Cognitive Deficits and their Underlying Structural and Functional Alterations in Mice Selectively Bred for High Stress Reactivity

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“Intellect is in itself a mode of exaggeration, and destroys the harmony of any face.”

Oscar Wilde

CONTENT

ABBREVIATIONS

2-DE	Two-dimensional gel electrophoresis
ANOVA	One factor analysis of variance
ATP	Adenosine-5'-triphosphate
BDNF	Brain derived neurotrophic factor
CgCtx	Cingulate cortex
CHESS	Chemical shift selective
CP	Caudate putamen
Cre	Creatine
CRH	Corticotrophin releasing hormone
CS	Conditioned stimulus
DAT	Dopamine transporter
D1R	Dopamine 1 receptor
D2R	Dopamine 2 receptor
ELISA	Enzyme-linked immunosorbent assay
FWHM	Full-with-at-half-maximum
FST	Forced swim test
^{1}H-MRS	Proton magnetic resonance spectroscopy
HPA	Hypothalamus-pituitary-adrenal
HR	High stress reactive
i.p	Intraperitoneally
IR	Intermediate stress reactive
ITI	Inter-trial interval
KWH-test	Kruskall-Wallis H-test
Li	Latent inhibition
LiCl	Lithium chloride
LTP	Long term potentiation

LR	Low stress reactive
MALDI	Matrix-assisted laser desorption/ionization
MEMRI	Manganese-enhanced magnetic resonance imaging
MD	Major Depression
$MnCl_2$	Manganse Chloride
MR	Magnetic resonance
MRI	Magnetic resonance imaging
MWU-test	Mann-Whitney U-test
MS	Mass spectrometry
N	Number of animals
NAc	Nucleus accumbens
NAA	N-acetylaspartate
NAAG	N-acetyl-aspartylglutamate
NaCl	Sodium chloride
No-PE	Non Pre-exposed
NOR	Novel object recognition
PFC	Prefrontal cortex
PE	Pre-exposed
pI/MW	Isoelectric point/molecular weight
ppm	Parts per million
RARE	Relaxation enhanced
ROI	Region of interest
RIA	Radioimmunoassay
SEM	Standard error of the mean
S/N	Signal to noise ratio
SNP	Single nucleotide polymorphism
SR	Stress reactivity
SRT	Stress reactivity test

SVZ	Subventricular zone
SZ	Schizophrenia
T	Total acrylamide-bisacrylamide monomer concentration
T1w	T1-weighted
T2w	T2-weighted
TOF	Time-of-flight mass spectrometer
US	Unconditioned stimulus
VTA	Ventral tegmental area
W-test	Wilcoxon-test

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their roman numerals.

I. **Knapman A**, Heinzmann J-M, Hellweg R, Holsboer F, Landgraf R and Touma C (in press). *Increased Stress Reactivity is Associated with Cognitive Deficits and Decreased Hippocampal Brain-Derived Neurotrophic Factor in a Mouse Model of Affective Disorders*. J Psychiatr Res. [Epub ahead of print]

II. **Knapman A**, Heinzmann J-M, Landgraf R, Holsboer F and Touma C (in press). *Modeling psychotic and cognitive symptoms of affective disorders: Disrupted latent inhibition and reversal learning deficits in highly stress reactive mice.* Neurobiol Learn Mem [Epub ahead of print]

III. **Knapman A***, Kaltwasser S*, Martins-de-Souza D, Holsboer F, Landgraf R, Turck CW, Czisch M and Touma C. *Increased stress reactivity is associated with decreased N-acetylapartate, neuronal activity and alterations in mitochondrial proteins.* Manuscript

*Equal contribution

SUMMARY

The hypothalamus-pituitary-adrenal (HPA) axis has repeatedly been found to be dysregulated in patients suffering from major depression (MD), as well as in patients suffering from schizophrenia (SZ). In this body of work, the relationship between stress reactivity and cognitive deficits, relevant to MD and SZ, has been investigated, using a mouse model of affective disorders, the “stress reactivity” (SR) mouse model. The SR mouse model consists of three lines of mice, selectively bred for high (HR), intermediate (IR), and low (LR) stress reactivity, respectively.

These three mouse lines were subjected to tests of prefrontal cortex- (PFC) and hippocampus-dependent memory, as well as a test of selective attention associated with psychotic behavior. In addition, mechanisms whereby increased stress reactivity may cause cognitive deficits were studied, using several different methods, including; *in-situ* hybridization, proton magnetic resonance spectroscopy (H^+-MRS), manganese enhance magnetic resonance imaging (MEMRI) and proteomics.

The results revealed that HR mice have deficits in PFC- and hippocampus-dependent tasks, in similarity to patients suffering from MD and SZ. In addition, latent inhibition, a measure of selective attention associated with psychosis, was found to be disrupted in HR mice. Furthermore, H^+-MRS revealed that HR mice have decreased levels of N-acetylaspartate, a marker of neuronal integrity, in the hippocampus and PFC. The activity of the hippocampal neurons was additionally reduced as measured by MEMRI. These findings also coincide well with finding in patients suffering from MD and SZ.

In contrast, neither the volume of the hippocampus, nor the volume of the PFC was reduced in HR mice in comparison to the other lines. This implies that the reasons for a less well functioning hippocampus and PFC in the HR mice is not due to a global shrinkage of the structures, but rather due to more subtle changes on a cellular level. We have found that these changes include decreased BDNF levels in the hippocampus and alterations of the PFC dopaminergic (DA) system, as well as alterations in proteins involved in energy metabolism in the hippocampus. Interestingly, similar changes have also been reported in patients suffering from SZ and MD, in relation to specific cognitive deficits.

Taken together, the findings presented here provide construct and face validity for the HR mouse line as a model for the cognitive deficits in SZ and MD. These experiments are also the first to show that a genetic predisposition for an increased in stress reactivity, without repeatedly subjecting the animals to a stressor or synthetic glucocorticoids, is sufficient to induce hippocampal and PFC dysfunction. The SR mouse model could thus be used in future studies to further our understanding of the etiology of cognitive deficits in MD and SZ and be a promising tool in the search for new treatment strategies for these disorders.

AIMS OF THE THESIS

The aim of this thesis was to characterize the three lines of mice selectively bred for extremes in stress reactivity with regard to their cognitive phenotype. We furthermore aimed to investigate the morphology and functionality of brain areas relevant to cognition. Ultimately the goal was to gain insight into the role of an increased hypothalamus-pituitary-adrenal axis reactivity in the etiology of symptoms of major depression and schizophrenia, with an emphasis on cognitive deficits frequently observed in patients suffering from these disorders.

SPECIFIC AIMS

- To investigate hippocampus-dependent memory, prefrontal cortex-dependent memory and measures of psychotic-like behavior in the stress reactivity mouse model
- To investigate underlying mechanisms of cognitive deficits in highly stress reactive mice
- To find phenotypic similarities between the stress reactivity mouse lines and patients suffering from major depression or schizophrenia
- To elucidate the underlying biological mechanisms of these phenotypic similarities
- To use the stress reactivity mouse model to investigate new targets to treat major depression and schizophrenia
- To use the stress reactivity mouse model to further our understanding of the etiology of major depression and schizophrenia

INTRODUCTION

MAJOR DEPRESSION

Major depression (MD) is a highly debilitating disorder, with a lifetime prevalence estimated to between 13 and 17 percent in Europe and the United States of America (Carta et al., 1995). It is a life threatening disorder, as MD is the most important risk factor for suicide, with two thirds of suicides being committed by patients suffering from MD (Sartorius, 2001). MD is also one of the leading causes of disability worldwide and causes substantial socioeconomic losses (Wong and Licinio, 2001). The US economy alone is estimated to loose 43 billion dollars per year due to MD (Greenberg et al., 1993). One of the reasons for these large economical losses is that for a large portion of the patients we are still lacking successful treatment options that allow the patients to resume a fully functioning life.

Symptoms of Major Depression

MD is a very heterogeneous disorder, which may partly explain why it is such a difficult disorder to treat. MD is characterized in the diagnostic and statistical manual of mental disorders (DSM) (APA, 2000) by a certain duration and number of symptoms, that cause impairment and dysfunction, distinguishing the disorder from normal sadness or grief. These symptoms include; depressed mood, feelings of worthlessness, diminished interest or pleasure in activities, large change in appetite or weight gain, psychomotor agitation or slowness of movement, fatigue and recurrent thoughts of death (APA, 2000).

Apart from these core symptoms of MD, patients also suffer from cognitive deficits. These cognitive deficits are mainly in the realm of executive functioning (Austin et al., 2001; Porter et al., 2003; Reppermund et al., 2007) and verbal memory deficits (Reppermund et al., 2007). Interestingly, the cognitive symptoms have been reported to precede the onset of MD, (Airaksinen et al., 2007) and often remain when the patients' other symptoms have successfully been treated (Reppermund et al., 2007). This raises the question whether the symptoms are a more integral part of the disease's development, rather than a byproduct of MD.

In addition to the affective and cognitive symptoms of MD, there are also physiological alterations that are characteristic of the disease. The most prominent and robust finding is an

increased hypothalamus-pituitary-adrenal (HPA) axis activity (Holsboer, 2000; Ising et al., 2005). Many studies have shown that excessive exposure to glucocorticoids has a detrimental effect on neurons (Sapolsky et al., 1990; Watanabe et al., 1992; Sousa et al., 1999; McKittrick et al., 2000; Lyons et al., 2007). Brain areas involved in cognition, such as the hippocampus and PFC have abundant glucocorticoid receptors. Thus, an excess exposure to glucocorticoids has been implicated in the cognitive deficits in depression. The exact mechanism by which the damage occurs to these areas is however still not precisely known.

Subtypes of Major Depression

Due to the heterogeneity of MD, certain subtypes of depression have been proposed. DSM-IV lists two distinct clinical depressive syndromes; melancholic and atypical depression. These two types of depression are the antithesis of one another in many aspects. The term atypical depression stems from a group of depressed patients who did not respond to typical antidepressants, however, they did respond to monoamine oxidase inhibitors (MAOIs) (Parker et al., 2002). These patients were less likely to report early morning wakening or worsened mood in the morning and were also less likely to have a pattern of clinical features consistent with “the more classical endogenous depression” (Parker et al., 2002). The DSM-IV criteria for atypical features also include hypersomnia and hyperphagia. Atypical depression has furthermore been associated with being overweight and aggressive personality traits (Nierenberg et al., 1998; Hasler et al., 2004). Interestingly, it has also been reported that patients suffering from atypical depression more commonly have a hypoactive HPA axis rather than a hyperactive HPA axis as is most commonly associated with MD (Gold et al., 1995).

Increased HPA axis activity on the other hand has more specifically been associated with melancholic depression (Gold and Chrousos, 2002). Melancholic depression in contrast to atypical depression features hyperarousal, decreased sleep, decreased food intake and decreased body mass (Gold et al., 2002). Psychotic MD (PMD) has also been more strongly associated with an increased HPA axis activity than non psychotic MD (NPMD) (Gomez et al., 2006). PMD also has other specific features that distinguish it from NPMD. Patients with psychotic features often have a higher depression score, more frequent psychomotor difficulties, increased feelings of guilt and are typically more difficult to treat (Keller et al., 2007). The course of the depressive episodes has also been found to be different in those who exhibit psychotic features. PMD

patients often have a longer duration of episodes (Coryell et al., 1987; Maj et al., 2007) and a greater likelihood of recurrent depression (Lykouras et al., 1986; Aronson et al., 1988). Cognitive deficits are also more prominent in PMD (Cornell et al., 1984; Austin et al., 1999; Basso and Bornstein, 1999)

Treatment Options for Major Depression

The most common first-line treatment for MD are selective serotonin reuptake inhibitors (SSRIs) (Koenig and Thase, 2009; Rakofsky et al., 2009). SSRIs are however not always successful in treating the symptoms of MD and also have a myriad of side-effects, such as sexual dysfunction, dry mouth and weight gain (Koenig and Thase, 2009). Due to the many side effects there is a high incidence of non-compliance in patients. In cases where SSRIs are ineffective, other types of drugs can be used including combined serotonin-norepinephrine reuptake inhibitors or tricyclic antidepressants (Shelton et al., ; Koenig and Thase, 2009; Rakofsky et al., 2009). Tricyclic antidepressants can however exasperate cardiac diseases, so they should be used with caution (Haanpaa et al.). PMD is most commonly treated with a combination of SSRIs and antipsychotics (Goodwin et al., 2009). Monoamine oxidase inhibitors (MAOIs) are in many cases more effective in treating atypical depression, but they have interactions with common foods, including cheese and certain types of meat (Fiedorowicz and Swartz, 2004). It is furthermore possible to commit suicide by over-dose of MAOIs, which is not ideal for the treatment of depression (Fiedorowicz and Swartz, 2004). Some patients do not respond well to any of the above mentioned treatment options, and furthermore, there is a delayed response to antidepressant treatment, which can be of crucial importance when dealing with suicidal patients. It is therefore apparent that the there is a need for better treatment strategies for treating MD.

SCHIZOPHRENIA

The lifetime risk of SZ is approximately one percent, with a predominant genetic influence (Sartorius et al., 1986; Cannon and Jones, 1996). Most patients continue to show some degree of incapacity and need to be medicated for the rest of their lives. The cost in loss of productivity as well astreatment costs for SZ are therefore substantial (Andreasen, 1995).

Symptoms of Schizophrenia

The diagnosis of SZ is based upon the presences of certain so-called “positive symptoms”, that include delusions, hallucinations and thought disorder, as well as “negative symptoms”, such as avolition, alogia and affective flattening (Andreasen, 1995). According to the DSM-IV these symptoms must be present for a minimum of six months and may not be secondary to any other disorder, such as depression or substance abuse (APA, 2000). There are also physiological alterations characteristic of SZ, including ventricular enlargement and decreased cortical and hippocampal volume (Harrison, 1999). Interestingly, an increased HPA axis reactivity has also been reported in patients suffering from SZ (Muck-Seler et al., 2004; Ryan et al., 2004; Gallagher et al., 2007; Ritsner et al., 2007).

Treatment Options for Schizophrenia

The first generation of antipsychotics, the so-called “typical antipsychotics” are dopamine 2 receptor (D2R) inhibitors (Snyder and Murphy, 2008). They are relatively effective in treating positive symptoms, however, they are known to cause extrapyramidal movement disorders (Snyder and Murphy, 2008). The second generation of antipsychotics, “atypical antipsychotics”, inhibit D2Rs as well as other receptors including 5-hydroxytryptamine 2A receptors (Snyder and Murphy, 2008). The atypical antipsychotics are less likely to cause extrapyramidal side effects, however they cause other side-effects, such as weight gain, prolactin and glucose elevation, and sedation (Snyder and Murphy, 2008). A striking 74 percent of patients discontinue the use of their medication within 18 months of treatment, due to these side effects or poor efficacy of the drugs (Lieberman et al., 2005), leading to the need for improved pharmacological treatment options for patients suffering from SZ.

SIMILARITIES BETWEEN MAJOR DEPRESSION AND SCHIZOPHRENIA

Cognitive deficits in MD are more predominant in patients suffering from melancholic depression or who have psychotic-like features (Cornell et al., 1984; Austin et al., 1999; Basso and Bornstein, 1999), which leads to a parallel to schizophrenia (SZ), a disorder characterized by psychotic episodes along with several well characterized cognitive deficits (APA, 2000). These deficits include prefrontal cortex (PFC) dependent tasks (Goto et al., ; Floresco et al., 2009) in

similarity to patients suffering from MD (Austin et al., 1999; Merriam et al., 1999), as well as deficits in sensorymotor gating (Braff et al., 1978; Perry et al., 2002; Martinez-Gras et al., 2009) and the ability to ignore irrelevant stimuli in paradigms such as latent inhibition (Baruch et al., 1988; Guterman et al., 1996; Rascle et al., 2001; Schmidt-Hansen et al., 2009). Furthermore, patients suffering from melancholic or PMD and patients suffering from SZ have been reported to have an increased HPA axis reactivity (Holsboer, 2000; Muck-Seler et al., 2004; Ryan et al., 2004; Ising et al., 2005; Gallagher et al., 2007; Ritsner et al., 2007). This provides a possible common underlying mechanism for the cognitive deficits in both disorders. In this body of work, we have therefore studied these cognitive symptoms as a phenotypic endpoint relevant to both SZ and MD.

MODELING PSYCHIATRIC DISEASES IN MICE

Psychiatric diseases are among the most difficult diseases to model due to the complex nature of the symptoms that are mainly in the realm of higher order cognitive processes. A good animal model of a disorder should provide face-, construct-, and predictive- validity (Anisman and Matheson, 2005). The face validity of a mouse model pertains to the similarity of the phenotype of the mouse model to the symptoms of the disease it is to model. Construct validity refers to the animal model of a disorder and the patients suffering from this disorder having a common underlying mechanism of these symptoms. Finally, predictive validity concerns the ability of known treatment strategies to reverse the symptoms in the mouse model in similarity to clinical observations.

There are several models used to test “depression-like” behavior in mice. The test we have used here, the forced swim test (FST), is the most common test of face- and predictive validity of mouse models of depression (Porsolt et al., 1977; Cryan and Holmes, 2005). In this test, the mice are placed in a cylinder of water for six minutes. At first, the animal will actively try to escape by struggling and swimming, but will eventually “give up” and adopt a more passive coping strategy, i.e. floating. An increased amount of time spent floating and a shorter latency to start floating is interpreted as increased “depression-like” behavior (Porsolt et al., 1977; Cryan and Holmes, 2005). Increased struggling behavior, on the other hand, indicates a more active coping strategy and hyperarousal (Porsolt et al., 1977; Cryan and Holmes, 2005).

In modeling SZ, the main focus in providing face validity for a model has been on the cognitive deficits associated with psychotic behavior, as delusions and hallucinations are not possible to assess in mice. One test used to assess selective attention deficits associated with psychotic behavior, is latent inhibition (Li) (Lubow, 2005). Li is the phenomenon whereby the pre-exposure to the to-be conditioned stimulus, retards subsequent pairing of the unconditioned stimulus to the conditioned stimulus (Lubow, 1973) and has been associated specifically with psychotic episodes in SZ (Lubow et al., 2000).

THE STRESS REACTIVITY MOUSE MODEL

The stress reactivity (SR) mouse model was established to model on of the core neuroencdocrine symptoms of MD. This model consists of three lines of mice that are selectively bred for high (HR), intermediate (IR) or low (LR) stress reactivity. This was achieved by taking a founder population of CD-1 mice and testing their plasma corticosterone increase in response to a 15-min restraint stressor. Male and female mice with extremely high HPA axis reactivity were selected and mated to create the HR mouse line and mice with extremely low stress reactivity were mated in order to create the LR line. This procedure was then repeated for each generation. The third line, the IR line was selected according to an intermediate stress reactivity, representative of the average CD-1 mouse and serves as a control group with the same inbreeding status as the HR and LR mice.

The stress reactivity mouse model has previously been established as a mouse model of MD (Touma et al., 2008; Touma et al., 2009). The highly stress reactive mice show robustly increased corticosterone levels in the plasma and hippocampus in response to a standardized stressor, in comparison to IR and LR mice (Touma et al., 2008; Heinzmann et al., under revision). In accordance with this, adrenocorticotropic hormone (ACTH) levels were also increased in response to a standardized stressor in HR mice. The HR mice also have an increased adrenal gland weight in comparison to IR and LR mice. Furthermore, the diurnal rhythm of glucocorticoid secretion of the three mouse lines has been investigated, using a non-invasive technique measuring corticosterone metabolites in fecal samples (Touma et al., 2008; Touma et al., 2009). This showed that the circadian rhythm of the HR mice was flattened with an increased nadir in comparison to the other two lines. The circadian peak of the corticosterone secretion, however, did not differ between the three lines. In similarity to patients suffering from MD, the

HR mice also have an increased response to the dexamethasone/ corticotropin releasing hormone (CRH) test. Interestingly, the CRH and arginine-vassopressin levels in the paraventricular nucleus of the HR mice did not differ between the three lines.

In the FST, HR mice show increased struggling and decreased floating in comparison to IR and LR mice, indicative of increased arousal and a hyper-active coping style (Touma et al., 2008; Knapman et al., 2009). The HR mice are thus similar to the melancholic and psychotic subtype of depression, whereas the LR mice are more similar to the atypical subtype of depression. The LR mice also show other similarities to patients suffering from atypical depression, such as increased body weight in the absence of increased food intake and increased social aggression (Touma et al., 2008). Furthermore, the sleep architecture of the HR mice is similar to patients suffering from MD with increased sleep fragmentation, increased rapid eye movement sleep and decreased slow wave activity (Touma et al., 2009). It should also be noted that the three lines do not differ in anxiety related behavior, as differences in anxiety would be a confounding factor in many of the behavioral tests presented here (Touma et al., 2008).

AIMS AND SCOPE OF THE THESIS

The studies presented here aimed to further characterize the stress reactivity mouse model with regard to its cognitive phenotype, in order to better understand the link between stress reactivity and cognitive deficits and to provide construct and face validity for the HR mouse line as a model MD and SZ. To this end, mice from the three breeding lines were subjected to a number of learning and memory paradigms. Firstly, we aimed to assess the hippocampus-dependent memory. This was achieved by using two tasks that take advantage of the mice innate preference for novelty, namely the novel object recognition (NOR) task and the Y-maze task. The first task, the NOR task is used to assess object memory which is believed to be dependent on the hippocampal formation and adjacent areas such as the perirhinal cortex (Dere et al., 2007). The second task, the Y-maze task, is a spatial learning task that lesion studies have revealed to be hippocampus-dependent (Sanderson et al., 2009) (see paper I).

Assessing executive functioning in mice in a similar manner to the tests conducted clinically is not feasible. However, tests that employ the same brain areas as executive functioning tasks, namely the PFC have been designed for mice and other rodents. One of the most common of these is a reversal learning task in a water filled T-maze. This test was not

suitable to subject the SR mouse model to though; as the swimming-induced stress would cause an acute effect on memory that would severely confound our results. In order to assess PFC-dependent tasks in the SR mouse model, we therefore designed a dry-land version of the T-maze reversal learning task involving a minimal amount of stress (see paper II). Reversal learning provides an analogue to the Wisconsin Card Sorting test, which is commonly used to assess medial PFC function in humans (Lie et al., 2006). Patients suffering from both SZ and MD have been found to have deficits in performing this task (Volz et al., 1997; Merriam et al., 1999; Rabin et al., 2009; Shirayama et al., 2009).

As cognitive deficits are more commonly observed in patients exhibiting psychotic symptoms or suffering from melancholic depression (Cornell et al., 1984; Austin et al., 1999; Basso and Bornstein, 1999), it was also of importance to assess psychotic-like behavior in the SR mouse model. A paradigm that has been shown to assess selective attention deficits associated with psychotic symptoms in mice is Li (Baruch et al., 1988; Guterman et al., 1996; Rascle et al., 2001; Schmidt-Hansen et al., 2009). Li is the phenomenon whereby the pre-exposure of a to-be conditioned stimulus (CS) retards the learning of the subsequent CS –unconditioned stimulus (US) pairing (Lubow, 1973). Li can be assessed in several ways. In order to test the SR mouse model, a conditioned taste aversion paradigm was chosen, where the CS was sucrose solution and the US was sickness, induced by a lithium chloride injection (see paper II).

Once the cognitive phenotype of the SR mouse lines had been assessed, the underlying mechanisms of the differences in behavior between the three lines were investigated using a number of different methods. In the first instance, brain-derived neurotrophic factor (BDNF) was measured using an enzyme-linked immunosorbent assay (ELISA) (see paper I). BDNF has been shown to be involved in cognitive processes and is regulated by glucocorticoids, making it an interesting candidate to explain differences in the cognitive performance of the SR mouse lines. Furthermore, depressed patients have been reported to have reduced plasma BDNF levels, which are normalized in response to antidepressant treatment (Nibuya et al., 1996; Santarelli et al., 2003; Lee et al., 2007; Huang et al., 2008; Monteleone et al., 2008). BDNF is therefore a promising target to investigate in the search for drug targets able to improve cognitive abilities in depressed patients.

