Behavioral phenotyping, gene expression profiles,

and cognitive aspects in a mouse model of trait anxiety

Dissertation

an der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

vorgelegt von

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München, 15. Januar 2008

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Tag der mündlichen Prüfung: 05.06.2008

Diese Arbeit ist meinen Eltern gewidmet.

Summary

Anxiety reflects a fundamental emotion, essential for survival. However, if it occurs unpredictably and exaggerated for a long period of time, it becomes pathological, confining a normal course of life. Anxiety disorders are among the most disabling psychiatric diseases, with increasing incidence. They are complex and occur as a combination of both, inherited and stress-related phenomena, whose origin and underlying mechanisms are still poorly understood. Besides clinical studies, extensive preclinical research is strongly focusing on the genetic, environmental, and developmental underpinnings of both, "physiological" and "pathological" anxiety.

Thus, in the year 2000, two mouse lines were generated by bi-directional selective inbreeding, reflecting extremes in trait anxiety. These phenotypic extremes, independent of gender, display either high (HAB) or low (LAB) anxiety-related behavior as measured in the elevated plus-maze test and a variety of other paradigms. Since anxiety is not considered as a single entity, but covers multiple facets, the studies presented in this thesis address behavioral, neuroendocrine, genetic, developmental as well as cognitive aspects in this mouse model of trait anxiety.

Comprehensive phenotyping confirmed the phenotypic divergence of the mouse lines. Although selection pressure was only exerted on anxiety-related behavior, the mouse lines exhibited comorbid depression-like and altered explorative behavior. Moreover, expression profiling of genes well described in the regulation of emotionality at the level of the hypothalamo-pituitary-adrenocortical axis and synaptic neurotransmission, as well as pharmacological intervention, highlighted arginine-vasopressin (AVP), corticotropin-releasing hormone (CRH), and synaptotagmin 4 (*Syt4*) as potential mediators contributing to the observed behavioral differences. AVP has been identified to be under-expressed in several brain regions of LAB mice associated with their non-anxious and non-depression-like behaviors. In addition, several genetic polymorphisms have been identified that are likely to play a critical role in the AVP under-expression of these animals. In contrast, the highly anxious HAB animals revealed a CRH over-expression in various brain areas. The significance of CRH over-expression in mediating the HAB-specific phenotype was pharmacologically validated via CRH receptor 1 antagonist administration. Synaptic release, indicated by *Syt4* expression, was found to be altered in both inbred mouse lines in opposite directions, indicating a dysregulation in both extremes of trait anxiety. Furthermore, glyoxalase1 (Glx1), a cellular detoxification enzyme, has been identified to be differently expressed already at early postnatal developmental stages in association with the phenotypic

divergence. Thus, Glx1 might act as a biomarker suitable for the early prediction of pathological anxiety.

As anxiety disorders have often been described to be accompanied by alterations in cognitive abilities, this coherency was also addressed in the HAB/LAB model. Indeed, HAB mice showed a superior ability in a social learning paradigm and displayed delayed extinction in a classical fear-conditioning study, the latter being similarly observed in patients suffering from posttraumatic stress disorder.

Taken together, the HAB/LAB mouse model covers many clinical core symptoms of anxiety disorders at different levels, including behavioral emotionality, gene expression, and cognitive alterations. Therefore, it provides a valuable and promising tool to elucidate the neurobiological basis of the continuum from vital to pathological anxiety.

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1   General introduction

The brain, and the brain alone, is the source of our pleasures, joys, laughter, and amusement, as well as our sorrow, pain, grief, and tears. It is especially the organ we use to think and learn, see and hear, to distinguish the ugly from the beautiful, the bad from the good, and the pleasant from the unpleasant. The brain is also the seat of madness and delirium, of the fear and terrors which assail by night or by day, of sleeplessness, awkward mistakes and thoughts that will not come, of pointless anxieties, forgetfulness, and eccentricities.

Hippocrates, ca. 400 BC

1.1   Neuropsychiatry

All human experience, emotion, motivation, behavior and cognitive functions are the products of brain functions. The clinical discipline of neuropsychiatry is the basis of contemporary approaches to understand human behavior as well as the effects of brain dysregulation (Cummings and Mega, 2003). It is devoted to the neurobiological basis, optimal assessment, natural history, and the most efficacious treatment of disorders (Cummings and Hegarty, 1994; Kendler, 2005). Furthermore, neuropsychiatry does not only integrate the interplay between environmental influences on the nervous system during the development of the individual and throughout adulthood and old age, but seeks for the mechanisms underlying the disorders of the central nervous system (CNS) that are responsible for abnormal behavior. This discipline combines both the psychiatric manifestation of neurological diseases and the neurobiology of psychiatric diseases, which evolved from biological psychiatry and behavioral neurology. The main focus of biological psychiatry is the identification of biological treatments for psychiatric diseases, focusing on the chemistry of behavior, by increasing our knowledge of transmitter systems and signal cascades, but not emphasizing the neuroanatomy of behavior or the relationship of CNS lesions to behavioral disorders. Behavioral neurology, in contrast, provides a detailed description of, for example memory disturbances, visuospatial abnormalities or dementia as a consequence of brain damage or degenerative CNS disease. Probing the mental status is used to aid in the neuroanatomical interpretation of deficit disorders, but does not focus on the symptoms of neuropsychiatric disorders such as depression, mania or personality alterations associated with brain dysfunction (Yodofsky and Hales, 2002). Due to the limitations of both fields of research, a combination of both provides a comprehensive knowledge of the relationship of brain and behavior in addition to the recent advantages in

neuroscience. Neurologic and psychiatric research approaches are moving closer together in the tools they apply, the questions they ask, and the theoretical frameworks they employ. The interests of neurology and psychiatry converge within the framework of modern neuroscience. Further progress in understanding brain diseases and behavior demands a more comprehensive integration of these fields (Price et al., 2000; Martin, 2002). Brain disorders, in contrast to other medical counterparts, are manifested by alterations in behavior and experience of the patient; in many ways they are disorders of the person rather than disorders that happen to the individual (Cummings and Mega, 2003).

1.2   Anxiety disorders

According to the standardized diagnostic criteria of the American Psychiatric Association´s Diagnostic and Statistical Manual of Mental Disorders, the subtypes of anxiety states include panic disorders, agoraphobia, specific phobia, social phobia, generalized or overanxious disorder, separation anxiety, obsessive-compulsive disorder, posttraumatic stress disorder (PTSD), and antisocial disorder (Sadock and Sadock, 2005). Since the 1990s, there has been an increasing interest in anxiety disorders caused by the finding that they emerged to be the most prevalent mental disorder in the general population (Kessler et al., 1994; Kessler et al., 2001; Kessler et al., 2005a; Kessler et al., 2005b; Sadock and Sadock, 2005). The lifetime prevalence estimated in the USA and Europe ranks from 13.6% up to 28.8%, with women being affected twice as often as men (Kessler et al., 1994; Cummings and Mega, 2003; Alonso et al., 2004; Bystritsky, 2006). This means that roughly one out of three people will meet the criteria for an anxiety disorder at least once in their lifetime. Anxiety disorders are among the most disabling neuropsychiatric diseases. Anxiety is claimed to be pathological when it occurs unpredictably, as discrete attacks, without identifiable triggers or a non-relative anxiety response over a long period of time. It results in symptoms such as sweating, trembling or shaking, chest pain, chills, palpitations, and shortness of breath (Sadock and Sadock, 2005). These symptoms are of such tremendous emotional intrusiveness that they disable the patients and overshadow them with feeling of fear, dread, failure, or even death, making any normal life impossible (Cummings and Mega, 2003). Furthermore, there is a large overlap and comorbidity with other disorders, especially with major depression. About 60% of patients with anxiety disorders additionally suffer from depression, which makes a certain similarity of underlying mechanisms rather likely (Landgraf, 2001; Lieb et al., 2005). Although several epidemiological studies revealed the prevalence of this group of disorders, they remain poorly understood, understudied, and

inadequately treated. There are evidence-based treatments available with the efficiency of psychological and biological treatment between 60 and 85% for anxiety disorders. When anxiety and depression disorders are taken together, only half of the patients who are seriously affected and around 25% of those with mild mental disorder receive adequate treatment (Demyttenaere et al., 2004; Kessler et al., 2005b; Bystritsky, 2006). Furthermore, this class of disease, due to its high social and economic burden, decreases productivity and increases morbidity, mortality, and drug abuse in a wide range of the population (Leon et al., 1995; Ustun et al., 2004; Bystritsky, 2006).

Before going into detail concerning already described brain dysregulations underlying anxiety and affective disorders one has to define what a "normal" reaction to a variety of different stressors is including. In the last decade, there has been a rapid progress in identifying the neurobiological basis of anxiety and fear. Specific neurochemical and neuropeptide systems have been demonstrated to play an important role in the behaviors associated with fear and anxiety-producing stimuli (Sadock and Sadock, 2005).

1.3   Stressors and stress response

One of the life´s main characteristics is maintaining a complex and dynamic equilibrium within the body (= homeostasis) which can be disturbed by internal or external challenges called stressors (Tsigos and Chrousos, 2002). Stress was first described by Hans Seyle in 1936 as "the nonspecific response of the body to any demand", altering the internal milieu with the neuroendocrine, cardiovascular, immune, and gastrointestial systems being the first to experience functional changes. Stressors can therefore be defined as conditions that endanger, or are perceived to endanger the survival of an individual. They can roughly be grouped into three categories: a) psychological stressors that are based on innate or learned responses to a threat of an impending aversive condition such as fear or anxiety; b) stressors consisting of a physical stimulus accompanied by a strong psychological component such as pain or immobilization and c) stressors which affect the cardiovascular homeostasis by exercise or heat exposure (Selye, 1936; Van de Kar and Blair, 1999; Carrasco and Van de Kar, 2003). Due to possible misinterpretation of the terms "psychological" and "physiological" which could refer to the experimenters´ control of stressor interpretation by the animal, an alternative classification has been suggested to avoid anthropocentric stressor categorization (Engelmann et al., 2004). *Real threats* (or *"systemic" stressors*) include for example pain (visceral and somatic), neuronal, or humoral homeostatic signals or humoral inflammatory signals. They represent genuine homeostatic

challenges and activate stress centers in the hypothalamus of the brain via somatic, visceral, or circumventricular sensory pathways. *Predicted threats* (or *"neurogenic" or "progressive" stressors*) contain the innate or learnt capability of anticipating/recognizing predators or dangers associated with novel a environment. These kinds of stressors are mainly processed by limbic brain areas, such as the amygdala, hippocampus, and prefrontal cortex (PFC). Most of the naturally occuring stressors include, at least partially, aspects of both categories (Sawchenko et al., 2000; Herman et al., 2003; Engelmann et al., 2004). Nevertheless, independent of categorization hypothesis, most stressors have in common that they lead to adaptive responses to gain a new setpoint of homeostasis, which is termed allostasis (Engelmann and Ludwig, 2004). During threatening situations, two neuroendocrine protection systems get activated: one that is very rapidly responding, the sympathetic-adrenomedullary (SAM) system and one with an increased reaction latency, the hypothalamo-pituitary-adrenocortical (HPA) axis (Tsigos and Chrousos, 2002; Förstl et al., 2006). Alarm reactions, after a sudden aversive situation, include an immediate non-specific behavioral response (such as startle) followed by a specific response (e.g. flight) and are primarily associated with the SAM system, nuclei of the brainstem, the vagal nerve, and the medulla of the adrenal gland. A variety of physiological parameters go together with behavioral responses, enabling the individual to successfully prepare the body to perform the appropriate reaction to the stressor by *active coping*. Many of the autonomic changes are produced by sympathetic and parasympathetic neural systems via adrenaline and noradrenaline release from the adrenal medulla into the blood circulation. The activation of both systems leads for example to an increase in blood pressure and pulse beat (via stimulation of the locus coeruleus) as well as an increase in respiratory rate (via activation of the nucleus parabrachialis), gluconeogenesis and lipolysis to provide the body with required oxygen and nutrients (Charney et al., 1998; Tsigos and Chrousos, 2002; Engelmann et al., 2004; Charmandari et al., 2005; Förstl et al., 2006).

On the other hand, whenever an encounter is perceived as being aversive and cannot be brought under control via a fight/flight reaction, *passive coping* occurs which is linked to HPA axis activation, followed by a hormonal change to allow adaptive redirection of both energy and behavior (Engelmann et al., 2004). The HPA axis, besides its involvement in circadian rhythm as well as in metabolic function, acts as the main central neuroendocrine system of the stress response and is, together with the hypothalamic-neurohypophysial system (HNS), part of the hypothalamo-pituitary system. The hypothalamus controls the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which, in turn, stimulates the

secretion of glucocorticoid hormones (mainly 95% cortisol in humans and 95% corticosterone (Cort) in mice) from the adrenal cortex, which are the final effectors of the HPA axis (Tsigos and Chrousos, 2002; Charmandari et al., 2005).

Hypothalamo-pituitary-adrenocortical (HPA) axis
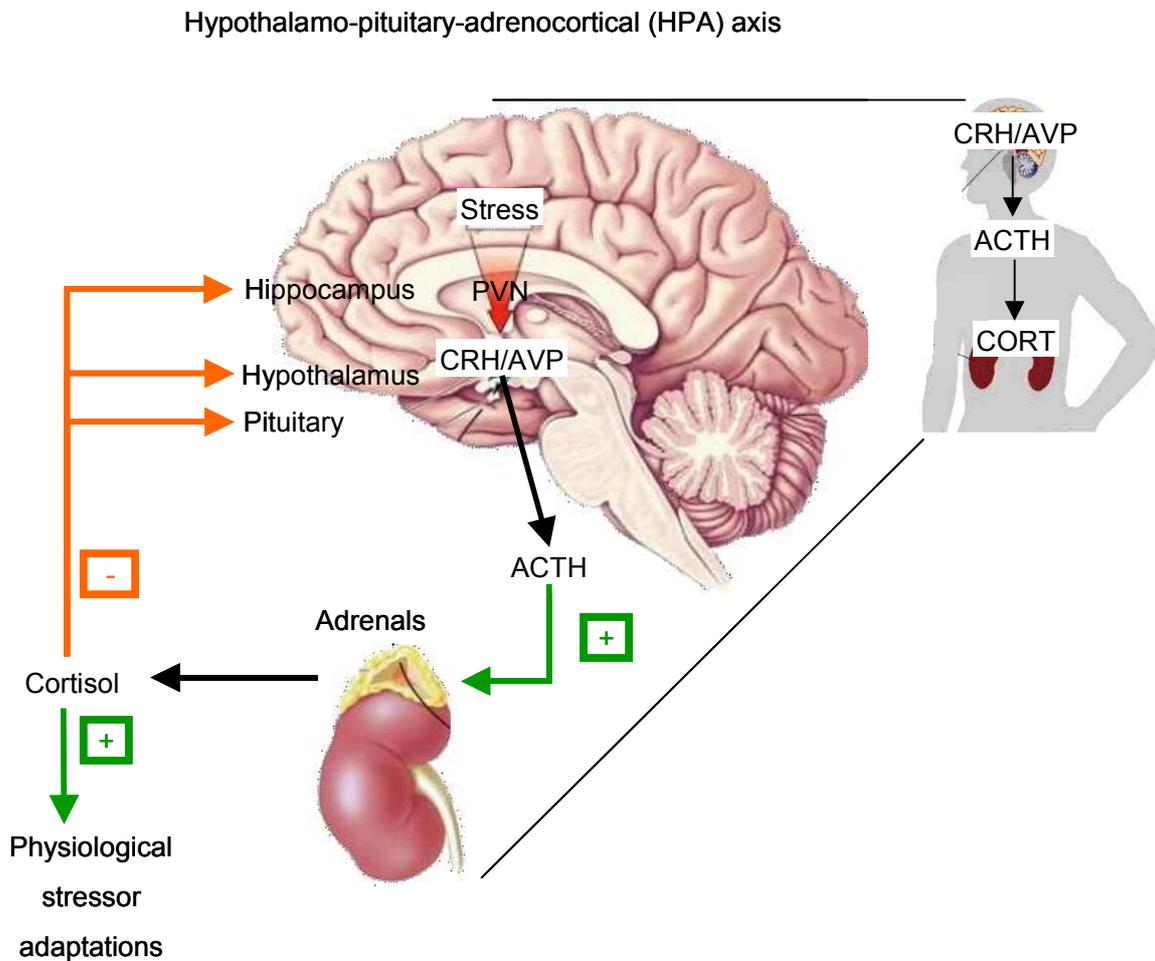
Figure 1.1:   Schematic overview of the location, components, and function of the
               hypothalamo-pituitary-adrenocortical (HPA) axis. Paraventricular nucleus
               (PVN) of the hypothalamus; Arginine-vasopressin (AVP); Corticotropin-
               releasing hormone (CRH); Adrenocorticotropic hormone (ACTH). This figure
               has been kindly provided by Dr. Elisabeth Frank.

The central control of the glucocorticoid secretion is in principle actively regulated by a selected population of neurosecretory neurons in the hypothalamic paraventricular nucleus (PVN), a region flanking the third ventricle from both sides. The PVN in humans and rats can anatomically be divided into several subunits including the posterior magnocellular region,

dorsolateral medial parvocellular zone, ventral medial parvocellular and dorsal parvocellular subregions (Herman et al., 2002). PVN projecting neurons are localized in regions that receive first- or second-order inputs from somatic nociceptors, visceral afferents, or humoral sensory pathways. These rapid and reflexive activations are evoked by e.g. adrenergic and noradrengergic innervations mainly from the nucleus of the solitary tract, serotonergic projections from the raphe nuclei, γ-aminobutyric acid (GABA)-ergic neurons from numerous regions of the hypothalamus, thalamic sensory nuclei and/or direct non-hypothalamic forebrain inputs such as the bed nucleus of the stria terminalis (BNST). Additional information is derived from the fluid and electrolyte status by way of the subfornical organ/lamina terminalis system. Indirect projections are largely confined to regions critical for emotional responses and memory, such as hippocampus, PFC, amygdala, lateral septum (LS), and thalamus. Furthermore, the intrinsic organization of the PVN is positioned for the cross-talk between hypophysial, neurohypophysial, and preautonomic cell populations (Herman and Cullinan, 1997; Herman et al., 2003). Upon stimulation by stress, parvocellular neurons of the PVN that express corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) project axonal terminals towards the zona externa of the median eminence, where they stimulus-dependently release these two main ACTH secretagogues into the portal blood of the anterior pituitary. CRH mediates its effect via the CRH receptor 1 (CRHR1), a G-protein that releases cyclic adenosine monophosphate (cAMP) by adenylylcyclase action, which in addition phosphorylates protein kinase A that evokes the synthesis of proopiomelanocortin (POMC) in anterior pituitary cells. AVP potentiates this effect, by activating the G-protein coupled AVP receptor 1b (V1b) receptor leading to the activation of a phosphatidylinositol pathway that via protein kinase C stimulates POMC expression (Birnbaumer, 2000; Klinke and Silbernagel, 2000). The glandotropic hormone ACTH is encoded by one splicing variant from the precursor POMC that additionally encodes for the opioid peptide β-endorphine and α-melanocortin stimulating hormone. ACTH is transported through the systematic circulation and binds to membrane-bound receptors in a cAMP-dependent manner, leading to the one-site synthesis (cholesterine-derived) and secretion of corticoids, especially glucocorticoids from the zona fasciculata of the adrenal cortex. The final effects of glucocorticoid actions include energy mobilization (glyolysis) in the liver, suppression of innate immunity in immune organs, inhibition of bone and muscle growth, potentiation of sympathetic-nervoussystem-mediated vasoconstriction, proteolysis, lipolysis, and suppression of reproductive functions (Munck et al., 1984; McEwen and Stellar, 1993). This wide range of effects led to the assumption that glucocorticoids restore

homeostasis, e.g. increasing glucose can refill lost energy stores or the inhibition of other hormonal systems reduces loss of energy on processes, not related to the immediate challenge (Herman et al., 2003). Furthermore, they increase the perception of acoustic, tactile, and olfactory stimuli. However, adequate control of glucocorticoids needs to be accomplished and such `negative feedback` is efficiently exerted by healthy organisms. Due to their high lipophility, glucocorticoids can pass the blood-brain-barrier without resistance and bind to glucocorticoid (GR) and mineralocorticoid (MR) receptor in the pituitary, hypothalamus (mainly PVN), and hippocampus. They inhibit the expression of AVP, CRH, and ACTH by complex-binding of cortisol and cytoplasmatic receptors that diffuse into the cell nucleus. By diffusion into the cell nucleus, cortisol is released from the complex and binds to specific DNA sequences to inhibit gene transcription (Lodish et al., 2001). Two kinds of feedback reactions are reported, one that reacts to the cortisol increase in the plasma within a few minutes and the other one that responds to later elevated cortisol concentrations. Additionally, an inhibition of HPA axis action is reported via AVP release within the PVN after emotional stressors, which provides a negative tonus on ACTH secretion (Wotjak et al., 1996). Taken together, these negative feedback mechanisms are essential for determining the stress response and to re-establish body homeostasis. Another system that belongs to the hypothalamo-pituitary system is the HNS. Magnocellular cells of the PVN and the supraoptic nucleus (SON) project through the zona interna of the median eminence to the posterior pituitary to release AVP and oxytocin (OXT) from axonal terminals into the blood circulation. OXT is, among others, involved in uterus muscle contraction at parturition and the milk injection, whereas AVP contributes to vasoconstriction and the maintenance of a physiological plasma osmolality by regulating water reuptake in the kidney (Klinke and Silbernagel, 2000). It has also been reported that these two neuropeptides contribute to HPA axis activation upon stress by modulating the activity of parvocellular PVN neurons via the release of AVP and OXT from magnocellular PVN neurons. Furthermore, they influence the secretion of ACTH at the level of the median eminence and the posterior pituitary (Engelmann et al., 2004). While stress hormone secretion during hostile conditions benefits survival, a long-term dysregulation of the involved systems can lead to psychiatric diseases, such as anxiety disorders and/or depression (Charney and Deutch, 1996; Van de Kar and Blair, 1999).

1.4   Pathological changes in anxiety and depression disorders

Importantly, anxiety and depression, being dramatic and debilitating multi-facetic psychiatric illnesses demonstrate marked overlap and co-occurence (Landgraf, 2001). Many of their symptoms are similar, which makes them difficult to distinguish and an overlap of underlying mechanisms contributing to these pathologies rather likely (Kalueff et al., 2007).

One of the best documented description in the research on anxiety disorders and depression is the finding of the dysregulation of the HPA axis (Tichomirowa et al., 2001). Prominent HPA axis abnormalities among depressed patients are an increased number of ACTH-secretory episodes followed by increased cortisol-secretory episodes, elevated urinary cortisol, and increased cortisol and CRH levels in the cerebrospinal fluid (CSF) (Holsboer and Barden, 1996). 30-60% of depressed patients show a biological anomaly in HPA axis acitivity, with 60% of acute depressed patient displaying changes in ACTH and cortisol secretion (Rubin et al., 2001; Ising et al., 2005). Hypoactivation of the stress system is characterized by the chronically reduced secretion of CRH and noradrenaline and has been reported in patient suffering from atypical depression (Charmandari et al., 2005). In contrast, hyperactivation of the HPA axis and the SAM system is manifested in melancholic depression (Engelmann et al., 2004). Severe anxiety and depression can result from exaggerated stimulation of one or more of the CRH-regulated pathways via CRHR1 and CRHR2 receptors (Carrasco and Van de Kar, 2003). High CRH concentrations have been observed in postmortem studies of severely depressed suicide victims, accompanied by an increased number of CRH-expressing neurons and a decreased amount of CRH receptors in several brain region, thus suggesting that the CRH system plays a leading role in the etiology of major depression as well as in anxiety disorders (Raadsheer et al., 1994; Arborelius et al., 1999; Strohle, 2003; Bale, 2006). Similar effects have been reported for AVP, with elevated AVP levels inducing depressive symptomatology and an increased number of AVP-containing PVN neurons in postmortem studies of depressed subjects (Purba et al., 1996; Muller et al., 2000; Scott and Dinan, 2002). Additionally a decreased expression of MR in the hippocampus has been reported in depressed patients (de Kloet et al., 1998), leading to an increased CRH expression in the PVN and elevated basal Cort levels, caused by a lack of feedback inhibition. This hypercortisolism over a long period of time can lead to a reduction of GR expression in the hippocampus and PVN, resulting in a "glucocorticoid resistance" (Pariante and Miller, 2001; Makino et al., 2002). The dexamethasone/CRH test allows the functional control of the HPA axis activity to be measured at both the hypothalamic and the anterior pituitary level. For instance, after

administration of the synthetic glucocorticoid antagonist dexamethasone the test would reveal a dysfunction of the negative glucocorticoid feedback via decreased suppression of ACTH secretion. Application of synthetic CRH accompanied by stimulation of corticotropic neurons, indicate disturbances at the level of the anterior pituitary. Depressed patients show decreased suppression of basal and CRH-induced ACTH release indicative of a reduced efficiency of feedback mechanisms as well as an increased stimulation of anterior pituitary cells leading to HPA axis hyperactivity (Holsboer, 1989; Holsboer and Barden, 1996). Additionally, this test, measuring HPA axis dynamics, can act as a good indicator for relapse probability. Increased cortisol levels, despite psychopathological recovery, are highly associated with an increased risk of relapse within the following six months (Zobel et al., 1999). Other neurocircuitry data suggest a crucial involvement of GABA-containing pathways in comprising HPA axis abnormalities observed in human stress pathologies (Herman and Cullinan, 1997). Long lasting increased cortisol concentrations may also lead to modifications of certain neuronal circuits causing symptoms of depression (Nemeroff, 1996). Beside the HPA axis, there are several other systems, e.g. serotinergic, noradrenergic, dopaminergic, or cholinergic circuits, that are likely to be involved in these psychiatric diseases. In addition, there are additional peptides, such as neuropeptide Y, galanin, cholecystokinin or opioid peptides that have gained interest in recent research (Holmes et al., 2003).

Epidemiologic studies revealed that about 40-50% of the risk for depression is genetically determined, which makes this disease, among others (e.g. asthma, hypertension), a highly heritable disorder (Fava and Kendler, 2000; Nestler et al., 2002a). Similar findings have been reported for panic disorders, where family studies showed an increased rate of effected first-grade relatives of patients suffering from panic disorders (Finn and Smoller, 2001; Bandelow et al., 2002). Twin studies reported a significantly higher risk for developing depression or panic disorder in monozygotic compared to dizygotic twins (Kendler et al., 1993; Smoller and Finn, 2003). This increased vulnerability also holds true for the risk of the comorbidity of anxiety disorders and depression (Lieb et al., 2002). In contrast, for generalized anxiety disorder as well as for social phobia, twin studies seem to be more inconsistent and current models are more focused on the combination of environmental and genetic factors contributing to these psychiatric diseases (Rosenbaum et al., 1992; Heim and Nemeroff, 2001; Förstl et al., 2006). These environmental factors include events like chronic stress of psychological or physiological origin (like mobbing or chronic inflammation), traumatic events (e.g. loss of a beloved person), and aversive early-life events (such as

neglect or sexual abuse) (Heim and Nemeroff, 2001; Sadock and Sadock, 2005). In the past years there is also an increasing interest in epigenetic phenomena induced by environmental factors resulting in e.g. histone acetylation/methylation or cytosine methylation. It has been demonstrated in twin studies that these DNA-protein interactions decisively change gene expression and can be correlated to certain phenotypic changes (Cardno and Gottesman, 2000; Kato et al., 2005). Research on genetic predisposition succeeded in identifying single-nucleotide polymorphisms (SNPs) pointing to a crucial involvement in controlling HPA and SAM axis activity (Ising and Holsboer, 2006). Furthermore, there is an ongoing effort in running unbiased approaches, such as microarray studies or linkage analysis to identify further genes underlying mental disorders. Therefore, it is important to annotate that the genetic blueprint (nature) and the biographic impact (nurture) interact and that in most cases neither one alone can lead to the development of the clinical phenotype (Sillaber and Holsboer, 2004). In some diseases, such as red-green color-vision deficiency, it is well described that the unequal recombination of two pigment genes leads to gene deletion or the formation of hybrid genes that explain the majority of the common red-green color-vision deficiencies (Deeb, 2004). This disease, among others, acts as one example, where a disease or deficiency is restricted to one or a manageable group of genes. Psychiatric diseases, in contrast, are among the most complex diseases due to their multigenic background, with single genes mainly producing small effects and being hard to detect. Pharmacogenomics is not only working on underlying genes and the numerous genetic variations that have been shown to affect disease susceptibility and drug response, but also tries to improve therapy on the basis of genetic information for each patient by focusing on the individual, sex-specific differences, and treatment outcome (Pinsonneault and Sadee, 2003).

1.5   Current pharmacological treatment

Clinical therapy mainly makes use of drugs that have been discovered by serendipity and empirically developed classes of substances. Treatment of depression was revolutionized about 50 years ago by the introduction of the two effective antidepressants: the tricyclic antidepressants and the monoamine oxidase (MAO) inhibitors. The original tricyclic agent, such as imipramine was discovered from antihistamine research, whereas an early MAO inhibitor, e.g. iproniazid, was derived from studies on antitubercular drugs. Both display antidepressive effects by increasing the bioactive amount of noradrenaline and serotonin via inhibition of a degrading enzyme or primary inhibition of neuronal reuptake. The

antidepressive effect of these two substance classes provided for the first time medical treatment of depression. The discovery and success of these antidepressants led to the development of the so called second generation medications, including selective serotonin reuptake inhibitors (SSRIs, like paroxetine or citalopram), selective noradrenaline reuptake inhibitors (NRIs, like reboxetine), or a combination of both (SNRIs, e.g. Venlafaxin) (Forth et al., 1998; Möller et al., 2002; Nestler et al., 2002b). Different classes of antidepressants are also the first-choice in treating anxiety disorders (Strohle, 2003), but additionally they are treated with benzodiazepines, like diazepam (valium) and buspirone, both representing tranquilizers or glutamate receptor ligands (Briley and Nutt, 2000). Most benzodiazepines assist the impact of GABA, whereas buspirone inhibits 5-hydroxytryptamine (5-HT, serotonin) neurons by stimulation of somatodendritic 5-HT$_{1A}$ autoreceptors (Forth et al., 1998).

The onset of the antidepressive or anxiolytic effects can last at least four weeks until they achieve any symptomatical relief and are, beside tolerance development and addiction, often accompanied by a variety of side effects, including sedative effects, fatigue, increased appetite, dizziness, sleep disturbances and agitation, diarrhea, nausea, and sexual dysregulation. These side effects reduce the patients´ quality of life by worsening familial, social, and professional abilities (Strohle, 2003; Cassano and Fava, 2004). Furthermore, there is still a high number of patients that are resistant to any kind of medication and less than 50% of patients demonstrate complete remission to the initial therapy with any type of antidepressant (Nestler et al., 2002a; Moller, 2004; Bystritsky, 2006). Besides insufficient drug treatment and diagnostic parameters, we also lack objective diagnostic tests to identify and categorize patients to allow early diagnosis and concerted treatment. The research of the past years reduced the number of side effects, but has not decisively improved the treament of patients (Nestler et al., 2002a; Nestler et al., 2002b).

Taken together, the current medications for anxiety disorders as well as for depression are not satisfying in terms of discharging the patient to allow them a constant healthy and normal everday life. Therefore, intensive resesrach on identifying genetic determinants of abnormal emotionality and the discovery of novel targets, biomarkers, and treatments of psychiatric diseases is urgently needed (Nestler et al., 2002b).

1.6   Human research and the necessity of animal models

For several reasons, beside ethical ones, humans are less-than-ideal for research on neuropeptides. First, although blood samples are easy to obtain for determining plasma

hormone concentrations, peripheral and CNS peptide release are independently regulated. The high amount of plasma peptidases together with the blood-brain barrier make an infer of CNS peptide physiology from plasma hormone concentrations rather impossible. Furthermore, the use of biopsy to directly assess tissue peptide concentrations is not ideal, as it can not be routinely repeated, is limited to superficial structures, and holds the risk of potential morbidity. The cerebrospinal fluid (CSF) is more appropriate for substance detection because it reflects extracellular transmitter concentrations due to its direct CNS contact and is separated from peripheral sources by the blood-brain barrier. The limitations of human CSF studies comprise a lack of information about the regional CNS source of peptide change and the difficulty to obtain CSF from healthy controls. Postmortem studies have been informative, but are unreliable in terms of postmortem delay, previous drug treatment, and coexisting illnesses. Additionally, choosing the right controls, that are not only matched for age and gender, but also for health parameters and other demographic variables is complicated, because there is a high heterogenity among the human research population (Sadock and Sadock, 2005).

Taken all these disadvantages together, animal models have been proven to be essential and necessary tools for any medical achievements and they are of increasing importance in psychiatric research (McKinney, 2001). Animals can be used in studies specially designed to induce psychopathology via experimental manipulations, such as brain lesion or intracerebroventricular (icv) drug injection. They can be bred, reared, maintained, and observed under standardized laboratory conditions, which allows a better scientific control over environmental influences or provide the basis for specific manipulation either genetically or environmentally. Moreover, animals, especially rodents, due to their shorter natural life span allow the assessment of long-term effects of any planned intervention and treatment. Even "intergenerational" consequences of a particular pathology or the efficiency of treatments can be obtained (Sadock and Sadock, 2005). Ideally, animal models should mimic the specific conditions in humans regarding disease etiology, symptomatology, treatment, and biological basis (McKinney, 2001). Due to higher cognitive levels such as motivation or self reflection in humans, it is obviously rather unlikely that any animal model could ever reproduce every feature of a particular human psychopathological disorder, but current research is making use of several strategies and different models to overcome this problem. There are three main criteria to be fullfilled by a valid animal model. The causal conditions (*construct* validity) and the diagnosed symptoms (*face* validity) should be similar to those found in humans and pharmacological treatment of the animals should result in

equal or similar quantifiable effects to those observed in patients (*predictive* validity) (McKinney, 2001). Meeting such requirements is difficult, because a high number of cardinal symptoms (e.g. feelings of worthlessness or suicidal ideation) are defined by subjective verbal report, which is impossible to measure in animals. However, features such as weight loss and appetite, anhedonia, sleep disturbances, or psychomotor changes can be assessed quite well in animals along with the use of a variety of behavioral tests reflecting emotionality (Sillaber and Holsboer, 2004).

Many laboratories use rodents as experimental animals, because they can be bred and housed easily, are phylogenetically close to humans (Sprott and Ramirez, 1997), and share many analogous physiological and behavioral parameters with the human organism (Ohl, 2005). Mice for example have a 92% exonal sequence homology with humans in common (Brudno et al., 2004).

There are several strategies inducing symptoms of psychopathologies in rodents. For instance, exposing the animals to chronic stress by sequential applications of unpredictable stressful conditions (Overmier and Seligman, 1967; Schmidt et al., 2007) or early life stressors such as maternal deprivation (McKinney and Bunney, 1969; Schmidt et al., 2003). Others focus on behavioral changes by virtue of different environmental influences such as rearing style (Weaver et al., 2004) or enriched environment (Friske and Gammie, 2005). Although these models can successfully mimic stress-related pathologies in humans, such as consequences of early neglect, they ignore the influence of genetic factors (Sillaber and Holsboer, 2004). Another approach is therefore the development of genetically engineered mice, by producing a conventional knockout animal, lacking the target gene from an early developmental stage on or the development of conditional knockouts where the deletion of the gene is regionally and temporally restricted. Besides the identification of the involvement of single genes in central dysregulation and behavioral changes, these strategies are accompanied by several disadvantages. Conventional knockouts can result in lethality early in development or unpredictable alterations in gene expression and compensating effects resulting in false positive or negative findings. The conditional knockout, as well as other new techniques like antisense-targeting or specific manipulation via adeno-associated virus application, allow a more direct and temporal control of a certain gene in a specific brain region, however, some drawbacks still remain concerning correct analysis, interpretation, and the disregard of the multigenic background (Plomin and Crabbe, 2000; Lightman et al., 2002; Sillaber and Holsboer, 2004). A third main focus is the bidirectional inbreeding of animals, selected due to a certain phenotype, resulting in behavioral extremes, reflecting

symptoms of a defined psychiatric disease. There are different approaches such as selecting and inbreeding animals according to their passive coping style (Vaugeois et al., 1997) or their stress-induced Cort increase, mimicking endophenotypes of affective disorders (Touma et al., 2006). The use of selective breeding increases the representation of genetic factors associated with a certain trait by shifting the respective phenotype from the strain´s average (Kromer et al., 2005).

Animals from the high (HAB) and low anxiety-related behavior (LAB) rat model were selected according to their anxiety-related behavior by using the elevated plus-maze (EPM) test. After inbreeding over several generations these breeding lines exhibits symptoms of pathological anxiety (Liebsch et al., 1997; Liebsch et al., 1998; Landgraf and Wigger, 2002). The two rat lines feature a high genetic homogenity and fullfill all three formerly mentioned criteria of a valid animal model, allowing the investigation of multiple genetic and environmental factors contributing to the animals´ trait anxiety and depression-like behavior (Wigger et al., 2001; Keck et al., 2003b; Landgraf, 2003; Murgatroyd et al., 2004). However by virtue of selection, accompanied by genetic drift, co-segregation of genes, dispensable for anxiety- and depression-like behavior, is possible and leads to false positive results correlated to psychopathologies. Furthermore, a reduction related to the genetic background or a loss of underlying genes can occur. This pitfall could be circumvented by running different sublines. As neither mice nor rats or other species used for research, can reflect entire human psychopathologies, it is necessary to use several research strategies, trying to close the lack of invasive methods in clinical research, by identifying linked neuronal structures, circuits, and genes (Cryan and Mombereau, 2004).

