multidrug resistant cells by inhibiting the drug efflux transporters in those cells (Peer *et al.*, 2004). This is consistent with studies reporting that in addition to anticancer drugs, Flx induced a substantial increase in intracellular GC levels as well as GC-induced GR activation due to inhibition of membrane steroid transporters (Pariante *et al.*, 2003). It has been proposed that antidepressants could inhibit the steroid transporters localised on the BBB and in neurons, and thus increase the access of GCs to the brain and the GC-mediated negative feedback on the HPA axis (Pariante *et al.*, 2003). Hence, Flx might have facilitated the access of GCs such as Dex into cells of the PVN, the hippocampus and/or the pituitary of HR, IR and LR mice, thereby increasing GR-mediated feedback mechanisms which, eventually, enhanced the Dex-mediated

suppressive effect on plasma CORT concentrations. Future studies should investigate the enhanced activation of the GR after Flx treatment in more detail, including mechanisms involved in the translocation of the ligand-activated GR from the cytosol into the nucleus (Spencer *et al.*, 2000). This is of interest since our data demonstrate that in the hippocampus of LR animals, which showed the lowest *Abcb1b* gene expression, Flx induced the most prominent Dex-mediated CORT suppression which paralleled an increased expression of GR target genes known to have a glucocorticoid response element (GRE) in their promoter region, e.g. *Abcb1a*, *Fkbp5* and *Gilz* (Wang *et al.*, 2004; Narang *et al.*, 2008) (see Table 5).

Moreover, it has been shown that an increased GR activation after treatment with the SSRI sertraline induced neurogenesis in a human hippocampal progenitor cell line (Anacker *et al.*, 2011). In addition, chronic Flx treatment stimulated maturation and synaptic plasticity of adult-born hippocampal granule cells (Duman *et al.*, 2001; Wang *et al.*, 2008), which was associated with an enhanced hippocampus-dependent negative feedback regulation of the HPA axis in mice (Surget *et al.*, 2011). This suggests that a restored negative feedback regulation of the HPA axis might not only be mediated by enhanced GR signalling *per se* but also by a GR-mediated induction of neurogenesis in the hippocampus after chronic antidepressant treatment, resulting in a functional HPA axis regulation through a reinstated neuronal network.

In this respect, studies by Knapman *et al.* revealed decreased expression of brain-derived neurotrophic factor (BDNF) with concomitantly decreased hippocampal N-acetylaspartate (NAA) levels, a marker for neuronal integrity, in the hippocampus of HR animals (Knapman *et al.*, 2010a; Knapman *et al.*, 2012). Accordingly, it has also been shown that high levels of GCs prevent cell proliferation in the hippocampus of rats (Gould *et al.*, 1992) and monkeys (Gould *et al.*, 1998). Interestingly, Castren and colleagues reported that antidepressant drugs elicit neurotrophic effects particularly in the hippocampus (Castren, 2004). Furthermore, this antidepressant-induced increase in cell proliferation has been associated with an increased expression of BDNF (Nibuya *et al.*, 1995; Sairanen *et al.*, 2005), which was found to be essential for neurogenesis (Lee *et al.*, 2009). Thus, future studies should address the question whether the restored negative feedback inhibition of the HPA axis in HR animals was due to a Flx-dependent increase in hippocampal neurogenesis, potentially associated with augmented hippocampal BDNF levels in these mice.

Effects of fluoxetine on spatial memory

Studies by Knapman and colleagues also revealed a reduced memory performance in HR animals, which was linked to the increased stress reactivity of these animals (Knapman *et al.*, 2010a,b). Consistently, it has been reported that increased glucocorticoid levels impair memory retrieval (Conrad *et al.*, 1996; de Quervain *et al.*, 1998; Roozendaal, 2000). In contrast, antidepressant treatment markedly ameliorated GC-induced cognitive deficits in mice (Flood *et al.*, 1987; Song *et al.*, 2006) and rats (Oitzl *et al.*, 1998). Thus, we investigated whether chronic treatment with Flx modulated the spatial memory performance in animals of the SR mouse model. In particular, we were interested whether Flx would be able to restore the observed cognitive deficits of HR mice, since these mice have been shown to perform worse in hippocampus-dependent memory tasks such as the Y-maze test, compared to IR and LR animals (Knapman *et al.*, 2010a; Knapman *et al.*, 2012). Indeed, we could observe a significantly enhanced discrimination ratio in Flx-treated HR animals compared to their Veh-treated littermates (see Figure 29). Thus, the accuracy to visit and explore the novel arm in the retrieval phase was apparently increased in Flx-treated HR animals. This is consistent with other studies reporting an increased cognitive performance after Flx treatment (Flood *et al.*, 1987; Grote *et al.*, 2005; Song *et al.*, 2006; Li *et al.*, 2009), potentially via a BDNF-mediated mechanism resulting in increased hippocampal neurogenesis (Nibuya *et al.*, 1995; Sairanen *et al.*, 2005). However, these results have to be interpreted with care. In our experiment, IR and LR animals spent similar amounts of time in the novel as well as in the familiar arms during the retrieval phase (see Table 7). This was unexpected since in previous studies by Knapman and colleagues, the animals of these two mouse lines consistently spent more time exploring the novel arm compared to the familiar arms (Knapman *et al.*, 2010a; Knapman *et al.*, 2012). Thus, we cannot exclude that the behavioural readout in the Y-maze test was obscured. We do not have a plausible explanation for the failure of animals of the IR and LR line to discriminate between the novel and familiar arms during the retrieval phase of the testing period. Maybe the animals' performance in the Y-maze test was affected by the number of experimenters handling the animals during the test. In the studies by Knapman and colleagues, all mice were handled and tested by only one experimenter, whereas in our study three scientists performed the tests due to the high number of experimental animals. Moreover, Knapman and colleagues tested the animals under naïve conditions, whereas in this study, we chronically injected all experimental