Several reports using proton magnetic resonance spectroscopy (^{1}H-MRS) have also demonstrated that patients suffering from psychiatric disorders have decreased amounts of N-acetylaspartate (NAA) in several brain areas relevant to cognitive performance, including the

hippocampus (Buckley et al., 1994; Bertolino et al., 1998; Gonul et al., 2006). NAA is a metabolite exclusively found in neurons (Birken and Oldendorf, 1989; Moffett et al., 1991), which is decreased in association with cellular dysfunction and neuronal loss (Demougeot et al., 2001). NAA is therefore considered to be a maker of neuronal integrity. We therefore assessed the NAA levels in the hippocampus and PFC of the SR mouse lines (see paper III).

Studies conducted on humans, non-human primates and rodents have previously shown that excess glucocorticoid exposure can reduce both the volume and activity, measured by blood flow, of the hippocampus (Endo et al., 1997; Bremner et al., 2000; Czeh et al., 2006). Therefore, in addition, mice from the three SR lines were injected with Mn^{2+} in order to assess the manganese enhanced signal intensity, as a measure of neuronal activity of these brain areas and to enable voxel-based morphometric studies to measure if increased glucocorticoid exposure decreased the volume of relevant brain areas, such as the hippocampus and PFC (see paper III).

The dopaminergic system was furthermore investigated, as the mesolimbic dopamine system is strongly linked to Li and psychotic behavior in psychiatric patients (Crow et al., 1976; Solomon and Staton, 1982; Weiner, 1990; Davis et al., 1991; Young et al., 1993; Lubow, 2005). The PFC dopaminergic system reportedly also sub-serves the optimal functioning of the PFC (Cropley et al., 2006), which is relevant to the reversal learning task. *In situ* hybridization histochemistry was employed to semi-quantify the dopamine 1 receptor (D1R), dopamine 2 receptor (D2R) and dopamine transporter (DAT) mRNA levels in the mesolimbic area and PFC, of mice from the three breeding lines (see paper II). The dopaminergic system also represents an interesting candidate in the search for new drug targets that can be used to treat the cognitive deficits in MD, as well as treatment resistant MD, as atypical antipsychotics have shown great promise in treating depressed patients who do not respond to typical antidepressants (Quintin and Thomas, 2004).

Once certain cognitive and molecular differences were established between the three breeding lines of the SR mouse model, the hippocampal proteome was screened in order to use an unbiased approach to find new protein candidates or pathways of relevance to MD and SZ (see paperIII).

DISCUSSION

In this body of work, the stress reactivity (SR) mouse line has been characterized with respect to cognitive abilities and underlying functional alterations, with the aim of modeling cognitive symptoms of psychiatric diseases in order to gain insight into the molecular underpinnings of theses symptoms. The highly stress reactive (HR) mouse line show several cognitive deficits which are similar to those observed in patients suffering from major depression (MD) and schizophrenia (SZ). These cognitive deficits include hippocampus-dependent tasks (papers I & III), prefrontal cortex- (PFC) dependent tasks (see papers II & III) and disrupted latent inhibition (Li) (see paper II). Furthermore, the HR mice display similar alterations in brain structures that are presumed to be responsible for the cognitive deficits in MD and SZ, including decreased brain-derived neurotrophic factor (BDNF) levels in the hippocampus (see paper I), decreased N-acetylaspartate (NAA) levels in the Hippocampus and PFC (see paper III) and alterations of the dopaminergic system (see paper II). In addition, proteins involved in energy metabolism have been found to differ between HR and low (LR) stress reactive mice, which proposes a mechanism whereby glucocorticoids may damage the brain (paper III).

A GENETIC PREDISPOSTION FOR INCREASED STRESS REACTIVITY

The HR mice are unique in the aspect that they are genetically predisposed for an increased hypothalamus-pituitary-adrenal (HPA) axis reactivity. The brains of HR mice are not exposed to excess glucocorticoids by means of repeatedly exposing them to synthetic glucocorticoids or specific stressors, instead each exposure to a normal life event that causes an activation of the HPA axis, such as an agonistic interaction with a conspecific, handling or cage changing, would lead to a higher exposure of the brain to glucocorticoids in HR mice compared to LR or IR mice. This resembles a more natural situation, as vulnerability to affective disorders in genetically predisposed individuals is believed to be related to heightened stress sensitivity (Nemeroff and Vale, 2005).

MODELLING COGNITIVE DEFICITS IN PSYCHIATRIC DISORDERS

We have shown that HR mice have deficits in two hippocampus-dependent tests, the novel object recognition task and the Y-maze task (see papers I & III). Furthermore, HR mice showed reversal learning deficits indicative of poor PFC function (see papers II & III). In similarity, patients suffering from MD commonly exhibit deficits in hippocampus-dependent tasks, such as verbal memory as well as deficits in PFC-dependent executive functioning tasks (Austin et al., 2001; Porter et al., 2003; Reppermund et al., 2007; Rabin et al., 2009; Reppermund et al., 2009; Shirayama et al., 2009). Interestingly these cognitive deficits are reportedly worse in patients with psychotic MD (Cornell et al., 1984; Austin et al., 1999; Basso and Bornstein, 1999). This is of particular interest as HR mice additionally show cognitive deficits associated with psychotic behavior, namely disrupted Li (see paper II) (Gray et al., 1992; Gray et al., 1995; Lubow et al., 2000; Rascle et al., 2001). The HR mice also demonstrate certain morphological alterations that are specifically related to patients suffering from SZ, a disorder with prominent psychotic features. These alterations include increased dopamine 2 receptors in the NAc and decreased dopamine 1 receptors in the PFC in HR mice in comparison to LR mice (see paper II), which mimics what is observed in patients suffering from SZ (Carlsson and Lindqvist, 1963; Carlsson et al., 2000; Mawlawi et al., 2001; Abi-Dargham, 2003; Alves Fda et al., 2008). HR mice thus demonstrate both construct and face validity as a model of psychotic behavior.

Interestingly, patients suffering from MD as well as patients suffering from SZ have repeatedly been reported to have an increased HPA axis activity (Holsboer, 2000; Muck-Seler et al., 2004; Ryan et al., 2004; Ising et al., 2005; Ritsner et al., 2007). In patients suffering from MD this is specifically associated with memory deficits, psychotic features and the melancholic subtype of depression (Belanoff et al., 2001; Gold and Chrousos, 2002; Bremner et al., 2004; Gomez et al., 2006).

Cognitive deficits and psychotic behavior can thus be seen as a cluster of symptoms associated with an increased HPA axis activity in psychiatric diseases including SZ, melancholic MD and psychotic MD. Our findings furthermore suggest that these symptoms have a common biological underpinnings brought about by excessive glucocorticoid exposure. The HR mice can be used as a model of these specific phenotypic endpoints relevant to MD and SZ and to discover by which means glucocorticoids contribute to these symptoms.

THE EFFECT OF EXCESS GLUCOCORTICOID EXPOSURE ON THE HIPPOCAMPUS

Excess glucocorticoid exposure has been associated with global changes to the hippocampus, including decreased birth of new granular cells in the dentate gyrus of the hippocampus, dendritic atrophy in the CA3 region of the hippocampus and reduced hippocampal volume (Sapolsky et al., 1990; Watanabe et al., 1992; Sousa et al., 1999; McKittrick et al., 2000; Lyons et al., 2007). Clinical studies have found contradicting results regarding hippocampal volume in MD with some studies indicating that patients suffering from unipolar depression have reduced hippocampal volume in comparison with healthy controls (Bremner et al., 2000; Campbell et al., 2004; Kaymak et al., 2009; McKinnon et al., 2009) and others reporting no such difference (Keller et al., 2008; Bearden et al., 2009; Kronmuller et al., 2009).

We therefore investigated if the increased glucocorticoid exposure of the hippocampus of HR mice had led to global morphological changes. This study revealed that the volume of the hippocampus was not decreased in HR mice in comparison to IR and LR mice (see paper III). However, both BDNF (see paper I) and NAA levels were decreased in the hippocampus of HR mice (see paper III). This indicates that the deficits in hippocampus-dependent learning tasks observed in HR mice are presumably not due to a global deterioration of the entire hippocampal structure, but are more likely due to more discrete morphological changes or changes occurring on the level of cellular processes.

THE EFFECT OF GLUCOCORTICOID EXPOSURE ON CELLULAR FUNCTIONS

There are theories as to how excess glucocorticoids lead to suboptimally functioning neurons. One central dogma in these theories is calcium induced neuronal cell damage, via glutamatergic hyper-activation, as glucocorticoids increase free cytosolic calcium (Elliott and Sapolsky, 1992, 1993). The majority of receptors at the synapses of the hippocampus are glutamatergic (Lee et al., 1991). Binding of glutamate to glutamate receptors activates *N*-methyl-*D*-aspartate receptors, which in turn leads to the mobilization of free cytosolic calcium. This calcium is believed to be responsible for activating the long-term changes that constitute “memory”. However, if the amounts of glutamate are excessive, this calcium mobilization produces an over-activity of calcium-dependent enzymes that leads to cytoskeletal degradation, protein malfolding and

oxygen radical generation. These processes together are detrimental for the cells functioning (Lee et al., 2002).

Energy availability is also believed to be a key regulatory mechanisms in neuronal function (Beal et al., 1993; Turski and Turski, 1993). As a strategy to divert energy toward using muscles during a stressor, glucocorticoids inhibit glucose transport in various peripheral tissues (Munck, 1971). A similar mechanism has been proposed to occur in the hippocampus. Glucocorticoids decrease glucose uptake in cultured neurons and glia cells, and decrease local glucose utilization in the hippocampus (Kadekaro et al., 1988; Horner et al., 1990; Virgin et al., 1991; Freo et al., 1992; Doyle et al., 1993). This reduction in glucose reduces the capacity of neurons and glia cells to remove glutamate from the synaptic cleft and the capacity of the cells to remove calcium from the cytoplasm, as both of these processes are highly energy costly (Lee et al., 2002). Alterations in proteins involved in energy metabolism in HR mice could thus potentially be a key factor in the decreased functioning of the hippocampus.

MITOCHONDRIAL DYSFUNCTION IN PSYCHIATRIC DISORDERS

The hippocampal proteomes of HR and LR mice were compared in order to use an unbiased approach to investigate if hippocampal-dependent learning deficits and decreased NAA levels were dependent on alterations in specific cellular processes. This comparison revealed differences in numerous proteins involved in energy metabolism (see paper III). Interestingly, these proteins appear to directly or indirectly be inducers of apoptosis and are up-regulated in HR mice in most cases.

Mitochondrial dysfunction in psychiatric disorders has been the focus of many recent studies (Jou et al., 2009; Rezin et al., 2009a) as post mortem studies of patients suffering from MD and SZ have reported alterations in proteins involved in energy metabolism (Beasley et al., 2006; Martins-de-Souza et al., 2009). Studies using animal models of depression where rats are subjected to chronic stress have also reported mitochondrial dysfunction and inhibition of the mitochondrial respiratory chain (Madrigal et al., 2001; Rezin et al., 2008; Rezin et al., 2009b) thus confirming a link between glucocorticoid exposure and mitochondrial dysfunction.

The number and density of the mitochondria in patients suffering from psychiatric diseases have also been found to be altered. A study by Uranova and colleagues found a significant decrease in mitochondria number and density in the PFC and caudate nucleus of

postmortem brains of subjects suffering from SZ in comparison to controls (Uranova et al., 2001). It has also been suggested that drug treatment increases the number of mitochondria in patients suffering from SZ (Inuwa et al., 2005). Studies have also been conducted on animals implicating mitochondrial energy metabolism as a target for certain mood stabilizing drugs, such as lithium and valproate (Wang et al., 2004; Zarate et al., 2006).

The mitochondria are consequently a potential new target for drugs to treat MD and SZ. HR mice provide an excellent tool in the search for such drug targets and in the evaluation of new treatment strategies focusing on mitochondrial dysfunction.

THE NEUROTROPHIC HYPOTHESIS OF MAJOR DEPRESSION

Glucocorticoids have also been proposed to exert their detrimental effects on the hippocampus via other pathways than those mentioned above. The neurotrophic hypothesis of depression states that stress-induced vulnerability and the therapeutic action of antidepressant treatments occur via intracellular mechanisms that decrease or increase, respectively, neurotrophic factors necessary for the survival and function of particular neurons (Duman et al., 1997). The neurotrophic factor that has most prominently been implicated in MD is BDNF (Castren et al., 2007). BDNF is reportedly decreased in the plasma of depressed patients and the levels tend to increase in response to treatment with antidepressants (Nibuya et al., 1996; Santarelli et al., 2003; Lee et al., 2007; Huang et al., 2008; Monteleone et al., 2008).

Reduced BDNF levels have also been implicated in the pathophysiology of SZ. BDNF plasma levels and cerebrospinal fluid levels were significantly lower in first-episode patients diagnosed with SZ in comparison to healthy controls (Chen da et al., 2009; Pillai et al., 2010). Plasma levels of BDNF have also been shown to be decreased in chronic patients with SZ (Xiu et al., 2009). Interestingly, the plasma levels vary in accordance with the medication with which these patients were treated. Patients treated with risperidone had lower plasma levels of BDNF than those treated with clozepine and typical antipsychotics (Xiu et al., 2009). However, in contrast to MD the improvement of SZ symptoms has not been associated with increased BDNF levels (de Lucena et al.).

The decreased amounts of BDNF in the hippocampus of HR mice (see paper I) thus provide further support to the assumption that there is a common underlying mechanism of the cognitive deficits in MD, SZ and the cognitive deficits observed in HR mice, presumably brought

about by increased HPA axis activity and decreased BDNF levels. These findings also support the hypothesis that increased exposure to glucocorticoids leads to decreased BDNF levels and show that the HR mice can be used in the search for new treatment strategies aiming at increasing BDNF levels.

THE ASSOCIATION BETWEEN ENERGY METABOLISM, BRAIN-DERIVED NEUROTROPHIC FACTOR AND N-ACETYLASPARTATE LEVELS

It has been suggested that alterations of neurotrophic or neuroprotective factors may be the underlying cause of lower hippocampal NAA levels that have been reported in several psychiatric disorders including bipolar disorder (BPD) (Deicken et al., 2003) unipolar depression and SZ (Buckley et al., 1994; Bertolino et al., 1998; Gonul et al., 2006). This is particularly interesting as the HR mice not only have decreased BDNF in the hippocampus (see paper I), but were also found to have decreased NAA levels in the hippocampus (see paper III). A positive correlation between plasma BDNF and NAA levels in the anterior cingulate cortex has been reported in healthy controls (Lang et al., 2007). Furthermore, a single nucleotide polymorphism (SNP) that results in decreased BDNF levels has been associated with decreased NAA levels in humans (Gallinat et al., 2010). This SNP encodes a Valine or Methionine at amino acid 66 of the gene product, where the Methionine allele has been associated with increased risk of MD (Gatt et al., 2008), poorer episodic memory, abnormal hippocampal activation, assayed with fMRI, and lower hippocampal NAA, assayed with MRI spectroscopy (Egan et al., 2003). This demonstrates that the link between memory deficits, NAA levels and BDNF that we observe in HR mice is also present in patients, lending further support to the validity of this mouse model.

BDNF is known to inhibit apoptosis in the granular cells of the hippocampus (Kubo et al., 1995). Thus, decreased BDNF in the hippocampus of HR mice may be a mechanism whereby the apoptosis inducing proteins are upregulated in HR mice. Decreased NAA levels have also been proposed to be associated with decreased energy metabolism in neurons (Signoretti et al., 2001). Decreased mitochondrial function or increased apoptosis may thus be the connection between decreased BDNF levels and decreased NAA levels reported in patients, as well as in our mouse model.

ADVANTAGES OF THE STRESS REACTIVITY MODEL

There are several well established animal models of depression, however, the SR mouse model has the advantage of having a more natural etiology in comparison with models where mice are repeatedly stressed or models where genes are overexpressed or knocked out. A further advantage of the SR mouse model, in comparison with models using genetic engineering techniques, is that the SR mouse lines are derived from an outbred mouse line. This means that the phenotypes we observe are independent of a homogenous genetic background. This is an important factor to consider as the same gene knocked out in different mouse lines can lead to entirely different phenotypes (Banbury_Conference, 1997; Lesch and Mossner, 1998). The SR mouse model thus has the added advantage of being more analogous to psychiatric disorders in that the HR mice show similar phenotypic endpoints to patients suffering from MD and SZ, driven from a variable genetic background, where a multitude of genes contribute to the observed endophenotype.

The approach of breeding lines of mice that will develop a known disease-like state also allows the investigation of the development of the disease over time. The HR mice can be utilized in future studies to investigate if the hippocampus is functioning suboptimally at birth or if these differences occur as a cumulative effect of exposure to excess amounts of glucocorticoids across their lifespan. Top down approaches going from a known behavior to screening of the genome or proteome are a further advantage of the model. This approach allows for an unbiased approach to finding new candidate proteins or genes that are important in the development of affective disorders.

OUTLOOK AND CONCLUDING REMARKS

There are ample opportunities to put the stress reactivity mouse model to use in future experiments aimed at discovering the molecular underpinnings of affective disorders such as MD and SZ. As the behavioral phenotype has now been extensively characterized, along with certain similarities in underlying mechanisms of cognitive deficits in MD and SZ, future findings of genetic or proteomic differences between HR an LR mice may be extrapolated to be relevant to patients suffering from MD an SZ. The first screening of the hippocampal proteome has thus far produced some highly interesting candidates and systems for further investigation. As most of the proteins revealed to differ between HR and LR mice were involved in energy metabolism, specifically screening the mitochondrial proteome would be of great interest, as well as

sequencing the mitochondrial DNA in order to discover if the origin of differences in protein expression are brought about by differences on the DNA level, or by downstream mechanisms that regulate gene expression, such as transcription factors activated by glucocorticoids. Proteins that are found to differ in the adult could then be investigated early in life or in the embryonic stage, in order to discover if these protein differences are the cause or effect of the stress reactivity phenotype.

The results reported here show that the SR mouse lines have good construct and face validity as a mouse model of affective disorders and can be used to further our understanding of the underlying mechanisms of the symptoms of these disorders as well as in the search for better treatment options for SZ and MD.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt habe.
Es wurden nur die in der Arbeit ausdrücklich benannten Quellen und Hilfsmittel benutzt.
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Diese Dissertation wurde von Dr. Chadi Touma betreut.

....

Ort, Datum Alana Knapman

Erklärung zum Eigenanteil bei Koautorenschaft

Manuskript I:
Frau Knapmans Anteil an der Arbeit bestand
-in der Konzeption, Planung und Durchfuehrung der Verhaltensexperimente,
-in der Auswertung der Verhaltensdaten,
-in der statistischen Analyse aller Befunde,
-im selbstaendigen Verfassen und Einreichen des Manuskripts,
-in der Aenderung des Manuskripts nach den Vorschlaegen von Fachgutachtern und im Wiedereinreichen des Manuskripts.

Manuskript II:
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-in der Auswertung der Daten,
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-in der Auswertung der Verhaltens- und Proteinexpressionsdaten,
-in der statistischen Analyse aller Befunde,
-im Verfassen des Manuskripts.

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Increased stress reactivity is associated with cognitive deficits and decreased hippocampal brain-derived neurotrophic factor in a mouse model of affective disorders

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ABSTRACT

Cognitive deficits are a common feature of major depression (MD), with largely unknown biological underpinnings. In addition to the affective and cognitive symptoms of MD, a dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis is commonly observed in these patients. Increased plasma glucocorticoid levels are known to render the hippocampus susceptible to neuronal damage. This structure is important for learning and memory, creating a potential link between HPA axis dysregulation and cognitive deficits in depression. In order to further elucidate how altered stress responsiveness may contribute to the etiology of MD, three mouse lines with high (HR), intermediate (IR), or low (LR) stress reactivity were generated by selective breeding.

The aim of the present study was to investigate whether increased stress reactivity is associated with deficits in hippocampus-dependent memory tests. To this end, we subjected mice from the HR, IR, and LR breeding lines to tests of recognition memory, spatial memory, and depression-like behavior. In addition, measurements of brain-derived neurotrophic factor (BDNF) in the hippocampus and plasma of these animals were conducted.

Our results demonstrate that HR mice exhibit hippocampus-dependent memory deficits along with decreased hippocampal, but not plasma, BDNF levels. Thus, the stress reactivity mouse lines are a promising animal model of the cognitive deficits in MD with the unique feature of a genetic predisposition for an altered HPA axis reactivity, which provides the opportunity to explore the progression of the symptoms of MD, predisposing genetic factors as well as new treatment strategies.

1. Introduction

Patients suffering from MD often complain of cognitive impairment, mainly memory impairment and executive functioning deficits (Austin et al., 2001; Porter et al., 2003; Reppermund et al., 2007). It has been reported that divided attention and verbal working memory deficits were present in the majority of subjects, both on admission and at discharge (Reppermund et al., 2007). Furthermore, low episodic memory performance in healthy subjects has been associated with an increased risk of developing depression, and can thus be seen as a premorbid marker of MD (Airaksinen et al., 2007).

There are also physiological correlates of MD, such as a dysregulation of the hypothalamus–pituitary–adrenal (HPA) axis. MD is generally associated with a hyperresponsive HPA axis (Holsboer, 2000; Ising et al., 2005). However, there are also depressed patients exhibiting a hypofunction of the hypothalamic corticotrophin-releasing hormone (CRH) system along with other symptoms characteristic of this subtype of MD (Angst et al., 2002; Antonijevic, 2006; Gold and Chrousos, 2002; Gold et al., 1995). In the context of cognitive deficits in MD, it is relevant to distinguish between the patients with a hypoactive and hyper-active HPA axis as cognitive dysfunction, in particular memory deficits, have been associated with hypercortisolemia in depressed patients (Belanoff et al., 2001; Bremner et al., 2004). The relationship between memory dysfunction and hypercortisolemia is presumably brought about by the negative influence of glucocorticoids on the hippocampus, a structure that is important for learning and memory (Eichenbaum, 2000; Sapolsky et al., 1990; Scoville and Milner, 1957). Repeated stress and chronically increased glucocorticoid levels have been reported to be associated with a reduction in hippocampal volume, dendritic atrophy in the CA3 region of the hippocampus and reduced birth of new granule cells in the dentate gyrus of the hippocampus (Lyons et al., 2007; McKittrick et al., 2000; Sapolsky et al., 1990; Sousa et al., 1999; Watanabe et al., 1992). Using *in vivo* imaging, hippocampal volume has been found to be decreased in patients suffering from MD (Bremner et al.,

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2000; Frodl et al., 2006; Holsboer and Ising 2010; MacQueen et al., 2003; Sheline et al., 1996) and in patients suffering from Cushing's syndrome, where hypercortisolemia is a cardinal symptom (Starkman et al., 1992).

Glucocorticoids have an acute effect on memory processing. Stress and glucocorticoids are believed to enhance memory consolidation, whereas increased glucocorticoid levels impair memory retrieval (Buchanan and Lovallo, 2001; de Quervain et al., 1998; Flood et al., 1978; Holsboer and Ising, 2010; Kovacs et al., 1977; Kuhlmann and Wolf, 2006; Oitzl et al., 1998; Roozendaal, 2000). The effects of stress on memory function are also dependent on the type of memory being studied (Luethi et al., 2008). Furthermore, studies using a standardized psychosocial stressor have confirmed a correlation between cortisol reactivity and memory impairment in healthy adults (Kirschbaum et al., 1996).