1.7   The HAB/LAB mouse model

Although the HAB/LAB rat model is among the most powerful tools used to investigate behavioral, neurobiological, and neuroendocrine parameters as well as environmental influences on "trait" (continously, e.g. by genetic predisposition) anxiety, the use of genetic approaches is limited in rats (Lister, 1990; Finn et al., 2003; Landgraf and Wigger, 2003).

Modern genetic approaches applicable to mice make them an advantageous model organism to uncover functional candidate genes and gene products underlying trait anxiety and depression (Tarantino and Bucan, 2000). Furthermore, the mouse genome has been fully sequenced and numerous gene marker are available to detect genetic changes (Gregory et al., 2002; Waterston et al., 2002). Therefore, beginning in the year 2000, we

established a mouse model using the same breeding strategy for CD1 mice as formerly used for Wistar rats (Liebsch et al., 1997).
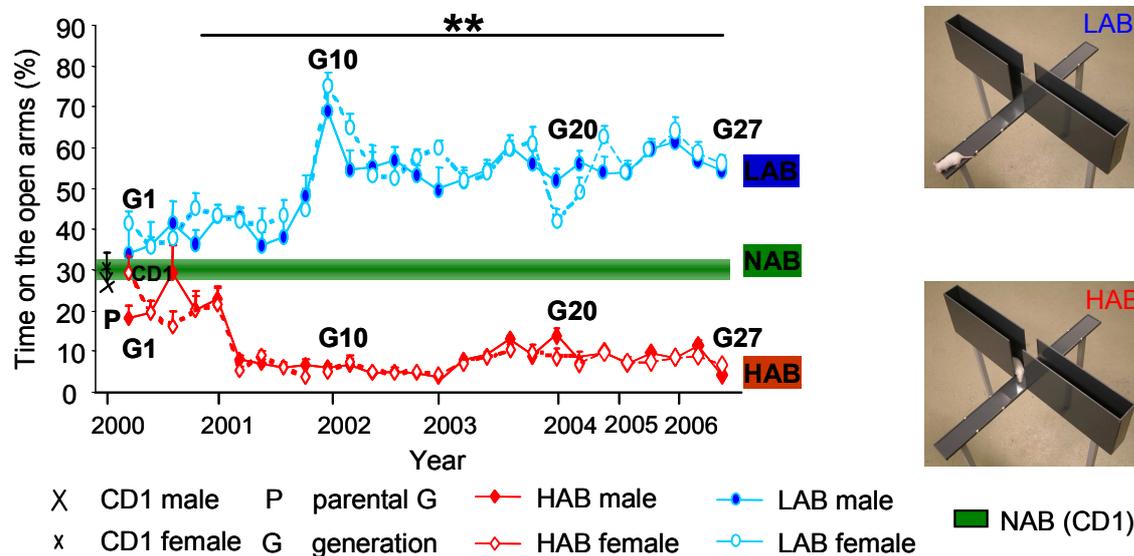
Figure 1.2:   *Breeding progress of the HAB/LAB mouse model. Left: Elevated plus-maze (EPM) data (% time spent on the open arms) of the parental generation and G1 to G27 of HAB and LAB mice, CD1 (NAB) mice as controls. Independent of gender, HAB and LAB animals differ significantly in their anxiety-related behavior (*** p<0.001, G1-G27) with NAB mice displaying an intermediate behavior (n=40-80 per line and generation). Right: Non-anxious LAB mice explore the a priori aversive open arms, whereas HAB animals spend most time in the protective closed arms.*

The EPM test is a common, unconditioned, and pharmacologically validated behavioral test measuring anxiety in rodents. It is a plus-shaped apparatus, elevated from the floor, consisting of two aversive open arms, two dark arms with protecting walls, and a neutral zone in the middle (see 3.2.2). It is based on the animals´ conflict between exploratory drive and their inborn anxiety of unprotected, elevated, and illuminated areas, with the time spent in the latter being the indirect index for high or low anxiety (Pellow et al., 1985; Lister, 1987). Around 250 male and female Swiss CD1 mice (Charles River, Sulzfeld, Germany) were used as the origin for selective and bidirectional breeding for anxiety-related behavior in the EPM test. According to the time spent on the open arms of the EPM, mice were bred with adequate partners to give rise to the behavioral extremes. Animals spending most of the test time and mice spending the least amount of time on the open arms established the

hyperanxious HAB and the hypoanxious LAB breeding lines respectively, which significantly differ in their EPM performance upon the fourth generation independently of gender (see Figure 1.1). These lines do not only differ in their spontaneous behavior on the EPM, but also in a variety of other behavioral tests, including additional tests measuring anxiety-related behavior such as the ultrasonic vocalization test, the dark-light box test, or open arm exposure, with HAB mice always displaying higher anxiety levels. Additionally, tests for depression-like behavior, including the forced swim and tail-suspension test, revealed a decreased depression-like behavior in LAB animals in comparison with HAB and "normal" anxiety-related behavior animals (NAB). Apart from the broad phenotyping and a pharmacological validation using diazepam in pups, glyoxalase 1, an enzyme involved in cellular detoxification, was identified as a biomarker indicative of trait anxiety (Kromer et al., 2005; Ditzen et al., 2006). Thus, the HAB/LAB mouse model is introduced as a new tool to investigate genetic predisposition, central dysregulation, and environmental factors contributing to pathological anxiety.

2   Aim and scope of the thesis

As anxiety is considered a multidimensional domain covering different levels, including behavioral, neuroendocrine, genetic, developmental as well as cognitive aspects, the following questions have been addressed by analyzing the HAB/LAB mouse model:

1) Do the two mouse lines of trait anxiety, beside their performance in the EPM test, differ in other behavioral tasks, including additional tests measuring anxiety-related behaviors as well as depression-like and explorative behaviors? Are there any associations between gene expression and the respective phenotypes?

2) During which developmental stages does the behavioral divergence of the mouse lines occur? Is there any association between gene expression and the onset of emotional differences?

3) Is there any linkage between extremes in trait anxiety and cognitive abilities in the HAB/LAB mouse model?

3   Animals, material, and methods

3.1   Animals

Animals of all lines, HAB, NAB, LAB, or cross-mated mice (F1, offsprings of reciprocal cross-mated HAB and LAB mice) were strictly treated in parallel concerning care, mating, and behavioral testing performed at the Max Planck Institute of Psychiatry, Munich. Standard laboratory conditions contained group housing with three to five animals per cage (type 2-macrolone, 25.5 x 19.5 x 13.8cm), in a 12-h light/dark schedule (lights on at 7am), with room temperature at 23 ± 2ºC, 60% humidity, food (Nr. 1314, Altromin GmbH), and tap water ad libitum. The animal studies were both approved by local authorities and conducted according to current regulations for animal experimentation in Germany and the European Union (European Communities Council Directive 86/609/EEC).

Data presented were obtained from male animals from generation 14 - 25. All tests were carried out at the age of seven to twelve weeks, with an initial EPM test procedure. Surgeries and the additional behavioral test paradigms were separated by at least 2 days recovery time. All behavioral testings have been performed between 8am and 1pm (Kromer et al., 2005).

3.2   Behavioral tests for emotionality

3.2.1   Ultrasonic vocalization test

It has been suggested that the number of ultrasonic vocalization (USV) calls induced by separation and isolation can be considered as a measure of separation anxiety and can be predictive of adult emotionality (Dichter et al., 1996; Brunelli, 2005; D'Amato et al., 2005). Therefore, as an early test for anxiety-related behavior, following the developmental pathways of emotional extremes, the USV was used to monitor the phenotypes in our breeding lines (Kromer et al., 2005). Each pup got separated from the dam and was gently placed onto a Petri dish (diameter: 15cm; wall height: 1.5cm; kept at a constant temperature of 23ºC by a water bath below the dish) without olfactory or auditory contact to its litter. Using a Mini-3 Bat detector (Ultrasonic Advice, London, UK), fixed about 10cm above the pup, ultrasonic vocalization was recorded for five min using a WM-D6C tape recorder (Sony Professional, Cologne, Germany). For analysis, the number of vocalization calls at 70kHz were recorded and quantified by the Eventlog 1.0 program (EMCO Software, R. Henderson, Germany, 1986). As a measure of locomotor activity the Petri dish was divided into 2 x 2cm squares and the line crossings (both forepaws and shoulders across the line) were directly

counted during the 5-min period (Figure 3.1). Furthermore, rotations defined as 360º movements of the pup were quantified. At the end of the trial each animal was weighed and the dish cleaned with an 80% alcohol solution.
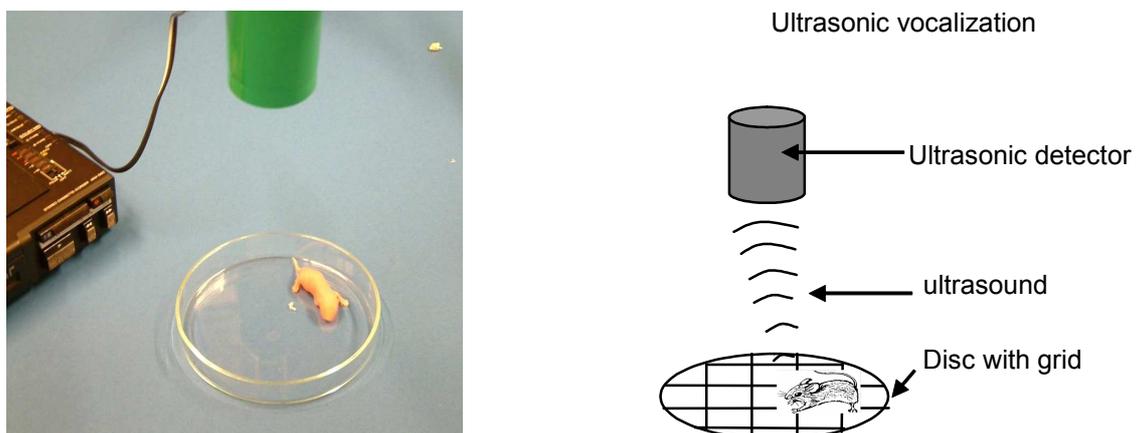
Figure 3.1:   Ultrasonic vocalization test. Left: Experimental setup. Right: Schematic overview of the recording of ultrasound by the ultrasonic detector and the grid on the Petri disc to define locomotor activity.

3.2.2   Elevated plus-maze test

The elevated plus-maze (EPM) test is based on creating a conflict between the mice´s exploratory drive and its innate fear of illuminated, unprotected and heightened areas (Lister, 1987). It consists of a plus-shaped platform elevated 37cm above the floor, with two open (30 x 5cm), two closed (30 x 5 x 15cm) arms and a connecting central zone (5 x 5cm) made of grey PVC with the whole apparatus being surrounded by a black curtain to prevent the experimental animal from visual or auditory cues (Figure 3.2). The open arms were lit by white light of 300lux, the neutral zone by 60lux and the closed arms by 5lux. The maze was cleaned with water containing detergent before the introduction of each mouse.

Animals were directly transferred from their homecage to the test apparatus, starting with the animal placed on the central part facing one of the closed arms. During a 5-min exposure, following parameters were recorded by means of a video/computer system (Plus-maze V2.0, Ernst Fricke, Germany, 1993) by an observer blind to line and/or treatment: the number of entries into open and closed arms (both forepaws and shoulders within the arm), the percentage time spent on the open arms (ratio of time spent on the open arms to total time spent on all arms), the full entries (all four paws within the arm) into the open arm and the

latency to the first entry into an open arm (Kromer et al., 2005). All animals have been weighed afterwards.
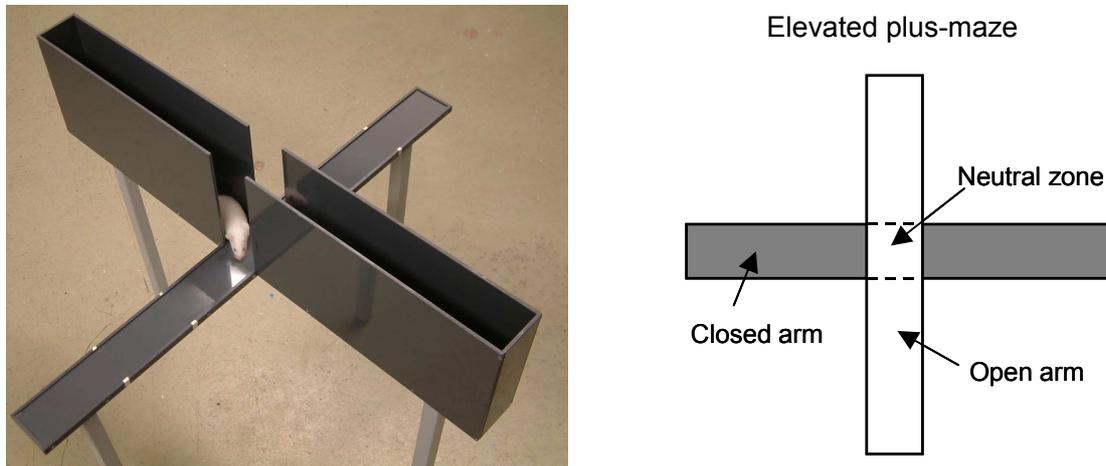
Figure 3.2:   Elevated plus-maze test. Left: Experimental setup. Right: Schematic overview of the defined areas of the maze used for the behavioral analysis.

3.2.3   Open arm exposure

To monitor the animals´ behavior in an unavoidable mild stress situation, one open arm (OA) of the EPM was separated from the central compartment by a plastic board to prevent the test animal from leaving the open arm. The arm was illuminated by 100lux and divided by small white stripes, fixed on the edge of the arm, into a proximal, medial and distal part. At the beginning of the experiment, each animal was placed onto the distal part of the OA facing the plastic board (Figure 3.3). After each trial the arm was cleaned with water containing detergent. During the 5-min test, the number of entries and time spent in the distal, medial, and proximal zone, locomotor activity (indirect measurement via entries to the different parts of the open arm), and time of exploratory head movement (whole head outside the bounds of the arm) was recorded and quantified by using the Eventlog 1.0 computer system (EMCO Software). A compartment was defined to be entered if the animals´ front paws and shoulders were inside the respective part. The open arm was cleaned with water containing detergent before the introduction of each mouse.
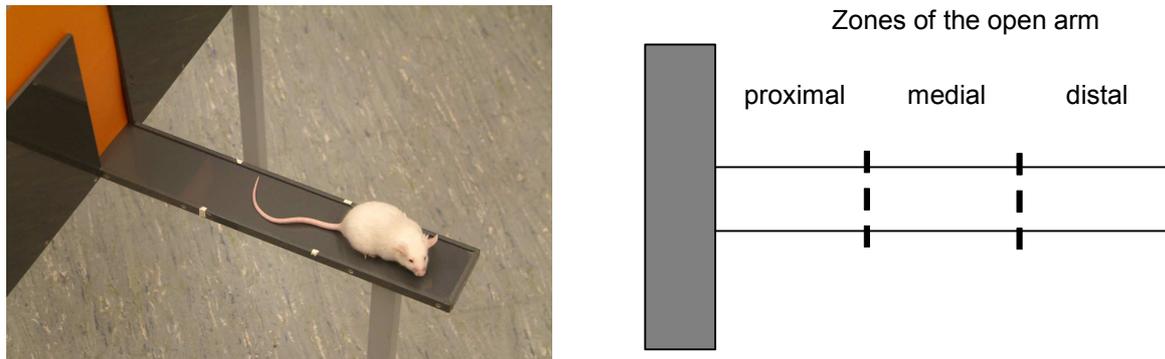
Figure 3.3:   *Open arm exposure. Left: Experimental setup. Right: Schematic overview of the defined areas of the open arm used for the behavioral analysis.*

3.2.4   Dark-light box test

The dark/light (DaLi) paradigm, as an additional behavioral task measuring anxiety-related behavior, is based on the innate aversion of rodents to brightly illuminated areas and on their spontaneous exploratory behavior, applying a mild stressor including novel environment and light (Hascoet et al., 2001). The dark-light box, open topped, consisted of an secure, small, black PVC compartment (15 x 28 x 27cm; 60lux) and a aversive, illuminated, white PVC (48 x 28 x 27cm; 300lux) part, connected by an aperture (5 x 7cm) (Figure 3.4). At the beginning of the task, each animal was placed into one edge of the dark compartment, facing the wall. During the 5-min test procedure, the percentage of time each mouse spent in the lit or dark compartment, as well as the rearings (raising the front legs), were scored. To quantify locomotor activity, the ground of the dark compartment has been divided into two parts of equal size, whereas the light compartment was divided into four squares of same size in order to evaluate the line-crossings. A mouse was defined to have entered a compartment or crossed a line when both front paws and shoulders were inside the respective part or square. Scoring was performed by an observer blind to line and treatment using the computer program Eventlog 1.0 (EMCO Software).

Dark-Light box

Compartment

lit   dark

Figure 3.4:   Dark-light box test: Left: Experimental setup. Right: Schematic overview of the dark and the lit compartment of the behavioral test, which are connected by a tunnel and the division of each compartment used to define locomotor activity.

3.2.5   Forced Swim test

The forced swim (FS) test is the most widely used test to measure depression-like behavior as well as to screen antidepressants in rats and mice (Porsolt et al., 1977; Porsolt et al., 1978; El Yacoubi and Vaugeois, 2007). It is described to be a strong stressor, because the animals are facing a psychological and physiological challenge, especially mice which are not supposed to swim in their natural environment. In this paradigm, the animal is forced to swim in a glass cylinder half-filled with water so that the animal cannot touch the bottom with its hind paws or tail (Figure 3.5). In the beginning the animals try to escape by swimming, which is impossible, and after a while they assume an immobile posture called "floating" or "immobility" (Sillaber and Holsboer, 2004). This phenomenon was originally termed "behavioral despair" (Porsolt et al., 1978) or searching-waiting strategy, where the animals change between active and passive coping style, with the amount of passive behavior reflecting depression-like behavior (Thierry et al., 1984).

For testing, each individual was placed into a 2 liter glass beaker (radius: 11cm, height: 23,5cm) filled up to a height of 15cm (1.6 liter) with 23ºC tap water. After 6-min swimming, the animal was gently dried with a cloth towel and returned to the home cage. The duration of floating (immobility of all four extremities) phase was scored by an observer blind to line and treatment using the computer program Eventlog 1.0 (EMCO Software).
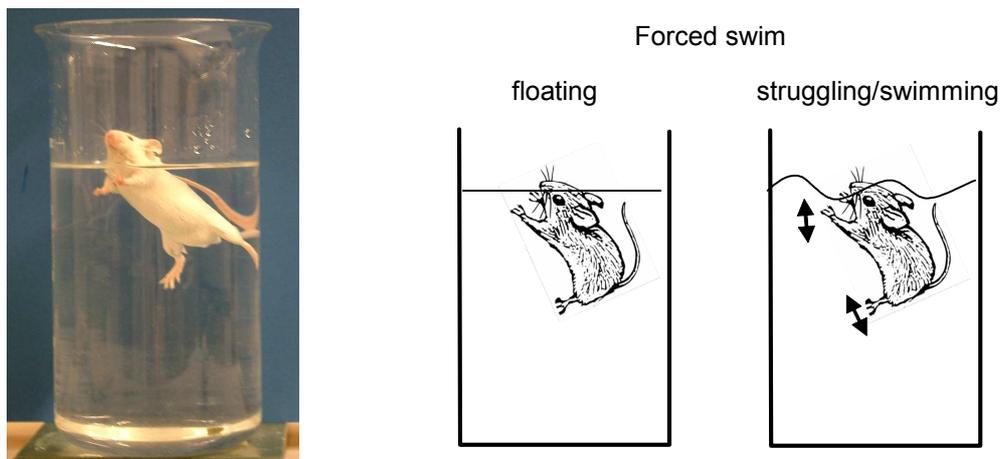
Figure 3.5:   *Forced swim test: Left: Experimental setup. Right: Schematic overview of the behavioral parameters analyzed. Floating, as a form of immobility, is reflecting passive coping, whereas struggling/swimming is indicative of active stress coping strategy.*

3.2.6   Tail-suspension test

The tail-suspension test (TST) is conceptually similar to the FS test, as both have a common theoretical basis and similar behavioral outcome (Steru et al., 1985). Mice, suspended by their tail, immediately engage in active, escape-orientated behavior, followed progressively by increasing periods of immobility, indicative of depression-like behavior (El Yacoubi and Vaugeois, 2007). Due to the fact that the animals are not exposed to a strong physiological challenge (e.g. hypothermia by water exposure), this paradigm is supposed to be less stressful compared to the FS test (Sillaber and Holsboer, 2004).

The testing apparatus consists of a horizontal plastic rod (length 75cm) at a height of 75cm with four vertical rods (15cm). Animals were suspended by their tail-tip at a height of 35cm above the ground (measured from ground to head) by an adhesive tape for six min (Figure 3.6). Four animals were tested at the same time. Each trial was videotaped and the immobility time (immobility of all four extremities) and the latency to the first immobility phase were analyzed by an observer blind to line and treatment using the computer program Eventlog 1.0 (EMCO Software).
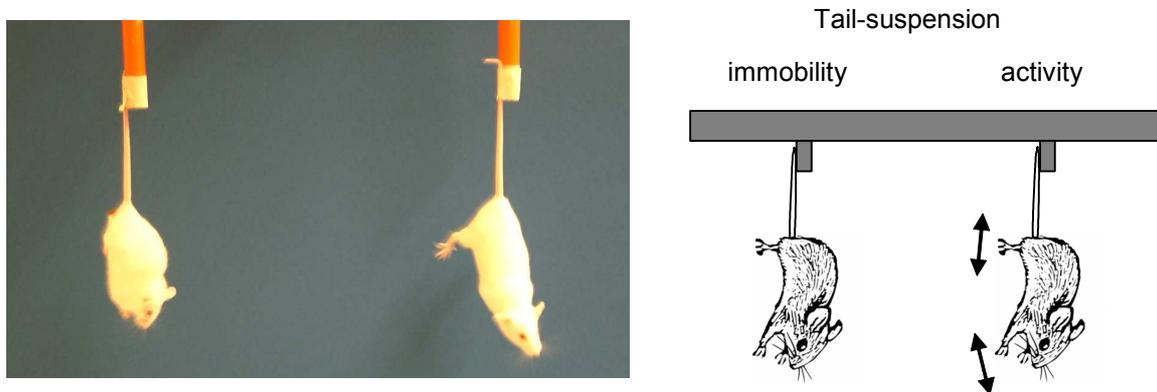
Figure 3.6:   *Tail-suspension test: Left: Experimental setup. Right: Schematic overview of*
                  behavioral parameters investigated, namely immobility and activity.

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3.2.7   Novel cage behavior

To monitor the animals´ spontaneous behavior in a novel stress-mild environment, each animal was transferred to a transparent experimental cage (plexiglas 38 x 22 x 35cm, 250lux), providing food pellets and water ad libitum, and behavior was analyzed during 15-min exposure. The novel cage (NC) was optically divided into an inner and outer (additionally divided into four parts each to allow quantification of outer line crossings) compartment in order to define the time spent in the more protective outer (thigmotaxis) or the more aversive unprotected inner part (see Figure 3.7). Additionally, the number and latency of line crossings ("two front paw and shoulder criterion"), latency to the first inner part entry, number of rearings (raising the front legs), grooming time, and time spent digging the saw dust (a kind of displacement activity due to an aversive situation) was measured by an observer blind to breeding line by the use of the computer program Eventlog 1.0 (EMCO Software).

Novel cage

Outer compartment
(four segments)
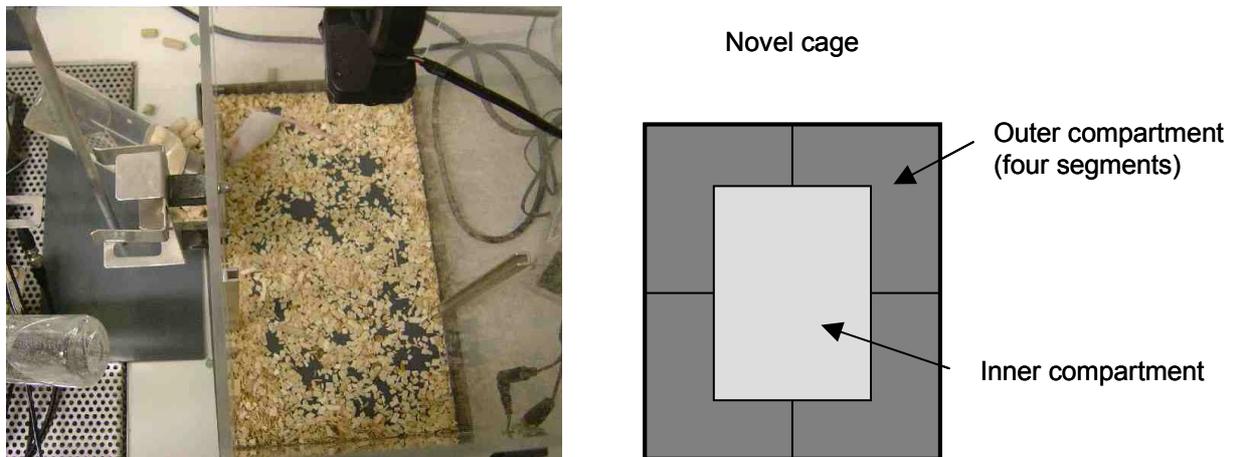
Inner compartment

Figure 3.7:   Novel cage behavior. Left: Experimental setup. Right: Schematic overview of the division of the cage in inner and outer compartment and the additional subdivision to define locomotor activity.

3.3   Tests for cognition

3.3.1   Social recognition test

The social recognition (SR) test is a learning and memory task, testing social memory abilities in rodents and has been established by Thor and Holloway in 1982. Former experiments have shown that a rodent is more interested in an unfamiliar, novel conspecific accompanied by a increased olfactory exploration, compared to a familiar conspecific (Thor and Holloway, 1982). Normally, reintroduction (introducing the familiar stimulus animal once more) of a familiar stimulus animal, results in a decrease in olfactory investigation indicative of functional social memory processes, whereas the introduction of a novel stimulus increases sniffing time. Presenting a novel stimulus animal excludes effects of tiredness of the experimental animal and lack of interest on a social stimulus which might influence the results (Ferguson et al., 2000b; Bielsky et al., 2004). Using this information, we performed a variety of pre-experiments by changing exposure times, social stimuli (including used female bedding, juveniles, and ovariectomized females) and inter-exposure intervals (IEIs), and introduce the following modified protocol suitable for HAB, NAB, and LAB male mice:

Pretraining: Experimental animals, group-housed, were transferred to a transparent experimental cage (plexiglas 38 x 22 x 35cm, 250lux) each, which contained an empty, perforated 50ml plastic tube (Sarstedt, Nümbrecht, Germany), which will later contain the stimulus animal. The animals habituated to the novel environment and the plastic tube for 40min. Furthermore, 24h prior to experiment, the animal was five times introduced to an

ovariectomized female (stimulus animal) protected in the perforated plastic tube, for 4min. To exclude that the later olfactory investigation in the stimulus animal is influenced by the animals´exploration of an unknown object, the animal is introduced to the empty plastic tube before. Avoiding anxiety-based influences on the results, induced by the novel environment, the experimental animals were habituated to the test situation itself by introducing them to a stimulus animal. The ovariectomized females were presented in these perforated plastic tubes to protect them from attacks of the experimental animals on one hand and on the other hand to provide a strict one-sided contact which is not influenced by behavioral or activity-based differences from the stimulus animal.

Experiment: After transfer to the experimental cage and 40min habituation, the experimental animal was introduced to the stimulus animal (First, F) for 4min. After 15min IEI the same animal was reexposed and the whole procedure repeated four times. In the 5th trial an novel (Novel, N) ovariectomized female was introduced for 4min. The time and latency to the first olfactory investigation, mostly in the facial or anogenital region of the stimulus animal, was quantified by using the computer program Eventlog 1.0 (EMCO Software) by an observer blind to the line.

Social recognition
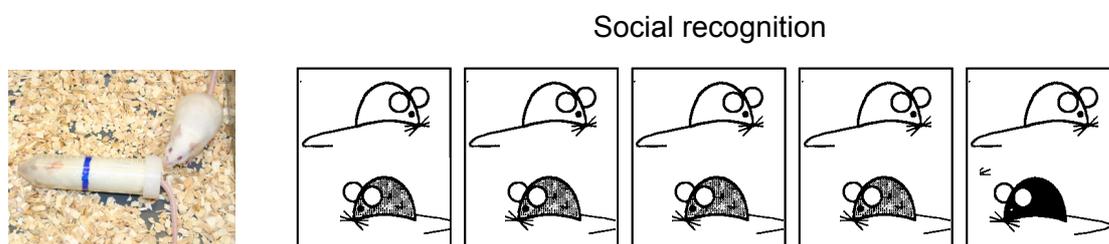
Figure 3.8:   Social recognition task. Left: Experimental setup. Right: Schematic overview
               of the experimental paradigm. The first (gray) stimulus animal was introduced
               to the experimental animal (white) for four min and for four times with an
               interexposure interval of 15min, whereas in the fifth exposure a novel stimulus
               (black) is introduced.

3.3.2   Social discrimination test

The social discrimination test (SD) was originally developed in our laboratory (Engelmann et al., 1995) to investigate olfactory-based learning and short-term memory processes in adult rats in a social context. Compared to the SR test, this task is more challenging for the experimental animals, because they have to discriminate between two stimulus animals at the same time. The hypothesis behind this test is similar to the SR test. If an animal is still

able to remember the first (familiar) stimulus animal, it will spend more time in olfactory exploration of the novel animal. Several modifications have been done in order to further standardize and adapt the protocol for mice (mentioned in 3.1).

Pretraining: see Social recognition (3.3.1)

Experiment: After transfer to the experimental cages and 40min of habituation, the experimental animal was introduced to the first stimulus animal, protected in a perforated plastic tube for five min. After IEIs of 15min, 30min, 2h, or 4h respectively, the first (First, F) ovariectomized female was reintroduced for five min to the test mouse together with a second (Novel, N) stimulus animal (also in a plastic tube). Each experimental animal underwent the four different IEIs with a consequent change in stimulus animals per run, i.e. each mouse was presented with new stimulus females during each trial. All experiments were performed between 8am and 3pm and videotaped for later analysis. The duration of olfactory investigation towards the respective stimulus animal in both sessions was quantified by an observer blind to the breeding line using the computer software Eventlog 1.0 (EMCO Software).

The total investigation time during the first exposure was quantified to exclude nonspecific effects on learning due to line differences. According to Engelmann et al. 1995, a significantly increased olfactory investigation of the novel stimulus female during the second exposure was taken as parameter of the animals´ social discrimination ability.

Figure 3.9:   Social discriminination test. Left: Experimental setup. Right: The first (gray) stimulus animal was introduced to the experimental animal (white) for five min whereas in the second exposure after different inter-exposure intervals the first (gray) and a novel, unfamiliar stimulus animal (black) are introduced.

3.3.3   Cued fear conditioning paradigm

Pavlovian fear conditioning (FC) and its extinction are related paradigms, which are used in laboratory animals to model the mechanisms of human fear learning and its reversal (Walker et al., 2002; Tamminga, 2006). Classical fear conditioning (FC) occurs when an affectively neutral stimulus such as a tone (conditioned stimulus, CS) is paired with a noxious aversive stimulus, such as footshock (unconditioned stimulus, US). During conditioning an association between CS and US is formed, which afterwards enables the previously neutral CS to elicit several fear-related behavioral changes, such as freezing or potentiation of the acoustic startle response. The fear-eliciting properties of the CS in the absence of the US extinguishes after repeatedly presenting the CS, indicating extinction as relearning (Walker et al., 2002). Corticosterone (Cort) and D-Cycloserine (D-cycl), a partial NMDA receptor agonist, are both described to facilitate extinction and are used to pharmacologically manipulate extinction in HAB and NAB mice (Ledgerwood et al., 2005; Cai et al., 2006; Lee et al., 2006).

Contexts: Two contexts, A and B, were used for the cued fear conditioning paradigm. A fear conditioning chamber (26 x 30 x 32cm; Coulbourn Instruments, Allentown, PA, USA) served as context A, whereas a standard sawdust-free type 2-macrolone cage (25.5 x 19.5 x 13.8cm) was used as context B. To maximally reduce the contribution of the context to cued fear conditioning, tactile, visual and olfactory cues were different in context B in comparison to context A. Thus, context A was equipped with a metal floor grid (rods spaced 1.5cm), with two plexiglas and two rough metal walls, the illumination was bright light of 300lux, and the context was cleaned with water containing detergent after each animal. In contrast, context B had a smooth PVC surface, was illuminated by dim red light approximately 5-10lux, and was wiped out with methanol after each session. Video cameras were mounted above each context and connected to a standard video recorder for recording and later scoring of freezing behavior (Maren, 2005a, b; Phelps and LeDoux, 2005).

Auditory stimuli for the cued fear conditioning task were delivered via a speaker (Coulbourn Instruments, Allentown, PA, USA) mounted approximately 20 cm above the contexts. US were delivered via an interface connected to the metal grid of context A. Experiments were conduced between 9am and 5pm, with the experimental groups being equally distributed through the time period of testing to avoid circadian influences on behavior.

Acquisition (day0 (d0): The experimental animals were transferred to context A and habituated to the acquisition chamber for 2min. Fear acquisition was elicited by introducing the experimental animals to the audible CS, a white noise of 80dB for 2min. The conditioning

stimulus, a mild but aversive footshock of 0.7mA was applied for 2sec co-terminated with the last 2sec of tone presentation. The tone-shock paradigm was in total presented for five times with a 2min inter-trial interval (stimulus-free period) in between. Following the CS-US pairing, the animals were kept 2min in the acquisition chambers and transferred back to their home cages afterwards.

Pharmacological treatment: 30min prior to the extinction protocol, animals were i.p. injected with a 45% cyclodextrine solution (Sigma Aldrich, Gemany, control groups), 10mg/kg corticosterone (Cort, Sigma Aldrich, Germany, Cort-treatment group) dissolved in 45% cyclodextrine solution or 30mg/kg D-cycloserine (D-cycl, Sigma Aldrich, Germany, D-cycloserine-treatment group) dissolved in 45% cyclodextrine solution. According to the animals´ body weight a volume of 300-380µl was administered.

Extinction training (d1): After 24h of memory consolidation extinction was performed in context B. Mice were again habituated to the novel environment for 2min and received 16 CS (Tone) presentations (2min, 80dB, white noise, 5sec inter-stimulus interval). The experimental animals were returned to their home cages 2min after the final CS presentation.

Retention (d6): To examine long-term memory storage, the experimental animals were again introduced and habituated (2min) to context B. Mice received eight CS presentations (2min, 80dB, white noise, 5 sec inter-stimulus interval) and 2min after the last tone presentation they were transferred to their home cages.

Analysis: During habituation and the last 2min after final CS presentation several behavioral parameters were quantified, including immobility, freezing, rearing and grooming time as well as locomotor activity. During CS presentations, we focused only on freezing time, as one of the most important parameters reflecting fear response. Behavior was quantified by using the computer program Eventlog 1.0 (EMCO Software) by an observer blind to the line and treatment.

Cued fear conditioning paradigm
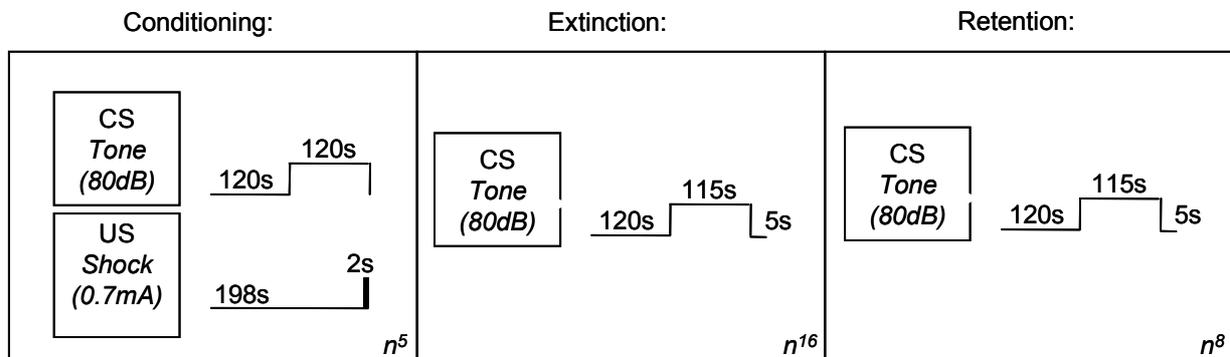
Figure 3.10:   Overview of the applied fear conditioning protocol. As a conditioning stimulus (CS) a tone (80dB) was used, whereas a footshock (0.7mA) was applied as the unconditioned stimulus (US).

3.4   Molecular techniques

3.4.1   In situ hybridization

The expression of *Avp*, *Crh*, MR, GR, *Oxt*, Synaptotagmin 4 (*Syt 4*), and brain-derived neurotrophic factor (BDNF) was assessed by in situ hybridization (ISH) using either oligonucleotide, or ribonucleotide probes. This technique is used to qualitatively and quantitatively detect gene expression in brain slides by adjusting high specific nucleotide mRNA probes. Furthermore, it provides information about the involvement of neuropeptides, receptors or other cellular components in mediating behavioral phenotypes. We investigated the expression of AVP, CRH, GR, and MR, two neuropeptides and two receptors, on different levels of the brain, largely involved in the regulation of behavior and HPA axis function (see 1.3). OXT as well as BDNF are also well described to contribute to anxiety- and depression-like behavior (Arletti and Bertolini, 1987; Bosch et al., 2005; Martinowich et al., 2007). Syt 4 is a Ca^{2+}-sensor protein involved in regulating neurotransmitter exocytosis and in tuning the fusion mechanisms (Lodish et al., 2001), and has been implicated in anxiety- and depression-like behavior (Schwab et al., 2001; Ferguson et al., 2004).