animals over a time period of 28 days before subjecting them to the Y-maze task. Thus, the injection procedure *per se* might have interfered with the outcome of this test. This is supported by our results demonstrating a slight increase in neuronal *C-fos* expression in the brain of chronically injected mice compared to naïve control mice, although this increase was not statistically significant.

Stress-induced neuronal activation in the brain

In our final experiment we aimed to find neuronal correlates in the CNS of HR, IR and LR animals reflecting a central origin of the differences in the neuroendocrine phenotypes of these animals. Furthermore, we tested whether potential differences in neuronal activity would be altered by chronic Flx treatment. To this end, we used *C-fos in-situ* hybridisation to determine the neuronal activation in different brain regions known to be involved in the regulation of HPA axis activity. The immediate early gene *C-fos* has been used as an indicator for neuronal activation in response to various forms of stress such as restraint or swim stress (Cullinan *et al.*, 1995). Moreover, it has been shown that *C-fos* mRNA expression in different brain regions is not affected by the amount of circulating glucocorticoids (Melia *et al.*, 1994; Helmreich *et al.*, 1996; Weiser *et al.*, 2011). This was of importance for our studies since the differences in stress reactivity between HR, IR and LR mice would have been a major confounding factor in the analysis of the *C-fos* signal. To induce *C-fos* expression in the experimental animals, we subjected them to 15 min of restraint stress and finally sacrificed them 45 min after the cessation of the stressor to determine *C-fos* mRNA expression. We expected a strong *C-fos* signal at this time point (60 min after the onset of the stressor), since studies by Cullinan and colleagues had detected the highest *C-fos* signals within 30 min to 60 min after the end of a 30-min restraint period or a 6-min FST in rats (Cullinan *et al.*, 1995). In addition, we determined plasma CORT concentrations immediately before, straight after and 45 min after the 15 min restraint stress as a neuroendocrine readout to assess stress-induced HPA axis activation.

Hypothalamus-pituitary-adrenal axis reactivity in response to restraint stress

HR, IR and LR animals showed the expected line-specific differences regarding the increase of plasma CORT concentrations in response to the 15 min restraint stressor. Moreover, Flx treatment did not affect the stress-induced rise in plasma CORT levels (see Figure 30). Consistently, also no differences in plasma CORT concentrations were

observed in response to 6 min of forced swimming between Flx- and Veh-treated animals of all three lines (see Figure 26). Thus, independently of the stressor, the fast feedback mechanisms of the HPA axis were obviously not modulated by chronic Flx treatment. In contrast, Flx-treated LR mice showed a significant decrease in plasma CORT levels 45 min after the cessation of the restraint period compared to their Veh-treated littermates. This suggests that to this time point, Flx treatment enhanced the delayed, or at least the intermediate, feedback mechanisms (Keller-Wood *et al.*, 1984; Watts, 2005). These findings are in accordance with our data investigating the impact of chronic Flx treatment on the functional regulation of the HPA axis in the Dex/CRH test (see Figure 27). There, a similarly reduced CORT surge after CRH stimulation was observed in Flx treated animals, particularly in the LR line, which was attributed to an enhanced Dex-mediated suppression of HPA axis activity. Thus, we suggest a common underlying molecular basis since an endogenous, anticipatory stressor (e.g. FST) as well as an exogenously applied, pharmacological stressor (Dex treatment) induced similar effects on the delayed HPA axis negative feedback. This common mechanism is presumably constituted by an enhanced GC-mediated activation of the GR brought about by the chronic Flx treatment.

Acute stress induces differences in PVN excitation in HR, IR and LR mice

Regarding the *C-fos* mRNA expression in response to 15 min of restraint stress, a significant increase was observed in all investigated brain areas of HR, IR and LR mice (see Table 8). Thus, the stressor strongly induced neuronal activation in limbic forebrain structures such as the hippocampus, the prefrontal cortex (PFC) and the basolateral amygdala (BLA) which are involved in stress perception (Herman *et al.*, 2003). Moreover, there was a significant difference regarding the neuronal activation in the PVN of the animals, with HR mice showing the strongest activation, while in LR animals the *C-fos* signal was less pronounced (see Figure 32). These differences, however, were not affected by Flx, since Flx-treated as well as Veh-treated animals presented a similar neuronal activation of the PVN (see Table 8 and Figure 32).