Moreover, studies on rodents have shown that glucocorticoid excess decreases the level of brain-derived neurotrophic factor (BDNF) in the hippocampus (Jacobsen and Mork, 2006; Murakami et al., 2005; Schaaf et al., 1998, 1997), which has been strongly implicated in the regulation of hippocampal long-term potentiation (LTP) (Chen et al., 1999; Korte et al., 1996; Patterson et al., 1996). Reduced hippocampal BDNF levels have furthermore been shown to impair memory performance in a number of animal studies (Gorski et al., 2003; Monteggia et al., 2004; Schaaf et al., 2001). Interestingly, BDNF levels are also reduced in the plasma of depressed patients and tend to increase in response to antidepressant treatment (Huang et al., 2008; Lee et al., 2007; Monteleone et al., 2008; Nibuya et al., 1996; Santarelli et al., 2003). It is believed that said increase in BDNF may play a key role in the effectiveness of the antidepressant treatment (Wang et al., 2008). However, the changes in brain BDNF concentration demonstrated in animal studies and the plasma BDNF concentrations of patients may merely reflect the treatment-induced decrease in plasma corticosterone/cortisol level (Greden et al., 1983; Holsboer et al., 1982; Ising et al., 2007). Furthermore, low plasma BDNF levels have been associated with memory impairment in MD (Grassi-Oliveira et al., 2008). However, it is important to note that plasma BDNF levels are not necessarily a reflection of brain BDNF levels.

The aim of the present study was to further the understanding of the relationship between HPA axis hyperresponsiveness or hyporesponsiveness and memory function. We approached this question by using a recently established animal model generated by selectively inbreeding mice for extremes in stress reactivity (Touma et al., 2008). A founder population of CD-1 mice was subjected to a standardized stressor (15 min restraint stress) and their HPA axis reactivity in response to this stressor was determined by subtracting the corticosterone level of the initial blood sample, taken before the stressor, from the corticosterone level of a blood sample taken immediately after the restraint stress. Males and females with very high stress reactivity (HR) were then mated, as were males and females with extremely low stress reactivity (LR). Their offspring were tested for their stress reactivity in the same manner and so forth for each generation to come (for details see (Touma et al., 2008)). An intermediate (IR) reactivity line was additionally established to serve as a control group with the same inbreeding status as the other two lines. The IR mice present a corticosterone response similar to the mean of the founder population of CD-1 mice (Touma et al., 2008).

These mouse lines have previously been characterized with respect to several endophenotypes relevant to MD. In summary, the HR mice display several characteristics found in patients suffering from melancholic depression, such as a hyperreactive HPA axis, a flattened circadian glucocorticoid rhythm, a hyper-active coping style, increased rapid eye movement sleep, and decreased body weight. On the other hand the LR mice display similarities to the atypical subtype of depression, such as a hyporeactive HPA axis, a more passive coping style, increased aggressive behavior and increased body weight (Touma et al., 2008, 2009). In order to investigate the cognitive abilities of this stress reactivity mouse model, we implemented a novel object recognition test to assess recognition memory and a Y-maze free choice exploration paradigm to assess spatial learning (Dellu et al., 2000). Furthermore, coping behavior was assessed using the forced swim test (FST). When the behavioral testing was completed the brains were collected and the amount of BDNF in the hippocampus was quantified.

The great advantage of this mouse model over others investigating the role of the HPA axis in depression is that it allows for the investigation of two pathological states i.e. an extremely high HPA axis reactivity and an extremely low HPA axis reactivity. In addition, it is possible to apply a top down approach and go from a known endophenotype to investigate the genotype. Another unique advantage is that knowing that the animals from a specific line will develop a certain phenotype enables the possibility to study the progression of the symptoms of the disease.

2. Methods

2.1. Subjects

Animals derived from the eighth generation of the stress reactivity (SR) mouse model (for details see (Touma et al., 2008)) were used in these experiments. Twelve male and 12 female mice from the HR, IR, and LR breeding lines, were selected according to their corticosterone increase in the stress reactivity test (SRT) (described below). In order to control for litter effects the mice were selected from a minimum of six different litters per line. During the behavioral testing period, animals were single housed in transparent polycarbonate cages (standard macrolon cages type II, $26 \times 20 \times 14$ cm) with food and water available *ad libitum*. A 12:12 h light–dark cycle with lights on at 8 a.m. was maintained throughout breeding and testing. The housing rooms and experimental rooms were kept at a constant temperature (22 ± 1 °C) and humidity ($55 \pm 10\%$). Animals were between 3 and 5 months of age during the period of behavioral testing. All experiments were performed during the trough of the circadian rhythm of glucocorticoid secretion (between 9 and 12 a.m.) and the order of testing was counterbalanced across the different breeding lines.

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

2.2. Stress reactivity test and plasma corticosterone measurements

At the age of approximately 8 weeks, the mice were subjected to a stress reactivity test (SRT) in order to assess the reactivity of their hypothalamic–pituitary–adrenal (HPA) axis. The SRT is described in detail elsewhere (Touma et al., 2008). Briefly, an initial blood sample for corticosterone measurement was collected from a small incision in the ventral tail vessel. The mouse was then placed in a restraint tube for 15 min. Following the restraint stress, a second blood sample, the reaction sample, was obtained from a second incision in the tail vessel. The corticosterone increase in response to this stressor was calculated by subtracting the corticosterone concentration in the initial sample from the corticosterone concentration in the reaction sample.

Plasma corticosterone concentrations were determined using a commercial radioimmunoassay (RIA) kit (MP Biomedicals, Solon, Ohio, USA), with slight modifications to the manufacturer's

instructions (for details see (Touma et al., 2008)). Inter- and intra-assay coefficients of variation were both below 10%.

2.3. Y-maze Test

The Y-maze was used to assess spatial memory (Dellu et al., 2000). The apparatus used for this test was Y-shaped and constructed from grey plastic. It consisted of three 11 cm wide and 30 cm long arms with 15 cm high walls at equal distance from each other, connected by a central zone. The spatial orientation of the mice was facilitated by each individual arm bearing a different symbol on the walls in the shape of a bar, a plus or a triangle.

During the acquisition phase one arm was blocked by a plastic partition wall. The mouse was placed in the central area of the maze facing the corner connecting the two accessible arms to ensure that the animal was not influenced to choose a specific arm due to the direction in which it was facing when placed in the maze. The animal was then allowed to explore the maze for 10 min. The maze was cleaned with soapy water and alcohol and thoroughly dried between test animals. After an inter-trial interval (ITI) of 60 min, where the mouse was returned to its home cage, the mouse was placed back in the maze, this time with the opportunity to explore all three arms. This retrieval phase lasted for 5 min.

If the mouse is able to discriminate between the novel and familiar arms, a preference towards exploring the arm that was blocked during the acquisition phase, i.e. the novel arm, will be reflected in an increased amount of time spent in this arm. The animals' movements were tracked using ANY-maze software (ANY-maze, Stoelting Co., IL, USA). To eliminate the effect of differences in locomotion between the lines, the mean percent time spent in the familiar arms was compared to the percent time spent in the novel arm. If the animals spent significantly more time in the novel arm compared to the mean of the familiar arms, they were assumed to have remembered the arms they had previously been allowed to explore.

2.4. Novel object recognition test

The novel object recognition (NOR) test assesses the animal's visual and tactile recognition memory and takes advantage of rodents innate behavior to spend more time investigating a novel object than one previously encountered (reviewed in (Eichenbaum et al., 2007)). The NOR test was carried out in black plastic testing cages measuring 21.5 × 38 × 35 cm, covered with a thin layer of sawdust. Animals were habituated to the test cage for 1 h preceding the acquisition phase. During the acquisition phase of the test, mice were presented with an object and allowed to explore it for the duration of 5 min. The retrieval phase consisted of a copy of the familiar object, the one that was presented during the acquisition phase, being presented together with a novel object. The mice were then allowed to explore both objects for 5 min. During the inter-trial interval (ITI) between the acquisition phase and the test phase, mice were left undisturbed in the testing cages.

Each animal was tested with three different ITIs; 30, 60, and 120 min, with 2 days between test days. Three pairs of objects were used for the test (Supplementary Fig. 1). All objects were of similar size and constructed from Lego blocks (LEGO®, Lego Group, Bilund Denmark). A different group of mice were used to verify that there was no innate preference or aversion to any of the chosen objects (data not shown). Any individual mouse was only presented with a certain pair of objects on one occasion. Which object was the novel object and which was the familiar object as well as the use of the different pairs was counterbalanced across the groups and ITIs.

The amount of time spent exploring each of the objects was videotaped and subsequently manually scored by a trained observer blind to the animals' experimental groups. Exploration was defined as the animal having a proximity to the object of no more than one centimeter with its head facing the object. Animals with an exploration time below 10 s during the acquisition phase or retrieval phase were excluded from the analysis. The animals were assumed to have remembered the familiar object if significantly more time was spent exploring the novel object.

2.5. Forced swim test

In order to assess coping strategies in aversive situations, the forced swim test (FST) was used (Porsolt et al., 1977). Each mouse was placed in a glass cylinder (12 cm in diameter and 24 cm high) filled to two thirds with 23 °C water for 6 min. All sessions were video recorded, and the animals' behavior was subsequently scored by a trained observer to determine the amount of time spent swimming, floating and struggling. For precise behavioral definitions (see Touma et al., 2008). In this test, an increased amount of immobility is classically regarded as behavioral despair, which can be interpreted as depression-like behavior.

2.6. Brain collection

The brains and plasma samples of all experimental animals were collected 4 weeks after the last behavioral test. Animals were briefly anesthetized with isoflurane before decapitation. The brains were quickly removed and cut in half separating the left and right hemisphere, in order to collect the hippocampus. Due to the possibility of lateralization, the left hippocampus was dissected from half of the animals from each group and the right hippocampus from the other half. All samples were stored at −80 °C until they were further processed.

2.7. Determination of BDNF protein

The content of endogenous BDNF was quantified in the plasma and hippocampal samples via an enzyme-linked immunosorbent assay (ELISA) using commercially available antibodies (Promega, Madison, WI, USA). The procedure is described in detail elsewhere (Hellweg et al., 2006, 2003). One IR male died before brain collection and one sample per line out of the males had too small amounts of tissue to be considered a valid measurement. Within the females, two samples from the IR line were excluded due to the same reason.

2.8. Statistical analysis

Non-parametric statistics were used to analyze all behavioral tests as the data was not normally distributed in all cases. Differences between more than two independent samples were compared using the Kruskal–Wallis H-test (KWH-test). When significance was reached, *post-hoc* comparisons between two independent groups were performed using Mann–Whitney U-tests (MWU-test). Bonferroni correction was applied to correct for repeated testing. In order to compare two dependent samples, the Wilcoxon-test (W-test) was applied. Corticosterone and BDNF measurements were analyzed using parametric statistics, as these data sets were found to be normally distributed. In order to compare differences between more than two independent samples, one factor analysis of variance (ANOVA) was followed by Tukey's *post-hoc* test. The association between the corticosterone increase in the SRT and hippocampal BDNF levels was analyzed using Pearson's correlation coefficient.

All statistical analyses were performed using SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA) and statistical significance was set at $p < 0.05$, however, $p < 0.1$ was considered a trend. Two-tailed p-values were reported in all cases except for the Y-maze test and the object recognition test, in which the outcome was predicted with a one-sided hypothesis, making the use of a one-tailed p-value appropriate.

3. Results

3.1. Stress reactivity test

Highly significant differences in the restraint stress-induced corticosterone increase were present between the three lines in males and females (males: $F_{(2,33)} = 112.0$, $p < 0.001$; females: $F_{(2,33)} = 304.5$, $p < 0.001$; Tukey's *post-hoc*; males: all $p < 0.001$; females: all $p < 0.001$; Fig. 1). This is in accordance with findings in previous generations of the stress reactivity mouse model (Touma et al., 2008). The three lines furthermore differed in their corticosterone levels in the initial blood sample taken in the SRT, with the highest levels found in the HR mice and the lowest levels in the LR mice (mean ± SEM: males: HR: 6.1 ± 2.2 ng/ml, IR: 4.7 ± 1.7 ng/ml, LR: 1.5 ± 0.2 ng/ml; females: HR: 41.6 ± 8.7 ng/ml, IR: 30.7 ± 4.3 ng/ml, LR: 9.7 ± 2.7 ng/ml). These effects were statistically significant (3(line) × 2(sex) ANOVA, $F_{2,66\ \text{line}} = 9.434$, $p < 0.001$, $F_{1,66\ \text{sex}} = 44.279$, $p < 0.001$, $F_{2,66\ \text{line} \times \text{sex}} = 5.232$, $p = 0.008$; Tukey's *post-hoc;* males: all $p > 0.1$; females: HR vs. LR: $p < 0.01$, HR vs. IR: $p < 0.1$, IR vs. LR: $p < 0.05$). This is in accordance with findings in previous generations and can be attributed to the increased trough levels of the circadian rhythm of glucocorticoid secretion observed in HR mice (Touma et al., 2008, 2009).

3.2. Y-maze test

Male LR mice displayed a superior performance in the Y-maze spatial learning test compared to both IR and HR mice. After the 1 h inter-trial interval (ITI), only the LR mice spent a significantly larger percent time exploring the novel arm than the mean of the familiar arms, demonstrating a recognition of the previously explored familiar arms (W-test, $Z = -2.20$, $p < 0.05$; Fig. 2). Male IR mice showed a tendency toward spending a larger percent of their time exploring the novel arm compared to the familiar arms, but statistical significance only reached trend values (W-test, $Z = -1.67$, $p < 0.1$; Fig. 2). Male HR mice were not able to distinguish between the novel and familiar arms (W-test, $Z = -0.86$, $p > 0.1$; Fig. 2).

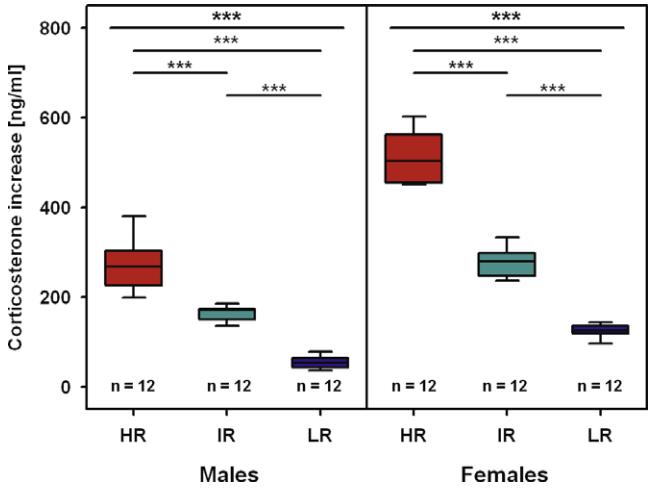

Fig. 1. Corticosterone increase in response to 15 min restraint in male and female mice selectively bred for high (HR), intermediate (IR), and low (LR) stress reactivity. The corticosterone increase differs significantly between the three lines in both male and female mice (ANOVA followed by Tukey's *post-hoc* test, all $p < 0.001^{***}$). Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers.

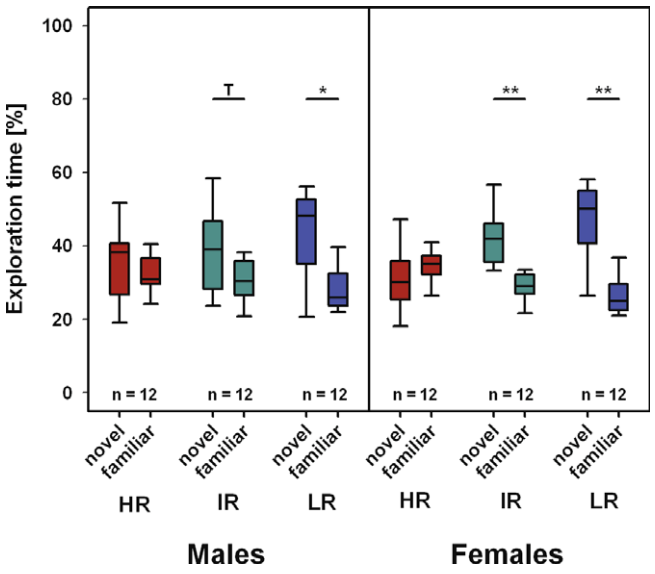

Fig. 2. Percent time spent exploring the novel arm compared to the mean of the two familiar arms in the Y-maze spatial learning task in male and female mice selectively bred for high (HR), intermediate (IR), and low (LR) stress reactivity. Significantly more time spent exploring the novel arm indicates that the animal remembered the familiar arm (W-test, $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.1$T). Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers.

In accordance with the results of the males, female LR and IR mice were able to distinguish between the novel and familiar arms of the maze and spent significantly more time in the novel arm compared to the mean of the familiar arms (W-test, IR: $Z = -2.98$, $p < 0.01$; LR: $Z = -2.75$, $p < 0.01$; Fig. 2), whereas HR mice were not able to do so (W-test, $Z = -0.94$, $p > 0.1$; Fig. 2).

3.3. Novel object recognition test

After an inter-trial interval (ITI) of 30 min, only the LR mice of both sexes were able to distinguish between the novel and the familiar object, demonstrated by them spending significantly more time exploring the novel object than the familiar object (W-test, males: $Z = -1.80$, $p < 0.05$ and females: $Z = -2.00$, $p < 0.05$; Fig. 3A). Female IR mice tended to explore the novel object more than the familiar one, but statistical significance only reached trend values (W-test, $Z = -1.80$, $p < 0.1$; Fig. 3A). None of the groups were able to distinguish between the novel and the familiar object after an ITI of 1 h (Fig. 3B). However, after an ITI of 2 h the male LR and IR mice were able to remember the familiar object and spent significantly more time exploring the novel object (W-test, IR: $Z = -1.84$, $p < 0.05$; LR: $Z = -1.96$, $p < 0.05$; Fig. 3C).

The overall mean exploration times of males and females were relatively high in comparison to many other studies using CD-1 mice or different inbred strains. Males explored on average 77 s and females 53 s during the acquisition and retrieval phase. Absolute exploration times during the acquisition phase or the retrieval phase did not differ significantly between the three lines in males or females for any ITI. In males and females each ITI was compared separately (KWH-test, $H = 0.1–5.2$, df = 2, all $p > 0.05$). In males, during the retrieval phases corresponding to the three different

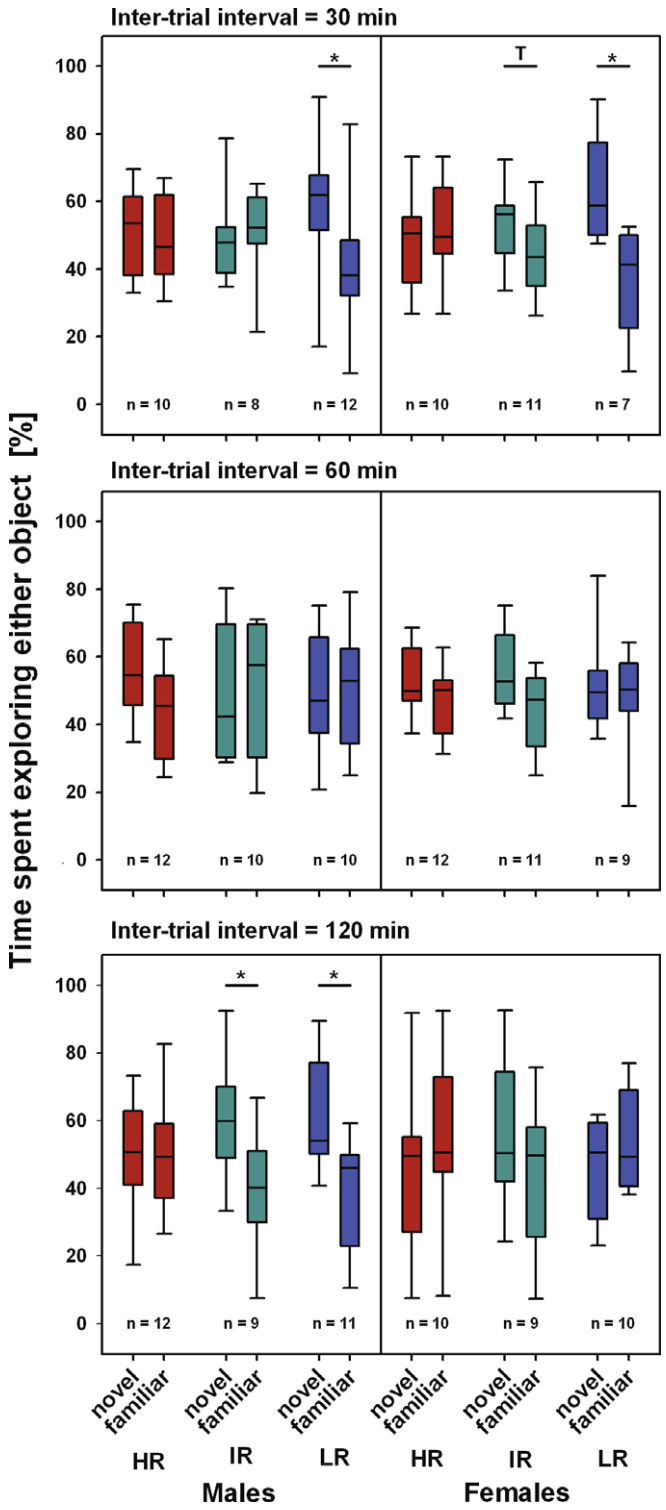

Fig. 3. Time spent exploring a novel vs. a familiar object in male and female high (HR), intermediate (IR), and low (LR) stress reactivity mice. Significantly more time spent exploring the novel object is interpreted as recalling the familiar object (W-test, $p < 0.05^{*}$, $p < 0.1$T). Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers.

ITIs, the mean total exploration times ranged between 72–89 s, 84–124 s and 56–78 s for HR, IR, and LR mice, respectively. In females, the mean exploration times during the retrieval phases were slightly lower ranging from 49–66 s, 63–66 s to 48–75 s for HR, IR, and LR mice, respectively.

3.4. Forced swim test

Both male and female HR mice spent significantly less time immobile in the FST than IR or LR mice (KWH-test, males: $H = 15.6$, df = 2, $p < 0.001$; females: $H = 11.2$, df = 2, $p < 0.01$; *post-hoc* MWU-tests, males: $U = 10$–17, HR vs. IR: $p < 0.05$, HR vs. LR: $p < 0.001$; females: $U = 19$–30, HR vs. IR: $p < 0.05$, HR vs. LR: $p < 0.001$; Fig. 4A). Male HR mice also exhibited significantly more struggling behavior than IR or LR mice (KWH-test, $H = 13.4$, df = 2, $p < 0.01$; *post-hoc* MWU-tests, $U = 12$–20, HR vs. IR: $p < 0.05$, HR vs. LR $p < 0.01$; Fig. 4B). Differences in struggling times between the three lines in the females only reached trend values (KWH-test, $H = 5.4$, df = 2, $p < 0.1$), but the direction of the results resembled that of the males. Male mice did not differ significantly between the groups in the amount of time spent swimming. However, female HR mice spent significantly more time swimming than female LR mice (KWH-test, $H = 6.6$, df = 2, $p < 0.05$; *post-hoc* MWU-tests, HR vs. LR: $U = 29$, $p < 0.05$).