Animals were sacrificed by an over-dose of isoflurane (Curamed Pharma, Germany) between 9am and 1pm. The brains were removed, shock-frozen in N-methylbutane (Roth, Germany) and stored at -20°C until sectioning in 14µm slices by a cryocut in the coronal plane (Microm HM 500, Germany). Several sets of sections at PVN, amygdala and dorsal hippocampus level were taken and used for in situ hybridization according to former

protocols. If not particularly mentioned, the substances used for the following molecular techniques were purchased from Sigma-Aldrich, Germany.

Anatomical locations
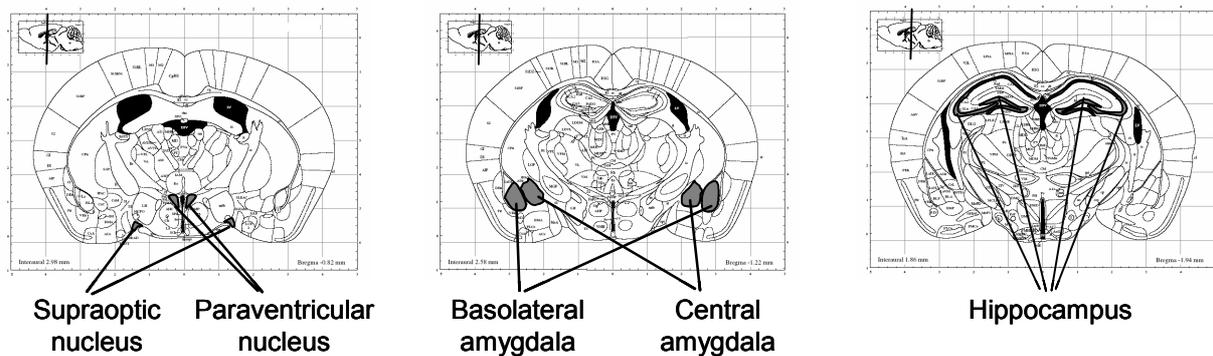
Figure 3.11:   Overview of the anatomical locations of the brain regions investigated in the following in situ hybridization and real-time PCR studies.

3.4.1.1 ISH measuring *Avp*, *Oxt*, and BDNF mRNA

To detect mRNA levels via oligonucleotide probes, we used unfixed tissue (Dagerlind et al., 1992). Briefly, for each animal one set of sections were used to detect one gene. Slices were dehydrated in increasing ethanol concentrations, degreased with chloroform, rinsed in ethanol, and air-dried afterwards. A highly specific oligonucleotide probe (see Table 3.1) directed against each of these three genes was used for hybridization. The oligonucleotides were labeled with ^{35}S by using ^{35}S-ATP (NEN DuPont, Germany) and terminal transferase (Tdt, Boehringer, Germany), including purification by tRNA precipitation. Tissue sections (5 sections per slide) were saturated with 100µl of hybridization buffer (deionized formamide, 20x SSC (standard saline citrate), dextran sulfate, 0.2M Na-phoshate buffer, Dehnhard´s solution, 20% sarcosyl solution, salmon sperm DNA, 5M DTT (DL-Dithiothreitol)) containing 10^6 cpm ^{35}S-labeled oligoprobe. Coverslipped sections were incubated in a humid chamber for 18–22h at 45ºC. After several washes in 1x SSC, sections were dehydrated and air-dried before they were exposed to radiation-sensitive films (Muller et al., 2003; Wigger et al., 2004). For data analysis, see 4.1.3.

Table 3.1:   *Nucleotide sequences used to detect Avp (Ivell and Richter, 1984; Villar et al.,*
                 1994), Oxt (Ivell and Richter, 1984), and BDNF (Sterlemann and Schmidt,
                 unpublished).

Gene	Nucleotide sequence
Avp	5´gggcttggcagaatccacggactcttgtgtcccagccagctgtaccag 3´
Oxt	5´ctcggagaaggcagactcagggtcgcaggcggggtcggtgcggcagcc 3´
BDNF	5´agttccagtgcctttttgtctatgcccctgcagccttccttggtgt 3´

3.4.1.2   ISH measuring *Crh*, MR, GR, and *Syt4* mRNA

ISH using ^{35}S-UTP-labeled ribonucleotide probes to detect *Crh*, MR, GR, and *Syt4* mRNA
was performed as described previously (Schmidt et al., 2002; Muller et al., 2003). Briefly,
sets of sections for each riboprobe ISH were fixed in 4% paraformaldehyde and acetylated in
0.25% acetic anhydride in 0.1M triethanolamine/HCl. Afterwards, slides were dehydrated in
increasing ethanol concentrations, degreased with chloroform, rinsed in ethanol and air-dried
afterwards. The antisense cRNA probes for *Crh* (356 base pairs, bp), GR (520 bp), MR (750
bp), and *Syt4* (470 bp) were transcribed from a linearized plasmid and labeled using either
SP6 (polymerase for *Crh*, MR, and *Syt4*) or T7 (polymerase for GR) transcription systems in
a standard labeling reaction mixture consisting: 1.5µg of linearized plasmid, 1x transcription
buffer, 0.12mCi of [^{35}S]UTP, 1mM NTPs, 16.7mM DTT, 40U of RNAse inhibitor, and 20U of
the appropriate polymerase. The reaction mix was incubated at 37ºC for 3h and the labeled
probe was subsequently separated from free nucleotides via Qiagen spin columns (Muller et
al. 2003). Tissue sections (5 sections per slide) were saturated with 100µl of hybridization
buffer (Tris HCl, EDTA, NaCl, formamide, 5M DTT, Dehnhard´s solution, DEPC H_2O, 50%
dextran sulfate) containing 10^6 cpm ^{35}S-labeled riboprobe. Brain sections were coverslipped
and incubated in humid chambers over night, approximately 18–22h at 55ºC. On the
following day, slides were rinsed in 2x SSC, treated with RNAse A (20mg/l), washed by
decreasingly concentrated SSC solutions, dehydrated by increasing ethanol concentration,
and air-dried before exposure to the radiation-sensitive films (for data analysis, see below).

3.4.1.3   Data analysis

For all ISHs, sections were exposed to a Kodak BioMax MR film (Eastman Kodak Co., USA) for 1-14 days, fixed, and developed afterwards. The films were digitized and the radiation-induced blackening of different brain region was quantified by means of image analysis using the computer software Optimas (Version 5.22, Optimas Corp., USA) or Scion Image (Version 4.0.3.2, Scion Corporation, USA). Autoradiograms were analyzed by using the computer-assisted optical density readings (relative grey intensity as a measure of relative expression) of an area as well as the relative size of labeled area. For each individual, three to six brain sections were quantified by an observer blind to the breeding line and the highest expression (hybridization signal of a certain region minus the background signal of a nearby structure that does not express the gene of interest) was used for the calculation of each mRNA expression, respectively.

3.4.2   Quantitative real-time PCR measuring *Avp* and *Syt4*

Tissue sampling for quantitative real-time PCR (qRTPCR):

For an additional measurement of mRNA levels, the tissue micropuncture technique (Czibere, unpublished) was applied to acquire tissue samples from specific regions with micropunchers of 0.5 and 1mm diameter (Fine Science Tools, Germany). Punches were collected from Bregma –0.56mm to -0.96mm medially 0.8mm above the ventral tissue edge around the dorsal end of the 3rd ventricle (Ø=1mm; PVN) and bilaterally from the optic tract (Ø=0.5mm) to acquire tissue from the SON. Central amygdala (CeA) tissue samples were collected bilaterally from Bregma –0.96mm to –1.36mm dorsomedially from the ventral end of the external capsule. The basolateral amygdala (BLA) was collected from Bregma -1.36mm to -1.76mm from in between the bifurcation of the external capsule (Paxinos and Franklin, 2001). Tissue punches were kept on -80°C until further processing.

RNA extraction and reverse transcription from tissue punches:

Tissue punches were homogenized with a pipet in 300µl Trizol (Tri Reagent), then 30µl bidistilled water, 1µl linear acrylamide (Ambion, TX), and 60µl chloroform were added before continuing with a standard protocol using n-propanol for RNA precipitation overnight. The amount of total RNA yielded from 0.3-1.5µg.

Not more than 1µg of total RNA was reverse transcribed with Superscript II (Invitrogen) after DNAse treatment. All steps required for reverse transcription were performed according to the manufacturer's protocol. For quality control, a small aliquot of cDNA was analyzed on agarose gel.

Quantitative PCR analysis:

cDNA gene transcripts were analyzed by qRTPCR, using the LightCycler® FastStart DNA MasterPLUS SYBR Green I reagent (Roche Diagnostics GmbH, Germany) according to manufacturer's instructions and the respective oligonucleotide primers for *Avp*, *Syt4,* and the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt*). Experiments were performed in duplicates on the Lightcycler®2.0 instrument (Roche Diagnostics GmbH) under the following PCR conditions: initial denaturation; 40 cycles of denaturation, annealing, and elongation (see Table 3.2 for primer sequences and Table 3.3 reaction conditions in the LightCycler® for *Syt4, Avp*, and the housekeeping genes *Gapdh* and *Hprt* for reaction conditions)*.* Fluorescence was assessed after each cycle after elongation phase. At the end of every run a melting curve (50-95°C with 0.1°C/sec) was generated to control for the quality of the PCR product. Crossing points (Cp) were calculated with the LightCycler®Software 4.0 (Roche Diagnostics GmbH, Germany) using the absolute quantification fit points method. Threshold and noise band were set in all compared runs to the same level.

Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using the real PCR efficiency calculated from an external standard curve. Cp were normalized to the housekeeping genes *Gapdh* and *Hprt*, respectively, and values calculated relative to the expression mean of LAB mice.

Table 3.2:   *Primer sequences used for the genes of interest and the housekeeping genes.*

Primer/ Gene	Forward primer	Reverse primer
Avp	5′ tcgccaggatgctcaacac 3′	5′ ttggtccgaagcagcgtc 3′
Syt4	5′ tgatgtcattggagaagtcctg 3′	5′ accacagtgagcgtgtttgt 3′
Gapdh	5′ ccatcaccatcttccaggagcgag 3′	5′ gatggcatggactgtggtcatgag 3′
Hprt	5′ gtcaagggcatatccaacaacaaac 3′	5′ cctgctggattacattaaagcactg 3′

Table 3.3:   *Reaction conditions in the LightCycler® for the analysis of Syt4, Avp, and the*
                housekeeping genes Gapdh and Hprt.

Gene/region	Preincubation	Amplification		
		Denaturation	Annealing	Elongation
Syt4 PVN/ACeA/BLA	95ºC/15min	95ºC/5sec	58ºC/5sec	72ºC/8sec
Avp PVN/CeA/SON	95ºC/10min	95ºC/10sec	56ºC/4sec	72ºC/7sec
Hprt PVN/BLA/ACeA/SON	95ºC/10min	95ºC/10sec	57ºC/5sec	72ºC/8sec
Gapdh PVN/BLA/ACeA/SON	95ºC/10min	95ºC/10sec	65ºC/5sec	72ºC/13sec

3.4.3   Double-immunohistochemistry detecting AVP and CRH

To get insight into neuropeptides, potentially involved in mediating anxiety- and depression-like behavior on peptide levels, immunofluorescence staining (Hetzenauer et al., 2006) was used to visualize AVP and CRH.

Transcardiac perfusion:

Animals were deeply anesthetized by an overdose of sodium pentobarbital (200mg/kg) by i.p. injection prior to cardiac perfusion. To expose the heart an abdominal incision was made and the right atrium was cut to insert a butterfly needle into the left ventricle. Subsequently, 20ml of 0.9% saline was slowly injected into the heart followed by 20ml of 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed and post-fixed at 4°C overnight in 4% paraformaldehyde in 0.1M phosphate buffer.

Double labeling CRH and AVP:

24h after post-fixation, 50μm sections were cut on the vibratome (Series 1000, Ted Pella Inc., USA). Sections were preincubated with 5% normal donkey serum (Jackson ImmunoResearch, PA) in immunobuffer for 30min and subsequently incubated for at least 48h at room temperature with a cocktail of polyclonal primary antibodies directed against CRH (guinea pig, Peninsula Laboratories, USA) and AVP (rabbit, Peninsula Laboratories, USA). The final dilution of the primary antibodies in immunobuffer supplemented with 5% normal donkey serum was 1:2000 (CRH) and 1:10.000 (AVP), respectively. Following 3 washing steps with immunobuffer (10min each), the sections were incubated at room temperature for 2.5h with a secondary antibody cocktail containing a donkey Cy3 (Cyanine 3)-conjugated antiserum directed against guinea pig immunoglobulins to stain CRH and a donkey Cy2 (Cyanine 2)-conjugated antiserum directed against rabbit immunoglobulins to stain AVP (all antibodies: Jackson Immuno Research Laboratories, USA). The final dilution of the secondary antibodies in immunobuffer containing 1% normal donkey serum was 1:100

(Cy2) and 1:400 (Cy3). From the incubation step with the fluorescent dyes onwards, care was taken to carry out all steps in the dark. After 3 washing steps with Tris buffer, the sections were finally mounted on slides, dried and coverslipped. An Olympus BX40 fluorescence microscope equipped with the following filter cubes was used to detect the staining: U-M41001 (excitation filter 455-495nm, dichroic mirror 505nm, emission filter 510-555nm) for Cy2 (excitation maximum 492nm, emission maximum 510nm) and U-M41007 (excitation filter 530-560nm, dichroic mirror 565nm, emission filter 575-645nm) for Cy3 (excitation maximum 550nm, emission maximum 570nm). Negative controls were performed by omission of the primary antibody. No staining was observed in any of these control sections.

Images were recorded by using a digital camera (Olympus DP50) and analySIS® image processing software (Soft Imaging Systems, Germany) with constant light conditions for all AVP or CRH slides. For semi-quantification, pictures were black/white converted (Corel Draw, Corel Corp., Canada) to admit image analysis by the optical software Optimas. For data analysis details see 3.4.1.3.

3.4.4   Glyoxalase 1 quantification by western blot

Glyoxalase 1 (Glx1) is an enzyme in the cytosolic fraction of cells and tissues of many organisms. Although is function is not fully described, it plays an important role in cellular detoxification processes and dysregulation of these enzyme has been described to be involved in psychopathologic mechanisms, including Alzheimer´s disease and anxiety-related behavior (Chen et al., 2004; Hovatta et al., 2005; Kromer et al., 2005) .

The Western blot (WB) analysis allows us the screen a number of blood samples obtained from HAB, NAB, and LAB animals during different developmental stages (postnatal day (pnd) 5, 12, 28, 54) and to determine the glyoxalase protein amount. Animals were sacrificed directly after behavioral testing or under basal conditions and blood samples were collected in EDTA tubes supplemented with Trasylol. Samples were centrifuged for 10min at 4,000rpm and 4°C. The whole amount of plasma was isolated to determine ACTH and Cort (see 4.5), whereas 20µl to 50µl of the red blood cell pellet was used for the WB quantification. After several washes in PBS (phoshate buffered saline), the red cell pellet was frozen at -80°C until use. For cell lysis, the pellets were thawn on ice, water containing 1mM PMSF (Phenylmethansulfonylfluoride) was added and the suspension was mixed. After removing the cellular debris, the supernatant was stored in aliquots at -80°C. For WB, 100µg of total protein from each lysate was run in each gel lane. Electrophoresis was performed on a 15%

miniature gel (Bio-Rad), and the proteins were transferred to an Immobilon PVDF (polyvinylidene difluoride) membrane (Millipore, Bedford, MA) at 100V for 1h with cooling. The membrane was treated with 5% Carnation instant nonfat dry milk (Nestle, Vevey, Switzerland) in TBS plus Triton X-100 (TBST) overnight and rinsed in TBST. Afterwards the membrane was incubated with Glx1 antiserum (kindly provided by Dr. Kenneth Tew, Fox Chase Cancer Center, Philadelphia, PA) at a 1:2000 dilution in TBST for 2h at room temperature and washed with water and TBST for 15min. Incubation time with protein A horseradish peroxidase (Amersham Biosciences) was 40min at room temperature, with the membrane being washed with water and TBST afterwards. Finally, the membrane was incubated with ECL mixture (Amersham Biosciences) for 1min and exposed to ECL film (Amersham Biosciences). The membrane was scanned and the signal intensity of the Glx1 band (optical density) assessed by QuantityOne software (Bio-Rad) (Ditzen et al, 2006; Kromer et al., 2005).

Figure 3.12:   Representative western blot, performed in red blood cell samples of male HAB, NAB, and LAB mice at postnatal day 5. kD = KiloDalton.

3.4.5   Radioimmunoassay measuring ACTH and Cort

ACTH and Cort, two hormones released as a consequence of HPA axis activation, (see 1.3) were assessed using commercial available radioimmunoassay (RIA) kits (MP Biomedicals, USA). Blood samples were collected, centrifuged and plasma isolated as described in 3.4.4. The plasma samples were measured according to the manufacturers´ instructions with slight modifications (half of the recommended volume of all ingredients was used). For Cort analysis, 10µl of plasma was used (1:13.5 dilution), while 25µl of plasma was directly used for a single estimation in the ACTH RIA, yielding a sensitivity of 1ng/ml and 40pg/ml, respectively. The RIA is based on the competition between ^{125}I-labeled ACTH or Cort and the ACTH or Cort in the plasma sample for a defined amount of ACTH or Cort specific antibody. The amount of bound ^{125}I-labeled ACTH or Cort is therefore inverted proportional to the unlabeled ACTH or Cort of the plasma sample. By use of a double-antibody method, the unbound ^{125}I-labeled ACTH or Cort gets bound by the secondary antibody and the radioactivity of the pellet is measured by Gamma-counter. Due to samples with known ACTH

or Cort concentration a calibration curve was generated and determined samples within this curve (and within 10-90% antibody binding values) were accepted for calculation. Cort samples were analyzed in double-estimation and coefficients of variants below 10% were accepted for calculation.

3.5   Pharmacological validation

3.5.1   Intracerebroventricular application of AVP and CRH

To investigate the impact of AVP and CRH on behavior, mice were implanted with an icv guiding canula to inject AVP and CRH simultaneously.

Surgery:

A guiding canula (1.2cm, 25G, suitable for a 27G injection system) was implanted into the lateral ventricle under isoflurane (Curamed Pharma, Germany) anesthesia. Mice were fixed in a stereotaxic frame (Typ 516000, TSE GmbH, Germany) and the brain surface was exposed. As implantation locus 0.03mm caudal and 0.1mm lateral to the bregma (Paxinos and Franklin, 2001) was chosen. A hole was drilled to ventrally implant the guiding canula 1.5mm into the brain. The canula was fixed in the skull by two screws (M1*3, stainless steel, Schrauben Preisinger, Germany) and dental acrylic (Kallocryl®, Speiko®, Germany). The wound was medicated with iodine afterwards and for recovery up to four animals were housed in type 2-macrolone cages for one week.

Intracerebroventricluar (icv) administration:

On the experimental day, animals were treated with either 1µl solution containing 1µg/µl AVP (Sigma-Aldrich, Germany) and 1µg/µl CRH (Ferring, Germany) or 1µl 0.9%saline. 30min prior to the behavioral testing, animals were injected by using a 25G injection canula (Braun, Germany) connected to a syringe, 1.5mm longer than the guiding canula, directly into the lateral ventricle. After performing the behavioral tasks (EPM and TST, with 10min in between), animals were lethally anesthetized, injected with 1µl ink solution and the brains removed to examine carefully the anatomically localization of the injection (see Figure 3.13).
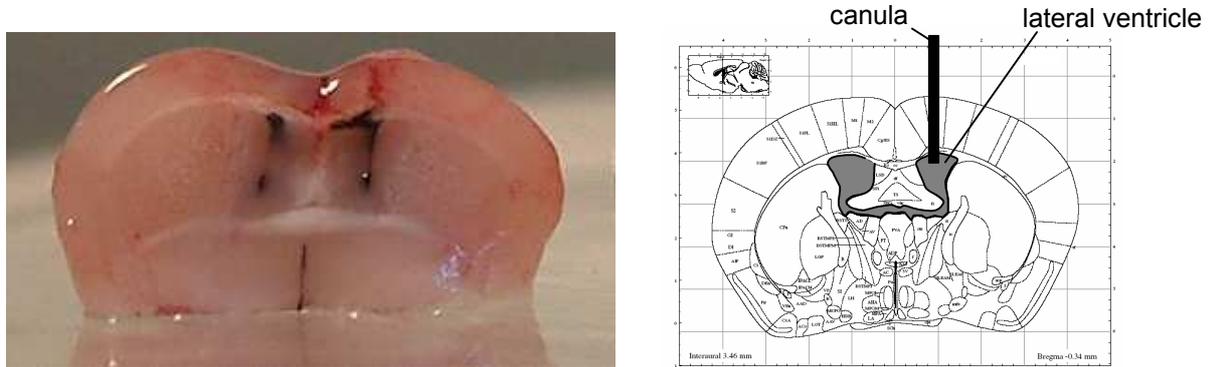
Figure 3.13:   Verification of probe localization. Left: Valid localization of the probe in the lateral ventricle labeled with ink. Right: Coronal brain section demonstrating the implantation locus.

3.5.2   Subchronical intraperitoneal administration of a CRHR1 antagonist (DMP696)

To examine the influence of CRH on emotionality, animals were intraperitoneal (i.p.) injected with a CRH receptor 1 (CRHR1) antagonist.

According to former experiments (Nielsen et al., 2004), animals were treated three times (subchronically) with 10mg/kg CRHR1 antagonist (DMP696 (4-(1,3-Dimethoxyprop-2-ylamine)-2,7-dimethyl-8-(2,4-dichlorophenyl)-pyrazolo[1,5-*a*]-1,3,5-triazine, donated by Bristol-Myers Squibb, USA) dissolved in PEG400 (Polyethylenglykol 400), DMSO (Dimethylsulfoxid), Tween 80, and saline), or a control solution (PEG, DMSO, Tween 80, saline). I.p. administration took place 24h, 12h, and 30min prior to behavioral testing. 30min after the last injection the EPM test and the TST (10min after the EPM test) were performed to investigate anxiety-related and depression-like behavior.

3.6   Statistical analysis

The data presented are shown in means $\pm$ SEM (standard error of the mean), analyzed by using SPSS 12.0. Significance was accepted with $p < 0.05$. *Post-hoc* tests were only performed after acceptance of significant p-values in the proceeding statistical tests (for overview of applied statistical tests, see Table 3.4).

Table 3.4:   Overview of the exerted statistical analyses.

Type of data	Test classification	Test	*Post hoc* test	Bonferroni correction for multiple comparisons
Two independent groups	nonparametric	Mann-Whitney U-test (MWU)	-	-
More than two independent groups	nonparametric	Kruskal-Wallis *H*-test (KWH)	MWU	sequential
Two dependent groups	nonparametric	Wilcoxon test	-	-
More than two dependent groups	nonparametric	Friedman-test	Wilcoxon test	sequential
More than two dependent groups	parametric	Analysis of Variance (ANOVA)	MWU	sequential
Correlation analysis of two factors	nonparametric	Spearman (two-sided)	-	-

4   Behavioral phenotyping and candidate genes of emotionality

4.1   Introduction

By definition, a proper animal model (see 1.6), for the investigation of basic principles underlying trait anxiety at a behavioral, neuroendocrine, and genetic level, should stand for a close approximation to core symptoms of anxiety and often comorbid depression disorders found in psychiatric patients (Finn et al., 2003; Cryan and Mombereau, 2004; Gordon and Hen, 2004). Ensuring face validity in the HAB/LAB mouse model, a variety of behavioral paradigms have been used in addition to the EPM test, to demonstrate the stable phenotypic divergence between the breeding lines and its persistence over generations. To confirm the differences in anxiety-related behavior, the open arm exposure (OA) and dark-light box test (DaLi) test were conducted, whereas the tail-suspension (TST) and forced swim (FS) tests were used to measure depression-like behavior. Furthermore, novel-cage (NC) behavior was monitored to further examine explorative behavior (for additional information concerning the behavioral task used, see 3.2).

Due to the multigenic background of psychiatric diseases and to confirm the models´ construct validity, a selection of gene expression profiles and gene products was analyzed under basal conditions. Further, a pharmacological validation was used to confirm the contribution of major candidates in mediating anxiety- and depression-like behavior. These studies focus on genes that have already been described in animal models and/or human studies to be causally involved in the pathology of anxiety and depression disorders (for detailed information, see below). The targets include neuropeptides, receptors, and proteins on the level of the HPA axis as well as in synaptic neurotransmission.

AVP

The chemical structure of the nonapeptide AVP has been firstly described in 1953 (Vigneaud et al., 1953). From an evolutionary perspective, AVP, similar to OXT, derives from vasotocin, prevailing in non-mammals (Acher and Chauvet, 1988; Frank and Landgraf, 2008). The gene encoding AVP, composed of three exons, is located on chromosome 20 (human) and 2 (Mus musculus), respectively (http://www.ncbi.nlm.nih.gov/). Exon 1 encodes the signal peptide, AVP, and the N-terminal part of neurophysin II. The second exon covers the central region of neurophysin II and exon 3 the C-terminal region of neurophysin II and the glycoprotein copeptin (Land et al., 1982). After multiple posttranslational modifications in the endoplasmatic reticulum, on its way across the Golgi apparatus, packed in large dense core vesicles, AVP finally reaches the axonal terminals as sites of release. As described before (see 1.3), in the hypothalamus, AVP is mainly expressed in parvo- and magnocellular

neurons of the PVN and SON where it contributes, together with OXT and/or CRH, to the regulation of the HNS or HPA axis activity (Engelmann et al., 2004). Furthermore, vasopressinergic fibers projecting to a variety of brain areas, including the hippocampus, septum, amygdala, and several brainstem areas, and the somatodendritic release of magnocellular neurons allow AVP to act as neurotransmitter (Buijs et al., 1991) and more importantly, as a neuromodulator on receptors at various distances from its site of release (Landgraf and Neumann, 2004; Ludwig et al., 2005). The BNST and the medial amygdala (MeA) are reported to be the two major extrahypothalamic vasopressinergic sources, which project to several brain regions, such as the LS, locus coeruleus (LC), or ventral hypothalamus (Hallbeck et al., 1999). AVP expression in the suprachiasmatic nucleus (SCN) has a defined role in the regulation of the circadian rhythm (Kalsbeek et al., 1995). AVP acts via its G-protein coupled AVP receptor 1a (V1a), V1b, and V2 receptors. The V2 receptor is primarily expressed in the renal collecting duct and is involved in the regulation of the osmo-induced water and sodium retention in the kidney (Zingg, 1996). The V1a (besides its presence in a variety of body tissues) and V1b receptors are widely expressed within the brain, with the V1a receptor present, among others, in the hypothalamus, septum, hippocampus, CeA, and cerebellum (Ostrowski et al., 1994), whereas the V1b is primarily located in the adenohypophysis, hippocampus, the external zone of the median eminence, and around the cerebroventricular system (Hernando et al., 2001). In summary, AVP and its multiple functions (Frank and Landgraf, 2008) can roughly be grouped into two systems. The peripheral AVP-system is responsible for classic neuroendocrine functions including antidiuresis, glycogen metabolism, or vasoconstriction, whereas the central system comprises the sites of AVP synthesis and release within the brain. In the latter system, AVP acts as a neuromodulator/neurotransmitter involved in a variety of central nervous functions, including learning/memory, neuroendocrine reactivity, circadian rhythmicity, autonomic functions, social behavior, and emotionality (Ring, 2005; Landgraf, 2006). In 1978, the role of vasopressinergic disturbances in the pathophysiology of affective disorders has been hypothesized for the first time in humans (Gold et al., 1978). Preclinical and clinical observations support a correlation between AVP and anxiety/depression disorders (Landgraf et al., 1995; van Londen et al., 1997; Bhattacharya et al., 1998; Scott and Dinan, 2002), altogether pointing to an anxiogenic and depressive effect of centrally released AVP.

OXT

The nonapeptide OXT was identified as a neurohormone, which is, together with AVP, involved in the regulation of the HNS (Bargmann and Scharrer, 1951) and is known to act in

several brain regions on various complex behaviors (Reijmers et al., 1998; Wang et al., 1998; Insel and Young, 2001). The *Oxt* gene, containing three exons, is located on the same chromosome as *Avp,* but in opposite translational orientations (Burbach et al., 2001). The two neuropeptides AVP and OXT differ from each other in only two of nine amino acids and due to their high homology, OXT is often used as a control gene if AVP mRNA is investigated (Carter, 2003; Wigger et al., 2004). The OXT receptor is localized in the areas of the CNS, foremost in the brainstem, MeA, and LS, related to its functional role (Barberis and Tribollet, 1996).

OXT is mainly expressed in magnocellular neurons of the PVN and SON, but also in a variety of other brain regions, including the septum or the amygdala (Landgraf and Neumann, 2004). Beside its role in mammalian birth and lactation, OXT is a well acknowledged neuromodulator/neurotransmitter, which has, beside other functions, been implicated in social behaviors, including parental behavior, formation of social bondings, social memory, and stress coping. It is reactive to a variety of stressors and plays a role in the regulation of the central, as HPA axis activity as well as the autonomic nervous system, like immune and cardiovascular functions (Insel and Young, 2001; Carter, 2003; Landgraf and Neumann, 2004; Carter et al., 2007). Moreover, it has been shown that OXT is not only linked to anxiety-related behavior (Bosch et al., 2005), but has also antidepressive effects (Arletti and Bertolini, 1987; Scantamburlo et al., 2007), which increases its clinical relevance and importance.

CRH

In 1981, CRH, a 41 amino acid peptide, has been identified as a main physiological factor involved in the regulation of HPA axis activity (for review see (Vale et al., 1981; Owens and Nemeroff, 1991). The *Crh* gene is located on chromosome 8 in humans and on chromosome 3 in mice (http://www.ncbi.nlm.nih.gov/). The main expression sites of CRH include the PVN, the amygdalar complex and the extended amgdala, the olfactory bulb, certain thalamic nuclei, the hippocampus, pars compacta of the substantia nigra, the periaqueductal grey (PAG), LC, the nucleus of the solitary tract, dorsal and ventral parabrachial nuclei, the cortex, and the deep cerebellar nuclei of the cerebellum (Swanson et al., 1983; Dunn and Berridge, 1990). Beside its involvement in HPA axis regulation and dysregulation (for details see 1.3; 1.4), administration of CRH into various animal models indicated that this neuropeptide has a variety of additional endocrine, physiological, neurochemical, and behavioral functions independently of its HPA axis function. Many effects of CRH resemble those observed under stress exposure, suggesting CRH to act as an endogenous mediator

of these responses (Dunn and Berridge, 1990). CRH has been shown to modulate several neurotransmitter systems, including glutamate, dopamine, serotonine, and noradrenaline (Lavicky and Dunn, 1993; Price and Lucki, 2001; Valentino and Commons, 2005), with its modulation on the serotinergic and noradrenergic system being implicated in affective and anxiety responses (Koob, 1999; Charney, 2004). Furthermore, CRH is well situated in the modulation of cognition, startle response, defensive behavior, and emotion (Risbrough et al., 2003; Risbrough and Stein, 2006). CRH acts via at least two known G-protein-coupled receptors, CRHR1 and CRHR2 (for review see (Dautzenberg and Hauger, 2002; Eckart et al., 2002)). In rodents, both receptor subtypes are expressed in discrete nuclei of the neocortex, amygdala and extended amygdala (BNST), nucleus accumbens, hypothalamus, pituitary, and sensory relay nuclei (van Pett et al., 2000). This distribution pattern led to the hypothesis that CRH may play a role in sensory processing and associations, defensive or anxious responses, and cognition (Risbrough and Stein, 2006). The role of the CRHR2 in anxiety is not well understood, as there is evidence for both anxiolytic and anxiogenic functions after receptor activation or gene depletion (Bale et al., 2000; Coste et al., 2000; Bakshi et al., 2002). In contrast, by receptor blockade or gene deletion studies, the CRHR1 receptor has been well described to act as a primary mediator of stress (Reul and Holsboer, 2002). Several CRHR1 antagonist studies revealed reduced anxiety-related responses and depression-like phenotypes (Habib et al., 2000; Keck et al., 2001). These findings together with clinical reports (Holsboer and Barden, 1996), support CRH and its receptors as potential candidates for pharmacotherapy of anxiety and depression disorders (Holsboer, 1989; Timpl et al., 1998; Holsboer, 1999; Dautzenberg and Hauger, 2002; Reul and Holsboer, 2002; Risbrough and Stein, 2006).

GR and MR

Glucocorticoids regulate stress response (see 1.3) and influence learning/memory via two receptors types, GR and MR, in the brain (Brinks et al., 2007). The gene encoding GR is located on chromosome 5 in humans and on chromosome 18 in mice, whereas MR is located on chromosome 4 in humans and on chromosome 8 in mice (http://www.ncbi.nlm.nih.gov/). Both receptors are expressed in brain regions involved in the regulation of emotionality, learning, and memory. MRs are present in the hippocampus and to a less extent in the PFC, amygdalae, and PVN. The GR is detectable throughout the brain at high levels in the hippocampus and PVN (de Kloet et al., 1991; Diorio et al., 1993; Patel et al., 2000). Additionally, these receptor types have a differential affinity for Cort: MR has a tenfold higher affinity compared to GR, resulting in predominant MR occupation under low

basal Cort levels and increasing GR activation by elevated Cort concentrations as a consequence of stress or circadian rhythm of the HPA axis (Reul and de Kloet, 1985; de Kloet et al., 1990). From these data the hypothesis evolved that the MR is regulating the tonic activity of the HPA axis, while the additional occupation of GR is essential for the recovery phase following HPA axis activation (de Kloet et al., 1998; de Kloet et al., 2005). These cytosolic receptors can modify gene expression at least by two different pathways. First, binding to specific DNA motifs in the regulatory promoter regions of genes, the so-called glucocorticoid response element, can lead to expression or repression of these genes (Drouin et al., 1993). Second, activated MRs and GRs can interact with other transcription factors to modulate gene expression (Gottlicher et al., 1998).

Beside their role in HPA axis inhibition, MRs and GRs have been shown to regulate anxiety-related behavior and to modulate learning and memory (for review see (Gass et al., 2001)). It has been demonstrated that GRs are involved in the storage of information, whereas the MR is responsible for the interpretation of environmental signals and selection of an appropriate behavioral response (Oitzl et al., 1997a; Oitzl et al., 1997b; Oitzl et al., 1998).

<u>BDNF</u>

BDNF is the most widely expressed member of the nerve growth factor family of growth regulators, summarized as neurotrophins. They play a crucial role in the development of the brain and continue to have a seminal role in shaping plasticity in the mature nervous system (Thoenen, 1995). BDNF is most abundant among the neurotrophins and is expressed at highest levels in the hippocampus and the cerebral cortex (Schmidt-Kastner et al., 1996; Conner et al., 1997). The gene encoding BDNF is located on chromosome 11 in humans and on chromosome 2 in mice (http://www.ncbi.nlm.nih.gov/). The BDNF gene has a complex structure with four different splicing variants, and one common 3´coding exon that generates the mature BDNF (Timmusk et al., 1993). Its effect is mediated through the stimulation of a tyrosine kinase-coupled receptor, known as tropomyosin receptor kinase (trkB for BDNF), that signals through the MAP kinase signalling cascade (Nair and Vaidya, 2006). BDNF acts as one key contributor in the development, survival, maintenance, and plasticity of CNS neurons (Thoenen, 1995). BDNF has also been shown to elicit rapid action potentials thus influencing neuronal excitability (Kafitz et al., 1999) and it has a prominent role in the activity-dependent synaptic plasticity, like long-term potentiation (Bramham and Messaoudi, 2005). Since it plays a critical role in the maintenance of dendritic arboration in a variety of neurons (McAllister et al., 1997; Horch et al., 1999), its deficiency may result in retraction or atrophy of dendrites. The "neurotrophin hypothesis of depression" is largely

based on the finding that decrease in hippocampal BDNF levels is correlated with stress-induced depressive disorders and that antidepressant treatment enhances the expression of BDNF (Duman and Monteggia, 2006), but a number of key questions remain to be fully addressed.

SYT4

Many of the current treatments for psychiatric diseases utilize drugs that increase the levels or activity of neurotransmitters, for example serotonin-reuptake inhibitors that increase serotonin levels in the synaptic cleft (Stokes and Holtz, 1997). The mode of action of such substances suggest that presynaptic function and neurotransmitter levels may be altered in patients suffering from psychiatric diseases, which was shown in patients with schizophrenia (Kugaya et al., 2000).

As such, it has been suggested that some pathologies might, at least in part, have presynaptic basis, suggesting that these psychopathologies might be based on disturbances at the presynapse (Mirnics et al., 2001; Spedding et al., 2003). Furthermore, a microarray study in HAB/LAB mice, performed in a variety of brain regions, revealed differences in the expression of several candidates involved in synaptic release. These genes include, among others, *Syt4/11, syntaxin7/12, Rab3/6, Snap25,* and *calnexin* (Czibere, unpublished)*,* which are involved in the docking, and fusion of synaptic vesicles and the synaptic release (for a detailed overview see (Lodish et al., 2001)).