In mammals, the stress response comprises the activation of the HPA axis which is driven by a neuronal signal originating from the PVN, eventually resulting in a release of stress hormones such as CORT from the adrenal cortex. Thus, our results demonstrate that the differences in stress reactivity between HR, IR and LR animals were not only a peripheral phenomenon brought about by a differential ACTH release from the pituitary or an altered adrenal sensitivity towards ACTH, but also have a neuronal correlate in the CNS

of the animals. However, the PVN is suggested to be an 'executing' brain area and the perception of stressors is mediated by other brain regions (Herman *et al.*, 2005). PVN neurons are activated by neuronal inputs from a number of sources (see Figure 33).

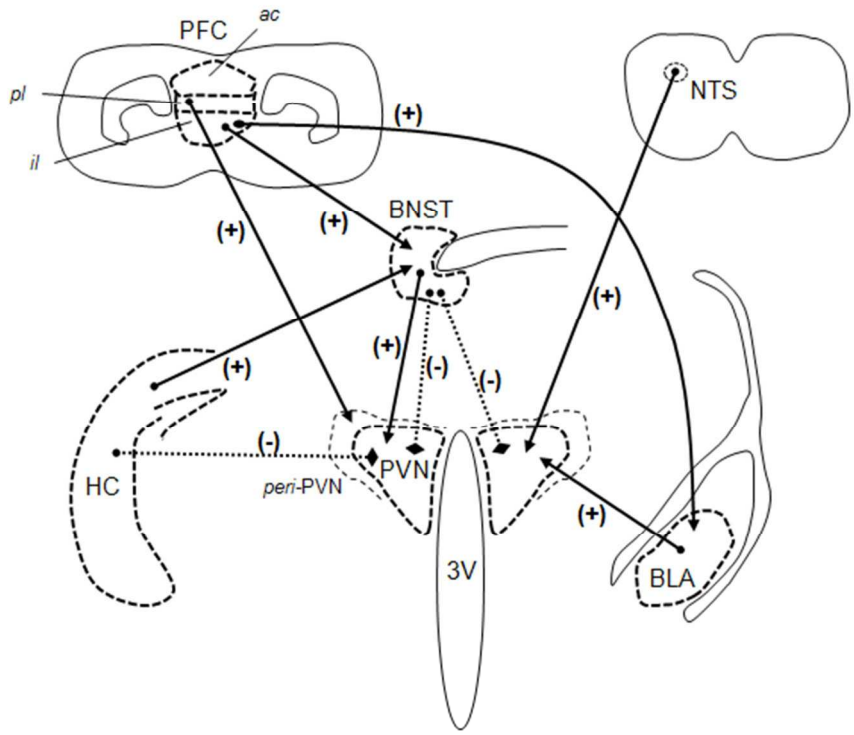

Figure 33: Simplified diagrammic representation of direct limbic stress-integrative pathways to the paraventricular nucleus of the hypothalamus (PVN) and the *peri*-PVN region from the prefrontal cortex (PFC), basolateral amygdala (BLA) and hippocampus (HC) as well as from brainstem regions such as the nucleus of the solitary tract (NTS). Indirect projections of these brain regions via transsynaptic intermediaries such as the bed nucleus of the stria terminalis (BNST) are also presented. Excitatory projections are indicated by (+) and filled lines ending in arrows, inhibitory projections are indicated by (-) and dotted lines ending in squares. *ac*, anterior cingulate cortex; *pl*, prelimbic cortex; *il*, infralimbic cortex; 3V, third ventricle. Figure modified from (Herman *et al.*, 2005).

Brain stem areas signalling systemic challenge (e.g. humoral homeostatic imbalance) such as the nucleus of the solitary tract (NTS) or the circumventricular organs provide direct excitation to the PVN, whereas anticipatory HPA axis responses are mediated by multisynaptic limbic forebrain circuits (Ulrich-Lai *et al.*, 2009; Myers *et al.*, 2012). This suggests that the activation of the PVN did not result from an intrinsic stress-induced stimulus but was rather evoked by superior brain structures known to project neuronal efferents to the PVN such as the hippocampus (Sapolsky *et al.*, 1984; Herman *et al.*, 1989; Jacobson *et al.*, 1991), the BLA (Jones *et al.*, 2011; Myers *et al.*, 2012) and the PFC (Spencer *et al.*, 2005; Radley *et al.*, 2006). Hence, we determined the neuronal

activation in these brain areas in order to investigate their implications in the excitation of the PVN.