3.5. BDNF measurements

BDNF content in the hippocampus of HR, IR, and LR mice differed significantly between the three lines and between the sexes,

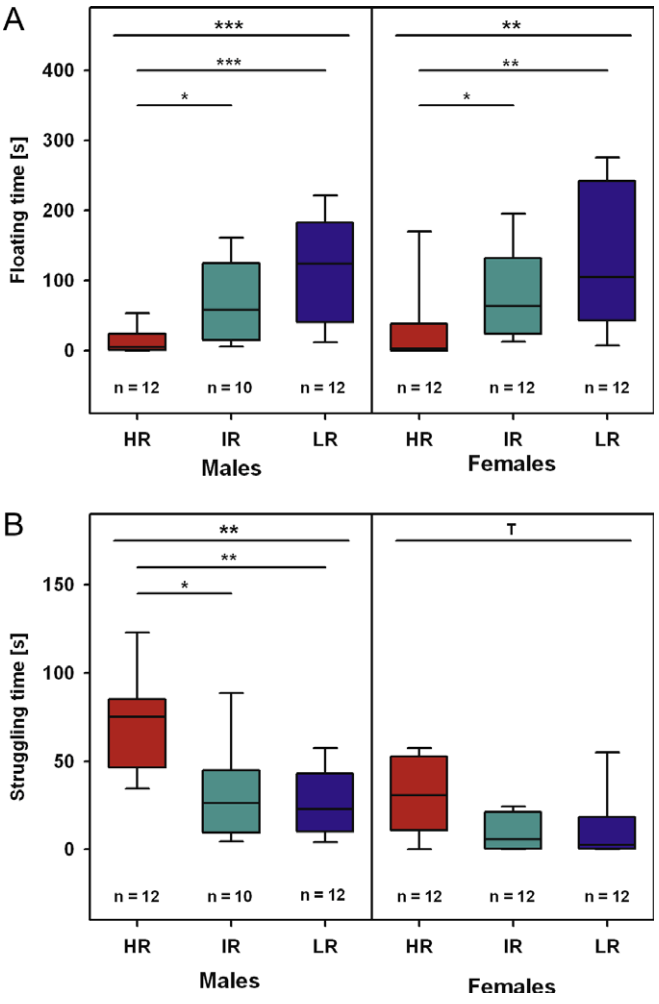

Fig. 4. Amount of time spent floating (A) and struggling (B) in the forced swim test in mice selectively bred for high (HR), intermediate (IR), and low (LR) stress reactivity (KWH-test, *post-hoc* MWU-tests; $p < 0.1$T, $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.001^{***}$). Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers.

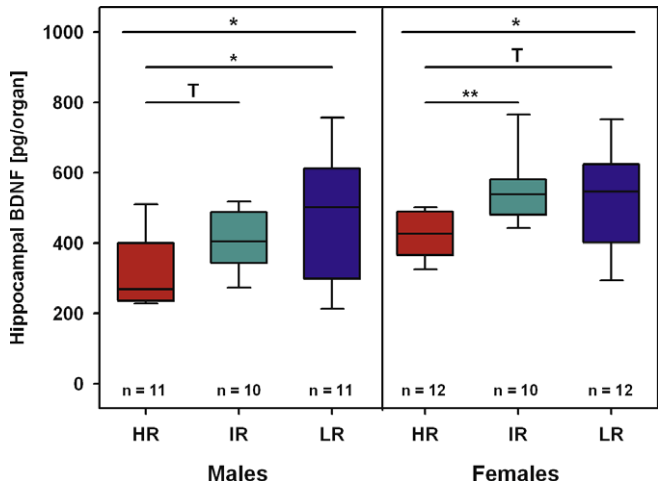

Fig. 5. Hippocampal brain-derived neurotrophic factor (BDNF) levels in male and female mice with high (HR), intermediate (IR), and low (LR) stress reactivity (ANOVA, Tukey's *post-hoc* test; $p < 0.1$ T, $p < 0.05$ *, $p < 0.01$ **). Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers.

but there was no significant interaction between line and sex (3(line) × 2(sex) ANOVA, $F_{2,60\ \text{line}} = 8.313$, $p = 0.001$, $F_{1,60\ \text{sex}} = 10.728$, $p = 0.002$, $F_{2,60\ \text{line} \times \text{sex}} = 0.892$, $p = 0.415$). In both males and females LR mice express the highest levels of BDNF and HR mice express the lowest levels (Fig. 5). *Post-hoc* analysis revealed that male HR mice express significantly lower levels of BDNF than LR mice, whereas there was only a tendency towards lower levels in comparison to IR mice (HR vs. IR: $p < 0.1$, HR vs. LR: $p < 0.05$; Fig. 5). Female HR mice also expressed significantly less BDNF in the hippocampus than IR mice and had a tendency towards lower levels compared to LR mice (HR vs. IR: $p < 0.05$, HR vs. LR: $p < 0.1$; Fig. 5).

Plasma BDNF levels, however, did not differ significantly between the three lines, nor did they differ between the sexes (mean ± SEM: HR: 42.6 ± 7.1 pg/ml, IR: 39.3 ± 6.3 pg/ml, LR: 26.9 ± 2.5 pg/ml; 3(line) × 2(sex) ANOVA, $F_{2,64\ \text{line}} = 2.167$, $p = 0.123$, $F_{1,64\ \text{sex}} = 0.103$, $p = 0.750$, $F_{2,64\ \text{line} \times \text{sex}} = 1.55$, $p = 0.321$).

In order to evaluate if corticosterone increase in the SRT is associated with hippocampal BDNF levels, covariance between the two factors were analyzed using Pearson's correlation coefficient. This revealed a significant negative correlation between corticosterone increase in the SRT and hippocampal BDNF levels in both males and females (males: $r = -0.53$, $p < 0.01$; females: $r = -0.37$, $p < 0.05$; Fig. 6).

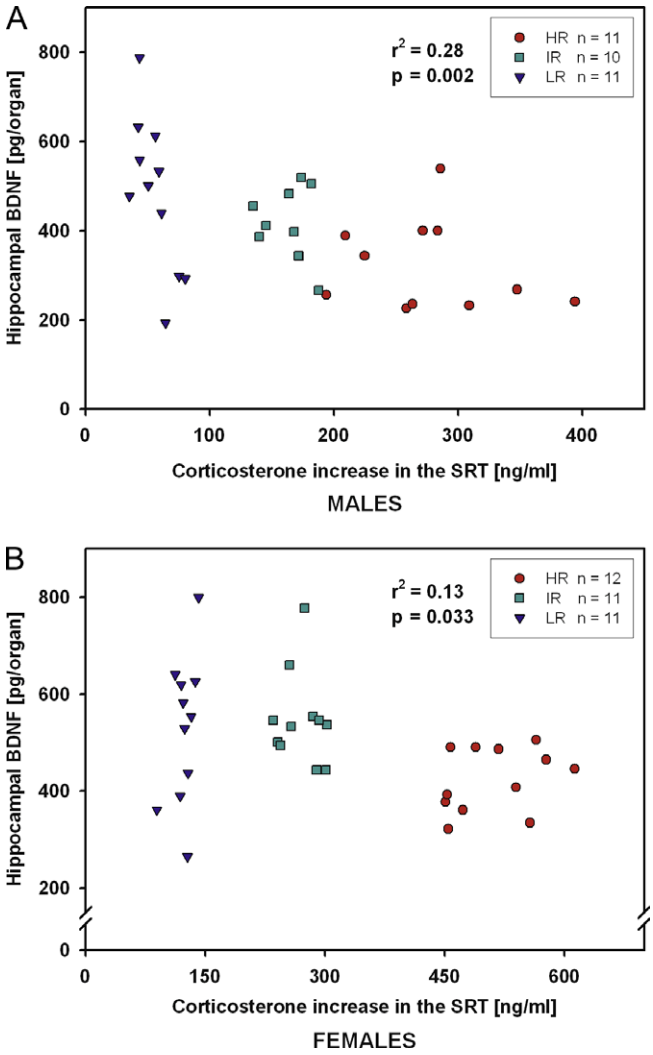

Fig. 6. Association between corticosterone increase in the stress reactivity test (SRT) and brain-derived neurotrophic factor (BDNF) content in the hippocampus in males (A) and females (B) selectively bred for high (HR), intermediate (IR), and low (LR) stress reactivity (Pearson's correlation coefficient).

4. Discussion

In this study we have demonstrated that highly (HR) stress reactive mice suffer from object memory impairment and spatial memory impairment, whereas mice with a low (LR) responsiveness to stress have a superior memory compared to intermediate (IR) stress reactive mice. These results are paralleled by findings of decreased hippocampal BDNF contents in HR mice and increased hippocampal BDNF contents in LR mice in comparison to IR animals. Taken together, these results demonstrate that HR mice display cognitive deficits, which could be explained by decreased BDNF levels in the hippocampus. Furthermore, the HR/IR/LR mouse lines differ significantly in their coping strategies in the forced swim test (FST).

According to our hypothesis, HR mice have an inferior performance in memory tests due to chronic exposure to corticosteroids. We propose that this is due to the fact that on any event that elicits a physiological stress response such as handling, cage changing or unavoidable noise in the animal housing facility, the hippocampus of an HR mouse would cumulatively be subjected to larger amounts of glucocorticoids than that of an IR or LR mouse. This in turn could lead to long-term functional impairment of the hippocampal neurons in the HR mice.

The inferior memory of the HR mice is demonstrated by the results of the Y-maze test. This is an intervalic recognition test, using a similar approach to the procedure used in human memory studies (Dellu et al., 1992). A study using hippocampally lesioned mice has shown that novelty preference in the Y-maze is hippocampus-dependent (Sanderson et al., 2009). Neither male nor female HR mice were able to successfully distinguish between novel and familiar arms in this spatial learning test, whereas LR males and females were able to do so (see Fig. 2). IR females were also able to successfully discriminate between the novel and familiar arms of the Y-maze, whereas IR males only showed a tendency towards spending more time in the novel arm during the retrieval phase.

The results of the novel object recognition (NOR) test are in accordance with the results of the Y-maze test, with HR mice exhibiting memory deficits and LR mice showing evidence of a superior memory performance (see Fig. 3). It is, however,

important to note that chronic effects of glucocorticoids on learning and memory can be masked by acute effects, if the testing situation itself is stressful. In such cases, the acute stress response could interfere with memory retrieval. Although being subjected to a novel environment and introduced to new objects is not a severe stressor *per se*, it will, however, lead to a state of arousal in the animal, which in turn will activate the HPA axis (Okuda et al., 2004). Furthermore, it has been shown that the acquisition phase of the NOR test leads to an increase in corticosterone well above baseline levels (Okuda et al., 2004; Palchykova et al., 2006). This is apparent in the case of the 1 h inter-trial interval (ITI) of the NOR test, where male LR mice failed to distinguish between the two objects, although they showed a preference toward the novel object with a 1/2 h ITI and a 2 h ITI. This finding is in concordance with the duration and peak of the corticosterone stress response of these mouse lines as measured by microdialysis in the hippocampus (Heinzmann et al., in preparation), which indicates that the retrieval phase of the 1 h ITI coincides with the peak of the stress response, with glucocorticoids presumably reaching levels that interfere with memory retrieval. The male IR mice were similarly unable to distinguish between the two objects after 1 h, but were able to do so after 2 h. Furthermore, the IR mice were unable to distinguish between the two objects after 1/2 h, which could be accounted for by the greater rise in glucocorticoid levels in IR mice compared to LR mice. However, this acute effect does not confound the entire experiment, as by the retrieval phase of the 2 h ITI all lines should be back to baseline corticosterone levels, meaning that the inability of the HR mice to distinguish between a novel and a familiar object presumably does not reflect the acute effects of stress, but is more likely to reflect a chronic exposure to increased levels of glucocorticoids.

None of the female mice were able to distinguish between the novel and the familiar object after one or 2 h, whereas both IR and LR male mice were able to do so after 2 h (see Fig. 3). This is in contradiction to most other findings, where male and female mice have been shown to perform the test equally well (Bredy et al., 2004; Chen et al., 2004; Clinton et al., 2007; Tordera et al., 2007; Villasana et al., 2006). However, most of these studies used C57BL/6 or transgenic mice on a mixed background. This could account for the differences between these results reported here and the results of other research groups. Furthermore, the length of ITIs used vary markedly between the studies with many experiments using ITIs of 15 min or less and others using ITIs of 1 and 24 h, but not investigating the time periods in-between. This does not coincide with the time frame of the ITIs used in our experiments, which could further explain the discrepancy between the results.

The NOR test and the Y-maze test were designed in every conceivable way to not be aversive or stressful to the animals. The tests were performed under low lighting conditions and the animals were habituated to the test setting when possible. It is, however, not possible to design a test that does not elicit any stress or arousal in the animals as this will occur as soon as they are removed from their home cage. Therefore, we can't exclude the possibility that acute effects of the stress response are contributing to the cognitive deficits we observe in the HR mice. A decrease in BDNF in the hippocampus of the HR mice however does support the notion that there are changes in the hippocampus of these animals making an acute effect of glucocorticoids less plausible than a chronic effect. Increased basal corticosterone levels in the HR mice (Touma et al., 2008, 2009) may also contribute to the cognitive deficits observed in the HR mice.

Interestingly, it has been shown that cognitive deficits in depression often remain when patients have been alleviated from their psychopathological and neuropsychological symptoms (Reppermund et al., 2009, 2007), although their cortisol response to the dexamethasone/corticotrophin-releasing hormone test has decreased at remission (Reppermund et al., 2007). A dissociation between HPA system dysregulation and its impact on cognitive function in depressed patients has therefore been postulated (Reppermund et al., 2007). This supports the idea that depressed patients' cognitive deficits are not due to an acute effect of an increased HPA axis reactivity. It would therefore be interesting to normalize the reactivity of the HPA axis reactivity of the HR mice and examine if the cognitive deficits remain. To this end, experiments using a CRH-R1 antagonist are currently underway.

Although MD is more commonly associated with a hyper-active HPA axis, MD is a heterogeneous disease, and one must consider the different endophenotypes when attempting to model this disorder in animals. Indeed, the atypical subtype of depression is associated with a hypoactive HPA axis and a more lethargic phenotype in comparison to melancholic or psychotic depression, which is associated with a hyper-active HPA axis, agitation and hyperarousal (Angst et al., 2002; Antonijevic, 2006; Gold and Chrousos, 2002; Hasler et al., 2004; Nierenberg et al., 1998). It is furthermore of vital importance to consider this when interpreting data of tests attempting to model depression-like behavior in animals. The forced swim test (FST) is a test classically used in rodents to screen for antidepressive effects of substances (Cryan and Holmes, 2005), it should however be stressed that the FST is not a test of depression, but rather gives an indication of the animal's coping strategy in an aversive situation. In this context, it is important to take into account that different subtypes of depression would be characterized by different coping styles. A model of the spectrum of depression represented by a more agitated and hyperaroused state would be expected to struggle more and float less than the control group, whereas a model of the atypical subtype of depression would here be represented by a less active coping style, i.e. less struggling and more floating compared to the control group. In accordance with this, LR mice spent significantly more time floating than the HR mice, indicating a more passive coping style and increased behavioral despair, whereas the HR mice exhibit decreased floating in comparison to the IR and LR mice in both males and females (Fig. 4A). These results are in concordance with previous findings from studies of these mouse lines (Touma et al., 2008).

Examining the two pathological states, hyper-active or hypoactive HPA axis in comparison to a control group of mice with a "normal" HPA axis response enables us to attempt to model both these subtypes of MD and consider them separately in regard to endopheonotypes that differ between patients suffering from different subtypes of MD, such as cognitive abilities and coping styles. The LR mouse line therefore could model this passive phenotype in association with a hypoactive HPA axis in similarity to atypical depression, whereas the HR mice are characterized by a highly increased HPA axis reactivity and a hyper-active coping style in similarity to the melancholic subtype of depression. Furthermore, memory deficits have been associated with hypercortisolemia in depressed patients (Belanoff et al., 2001; Bremner et al., 2004). Most studies of cognitive deficits in depression only differentiate between unipolar and bipolar depression. However, when subtypes of depression such as atypical and melancholic are considered, the cognitive deficits are predominantly found in patients suffering from the melancholic subtype of depression (Exner et al., 2009), which also coincides with our findings that the HR mice have cognitive deficits. Previous studies of the stress reactivity mouse model have also demonstrated alterations in the emotional behavior, neuroendocrine phenotype and sleep architecture of the HR and LR mice revealing further similarities to these different subtypes of depression (Touma et al., 2008, 2009).

Increased stress reactivity is presumably linked to cognitive deficits via up- or down-regulation of genes involved in learning and memory processes. BDNF is a prime candidate for investigation

in this context, as it has repeatedly been demonstrated to be involved in learning and memory processes, and glucocorticoids regulate BDNF synthesis in the hippocampus (Chen et al., 1999; Egan et al., 2003; Jacobsen and Mork, 2006; Korte et al., 1996; Lindholm et al., 1994; Patterson et al., 1996). As predicted, HR mice of both sexes had reduced amounts of BDNF content in the hippocampus in comparison to the other lines (see Fig. 5). Furthermore, the corticosterone increase in response to the SRT was negatively correlated to hippocampal BDNF across all three lines (see Fig. 6). Albeit this is a relatively weak correlation and to prove a causal relationship between the cognitive deficits described in the HR mice and the decreased BDNF levels further experiments would have to be performed.

There are a myriad of ways that decreased BDNF levels in the hippocampus could lead to cognitive deficits. BDNF influences neuronal excitability and has been implicated in synaptic plasticity events such as LTP (Figurov et al., 1996; Patterson et al., 1996). Moreover, BDNF increases neuronal survival (Kubo et al., 1995; Sossin and Barker, 2007). The functional integrity of the neurons may also be influenced by BDNF, as a single nucleotide polymorphism (SNP) in the BDNF gene, that leads to reduced intracellular trafficking and secretion of BDNF, has been reported to be associated with decreased hippocampal N-acetylaspartate levels, a measure of neuronal integrity in humans (Egan et al., 2003; Stern et al., 2008). The mechanism by which BDNF influences cognitive abilities in the stress reactivity mouse model, therefore, needs to be further investigated.

BDNF has also been implicated in the pathophysiology of MD. Depressed patients reportedly have lower plasma levels of BDNF, which has been associated with memory impairment (Grassi-Oliveira et al., 2008; Lee et al., 2007; Monteleone et al., 2008). It is, however, unclear whether there is an association between hippocampal BDNF levels and plasma BDNF levels in humans. A correlation between serum and cortical BDNF has been reported in rats (Karege et al., 2002). However, our findings indicate that there is no such association, as the BDNF differences observed in the hippocampus are not found in the plasma of our three mouse lines. This discrepancy could be explained by the multitude of factors able to regulate BDNF transcription and release. A SNP affecting the levels of BDNF would likely be found in both the neurons and platelets, the main source of BDNF in the blood, leading to a similar expression of BDNF in the plasma and the brain. On the other hand, if BDNF alterations were due to local hormone regulation, as we are assuming is the case here, the plasma BDNF levels need not reflect the brain BDNF levels. In MD patients, antidepressant treatment has furthermore been shown to increase BDNF levels and is suggested to be an important mechanism by which antidepressants alleviate affective symptoms (Huang et al., 2008). The stress reactivity mouse model could therefore be used to test new pharmaceutical compounds' abilities to increase BDNF levels and reverse affective and cognitive deficits.

All the experiments in this study were conducted in male and female mice revealing similar findings in both sexes. We would like to emphasize the importance of also using female mice when attempting to model symptoms of depression, as women have a higher lifetime prevalence of depression than men, even though the estrous cycle of females may complicate the interpretation of the results. Due to the fact that monitoring the estrous cycle in mice is relatively stressful, we assumed that the number of females in estrus and proestrus would be counterbalanced across the three lines as it is highly unlikely that their estrous cycles would be synchronized as the females were single housed.

In this study we have investigated the cognitive abilities of mice with both hyperreactive and hyporeactive HPA axes, as a dysregulation of the HPA axis in either direction is relevant to depression. The stress reactivity mouse model furthermore has the advantage over other mouse models of depression featuring stress or glucocorticoid exposure that it simulates the situation where the organism would be genetically predisposed to react in a detrimental way to an environmental stressor. This allows us to investigate the progression of the symptoms of the disease as well as predisposing genetic and environmental factors in our future research. The results provided here, indicating a relationship between altered stress reactivity, cognitive abilities, coping strategies, and hippocampal BDNF levels are of significant importance and demonstrate that the stress reactivity mouse model can be utilized in the search for putative new drug targets and the testing of such compounds, as well as in furthering our knowledge of the underlying mechanisms of the cognitive deficits in MD.

Disclosure/conflict of interest

A. Knapman, J.-M. Heinzmann, R. Hellweg, F. Holsboer, R. Landgraf and C. Touma report no conflict of interest associated with the content of this paper and have no financial interest regarding any of its content.

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Contributors

A. Knapman, C. Touma designed the experiments. A. Knapman, J.-M. Heinzmann, C. Touma and R. Hellweg performed the experiments. A. Knapman analyzed the data. F. Holsboer and R. Landgraf contributed materials/analysis tools. A. Knapman wrote the manuscript. All authors contributed to and have approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpsychires.2009.11.014.

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Modeling psychotic and cognitive symptoms of affective disorders: Disrupted latent inhibition and reversal learning deficits in highly stress reactive mice

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ABSTRACT

Increased stress reactivity has repeatedly been reported in patients suffering from psychiatric diseases including schizophrenia and major depression. These disorders also have other symptoms in common, such as cognitive deficits and psychotic-like behavior. We have therefore investigated if increased stress reactivity is associated with these phenotypic endpoints in an animal model of affective disorders. The stress reactivity mouse model used in this study consists of three CD-1-derived mouse lines, that have been selectively bred for high (HR), intermediate (IR) or low (LR) stress reactivity. Male mice from these three breeding lines were subjected to a reversal learning task and latent inhibition (Li) was assessed using a conditioned taste aversion paradigm. Furthermore, as the dopaminergic system is involved in both Li and reversal learning, the dopamine 1 receptor (D1R), dopamine 2 receptor (D2R) and dopamine transporter (DAT) mRNA expression levels were assessed in relevant brain areas of these animals. The results demonstrate that HR mice show perseveration in the reversal learning task and have disrupted Li. Furthermore, compared to LR mice, HR mice have decreased D2R mRNA levels in the ventral tegmental area, as well as decreased D1R mRNA levels in the cingulate cortex, and an increased expression of D2R mRNA in the nucleus accumbens. Taken together, these results demonstrate that the HR mice display cognitive deficits associated with psychotic-like behavior, similar to those observed in patients suffering from schizophrenia and major depression and could be utilized in the search for better treatment strategies for these symptoms of psychiatric disorders.

1. Introduction

Although many treatment strategies are available, major depression (MD) is not a fully treatable disorder (Holsboer & Ising, 2010; Wong & Licinio, 2001). This particularly applies to the symptoms in the cognitive realm that often subside when the affective symptoms of the disease have successfully been treated (Reppermund, Ising, Lucae, & Zihl, 2009; Reppermund et al., 2007). Typical antidepressants also lack the capacity to treat the psychotic symptoms of major MD (Holtzheimer & Nemeroff, 2006). It is therefore of great importance to better understand the mechanisms underlying these cognitive deficits and psychotic symptoms and to explore new potential targets for their treatment.

Drugs focusing on the dopaminergic system, such as atypical antipsychotics, have shown great promise in treating depressed patients who do not respond to typical antidepressants (Quintin & Thomas, 2004). Atypical antipsychotics are believed to exert their effect by increasing dopaminergic activity in the prefrontal cortex (PFC) implicating the dopaminergic system in these symptoms (Ichikawa, Li, Dai, & Meltzer, 2002; Kuroki, Meltzer, & Ichikawa, 1999; Rollema, Lu, Schmidt, & Zorn, 1997). The dopaminergic system subserves an optimal neuronal function in the PFC (Cropley, Fujita, Innis, & Nathan, 2006), which plays a key role in executive functioning tasks (Carpenter, Just, & Reichle, 2000; Petrides, 1994; Robbins & Arnsten, 2009). Fittingly, executive functioning tasks are the cognitive tasks that depressed patients and schizophrenic patients display the most deficits in (Austin, Mitchell, & Goodwin, 2001; Porter, Gallagher, Thompson, & Young, 2003; Rabin, Sacco, & George, 2009; Reppermund et al., 2007, 2009; Shirayama et al., 2009). Thus, it appears that psychotic MD and schizophrenia (SZ) share certain symptoms and possibly have similar underlying biological underpinnings causing these symptoms. It was therefore our aim to study these symptoms as phenotypic endpoint relevant to both disorders. The focus of this study was the dopaminergic system and the behaviors likely to be subserved by the dopaminergic system in the context of these symptoms.