Synaptotagmins comprise a large family of proteins that regulate vesicle trafficking in neurons and are widely considered the presynaptic "calcium sensor" in neuronal exocytosis (Chapman, 2002; Sudhof, 2002). They are widely evolutionary conserved, and 15 isoforms , have been identified (Ting et al., 2006). The gene encoding *Syt4* is located on chromosome 18 in humans and mice (http://www.ncbi.nlm.nih.gov/). SYT4 is a secretory vesicle protein that is inducible in rodent cells as well as in the rat hippocampus by agents that elevate intracellular calcium and cAMP levels (Ferguson et al., 1999) and is broadly expressed in the brains of both rodents (Vician et al., 1995) and humans (Ferguson et al., 2000a). Additionally, it appears to regulate synaptic function (Littleton et al., 1999; Wang et al., 2001), but its specific role and function are not fully understood and controversial discussed (Ting et al., 2006). Nevertheless, in humans, SYT4 maps to a chromosomal region associated with psychiatric disease and was identified to be down regulated in a screen for transcripts and in patients with mental illness (Ferguson et al., 2000a).
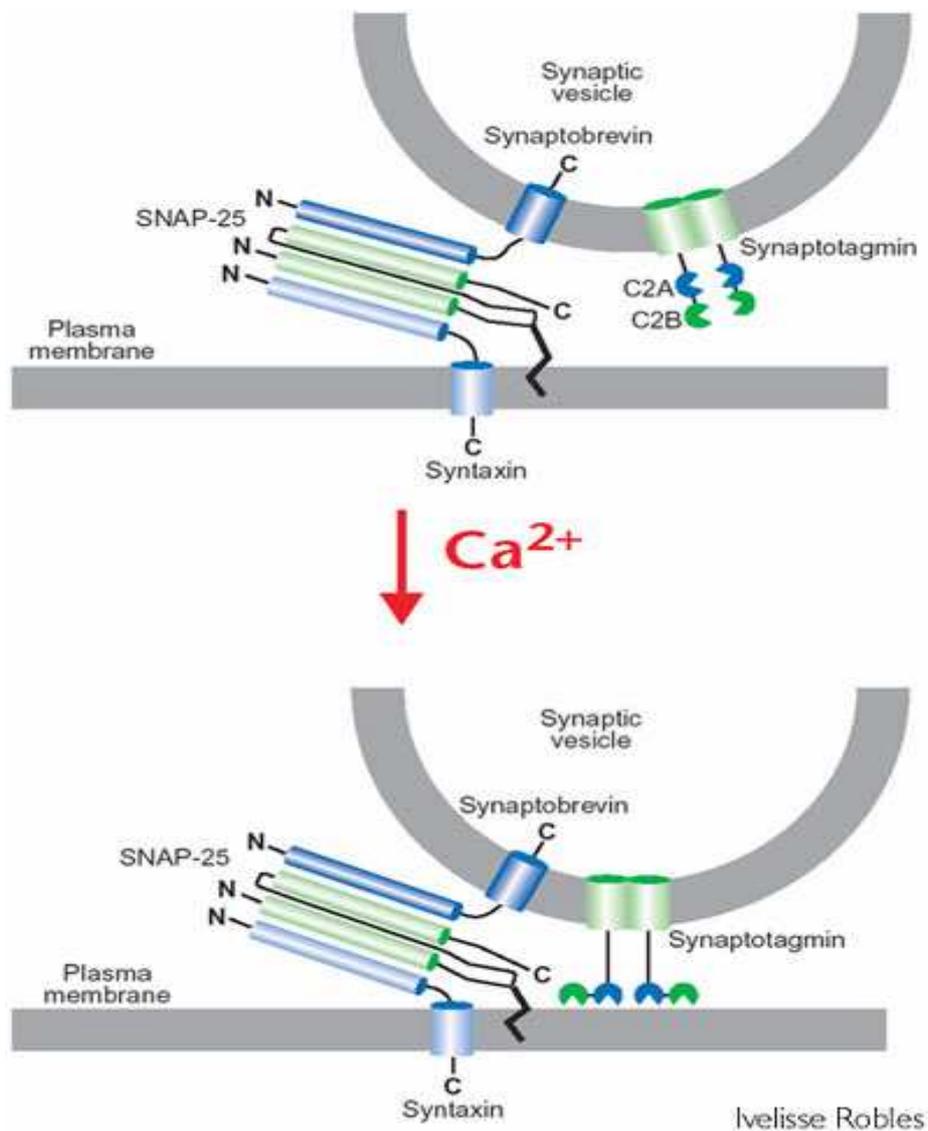
Figure 4.1.   *A schematic description of the elements of the fusion and calcium-sensing*
machinery of fast neurotransmitter release, showing interactions between v-
SNARE, synaptobrevin, in the synaptic vesicle membrane and t-SNAREs,
SNAP-25 and syntaxin, in the plasma membrane. Synaptotagmin is shown as
a dimer, as it is known to oligomerize in the membrane. Once calcium
increases, the two C2 domains form a complex with phospholipids of the
plasma membrane. Interactions between the C2B domain of synaptotagmin
and SNAP-25 could link synaptotagmin to the fusion machinery, catalyzing
fusion in a calcium-dependent manner (overview by Ivelisse Robles).

4.2   Results

4.2.1   Behavioral phenotyping

EPM behavior

In the EPM test at seven weeks of age, HAB, LAB, and NAB or F1, respectively (Figure 4.2) showed a significant difference in the percentage of the time spent on the open arms of the EPM ($p < 0.001$). Post hoc group comparisons revealed a significant difference between all mouse lines tested ($p < 0.001$ for HAB; NAB, and LAB; $p < 0.01$ for HAB, F1, and LAB). HAB mice exhibited the most anxious behavior in comparison to LAB animals, whereas NAB and F1 mice revealed intermediate behavior. Similar behavioral differences have been shown by additional behavioral measures (Table 4.1 and Table 4.2), including latency to the first open arm entry and full open arm entries. Interestingly, the number of total arm entries did not differ between HAB and NAB, but NAB as well as F1 animals revealed an increased number of arm entries (Table 4.1 and Table 4.2). Moreover, NAB and F1 mice exhibited significant increased bodyweight in comparison to the inbreeding lines.
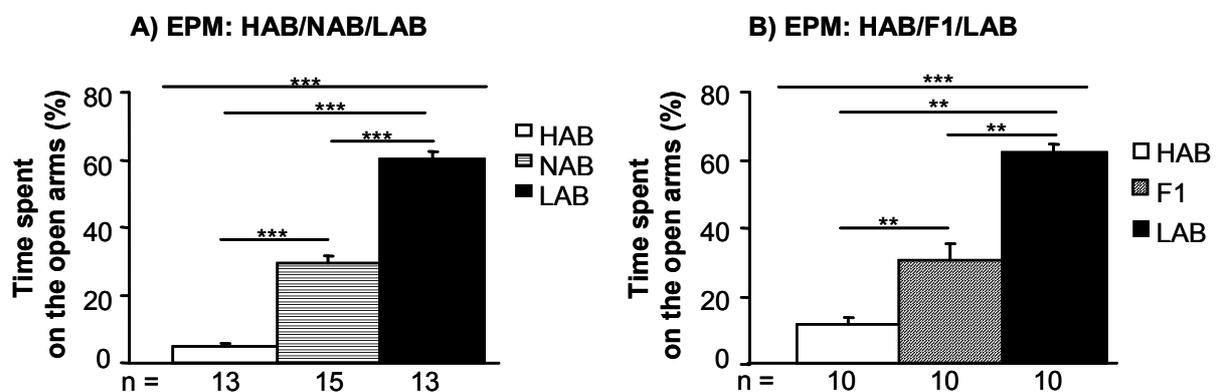
Figure 4.2:   Anxiety-related behavior in the elevated plus-maze (EPM) test in A) HAB, LAB, NAB or B) F1 mice at seven weeks of age, addressed in separate experiments. HAB mice spent significantly less time on the open arms of the EPM in comparison to LAB mice, whereas NAB and F1 animals exhibited an intermediate phenotype.***p<0.001 for three group comparisons and the post hoc comparisons in A), **p<0.01 for all post hoc comparisons in B).

Table 4.1:   *Additional behavioral parameters measured between HAB, NAB, and LAB*
          *animals in the elevated plus-maze test. T p <0.1; *p<0.05, **p<0.01, and*
          ****p<0.001 for three group comparison (KWH) and post hoc tests.*

	Behavorial parameter			Physiological parameter
	Latency to the first open arm entry (sec)	Full open arm entries (n)	Total arm entries (n)	Body weight (g)
HAB	81.6 ± 16.1	0.00 ± 0.00	21.8 ± 1.69	25.4 ± 0.58
NAB	20.5 ± 2.64	2.53 ± 0.70	28.6 ± 1.29	30.9 ± 0.34
LAB	31.9 ± 4.93	12.2 ± 1.22	22.3 ± 1.73	27.9 ± 0.72
KWH	**	***	**	***
HAB vs. LAB	*	***	n.s.	T
HAB vs. NAB	**	**	*	***
LAB vs. NAB	*	***	*	***

Table 4.2:   *Additional behavioral parameters measured between HAB, F1, and LAB*
          *animals in the elevated plus-maze test. **p<0.01 and ***p<0.001 for three*
          group comparison (KWH) and post hoc tests.

	Behavorial parameter			Physiological parameter
	Latency to the first open arm entry (sec)	Full open arm entries (n)	Total arm entries (n)	Body weight (g)
HAB	69.0 ± 13.1	0.0 ± 0.00	23.8 ± 2.26	30.5 ± 0.44
F1	21.2 ± 1.48	3.1 ± 0.75	35.8 ± 1.15	37.0 ± 0.60
LAB	31.3 ± 6.41	8.8 ± 1.31	26.8 ± 2.16	32.2 ± 0.73
KWH	***	***	***	***
HAB vs. LAB	***	***	n.s.	n.s.
HAB vs. F1	***	***	***	***
LAB vs. F1	n.s.	**	**	***

Open arm exposure

In the OA exposure HAB mice revealed an increased time spent in the less aversive proximal part of OA (Figure 4.3 A; KWH: p<0.001; p<0.05 vs. NAB, LAB; p<0.01 NAB vs. LAB) and exhibited less explorative behavior indicated by a decreased number of explorative

head movements (Figure 4.3 B; KWH: p<0.001; T p<0.1 vs. NAB; p<0.01 NAB vs. HAB; p<0.001 vs. HAB) relative to LAB mice, whereas NAB animals exhibited an intermediate behavior. Additional behavioral parameters obtained revealed similar behavioral differences (Table 4.3).
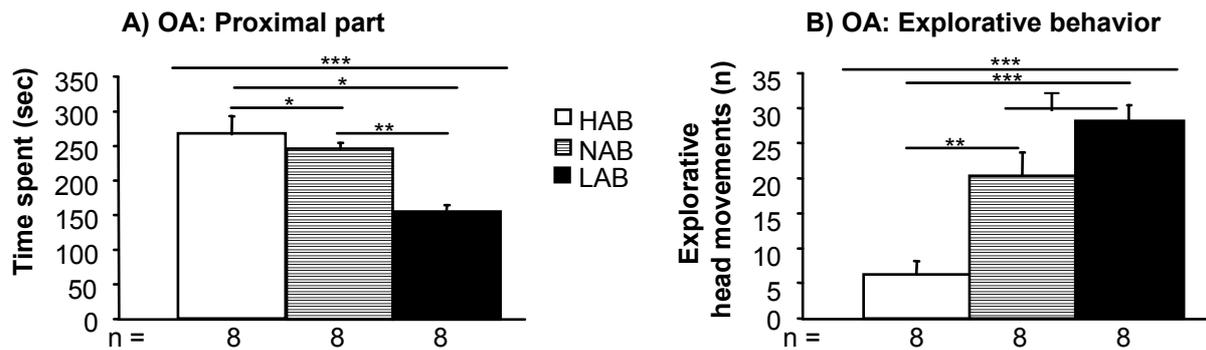
Figure 4.3:   Anxiety-related and explorative behaviors in HAB, NAB, and LAB mice measured during open arm exposure (OA). A) HAB spent significant more time in the less aversive proximal part of the OA and B) exhibited decreased explorative head movements in comparison to LAB mice, with NAB animals being in between both extremes. ***p<0.001 for three group comparison. Post hoc comparisons T p<0.1, *p<0.05; **p<0.01; **p<0.01 and ***p<0.001.

Table 4.3:   Additional behavioral parameters measured during open arm exposure in HAB, NAB, and LAB animals. T p<0.1, *p<0.05, **p<0.0 and ***p<0.001 for three group comparison (KWH) and post hoc tests.

	Behavorial parameter			
	Time spent in the medial part of the arm (sec)	Time spent in the distal part of the arm (sec)	Zone crossings (n)	Rearings (n)
HAB	9.23 ± 6.11	22.0 ± 20.23	2.5 ± 0.82	0.4 ± 0.26
NAB	24.8 ± 3.83	24.3 ± 5.96	18.0 ± 3.60	2.6 ± 0.80
LAB	49.9 ± 3.69	85.7 ± 10.08	43.4 ± 2.11	5.6 ± 1.57
KWH	***	**	***	**
HAB vs. LAB	**	*	***	**
HAB vs. NAB	*	T	***	**
LAB vs. NAB	***	***	***	n.s.

Dark light box test

HAB mice revealed decreased time spent in the light compartment of the DaLi (Figure 4.4 A; KWH: p<0.01; p<0.05 vs. NAB; p<0.01 vs. LAB;), exhibited less locomotor activity (Figure 4.4 B; KWH: p<0.01; T p<0.1 vs. NAB; LAB; p<0.05 NAB vs. LAB;), and showed less explorative behavior (Figure 4.4 C; KWH: p<0.01; p<0.05 vs. LAB, p<0.001 vs. NAB) in comparison to NAB and LAB animals.
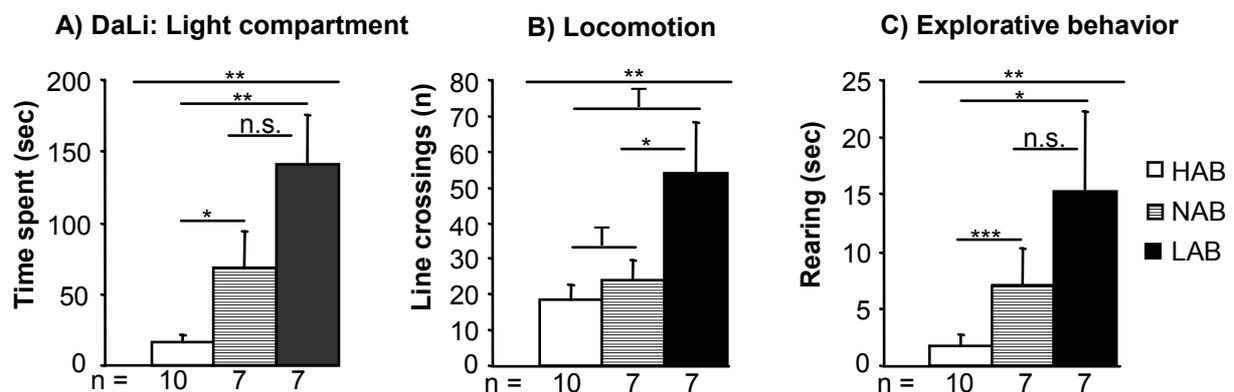
Figure 4.4:   Anxiety-related and explorative behavior investigated in the dark light box
               (DaLi) test in HAB, NAB, and LAB male mice. A) HAB spent significant less
               time in aversive light B) exhibited decreased locomotion and C) less
               explorative rearings in comparison to LAB mice, with NAB animals being in
               between both extremes. **p<0.01 for three group comparison. Post hoc
               comparisons T p<0.1,* p<0.05; **p<0.01 and ***p<0.001.

Novel cage behavior

The NC observations revealed a decreased time spent in the aversive inner part of the NC (Figure 4.5 A; KWH: p<0.001; p<0.01 vs. NAB, LAB), diminished explorative behavior (Figure 4.5 B; KWH: p<0.001; p<0.001 vs. NAB, LAB) and locomotion (Figure 4.5 C; KWH: p<0.05; p<0.001 vs. LAB, T p<0.1 NAB vs. LAB) in HAB mice relative to NAB and/or LAB animals. LAB mice exhibited less digging than the two other groups (Figure 4.5 D; KWH: p<0.01; p<0.01 vs. HAB, NAB).
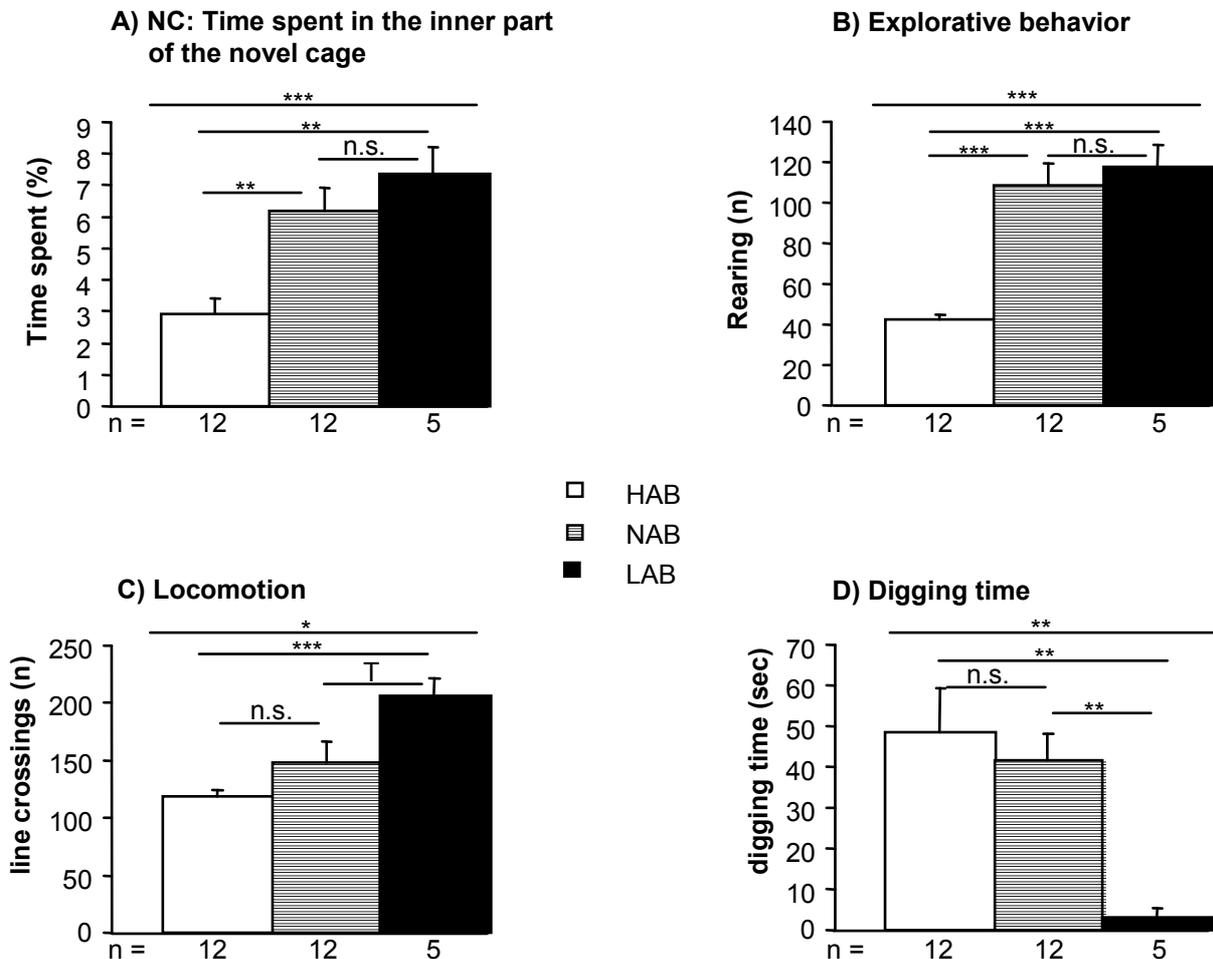
Figure 4.5: *Behavioral parameters obtained during novel cage (NC) observations in HAB, NAB, and LAB mice. HAB animals exhibited A) less time spent in the aversive inner part of the NC, B) less explorative rearings, and C) locomotion relative to LAB mice. D) LAB animals revealed less digging behavior in comparison to HAB and NAB. **p<0.01 and ***p<0.001 for three group comparisons. Post hoc comparisons T p<0.1, **p<0.01 and ***p<0.001.*

Forced swim and tail-suspension test

LAB mice exhibited a decreased immobility time in the FS as well as in the TST (Figure 4.6 A/B; KWH: p<0.001; p<0.001 vs. HAB, NAB), whereas HAB and NAB did not reveal any differences between each other. F1 mice showed an intermediate phenotype in comparison with HAB and LAB animals (Figure 4.6 B; KWH: p<0.001; p<0.01 vs. F1, LAB, T p<0.1 LAB vs. F1).
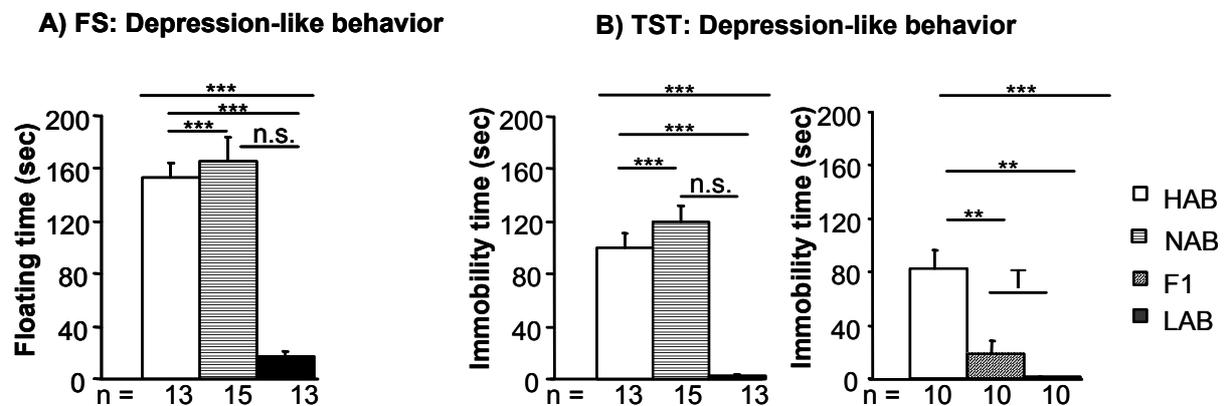
*Figure 4.6:   Depression-like behavior in the forced swim (FS) and the tail-suspension (TST) test in HAB, LAB, NAB or F1 mice. LAB animals revealed a significant decreased immobility in both the TST and FS test compared to the other groups. HAB and NAB animals did not differ, whereas F1 mice were in between HAB and LAB. ***p<0.001 for three group comparison. Post hoc comparisons T p<0.1, **p<0.01 and ***p<0.001.*

4.2.2   Expression profiling

Avp and *Oxt* expression

As depicted by ISH, LAB mice exhibited a decreased expression of *Avp* in the PVN (Figure 4.7 A; KWH: $p<0.001$; $p<0.01$ vs. NAB, $p<0.001$ vs. HAB) and SON (Figure 4.7 B; KWH: $p<0.01$; $p<0.05$ vs. HAB, NAB) in comparison to HAB and LAB animals. *Oxt* mRNA in the PVN (Figure 4.6 C) did not differ between the lines.

LAB animals showed decreased *Avp* mRNA in the PVN, SON, and CeA relative to HAB mice under basal conditions, indicated by qRTPCR (Figure 4.8, $p<0.05$).

Correlation analysis between anxiety-related behavior (Figure 4.9 A) and *Avp* mRNA in the PVN revealed a significant negative correlation between the time spent on the open arms of the EPM and the relative *Avp* expression in HAB, F1, and LAB mice under basal condition ($p<0.001$, $r=-0.619$). Moreover, a significant positive correlation was found between immobility time in the TST, indicative of depression-like behavior, and *Avp* expression in the PVN of HAB, F1, and LAB animals under basal conditions (Figure 4.9 B; $p<0.001$, $r=0.655$).

Moreover, LAB mice revealed a tendency towards reduced *Avp* mRNA in the SON relative to HAB and F1 mice, measured by ISH (Figure 4.10; KWH: $p<0.05$; T $p<0.1$ vs. LAB).
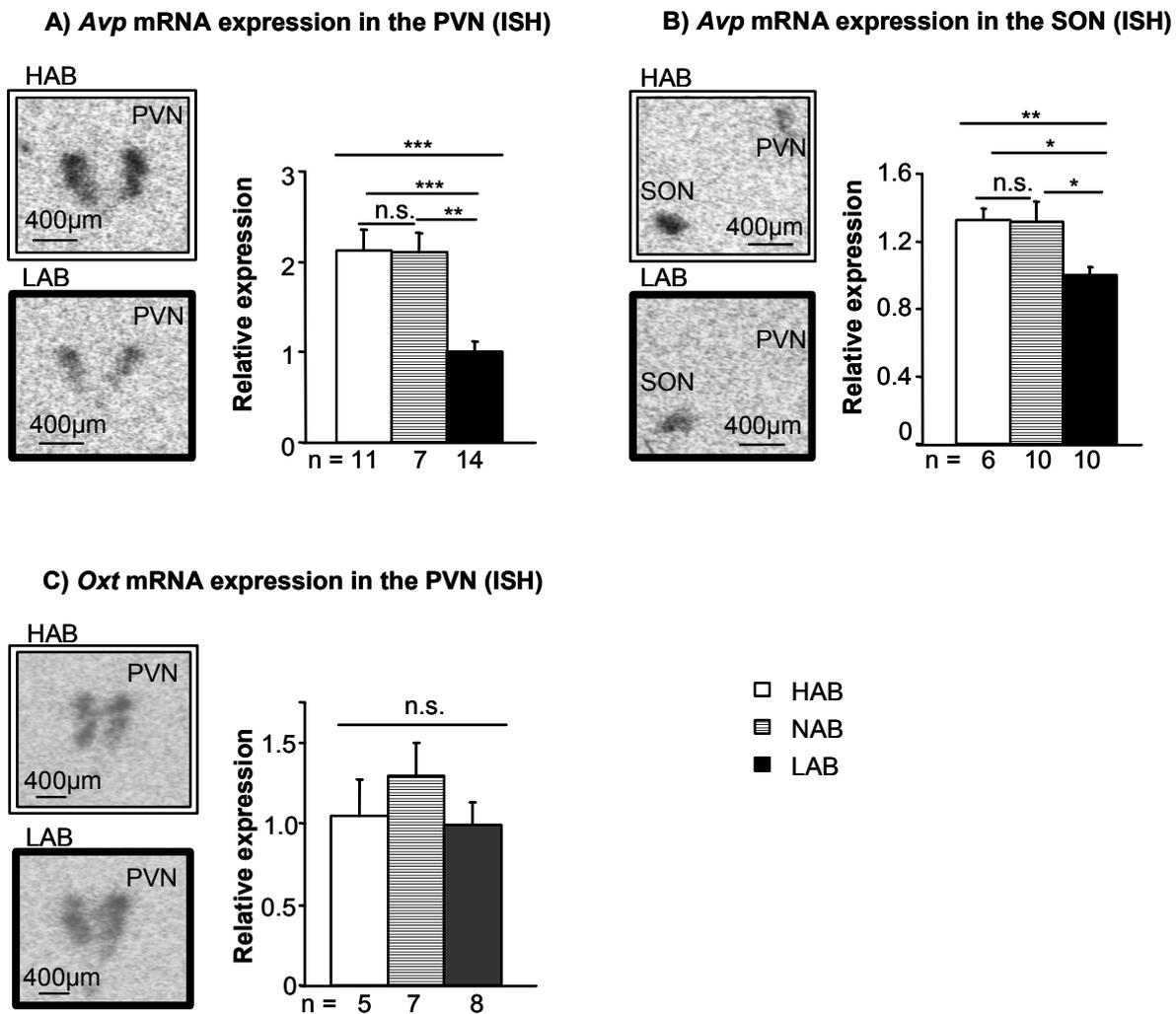
Figure 4.7:   Expression profiles of arginine-vasopressin (Avp) and oxytocin (Oxt) in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON), measured by in situ hybridization (ISH), in HAB, NAB, and LAB mice under basal conditions. LAB exhibited a significant decrease in AVP mRNA in A) the PVN and B) the SON compared to HAB and NAB. OXT mRNA did not differ between the lines. ***p<0.001and **p<0.01 for three group comparison. Post hoc comparison * p<0.05, **p<0.01 and ***p<0.001.
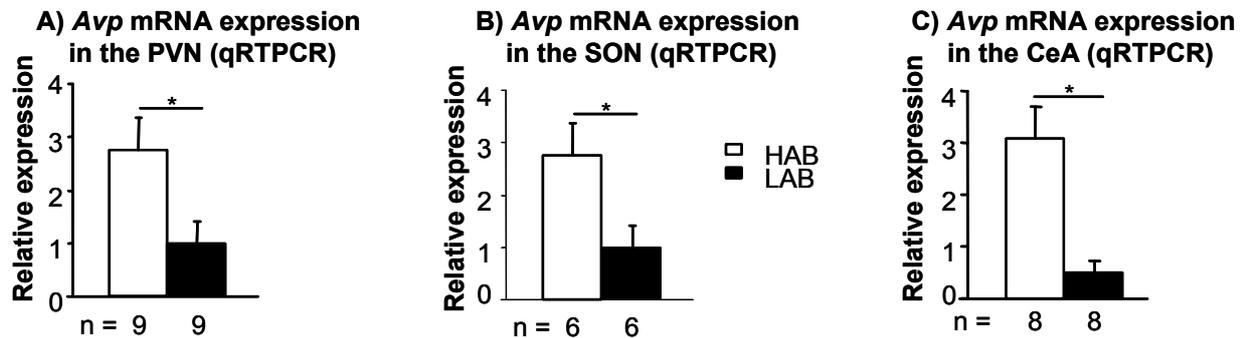
Figure 4.8:   *Expression of arginine-vasopressin (Avp), measured by quantitative real-time PCR (qRTPCR), in the paraventricular nucleus (PVN), the supraoptic nucleus (SON), and the central amygdala (CeA) of HAB, NAB, and LAB mice under basal conditions. LAB animals revealed a decreased expression of Avp in the PVN, SON, and CeA relative to HAB mice. *p<0.05 (these data have been obtained in close collaboration with Cornelia Graf and Ludwig Czibere).*
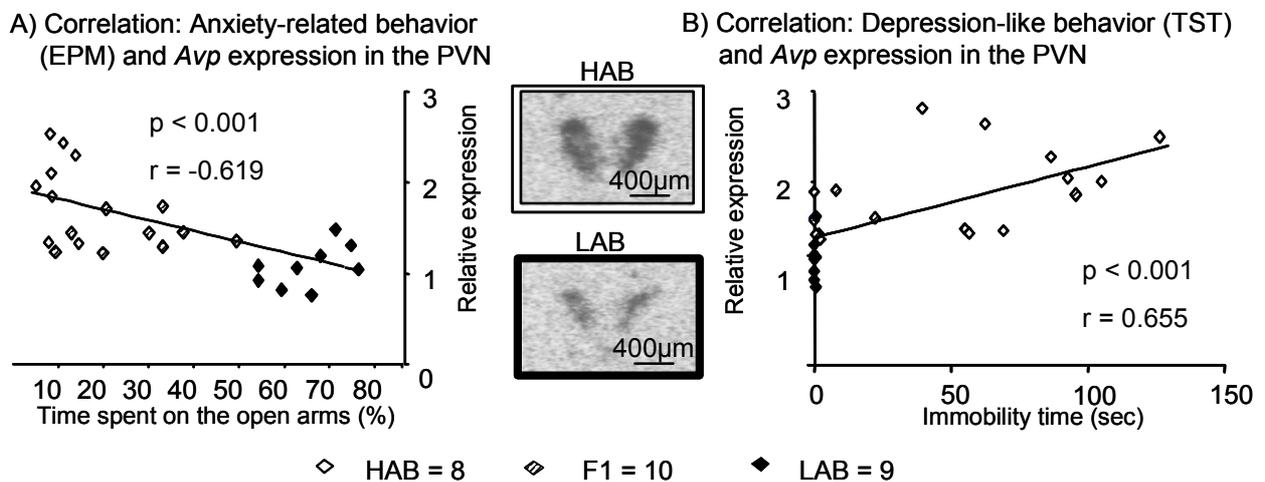
Figure 4.9:   *Correlation between A) anxiety-related behavior measured on the elevated plus-maze (EPM), B) depression-like behavior measured in the tail-suspension test (TST), and arginine-vasopressin (Avp) mRNA expression in the paraventricular nucleus (PVN) of HAB, F1, and LAB male mice, measured by in situ hybridization under basal conditions.*
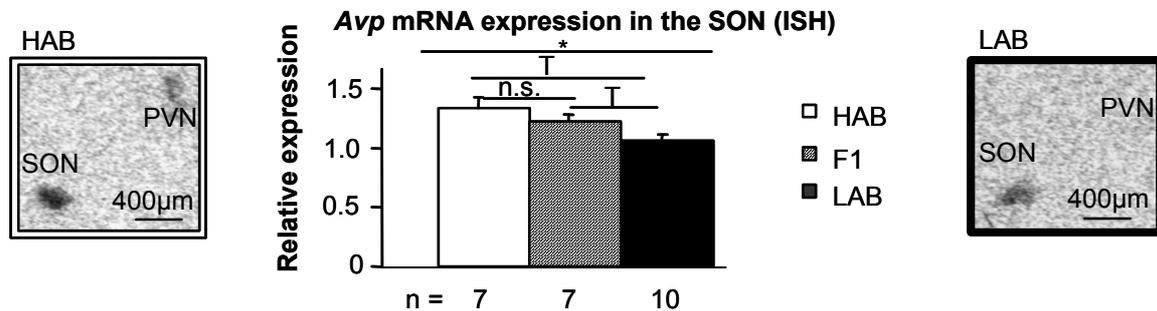
Figure 4.10:   Expression of arginine-vasopression (Avp) in the supraoptic nucleus (SON) of HAB, F1, and LAB mice under basal conditions, measured by in situ hybridization (ISH). LAB mice showed a tendency towards less Avp in the SON in comparison to HAB and F1 mice. *p<0.05 for three group comparison and T p<0.1 for post hoc tests.

Crh expression

HAB mice exhibited significantly increased Crh mRNA under basal conditions in the PVN in comparison to NAB and LAB (Figure 4.11 A; KWH: p<0.05; p<0.5 vs. LAB, T p<0.1 vs. NAB) as well as in the CeA relative to LAB (Figure 4.11 B; p<0.5 vs. LAB).
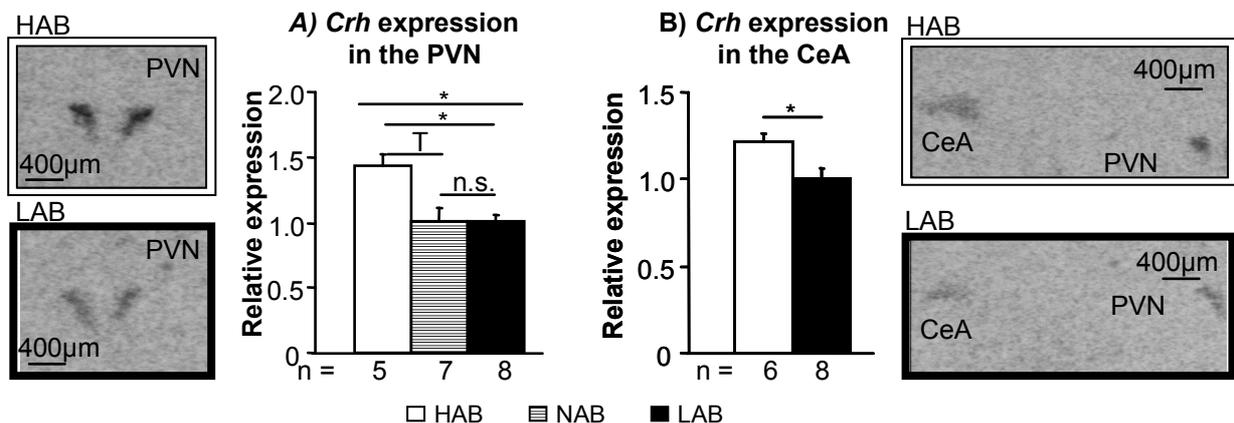
Figure 4.11:   Expression of corticotropin-releasing hormone (Crh) in the paraventricular nucleus (PVN) in HAB, NAB, and LAB and in the central amygdala (CeA) of HAB and LAB male mice under basal conditions, measured by in situ hybridization (ISH). HAB mice revealed increased Crh mRNA in the PVN and CeA in comparison to NAB and LAB animals. *p<0.05 for three group comparison. Post hoc comparisons T p<0.1 and *p<0.5.

GR, MR, *Syt4*, and BDNF expression

HAB animals exhibited increased GR mRNA levels in the hippocampus under basal conditions in comparison to NAB and LAB (Figure 4.12 A, KWH: p<0.01; p<0.01 vs. NAB, LAB), whereas the cortex did not reveal any differences between the lines investigated (Figure 4.12 B).

MR and BDNF mRNA levels, measured by ISH, failed to reveal any differences between HAB, NAB, and LAB animals under basal conditions (Figure 4.13).

Syt4 tended to be increased in HAB mice in comparison to LAB mice (Figure 4.14) in the BLA (p=0.05 vs. LAB) and CeA (T p<0.1 vs. LAB) under basal conditions, revealed by ISH.