The applied 15-min restraint stressor, however, did not induce a significantly different neuronal activation between the three lines in most of the investigated limbic brain regions or subregions (see Table 8). Only in the CA3 subregion of the dorsal hippocampus (dHC) and in the dentate gyrus of the ventral hippocampus (vHC) of Veh-treated animals, a significant difference could be observed between the three lines. HR animals presented a lower stress-induced activation of these two hippocampal subregions compared to LR mice. This is of interest since it has been shown in a number of studies that the hippocampus exerts inhibitory actions on the HPA axis (Jacobson *et al.*, 1991; Herman *et al.*, 1997). Hippocampal stimulation has been shown to decrease GC secretion in rats and humans suggesting that activation of this region is sufficient to inhibit HPA response (Rubin *et al.*, 1966; Dunn *et al.*, 1984). Thus, a stronger activation of the hippocampus (as seen in LR mice) would increase the inhibitory tone on PVN neurons which would, in turn, result in more dominant inhibition of CORT release. In contrast, a dampened inhibition of the PVN would cause an increased release of ACTH secretagogues (CRH and AVP) and eventually lead to an enhanced CORT secretion (as observed in HR mice). Furthermore, although not statistically significant, a comparison between the mean values of the *C-fos* expression in different subregions of the vHC and dHC of HR and LR mice revealed a tendency towards a more active hippocampus in LR animals, supporting the more prominent inhibitory role of this brain region on PVN activity in LR mice. It should be noted, that the dentate gyrus (DG) of both, the dHC and the vHC of HR, IR and LR mice, already showed a prominent neuronal activity under unstressed conditions. Moreover, restraint stress induced only a marginal increase in *C-fos* expression in this brain area (see Table 8 in the results section and Table 5 in the appendix). Consistently, a prominent neuronal activity in the hippocampus under unstressed conditions has also been reported in other studies (Cullinan *et al.*, 1995; Herdegen *et al.*, 1995). However, considering the multitude of brain areas integrating information into the hippocampus, its role in the processing of environmental stimuli and its profound regulatory functions regarding the basal and stress-induced HPA axis tone, a prominent neuronal activity in the hippocampus already under basal conditions is not surprising.

Similar to the hippocampus, the PFC also projects to the PVN, modulating the activation of the HPA axis, albeit in a more complex fashion (Herman *et al.*, 2005; Spencer *et al.*,

2005; Radley *et al.*, 2006; Myers *et al.*, 2012). For instance, the infralimbic cortex projects to the bed nucleus of the stria terminalis (BNST) and amygdala which are implicated in stress excitation, whereas the prelimbic cortex does not target these regions but projects to the peri-PVN region, an area which is implicated in stress inhibition (Sesack *et al.*, 1989; Hurley *et al.*, 1991; Herman *et al.*, 2005). Thus, the role of the PFC in HPA axis activation remains less clearly defined. Furthermore, the implication of the PFC in HPA axis activation seems to be stressor specific (Herman *et al.*, 2005). However, a between-line comparison of the *C-fos* signal in the PFC of HR, IR and LR animals did not yield significant differences in both treatment groups (Flx and Veh). Therefore, we suggest that the PFC and its subregions were marginally contributing to the differences in PVN excitation between the three mouse lines.

In contrast to the hippocampus and the PFC, the BLA is believed to activate the HPA axis (Herman *et al.*, 2005). The stimulatory role of the BLA on HPA axis activity has been investigated in studies showing that lesions of the BLA dampened the HPA axis response to stressors (Bhatnagar *et al.*, 2004), whereas intra-BLA CRH injections increased adrenal GC secretion (Daniels *et al.*, 2004). This indicates that the BLA might provide feedforward regulation of the HPA axis. In the animals of the SR mouse model, Flx-treated HR mice revealed a significantly increased *C-fos* mRNA expression in this brain region compared to LR animals of the same group. This suggests a more pronounced excitatory input of the BLA to the PVN in Flx-treated HR mice. A tendency in the same direction could be observed in Veh-treated animals, although statistical significance was not reached in this group (HR>IR>LR, $p=0.288$).

Taken together, our data clearly demonstrate that the distinct neuroendocrine phenotypes of the different breeding lines of the SR mouse model do not exclusively arise from a peripheral source but are driven by a neuronal signal originating from the PVN. Furthermore, we could show that the perception and interpretation of stressful stimuli might have induced a differential integration of hippocampal, amygdalar and prefrontal cortical information in the PVN, resulting in the observed differences in PVN excitation between HR, IR and LR animals. However, we could not detect an impact of chronic Flx treatment on the neuronal activation of the investigated limbic forebrain structures as well as on the PVN. It should be emphasised here, that the neuronal circuitry of stress in the brain includes many more regions than the areas that were investigated in this study, which could be influenced by antidepressant treatment, including important subcortical relay stations modulating the input from limbic structures to the PVN, e.g. the BNST

(Cullinan *et al.*, 1993; Crane *et al.*, 2003; Spencer *et al.*, 2005; Choi *et al.*, 2007). In addition, there is a considerable overlap of innervation fields from inhibitory (hippocampus, partly PFC) and excitatory (BLA) structures which imply that limbic information might be summated at subcortical sites (Herman *et al.*, 2005). Hence, it might be possible that the differences in neuronal activity of the PVN were brought about by excitatory or inhibitory inputs from several limbic brain areas, which only showed marginal differences between HR, IR and LR animals in the respective brain region *per se* but together had a cumulative effect on the neuronal activation of the PVN. How these subcortical intermediaries, as well as the subregions and nuclei of a given limbic brain region, might be involved in the perception and interpretation of stressful stimuli and HPA axis activation in animals of the SR mouse model, should be addressed in future studies.