Dopamine plays a key role in the regulation of latent inhibition (Li). Li is the phenomenon whereby pre-exposure to the to-be conditioned stimulus retards the learning of subsequent pairing of the unconditioned stimulus (US) and the conditioned stimulus (CS) (Lubow, 1973). Disrupted Li is strongly associated with an increased dopaminergic activity in the mesolimbic area (Lubow, 2005;

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Solomon & Staton, 1982; Weiner, 1990; Young, Joseph, & Gray, 1993) and is considered to be a model of the inability to ignore irrelevant stimuli associated with schizotypy (Baruch, Hemsley, & Gray, 1988; Guterman, Josiassen, Bashore, Johnson, & Lubow, 1996; Rascle et al., 2001; Schmidt-Hansen, Killcross, & Honey, 2009).

In addition to the affective and cognitive symptoms, dysregulation of the hypothalamus–pituitary–adrenal (HPA) axis is commonly observed in patients suffering from MD (de Kloet, Joels, & Holsboer, 2005; Holsboer, 2000; Holsboer & Ising, 2010; Ising et al., 2005), but has also been reported in patients suffering from SZ (Gallagher, Watson, Smith, Young, & Ferrier, 2007; Muck-Seler et al., 2004; Ritsner et al., 2007; Ryan, Sharifi, Condren, & Thakore, 2004). An animal model of affective disorders, the "stress reactivity" mouse model, was therefore established using a selective breeding approach to generate mice with high, intermediate or low stress reactivity (Touma et al., 2008). Briefly, a founder population of CD-1 mice was subjected to a standardized stressor (15-min restraint), and the increase of plasma corticosterone concentrations in response to this stressor was determined. Males and females with very high stress reactivity (HR) were then mated, as were males and females with very low stress reactivity (LR). Their offspring were tested for their stress reactivity in the same manner and so forth for each generation to come (for details see (Touma et al., 2008)). An intermediate reactivity (IR) line was additionally established to serve as a control group with the same inbreeding status as the other two lines. The IR mice present a corticosterone response similar to the mean of the founder population of CD-1 mice (Touma et al., 2008). In this study, these three mouse lines have been utilized to investigate the effect of a hyperactive vs. hypoactive HPA axis reactivity on the dopaminergic system as well as behaviors subserved by the dopaminergic system that are relevant to the psychotic and cognitive deficits observed in MD and SZ. Ultimately, we aim to provide a mouse model for cognitive deficits and psychotic symptoms that could be utilized in the search for better treatment options for these symptoms.

To this end, mice from the stress reactivity model were subjected to a reversal learning task, and Li was assessed in a conditioned taste aversion paradigm. The brains of these animals were subsequently analyzed via *in situ* hybridization to measure dopamine 1 receptor (D1R), dopamine receptor 2 (D2R) and dopamine transporter (DAT) mRNA levels in relevant brain areas.

2. Methods

2.1. Subjects

A total of 48 male mice were used in these experiments. The mice originated from the 11th generation of the stress reactivity mouse model. This mouse model consists of three CD-1-derived mouse lines, selectively bred for high (HR), intermediate (IR) or low (LR) stress reactivity, respectively (Touma et al., 2008). Stress reactivity was determined by the method described below. From each breeding line, HR, IR, and LR, 16 male mice were selected according to their plasma corticosterone increase in the SRT. Within each breeding line, two independent sub-lines, A and B, exist. These sub-lines were never interbred and serve as a replication of the breeding protocol conducted in parallel. The 16 mice of each group comprised of 8 mice from each sub-line. Two weeks prior to the onset of behavioral testing all animals were single housed under standard laboratory conditions in transparent polycarbonate cages (standard macrolon cages type II, $38 \times 22 \times 15$ cm) with food and water available *ad libitum*. Testing and housing rooms were maintained on a 12:12 h light–dark cycle with a constant temperature and humidity of 22 ± 1 °C and $55 \pm 10\%$, respectively. All behavioral tests and hormone measurements were conducted during the first 4 h of the light phase when the animals were still relatively active and corticosterone levels are at their circadian trough. The presented work complies with current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC). All experiments were announced to the appropriate local authority and were approved by the 'Animal Welfare Officer' of the Max Planck Institute of Psychiatry.

2.2. Stress reactivity test

All the mice used in these experiments were tested in the "stress reactivity test" (SRT) at the age of approximately eight weeks. The test comprises of an initial blood sample collected from a small incision in the ventral tail vessel, followed by 15 min of restraint stress and finally a reaction blood sample (for details see Touma et al., 2008), collected from a second incision in the ventral tail vessel immediately after the restraint stressor. Corticosterone levels in the blood plasma were analyzed as described below. The test was performed during the first hours of the light phase when corticosterone levels are at their trough level.

2.3. Plasma corticosterone measurements

A radioimmunoassay (RIA) kit (MP Biomedicals, Solon, Ohio, USA) was used with a slight modification to the manufacturer's instructions to determine corticosterone levels in the plasma samples. Only half of the recommended volumes were used for all components to increase the amount of samples that could be analyzed per kit. From the initial sample, 10 μl of plasma was diluted 1:13.5, and for the reaction sample, 10 μl of plasma was diluted 1:100. The difference in dilution ensures that the samples are within the linear part of the standard curve. Inter- and intra-assay coefficients of variation were both below 10%.

2.4. Reversal learning task

Reversal learning is a useful task to test the functional integrity of the PFC in patients and animal models of psychiatric diseases (Clark, Cools, & Robbins, 2004).The mice were tested in the reversal learning task at the age of approximately 16 weeks. A *T*-maze with three 50 cm long arms made out of Plexiglas, mounted on a table with the height of 50 cm was used for this test. The arms could individually be opened or closed via transparent Plexiglas pulley doors. The walls of the start arm were covered in black paper with diagonal white stripes; one of the goal arm's walls was white and the other black. At the end of one of the goal arms an escape tunnel made of chicken wire with a diameter of 5 cm was present leading back to the mouse's home cage which was placed on the floor to ensure that the mouse could neither smell nor see its cage from the center of the maze. At the end of the other arm a 'dummy' tunnel was present that consisted of an identical chicken wire tunnel with a dead end after 20 cm to ensure that the two arms looked identical from the center of the maze where the mouse made its choice of which arm to enter. The mouse was placed in a Plexiglas start box with a trap door. The start box was placed in the beginning of the start arm and the trap door was opened. When the mouse reached the end of the start arm and entered the center zone the pulley door to the start arm was closed so the mouse must choose one of the goal arms. When the mouse had selected one of the goal arms the door to that arm was closed. If the mouse entered the correct arm it was allowed to proceed to its home cage via the escape tunnel. If the incorrect arm was selected the mouse received a mildly aversive air-puff and was left in the arm for 30 s, and subsequently returned to its home cage. The mice were trained to find their home cage by turning left or right and selecting the correct goal arm. They were tested in batches of three (one mouse

from each breeding line) with an inter-trial interval of approximately 5 min. Which arm was the goal arm was counterbalanced across the groups. When the mouse had achieved to select the correct arm 8 out of 10 times, it had reached the learning criterion and was left undisturbed until the next day. As the inter-trial interval was comparatively short the mice were retested 24 h later to ensure that they still recalled where the correct arm was and to see if the groups differed in memory retention, as such differences would confound the subsequent reversal task. On the third day, 24 h after the retention trial, the goal arm with the escape tunnel was moved to the opposite arm and the mouse had to learn the new position of the arm. The learning criterion of eight out of ten correct trials was again applied. The number of trial to reach the learning criterion for each stage of the test (acquisition, retrieval and reversal) was recorded and subsequently used to statistically compare the three breeding lines. Twelve animals per line were tested in this paradigm. If an animal failed to reach the learning criterion by 30 trials or needed more trials to reach the learning criterion the second day (the retrieval phase) it was excluded from the analysis at all stages of the test. The final number of animals (*N*) for each group is presented along with the outcome of statistical analysis in Section 3.

2.5. Latent inhibition

Latent inhibition (Li) is the phenomenon whereby pre-exposure to the to-be conditioned stimulus retards the learning of subsequent pairing of the unconditioned stimulus (US) and the conditioned stimulus (CS) (Lubow, 1973). Li was assessed when the animals were approximately 21 weeks old. The mice were habituated to water deprivation over a period of 4 days to reduce the stress of water deprivation. During this period they were given the opportunity to drink from either of two water bottles, one placed on the left and one placed on the right side of the cage-lid, for 30 min twice daily with an interval of 4 h. By so means, we could deduce if any mouse had a preference for the right or the left bottle. Custom made non-drip water bottles were used and the bottles were weighed before and after each drinking session with a precision of 0.1 g to measure the amount of fluid consumed. On the fifth day, the pre-exposure day, the three groups of mice consisting of 16 HR, IR and LR mice, respectively, were split into two groups each, leading to six groups of eight mice. One group from each line, the pre-exposed (PE) group, was given a 10% sucrose solution in both bottles during the first 30-min drinking session. The other group, the non pre-exposed group (No PE), was given tap water as during the habituation period. On day six of the experiment, the conditioning day, all animals were given sucrose solution to drink, the conditioned stimulus (CS), and were injected with a volume of 2% of the body weight 0.25 M lithium chloride (LiCl) dissolved in 0.9% sodium chloride (NaCl) solution intraperitoneally, five minutes after the drinking session in order to induce nausea, the unconditioned stimulus (US). On the second drinking session of the day, all mice were again given tap water in both bottles. On day seven of the experiment, the test day, all animals were given the choice between tap water and sucrose solution. The stronger the CS–US pairing the less sucrose solution an animal will drink. A weaker CS–US pairing is to be expected in the PE group compared to the No PE group if the animals exhibit latent inhibition. In order to make this comparison, a ratio of how much water compared to sucrose was consumed was calculated ((water consumption – sucrose solution consumption)/total volume consumed) for each group. This ratio or discrimination index was then used to statistically compare the PE and No PE group within each breeding line. If an animal showed a side preference during the habituation period the bottle containing water was placed on that side in the PE group and the sucrose was placed on that side in the No PE group, so that any bias due to a side preference would be in an unfavorable direction making it unlikely to produce a false positive result due to side preferences of the animals.

2.6. Brain collection

The experimental animals were left undisturbed for three weeks after all behavioral tests were completed before the brain collection took place. The animals were approximately 25 weeks old at this time. Animals were briefly anaesthetized with isoflurane and then rapidly decapitated. The brain was removed from the scull and immediately frozen in n-methylbutane cooled on dry ice, within three minutes of touching the animals' cage, to ensure that subsequent mRNA measurements reflected baseline levels. The brains were stored at −80 °C until further processing for *in situ* hybridization.

2.7. In situ hybridization histochemistry

Brains were cut in 14 μm thick slices using a MICROM HM 560 cryostat (MICROM International GmbH, Walldorf, Germany) and freeze mounted onto glass slides. The cut brains were stored at −20 °C and further processed approximately one week later. The details of the *in situ* hybridization procedure are described elsewhere (Wigger et al., 2004). In brief, sections were fixed in 4% paraformaldehyde, washed in 10 (PBS, followed by acetic anhydride and passed through a series of ethanol in increasingly high concentration from 65% to 100%, then briefly bathed in chloroform and then again in 100% ethanol. After the slides had been air dried for approximately 1 h, 10 μl of hybridization mix was applied to each slide, including a ^{35}S radio-labeled probe calculated to 1 million counts of radioactivity per slide. The probes used were designed to recognize the following D1 dopamine receptor (D1R) (Dearry et al., 1990; Gross et al., 2003), D2 dopamine receptor (D2R, mix of three oligonucleotides) (Dal Toso et al., 1989; Gross et al., 2003), and dopamine transporter (DAT) (Jaber et al., 1999). The slides were covered with cover-slips and sealed into a hybridization chamber, which was incubated at 45 °C over night (18–22 h). The cover-slips were then removed in 1× SSC buffer (diluted from 20× SSC; 175 g NaCl, 88 g tri-Na–Citrate dehydrate) and the slides were subsequently bathed in 1× SSC 4 times 15 min at 45 °C, allowing the slides to come to room temperature during the last bath. Finally, the slides were passed through a graded series of ethanol yet again (65%, 95% and 100%) and left to air dry for several hours before they were placed on radiation-sensitive films (Kodak BioMax, Eastman Kodak Co., Rochester, New York, USA) for 4 days.

Films were subsequently scanned and the signal intensity was estimated for each region of interest; nucleus accumbens (NAc), caudate–putamen (CP), ventral tegmental area (VTA), and cingulate cortex (CgCtx), using ImageJ software (National Institute of Health). For each region, two to four brain sections were analyzed. In addition, for each brain slice, a background signal consisting of a nearby region without specific labeling was estimated and subtracted from the hybridization signal of each region of interest. If there was not more than one quantifiable slice from an animal it was excluded from the analysis. The final number of animals (*N*) included in the statistical analysis is presented together with the statistics in the results section.

2.8. Statistical analysis

The data was exclusively analyzed using non-parametric statistics as a normal distribution and variance homogeneity of the data could not always be assumed. The Kruskal–Wallis *H*-test (KWH-test) was used to compare more than two independent samples.

Post-hoc tests and group comparisons of two independent were performed using the Mann Whitney *U*-test (MWU-test). Sequential Bonferroni (Bf) correction was applied to correct for multiple testing, when appropriate. In order to compare two dependent samples the Wilcoxon test (*W*-test) was applied. The statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). Two tailed *p*-values are reported in all cases and statistical significance was set to $p < 0.05$. *p*-values between 0.05 and 0.1 were reported as trends.

3. Results

3.1. Stress reactivity test

The differences in the restraint stress-induced corticosterone increase were highly significant between the three breeding lines (KWH-test: HR: $N = 16$, IR: $N = 16$, LR: $N = 16$, $H = 41.80$, $df = 2$, $p < 0.001$; *post-hoc* MWU-tests: all $U = 0$, all $p < 0.001$), with HR mice having the highest increase (mean ± SEM: 400 ± 12.5 ng/ml), LR mice the lowest (mean ± SEM: 52.3 ± 3.0 ng/ml) and IR mice being intermediate (mean ± SEM: 176.7 ± 3.5 ng/ml). These findings are in accordance with previous generations of the stress reactivity mouse model (Touma et al., 2008, 2009). Furthermore, the three lines differed in their corticosterone levels in the initial blood sample taken in the SRT, with the highest levels found in the HR mice and the lowest levels in the LR mice (mean ± SEM: HR: 8.4 ± 21.9 ng/ml, IR: 3.8 ± 1.1 ng/ml, LR: 1.1 ± 0.1 ng/ml). These effects were statistically significant (KWH-test: HR: $N = 16$, IR: $N = 16$, LR: $N = 16$, $H = 19.86$, $df = 2$, $p < 0.001$; *post-hoc* MWU-tests: $U = 25–92$, HR vs. IR: $p > 0.1$, HR vs. LR: $p < 0.001$, IR vs. LR: $p < 0.001$). This is in accordance with findings in previous generations and can be attributed to the increased trough levels of the circadian rhythm of glucocorticoid secretion observed in HR mice (Touma et al., 2008, 2009).

3.2. Reversal learning task

Mice from the three breeding lines did not significantly differ in their acquisition of the task (KWH-test: HR: $N = 9$, IR: $N = 9$, LR: $N = 8$, $H = 0.01$, $df = 2$, $p > 0.1$; Fig. 1), nor did they differ in their retention of the task (KWH-test: HR: $N = 9$, IR: $N = 9$, LR: $N = 8$, $H = 4.06$, $df = 2$, $p > 0.1$; Fig. 1) on the subsequent day. However, in the reversal phase of the test, on the third day, HR mice needed significantly more trials to reach the learning criterion than LR mice, and IR mice tended towards needing more trials to reach the learning criterion than LR mice (KWH-test: HR: $N = 9$, IR: $N = 9$, LR: $N = 8$, $H = 6.19$, $df = 2$, $p < 0.05$; *post-hoc* MWU-tests: $U = 10–38$, HR vs. LR: $p < 0.05$; HR vs. IR: $p > 0.1$; IR vs. LR: $p < 0.1$; Fig. 1).

3.3. Latent inhibition

In IR and LR mice, the discrimination ratio ((water consumption – sucrose solution consumption)/total volume consumed) of the PE group was significantly higher in the nonpre-exposed group than the pre-exposed group (MWU-test: IR: both $N = 8$, $U = 7$, $p < 0.01$; LR: both $N = 8$, $U = 12$, $p < 0.05$; Fig. 2), indicating a higher preference towards water in the non pre-exposed group, which is to be expected if animals exhibit Li. However, in HR mice, the discrimination ratio between PE and No PE groups did not significantly differ (MWU-test: both $N = 8$, $U = 19$, $p > 0.1$; Fig. 2), indicating that HR mice display disrupted Li.

3.4. In situ hybridization

All probes used for *in situ* hybridization showed a specific binding with a low background signal intensity (see Fig. 3). HR mice had decreased levels of D1R mRNA in the CgCtx compared to LR mice (MWU-tests: HR: $N = 11$, LR: $N = 10$, $U = 23$, $p < 0.05$; Fig. 4a). However, there were no significant differences between the HR and LR mice in D1R mRNA levels in the NAc or CP (MWU-test: NAc: HR: $N = 11$, LR: $N = 10$, $U = 45$, $p > 0.1$; CP: HR: $N = 11$, LR: $N = 10$, $U = 41$, $p > 0.1$ Fig. 4b and c). Furthermore, no significant differences between HR and LR mice were detected in the expression of D2R mRNA in the cingulate cortex (MWU-tests: HR: $N = 16$, LR: $N = 16$, $U = 133$, $p > 0.1$; Fig. 5a). HR mice did however have increased levels of D2R mRNA in the NAc compared to LR mice (MWU-tests: HR: $N = 14$, LR: $N = 15$, $U = 56$, $p < 0.05$; Fig. 5b), as well as a tendency towards increased levels of D2R mRNA in the

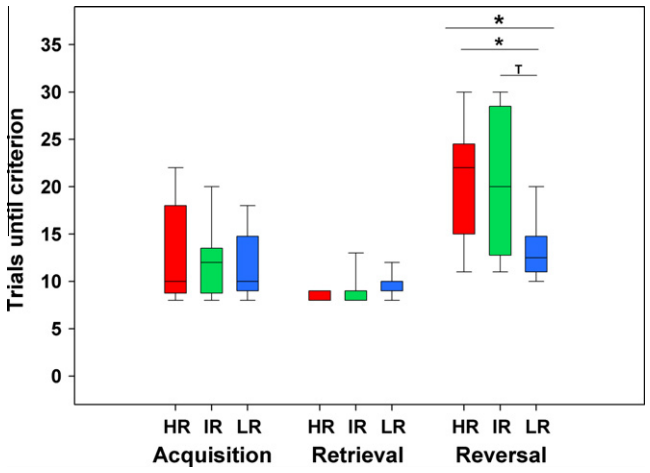

Fig. 1. Reversal learning task in mice selectively bred for high (HR), intermediate (IR) and low (LR) stress reactivity. The number of trials necessary to reach the learning criterion of 8/10 correct trials for each stage of the test, acquisition, retrieval and reversal, are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. Each phase of the test was statistically analyzed separately using the KWH-test (results given above the line encompassing the perimeter of all three box plots) followed by *post-hoc* MWU-tests (results given above the line connecting the middle of the two box plots being compared) where appropriate (Bf corrected $p < 0.05^{*}$, $p < 0.1$ T).

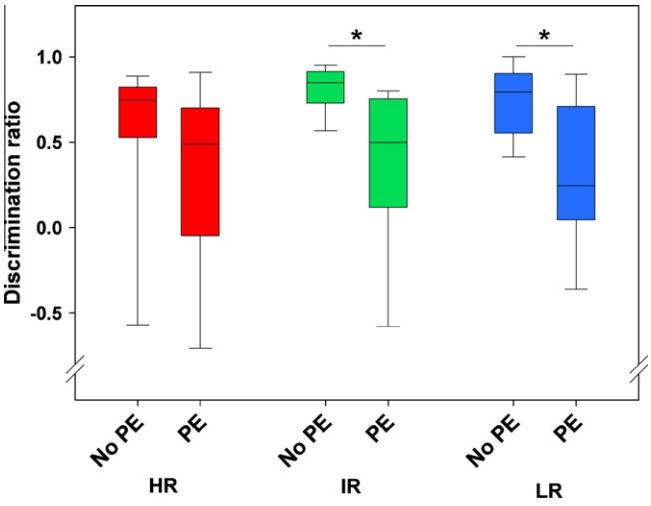

Fig. 2. Latent inhibition in a conditioned taste aversion paradigm in mice selectively bred for high (HR), intermediate (IR) and low (LR) stress reactivity. Within each line the discrimination ratio of water to sucrose solution of animals pre-exposed (PE) to the "to be conditioned stimulus" (sucrose solution) is compared to a nonpre-exposed (No PE) group (*W*-test, $p < 0.01^{**}$, $p < 0.05^{*}$). Data are depicted as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers.

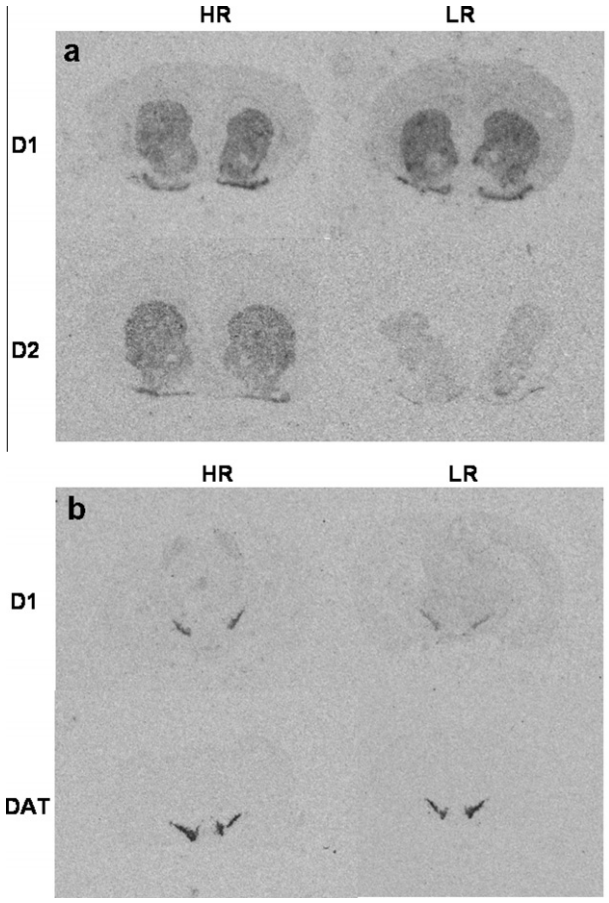

Fig. 3. Representative brain sections of high (HR) and low (LR) stress reactive mice hybridized with dopamine 1 receptor (D1R), dopamine 2 receptor (D2R) or dopamine transporter (DAT) oligoprobes, showing the striatum and cingulate cortex (a) and the ventral tegmental area (b).