Moreover, qRTPCR exhibited increased *Syt4* mRNA levels in HAB mice relative to NAB and LAB in the BLA (Figure 4.15 A; KWH: p<0.05; p<0.5 vs. NAB, T p<0.1 vs. LAB) and in the CeA BLA (Figure 4.15 B; KWH: p<0.05; p<0.5 vs. LAB, T p<0.1 vs. NAB). LAB, in contrast, revealed decreased *Syt4* expression in the PVN relative to the two other lines (Figure 4.15 C; KWH: p<0.05; p<0.05 vs. HAB and NAB).
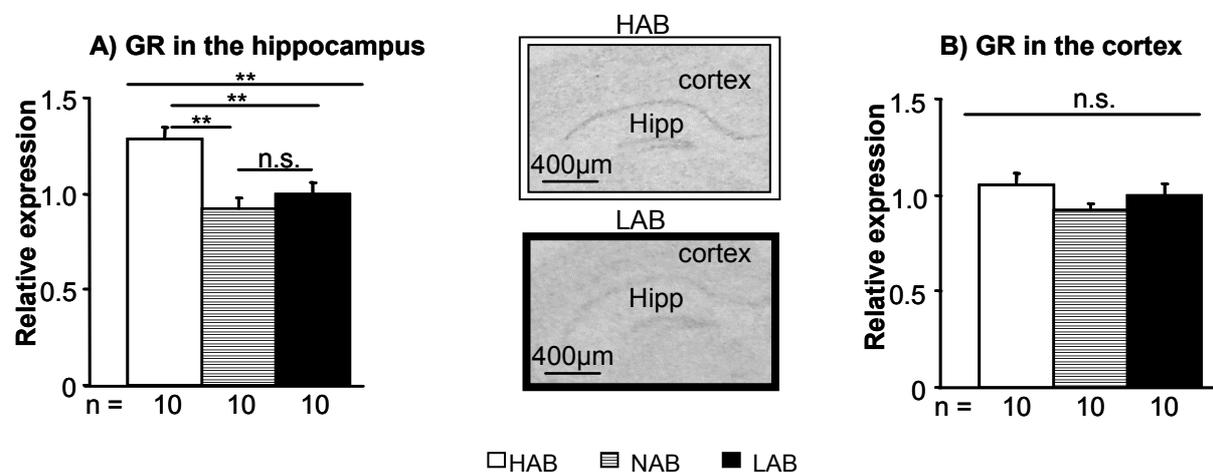
Figure 4.12:   Glucocorticoid receptor (GR) expression in the hippocampus and cortex of HAB, NAB, and LAB male mice under basal conditions, measured by in situ hybridization. HAB mice showed increased GR mRNA levels in the hippocampus in comparison to NAB and LAB animals, whereas the cortex did not reveal any expression differences between the lines. **p<0.01 for three group comparison. **p<0.01 vs. NAB and LAB.

A) MR in the hippocampus

B) BDNF in the hippocampus
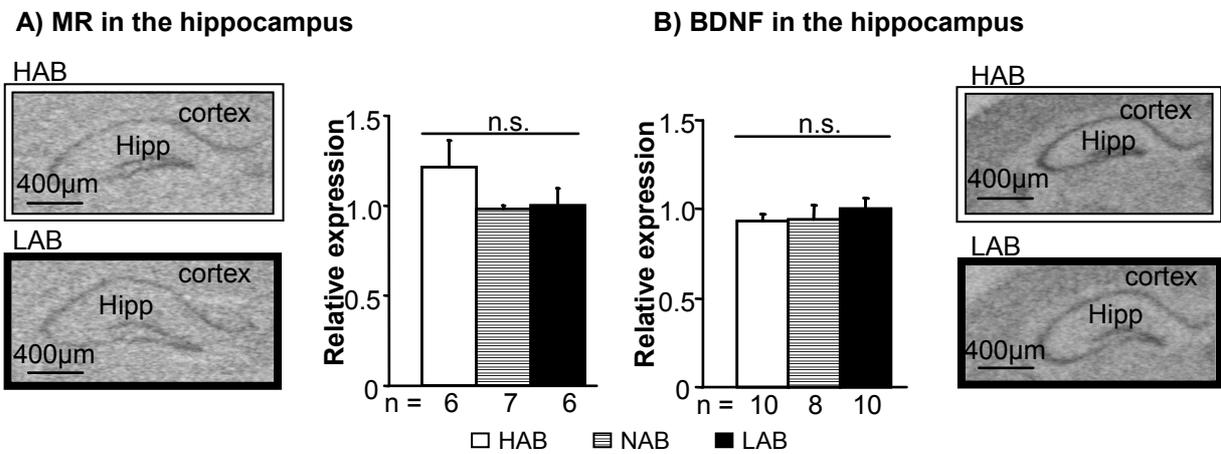
Figure 4.13:   *Expression of mineralocorticoid receptor (MR) and brain-derived neurotrophic factor (BDNF) mRNA in the hippocampi of HAB, NAB, and LAB mice under basal conditions, measured by in situ hybridization. MR as well as BDNF expression failed to reveal any differences between the three groups investigated.*

Syt4 in the BLA (ISH)

Syt4 in the CeA (ISH)
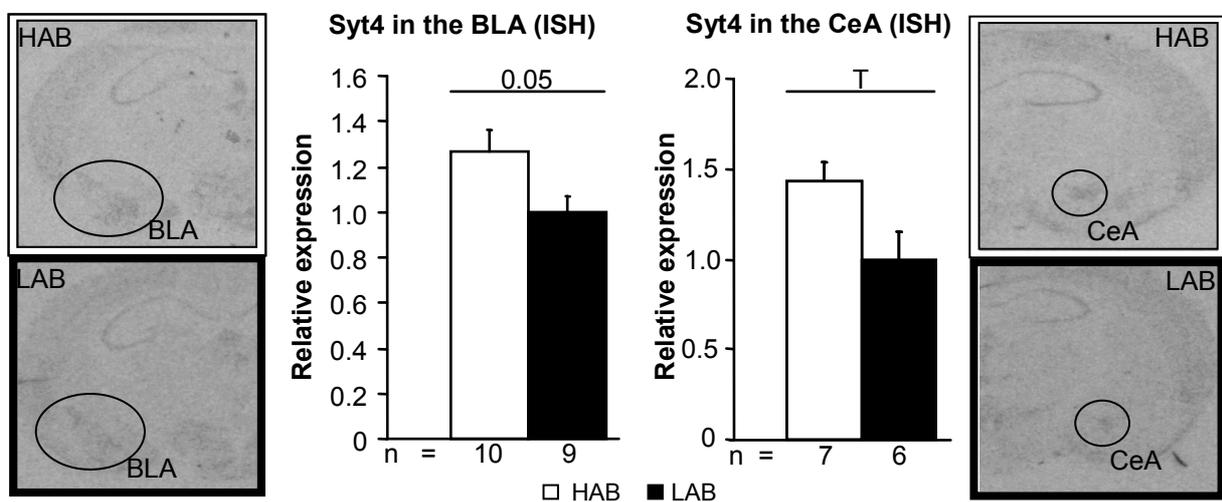
Figure 4.14:   *Synaptotagmin4 (Syt4) expression in the basolateral (BLA) and central amydgala (CeA) of HAB and LAB mice under basal conditions, measured by in situ hybridization (ISH). HAB mice showed a trend towards increased Syt4 expression in both brain regions. p=0.5 and T p<0.1 vs. LAB.*

A) Sy4 in the BLA (qRTPCR) B) Syt4 in the CeA (qRTPCR) C) Syt4 in the PVN (qRTPCR)

□ HAB   ▤ NAB   ■ LAB
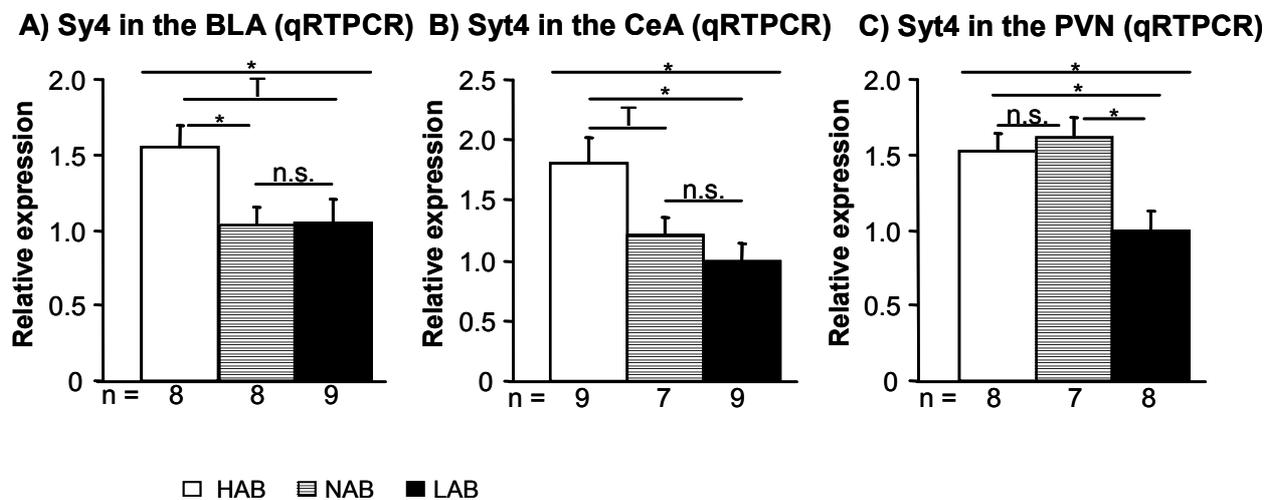
Figure 4.15:   Synaptotagmin4 (Syt4) expression in A) the basolateral (BLA), B) central

amydgala (CeA), and C) the paraventricular nucleus (PVN) of HAB and LAB

mice under basal conditions, measured by quantitative real-time PCR

(qRTPCR). HAB animals revealed a significant over-expression of Syt4 in the

BLA and CeA relative to NAB and LAB, whereas LAB mice exhibited a

decreased Syt4 mRNA level in the PVN in comparison to the two other

groups. *p<0.5 for three group comparison. Post hoc comparisons T p<0.1

and *p<0.5 (these data have been obtained in close collaboration with

Cornelia Graf and Ludwig Czibere).

4.2.3   Immunohistochemistry

AVP

Fluorescent antibody-staining of AVP detectable in the PVN, the PVN-ascending axonal
projections to the median eminence, the SCN, and the SON of HAB and LAB under basal
conditions (Figure 4.16).

Semiquantification of immunohistochemical AVP antibody-staining in HAB, NAB, and LAB
mice under basal conditions revealed a decreased amount of AVP in the PVN (Figure 4.17
A; KWH: $p<0.01$; $p<0.05$ vs. NAB, $p<0.001$ vs. HAB) and PVN-ascending axonal projections
of LAB mice relative to NAB and HAB animals (Figure 4.17 B; KWH: $p<0.001$; $p<0.01$ vs.
NAB, $p<0.001$ vs. HAB).
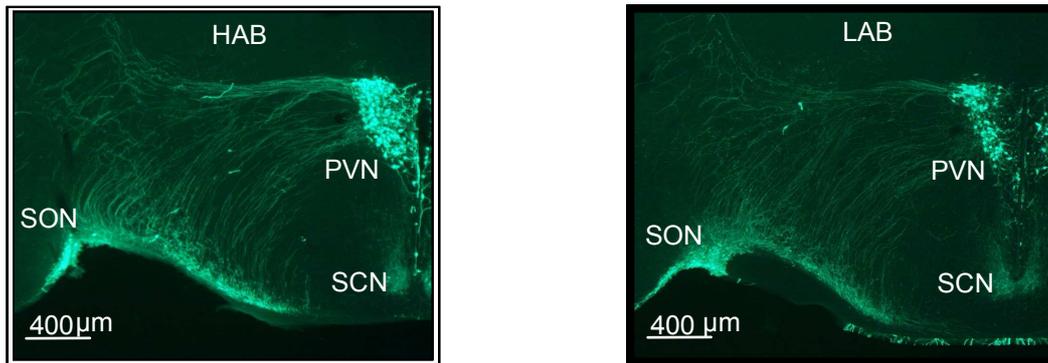
Figure 4.16:   *A representative section of fluorescent antibody-staining of arginine-vasopressin (AVP), depicted in the paraventricular nucleus (PVN), PVN-ascending axonal projections to the median eminence, suprachiasmatic nucleus (SCN), and supraoptic nucleus (SON) in HAB and LAB male mice under basal conditions.*
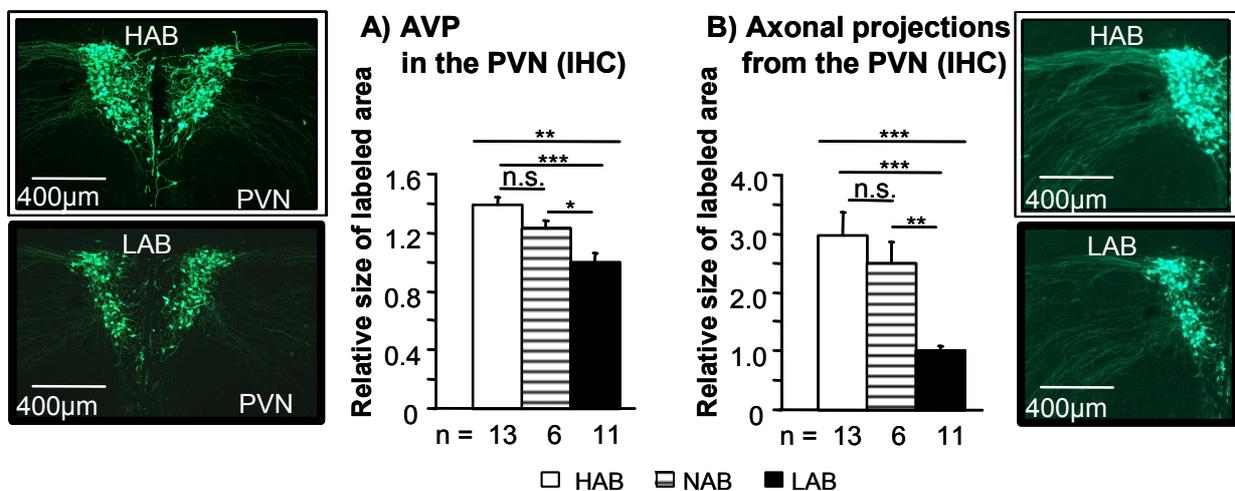
Figure 4.17:   *Immunohistochemistry (IHC) of arginine-vasopressin (AVP): Semi-quantification of the relative size of AVP-labeled area A) within the paraventricular nucleus (PVN) and B) of AVP-containing axonal projections descending from the PVN measured in HAB, NAB, and LAB male mice under basal conditions. LAB animals exhibited decreased amount of AVP in the PVN as well as in PVN-ascending axonal projections compared to HAB and NAB mice. **p<0.01 and ***p<0.001 for three group comparison. Post hoc comparisons *p<0.01, **p<0.01, and ***p<0.001.*

CRH

Semiquantification of immunohistochemical CRH antibody-staining in HAB, NAB, and LAB mice under basal conditions, revealed a trend towards increased CRH in the PVN of HAB mice relative to NAB and LAB (Figure 4.18, KWH: p<0.1)
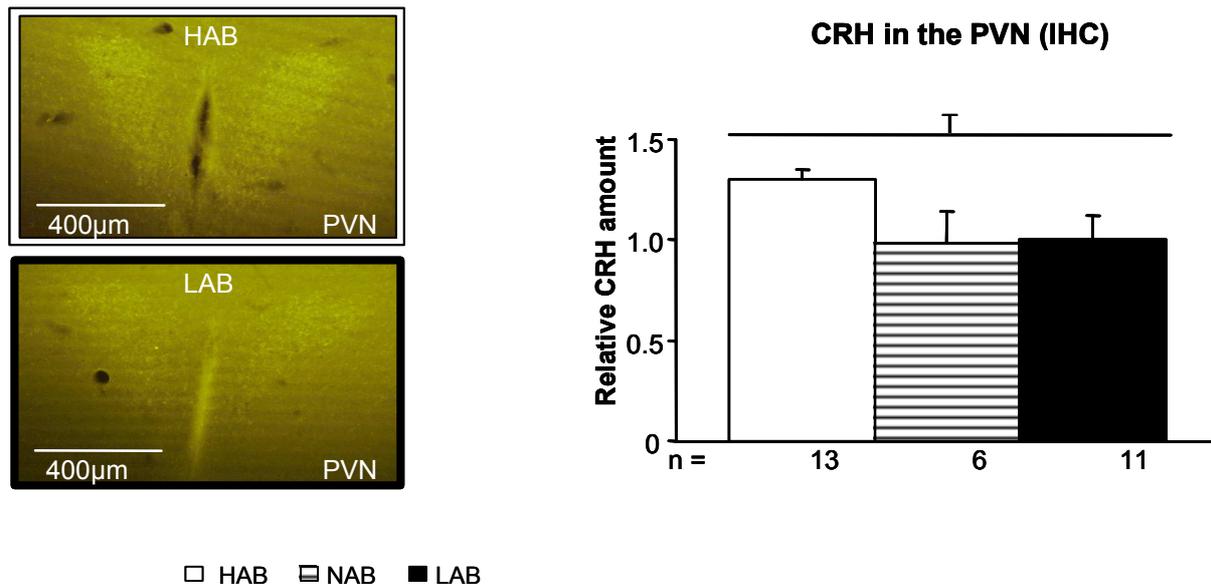
Figure 4.18:   *Immunohistochemistry (IHC) of corticotropin-releasing hormone (CRH): Semi-quantification of the relative CRH amount within the paraventricular nucleus (PVN) of HAB, NAB, and LAB male mice under basal conditions. HAB mice showed a tendency towards increased CRH in the PVN relative to NAB and LAB. T p<0.1 for three group comparison.*

4.2.4   Pharmacological validation

Intracerebroventricular administration of AVP/CRH

Icv administration of AVP/CRH resulted in a trend to reduced % time spent on the open arms (T p<0.1) and a significant decrease in the number of open arm entries in the EPM test in NABs in comparison to the control group (p<0.5). In HAB and LAB mice anxiety-related behavior remained unaltered. However, AVP/CRH administration significantly reduced the immobility time in the TST in comparison to the respective control groups in all three lines (Figure 4.19; p<0.5, p<0.01 vs. respective control group.
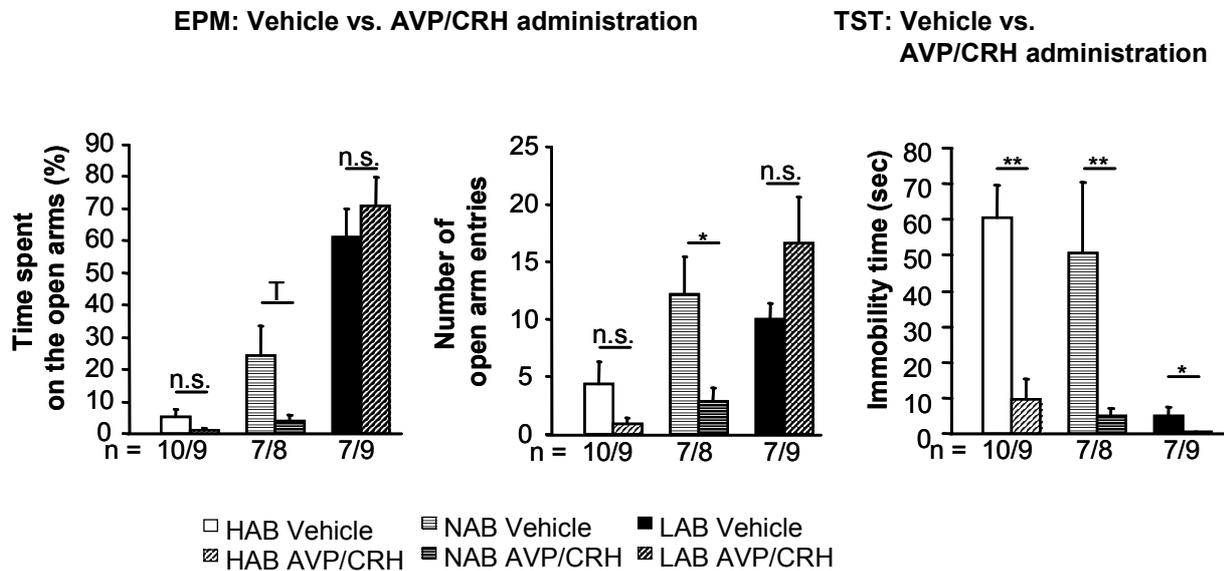
Figure 4.19:   Anxiety- and depression-like behavior in the elevated plus-maze (EPM) and
tail-suspension test (TST) in HAB, NAB, and LAB male mice
intracerebroventricularly (icv) treated with arginine-vasopressin and
corticotropin-releasing hormone (AVP/CRH) or vehicle (Ringer solution). NAB
mice showed a tendency towards reduced time spent on the open arms and
significantly decreased number of open arm entries upon AVP/CRH
treatment, whereas no changes in anxiety-related behavior were observed in
HAB and LAB mice. AVP/CRH treatment significantly decreased the
immobility time in the TST in comparison to the respective vehicle group in all
three lines. T p<0.1, *p<0.5, and **p<0.01 versus respective vehicle group.

Intraperitoneal administration of a CRHR1 antagonist

I.p. administration of the CRHR1 antagonist, DMP696, resulted in an anxiolytic effect
exclusively in HAB mice. Upon DMP696 treatment, HAB animals showed a significant
increase in the time spent on the open arm as well as in the number of open arm entries
(p<0.5). Depression-like behavior remained unaltered in both mouse lines investigated
(Figure 4.20).
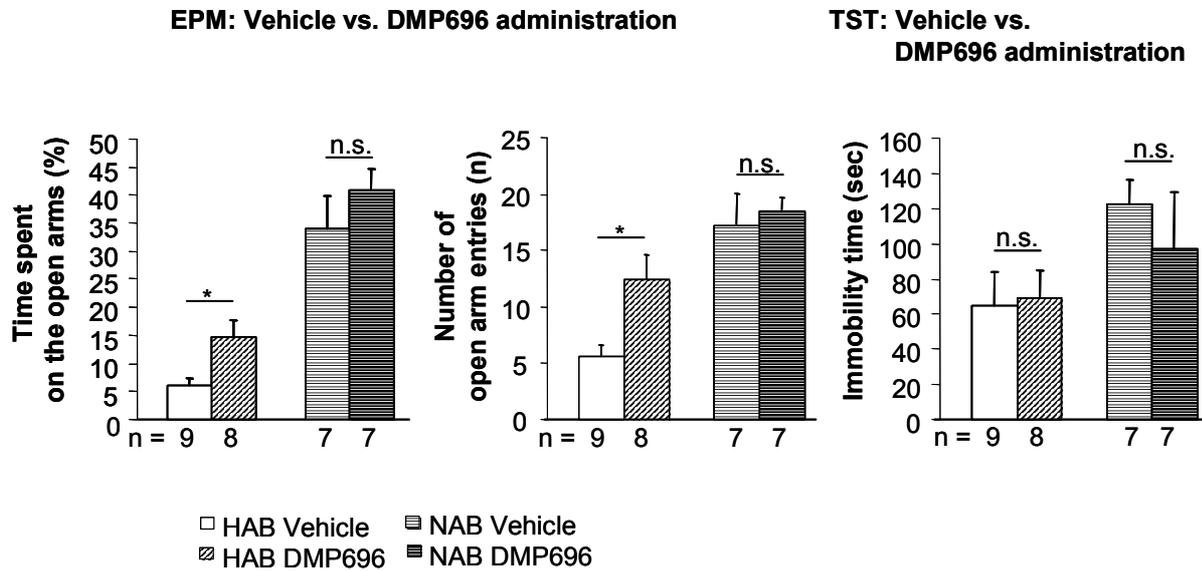
Figure 4.20:   *Anxiety- and depression-like behaviors in the elevated plus-maze (EPM) and tail-suspension (TST) test in HAB and NAB male mice, intraperitoneally (i.p.) treated with the CRH receptor 1 antagonist (CRHR1), DMP696, or vehicle (Ringer solution). Only HAB animals treated with DMP696 showed a significant reduction in anxiety-related behavior, indicated by a significant increase in time spent and number of open arm entries on the EPM. Depression-like behavior remained unchanged in both groups. *p<0.5 vs. control group.*

4.3   Discussion

Behavioral screening of HAB, NAB or F1, and LAB animals revealed a significantly increased anxiety-related behavior in HAB animal relative to LAB mice that displayed decreased anxiety-related behavior, compared to NAB and F1 animals which showed an intermediate phenotype in different tests measuring anxiety-related behavior in rodents. Furthermore, HAB animals exhibited decreased explorative behavior, compared to the two other lines. Interestingly, LABs revealed a highly significant decreased immobility time in comparison to HAB and NAB animals, with F1 being in between HAB and NAB in tests for depression-like behavior. Applying ISH and qRTPCR, decreased *Avp* mRNA expression in LAB mice in the PVN, CeA, and SON compared to HAB animals under basal conditions has been detected. NAB mice showed an *Avp* expression indistinguishable from HAB in the PVN and SON, whereas F1 animals exhibited an intermediate expression pattern relative to HAB and LAB. The *Avp* expression profile measured in HAB, F1, and LAB mice revealed a significant correlation with anxiety- as well as depression-like behaviors. Despite a high sequence homology*,* the differences in *Avp* expression were not accompanied by a differential expression of *Oxt.*

An AVP deficit in LAB animals was further confirmed by a diminished amount of both AVP in the PVN and descending axonal projections from the PVN under basal conditions, measured by fluorescent antibody-staining. Interestingly, *Crh* expression was significantly elevated in HAB mice in comparison to NAB and LAB and additionally in the CeA compared to LAB animals under basal conditions. These results were confirmed by immunohistochemistry in the PVN of HAB, NAB, and LAB mice. By treating HAB and NAB mice with a CRHR1 antagonist, DMP696, a significant anxiolytic effect was measured exclusively in HABs. Furthermore, icv injection of AVP and CRH in all three mouse lines exhibited an anxiogenic effect in NAB animals in the EPM test as well as a decreased immobility time measured in the TST in all three lines. GR expression in the hippocampus was increased in HAB mice compared to NAB and LAB, whereas GR expression in the cortex, as well as MR and BDNF expression in the hippocampus failed to reveal any differences between the lines. As indicated by ISH and confirmed by qRTPCR, *Syt4* was found to be significantly increased in the BLA and CeA of HAB animals in comparison to LAB and/or NAB, whereas a decreased expression of *Syt4* was revealed in LAB mice in the PVN.

As previously described by Krömer et al. and similar to HAB/LAB rats (Landgraf and Wigger, 2002; Murgatroyd et al., 2004; Wigger et al., 2004; Kromer et al., 2005), selective and bidirectional inbreeding resulted in two lines significantly and stably differing in their anxiety-

related behavior measured on the EPM test from the fourth generation on. HAB mice spent less time on the open arms, revealed increased latency to the first open arm entry, and no full open arm entries in comparison to LAB mice, with NAB and F1 animals displaying an intermediate phenotype. NAB and F1 mice exhibited increased bodyweight at seven weeks of age and locomotor activity, indicated by the number of total arm entries in the EPM test, whereas HAB and LAB animals did not differ. The decrease in bodyweight and locomotion, measured in the two breeding lines, reflect unavoidable consequences of an inbreeding status. However, these differences are unlikely to influence the phenotype of these animals as the two inbreeding lines reflect extremes in anxiety-related behavior independent of bodyweight and locomotion. Furthermore, the behavioral divergence between HAB and LAB mice as a result of behavioral selection and inbreeding is not based on or contaminated by line-specific differences in locomotor activity. The degree of avoidance of aversive compartments has been considered a measure of genetic predisposition to anxiety and is also predictive for stress coping (Ducottet and Belzung, 2004) and behavior in other tests (Trullas and Skolnick, 1993; Henderson et al., 2004). Indeed, the OA exposure, the DaLi test, and the NC observations revealed significant differences in anxiety-related as well as in explorative behavior, indicated by a decreased time spent in unprotected and/or illuminated areas and diminished locomotor activity, exploratory head movements, and rearings of HAB animals. It has been described that changes in locomotor activity and explorative behavior depend on the test situation (File, 2001). Reduced locomotion as well as suppression of explorative behavior reflects consequences of anxiety in a variety of behavioral tasks (Suaudeau et al., 2000; Do-Rego et al., 2002) and vica versa (Ferguson et al., 2004). Furthermore, a) the suppression of locomotor activity in low and moderate anxiogenic regions (e.g. OA exposure, NC observations), b) the shift towards less time spent and less activity in highly anxiogenic areas (e.g. open arms of the EPM or lit compartment in the DaLi test, c) the reduction of rearing behavior (e.g. in OA exposure, DaLi test or NC observations), d) increased latencies to explore novel areas (e.g. first entry of the open arm of the EPM), and e) increased autonomic responses (Singewald, unpublished) have all been identified as cross-test dimensions of anxiety which can be genetically separated (Henderson et al., 2004). Interestingly, all these dimensions of anxiety are reflected in the HAB/LAB mouse lines. Similar results have been reported from HAB/LAB rats (Henniger et al., 2000; Salome et al., 2004), underlining that the phenotypic divergence is not restricted to a given species and to the behavioral test used as the selection criterion. Rather, it is indicative that the HAB mice´/rats´ behavior was driven by anxiety, whereas the LAB

mice´/rats´ behavior was mainly explained by locomotor activity (Salome et al., 2006), with NAB animals being in between both extremes. Moreover, HABs and LABs displayed behaviors that reflected distinct coping strategies confirming anxiogenic effects even after a mild stress exposure in HAB mice and non-anxious explorative behavior in LABs. These results underline the hyper- and hypo-emotionality reflected in these animals. Furthermore, in both, the FS and the TST, indicative of depression-like behavior, LABs showed lower scores of immobility than HAB and NAB mice, with F1 animals being in between HABs and LABs. Although it has been described that these two tests do not necessarily share the same neurochemical pathways (Bai et al., 2001), both have been successfully used to measure and pharmacologically validate depression-like behavior (Cryan et al., 2002; Yoshikawa et al., 2002; Cryan and Mombereau, 2004). Furthermore, a correlation between anxiety- and depression-like behaviors has been described several times (Levine et al., 2001; Landgraf and Wigger, 2003). Indeed, in the HAB/LAB mouse model, these two tests do not only show a significant correlation to each other, but are also in close association with anxiety-related behavior. This is an intriguing finding as no selection pressure was exerted on depression-related indices, resulting in a comorbidity of anxiety- and depression-like behaviors in these animals, resembling the clinical situation of a high comorbidity between both psychopathologies (Levine et al., 2001; Kromer et al., 2005).

AVP and OXT

In accordance with findings from the HAB/LAB rat model (Murgatroyd et al., 2004), we found *Avp* mRNA and neuropeptide levels in the PVN significantly differing between HAB and LAB mice under basal conditions. The expression in HAB and LAB animals is in strict association with their respective behavior. In contrast to HAB/LAB rats (Wigger et al., 2004), this difference in *Avp* expression between the HAB/LAB mouse lines seems not to be restricted to the hypothalamic PVN, but has additionally been confirmed for both the SON and the CeA.

Due to the high sequence homology of *Oxt* to *Avp* (Ivell and Richter, 1984) and its contribution to anxiety-related behavior (Windle et al., 1997; Neumann et al., 2000b; Neumann et al., 2000a; Bale et al., 2001), OXT is considered as another neuropeptide, potentially involved in emotionality. In contrast to AVP, *Oxt* mRNA levels as well as the basal and hypertonically stimulated release of OXT within the PVN (Keßler, unpublished) were undistinguishable in all three lines. This makes, in similarity with the HAB/LAB rat model (Wigger et al., 2004), an involvement of OXT in the behavioral alterations of our mouse

model rather unlikely, thus further underscoring the specifity of our bi-directional inbreeding approach.

Several independent groups reported an association between the V1a receptor and anxiety-related behavior. V1a receptor knockout mice showed impaired social interaction, social recognition, and reduced anxiety-related behavior (Bielsky et al., 2004; Bielsky et al., 2005; Egashira et al., 2007). In contrast, a V1a receptor study revealed no differences between HAB/LAB mice in a variety of anxiety-related brain regions, suggesting that line-specific divergences in behavior and expression are not associated with a lack of V1a receptors and a thereby resulting in a compensatory expression effect in either line, but are rather a consequence of the line-specific gene and promoter/enhancer sequence (Bunck et al.).

Interestingly, in contrast to the HAB/LAB rat model (Murgatroyd et al., 2004), LAB mice revealed decreased *Avp* mRNA in the PVN and SON in comparison to HAB and NAB animals. Therefore, if a deficit in *Avp* expression really contributes to non-anxiety and reduced depression-like behavior of LAB mice, it seemed worth trying to identify the molecular-genetic underpinnings of this phenomenon.
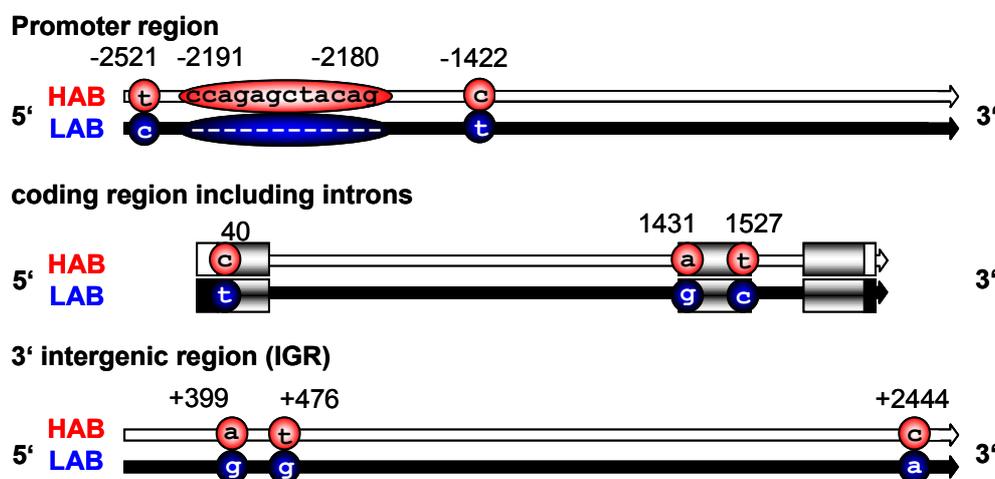
Figure 4.21:   Arginine-vasopressin (Avp) gene sequence of HAB and LAB mice.
                Polymorphic sites are indicated with positions from transcription start (-1 to -2600bp) in the promoter region (two SNP´s and deletion in LABs); within the Avp coding sequence also from transcription start (1 to 1960bp; three SNP´s); in the intergenic region between Avp and Oxt from the end of the last exon (+1 to +2600bp; three SNP´s). Exons and untranslated regions (UTRs) are indicated by boxes (exons shaded, UTRs completely filled black or white)(Bunck et al.) .

Sequencing of the *Avp* gene, the upstream promoter, and the downstream enhancer region resulted in the identification of nine polymorphic loci differing between the HAB and LAB lines, including eight single nucleotide polymorphisms (SNPs) and a 12bp deletion in LAB mice (Bunck et al.). Out of nine polymorphic loci identified between HAB and LAB mice, each structural parts, the upstream promoter, the gene locus, and the downstream enhancer region (IGR), contained three polymorphisms each (Figure 4.19). The three SNPs in the gene locus were located in the coding sequence. There were no polymorphisms identified in the non-coding (untranslated region and intronic) sequence. Two of these are silent mutations (A(1431)G and T(1527)C), whereas the third one (C(40)T) causes a substitution of alanine to valine in the third amino acid of the AVP signal peptide. At present 56 dominant or recessive SNPs in the AVP precursor gene have been identified and described to inhibit processing and trafficking of the AVP precursor and as a consequence causing an AVP deficit (Christensen and Rittig, 2006). This lack of AVP is associated with an inability of the kidney to properly retain water, thus provoking high fluid intake, hypotonic polyuria, and inadequate plasma osmolality (Verbalis, 2003), known as central diabetes insipidus (cDI). Indeed, the (C(40)T) SNP has been described as a genetic marker that co-segregated in a F2 panel with symptoms of cDI in LAB mice and partially with non-anxiety-related behavior (Keßler et al., 2007). The hypothesis of a deficit in processing and trafficking of AVP is further confirmed by the immunohistochemical findings. It is likely that the AVP antibody in the PVN can bind to the unprocessed prepropeptide, therefore revealing only a 1.4fold difference in the PVN between HAB and LAB. However, in PVN-descending axons, where the precursor is further processed in vesicles during their transport to the axonal terminals (Burbach et al., 2001), the difference occurs 3fold, indicating a disturbed processing in LAB and therefore a deficit in AVP. Indeed, this finding underlines the hypothesis, that due to the (C(40)T) SNP, the precursor gets stuck and accumulates in the endoplasmic reticulum, leading to an inadequate processing and lack of endogenous AVP and, consequently, to symptoms of cDI in LAB mice (Keßler et al., 2007). Interestingly, studies in Brattleboro rats and humans confirm that an AVP deficit may be accompanied by symptoms of cDI, reduced anxiety-related or attenuated depression-like behavior (Mlynarik et al., 2007), and signs of diminished agoraphobia as well as impaired memory processing (Bruins et al., 2006).