5 Conclusion and perspectives

Using a selective inbreeding approach, three mouse lines were generated with distinct differences in their HPA axis reactivity in response to a standardised, psychological stressor, the high (HR), the intermediate (IR) and the low (LR) reactivity line. We could show in our study that the HR and LR animals of the SR mouse model reveal alterations on all levels of the HPA axis - peripheral, central as well as regarding the regulation – similar to the pathophysiological conditions observed in MD patients. This is an intriguing finding since no selection pressure was exerted on other, upstream control centres of the HPA axis, but was limited to the selection of extremes in stress-induced CORT secretion from the adrenals. Moreover, we could demonstrate that antidepressant treatment restored regulatory functions of the HPA system, thereby highlighting the predictive validity of the SR mouse model in addition to its previously assessed face and construct validity.

Thus, the SR model is of special interest for the psychiatric research since it has been shown that a dysregulation of the HPA axis is a prominent risk factor for the development of psychiatric diseases (de Kloet *et al.*, 2005). Moreover, several studies also presented evidence that feedback resistance and mild hypercortisolism are already present in healthy subjects at genetic risk for depression, indicating that an imbalance in the HPA axis activity and negative feedback drive are preceeding the clinical manifestations of the disorder (Holsboer *et al.*, 1995; Modell *et al.*, 1998). Thus, molecular-genetic studies on the SR mouse model could yield promising insights into the mechanisms by which pathophysiological alterations of the HPA axis are linked to the increased risk of developing psychiatric diseases. Furthermore, the SR mouse model holds several advantages for psychiatric research due to the vulnerable genetic predisposition towards a HPA axis dysfunction of the three breeding lines. Compared to other mouse models, it is closely related to the natural aetiology of developing pathophysiologies which are associated with affective disorders such as MD. In contrast to genetically engineered or knockout models, animals of the SR mouse model are derived from CD-1 outbred founder population with a heterogenic background. Thus, by selective inbreeding, genetic factors which are associated with the respective phenotype are taken into account which,

in turn, considers the heritability of genetic risk factors in the aetiology of psychiatric diseases.

As already outlined in the respective paragraphs of the discussion, there is ample of opportunity to put the stress reactivity to use in future experiments aimed at discovering the molecular underpinnings of affective disorders such as MD.

In our study, we found significant differences in the adrenal sensitivity between the three mouse lines in absence of differences in ACTH receptor expression. In contrast, StAR was differentially regulated in HR, IR and LR animals. Thus, the adrenal presents an interesting target for further studies, including the investigation of adrenal and plasma cholesterol, the substrate for steroid biosynthesis. Moreover, a detailed characterisation of the enzymes involved in steroid biosynthesis would be mandatory, since adrenal dysfunctions, for example observed in patients suffering from an adrenal hyperfunction (Cushing's syndrome), are closely associated with psychiatric diseases (Gold *et al.*, 1986; Starkman *et al.*, 1986).

Moreover, we found significant differences in the stress-induced neuronal excitation of the PVN between the three lines. It has been shown that not only the neuroendocrine stress response is originating from this brain nucleus but the adrenal CORT secretion is likewise governed by direct innervation of PVN neurons (Engeland *et al.*, 2005), suggesting a synergistical activation of the adrenal by these two stress systems. Thus, the SR mouse model could provide a good model for investigating the role of the sympathetic nervous system in the stress response, particularly regarding the secretion of noradrenalin and adrenalin from the adrenals.

Furthermore, the SR mouse model could serve as a valuable tool to investigate the role of the Mdr1 Pgp in the activation and negative feedback regulation of the HPA axis since it has been shown that this transporter is able to restrict the access of glucocorticoids to the brain (Uhr *et al.*, 2002). In our study, significant differences in the expression of Mdr1b in the hippocampus between HR, IR and LR mice were found. Interestingly, preliminary data from our group also revealed differences in the sequence of the *Mdr1b* gene between the three lines. Therefore, it is unlikely that the differential expression of Mdr1b is a compensatory effect, but that this transporter might be causally involved in the differential HPA axis reactivity between the three mouse lines. In this respect, it should be noted that polymorphisms in the Mdr1 Pgp gene predicted antidepressant treatment response in depression (Uhr *et al.*, 2008), emphasising the important role of Mdr1 Pgp in psychopharmacology. Thus, pharmacokinetic studies on the SR mouse model could

contribute to the understanding of the mechanisms by which antidepressants penetrate the brain and hence improve the effectiveness of CNS drugs.