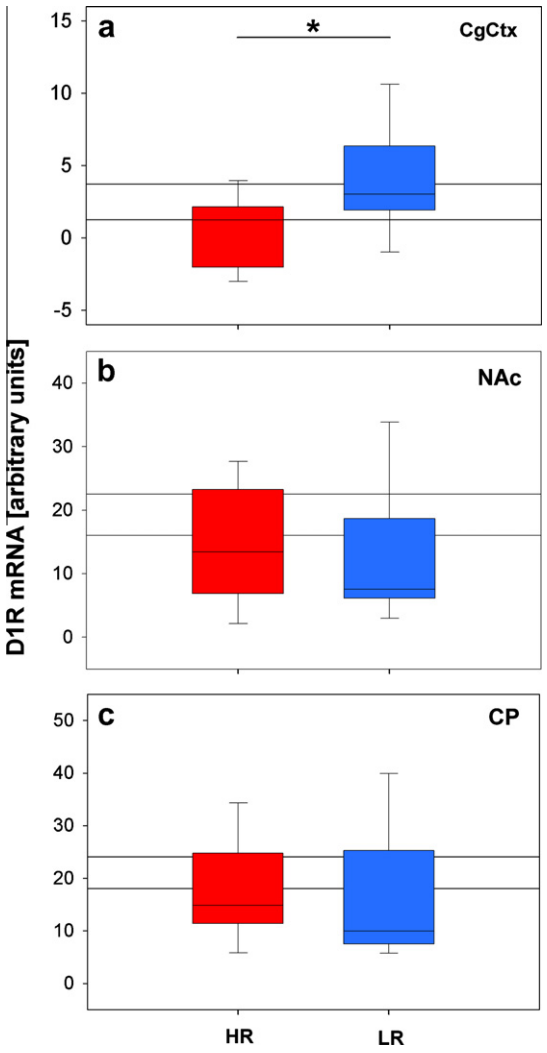

Fig. 4. Dopamine receptor 1 (D1R) mRNA levels in the (a) cingulate cortex (CgCtx), (b) nucleus accumbens (NAc), and (c) caudate–putamen (CP) of mice selectively bred for high (HR) and low (LR) stress reactivity, respectively. Data from HR and LR mice are depicted as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. As a reference the mean ± standard error of the mean of the intermediate (IR) stress reactive mice is depicted as the area between the horizontal lines. HR and LR mice were compared statistically using the MWU-test ($p < 0.05$).

CP (MWU-test: HR: $N = 16$, LR: $N = 15$, $U = 72$, $p < 0.1$; Fig. 5c). On the contrary, HR mice had significantly decreased D2R mRNA levels in the VTA compared to LR mice (MWU-test: HR: $N = 15$, LR: $N = 10$, $U = 36$, $p < 0.05$; Fig. 4d). DAT mRNA levels in the VTA did not significantly differ between HR and LR mice (MWU-test: HR: $N = 16$, LR: $N = 9$, $U = 54$, $p > 0.1$; Supplementary Fig. 1). Furthermore, if the results of the two independent sub-lines of HR and LR mice, respectively, were regarded separately, the observed alterations of the DA system were similar in both sub-lines (see Supplementary Figs. 2–4). This indicates that the alterations of the DA system are indeed associated with the stress reactivity phenotype and not brought about by effects of genetic drift.

4. Discussion

The aim of the present study was to examine if mice with increased stress reactivity display behaviors associated with cognitive and psychotic features of major depression and schizophrenia. Furthermore, the dopaminergic system of these mice was investigated in brain areas relevant to these behaviors. The experiments reported here demonstrate that mice selected for high stress reactivity have deficits in reversal learning and disrupted Li, along with congruent alterations in the expression of D1Rs and D2Rs in relevant brain areas. Apart from the brain areas we have focused on here, the PFC, NAc and VTA, the hippocampus has also been implicated in Li (Weiner & Feldon, 1997). Our previous studies have shown that HR mice have deficits in hippocampus-dependent cognitive tests (Knapman et al., 2009), such as the novel object recognition test and the *Y*-maze test. In addition, tests of anxiety and depression-like behavior have previously been performed on the stress reactivity mouse model, including elevated plus-maze and forced swimming (Touma et al., 2008). These tests indicate that the three mouse lines do not differ in measures of anxiety, however, there are robust differences in their coping strategy in other tests such as the forced swim test (Knapman et al., 2009; Touma et al., 2008). These findings along with reported differences in sleep architecture (Touma et al., 2009) and neuroendocrine measures have served to establish the stress reactivity moue model as a model of affective disorders.

Patients suffering from major depression and schizophrenia typically show deficits in PFC-dependent tasks, such as the Wisconsin Card sorting test (Rabin et al., 2009; Shirayama et al., 2009). Interestingly, these patients also often exhibit an increased HPA axis activity when subjected to the dexamethasone/corticotropin-releasing hormone test (Gallagher et al., 2007; Holsboer, Liebl, & Hofschuster, 1982; Holsboer, von Bardeleben, Wiedemann, Muller, & Stalla, 1987; Ising et al., 2005; Muck-Seler et al., 2004;

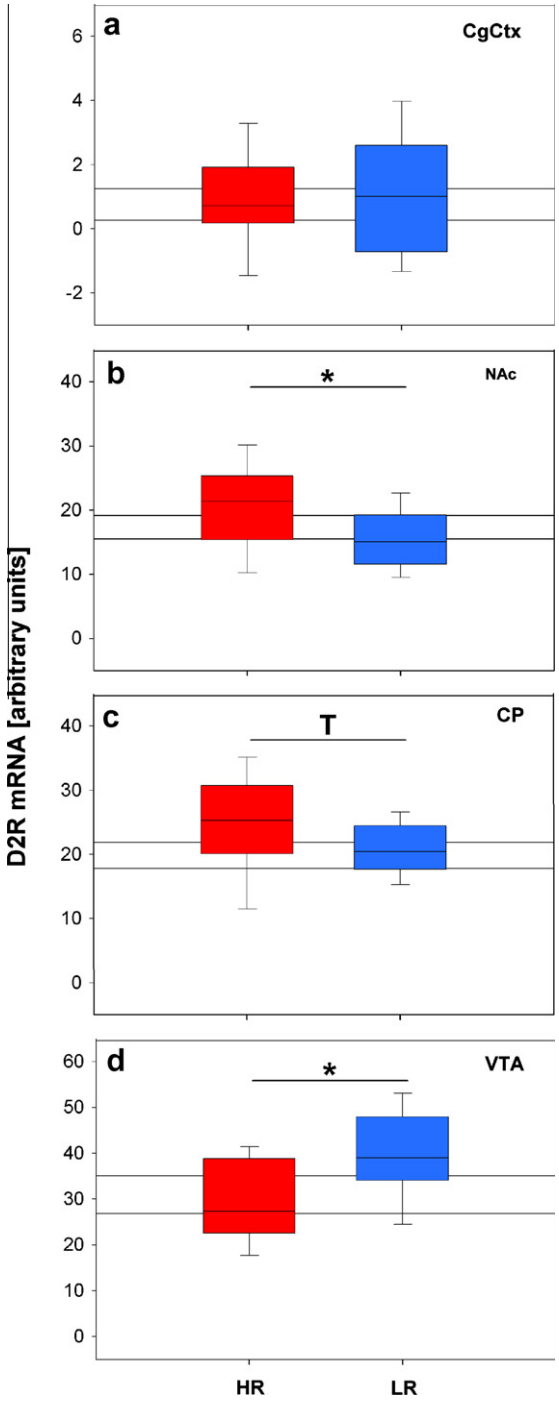

Fig. 5. Dopamine receptor 2 (D2R) mRNA levels in the (a) cingulate cortex (CgCtx), (b) nucleus accumbens (NAc), (c) caudate–putamen (CP), and (d) ventral tegmental area (VTA) of high (HR) and low (LR) stress reactive mice. Data from HR and LR mice are depicted as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. As a reference the mean ± standard error of the mean of the intermediate (IR) stress reactive mice is depicted as the area between the horizontal lines. HR and LR mice were compared statistically using the MWU-test ($p < 0.05^{*}$, $p < 0.1$T).

Ritsner et al., 2007; Ryan et al., 2004). This association between deficits in PFC-dependent tasks and stress reactivity, also shown in our mouse model, may be brought about by increased exposure of the PFC to glucocorticoids, as this region has an abundant expression of glucocorticoid receptors (Ahima & Harlan, 1990; Ahima, Krozowski, & Harlan, 1991; Aronsson et al., 1988). In addition, it is possible that the decreased amounts of D1R in the cingulate cortex of HR mice play a role in the decreased performance in the reversal learning task, as the dopaminergic system is known to subserve an optimal functioning of the PFC (Cropley et al., 2006). It is likely that the alterations of the dopaminergic system are a consequence of the genetically driven increased stress reactivity phenotype, rather than being due to the accidental selection of a genetic drift-related genotype unrelated to stress reactivity, as these changes in the dopaminergic system are observed in both of the two independent sub-lines of HR mice (see Supplementary Figs. 2-4). The VTA has also been implicated in learning processes where the outcome is not as predicted. The spiking activity of DA neurons in the VTA is higher in response to an unexpected reward and lower in response to a negative prediction error (Roesch, Calu, & Schoenbaum, 2007). Interestingly, HR mice have a reduced expression of D2R mRNA in the VTA compared to LR mice. Thus, one could speculate that the VTA of the HR mice is not correctly signaling prediction errors, which in turn would lead to difficulty in learning tasks where the outcome is not as predicted, such as the reversal learning task performed in this study. A lack of differences in learning and memory retention between the three lines in this phase of the test may appear to be contradictory to the previously published data demonstrating differences in the novel object recognition test and the *Y*-maze test. However, the reversal learning task was specifically designed to have a low degree of difficulty in the acquisition and retention phase as any difference between the lines at this phase of the test would confound the reversal phase of the test.

The HR mice furthermore display disrupted Li, which is also commonly observed in patients suffering from psychotic disorders (Gray, Pickering, Hemsley, Dawling, & Gray, 1992; Gray, Pilowsky, Gray, & Kerwin, 1995; Lubow, Kaplan, Abramovich, Rudnick, & Laor, 2000; Rascle et al., 2001). Li is a measure of selective attention, often used to model the cognitive deficits associated with psychotic behavior in animal models and patients. An increase in mesolimbic DA has been strongly implicated in disrupted Li (Solomon & Staton, 1982; Weiner, 1990; Young et al., 1993), and patients suffering from schizophrenia reportedly have an increased DA activity in the mesolimbic area along with increased amounts of D2Rs in the striatum (Abi-Dargham, 2003; Alves Fda, Figee, Vamelsvoort, Veltman, & de Haan, 2008; Carlsson & Lindqvist, 1963; Carlsson, Waters, Waters, & Carlsson, 2000; Mawlawi et al., 2001). Our findings of an increased expression of D2R mRNA in the HR mice, along with disrupted Li, thus demonstrate that the HR mice show similarities to psychotic features of schizophrenia and major depression.

Disrupted Li and perseveration in a reversal learning task are in a sense non- congruent, as disrupted Li implies that the animal is switching its behavior, when it should not, and in the reversal learning task it is not switching its behavior, when it should. However, from a neurobiological perspective, the situation of disrupted Li and reversal learning deficits may occur in the same individual, as the mechanisms behind the switching behavior in Li and the pathways involved in reversal learning are not believed to be identical (Clark et al., 2004; Floresco, Zhang, & Enomoto, 2009; Weiner, 1990). Interestingly, the final outcome of the response of the HR mice in the two behavioral tests used in this study is similar in that both perseveration and disrupted Li imply a lack of a capacity to modify a behavioral response in accordance to a new response contingency. This can be extrapolated to predict an inability to adapt to new and stressful situations in a way that is beneficial to the organism, thus causing a prolonged and more severe stress reaction to these situations. In theory, this could be a vicious circle, with increased stress reactivity leading to alterations in the brain, that render the organism incapable of adapting to a changing environment, which in turn would lead to more stress, that could

further impair the cognitive functions underlying appropriate behavioral modifications to a changing environment.

Rodent models of affective disorders where mice are subjected to stress, such as the chronic mild stress paradigm in mice and rats, or maternal deprivation in rats, have a behavioral phenotype similar to what we have observed in HR mice, and similar alterations of the DA system have been reported (Bielajew, Konkle, & Merali, 2002; Ellenbroek & Cools, 2002; Rentesi, Antoniou, Marselos, & Konstandi, 2009; Willner, 2005). Studies using the chronic mild stress model of major depression, which is also known to increase HPA axis reactivity in rats (Bielajew et al., 2002), have found both increased and decreased D2R binding in the striatum (Willner, 2005). Interestingly, D2R binding was found to be increased in mice/rats with an "anomalous" profile and decreased in mice with a "depressive" profile (Willner, 2005). One of the anomalous behavioral effects reported was a decreased amount of time floating in the forced swim test, which has also previously been reported in the HR mice (Knapman et al., 2009; Touma et al., 2008). The maternal deprivation model of affective disorders has also been confirmed to show cognitive deficits related to psychotic behavior (Ellenbroek & Cools, 2002), increased D2R protein levels in the striatum (Rentesi et al., 2009), as well an increased HPA axis reactivity in response to a stressor in rats (Aisa, Tordera, Lasheras, Del Rio, & Ramirez, 2007).

The advantage of our mouse model is that the HR mice simulate a situation where the organism is genetically predisposed to hyper-react to stressors, which is a more natural approach than the chronic mild stress paradigm or maternal deprivation paradigms. Interestingly, we have demonstrated here that the HR mice show behavioral and anatomical differences that are similar to the alterations described above to be observed in the chronic mild stress paradigm and maternal deprivation models, although the HR mice have not repeatedly been subjected to a stressor. We hypothesize that the increase in glucocorticoid exposure in response to the events that mice are normally subjected to, such as cage changing, unavoidable noise in the animal house or agonistic interactions with cage mates, is sufficient to induce the observed changes. The possibility to use a top down approach, starting with a known phenotype and studying the genotypic and molecular differences between HR and LR mice, is yet another advantage of the model. Furthermore, the majority of studies using the chronic mild stress paradigm or maternal deprivation use rats. Thus, the stress reactivity mouse model has the further advantage that it is based on mice, as the mouse genome is better annotated than the rat genome, which is advantageous when searching for genetic correlates of behavioral phenotypes.

Although we have not demonstrated a causal link between stress reactivity, the alterations to the DA system and the behavioral phenotype observed, these behavioral differences and alterations of the dopaminergic system are not likely to be due to genetic drift, i.e. accidentally selecting for genes unrelated to increased stress reactivity in the HR mice, as both sub-lines of HR mice display a similar phenotype. Furthermore, it has repeatedly been demonstrated that alterations of the DA system in concordance with our findings are the underlying mechanism of disrupted Li (Lubow, 2005; Solomon & Staton, 1982; Weiner, 1990; Young et al., 1993). In addition, D2Rs in the NAc and VTA have been strongly implicated in the expression of fear following fear conditioning, another form of learning involving conditioned associations (de Oliveira, Reimer, & Brandao, 2006, 2009; Martinez, Oliveira, Macedo, Molina, & Brandao, 2008). This strongly implicates the alterations to the DA system of the HR mice in their behavioral deficits.

In summary, the experiments presented here have shown for the first time that a genetic predisposition for increased stress reactivity, without specifically subjecting the animal to repeated stressors prior to testing, is sufficient to alter the DA system. In addition, the HR mice display concomitant deficits in behaviors subserved by the DA system, such as Li and reversal learning. These findings are important in understanding the relationship between HPA axis hyperactivity, cognitive deficits and psychotic symptoms in major depression and schizophrenia and demonstrate that the HR mice can be used to explore new targets for treating these disorders.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nlm.2010.04.010.

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III

Increased Stress Reactivity is Associated with Decreased Hippocampal N-Acetylaspartate, Neuronal Activity, and Alterations of Mitochondrial Proteins

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ABSTRACT

Patients suffering form major depression (MD) have repeatedly been reported to have a dysregulated hypothalamus-pituitary-adrenal (HPA) axis along with deficits in cognitive processes, which are related to hippocampal and prefrontal cortex (PFC) function. Whether these symptoms are causally related still remains unclear. In this study we have utilized three lines of mice selectively bred for high (HR), intermediate (IR), or low (LR) stress reactivity to probe the behavioral, functional, morphological and molecular consequences of an increased HPA axis reactivity on the hippocampus and PFC. We assessed their perfromance in hippocampus- and PFC-dependent cognitive tasks and determined the morphology, basal activity, and neuronal integrity of the hippocampus and prefrontal cortex. using *in-vivo* manganese-enhanced magnetic resonance imaging (MEMRI) and proton magnetic resonance spectroscopy (^{1}H-MRS). Additionally, the hippocampal proteome of HR and LR mice was compared. We found deficits in performance of hippocampus- and PFC-dependent tests in HR mice which could be related to decreased N-acetylaspartate (NAA) levels, a marker of neuronal integrity, in the right dorsal hippocampus and prefrontal cortex. Furthermore, the basal activity of the hippocampus as assessed by MEMRI was reduced in HR mice; however, the three lines did not differ in hippocampal volume. The majority of the proteins that were found to be differentially expressed in HR and LR mice were involved in energy metabolism, suggesting that mitochondrial dysfunction may be the underlying cause of the observed behavioral deficits and alterations in neuronal integrity and function.

INTRODUCTION

Increased stress reactivity has repeatedly been demonstrated in patients suffering from major depression (MD) and schizophrenia (SZ) (Holsboer, 2000; Muck-Seler et al., 2004; Ryan et al., 2004; de Kloet et al., 2005; Ising et al., 2005; Gallagher et al., 2007; Ritsner et al., 2007; Holsboer and Ising, 2010). It has been proposed that an excessive amount of circulating corticosteroids would be detrimental for specific brain areas, namely the hippocampus and prefrontal cortex (PFC) (Sapolsky et al., 1990; Swaab et al., 2005; Zhu et al., 2007; Radley et al., 2008). These brain areas are important in cognitive processes, such as learning, memory and executive functioning (Scoville and Milner, 1957; Petrides, 1994; Carpenter et al., 2000; Eichenbaum, 2000; Robbins and Arnsten, 2009). Interestingly, deficits in these cognitive processes have repeatedly been demonstrated in patients suffering from MD and SZ (Austin et al., 2001; Porter et al., 2003; Rabin et al., 2009; Shirayama et al., 2009) and have been linked to an increased hypothalamus-pituitary-adrenal (HPA) axis activity (Belanoff et al., 2001; Bremner et al., 2004).

We have here made use of a previously established mouse model of affective disorders, the stress reactivity (SR) mouse model, which consists of three CD-1-derived breeding lines that are selectively bred for high (HR), intermediate (IR), or low (LR) reactivity of the HPA axis (Touma et al., 2008). It has previously been demonstrated that the SR mouse model is a valid model of MD and that the HR mice have cognitive deficits associated with depression and psychotic behavior (Touma et al., 2008; Knapman et al., 2009; Touma et al., 2009; Knapman et al., in press-a).

In-vivo magnetic resonance (MR) spectroscopy was used to assess the levels of N-acetylaspartate (NAA) in the dorsal hippocampus

and PFC of the three breeding lines. NAA, which gives rise to a prominent signal in ^{1}H-MRS of the brain, is a metabolite exclusively found in neurons (Birken and Oldendorf, 1989; Moffett et al., 1991). Since NAA signal amplitude is decreased in association with both cellular dysfunction and neuronal loss (Demougeot et al., 2001), NAA is considered to be a marker of neuronal integrity. Patients suffering from MD and SZ have shown decreased values of the NAA/Creatin (Cre) ratio in the hippocampus and PFC (Buckley et al., 1994; Bertolino et al., 1998; Gonul et al., 2006). Since these brain areas are also likely to be affected by glucocorticoid exposure, we additionally investigated the volume and functional activity of brain areas likely to be affected by glucocorticoid exposure, namely the hippocampus and PFC, in the SR mouse model, using manganese enhanced magnetic resonance imaging (MEMRI). Manganese ions enters the cells through voltage gated calcium channels during depolarization, in an activity dependent manner. Their paramagnetic properties lead to a positive contrast enhancement in T1-weighted magnetic resonance images (Mendonca-Dias et al., 1983; Simpson et al., 1995). Manganese ions accumulate in the brain of freely behaving mice over an extended period of time, thus leading to pronounced signal enhancement in activated brain regions, which can finally be read-out under anaesthesia in the MR-scanner. These properties therefore allow an in-vivo assessment of neuronal activity. As intraperitoneally applied manganese especially enlightens the hippocampal formation, MEMRI also enables a semi-automatic determination of the hippocampal volume. Furthermore, the cognitive abilities of the SR mice have been assessed using specific hippocampus- and PFC-dependent tests. In addition, the hippocampal proteome of HR and LR mice was compared, in order to probe the underlying mechanism of altered hippocampal function.

METHODS

ANIMALS AND HOUSING CONDITIONS

A total of 92 adult male mice from the SR mouse model were used in these experiments. The SR mouse model consists of three independent CD-1-derived mouse lines. These three lines are selectively bred for high (HR), intermediate (IR) and low (LR) stress reactivity, respectively (Touma et al. 2008). Stress reactivity was determined by the stress reactivity test (SRT) described below. Within each breeding line, two independent sub-lines, A and B, exist. These sub-lines were never interbred and serve as a replication of the breeding protocol conducted in parallel (Touma et al., 2008). The mice of each group comprised of mice from both sub-line. Two weeks prior to the onset of behavioral testing all animals were single housed under standard laboratory conditions in transparent polycarbonate cages (standard macrolon cages type II, 38 × 22 × 15 cm) with food and water available *ad libitum*. Testing and housing rooms were maintained on a 12:12h light-dark cycle with a constant temperature and humidity of 22 ± 1 ^{o}C and 55 ± 10 %, respectively.

The mice used in the hippocampal spectroscopy study originated from the ninth breeding generation of the SR mouse model (N = 12 per breeding line). The mice used in the behavioral tests, hippocampal volume measurement, hippocampal activity measurement and PFC spectroscopy were derived from the twelfth breeding generation (N = 16 per breeding line). The mice used for the screening of the hippocampal proteome originated from the fourteenth breeding generation (N = 4 per breeding line).

All behavioral tests and hormone measurements were conducted during the first hours of the light phase when the animals were still relatively active and corticosterone levels are at their circadian trough. Magnetic resonance imaging (MRI) was conducted throughout the light phase. The time of day of scanning and behavioral testing was counterbalanced across the groups. The presented work complies with current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC). All experiments were announced to the appropriate local authority and were approved by the 'Animal Welfare Officer' of the Max Planck Institute of Psychiatry.

DETERMINATION OF HPA AXIS REACTIVITY

Stress Reactivity Test (SRT)

All mice used in these experiments underwent the SRT at the age of approximately eight weeks. The is comprised of an initial blood sample collected from a small incision in the ventral tail vessel, followed by a 15-min restraint stressor and finally a reaction blood sample, collected from a second incision in the ventral tail vessel immediately after the period of restraint (for details see (Touma et al., 2008)). Corticosterone levels in the plasma were analyzed as described below.

Plasma Corticosterone Measurements

A radioimmunoassay (RIA) kit (MP Biomedicals, Solon, Ohio, USA) was used with a slight modification to the manufacturer's instructions to determine corticosterone levels in the plasma samples (for details see (Touma et al., 2008)). From the initial sample, 10 µl of plasma were diluted 1:13.5, and for the reaction sample, 10 µl of plasma were diluted 1:100. The difference in dilution was used to ensure that the corticosterone concentrations of the samples were within the linear part of the standard curve. Inter- and intra-assay coefficients of variation were both below 10 %.

MAGNETIC RESONANCE MEASUREMENTS

Apparatus

At the age of 20 weeks, each animal underwent a proton magnetic resonance spectroscopy (^{1}H-MRS) scan and/or manganese enhanced magnetic resonance imaging (MEMRI). Mice were anesthetized with isoflurane (DeltaSelect, Germany), fixed and further kept under inhalation anesthesia with an isoflurane-oxygen mixture (1.5 – 1.9 vol % with an oxygen flow of 1.2 – 1.4 l/min). Head movements were prevented by fixing the frontal teeth with a surgical fiber. Body temperature was monitored with a rectal thermometer (Thermalert TH-5, Physitemp Instruments, USA) and kept between 34 – 36 °C using a custom build heating pad with warm water circulation. Pulse rate was continuously monitored by a plethysmographic pulse oxymeter (Nonin 8600V, Nonin Medical Inc., USA). All MR experiments were acquired on a 7 T Avance Biospec 70/30 scanner (BRUKER, Germany) using a saddle-shaped receive only coil.

Proton Magnetic Resonance Spectroscopy Measurements

Adjustment of the magnetic resonance (MR) system and acquisition of localizer images for definition of the 3D brain volume and for positioning the spectroscopic volume of interest was performed with a 2D relaxation enhanced (RARE) sequence (TR = 5000 ms, TE = 11.9 ms, Rare factor 6, TEeff = 38.8 ms, spatial resolution: 0.133 x 0.133 x 0.5 mm^3).