How do the identified polymorphisms translate into different expression profiles? The C(-1422)T SNP in the promoter region was not located in a transcription factor binding site, whereas the T(-2521)C SNP, and the Δ(-2180-2191) deletion were located directly in the center of a binding site for the nuclear factor 1 (NF-1), a well known transcription factor in

the brain that is promoting transcription in combination with C/EBPalpha or C/EBPbeta (Ji et al., 1999). Both polymorphic sites are also in direct neighborhood to a C/EBP binding site, but only the Δ(-2180-2191) site neighbors to a C/EBPbeta binding site. Even the T(-2521)C SNP might have a minor effect on reduced *Avp* expression in LABs, though it creates a binding site for NF-1 in LABs, but NF-1 might act as a repressor if a neighboring C/EBP binding site is lacking (Kraus et al., 2001). Although there is a C/EBPalpha binding site, C/EBPalpha is not expressed in the brain. Considering the deletion site, NF-1 could not bind to the Δ(-2180-2191) in animals with the LAB-specific sequence, thus resulting in a lower expression rate.

Also the SNPs in the IGR could have a major impact on expression, as formerly described (Fields et al., 2003). There is a repeat of motifs from that 178bp region between +370bp and +480bp in that two mouse line-specific SNPs were identified. The analysis of that region resulted in the identification of a binding site for c-Ets-2 near to a binding site of C/EBPbeta (Chakrabarty and Roberts, 2007). In the center of the c-Ets-2 binding site, we found the A(+399)G polymorphism that would allow to enhance transcription rate in the HAB-like but not in the LAB-like DNA sequence. As expected from variations in the gene sequence, we found similar differences in *Avp* expression in distinct nuclei of the hypothalamus. Also the polymorphisms in the coding region could lead to lower *Avp* mRNA content by negatively influencing mRNA secondary structure and stability. We can't rule out the possible influence of other polymorphisms in the coding region. Regarding mRNA secondary structure and stability, the C(40)T SNP is close to the translation start site, which is rather a conserved sequence with a relaxed secondary structure in most mRNAs, meaning it might probably not have a critical role for stability. But, taken the two other polymorphisms in the coding sequence - A(1431)G and T(1527)C - where each one is at the third degenerate codon site could lead to the hypothesis that the resulting mRNAs have different secondary structures, thus decreasing the stability of LAB sequence-specific mRNAs and finally leading to faster degradation (Shabalina et al., 2006).

Due to the similar expression of *Avp* in HAB and NAB, the allele frequency in an unselected NAB population of both the Δ(-2180-2191) deletion as well as of the C(40)T SNP that are most likely to be involved in either the regulation of gene expression or the processing of the AVP precursor (Keßler et al., 2007) has been investigated. Determining the most common genotypes regarding the described polymorphisms in the CD1 (NAB) mouse population – which the HAB and LAB animals are derived from – revealed that the HAB-specific gene SNPs represent the most common genetic variant. More than 70% of the NAB mice were

homozygous for the HAB-specific sequence, whereas less than 1% carried the LAB-specific allele homozygously. Furthermore, the two polymporphisms co-segregated in all analyzed samples (N>150), suggesting a strict linkage.

The fact that the NAB line's most common genotype in and around the *Avp* gene fully corresponds to the HAB-specific sequence in more than 73% of all cases, gives rise to the explanation of the often observed discrepancy between F1 and NAB mice, both being often used as controls (regarding depression-like and *Avp* expression phenotype). In contrast to strictly intermediate F1, unselected CD1 mice displayed in about 70% of all animals a HAB-like depression-like behavior in the TST, what has also been demonstrated recently in an unselected CD1 population (Touma et al., 2007).

Furthermore, strengthening the strict association of an expression pattern with the identified allele-specific SNPs in either line, allele-specific transcription analyzes were applied. It was shown that the *Avp* deficit in LAB animals is not primarily a result of differences in synaptic input or epistatic effects of other genes. By cross-mating HAB and LAB mice, we produced heterozygous F1 animals that host both HAB and LAB line-specific alleles in each cell. The analysis revealed a strongly decreased expression of the LAB-specific allele by 75% compared to the HAB-specific one. Indeed, these heterozygous F1 mice – in contrast to NABs – display a strictly intermediate anxiety-related and depression-like phenotype together with intermediate *Avp* expression in comparison to HAB and LAB animals. We therefore conclude that the line-specific expression of *Avp* is causally related to the line-specific polymorphisms (Bunck et al.). This confirms our hypothesis that distinct polymorphisms in a candidate gene may contribute to the phenotype of trait anxiety and depression. Indeed, an increased *Avp* mRNA expression has been shown in the PVN and SON of depressed patients (Purba et al., 1996; Meynen et al., 2006) and in the opposite extreme of the behavioral phenotypic scales in LAB mice, a decreased *Avp* expression in these brain regions could be associated with non-anxious and non-depression-like behavior. Additionally, whereas AVP in the CeA is described to be involved in immobility reflecting passive coping strategy (Roozendaal et al., 1992), in LAB animals the AVP deficit might contribute to their increased active coping style in comparison to HAB or NAB.

Although no genetic underpinnings in the HAB line concerning the *Avp* gene, in comparison to NAB animals, have been identified, its contribution to the hyper-anxious phenotype in HABs can not be excluded. As a microdialysis study in the PVN (Keßler et al., 2007) did not include NAB animals, it is still possible that their hyper-emotionality, reflected in a variety of behavioral tests in this line, is a consequence of an increased release of AVP in several

brain regions including the PVN and/or CeA. Indicated by studies in psychiatric patients, hyper-excitability in anxiety/fear circuits are expressed as pathological anxiety that is manifested in various anxiety disorders (Rosen and Schulkin, 1998). Indeed, by use of Fos antibody-staining after mild stress exposure (Muigg, in preparation) or manganese-enhanced magnet-resonance imaging (MEMRI) under basal conditions (Czisch, in preparation), several brain regions have been identified to be hyper-activated in HAB animals in comparison to NAB and LAB mice, including amygdalar nuclei or the LS. These regions include brain region described for their AVP release and contribution to the regulation of emotionality (Frank and Landgraf, 2008). Additionally, the HAB animals did not reveal any changes in anxiety-related behavior after AVP and CRH injection, indicating that their already high anxiety level measured in the EPM can not be further increased by anxiogenic drugs as a possible consequence of an already elevated release of AVP and CRH under untreated conditions, reflecting kind of a ceiling effect.

Furthermore, administration of an AVP V1a/b receptor antagonist tended to reduce anxiety-related behavior and significantly decreased depression-like behavior in HAB mice (Bunck et al.). The involvement of V1a/b receptor pathways in mediating anxiety-related behavior as well as passive coping strategies has formerly been described for HAB rats (Keck et al., 2002; Wigger et al., 2004) and in other laboratories (Griebel et al., 2002; Griebel and Gal, 2005). As we do not have any evidence for AVP or CRH release patterns being different between HAB and NAB, it can only be speculated that the single injection of AVP and CRH in NAB mice is involved in the anxiogenic effect measured by the increase in anxiety-related behavior exclusively in these animals. As mentioned above, it is possible that under untreated conditions, the AVP release in NABs is decreased compared to HAB mice, and therefore, an administration of AVP leads to the anxiogenic effect of these animals. However, in HAB mice according to their already high level of anxiety-related behavior, a further increase seems to be impossible. The anxiogenic effect of AVP administration could also been demonstrated in rats (Bhattacharya et al., 1998). Additionally, CRH has been described several times to be involved in alterations of anxiety- and depression-like behavior (see discussion below). LAB animals, in contrast, did not reveal any differences upon AVP and CRH injection indicative of their robust non-anxious behavior. As shown before, due to their lack of AVP in association with their phenotype, it is rather likely that by a single administration, the behavior in these animals can not be altered. One possible experimental design to rescue the phenotype of non-anxiety in these animals could be a chronic AVP treatment via osmotic minipumps over several days or even weeks, to examine alterations in

behavior. The advantage of a chronic administration of AVP via minipump implantation could be demonstrated by Bosch et al. (unpublished), resulting not only in alterations of maternal behavior, but also in anxiogenic behavior in the EPM test in LAB females.

Taken together, applying a similar breeding strategy in two different species (*Rattus norvegicus* and *Mus musculus*) ended up with similar findings concerning central expression and release of AVP, and in the identification of polymorphisms in and around the *Avp* gene, likely to be involved in mediating both anxiety-related and depression-like behavior. These results, together with human studies (Scott and Dinan, 2002) do not only ensure the construct validity of our mouse model, but additionally provide a strong impetus for *Avp* as a valuable diagnostic tool and therapeutic target in psychopathologies.

CRH

Interestingly, in similarity with expression profiles revealed in HAB/LAB rats, HAB and LAB mice significantly differed in their *Crh* expression in the PVN (Bosch et al., 2006). Although in the rat model is has not been clarified if the alteration in expression is restricted to HAB or LAB animals, as a comparison to Wistar rats is missing. In the mouse model, however, it could be shown that HAB mice exhibited an increased *Crh* expression as well as CRH amount in the PVN compared to NAB and LAB under basal conditions. Sequencing of the *Crh* gene in both animal models (Murgatroyd, Czibere, unpublished) did not reveal any SNPs or deletions in either line, suggesting that the differences in expression might be due to alterations in synaptic inputs, epigenetic factors, transcription factors, or epistatic effects of other genes. Especially, transcription factors interacting with cAMP response elements are an important factor in the regulation of *Crh* (Chen et al., 2000). Concerning gene sequencing, similar results have been found in human studies, revealing increased levels of CRH in the brain and spinal cord in depressed patients in contrast to control subjects, but no differences in the structure of the gene (Stratakis et al., 1997).

Furthermore, the over-expression of *Crh* in HAB mice is not only restricted to the PVN, but was also present in the CeA. Roles for both CRH-containing brain regions, the PVN and amygdala, in stress responsiveness have been reported (Cook, 2004). Injection of cytotoxic antibodies directed against CRH in the PVN has been found to block anxiety-related response in the EPM test provoked by social defeat (Menzaghi et al., 1992), whereas intra-amygdala infusion of CRH produced anxiogenic effects (Griebel, 1999). Furthermore, prenatally stressed rats, in comparison to control rats, but similar to HAB mice, displayed a hyperemotional state and increased anxiety-related behavior associated with an increased

CRH release in a time- and calcium-dependent manner (Cratty et al., 1995). These findings are in line with transgenic mice over-producing CRH, which showed increased anxiety-related behavior (Stenzel-Poore et al., 1994; van Gaalen et al., 2002). Several animal studies have illustrated the involvement of CRH downstream neurotransmitter targets, including serotonin and noradrenaline, in the profound neurocircuitry failure that may underlie maladaptive coping strategies (Bale, 2006). Indeed, the activity of the noradrenergic neuronal system has been observed to be increased during stress and anxiety in several animal species and states of anxiety and fear appear to be associated with an increase in noradrenaline release in humans as well (Charney et al., 1995). Additionally, anatomical evidence exists for the direct synaptic contact between CRH terminals and dendrites of noradrenergic cells in the LC (Arborelius et al., 1999). Stress- or CRH-induced increase in LC neuronal firing are blocked by CRHR antagonists (Valentino et al., 1993), suggesting that the anxiogenic effects of CRH are mediated through its action on the noradrenergic system via the LC (Butler et al., 1990). On the other hand, noradrenaline has been described to increase *Crh* mRNA in the PVN (Cole and Sawchenko, 2002). Therefore, the increased *Crh* mRNA in the PVN (Bosch et al., 2006) and LC (Plotsky et al., 2000) in HAB rats and increased *Crh* mRNA in the PVN and CeA of HAB mice together with the hyperactivation exhibited in the LC in both species (Salome et al., 2004), give rise to the hypothesis that the interaction between both system, the noradrenaline and the CRH system, might contribute to the hyper-emotionality observed in these animals.

Not all CRH projections have been clearly defined yet, but it is possible to describe connections that use CRH as a neurotransmitter that may be relevant for the explanation of neuroendocrine as well as behavioral alterations. First, and still speculative, a stress-related CRH-circuit has been proposed, including CRH input to the CeA, originating in the lateral hypothalamus, dorsal raphe, and intrinsic cells of the CeA (Gray, 1993). CRH cells located in the CeA in turn project to the BNST, the latter of which project to the PVN, subsequently leading to an activation of the HPA axis (Steckler and Holsboer, 1999). Second, CRH projections originating in the CeA, the PVN, and the BNST, terminating in the LC, may underlie the enhanced arousal in response to stressors that have an emotional component (Butler et al., 1990; Valentino et al., 1993) and possibly affect attentional processes, as the coeruleo-cortical projection has been shown to be critically involved in mediating selective attention (Carli et al., 1983; Cole and Robbins, 1992; Usher et al., 1999). Third, CRH projections from the CeA and the BNST to the PAG and the brainstem autonomic regions, from the hypothalamus to the LS, and from the inferior olivary nucleus to the cerebellum

have been discussed, in addition to other projection circuits, to mediate various types of behavior, including anxiety (Steckler and Holsboer, 1999). Although more circuits have been described, the ones mentioned before seem to be the most relevant ones to explain possible alterations in HAB animals contributing to their hyperemotionality due to changes in CRH. All the brain areas depicted in these circuitries have been highlighted by either over-expression of CRH or by hyperactivation, measured via Fos-staining after mild stress-exposure or MEMRI technique under basal conditions (Muigg, Czisch, in preparation), in HAB mice relative to NAB or LAB. These findings point to the involvement of CRH in a variety of neuronal circuits underlying specifically the hyper-anxious phenotype of HAB mice.

CRH as a contributor of anxiogenic effects (van Gaalen et al., 2002) revealed a significant increase in anxiety-related behavior in NAB mice. Although the animals have been injected with AVP and CRH simultaneously, according to their lower *Crh* expression in the PVN in comparison to HAB animals, it is rather likely that the observed anxiogenic effect is mediated by CRH, with the CRHR1 receptor-mediated pathways suggested to underlie the anxiogenic-like effects of CRH (Heinrichs et al., 1997). Furthermore, the anxiogenic properties of CRH have been proven several times (Steckler and Holsboer, 1999), accompanied by behavioral aspects such as increase in grooming, arousal, heart rate, and decreased explorative behavior or food intake (Britton et al., 1982). As mentioned above the robust non-anxiety of LAB mice was not altered by a single application of an anxiogenic substance, whereas the hyper-anxiety in HAB mice, with their already high level of AVP and CRH, can not be further increased. As confirmed by Conti et al., the sensitivity to respond to CRH is strongly depending on the animals´ respective genetic background (Conti et al., 1994).

In contrast to anxiety-related parameters, depression-like behavior was decreased in all three lines upon AVP/CRH injection into the lateral ventricle. As there are no anti-depressive effects reported after AVP administration, we hypothesize that the increase in activity is primarily due to CRH injection. Indeed, several series of experiments tested the hypothesis of a behavioral activation and anxiogenic effects produced by icv administration of CRH may be mediated by noradrenergic neurons in the brain-stem LC. In the modified Porsolt swim test, which similarly to the TST examines arousal and agitation in a stressful situation, a decreased immobility time was observed following CRH infusion directly into the LC or into the lateral ventricle. These results suggest that the behavioral effects of CRH in the LC might be related to arousal or stress-related effects, rather than to increased locomotor activity per se. Additionally, biochemical studies showed a significant increase in the concentration of the noradrenaline metabolites in areas of the LC´s forebrain projection,

such as the amygdala and posterior hypothalamus. These data suggest that CRH produces its behavioral activation and anxiogenic effects, at least in part, by increasing the activity of LC noradrenergic neurons (Butler et al., 1990). Several independent groups confirmed the behavioral alterations following icv administration of CRH and reported that no behavioral parameter was altered by prior administration of dexamethasone in a dose that blocked pituitary-adrenal activation to CRH. Therefore, it was hypothesized that behavioral effects of CRF are mediated by its action at central sites and not via an action on the pituitary-adrenocortical system (Britton et al., 1982). Nevertheless, the experimental design applied could reveal alterations in behavior, but the impact of each of the applied substances on the respective behavioral change could not be clearly defined. Therefore, for analysis of the detailed effects of AVP and CRH on behavior, the substances should be tested separately.

Treatment of HAB and NAB mice with a non-peptide CRHR1 antagonist DMP696 revealed a significant reduction of anxiety-related behavior exclusively in HAB animals, whereas the phenotype of NAB mice remained unchanged. Anxiolytic properties of CRHR1 receptor antagonists in a dose-dependent manner have been demonstrated in a variety of independent laboratories in several species (Steckler, 2005). The efficiency of treatments is strongly depending on the genetic background, indicated by studies in different mouse strains, with some strains being sensitive and others resistant to CRHR1 antagonist treatment (Conti et al., 1994). This is further underlined by inconsistent findings concerning anxiolytic and/or antidepressive effects revealed in different strains underlying different genetic underpinnings (Nielsen et al., 2004). Furthermore, previous studies using unselected animals, including those using DMP696 as an CRHR1 antagonist, demonstrate anxiolytic or antidepressive effects only after pre-exposure to stressors (Heinrichs et al., 1994; Schulz et al., 1996; Mansbach et al., 1997; Griebel et al., 1998; Deak et al., 1999; Okuyama et al., 1999). It has further been suggested that DMP696 is most effective in rodents that are hyperresponsive to stress and have increased CRH levels (Maciag et al., 2002). As both suggestions are reflected in the HAB phenotype, these animals, in contrast to unselected mice, provide a model that mimics clinical conditions observed in patients.

The result that only animals reflecting hyper-emotionality respond to a CRHR1 antagonist treatment, is consistent with findings revealed in the HAB rats (Keck et al., 2003a) as well as with decreased anxiety scores after R121919 treatment in depressed patients (Zobel et al., 2000). Furthermore, the anxiolytic effects caused by CRHR1 antagonists (Keck et al., 2001) in contrast to other anxiolytics like diazepam did not reveal differences in locomotor activity, indicative of sedative effects (Liebsch et al., 1998).

The anxiogenic effect of CRH is mediated via CRHR1 pathways, not CRHR2 (Heinrichs et al., 1997). In more detail, the two CRH receptor subtypes selectively mediate differential effects of endogenous CRH or CRH-related peptides at the brain level with the CRHR1 contributing predominantly to emotional behavior, e.g. anxiety-related behavior, and the CRHR2 being involved in the regulation of stress coping behavior, e.g. coping strategies (Liebsch et al., 1999). Furthermore, CRHR1 receptor antisense in the CeA reduced anxiety-related behavior, highlighting the CeA as an important center in the pathways underlying emotional integration (Liebsch et al., 1995). Indeed, CRHR1 deficient mice, similarly to LAB mice, revealed decreased anxiety-related behavior in the DaLi test without changes in locomotor activity and hyperactivity in the open field test, possibly due to a deficit in the CRHR1-mediated pathway in the CeA (Timpl et al., 1998). Besides the CeA, the BNST, the PFC, the cingulate cortex, the LC, and the PAG are proposed sites, where CRHR1 can modulate behavior (Steckler and Holsboer, 1999).

Taken together, these findings suggest that the interaction between endogenous CRH and its receptors in this limbic brain regions is crucial for the expression of anxiety-related behavior in response to stressful experience, pronounced in HAB mice, which highlights the CRHR1, especially the DMP696, as a promising candidate for therapeutical intervention in anxiety disorders.

MR and GR

Overexpression of MR in the forebrain has been described to decrease, directly or indirectly, anxiety-related behavior and to alter stress response in mice (Rozeboom et al., 2007). As MR mRNA did not reveal any differences in the hippocampi of HAB, NAB, and LAB mice, MR expression, is at first glance, is not contributing to the phenotypic differences observed in the three mouse lines, at least under basal conditions. Although alterations in the circadian rhythm have been revealed in HAB, NAB, and LAB mice, with LAB mice showing hyperactivity as well as a shifted circadian rhythm compared to the two other lines (Keßler, Singewald, unpublished), these differences seemingly do not affect the control of basal glucocorticoid levels and the maintenance of circadian rhythm of glucocorticoids via MR, at least as indicated by the timepoint of the measurement. This hypothesis is further confirmed by equal Cort levels under basal condition in HAB and LAB mice (see 5).

The forebrain GR plays an anxiogenic role in several genetic (Montkowski et al., 1995; Gass et al., 2001) or chronic stress animal models (Schmidt et al., 2007) of altered GR levels, mainly due to decreased hippocampal GR levels accompanied by disturbances in HPA axis

feedback. In HAB animals, however, significantly increased GR mRNA levels in the hippocampi were measured compared to NAB and LAB, whereas the cortex did not reveal any differences under basal conditions. Due to the discrepancy between cortical and hippocampal expression, it is rather unlikely that the differences in the hippocampal GR expression are a consequence of SNPs or deletion in the gene encoding GR. However, there is evidence that GR expression is altered by maternal behavior (Weaver et al., 2004). High amounts of pup licking and arched back nursing have been associated with increased GR expression in the hippocampus and changes in stress responsiveness (Szyf et al., 2005). Similar to this animal model, HAB mice revealed increased maternal care compared to LAB females (Keßler, in preparation), with the offspring displaying increased hippocampal GR expression, possibly due to DNA methylation and enhanced transcription binding in the GR promoter region as described by Szyf et al. (2005). As GR is mainly required for the stress response and the subsequent recovery of homeostasis via feedback (de Kloet et al., 1990; de Kloet et al., 1998), we challenged HAB and LAB mice by a 15-min restraint (Touma et al., 2007). Similar to the high-licking/-arched back nursing rat model, HAB mice showed a decreased Cort response after restraint stress in contrast to LAB mice, indicating a pronounced GR-mediated fast feedback mechanism in these animals. These data suggest that even in a mouse line with a strong genetic determination, epigenetic influences, e.g. via maternal care or enriched environment (Bunck, Baier, Touma, unpublished), are still possible. Although there is lack of evidence concerning the neuronal and molecular mechanisms underlying maternal behavior and consequently GR alterations, reversing the epigenetic programming and following GR downregulation together with a "normalization" of stress response, clearly implicated the linkage of the phenomena observed (Weaver et al., 2005). This linkage due to maternal programming has to be investigated in more detail in the HAB/LAB mouse model. However, compared to the high-licking/-arched back nursing rat model, alteration in GR expression is not accompanied by a decrease in anxiety-related behavior in HAB mice, suggesting other neuronal circuits, possibly the AVP and/or the CRH system, to contribute to the hyper-anxious phenotype in this mouse line.

BDNF

Several animal and human studies described a stress-induced reduction of BDNF mRNA, reduced hippocampal volume, and impaired cognitive functions in comparison to unstressed controls (Bremner et al., 1995; Karege et al., 2005; Murakami et al., 2005; Schmidt et al., 2007). In HAB, NAB, and LAB mice, however, BDNF mRNA failed to reveal any differences

in hippocampal expression. Although BDNF protein levels remain to be quantified to ensure equal BDNF level in all three lines, at first glance BDNF seems to be unaltered in the HAB/LAB mouse model, excluding BDNF as a major player underlying the extremes in emotionality. The discrepancy between numerous studies in rats and mice and the HAB/LAB model might be due to the differences in experimental designs as well as in breeding strategies. Our data again suggest that, extremes in the genetic disposition to anxiety, are related to specific circuitries.

Series of experiments that resulted in BDNF alterations, used chronic social stress paradigms (Schmidt et al., 2007), acute stressors (Murakami et al., 2005), or chronic Cort administration. These paradigms resulted in neuronal damage, decrease in BDNF, cognitive impairment and consequently increased depression-like behavior and/or anxiety-related behavior. Although not excluded primarily, HAB animals according to their hyper-emotionality, do not reveal physiological symptoms, such as increased Cort levels, indicative of chronic stress. The HAB/LAB mice, in contrast to stress-induced paradigms, are derived from a breeding protocol, selecting animals according to their basal trait anxiety. As the strategies used are addressing different aspects (induced vs. trait anxiety), it is perhaps not surprising that BDNF is unaltered in the HAB/LAB mice. Nevertheless, one interesting focus in the HAB/LAB mouse is to address the vulnerability to chronic stress´ effects in these mouse lines of extremes in trait anxiety.

SYT4

In *Syt4* knockout mice it could be demonstrated for the first time that a presynaptic molecule is able to alter behavior. These knockout mice, in comparison to wildtype animals, revealed reduced anxiety- and depression-like behavior in various tests as well as enhanced locomotion in the open field test (Ferguson et al., 2004). While particularly data from conventional knockout approaches are at times inconclusive, these findings are in confirmation with results depicted in HAB, NAB, and LAB mice. In the CeA and BLA *Syt4* mRNA was increased in HAB mice displaying increased anxiety-related behavior, in comparison to NAB and LAB mice which revealed decreased anxiety-related behavior measured by qRTPCR and ISH under basal conditions. Furthermore, LAB mice exhibited a decrease in *Syt4* in the PVN compared to the two other lines. However, as most studies on *Syt4* are focusing on the role of *Syt4* in the presynaptic fusion machinery, the mechanisms by which *Syt4* is able to alter behavior remain unclear. Due to its biochemical structure and compared to other synaptotagmins, SYT4 is considered non-functional for Ca^{2+}-dependent

interactions (Sullivan, 2007). However, SYT4 can also exhibit Ca^{2+}-dependent binding to SYT1 and, thus, it was proposed that elevated SYT4 could form part of the Ca^{2+} sensor to regulate neurotransmission (Ferguson et al., 1999; Littleton et al., 1999). These findings, in context with the impaired Ca^{2+} binding ability of SYT4, led to the hypothesis that SYT4 upregulation after seizure is a protective mechanism to reduce neuronal activity (Ferguson et al., 1999; Littleton et al., 1999). However, this hypothesis has been refuted by recent findings, showing that SYT4 does not act as an inhibitor of neurotransmitter release (Ting et al., 2006), indicating that the precise function of SYT4 is not well understood.

Nevertheless, to receive information about cerebral activity under basal conditions, MEMRI as a non-invasive technique was applied in HAB, NAB, and LAB animals. Injected Mn^{2+} ions do mimic Ca^{2+} and may therefore accumulate in activated cells, indicating differences in neuronal activation between the mouse lines. Indeed, an increased Mn^{2+} signal could be detected in the amygdala of HAB animals, relative to NAB and LAB, whereas a decreased activation was found exclusively in LAB mice (Czisch, in preparation), suggesting alterations in synaptic release in both, HAB and LAB mice. These patterns of activation are similar to the expression patterns found for *Syt4*, indicating that especially these brain regions are of major interest concerning changes in synaptic release, rather likely to contribute to the observed phenotypic differences in the HAB/LAB mouse model. However, the molecular mechanisms underlying these differences in synaptic release, including besides *Syt4* various candidates involved in presynaptic docking, fusion of synaptic vesicles, and release, revealed by microarray analysis (Czibere, unpublished), have to be investigated in more detail to gain insight into the functional impact of each protein as well as to reveal the complexity of presynaptic networks associated with synaptic release and, finally, psychopathologies. These findings are especially of interest as proteins such as SNAP, syntaxins, synaptotagmins, synaptobrevin and the SNARE proteins are likely to play an important role in somato-dendritic release and are still poorly understood (Lodish et al., 2001; Landgraf and Neumann, 2004). Furthermore, it is necessary to apply techniques such as microdialysis and electrophysiology to discover neurotransmitters possibly contributing to the behavioral alterations observed.

Taken together, by use of selective and bidirectional breeding, we established an animal model that does not only differ in anxiety-related behavior in the EPM test, used as selection criterion, but also in other tests reflecting anxiety-related behavior. Furthermore, these breeding lines of extremes in trait anxiety additionally vary in several other behavioral tasks,

including those reflecting depression-like behavior as well as explorative behavior. A hypothesis-driven selection of genes revealed in similarity to the HAB/LAB rat model and clinical studies differences in gene expression profiles, well described to contribute to anxiety and depression disorders. These findings strengthen face and construct validity for our mouse lines, whereas the pharmacological interventions, together with former results, confirm predictive validity (Kromer et al., 2005). Therefore, by meeting all three criteria required for a valid animal model, this mouse model provides a unique opportunity to focus on the mechanisms underlying trait anxiety and comorbid depression at different levels, including behavioral, developmental, cognitive, neuroendocrine, and genetic aspects.

5   Developmental aspects in a mouse model of trait anxiety

5.1   Introduction

Beside the identification of neuropeptides, transmitter systems, receptor alterations, and genetic underpinnings, contributing to the development of mental disorders, it is of increasing interest to identify biomarkers that allow the diagnosis at very early stages of the disease as well as to permit categorization and subgrouping of patients in a more reliable and consistent manner that consequently enable specific treatment. Biomarkers are measures of specific biological parameters that allow to classify the disease and predict the most effective treatment (LaBaer, 2005).

To get insight into developmental aspects in the HAB/LAB mouse model, concerning onset of emotional differences as well as central alterations underlying the phenotypic divergence observed during adulthood in these animals, early behavioral phenotyping, HPA axis components, and a potential biomarker, glyoxalase 1 (Glx1), have been investigated under basal conditions.

It has been suggested that the number of ultrasonic vocalization calls induced by separation and isolation can be considered as a measure of separation anxiety and can be predictive of adult emotionality (Dichter et al., 1996; Brunelli, 2005; D'Amato et al., 2005). Therefore, as an early test for anxiety-related behavior and to follow the developmental pathways of emotional extremes, the USV was used to monitor the phenotypes in our breeding lines (Kromer et al., 2005). Similarly, we focused on the expression profiles of *Avp* and *Crh*, two HPA axis mediators, during development, starting from postnatal day (pnd) 5 up to adulthood at seven weeks of age. To control for HPA axis alterations during development, also ACTH and Cort were measured. Moreover, Glx1 protein levels have been determined to confirm the importance of this enzyme as a reliable and predictive biomarker in the HAB/LAB mouse model (Kromer et al., 2005). Glx1 is part of the glyoxalase system, which is present in the cytosol of all cells. Its function is not entirely resembled, but it has been described to detoxify α-oxo-aldehydes, mostly methylglyoxal, but also glyoxal, which represent potent cytotoxic metabolites (Thornalley, 2003a). Glx1 catalyzes the chemical reaction of methylglyoxal and the cofactor glutathione to S-D-lactoylglutathione, which is, in turn, converted to D-lactate and glutathione by glyoxalase 2. Glx1 protects against glycation of proteins and nucleotides by methylglxoxal and similar dicarbonyls, possibly impairing physiological functions (Thornalley, 2006). Due to its ubiquitous expression, the glyoxal pathway is believed to be of fundamental importance for the cellular metabolism. Although this enzyme is at first glance not one of the usual suspects in transduction of mental

diseases, it attracts increasing attention for its role in psychopathologies, including Alzheimer´s disease (Chen et al., 2004) or anxiety-related behavior (Hovatta et al., 2005; Kromer et al., 2005). Furthermore, a possible connection between Glx1 and depression has been described in a linkage study of families with depressive disease, revealing evidence for an association between subgroups of unipolar affective disease and the Glx1 locus (Tanna et al., 1989).
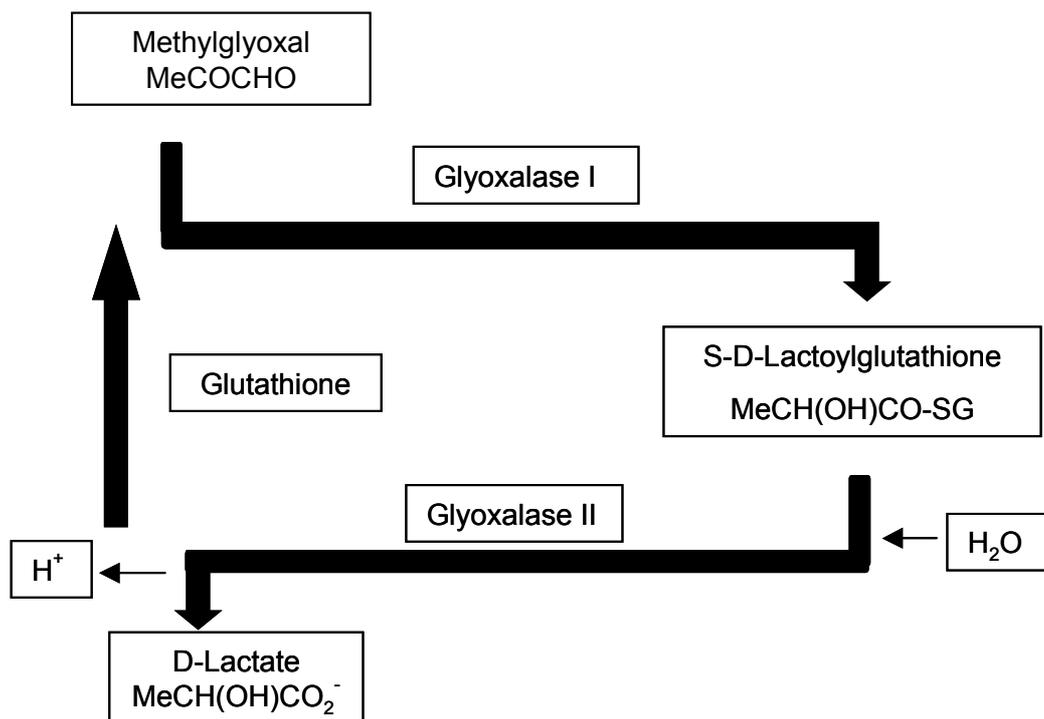
Figure 5.1:   The glyoxalase system. This system catalyzes the conversion of methylglyoxal to D-lactate via the intermediate S-D-lactoylglutathione. It involves two consecutive enzymatic reactions catalyzed by glyoxalase 1 and glyoxalase 2, respectively, and by catalytic portion of glutathione.

5.2 Results

USV test

USV test on pnd5 revealed significant more USV calls, locomotor activity, and number of rotations in HAB pups in comparison to LAB mice with NAB animals being in between both extremes. NAB pups weighed significantly more in comparison to HAB and LAB, with HAB exhibiting less bodyweight than LAB pups (Figure 5.2, $p < 0.001$ for all three group and post hoc comparisons).
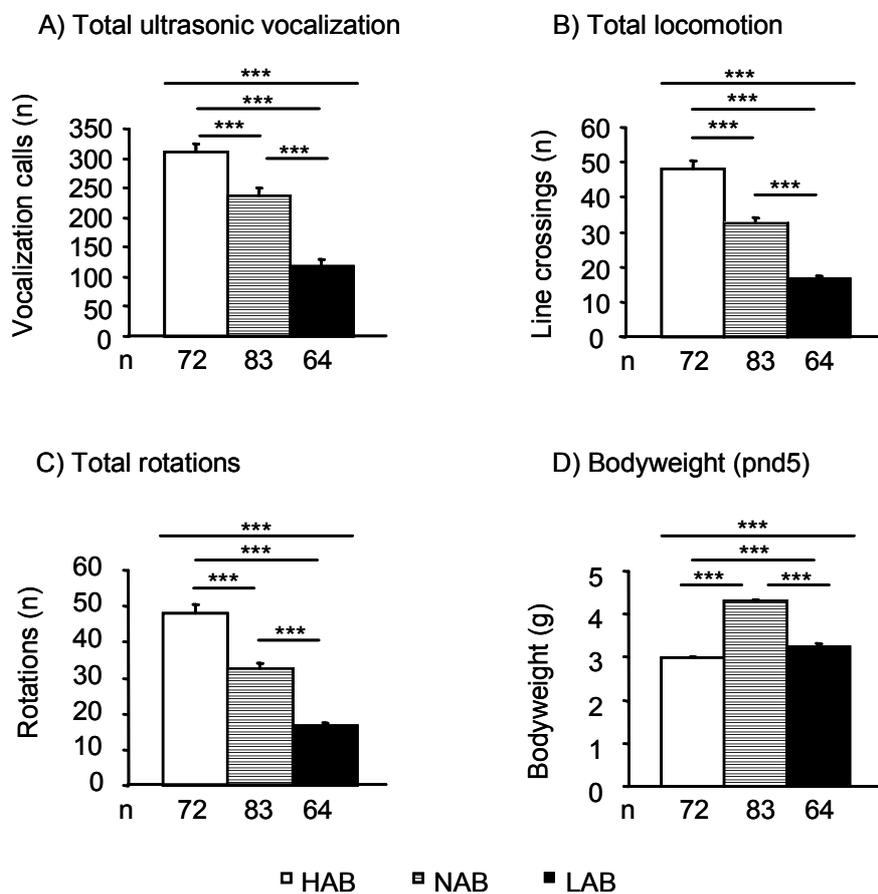
Figure 5.2:   Anxiety-related behavior measured in the ultrasonic vocalization (USV) test on postnatal day (pnd) 5 in HAB, NAB, and LAB pups. A) HAB mice emitted significant more USV calls, B) revealed increased locomotor activity and C) exhibited an increased number of rotations relative to LAB animals, with NAB pups being in between both extremes. D) NAB pups weighed significantly more in comparison to HAB and LAB, with HAB exhibiting less bodyweight than LAB pups. ***$p < 0.001$ for all three group and post hoc comparisons.

Moreover, detailed analysis of the USV test revealed a significant decline in USV calls (Figure 5.3 A; p<0.01 for NAB and LAB, p<0.001 for HAB) and locomotion (Figure 5.3 B; p<0.001 for all three lines) over the test time. Also the differences in USV between the groups remained significant during the test time (KWH: p<0.001; p<0.5, p<0.01, p<0.001 for post hoc tests), whereas the differences in locomotion disappeared at the fourth and fifth minute of test time between HAB and LAB (KWH: p<0.05, p<0.01 and p<0.001; similar p-values for post hoc tests).