In addition, it has been shown in several studies that psychiatric disorders such as bipolar disorder, major depression and schizophrenia are closely associated with impairments in cell energy metabolism, e.g. mitochondrial dysfunction, resulting in insufficient energy resources for the maintenance of functional cellular processes and physiological homeostasis (Shao *et al.*, 2008; Rezin *et al.*, 2009). Interestingly, hippocampal proteomic studies in HR, IR and LR mice identified several proteins, which were differentially expressed in HR and LR animals and were found to be involved in energy metabolism pathways (Knapman *et al.*, 2012). Furthermore, microarray- and Serial Analysis of Gene Expression (SAGE)-based expressional profiling approaches in hippocampal tissue of HR and LR mice revealed clusters of differentially regulated genes contributing to mitochondrial morphology and function (Widner-Andrä, 2011). This data strongly suggests an involvement of the mitochondria in the different endophenotypes of HR, IR and LR animals. Hence, a recently initiated study aims to investigate the mitochondrial proteome of the hippocampus of HR, IR and LR mice, allowing pathway analysis in order to identify candidate proteins involved in an altered mitochondrial energy metabolism in these mice. Thus, the SR mouse model could provide essential insights into the mechanisms linking mitochondrial dysfunction and a dysregulated HPA axis, thereby improving our understanding of the biological mechanisms of affective disorders.

Taken together, the results presented in this work as well as the findings of previous studies using HR, IR and LR mice, provide compelling evidence for the usefulness of the SR mouse model as valuable tool for elucidating the molecular and genetic underpinnings of affective disorders and therefore present an essential and indispensable tool for the future of psychiatric research.

6 Appendix

Table 1: List of used buffers for western blot analysis with their respective composition. Suppliers: (a) Carl Roth GmbH, Karlsruhe, Germany; (b) Merck KGaA, Darmstadt, Germany; (c) Sigma-Aldrich Chemie GmbH, Steinheim, Germany; (d) Roche Diagnostics Deutschland GmbH, Mannheim, Germany.

Name	Abbreviation	Composition	Supplier
Homogenisation buffer	H-buffer	50mM Tris (pH=7.5)	a
		150mM NaCl	b
		3.33mM EDTA	a
		Protease inhibitor mix (1 tablet on 10ml buffer)	d
Extraction buffer	E-buffer	50mM Tris (pH=7.5)	a
		150mM NaCl	b
		2% NP-40 (Igepal)	c
		2% Sodium Deoxycholate	a
		4% SDS	c
Radioimmunoprecipitation buffer	RIPA buffer	50mM Tris (pH=8.0)	a
		150mM NaCl	b
		0.1% SDS	c
		1.0% NP-40 (Igepal)	c
		0.5% Sodium Deoxycholate	a
		Protease inhibitor mix (1 tablet on 10ml buffer)	d
Protein loading buffer	PLB	5% SDS	c
		40% Glycerin	c
		160mM Tris (pH=6.8)	a
		5% β-mercaptoethanol	c
		Bromphenol blue	b
Electrophoresis buffer		2.5mM Tris	a
		19.2mM Glycine	c
		0.5% SDS	c
Wetblot buffer		2.5mM Tris	a
		190mM Glycine	c
		20% Methanol	a
Tris-buffered saline and Tween 20	TBST	10mM Tris	a
		150µM NaCl	b
		0.1% Tween 20	c

Table 2: Composition of the enhanced chemiluminescence solution. Suppliers: (a) Carl Roth GmbH, Karlsruhe, Germany; (b) Merck KGaA, Darmstadt, Germany; (c) Sigma-Aldrich Chemie GmbH, Steinheim, Germany; (d) Roche Diagnostics Deutschland GmbH, Mannheim, Germany.

Name	Composition	Amount	Mixing ratio	Supplier
ECL solution A	100mM Tris (pH=6.8)	200ml	10ml	a
	Luminol	50mg		a
ECL solution B	DMSO	10ml	1ml	c
	p-coumaric acid	11mg		c
30% H_2O_2			3µl	a

Table 3: Relative gene expression in the hippocampus of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII. Statistical parameters of the within-line comparisons, corresponding to Table 5 of the results section. Exact p values are given, asterisks represent the significance level (p≤0.05*, p≤0.01).**

		Veh		Flx		Within-line comparison (MWU-test)		
candidate	line	mean	SEM	mean	SEM	U	p	
Fkbp5	LR	0.76	0.04	0.95	0.05	8	0.007	**
Abcb1a	LR	0.80	0.05	1.09	0.10	10.5	0.014	*
Gilz	LR	0.95	0.08	1.27	0.08	9	0.009	**

Table 4: Relative gene expression in the pituitary of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XVII. Statistical parameters of the within-line comparisons, corresponding to Table 6 of the results section. Exact p values are given, asterisks represent the significance level (p≤0.05*, p≤0.01).**

		Veh		Flx		Within-line comparison (MWU-test)		
candidate	line	mean	SEM	mean	SEM	U	p	
Fkbp5	IR	1.00	0.10	1.68	0.16	8	0.007	**
Abcb1a	IR	1.00	0.13	1.89	0.24	10	0.012	*
Abcb1b	LR	0.57	0.06	0.91	0.12	15	0.043	*
Gilz	IR	1.00	0.05	1.41	0.12	11	0.016	*

Table 5: *C-fos* mRNA expression (optical density) in the brain of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XIX of control I (animals that received chronic injections but were not subjected to the SRT). Post-hoc MWU-tests were performed if the between-line comparison (KWH-test) yielded significant results. Exact p values are given, asterisks represent the significance levels (p≤0.05*). Significant differences between Veh and Flx treated animals of the HR, IR or LR line are indicated by bold numbers, the corresponding statistical details (MWU-test) are given in Table 6 of the appendix.