For ^{1}H-MRS, a PRESS sequence (TR = 5000 ms, TE = 17 ms, 256 averages, bandwidth 5 kHz, 2 K time domain points) with automated shimming and manual adjustment of water suppression using three chemical shift selective (CHESS) pulses was employed. Each spectrum was acquired in 21:20 minutes. The acquired spectra were analyzed by using LCModel 6.1-4 with a set of metabolite reference spectra acquired under identical experimental conditions. The following metabolites were used for spectral fitting: Creatine (Cre), γ-aminobutyric acid, glucose, glutamate, glutamineglycerophosphocholine, phosphocholine, myo-inositol, lactate, N-acetylaspartate (NAA), N-acetyl-aspartylglutamate (NAAG), phosphocreatine, scyllo-inositol and taurine, along with macromolecules and lipids. The fit was performed over a spectral range of 1.0 – 4.2 parts per million (ppm). To be included into the analysis, the respective metabolites needed a signal-to-noise (S/N) ratio in the left or right hippocampus smaller or equal to eight and a spectral fitting with a standard deviation smaller than 18%. For the PFC only spectra with a S/N-ratio smaller than or equal to four and a spectral fitting with a standard deviation smaller than 18% where included. Due to these criteria, three out of 33 spectra had to be excluded from the analysis of the left hippocampus, and nine out of 33 spectra from the analysis of the right hippocampus. For the analysis of the PFC, 16 out of 46 spectra had to be excluded due to the criteria specified above. For the hippocampal spectroscopic volumes, we found a full-with-at-half-maximum (FWHM) of 0.049 ± 0.003 ppm for the right hippocampus and 0.048 ± 0.002 ppm for the left hippocampus (mean ± standard error of the mean (SEM)). For the PFC, we found a FWHM of 0.058 ± 0.004 ppm. Metabolic concentrations are conservatively presented relative to Cre as an internal standard. Cre concentration are given in institutional units (i.u.) referenced aginst the unsupressed water signal from the spectroscopic volume.

Localization of the Volumes of Interest

The spectroscopic volume of interest for the dorsal hippocampi included mainly dorsal CA1 region and parts of the CA2 and CA3 regions and the dentate gyrus, reaching from approximately Bregma -1.3 mm to Bregma -2.8 mm according to the mouse brain atlas (Franklin and Paxinos, 1997). The spectroscopic volume was 5.625 µl (see Fig. 1a). Bilateral measurements of the hippocampus were performed.

The volume of interest for the PFC included mainly the prelimbic and infralimbic cortex and parts of the cingulate cortex, reaching from approximatly Bregma +2.3 mm to Bregma +1.3 mm according to the mouse brain atlas (Franklin and Paxinos, 1997). Here the spectroscopic volume was 3.00 µl (see Fig. 4a).

Manganese Enhanced Magnetic Resonance Imaging Measurements

Animals were injected intraperitoneally (i.p) with a 50 mM manganese chloride ($MnCl_2*4H_20$, Sigma, Steinheim, Germany) solution in 0.9% NaCl (pH 7). The injection protocol was based on the fractionated application scheme proposed by Gruenecker and colleagues. A total concentration of 180 mg/kg was injected in three fractionated doses of 60 mg/kg $MnCl_2$ with an inter-injection interval of 48 h.

Mice were scanned 24 hrs after the last injection. T1-weighted (T1w) brain images were acquired using a 3D gradient echo pulse sequence (TR = 50 ms, TE = 3.2 ms, matrix size = 128×106×106, zero filled to 128×128×128, field of view (FOV) = 16×16×18 mm^3, number of averages = 10, resulting in a spatial resolution of 125×125×140.6 μm^3 with a total measurement duration of 90 min). Additionally, T2-weighted (T2w) images were obtained using a RARE enhanced pulse sequence (TR = 1000 ms, TE = 10 ms, matrix size = 128×112×112 zero filled to 128×128×128, FOV = 16×16×18 mm^3, number of averages = 2, Rare factor = 16, TE_{eff} = 78.6 ms, resulting in a resolution of 125×125×140.6 μm^3, with a measuring time of around 30 min). T1w- and T2w-images were acquired using identical image orientation and geometry. Total measurement time was around two hours per animal.

Magnetic Resonance Imaging Data Processing

Images were reconstructed using Paravision software (Bruker BioSpin, Ettlingen, Germany) and transferred to standard ANALYZE format. Further post-processing was performed using SPM2 (www.fil.ion.ucl.ac.uk/spm). T1w- and T2w-images were first co-registered using affine transformations. Images were bias corrected to remove intensity gradients introduced by geometry of the surface coil. A representative T1w- and T2w-image, respectively, of one animal was selected that served as a first template for the generation of a customized second generator template.

For both T1w- and T2w-images, bias corrected images of all mice were normalized to the single animal template of the respective contrast. A group template was then produced based on an average of all normalized images of the first normalization step. Bias corrected raw images of all individual animals were then normalized to the group template.

For improved normalization of T1w-images, independent of extra-brain tissue as well as signal hyperintensity of large vessels typically found in T1w-images, a brain extraction step was performed first. This was based on normalized T2w-images. Due to the better contrast between parenchyma and other tissue types and no signal hyperintensity of large vessels compared to T1w-images, a brain extraction step could be performed as follows: A binary mask defining the intracranial vault without large vessels (total brain) was defined (MRIcro, www.sph.sc.edu/comd/rorden/mricro.html) on the T2w-group template, and transformed to native (co-registered) space of each individual animal (by inverted spatial normalization). Brain extracted images of the co-registered and bias-corrected T1w-images were then used for the normalization steps of T1w-images.

Regions of interest (ROIs) were defined on the T1w group template for selected structures, based on the mouse anatomical atlas (Franklin and Paxinos, 1997). As ROIs we defined the hippocampus, the prefrontal cortex and the ventricles. The extracted total brain volume, excluding ventricles, served as an individual reference. Binary ROI masks were back-transformed into native space as described for the total brain. Eventually, volume and intensity measurements of each ROI were performed on the bias-corrected raw T1w-images of each animal using in-house written software in IDL (www.creaso.com) and normalized to the total brain volume and intensity respectively.

BEHAVIORAL TESTS

Y-Maze Test

The mice were tested in a Y-maze test at the age of approximately 24 weeks. to assess hippocampus dependent spatial memory (Dellu et al., 2000). The details of the test are described elsewhere (Knapman et al., 2009). Briefly, the apparatus used for this test was Y-shaped and consists of three arms bearing a different symbol on the walls. During the acquisition phase, the animal was allowed to explore two of the arms for ten min. After an inter-trial interval of 30 min, where the mouse was returned to its home cage, the mouse was placed back in the maze for five minutes, this time with the opportunity to explore all three arms. The animals' movements were tracked using ANY-maze software (ANY-maze, Stoelting Co., IL, USA). If the animal spent significantly more time in the novel arm compared to the mean of the familiar arms, it was assumed to have remembered the arms it had previously been allowed to explore.

Reversal Learning Test

In order to test PFC function, the mice were tested in a reversal learning test at the age of approximately 18 weeks. Briefly, mice were placed in a T-maze with an escape tunnel at the end of one of the goal arms leading to the home cage. The mice were tested with an inter-trial interval of approximately 5 min until they reached the learning criterion of eight out of ten correct trials. The mice were retested 24 hrs later to see if the groups differed in memory retention. On the third day, i.e. 24 hrs after the retention trial, the goal arm with the escape tunnel was moved to the opposite arm and the mouse had to learn the new position of the arm. For detailes see (Knapman et al., in presss). In this paradigm 12 animals per line were tested. If an animal failed to reach the learning criterion by 30 trials or needed more trials to reach the learning criterion on the second day (the retrieval phase) it was excluded from the analysis at all stages of the test. The final number of animals (N) for each group is presented along with the outcome of statistical analysis in the results section.

PROTEOMICS

Sample Preparation

At the age of approximately 18 weeks, animals were briefly anesthetized with isoflurane and then rapidly decapitated. The brain was removed from the skull and the hippocampus was subsequently bilaterally dissected. The hippocampal tissue was then individually homogenized in 1.5 ml eppendorf tubes with glass spheres (Sample Grinding Kit, GE Healthcare) in 100 µl of so-called IEF buffer (7 M urea, 2M thiourea, 4% CHAPS, 2% ASB-14 and 70 mM DTT buffer) (for details see (Martins-de-Souza et al., 2007)). Samples were centrifuged at 17500 rcf for 10 min in order to separate the dissolved proteins from the spheres and lipids. The dissolved protein samples were individually transferred to fresh tubes, and 1 µl of protease inhibitor was added to the samples to inhibit protein degradation. Samples were mixed and stored at -80 ºC until further analysis.

Two–Dimensional Gel Electrophoresis

The individual hippocampal proteome of four HR and four LR mice were compared using two-dimensional gel electrophoresis (2-DE). The methods are described in detail elsewhere (Martins-de-Souza et al., 2009). Briefly, 650 µg of protein from HR or LR mouse hippocampus were applied to IPG gel strips with a nonlinear separation range of pH 3-10 prior to the second dimension in 12.5 % T (T = Total acrylamide-bisacrylamide monomer concentration) acrylamide gels that were stained using a modified colloidal coomassie blue protocol.

Determination of Protein Expression Differences and Protein Identification by Peptide Mass Fingerprinting

2-DE gel images were used for spot detection and pI/MW (isoelectric point/molecular weight) calibration using the PDQuest software (Bio-Rad, Hercules, CA, USA). One gel from a HR mouse could not be scanned due to the gel being excessively fragmentized, leaving a total of seven 2-DE profiles that were analyzed (4 LR mice and 3 HR mice). Corresponding spots were matched for all 2-DE profiles. Five protein spots that differed significantly between HR and LR mice were excised for mass spectrometry (MS) identification. Protein identification by peptide mass fingerprinting was done as previously described (Martins-de-Souza et al., 2009).

STATISTICS

The data was mainly analyzed using non-parametric statistics as a normal distribution and variance homogeneity of the data could not always be assumed. The Kruskal-Wallis H-test (KWH-test) was used to compare more than two independent samples. Post-hoc tests and group comparisons of two independent groups were performed using the Mann Whitney U-test (MWU-test), apart from proteomics data that was analyzed using the t-test. Sequential Bonferroni correction was applied to correct for multiple testing, when appropriate. In order to compare two dependent samples the Wilcoxon test (W-test) was applied. The statistical analysis was performed using SPSS software (SPSS Inc, Chicago, IL, USA). Two tailed p-values were reported in all cases and statistical significance was set to $p<0.05$. P-values between 0.05 and 0.1 were reported as trends.

RESULTS

STRESS REACTIVITY TEST

The differences in initial corticosterone levels and restraint stress-induced corticosterone increase were highly significant between the three breeding lines (HR>IR>LR) in all groups of experimental animals i.e. the animals from the ninth, twelfth and fourteenth breeding generation in both initial

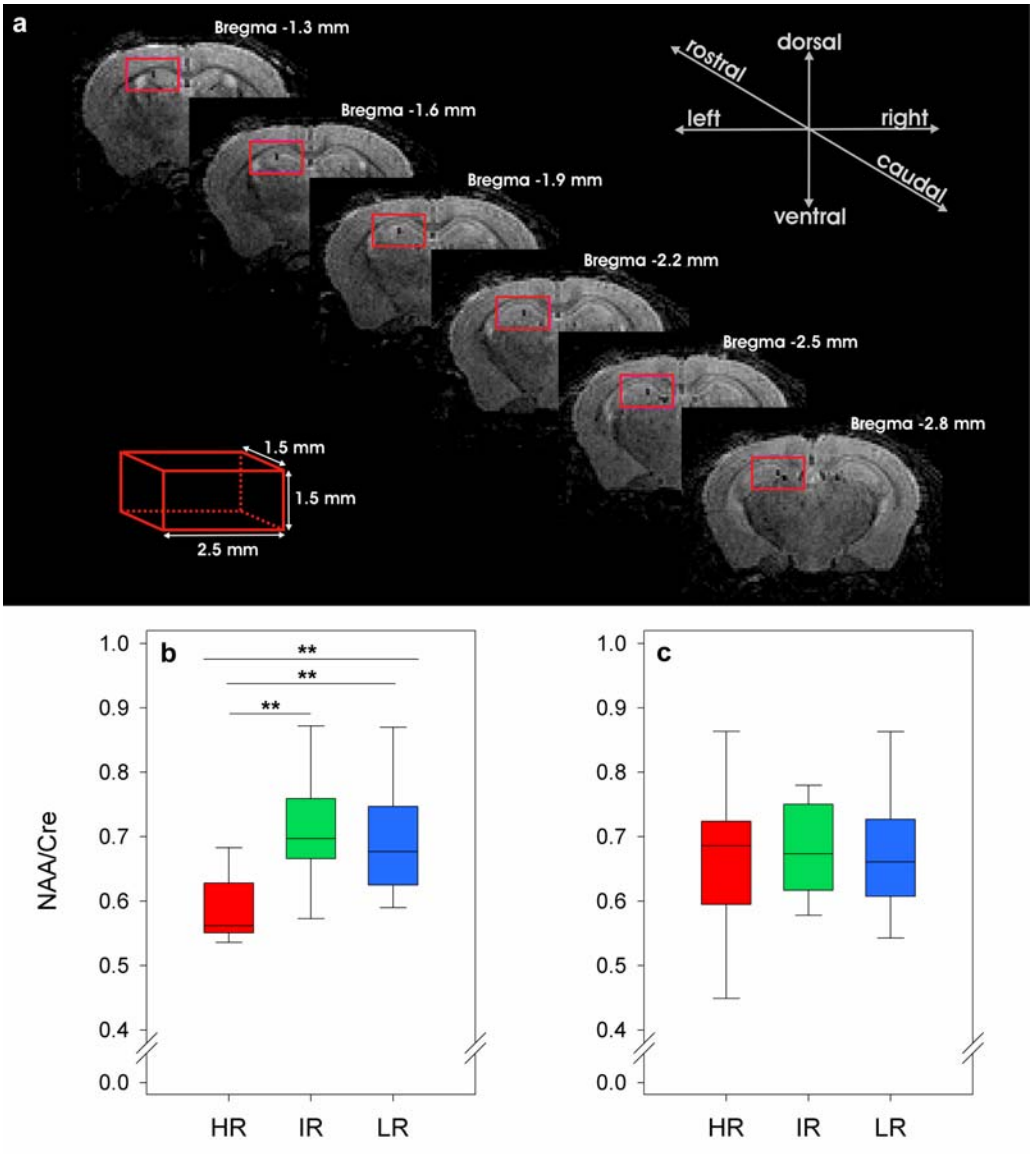

Fig. 1: Localization and volume of region of interest for hippocampal spectroscopic measurements (a) and N-acetylaspartate (NAA) levels normalized to Creatin (Cre) in the right (b) and left (c) hippocampus of mice selectively bred for high (HR), intermediate (IR) and low (LR) stress reactivity. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. Data was statistically analyzed using the KWH-test followed by *post-hoc* MWU-tests where appropriate (Bonferroni corrected $p<0.05$*, $p<0.01$**).

corticosterone plasma levels and stress induced increase in plasma corticosterone levels. See supplementary material for details.

MAGNETIC RESONANCE MEASUREMENTS

Hippocampal Spectroscopy

The three lines of mice significantly differed in their NAA/Cre levels in the right dorsal hippocampus, with HR mice demonstrating a reduced level compared to both IR and LR mice (KWH-test: HR: N = 9, IR: N = 7, LR: N = 9, H = 12.4, df = 2, p = 0.002; *post-hoc* MWU-tests: U = 4-27, HR *vs.* LR: p = 0.006; HR vs. IR: p = 0.008; IR vs. LR: p = 0.634; Fig. 1b). However, the NAA/Cre level in the left dorsal hippocampus did not significantly differ between the lines (KWH-test: HR: N = 11, IR: N = 8, LR: N = 10, H = 0.1, df = 2, p = 0.934; Fig. 1c). The levels of Cre furthermore did not differ between the three lines in the right (HR: 32400 ± 1268 i.u., IR: 31833 ± 1252 i.u, LR: 31089 ± 2711 i.u; KWH-test: HR: N = 9, IR: N = 7, LR: N = 9, H = 0.9, df = 2; p = 0.640) or left hippocampus (28709 ± 1126 i.u, IR: 29256 ± 1586 i.u, LR: 33840 ± 3659 i.u; KWH-test: HR: N = 11, IR: N = 8, LR: N = 10, H = 0.8, df = 2, p = 0.682)

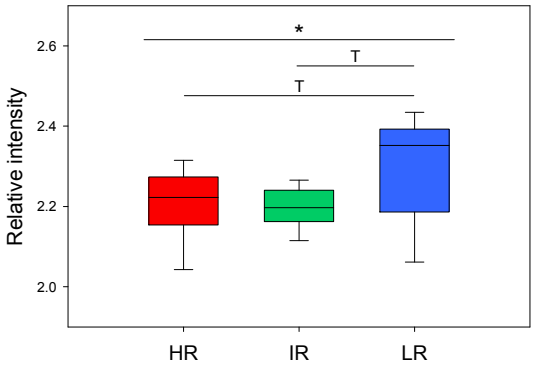

Fig.2: Relative signal intensity of the hippocampus of mice selectively bred for high (HR), intermediate (IR) and low (LR) stress reactivity. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. Data was statistically analyzed using the KWH-test followed by *post-hoc* MWU-tests where appropriate (Bonferroni corrected p<0.05*)

Hippocampal Signal Intensity and Volume

There was a significant difference between the three lines in the T1w-signal intensity due to manganese accumulation
in the hippocampus relative to the whole brain intensity. HR mice showed a lower signal intensity than LR mice and LR mice had a higher signal intensity than IR mice, this differences however only remained a strong trend values after Bonferroni correction (KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 6.8, df = 2, p<0.034; *post-hoc* MWU-tests: U = 55-90, HR *vs.* LR: p = 0.058; HR vs. IR: p = 0.533; IR vs. LR: p0.078; Fig. 2). The hippocampal volume however did not differ between the three lines (KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 0.1, df = 2, p = 0.700; Fig. 3).

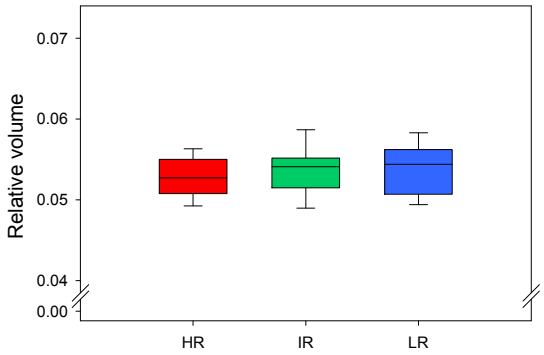

Fig. 3: Hippocampal volume relative to brain volume of mice selectively bred for high (HR), intermediate (IR) and low (LR) stress reactivity. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. Data was statistically analyzed using the KWH-test followed by *post-hoc* MWU-tests where appropriate.

Prefrontal Cortex Spectroscopy

The three mouse lines significantly differed in their NAA/Cre levels in the PFC, with the HR mice displaying significantly lower levels than the LR mice. Furthermore, the LR mice had higher NAA/Cre levels than IR mice, but this difference only reached statistical trend values after Bonferroni correction (KWH-test: HR: N = 12, IR: N = 11, LR: N = 11, H = 11.4, df = 2, p = 0.003; *post-hoc* MWU-tests: U = 55-90, HR *vs.* LR: p = 0.003; HR vs. IR: p = 0.104; IR vs. LR: p = 0.056; Fig. 4).

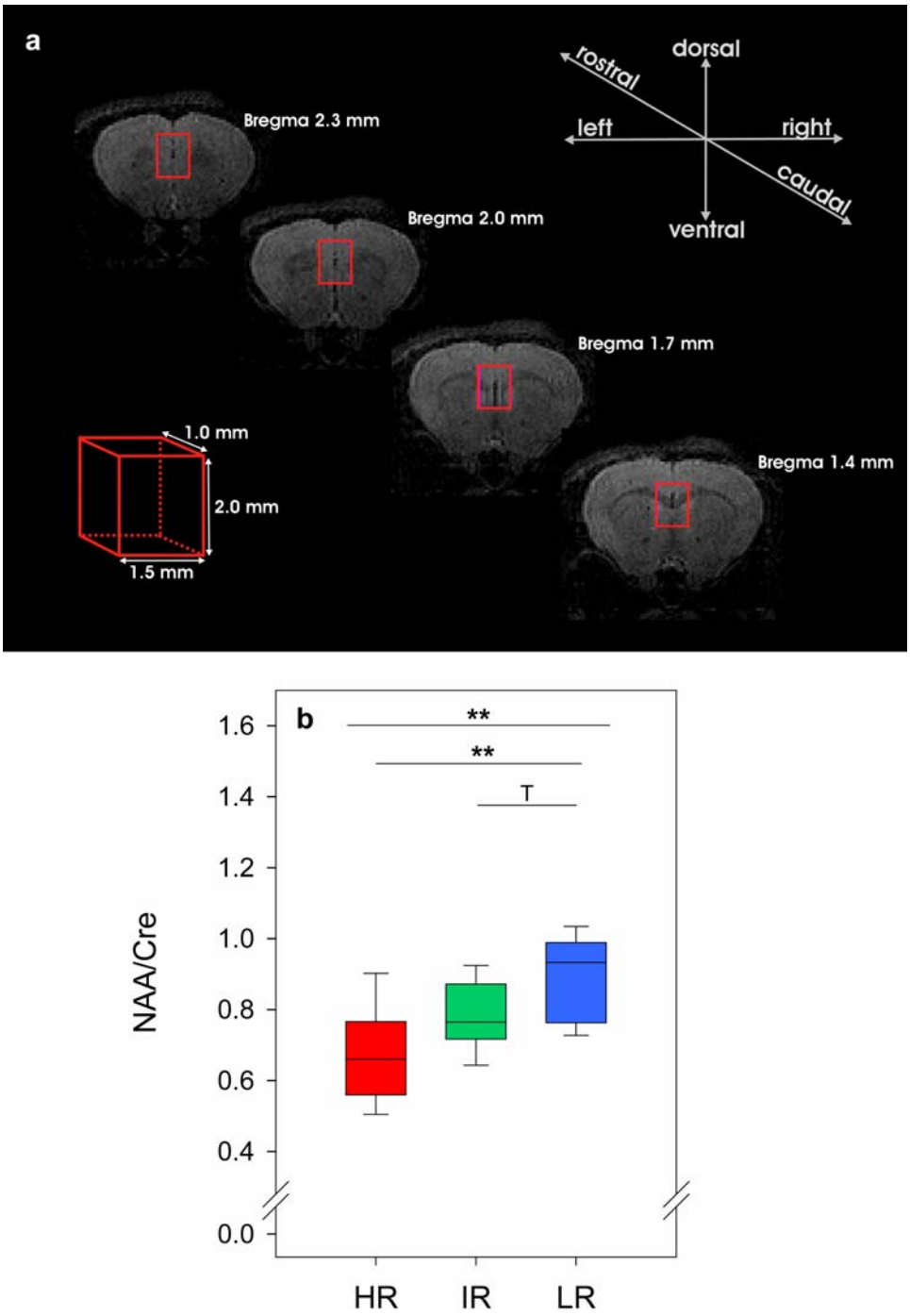

Fig. 4: Localization and volume of region of interest for prefrontalcortex spectroscopic measurements (a) and N-acetylaspartate (NAA) levels normalized to Creatin (Cre) in the prefrontal cortex of mice selectively bred for high (HR), intermediate (IR) and low (LR) stress reactivity. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. Data was statistically analyzed using the KWH-test followed by *post-hoc* MWU-tests where appropriate (Bonferroni corrected $p<0.01$**, $p<0.1$T).