A) Ultrasonic vocalization per minute   B) Locomotion per minute
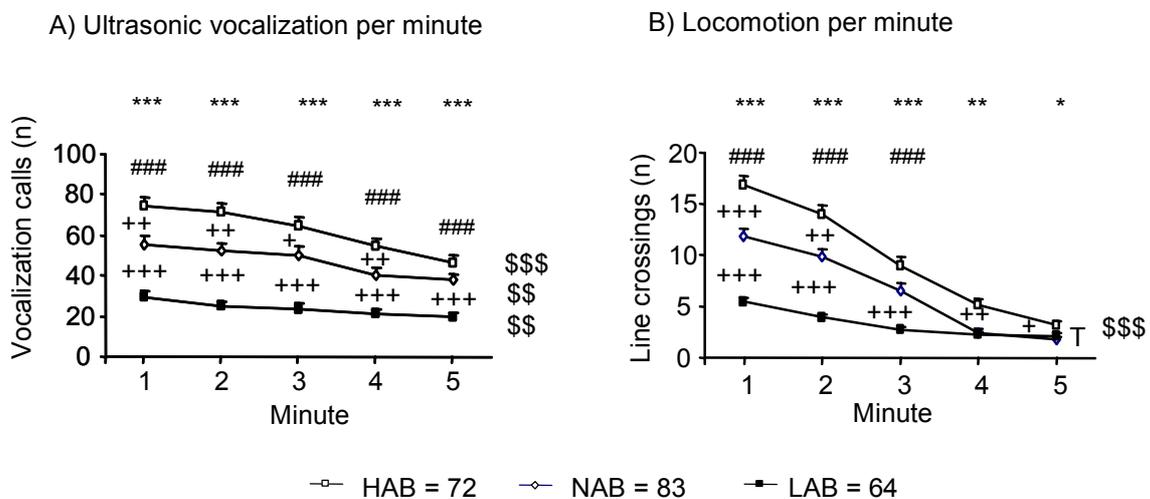
Figure 5.3:   *Detailed analysis of the ultrasonic vocalization (USV) calls and locomotor activity during 5min test time in HAB, NAB, and LAB pups. Both parameters USV calls and locomotion showed a decline during the test time in all three lines. The differences in USV calls were significant between all three lines during the whole test time, whereas the locomotor activity between HAB and LAB pups was similar during the fourth and fifth test minute. $$ p<0.01, $$$ p<0.001 (same line); *p<0.05, **p<0.01, ***p<0.001 (three group comparison for the same timepoint). ### p<0.001 and T p<0.1 for HAB vs. LAB (same time), + p<0.5, ++ p<0.01, +++ p<0.001 vs. HAB and LAB (same time) for post hoc tests.*

Bodyweight and Glx1 protein levels during development

Bodyweight was increasing in both HAB and LAB mice during development (p<0.001 for each line). The decreased body weight in HAB compared to LAB at pnd5 (p<0.001 for KWH and post hoc tests) was still present at pnd12 (p<0.05), but seemed to be caught up at later developmental stages (Figure 5.4 A). Glx1 protein levels already differed at pnd5 between

HAB and LAB, with NAB mice revealing intermediate levels (Figure 5.4 B; KWH: p<0.01; T p<0.1, p<0.5). However, in both HAB and LAB an increase in protein levels was detectable (p<0.05 for each line), with the Glx1 levels being constantly different between HAB and LAB (p<0.05, p<0.01).
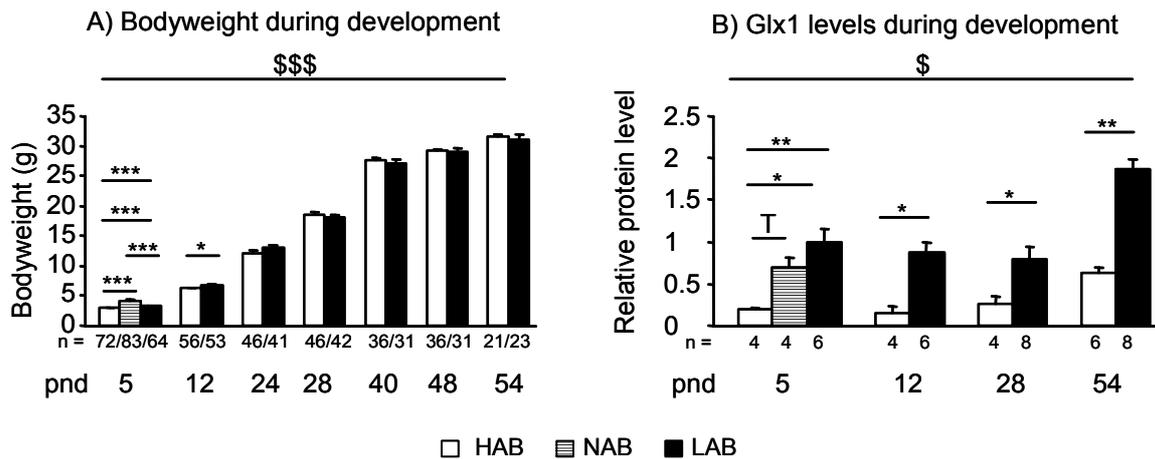
Figure 5.4:   *Bodyweight and glyoxalase1 (Glx1) protein levels, measured by western blot analysis in red blood cells, during development of HAB and LAB mice. Bodyweight obtained at postnatal day (pnd) 5, 12, 24, 28, 40, 48 and 54 revealed an steady increase in both HAB and LAB. The differences in bodyweight at postnatal day (pnd) 5 between HAB, NAB, and LAB, have already been shown in Figure 5.1 D and were additionally significant between HAB and LAB at pnd12. Glx1 protein levels were increasing during development and significantly differed from pnd5 on between HAB and LAB, with NAB being in between HAB and LAB at pnd5. $ p<0.5, $$$ (same line); *p<0.5, **p<0.01and ***p<0.001 (three group comparison and post hoc tests, same time).*

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Expression of *Avp* and *Crh* during development

Avp mRNA tended to be decreased in LAB from pnd5 on and was significant at pnd54 (Figure 5.5 A, T p<0.1, p<0.01). HAB mice revealed a significant increase in *Avp* mRNA between pnd5 and 12 (Figure 5.5 A, p<0.05). *Crh* mRNA levels decreased during development in both lines (Figure 5.5 B, p<0.01). HAB mice revealed a tendency towards

increased *Crh* expression between pnd5 and 12 (p<0.1). Moreover, HAB animals exhibited elevated *Crh* expression at pnd12 and 54 compared to LAB mice (Figure 5.5 B, p<0.01).

Figure 5.5:   *Expression of arginine-vasopressin (Avp) and corticotropin-releasing hormone (Crh) in the paraventricular nucleus (PVN) at postnatal day (pnd) 5, 12, 28, and 54 in HAB and LAB mice, measured by in situ hybridization. A) Avp mRNA tended to be decreased in LAB from pnd5 on and was significantly differently expressed at pnd54. HAB mice revealed a significant increase in Avp mRNA between pnd5 and 12. Crh mRNA levels decreased during development in both lines. HAB mice revealed a tendency towards increased Crh expression between pnd5 and 12. Moreover HAB animals exhibited increased Crh expression at pnd12 and 54 compared to LAB mice. A) T p<0.1, **p<0.01 vs. LAB (same age); + vs. HAB pnd12 (same line); B) $$ p<0.01 (same line); T <0.1vs. HAB pnd12 (same line), **p<0.01 vs. LAB (same age).*
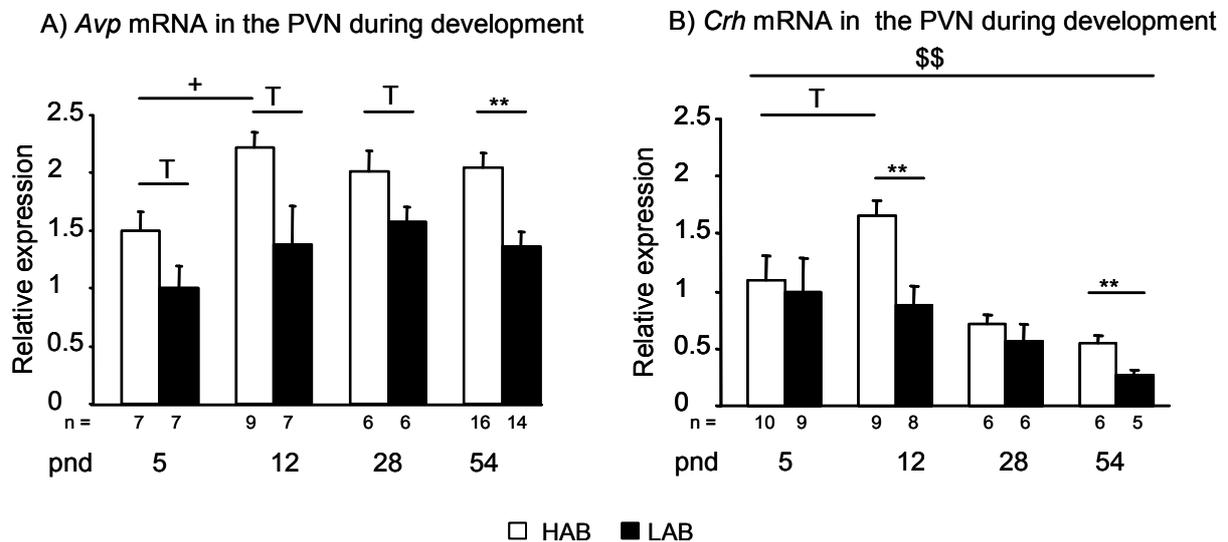
Plasma ACTH and Cort during development

ACTH was significantly altered in both lines during development (p<0.5). LAB showed a trend towards decreased ACTH from pnd5 to 12 (Figure 5.6 A, p<0.1). At pnd28 ACTH levels tended to be increased in HAB vs. LAB (p<0.1). From pnd5 to 12 both lines exhibited a Cort increase (Figure 5.6 B, p<0.01, p<0.05).
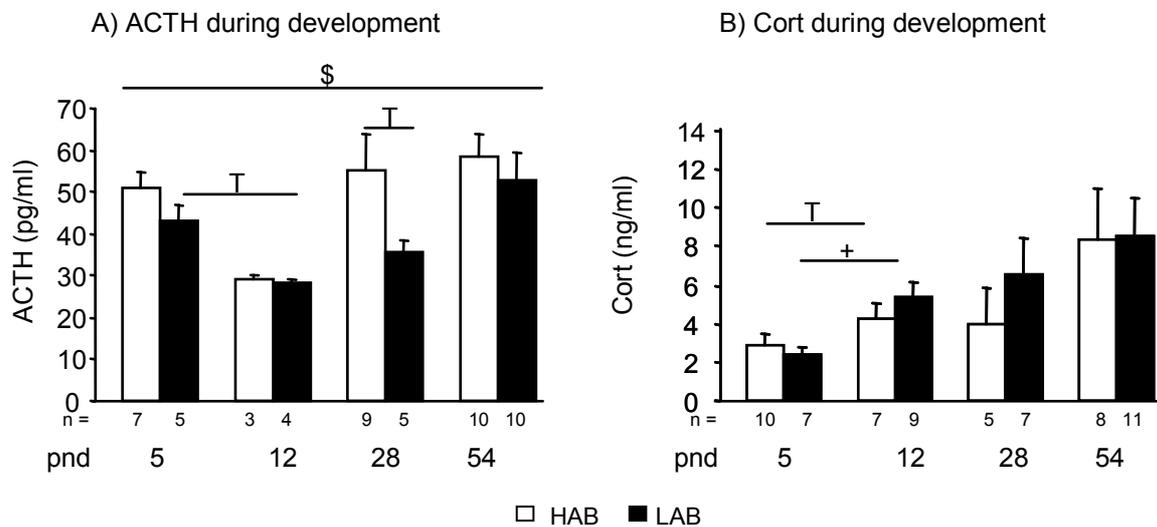
Figure 5.6:   *Adrenocorticotropic hormone (ACTH) and corticosterone (Cort) plasma*
concentrations at postnatal day (pnd) 5, 12, 28, and 54, measured by
radioimmunoassay, in HAB and LAB mice. A) ACTH was significantly altered
in both lines during development. LAB revealed a trend towards a decrease of
ACTH from pnd5 to 12. At pnd28 ACTH levels tended to be different between
HAB and LAB. B) From pnd5 to 12 both lines exhibited a Cort increase. A) $
p<0.05 (same line), T p<0.1 vs. LAB pnd12 (same line), T p<0.1 vs. HAB
pnd28 (same age). B) T p<0.1 vs. HAB pnd12, + p<0.05 vs. LAB pnd12
(same line).

5.3   Discussion

As early as on pnd5, emotional differences between HAB, NAB, and LAB pups were detectable, with HABs emitting more USV calls and showing elevated anxiety-induced locomotor activity, relative to LAB mice, indicative of increased anxiety-related behavior, whereas NAB mice revealed an intermediate phenotype. Moreover, Glx1 levels detected in red blood cells significantly differed between HAB and LAB pups at any timepoint measured during development. *Avp* mRNA under basal conditions tended to be reduced in LAB mice from pnd5 on, a difference that is getting highly significant, relative to HAB animals, at pnd54. Additionally, *Crh* expression exhibited a significant divergence between the two breeding lines at pnd12 as well as at pnd54 under basal conditions. Despite alterations in *Avp* and *Crh* expression in the PVN of HAB and NAB mice during development, no differences were detectable in plasma levels of ACTH or Cort under basal conditions.

Temperature has long been recognized as a primary factor in the stimulation of USV, acting as a signal to the pup that it is no longer in the nest (Allin and Banks, 1971). The effects of cooling on cardiac rate, oxygen consumption, respiratory rate, arterial pressure, and blood viscosity suggest that USV calls, produced by the forced expiration of air through a constricted larynx, are produced as part of a coordinated physiological response to homeostatic challenges (Blumberg et al., 2005). Furthermore, Blumberg et al. (2005) demonstrated an inhibition of USV by noradrenaline and consequently increased body temperature, suggesting the USV test as being inadequate to measure anxiety-related behavior in pups (Blumberg et al., 2005). However, we could demonstrate for the first time that the differences in USV in HAB, NAB, and LAB pups are primarily not a consequence of changes in body temperature, but reflecting distress and anxiety-related behavior. A decrease in body temperature, from nest temperature to 23ºC during experiment, is rather likely to stimulate USV in all three lines. Nevertheless, the differences in the number of USV calls were already present during the first minute of separation and stably differing during the 5-min separation. A further decrease in body temperature has been suggested to increase USV during the test time (Blumberg et al., 2005), but the contrary, an overall reduced USV, was measurable in all three mouse lines tested. Therefore, it was suggested that decreased body temperature might lead to reduced locomotor activity, indicated in all three groups, but is not directly influencing USV. A slow motion analysis of USV testing in CD1 mice revealed that emission of USV is connected to locomotion and head rising (Branchi et al., 2004). These observations are in accordance with our findings, as a high number of USV calls are

accompanied by increased locomotor activity and number of rotations in HABs, with comparable ratios in NAB and LAB pups. As pups vocalize during locomotion (Branchi et al., 2004), it is not surprising that a decline in one parameter is accompanied by a parallel decrease of the other one. This conjunction of vocalization and movement has been implicated in a variety of studies in different species (for details see (Branchi et al., 2004)). Furthermore, neither the observed differences in bodyweight between the inbred lines in comparison to NAB, similar to those measured during adulthood (see previous chapter), nor the difference between HAB and LAB at pnd5, seem to influence this behavior. The number of rotations has been considered as a parameter measuring exploration of the surrounding environment. At pnd5, the olfactory system represents the main sensory modality for infant mice to scan and perceive distal cues (Branchi et al., 2004). The increased number of rotations, together with the elevated USV in HAB pups, might reflect their increased active search for maternal protection as a consequence of their hyper-emotional status. Detailed analysis of the USV test in HAB/LAB pups led to the suggestion that the test per se, at least in our mouse model, can be limited to 1-min test intervals. As the emotional parameters measured were already significantly different during the first minute of separation, a prolonged separation, which is possibly accompanied by separation effects in the pup, is not necessary, at least in the HAB/LAB model.

These results describe an early phenotypic divergence between HAB and LAB, with the former revealing more USV calls than the latter, indicative of increased inborn anxiety-related behavior. Thus, together with the previous pharmacological validation (Kromer et al., 2005) and a variety of other studies, confirming the hypothesis that the USV test is a suitable paradigm to investigate separation anxiety. Moreover, the number of USV calls can be predictive of adult emotionality (Dichter et al., 1996; Brunelli et al., 1997; Wigger et al., 2001; Brunelli, 2005; Burgdorf et al., 2005). Furthermore, epidemiological/clinical studies showed that children who cry more in response to novelty at four month of age, are highly likely to become shy and inhibited in childhood (Brunelli, 2005).

The differences in bodyweight between HAB and LAB at pnd5 and pnd12 are due to varying pup numbers per litter between the lines. Although most of the litters of both lines have been standardized to eight pups per litter, LAB females showed a reproductive deficit and they deliver decreased numbers of pups (Keßler, unpublished). Therefore, as LAB mothers raised fewer pups, this might be advantageous for the offspring in terms of milk consumption, resulting in increased bodyweight. However, this difference seems to be caught up by HAB pups at around three weeks of age, possibly due to self-feeding during this age. As NAB

animals, being outbred mice, revealed a highly increased bodyweight at pnd5, suggesting a faster or at least divergent development, in comparison to the two inbred lines, they were not included in the further brain analysis.

Already at pnd5, *Avp* mRNA tended to be reduced in LAB, an effect that is getting highly significant during adulthood. As described in the previous chapter, several SNPs in the *Avp* gene have been identified, including a prominent Δ(-2180-2191) deletion in the promoter region as well as a (C(40)T) SNP in the signal peptide of the AVP precursor gene. Furthermore, we demonstrated that distinct polymorphisms in LAB mice contribute to decreased *Avp* expression (see previous chapter), already indicated at pnd5. AVP was shown to be expressed in the diencephalon already on embryonic day 13.5 and in a PVN-corresponding region on day 14.5 (Jing et al., 1998) and is slightly increasing during development as observed by *Avp* expression in the PVN of HAB mice. It is rather likely that a decreased amount or even constant lack of bioavailable AVP in LAB animals, through its release from synapses, somata, and dendrites of neurons (Wotjak et al., 1996; Landgraf et al., 2007), is shaping trait anxiety robustly from birth on, possible explaining the decreased anxiety-related behavior of LAB pups as early as on pnd5 in the USV test. Furthermore, the (C(40)T) SNP in the signal peptide has been associated with symptoms of cDI in LAB animals and decreased anxiety-related behavior in adult mice (Keßler et al., 2007). Mutations are described to change the three-dimensional structure of a protein by inducing substitutions or deletions of aminoacids, involved in the secondary structure or disulfide bridges (Christensen and Rittig, 2006). These structural alterations lead to inaccurate folding of the peptides and inadequate binding of AVP to its carrier protein. Consequently, misfold propeptide accumulates in the ER and leads to impaired processing, axonal transport, and, finally, AVP secretion. The accumulated mutant protein in the ER impedes not only the processing of the wildtype AVP propeptide, but also the processing of other essential proteins, finally causing cell death (Ito and Jameson, 1997; Ito et al., 1999). Both mechanisms cause a delayed onset and progressive cause of cDI. Our developmental results concerning *Avp* mRNA in the PVN and SON (see previous chapter), together with the onset of physiological symptoms of cDI in LAB mice suggest the mentioned molecular mechanisms underlying the progressive phenomena observed. Increased water intake and, consequently, elevated amount of urine, reflected by a low urine osmolality were detectable at five weeks of age in LAB, indicating the dramatic physiological consequences of cDI (Keßler, unpublished). These findings go along with the expression profile indicating a lack of *Avp* mRNA before adolescence and at the beginning of adulthood. This delayed onset of

cDI has also been described for patients (for review see (Frank and Landgraf, 2008)). In summary, it can be hypothesized that the genetic underpinnings of AVP deficit, detected in LAB mice, lead to a decreased expression of *Avp*, possibly from birth on and progressing to an AVP deficit in these animals, resulting in both symptoms of cDI and reduced anxiety.

As it has been shown in detail, CD1 mice undergo different phases of stress responsiveness during development (Schmidt et al., 2003). The first developmental stage of the HPA axis after birth is characterized by a so-called stress hypo-responsive phase. During this period, from pnd1 until pnd12, similar to our results, mice showed low basal levels of Cort, while basal ACTH was already present at concentration of adults (Schmidt et al., 2003) and no alterations in *Crh* expression were detectable in the PVN. At the end of this developmental phase, with the onset of the responsive phase, *Crh* expression in the PVN was revealed to be differently between HAB and LAB. *Crh* mRNA has been described to be already high at pnd1 in CD1 mice under basal, following a decreased expression at pnd12 (Schmidt et al., 2003), which is in accordance with LAB animals. However, HAB mice exhibited increased *Crh* expression in comparison to LAB. As pnd12 is the beginning of the stress-responsive phase, it is rather likely that this timepoint is also indicative of central modification relevant under basal conditions, displayed in HAB animals. Since no genetic variants have been identified in the *Crh* gene (Czibere, unpublished), it is presumable that the over-expression in HABs is influenced by non-genomic factors (see previous chapter). In unselected CD1 mice, decreased *Crh* expression at pnd12 is accompanied by an increased GR expression, pointing to a GR-mediated suppression of *Crh* in the PVN (Schmidt et al., 2003), possibly similar to LAB mice. Therefore, one possible explanation for the over-expression in HAB mice could be a deficiency in GR feedback mechanisms on *Crh* expression, possibly due to diminished GR activity in the PVN, at least at this developmental stage. Although this divergence in expression is stable under basal conditions in HAB and LAB animals, similar to former findings (see previous chapter), *Crh* expression profiles seem to be accessible for external stressors (Schmidt et al., 2002; Schmidt et al., 2004). Juveniles of both breeding lines are weaned from their mothers at pnd24 and are housed in groups of three to four littermates of the same sex. Beside the separation from the mother and the novel environment, animals might be stressed by setting up a social hierarchy. It has been demonstrated that external stressors, such as maternal deprivation, influence *Crh* expression (Schmidt et al., 2004). According to this data, *Crh* expression is decreased due to the stressor, a finding that could explain the rapid decrease of *Crh* mRNA from pnd12 to pnd28 in HAB mice in comparison to LAB. It is likely that the stress-induced decrease in *Crh*

in HABs is reflecting their increased emotional interpretation of "post-weaning" stress. However, similar effects were not detectable or absent in LAB animals, possibly indicating increased stress resistance in these mouse line.

During adulthood and under basal conditions at pnd54, the divergence in *Crh* expression became significant again. We therefore conclude that the CRH system is hyperactivated in HAB mice from an early developmental stage on, but still stressor accessible, confirming a non-genomic regulation in these animals.

Although the two key players of HPA axis activity, AVP and CRH, have been identified to be differently expressed in the two breeding lines, they do not seem to influence basal ACTH and Cort levels, neither in the early nor at later developmental stages. Similar results have been reported in CD1 mice during development, where CRH was not able to induce an ACTH or Cort response under basal conditions (Schmidt et al., 2003). Furthermore, exposing HAB/LAB animals to a mild stressor did not reveal any differences in Cort increase in HAB and LAB mice (Bunck and Touma, unpublished). Applying restraint stress as a strong stressor, exhibited a diminished Cort response in HAB mice in comparison to LAB (Touma, unpublished). Since many of the effects of CRH were found to be independent of HPA axis activation, an involvement of extrahypothalamic CRH receptors in mediating behavioral responses was suggested and proven by receptor distribution studies (Dunn and Berridge, 1990; Steckler and Holsboer, 1999). Furthermore, over the last two decades there is mounting evidence that AVP is shaping emotional responses upon dendritic release within the brain (Frank and Landgraf, 2008). Wotjak et al. (1996) demonstrated a local release of AVP in the PVN due to social defeat exposure, with no simultaneous release of AVP from the SON or into the circulation, indicating the function of centrally released AVP in emotionality, independent of HPA axis response (Wotjak et al., 1996) .

Therefore, in view of the wide range of CRH and AVP effects on anxiety and depression, we hypothesize that, similar but independent of HPA axis regulation, both neuropeptides shape behavioral phenomena linked to innate emotionality via their central somadendritic release. As only 30-60% of depressed patients show HPA axis alterations (Ising et al., 2005), the finding in HAB/LAB mice might be of additional clinical interest.

In accordance with former findings, Glx1 protein levels, measured in red blood cells of male mice, significantly differed between HAB and LAB mice in adulthood. Beside red blood cells, similar results were detected in various brain areas, including hypothalamus, amygdala, and the motor cortex. This divergence of Glx1 levels was not only true for male, but also for female mice of the respective line, with NAB and cross-mated animals revealing intermediate

protein levels. Moreover, in similarity with findings from HAB/LAB animals, less anxious BALB/c mice expressed more Glx1 than the more anxious C57BL/6 (Kromer et al., 2005). Furthermore, in the present study it could be demonstrated that Glx1 levels are not only a stable and reliable biomarker for the non-anxious versus anxious phenotype, but, as the differences are already present at pnd5, Glx1 might also be suitable as a marker for diagnosis even at early stages of disease. In contrast to AVP and CRH, Glx1 levels appeared to be significantly divergent as early as on pnd5, a difference which remains robust and stable throughout all measured timepoints during development. Moreover, there are hints that Glx1 levels, differently to the *Crh* expression, are not altered by external stressors, such as post-weaning stress, indicated by unchanged Glx levels at pnd28, pointing to a strong genetically based influence on Glx expression. Indeed, several SNPs between HAB and LAB mice have been identified by sequencing the promoter and coding region of the gene coding for Glx1 and their functional impact on Glx expression is currently under investigation (Prigl, Hambsch, Ahmad, unpublished). It is rather likely that the genetic predisposition might explain differences in cell metabolism contributing to either high or low anxiety-related behavior. Furthermore, a decreased amount of Glx1 protein could be demonstrated in patients suffering from anxiety disorders in comparison to healthy control subjects, implicating the clinical impact of Glx1 as a reliable protein biomarker of psychiatric diseases (Ditzen et al., 2006).

However, the Glx1 findings in the HAB/LAB mice are discordant to recent data, describing increased Glx1 levels in association with increased anxiety-related behavior (Hovatta et al., 2005). This discrepancy might have arisen due to the use of mouse strains that according to their different selection pressure exerted, vary in their genetic background. On the one hand, as CD1 mice were not included in a study by Hovatta et al. (2005), a direct comparison concerning Glx1 levels is not possible, on the other hand, the mouse strains used are differing in their anxiety-related but are not, as the HAB/LAB mouse, selected and inbred for either high or low anxiety-related behavior and therefore, resembling the clinical situation in patients (Kromer et al., 2005; Ditzen et al., 2006). Moreover, the behavioral phenotyping as well as the molecular techniques used were not comparable between both laboratories. Nevertheless, further investigations will focus on the alterations in dicarbonyl metabolism, possibly uncovering similar molecular mechanisms in both approaches (Thornalley, 2006).

Chronic exposure to methylglyoxal and/or glyoxal leads to higher Glx1 expression as a protective response to dicarbonyl stress in order to decrease tissue damage by methylglyoxal (McLellan, 1994; Thornalley, 2006). However, it is rather likely that increased

Glx1 levels insufficiently prevent increased protein damage (Thornalley, 2003b; Ahmed, 2005). Dicarbonyl-stress processes accompanied by increased Glx1 expression have been reported in P301L mutant tau transgenic mice and patients with confirmed Alzheimer´s disease in comparison to control subjects (Chen et al., 2004). Similar to these patients (Decker, 1995), LAB mice revealed higher Glx1 levels, indicative of an increased dicarbonyl stress and impaired cognitive function, indicated in the Morris water maze test (unpublished data) and social discrimination task (see following chapter). The possible involvement of increased methylglyoxal in cognitive impairment has recently been demonstrated, showing decreased hippocampal field EPSPs after methylglyoxal treatment (Eder, unpublished). Furthermore, treating NAB animals icv with methylglyoxal revealed a significant decrease in anxiety-related behavior measured in the EPM test (Hambsch, unpublished). These findings suggest an involvement of dicarbonyl metabolites in cognitive and behavioral changes. On the other hand, a lack of dicarbonyl metabolites and, consequently, decreased Glx1 levels might be indicative of a decreased cellular metabolism in HAB animals, possibly leading to high anxiety-related behavior. As these animals also exhibit increased passive coping strategies, the hypothesis of metabolic depression in major depression might be of interest in this context (Tsiouris, 2005; Landgraf et al., 2007). A disequilibrium in both directions, with either decreased or increased Glx1 levels in cell metabolism might lead to a pathological outcome in both HAB and LAB mice. However, to gain insight into respective mechanism, all components of the Glx-pathway have to be investigated in more detail, including methylglyoxal levels, Glx1 activity, the cofactor glutathione, as well as glutathione reductase, to get more insight into the metabolic turnover in each line. Secondly, genetic manipulations, such as virus-mediated gene transfer or siRNA techniques should be applied to test the association between Glx1 level and the respective phenotype. Additionally, the link between Glx1 expression in relation to neurotransmitter metabolism and neuronal activation has to be uncovered, to identify the missing link between Glx1 levels and anxiety, thereby providing predictive validity of Glx1 beyond that of a biomarker (Landgraf et al., 2007).

In summary, the early onset of alterations on a behavioral, expression, and metabolic level raised the hypothesis that the differences observed in HAB and LAB mice are mostly determined by genetic predispositions rather than consequences of non-genetically determined external stimuli. This strict genetic determination of the two extremes in emotionality could be further demonstrated by a cross-fostering study. Although maternal behavior significantly differed between HAB and LAB mothers, cross-fostered (HAB pups raised by a LAB mother and vice versa) animals failed to reveal emotional changes in a

variety of behavioral tests (Keßler, in preparation). In the HAB/LAB mouse model, we thus succeeded in accumulating genetic markers in association with the line-specific phenotype, which are relevant for anxiety and depression disorders (Landgraf et al., 2007).

6   Cognitive aspects in a mouse model of trait anxiety

6.1   Introduction

Shettleworth´s comprehensive definition of cognition is referring to "the mechanisms by which animals acquire, process, store, and react to information perceived from the environment (Shettleworth, 1998). This definition can incorporate many different types of information processing, ranging from sensory perception and mechanisms of associative learning to conscious, rational, linguistically based thought processes (Paul et al., 2005). Emotions in contrast refer to processes which are likely to have evolved from basic mechanisms that gave animals the ability to avoid harm/punishment and seek valuable resources/reward. Emotions include adaptive, behavioral, physiological and neural processes (Cardinal et al., 2002; Paul et al., 2005). Both phenomena, emotionality and cognition, are closely related. Emotions are determined by cognitive estimations and vice versa, emotions influence cognitive processes (Landgraf and Wigger, 2002). During the last years, this relationship between cognitive function, anxiety, and depression disorders with regard to clinical relevance has been demonstrated to play a major role in human and animal studies (McNaughton, 1997; Ohl et al., 2001; Ohl et al., 2003; Ohl, 2005; Reppermund et al., 2007; Schmidt et al., 2007). These findings lead us to the question, if extremes in trait anxiety are accompanied by alterations in cognitive abilities in our mouse model. As cognitive performance is strongly based on the emotional context and varies within different types of learning, two different paradigms were chosen to investigate the question of cognitive changes in HAB, LAB and/or NAB adult male mice.

The sense of smell is of paramount importance for rodents, in which odor discrimination and long-lasting olfactory memory permits adequate responses to predator and prey critical for survival (Shimshek et al., 2005). Moreover, recognition of a familiar individual provides the basis upon which all social relationships are built (Ferguson et al., 2002; Bielsky and Young, 2004). In a natural context, social memory is a unique form of memory, critical for reproduction, territorial defense, and establishment of hierarchies (Ferguson et al., 2002). Rats and mice are macrosmates for which storage and recall of information acquired via olfaction are necessary for a successful interaction with their living and non-living environment (for a rough overview about perception and processing of social cues see Fig.6.1), implying that investigation of such information processing provides access to learning and memory in these species (Richter et al., 2005). Information about volatile stimuli is mainly processed in the main olfactory system, whereas non-volatile stimuli are primarily processed in the accessory olfactory system. The main olfactory system consists of the main

olfactory bulb and the limbic structures, including areas such as the cortical nucleus of the amygdala and the piriform cortex.
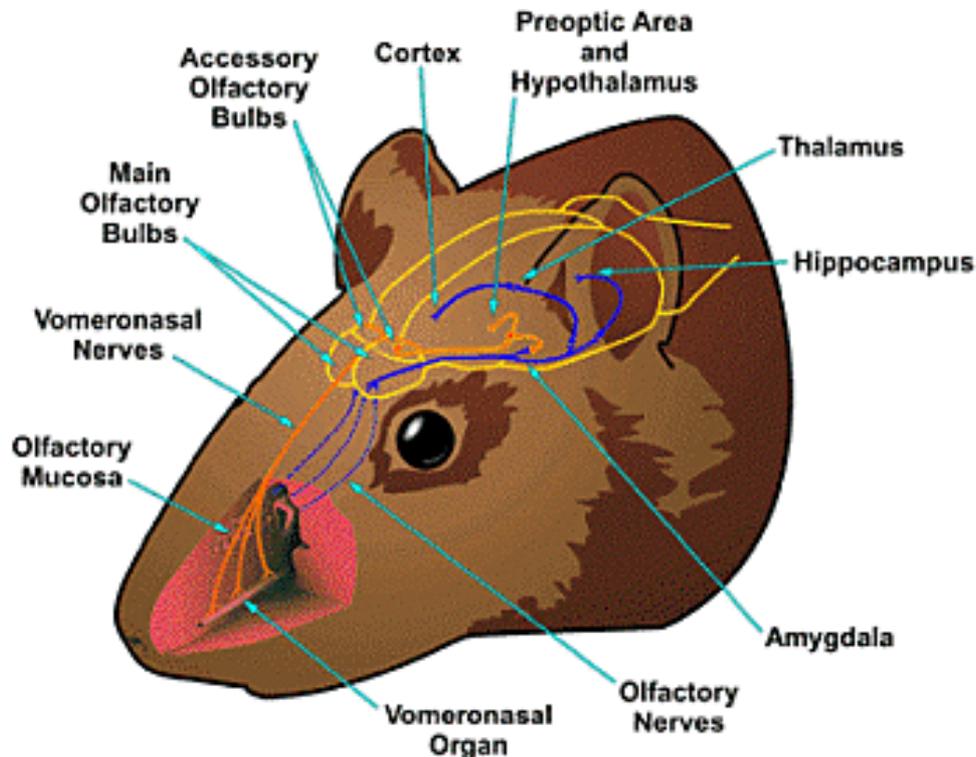
Figure 6.1:   A rough overview about tissues and brain structures involved in the perception and processing of social cues ([www.neuro.fsu.edu/research /vomeronasal/](www.neuro.fsu.edu/research/vomeronasal/) by Dr. Michael Meredith).

The accessory system, in contrast, is comprised of the accessory bulb, the MeA, the bed nuclei of the lateral olfactory tract and of the stria terminalis, the medial preoptic area, and the septum (Cooke et al., 1998; Richter et al., 2005). Based on mice´s/rats´ natural tendency to intensely investigate novel individuals, a simple laboratory test was established to investigate short-term, social recognition capacities (Thor and Holloway, 1982). In this task it could be demonstrated that a rodent is more interested in an unfamiliar, novel conspecific accompanied by increased olfactory exploration compared to a familiar conspecific. The original concept from 1982 has been modified several times. In our first experimental approach we used a modified design, the habituation-dishabituation paradigm, originally described by Winslow and Dluzen (Dluzen et al., 1998b; Dluzen et al., 1998a; Winslow and Insel, 2004) (see 3.3.1.) The experimental animal is introduced to the same ovariectomized stimulus animals four times for four minutes each trial. During the fifth trial, a new stimulus animal is presented. Reintroduction (introducing the familiar stimulus animal once more) of a familiar stimulus animal results in a decrease in olfactory investigation, indicative of

functional social memory processes, whereas the introduction of a novel stimulus increases sniffing time. Presenting a novel stimulus animal excludes effects of tiredness of the experimental animal and lack of interest on a social stimulus which might influence the results (Ferguson et al., 2000b; Bielsky et al., 2004). Additionally, as a more challenging task for the experimental animal, the social discrimination test (SD) was applied, which has been originally developed in our laboratory (Engelmann et al., 1995) to investigate olfactory-based learning and short-term memory processes in adult rats in a social context. Compared to the SR test, the animals have to discriminate between two stimulus animals at the same time during the second exposure. The hypothesis behind this test is similar to the SR test. If an animal is still able to remember the first (familiar) stimulus animal, it will spend more time in olfactory exploration of the novel animal. Several modifications have been introduced in order to further standardize and adapt the protocol for mice (mentioned in 3.3.2). Beside these two social, non-aversive, and stress-mild learning tasks, a cued fear conditioning paradigm was used to investigate the animals´ performance in a non-social, aversive task.

For many decades, classical conditioned fear based on the original paradigm in dogs developed by Ivan Petrovich Pavlov in 1927, has provided fertile ground to investigate learning and memory. The protocols used are robust, and the paradigm itself is of unquestionable relevance for humans and animals. Fear, or at least a comparable emotion, might be as old as the animal kingdom itself. Aversive learning is therefore ancient and well conserved, even measurable in simply structured organisms, such as Aplysia (Myers and Davis, 2002; Barad, 2006; Tamminga, 2006). However, fear is a mixed blessing for humans and animals. On one hand it is protective and absolutely necessary for survival, on the other hand indiscriminate fear displaces many crucial activities in life. In humans fear is often learned during traumatic episodes with fearful stimuli and is enhanced by genetic predisposition (Tamminga, 2006). However, when the fearful situation is overwhelming or the mediating neural systems are dysregulated, fearful situations can be learned, but not relearned, creating forms of anxiety (e.g. post traumatic stress disorder, generalized anxiety disorder), a phenomenon of high clinical relevance (Morgan et al., 1995; Fyer, 1998; Gorman et al., 2000). Therefore, understanding the precise neural mechanisms underlying fear conditioning and extinction, as relearning, is necessary for developing selective and effective treatment for these psychopathologies accompanying the inability to extinguish intense fear memory (Milad et al., 2006). Studies in animals highlight the amygdala nuclei, the hippocampus and the PFC as the major brain regions orchestrating memory consolidation and extinction (Tamminga, 2006).