				HR		IR		LR		between line comparison (KWH-test)		post-hoc MWU-test					
												HR vs IR		HR vs LR		IR vs LR	
brain region	subregion	N=HR/IR/LR	treatment	mean	SEM	mean	SEM	mean	SEM	H	p	U	p	U	p	U	p
PFC		4/4/4	Veh	9.74	3.13	13.09	3.49	9.45	2.34	0.5	0.779						
		4/4/2	Flx	4.69	1.74	19.54	8.49	14.35	3.88	5.6	0.059T						
PVN		4/4/4	Veh	11.47	1.03	9.62	1.26	10.49	1.60	1.6	0.437						
		4/4/2	Flx	9.92	1.40	14.11	3.70	14.64	3.94	2.2	0.327						
BLA		4/4/4	Veh	4.10	0.89	7.42	2.29	5.18	2.49	0.7	0.694						
		4/4/2	Flx	2.83	0.42	13.03	4.44	8.90	3.28	6.6	0.036*	0	0.063	0	0.128	3	0.643
dHC	CA1	4/4/4	Veh	12.92	3.64	16.54	4.40	14.64	4.38	0.2	0.874						
		4/4/2	Flx	10.70	1.77	13.76	3.62	11.59	0.03	0.7	0.683						
	CA2	4/4/4	Veh	9.84	2.25	13.73	3.47	12.96	3.04	0.5	0.779						
		4/4/2	Flx	9.68	1.40	10.38	2.79	12.08	1.01	0.327	0.849						
	CA3	4/4/4	Veh	13.56	3.09	15.84	3.20	15.05	2.94	0.4	0.794						
		4/4/2	Flx	9.91	0.40	17.54	2.66	14.46	0.84	6.8	0.032*	0	0.063	0	0.128	2	0.355
	DG	4/4/4	Veh	18.54	2.04	19.26	1.35	22.81	2.52	1.8	0.397						
		4/4/2	Flx	20.22	1.34	19.65	1.60	18.27	0.49	1.5	0.453						
vHC	CA1	4/4/4	Veh	**9.51**	**0.84**	8.71	1.28	10.72	1.67	0.4	0.794						
		4/4/2	Flx	**4.65***	**0.87**	11.97	1.59	10.03	0.79	6.8	0.032*	0	0.063	0	0.128	2	0.355
	CA3	4/4/4	Veh	**8.34**	**0.70**	**7.26**	**0.73**	8.25	0.90	1.4	0.491						
		4/4/2	Flx	**5.36***	**0.50**	**10.85***	**1.13**	8.14	2.39	6.0	0.048*	0	0.063	1	0.330	2	0.355
	DG	4/4/4	Veh	10.56	1.29	**8.08**	**0.86**	11.90	1.39	5.1	0.077T						
		4/4/2	Flx	7.39	0.48	**12.05***	**1.37**	12.23	3.12	6.6	0.036*	0	0.063	0	0.128	3	0.643

Table 6: *C-fos* mRNA expression (optical density) in the brain of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XIX of control I (animals that received chronic injections but were not subjected to the SRT). Statistical parameters of the within-line comparisons, corresponding to Table 5 in the appendix. Exact p values are given, asterisks represent the significance level (p≤0.05*).

			Veh		Flx		Within-line comparison (MWU-test)		
brain region	subregion	line	mean	SEM	mean	SEM	U	p	
vHC	CA1	HR	9.51	0.84	4.65	0.87	0	0.021	*
vHC	CA3	HR	8.34	0.70	5.36	0.50	0	0.021	*
vHC	CA3	IR	7.26	0.73	10.85	1.13	1	0.043	*
vHC	DG	IR	8.08	0.86	12.05	1.37	1	0.043	*

Table 7: *C-fos* mRNA expression (optical density) in the brain of high (HR), intermediate (IR) and low (LR) reactivity mice of Gen XIX which were subjected to the SRT. Statistical parameters of the within-line comparisons, corresponding to Table 8 of the results section. Exact p values are given, asterisks represent the significance level (p≤0.05*).