Prefrontal Cortex Signal Intensity

In contrast to the spectroscopy results the three lines did not differ in T1w-signal intensity of the cingulate cortex 1 and 2 (HR: 2.09 ± 0.09, IR: 2.08 ± 0.03, LR: 2.15 ± 0.04, KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 3.6, df = 2, p = 0.161), the infralimbic cortex (HR: 1.91 ± 0.03, IR: 1.95 ± 0.03, LR: 2.00 ± 0.02, KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 2.1, df = 2, p = 0.348) or the prelimbic cortex (HR: 2.09 ± 0.04, IR: 1.97 ± 0.03, LR: 2.02 ± 0.03, KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 3.8, df = 2, p = 0.147).

Volumes of the Ventricles

Interestingly, LR mice were revealed to have significantly larger relative lateral ventricle volume than HR and IR mice (KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 25.7, df = 2, p<0.001; *post-hoc* MWU-tests: U = 10-125, HR *vs.* LR: p<0.001; HR vs. IR: p = 0.533; IR vs. LR: p<0.001; Fig. 5) as well as a larger relative volume of the third ventricle in comparison to IR mice (HR: 6.8E-6 ± 4.7E-7 mm^3, IR: 5.8E-6 ± 2.4E-7 mm^3, LR: 8.1E-6 ± 2.4E-7 mm^3, KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 17.4, df = 2, p<0.001; *post-hoc* MWU-tests: U = 5-74, HR *vs.* LR: p = 0.092; HR vs. IR: p = 0.186; IR vs. LR: p<0.001).

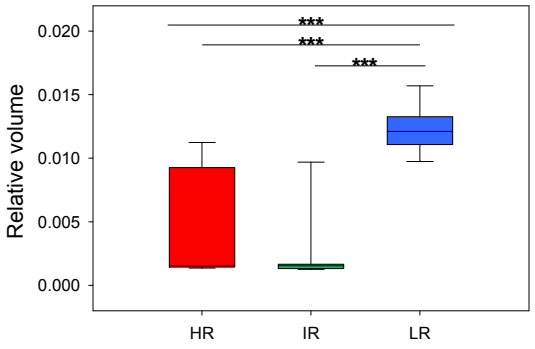

Fig. 5: Lateral ventricle volume of mice selectively bred for high (HR), intermediate (IR) and low (LR) stress reactivity. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. Data was statistically analyzed using the KWH-test followed by *post-hoc* MWU-tests where appropriate (p<0.001***).

Whole Brain Measures

The intracranial volume did not significantly differ between the three lines (HR: 537.1 ± 5.3 mm^3, IR: 537.1 ± 4.7 mm^3, LR: 532.3 ± 7.07 mm^3, KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 0.1, df = 2, p = 0.936). Despite the differences in lateral ventricle volume, the total volume of the brain (excluding the ventricles) did not differ between the three lines (HR: 5237.7 ± 5.7 mm^3, IR: 532 ± 4.7 mm^3, LR: 521.7 ± 6.9 mm^3, KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 1.2, df = 2, p = 0.554). The mean T1w signal intensity of the whole, as a measure of unspecific accumulataion of manganese, did not differ between the three lines (HR: 55.7 ± 3.1, IR: 50.1 ± 3.5, LR: 50.4 ± 2.0, KWH-test: HR: N = 14, IR: N = 15, LR: N = 15, H = 3.0, df = 2, p = 0.225).

BEHAVIORAL TESTS

Y-Maze Test

HR mice displayed an inferior performance in the Y-maze spatial learning test compared to IR and LR mice. Both LR and IR mice spent significantly more time exploring the novel arm than the familiar arms after a 30-min inter-trial-interval, demonstrating a recognition of the previously explored arms (W-test: IR: N = 12, Z = -2.19, p = 0.034; LR: N = 15, Z = -3.35, p = 0.001; Fig. 6). HR mice, however only tended to prefer the novel arm over the familiar one (W-test: N = 12, Z = -1.65, p = 0.099; Fig. 6). This indicates that the HR mice show deficits in this task.

Reversal Learning Test

Mice from the three breeding lines did not significantly differ in their acquisition of the task (KWH-test: HR: N = 11, IR: N = 11, LR: N = 8, H = 0.8, df = 2, p = 0.657; Fig. 7), nor did they differ in their retention of the task on the subsequent day (KWH-test: HR: N = 11, IR: N = 11, LR: N = 8, H = 3.9, df = 2, p = 0.140; Fig. 7). However, in the reversal phase of the test, HR mice needed significantly more trials to reach the learning criterion than LR mice (KWH-test: HR: N = 11, IR: N = 11, LR: N = 8, H = 7.0, df = 2, p = 0.025; *post-hoc* MWU-tests: U = 14-37, HR *vs.* LR: p = 0.027; HR vs. IR: p = 0.133; IR vs. LR: p = 0.258; Fig. 7).

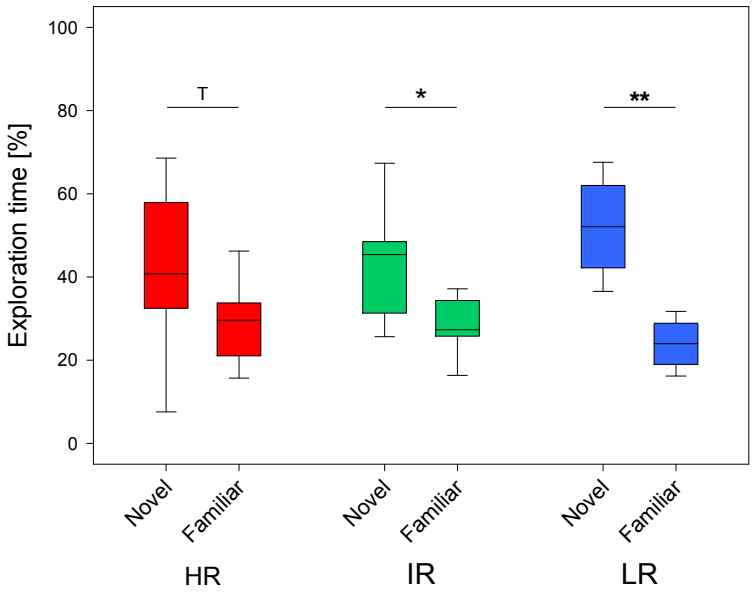

Fig. 6: Y-maze spatial learning test. Percent time spent exploring a novel arm *versus* the mea of two familiar arms of mice selectively bred for high (HR), intermediate (IR), and low (LR stress reactivity, respectively. Data are depicted as box plots showing medians (lines in th boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile ar indicated by the whiskers. Data was statistically analyzed using the W-test ($p<0.05$*, $p<0.1$T).

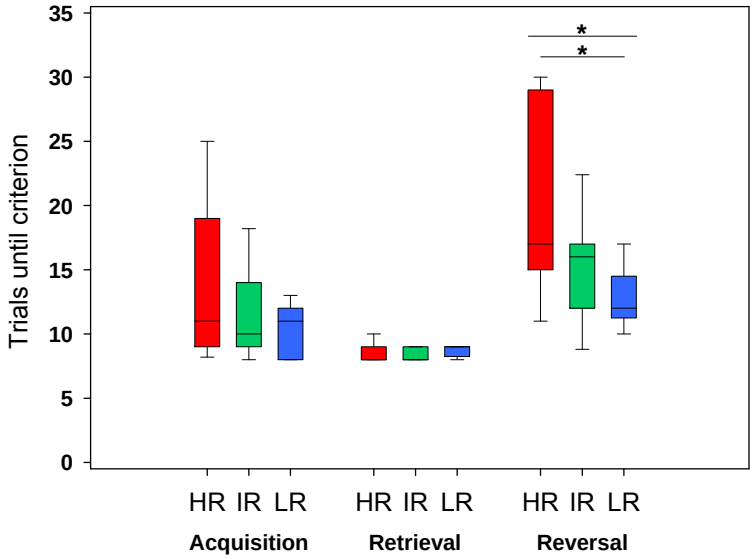

Fig. 7: Reversal learning test in mice selectively bred for high (HR), intermediate (IR), and low (LR) stress reactivity. The number of trials necessary to reach the learning criterion of 8/10 correct trials for each stage of the test, acquisition, retrieval and reversal, are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes). The 10% percentile and 90% percentile are indicated by the whiskers. Each phase of the test was statistically analyzed separately using the KWH-test followed (results given above the line encompassing the perimeter of all three box plots) by *post-hoc* MWU-tests (results given above the line connecting the middle of the box plots being compared) where appropriate

Tab. 1: Proteins differentially regulated in the hippocampus of high (HR) or low (LR) stress reactive mice. All proteins listed differ significantly in expression between HR and LR mice (t-test) with $p<0.05$. The arrows indicate an up (↑) or down (↓) regulation in HR mice in comparison to LR mice. The moleuclar weight (MW) and isoelectric point (pI) values were determined using LaGrange regression.

Protein name	Biological Process	MW	pI	Regulation in HR	Fold Change	MASCOT Score	ID Peptides	Coverage (%)
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	Cell communication; Signal transduction	38048	5.6	↑	3.50	181	19	59
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	Metabolism; Energy pathways	39254	6.41	↑	3.50	64	9	29
Cofilin-1	Cell growth and/or maintenance	18776	8.22	↓	-2.48	183	10	65
Glutamate dehydrogenase 1, mitochondrial	Metabolism; Energy pathways	61640	8.05	↑	18.41	242	26	44
Pyruvate kinase isozymes M1/M2	Metabolism; Energy pathways	58378	7.18	↑	4.02	188	18	38
Aconitate hydratase, mitochondrial	Metabolism; Energy pathways	86151	8.08	↑	5.09	278	25	37

HIPPOCAMPAL PROTEOME ANALYSIS

Five protein spots were found to differ between LR and HR mice. For a represenative gel please see Supplementary Fig. 1. All spots were successfully identified by MALDI-TOF/TOF. One of these spots contained two distinct proteins leading to a total of six identified proteins. These proteins are: cofilin-1 ($p = 0.003$, fold change LR × HR: -2.48), glutamate dehygrogenase 1 ($p<0.001$, fold change LR × HR: 18.41), pyruvate kinase isozymes M1/M2 ($p = 0.028$, fold change LR × HR: 4.02), aconitate hydratase ($p = 0.002$, fold change LR × HR: 5.09), guanine nucleotide-binding protein G(I)G(S)/G(T) subunit beta-2/ pyruvate dehydrogenase E1 subunit beta ($p = 0.027$, fold change LR × HR: 6.4). Their biological significance and molecular functions were ascertained using the Human Protein Reference Database (HPRD –http://www.hprd.org). The majority of these proteins were involved in metabolism/energy pathways. The results are summarized in table 1.

DISCUSSION

Using behavioral tests, MEMRI, ^{1}H-MRS, and proteomics we have here investigated the effects of increased stress reactivity on cognitive function, neuronal activity, integrity and hippocampal protein expression in a mouse model selectively bred for extremes in stress reactivity.

The HR mice are unique in the aspect that they are genetically predisposed to develop an increased HPA axis reactivity. Unlike most models of depression focusing on a dysrergulated HPA axis, the HR mice have not repeatedly been subjected to a defined experimental stressor, however, they are likely to be subjected to increased amounts of glucocorticoids in response to events such as hadling, cage changing or aggressive encounters with conspecifics. This provides a more natural model of HPA axis dysregulation in psychiatric diseases. HR mice furthermore have an increased circadian trough level of corticosterone, which also contributes to increased glucocorticoid exposure (Touma et al., 2008; Touma et al., 2009).

As expected, we found a reduction of NAA/Cre in both the dorsal hippocampus and the PFC of HR mice (see Fig. 1 & 4). NAA was initially believed to reflect neuronal loss (Sager et al., 1995), but this assumption has been questioned as decreased NAA levels were found to be reversible (Davie et al., 1994; Hugg et al., 1996; Kalra et al., 1998; Gonul et al., 2006; Block et al., 2009). This suggests that NAA levels are more strongly linked to the functional integrity of the neurons. Some studies conducted on depressed patients have reported a reduction in NAA/Cre in the prefrontal region in comparison to healthy controls (Gruber et al., 2003; Gonul et al., 2006) in concordance to our findings, whereas other studies have found no such difference (Coupland et al., 2005; Nery et al., 2009). These studies, however, did not investigate HPA axis activity in the patient sample, which may explain some of the discrepancies between the reported findings. Furthermore, several studies of patients suffering from SZ reported decreased NAA/Cre levels in the hippocampus compared to healthy controls (Bertolino et al., 1998; Blasi et al., 2004) in similarity to our findings.

A close relationship between glucose metabolism and NAA synthesis has been demonstrated in humans (Moreno et al., 2001). It has also been proposed that low NAA levels could reflect mitochondrial dysfunction (Clark, 1998; Signoretti et al., 2001). Together with our findings of altered protein expression of several mitochondrial proteins, this may suggest that the mitochondria of the HR mice could be damaged by increased glucocorticoid exposure during their life. Corticosterone has been shown to suppress adenosine-5'-triphosphate (ATP) production in mitochondria from hypothalamic cell cultures (Fujita et al., 2009) and hippocampal cell cultures (Brooke et al., 1998). Interestingly, the comparison of the HR and LR hippocampal proteome revealed that several proteins involved in energy metabolism were indeed differentially regulated between the lines. This is in accordance with post mortem studies in patients suffering from MD, SZ and bipolar disorder that have reported alterations in several proteins involved in energy metabolism (Iwamoto et al., 2005; Beasley et al., 2006; Martins-De-Souza et al., 2010).

Another protein found to be differentially expressed in HR and LR mice was cofilin 1, which has previously been demonstrated to inhibit glucocorticoid receptors (Ruegg et al., 2004). The biological function of an inhibitor of glucocorticoid receptors would most likely be to regulate the amount of glucocorticoid receptors that are activated in order to protect the cells from being damaged due to excess glucocorticoids (Rogatsky et al., 1999; Ruegg et al., 2004; Amaral et al., 2009). Thus, a decreased expression of cofilin 1 in HR mice might lead to an increased susceptibility to damage incurred by glucocorticoids, however, further experiments would have to be conducted to

substantiate this theory. Interestingly, cofilin 1 was also found to be down-regulated in the PFC of SZ brain tissue (Martins-de-Souza et al. 2009a).

In the context of NAA measurements and energy balance, it is important to note that the three lines did not differ in their absolute Cre levels, as the NAA values were normalized to Cre. Cre is known to be involved in maintaining the energy balance in the brain and is reported to be neuroprotective (Chaturvedi and Beal, 2008). This is in accordance with previously published data showing that there are no effects of glucocorticoid exposure on Cre levels in healthy subjects (Scheel et al., 2009).

The relative T1w-signal intensity in the hippocampus, as assessed by MEMRI, was also reduced in HR mice compared to LR mice. Manganese enters the cells via voltage-gated calcium channels (Simpson et al., 1995). Thus, the more active the neuron, the more the calcium channels will open and the more manganese will accumulate in the cell, leading to increased signal intensity. Therefore, it may be assumed that reduced T1w image intensity in the hippocampus of HR reflects a reduced activity of the hippocampal neurons.

Interestingly, we did not find any decrease in the volume of the hippocampus of HR mice, which implies that the volumetroc, functional, and metabolic markers show different aspects of detrimental effects of increased stress reactivity. Lower functional activity and reduced NAA levels in HR mice may primarily be caused by mitochondrial dysfunction, whereas hippocampal atrophy due to hypercortisolism is subtle. Clinical studies have found contradicting results regarding hippocampal volume in MD, with some studies indicating that patients suffering from unipolar depression have reduced hippocampal volume in comparison to healthy controls (Bremner et al., 2000; Campbell et al., 2004; Kaymak et al., 2009; McKinnon et al., 2009) and others reporting no such difference (Keller et al., 2008; Bearden et al., 2009; Kronmuller et al., 2009).

HR mice exhibited the lowest MEMRI signal intensity of the three breeding lines in the PFC, however, the intensity of this region was not significantly decreased in comparison to IR or LR mice. This does not coincide with the findings of decreased signal intensity along with decreased NAA/Cre levels in the hippocampus, however, the two measures need not coincide as they reflect different aspect of cellular functions.

The behavioral measures correspond well with the functional alterations of the hippocampus and prefrontal cortex. The hippocampus-dependent (Sanderson et al., 2009) spatial learning test used here, the Y-maze test, demonstrates that the HR mice have inferior spatial memory compared to the IR and LR mice. This has also been shown in previous studies (Knapman et al., 2009) Furthermore, the increased amount of trials needed by the HR mice to complete the reversal phase of the reversal learning test demonstrate that HR mice show perseverance, indicative of poor PFC function. This is accordance with previously published data (Knapman et al., in press-b). The results of the behavioral test fit well with the decreased amount of NAA/Cre in the prefrontal cortex and hippocampus of the HR mice.

Interestingly, the LR mice were serendipitously found to have larger lateral ventricles than both IR and HR mice. As LR mice perform better than IR and HR mice in cognitive tests and have a higher NAA concentration in the hippocampus and PFC in comparison to the other two breeding lines, it would appear that this increase in ventricle size does not lead to a reduced functioning of the brain structures in the vicinity of the ventricles. A possible positive aspect of enlarged ventricles could be that also the subventricular zone (SVZ) is enlarged, as it lies adjacent to the lateral walls of the lateral ventricles. Cells originating in the SVZ migrate to the corpus callosum, striatum, and fornix where they differentiate into oligodendrocytes (Menn et al., 2006). The ventricular system has furthermore been implicated in transporting nutrients and waste (Lowery and Sive, 2009), as well as carrying signaling molecules that regulate neurogenesis and cell survival (Miyan et al., 2003; Johanson et al., 2008). It should however be mentioned that most studies reporting enlarged ventricles have found larger ventricles in patients suffering from psychiatric diseases such as SZ (Chua and McKenna, 1995; Wright et al., 2000; Shenton et al., 2001) and MD (Shima et al., 1984). It is therefore important to distinguish between pathologically increased ventricles that may be causing internal hydrocephalus and ventricles that are simply larger without any loss of intracranial volume, as is the case of the LR mice. C57/BL-6 mice are for example generally better at cognitive tests than CD-1 mice (Dellu et al., 2000; Gerlai, 2002) and have larger ventricles (Li et al., 2009; Nag et al., 2009) than all three lines of mice examined here.

Taken together the alterations of the hippocampus and PFC of HR mice are very similar to what is observed in patients suffering from MD and SZ. Furthermore, the finding of an altered expression of several proteins involved in energy

metabolism in HR mice compared to LR mice lends support to the hypothesis of mitochondrial dysfunction in psychiatric diseases. The HR mice could thus be used in future studies probing the underlying mechanisms of hippocampal and PFC dysfunction in psychiatric diseases and be a promising tool in the search for new targets for the treatment of MD and SZ.

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SUPPLEMENTARY MATERIAL

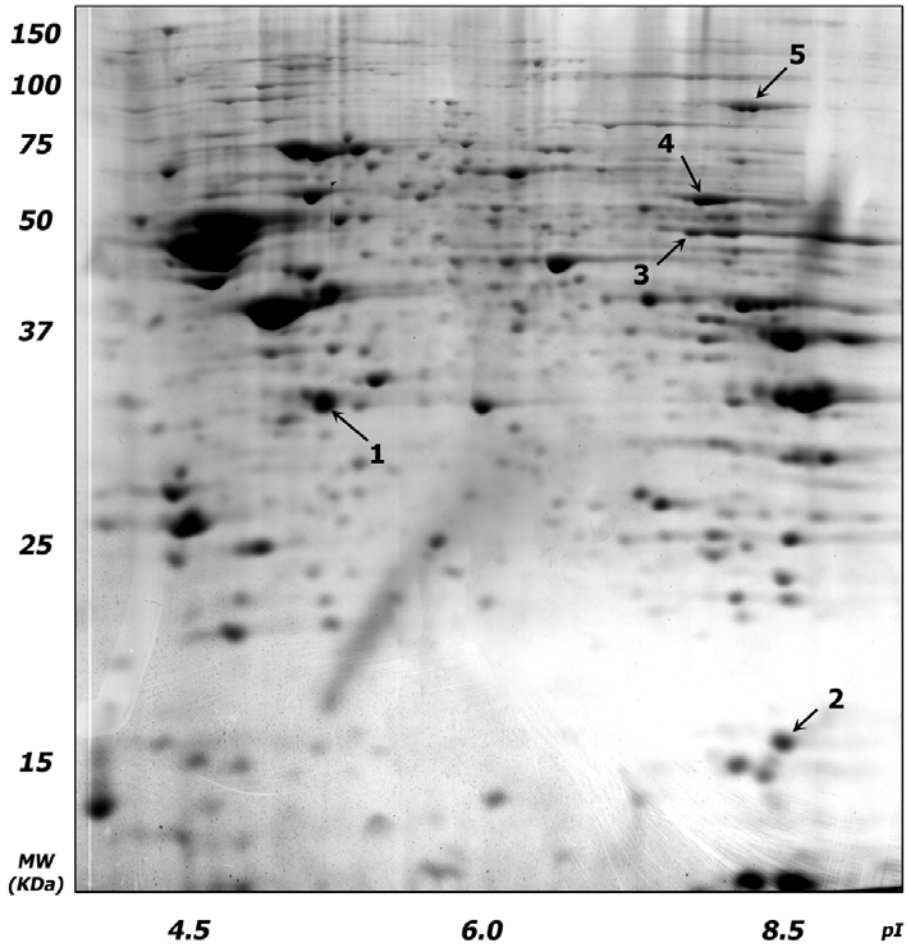

Fig. 1: Representative 2-dimentional gel indicating the identified spots; Guanine nucleotide binding protein subunit beta-2 and Pyruvate dehydrogenase E1 component subunit beta (spot 1), Cofillin 1 (spot 2), Glutamate dehydrogenase 1 (spot 3), Puyruvate kinase isozymes M1/M2 (spot 4) and Aconitate hydratase (spot 5).

Tab. 1: Initial plasma corticosterone levels and reaction plasma corticosterone levels (i.e. increase in response to a standardized stressor) in mice selectively bred for high (HR), intermediate (IR), and low (LR) stress reactivity. Mice are derived from breeding generations (Gen.) nine, twelve and fourteen. Data was statistically analyzed using the KWH-test followed by *post-hoc* MWU-tests where appropriate (Bonferroni corrected $p<0.05$*, $p<0.01$**, $p<0.001$***, >0.1 = T)

		Initial plasma corticosterone levels			**KWH-test**			***Post hoc* MWU-test**						
	N	**Mean ± SEM**			**Group Comparison**				**HR vs. LR**		**HR vs. IR**		**IR vs. LR**	
Gen	**HR/IR/LR**	**HR**	**IR**	**LR**	**H**	**p**		**U**	**p**		**p**		**p**	
9	12/12/12	14.4 ± 2.3	5.5 ± 1.8	2.1 ± 0.6	18	<0.001	***	3-46	<0.001	***	0.003	***	0.143	n.s
12	16/15/15	10.42 ± 2.4	3.52 ± 1.3	0.92 ± 0.1	18	<0.001	***	29-75	<0.001	***	0.078	T	0.002	**
14	4/0/4	10.5 ± 3.3		3.0 ± 1.9				2	0.083	T				

		Reaction plasma corticosterone levels			**KWH-test**			***Post hoc* MWU-test**						
	N	**Mean ± SEM**			**Group Comparison**				**HR vs. LR**		**HR vs. IR**		**IR vs. LR**	
Gen	**HR/IR/LR**	**HR**	**IR**	**LR**	**H**	**p**		**U**	**p**		**p**		**p**	
9	12/12/12	327.5 ± 8.4	184.1 ± 6.3	73.3 ± 4.2	31	<0.001	***	0	<0.001	***	<0.001	***	<0.001	***
12	16/15/15	376.2 ± 19.0	183.7 ± 3.7	78.02 ± 9.8	40	<0.001	***	0	<0.001	***	<0.001	***	<0.001	***
14	4/0/4	453.1 ± 35.3		49.8 ± 8.4				0	0.021	*				