			Veh		Flx		Within-line comparison (MWU-test)		
brain region	subregion	line	mean	SEM	mean	SEM	U	p	
dHC	CA2	LR	29.23	2.39	22.76	0.58	8.5	0.029	*
dHC	CA3	HR	21.20	1.70	25.32	1.25	15	0.043	*
vHC	CA3	LR	16.05	1.36	11.84	1.69	8	0.039	*

7 Lists

7.1 List of abbreviations

2-AG	2-arachidonoylglycerol
5-HT	serotonine
A	ampere
Abcb1a	ATP-binding cassette, sub-family B, member 1A
Abcb1b	ATP-binding cassette, sub-family B, member 1B
ACTH	adrenocorticotropic hormone
AMPT	α-methyl-*para*-tyrosine methyl ester
AVP	arginine vasopressine
BBB	blood brain barrier
BCA	bicinchoninic assay
BDNF	brain-derived neurotrophic factor
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
bp	base pairs
BW	body weight
CA	cornu ammonis
cAMP	cyclic adenosine monophosphate
CB 1	endocannabinoid receptor 1
CBG	corticosteroid binding globuline
cDNA	copy deoxyribonucleic acid
CeA	central amygdala
Cfl1	cofilin-1
CNS	central nervous system
CORT	corticosterone
CP	crossing point
CRH	corticotropin-releasing hormone
Crhr1	corticotropin-releasing hormone receptor 1
CVS	chronic variable stress
Dex	dexamethasone
df	degrees of freedom
DG	dentate gyrus
dHC	dorsal hippocampus
DNA	deoxyribonucleic acid
DSM IV	Diagnostic and Statistical Manual of Affective Disorders, 4th Edition
e.g.	for example
E-buffer	extraction buffer
ECL	enhanced chemoluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F	Friedman
FGD	familial glucocorticoid deficiency
Fkbp4	FK506 binding protein 4

Fkbp5/FKBP51	FK506 binding protein 5
Flx	fluoxetine
FST	forced swim test
g	rcf, relative centrifugal force
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
GC	glucocorticoid
Gilz	glucocorticoid induced leucine zipper
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
H-buffer	homogenisation buffer
HPA	hypothalamic-pituitary-adrenal
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HR	high reactivity
i.e.	that is
i.p.	intraperitoneal
IR	intermediate reactivity
ISH	*in-situ* hybridisation
kDa	kilodalton
KO	knockout
KWH	Kruskal-Wallis H
LC	locus coeruleus
LR	low reactivity
MAOI	monoamine oxidase inhibitor
MC2R	melanocortin 2 receptor
MD	major depression
Mdr1 Pgp	multidrug resistance 1 P-glycoprotein
Met	metyrapone
MR	mineralocorticoid receptor
MRAP	melanocortin 2 receptor accessory protein
mRNA	messenger ribonucleic acid
MWU	Mann-Whitney U
n.s.	not significant
NAA	N-acetylaspartate
NE	norepinephrine
Nr3c1	nuclear receptor subfamily 3, group C, member 1
Nr3c2	nuclear receptor subfamily 3, group C, member 2
NTS	nucleus of the solitary tract
OF	open field test
PFC	prefrontal cortex
PKA	protein kinase A
PLB	protein loading buffer
PNMT	phenylethanolamine-N-methyltransferase
Pomc	proopiomelanocortin
PTSD	post traumatic stress disorder
PVC	polyvinyl chloride
PVN	paraventricular nucleus of the hypothalamus
qPCR	quantitative real-time polymerase chain reaction
RIA	radioimmunoassay
RIPA	radioimmunoprecipitation assay

RNA	ribonucleic acid
SAGE	serial analysis of gene expression
SAM	sympathetic adrenomedullary
SCN	suprachiasmatic nucleus
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SNRI	serotonine norepinephrine reuptake inhibitor
SR	stress reactivity
SRT	stress reactivity test
SSRI	selective serotonine reuptake inhibitor
StAR	steroid acute regulatory protein
TBP	TATA-binding protein
TBST	tris buffered saline with tween
TCA	tricyclic antidepressant
TF	transferrin
TH	tyrosine hydroxylase
Tm	melting temperature
TRI	trizol
Tris	trisaminomethane
V	volt
Veh	vehicle
vHC	ventral hippocampus
W	Wilcoxon
WHO	World Health Organisation

7.2 List of figures

7.3 List of tables

8 References

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10 Curriculum vitae

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University

June 2012 – May 2013	**Post-Doc at the Max Planck Institute of Psychiatry in Munich**
October 2008 – June 2012	**PhD student at the Max Planck Institute of Psychiatry in Munich** Title of PhD thesis: "Central and peripheral aspects of hypothalamic-pituitary-adrenocortical (HPA) axis dysfunction: insights from mice selectively bred for extremes in stress reactivity"
October 2007 – June 2008	**Diploma thesis at the Max Planck Institute of Psychiatry in Munich** Title of diploma thesis: "Temporal aspects of hypothalamic-pituitary-adrenocortical (HPA) axis regulation: microdialysis studies on the HR/IR/LR mouse model"
October 2002 – July 2007	**Studies at the Ludwig Maximilian University Munich** Diploma in Biology Major subject: Neurobiology Minor subjects: Cell biology, Pharmacology/Toxicology

Conferences and Awards

Neurobiology of Stress Workshop, Boulder, Colorado, USA; June 15-18 2010
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Publications

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11 Declaration/Erklärung

Hiermit versichere ich, die vorliegende Dissertation selbstständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben. Alle Äußerungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet. Des Weiteren erkläre ich, dass ich nicht anderweitig versucht habe, eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen. Die vorliegende Dissertation liegt weder ganz noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

München, den 14. Juni 2012

Jan-Michael Heinzmann