Classical fear conditioning occurs when an affectively neutral stimulus such as a tone (conditioned stimulus, CS) is paired with a noxious aversive stimulus such as footshock (unconditioned stimulus, US). During conditioning an association between CS and US is formed, which afterwards enables the previously neutral CS to elicit several fear-related behavioral changes such as freezing or potentiation of the acoustic startle response. The fear-eliciting properties of the CS in the absence of the US extinguishes after repeatedly presenting the CS, pointing to extinction as a process of relearning (Walker et al., 2002).

Growing evidence suggests that fear memories are selectively sensitive to pharmacological intervention (Cai et al., 2006). Recent clinical studies showed that PTSD patients often reveal reduced cortisol levels (Yehuda, 2001) and daily cortisol administration reduced symptoms of traumatic memory in PTSD patients (Aerni et al., 2004). Moreover, D-Cycloserine, a partial N-methyl-D-aspartate (NMDA) receptor agonist at the glycine-binding site of the NMDA receptor, has been described to augment the extinction of conditioned fear (Ledgerwood et al., 2005; Cai et al., 2006; Lee et al., 2006). Based on these former findings, we applied both substances separately to pharmacologically manipulate extinction in HAB and CD1 mice.

6.2   Results

Social recognition

Indicated by the SR task, all three lines were able to recognize a known stimulus animal, indicated by their decline in olfactory investigation upon repeated exposure of the same stimulus (Figure 6.2, $p<0.01$, $p<0.001$ each line). Introduction of a novel stimulus significantly increased the sniffing time to the same amount as the first exposure (Figure 6.2, $p<0.05$, $p<0.001$).
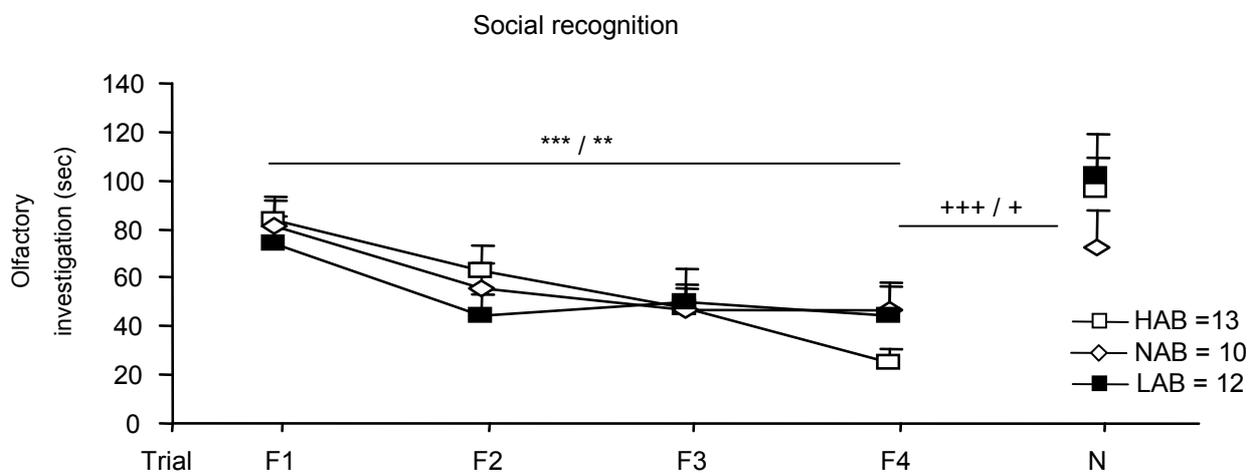
Figure 6.2:   *Social recognition task performed in HAB, NAB, and LAB male mice using ovariectomized females as stimulus animals. The first (F) stimulus animal was introduced to the experimental animal for four minutes and four times with an inter-exposure interval of 15min. All three groups showed a decline in olfactory investigation upon repeated presentation of the first animal, indicative of social recognition. Introduction of a novel (N) stimulus animal led to an olfactory investigation indistinguishable to the first introduction of the familiar animal. ***p<0.001 for HAB, **p<0.01 for NAB and LAB (same line); + p<0.05 LAB F4 vs. N, ++ p<0.01 HAB F4 vs. N:*

In the SD task, with an IEI of 15min, all three lines tested could discriminate between first and novel stimulus animal (Figure 6.3, $p<0.1$ for LAB, $p<0.5$ for NAB, $p<0.01$ for HAB). However, after 30min, LAB failed to distinguish between the two stimuli, whereas HAB and NAB could still discriminate ($p<0.05$ for NAB, $p<0.01$ for HAB). Interestingly, after 2h, only

HAB mice could discriminate between first and novel stimulus animal (p<0.05). This ability was vanished after 4h IEI even in HAB mice.
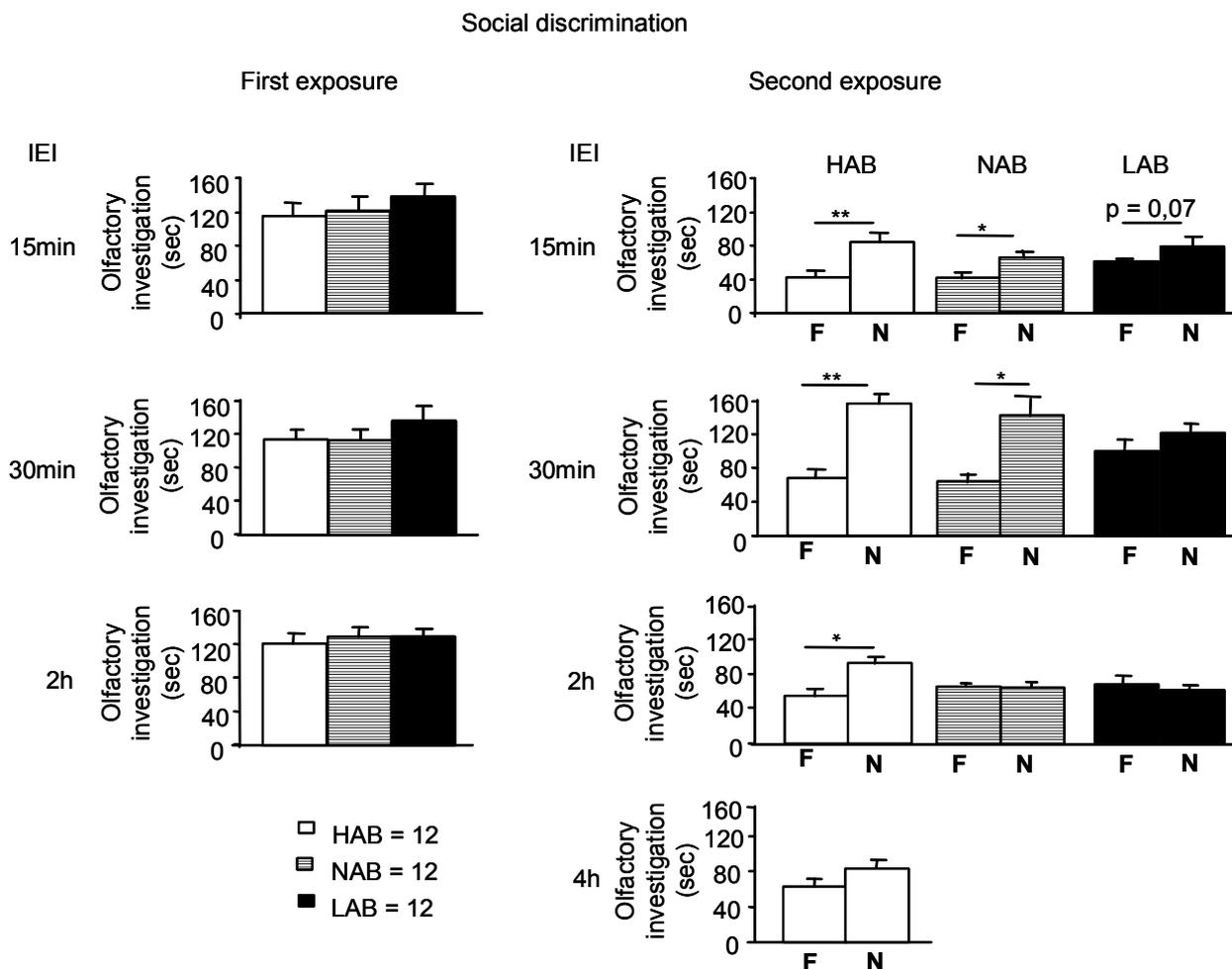
Figure 6.3:   Social discrimination test in HAB, NAB, and LAB male mice. Experimental animals have been introduced to the first (F) stimulus animal for 5min and after different interexposure intervals (IEIs), the F and a novel (N) stimulus were exposed at the same time. After 15min all three lines could discriminate between the two stimuli whereas, after 30min, LAB mice failed to discriminate. Interestingly, after 2h, only HAB animals could distinguish between the first and novel stimulus mouse. T p<0.01, * p<0.05 and **p<0.01 (vs. Novel stimulus).

Fear conditioning

Behavioral analysis during habituation to novel environment (context A) revealed increased freezing and immobility time as well as decreased locomotion and rearing in HAB mice relative to NAB animals (Table 6.1). This decreased explorative behavior in HAB animals could also be detected during habituation to the context B before extinction training (Table 6.2) and retention (Table 6.3).

Table 6.1:   Behavioral parameters obtained in control HAB and NAB mice during habituation to the fear conditioning (context A) chamber. ***p<0.001.

	Before acquisition		Statistics
	HAB (mean ± SEM)	NAB (mean ± SEM)	MWU
Freezing time (sec)	2.1 ± 0.9	0.0 ± 0.0	***
Immobilitiy time (sec)	67.1 ± 4.6	26.6 ± 3.2	***
Locomotion (sec)	40.1 ± 4.8	74.6 ± 3.4	***
Grooming time (sec)	4.4 ± 0.7	4.8 ± 0.5	n.s
Rearing time (sec)	0.6 ± 0.3	8.7 ± 0.9	***
Rearing (n)	2.5 ± 1.4	11.0 ± 1.0	***

Table 6.2:   Behavioral parameters obtained in control HAB and NAB mice during habituation to an empty macrolone cage (context B) before extinction training. *p<0.05 and **p<0.01.

	Before extinction training		Statistics
	HAB control (mean ± SEM)	NAB control (mean ± SEM)	MWU
Freezing time (sec)	2.7 ± 0.8	2.0 ± 0.9	n.s.
Immobilitiy time (sec)	17.3 ± 4.8	12.3 ± 3.1	n.s
Locomotion (sec)	72.6 ± 4.2	54.0 ± 2.3	**
Grooming time (sec)	4.4 ± 2.9	3.2 ± 1.6	n.s
Rearing time (sec)	13.5 ± 2.9	37.3 ± 4.1	**
Rearing (n)	15.1 ± 2.9	23.9 ± 1.7	*

Table 6.3:   *Behavioral parameters obtained in control HAB and NAB mice during*
                 habituation to an empty macrolone cage (context B) before retention. T p<0.1
                 *and **p<0.01.*

	Before extinction training		Statistics
	HAB control (mean ± SEM)	**NAB control (mean ± SEM)**	**MWU**
Freezing time (sec)	11.1 ± 2.6	7.8 ± 1.8	n.s.
Immobilitiy time (sec)	30.9 ± 4.8	19.6 ± 3.6	T
Locomotion (sec)	49.1 ± 4.3	43.4 ± 1.7	n.s.
Grooming time (sec)	9.4 ± 3.0	5.6 ± 2.0	n.s.
Rearing time (sec)	8.1 ± 2.7	30.4 ± 5.0	**
Rearing (n)	8.8 ± 1.9	21.4 ± 2.8	**

Both HAB and NAB animals acquired the tone-shock association during conditioning to a similar extent (Figure 6.4 A, $p<0.01$ (lines, times, interaction), with HAB mice showing a fast acquisition ($p<0.05$, $p<0.01$, $p<0.001$ between the groups). During extinction training control NAB mice exhibited a decline in freezing response upon the repeated tone presentation, whereas control HAB mice showed impaired extinction (Figure 6.4 B, $p<0.01$ (lines, time), T $p<0.1$, $p<0.05$, $p<0.01$, $p<0.001$ between the groups). Moreover, control HAB mice revealed significant more freezing relative to NAB animals during retention (Figure 6.4 C, $p<0.05$ (lines), T $p<0.1$, $p<0.05$ between the groups).
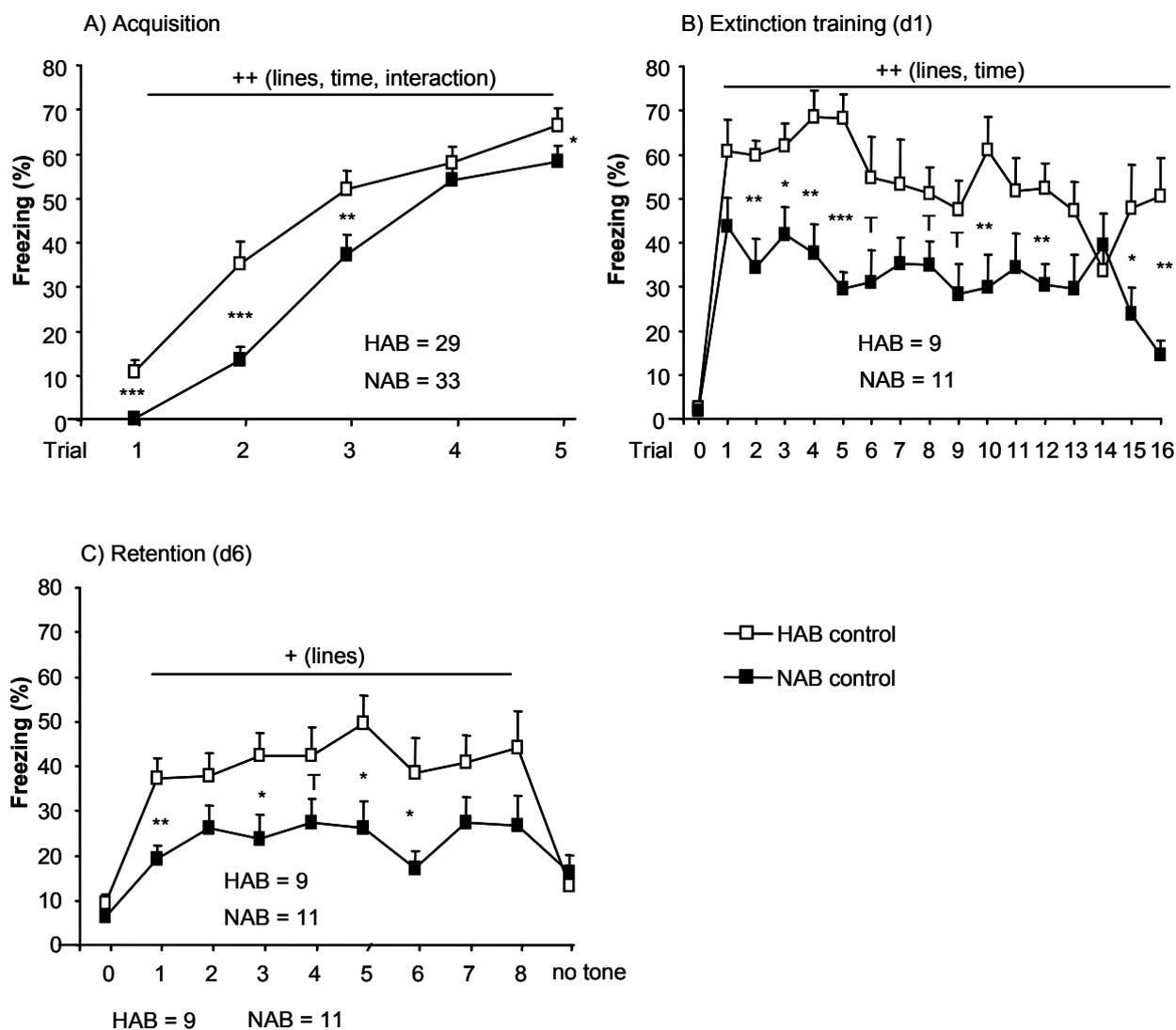
Figure 6.4:   *Fear conditioning in HAB and NAB male control animals. A) Both HAB and NAB animals acquired the tone-shock association during conditioning to a similar extent, with HAB mice showing a faster acquisition relative to NAB. B) During extinction training NAB exhibited a decline in freezing response upon the repeated tone presentation, whereas HAB mice showed impaired extinction. C) Moreover, HAB mice revealed significant more freezing compared to NAB animals during retention. A) ++ p<0.01 (lines, time, interaction), B) ++ p<0.01 (lines, time) and C) + p<0.5 (lines). T p<0.1, *p<0.5, **p<0.01 and ***p<0.001 vs. NAB (same tone).*

D-Cycloserine and Cort treatment

Behavioral parameters investigated during environmental habituation to context B, before extinction training and retention, did not reveal any relevant behavioral changes between HAB (control), HAB (D-cycl) or HAB (Cort) mice as well as for NAB (control), NAB (D-cycl) or NAB (Cort) animals (Table 6.4/6.5).

Table 6.4:   *Behavioral parameters obtained in A) HAB (control), HAB (D-cycl) or HAB (Cort) mice and B) NAB (control), NAB (D-cycl) or NAB (Cort) animals during habituation to an empty macrolone cage (context B) before extinction training.*

A)

	Before extinction training			Statistics
	HAB control (mean ± SEM)	HAB D-cycl (mean ± SEM)	HAB Cort (mean ± SEM)	KWH
Freezing time (sec)	2.7 ± 0.8	1.9 ± 0.8	6.4 ± 1.9	n.s.
Immobilitiy time (sec)	17.3 ± 4.8	19.8 ± 4.3	24.2 ± 4.6	n.s.
Locomotion (sec)	72.6 ± 4.2	78.0 ± 3.6	72.3 ± 4.7	n.s.
Grooming time (sec)	4.4 ± 2.9	0.6 ± 0.3	0.1 ± 0.1	n.s.
Rearing time (sec)	13.5 ± 2.9	10.9 ± 2.6	9.2 ± 1.6	n.s.
Rearing (n)	15.1 ± 2.9	13.5 ± 2.6	9.5 ± 1.5	n.s.

B)

	Before extinction training			Statistics	
	NAB control (mean ± SEM)	NAB D-cycl (mean ± SEM)	NAB Cort (mean ± SEM)	KWH	MWU
Freezing time (sec)	2.0 ± 0.9	2.4 ± 1.2	6.5 ± 2.4	n.s.	
Immobilitiy time (sec)	12.3 ± 3.1	8.4 ± 2.3	20.3 ± 4.8	n.s.	
Locomotion (sec)	54.0 ± 2.3	60.2 ± 2.9	48.7 ± 3.2	*	T (D-cycl vs. Cort)
Grooming time (sec)	3.2 ± 1.6	3.8 ± 1.6	7.3 ± 3.0	n.s.	
Rearing time (sec)	37.3 ± 4.1	33.5 ± 3.5	26.5 ± 4.0	n.s.	
Rearing (n)	23.9 ± 1.7	25.5 ± 1.5	21.5 ± 3.4	n.s.	

Table 6.5:   *Behavioral parameters obtained in A) HAB (control), HAB (D-cycl) or HAB (Cort) mice and B) NAB (control), NAB (D-cycl) or NAB (Cort) animals during habituation to an empty macrolone cage (context B) before retention.*

A)

	Before retention D6			Statistics
	NAB control (mean ± SEM)	NAB D-cycl (mean ± SEM)	NAB Cort (mean ± SEM)	KWH
Freezing time (sec)	7.8 ± 1.8	3.3 ± 1.1	4.5 ± 1.0	n.s
Immobilitiy time (sec)	19.6 ± 3.6	16.1 ± 3.8	22.8 ± 4.6	n.s
Locomotion (sec)	43.4 ± 1.7	50.5 ± 3.5	46.4 ± 2.3	n.s
Grooming time (sec)	5.6 ± 2.0	6.4 ± 2.2	5.8 ± 1.5	n.s
Rearing time (sec)	30.4 ± 5.0	31.4 ± 4.6	28.0 ± 3.0	n.s
Rearing (n)	21.4 ± 2.8	22.2 ± 2.4	19.6 ± 2.4	n.s

B)

	Before retention D6			Statistics
	HAB control (mean ± SEM)	HAB D-cycl (mean ± SEM)	HAB Cort (mean ± SEM)	KWH
Freezing time (sec)	11.1 ± 2.6	9.1 ± 1.7	9.6 ± 1.9	n.s.
Immobilitiy time (sec)	30.9 ± 4.8	33.8 ± 4.9	32.6 ± 4.1	n.s.
Locomotion (sec)	49.1 ± 4.3	54.1 ± 5.0	51.6 ± 3.7	n.s.
Grooming time (sec)	9.4 ± 3.0	5.2 ± 1.4	9.5 ± 1.7	n.s.
Rearing time (sec)	8.1 ± 2.7	7.9 ± 2.0	7.0 ± 1.8	n.s.
Rearing (n)	8.8 ± 1.9	9.5 ± 2.5	8.1 ± 1.8	n.s.

Freezing behavior during extinction behavior and retention remained unchanged after D-cycl and Cort treatment in HAB and NAB animals (Figure 6.5 and 6.6).
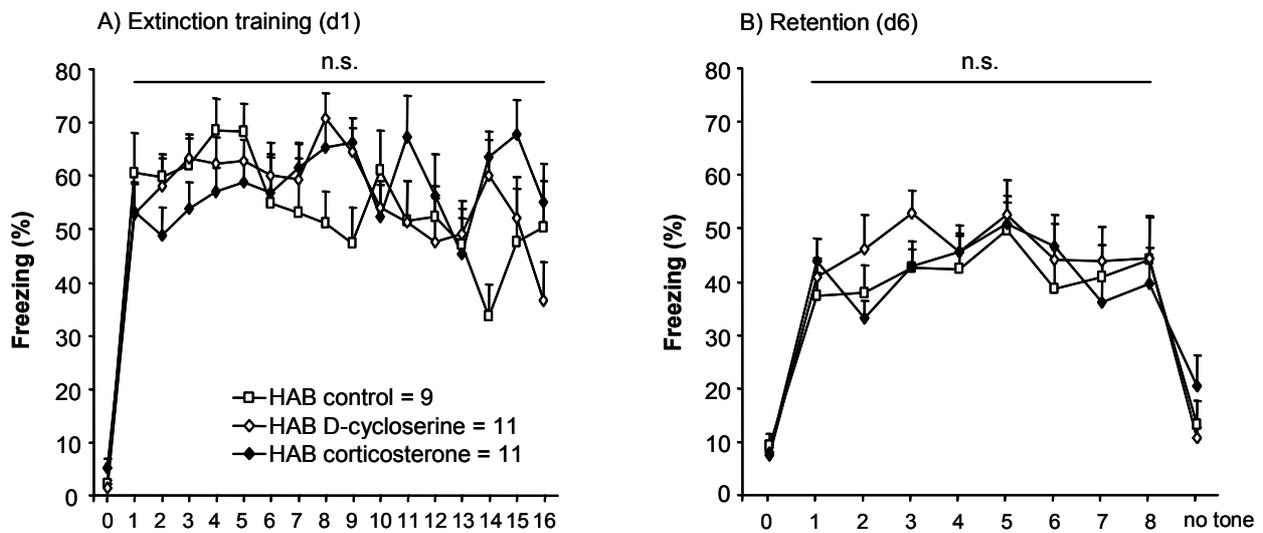
Figure 6.5:   *A) Extinction training and B) retention in HAB (control), HAB (D-cycloserine) or HAB (corticosterone) mice. Freezing neither during extinction training nor during retention was altered by D-cycloserine or corticosterone treatment.*
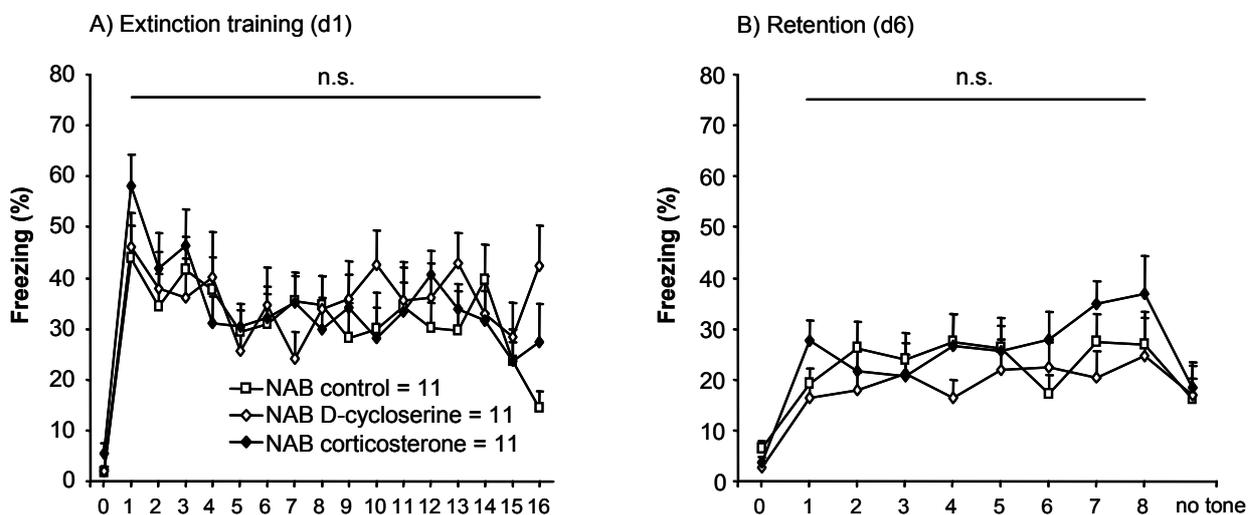
Figure 6.6:   *A) Extinction training and B) retention in NAB (control), NAB (D-cycloserine) or NAB (corticosterone) mice. No changes in freezing responses could be detected in the three groups during extinction training and retention after D-cycloserine or corticosterone treatment.*

6.3   Discussion

The decline in olfactory investigation of the familiar stimulus animal upon repeated presentation, indicate that all three mouse lines were able to recognize a familiar conspecific. In the SD test, only at an IEI of 15min all three groups can significantly or at least with a trend distinguished between the novel and the first stimulus animal. With an IEI of 30min, however, this discrimination ability got lost in LAB mice. Interestingly, after 2h, only HAB animals were able to discriminate between novel and first stimulus animal. This ability was not maintained after an IEI of 4h in HAB mice. In the FC paradigm during habituation to the FC chamber, HAB mice revealed increased freezing and immobility time as well as a decreased explorative behavior in comparison to NAB animals. However, both groups similarly acquired the tone-shock association, as proven by their increase in freezing response due to repeated tone-shock events. Moreover, control HABs exhibited fast acquisition during FC, a deficit in extinction and increased freezing response upon tone presentation during the retention trial performed six days (d6) after conditioning, in comparison to control NAB mice. Neither behavior during habituation phase before the extinction trial nor extinction or retention at d6 was altered by treatment with Cort or D-cycloserine in any line.

Social recognition / Social discrimination

By use of the modified experimental design for the SR test, it could be demonstrated that social memory was properly working in all three lines tested, indicated by the decline of olfactory investigation upon repeated presentation of the first stimulus animal. All animal revealed a learning curve (a decline in olfactory investigation), reflecting the ability to recognize a familiar mouse with an IEI of 15min. Moreover, as introduction of a novel stimulus was provoking similar exploration times as previously the first animal (during the first introduction), effects of tiredness or a lack of social interest are neglectable (Ferguson et al., 2000b; Bielsky et al., 2004). Additionally, as the total investigation times between the lines were very similar during the whole experiment, it is suggested that the perception and processing of olfactory cues per se is not impaired, possibly due to selection or inbreeding, in either line. An intact social memory is fundamental for social interaction in mammals (Bielsky and Young, 2004). Nevertheless, the specific neurochemical and neurophysiological processes underlying social recognition are not fully understood (Ferguson et al., 2002). Social memory, in contrast to other forms of learning such as contextual learning, is mainly based on the AVP and the OXT system (Ferguson et al., 2002). While OXT has been

described to be necessary for the initial processing, encoding and acquisition of social cues, AVP seem to be more related to memory consolidation, retention and recall of social memory (Young, 2002; Bielsky and Young, 2004; Winslow and Insel, 2004). Former results suggested that OXT reduces social memory abilities (Popik et al., 1991). However, it could be demonstrated that this finding is only true at high doses of OXT and opposite effects, improved social memory, has been shown for low doses of OXT (Popik et al., 1992; Dluzen et al., 1998b; Dluzen et al., 2000; Bielsky and Young, 2004). Moreover, studies in OXT knockout animals revealed impaired social recognition abilities, whereas other cognitive functions remained unchanged, implicating an involvement of OXT specifically in a social olfactory-based context (Dantzer et al., 1990; Winslow and Insel, 2004). As no differences in *Oxt* mRNA in the PVN (see 4.2.2) and additional OXT-containing brain region (Czibere, unpublished) were detectable in HAB, NAB, and LAB mice, together with their similar performance in the SR test, it is hypothesized that the OXT system is similarly and adequately functional in all three mouse lines.

For almost four decades it has been known that AVP is centrally affecting learning and memory (Ferguson et al., 2002). AVPs importance in the context of learning was first described by David de Wied in the 1960s. In his landmark report, removal of the posterior and intermediate lobes of the pituitary resulted in the facilitation of extinction of shuttle box learning in rats. In turn, an effect that could be normalized by administration of a crude pituitary extract of AVP (Ferguson et al., 2002; Ring, 2005). Moreover, it has been demonstrated that icv administration of AVP facilitates social memory, whereas receptor antagonists impair memory retrieval (Dantzer et al., 1987; Le Moal et al., 1987). AVP´s effects on learning processes are mainly attributed to the LS, which receives vasopressinergic projections from the BNST and the MeA (De Vries and Buijs, 1983; Frank and Landgraf, 2008). The importance of both the LS and the BNST have been underlined by agonizing (e.g. via AVP administration) or antagonizing (e.g., via AVP antisense) the AVP system and thereby improving or impairing social memory performance (Dantzer et al., 1988; van Wimersma Greidanus and Maigret, 1996; Frank and Landgraf, 2008). Our SR data indicate that, although HAB, NAB, and LAB significantly differ in their *Avp* expression and AVP amount in several brain regions (see 4.2.2), the amount of AVP, necessary to successfully perform a SR task, seem to be provided in all three lines. Nevertheless, the V1a receptor has been reported to be sufficient for normal social recognition and overexpression of the V1a receptor in the septum of rats is improving social discrimination abilities (Landgraf et al., 1995; Landgraf et al., 2003; Bielsky et al., 2005). Furthermore, it has been suggested

that the V1a receptor, beside V1b receptor with its weaker contribution, is mainly mediating memory processes within the brain (Wersinger et al., 2002). However, V1a receptor autoradiography in HAB and LAB mice did not reveal any differences in various brain areas analyzed (Bunck et al.), pointing to AVP itself rather than to its V1a receptor density as a critical variable. As AVP has been primarily implicated in memory consolidation, which requires a certain time and recall of cues (Winslow and Insel, 2004), it is not surprising that in the context of a simple SR task with its IEI of 15min, the role of AVP is less pronounced. Even in the SD task, which is more challenging for the experimental animals, as they have to discriminate between two stimulus mice presented at the same time, all three mouse lines are able to discriminate the novel from the first animal at an IEI of 15min. It could be demonstrated again that all three lines tested, independently of IEI, investigated a social cue to similar ratios, excluding aberrations in social interest and providing equal basis to store information. However, after 30min, LAB male mice failed to distinguish between novel and familiar stimuli. It is rather likely that with an increase IEI and difficult of the learning task per se, the involvement of AVP becomes more important, especially in terms of memory consolidation and recall.

It has been demonstrated before that AVP is facilitating social memory with increasing IEI (Dantzer et al., 1987). As previously described, LAB animals revealed decreased *Avp* mRNA levels in various brain areas, including the PVN, SON, and CeA (see 4.2.2) and a deficit in AVP in the PVN and PVN-ascending axonal projections compared to HAB and NAB mice (see 4.2.3). According to the genetic underpinnings identified in LAB mice (Bunck et al.), it is rather likely that this AVP deficiency is not restricted to the already mentioned brain regions, but does also effect AVP-containing areas such as the LS, BNST, MeA or medial preoptic area, circuits that has been well described in social memory (Ferguson et al., 2002; Frank and Landgraf, 2008). The lack of endogenous AVP in these animals is not only related to their non-anxious phenotype (Bunck et al.), but leads to symptoms of cDI (Keßler et al., 2007). Moreover, Brattleboro rats that have been reported to show symptoms of cDI (Kim and Schrier, 1998), similar to LAB mice, exhibited impaired social discrimination abilities in comparison to Long-Evans rats or HAB and NAB mice. Intra-septal administration of synthetic AVP in Brattleboro rats significantly improved social memory abilities (Engelmann and Landgraf, 1994). Similar to the observations in LAB mice and Brattleboro rats, the relationship between AVP deficits and cognitive impairment have been confirmed by a clinical study performed in patient suffering from familial neurohypophyseal DI. These subjects exhibited impaired retrieval processes and substained attention in several learning

tests as well as fewer signs of agoraphobia (Bruins et al., 2006). Due to this finding and together with the results obtained in Brattleboro rats and LAB mice, it is suggested that the AVP deficit is effecting emotionality, body water homeostasis, and cognitive functions. Moreover, it has to be investigated in more detail, if this dysregulation of the AVP system, beyond social memory, is directly or indirectly impairing other forms of learning, such as spatial learning or FC.

Similar to HAB rats (Frank and Landgraf, 2008), HAB mice outperformed LAB and normal anxiety animals in their abilities to remember a familiar conspecific. In HAB rats, a AVP promoter polymorphism has been identified and described to consequently lead to an AVP overexpression in these animals (Murgatroyd et al., 2004), whereas no genetic underpinnings have been found in HAB mice compared to CD1 animals (see 4.3). However, HAB mice (Muigg, in preparation) as well as HAB rats (Salome et al., 2004) showed a similar pattern of brain activation upon a mild stressor, measured by Fos-antibody staining. In HAB mice, these regions of hyperexcitability, beside others, include the LS, medial preoptic area, MeA, and BNST. This pattern points to an increased activation exactly in those neuronal circuit that are well described for AVP-mediated social memory (Ferguson et al., 2002). Moreover, a link between increased Fos expression and AVP has been demonstrated using *c-fos* antisense technique. Rats treated with *c-fos* antisense revealed reduced anxiety-related behavior compared to control animals (Lu et al., 2000). The gene encoding AVP comprises a AP-1 sequence in its regulatory domain, indicating that this gene may be a potential target for Fos-mediated gene expression (Kovacs, 1998).

Furthermore, this increased activation of specific brain regions, crucially involved in memory processes, has been confirmed even under basal conditions in HAB animals (Czisch, in preparation). Although the precise role of AVP, in terms of increased release of AVP, has to be investigated in more details, it is rather likely that a hyperactive AVP system in HABs is contributing, beside their hyperemotional phenotype, to their improved cognitive abilities. The data, derived from animal and clinical studies (Engelmann and Landgraf, 1994; Kim and Schrier, 1998; Grady and Keightley, 2002; Murgatroyd et al., 2004; Bruins et al., 2006; Frank and Landgraf, 2008; Keßler et al., 2007) strengthen the hypothesis of a striking association between cognition- and emotion-related phenomena, where AVP is rather likely to play a crucial role. However, the cellular mechanisms triggered by AVP during social recognition remain questionable and have to be investigated in more detail (Frank and Landgraf, 2008). Beside the well-known involvement of the AVP and the OXT system, there are several indications that dopaminergic and noradrenergic circuits contribute to social memory (Dluzen

and Kreutzberg, 1993; Guan et al., 1993; Guan and Dluzen, 1994; Ferguson et al., 2002). Indeed, both the nucleus accumbens and the LC as main regions synthesizing dopamine or noradrenaline have been depicted to be hyperactivated in HAB mice relative to NAB and LAB animals (Muigg, in preparation), highlighting both systems to be investigated in further studies. Moreover, it is rather likely that, according to their hyperemotional phenotype, these animals pay more attention to social cues accompanied by increased perception and processing of olfactory information which in turn, enables improved memory consolidation and recall. Indeed, depression has been reported to induce cognitive alteration with patient showing an increased focus on social problems, perception, and information processing of their "social" environment (Watson and Andrews, 2002). Furthermore, depressed patients revealed hypoactivation in areas involved in higher cognitive function (e.g. PFC), whereas structures mediating emotional and stress responses (e.g. amygdala) were abnormally activated during social cognition (Grady and Keightley, 2002). Nevertheless, it has not been excluded that cognitive alterations in turn provide the neurobiological basis to develop various psychopathologies. One suggestion, derived from some instances of clinical anxiety, is the hyperactivation of the septo-hippocampal system, which produces cognitive dysfunction (McNaughton, 1997).

By use of the SR test, it could be demonstrated that social recognition of one stimulus is adequately working in all three mouse lines tested. The more challenging SD test, however, revealed significant differences in cognitive abilities between the lines. Therefore, it is suggested that both tests should be applied to appropriately investigate general social recognition abilities and more challenging social discrimination abilities in rodents to reveal possible alterations in social memory.

Fear Conditioning

As LAB mice already exhibited impaired cognitive abilities in the SD task and preliminary experiments implicated that these animals do not show any freezing response during acquisition (Singewald, unpublished), they were not included in the present FC paradigm.

During 2min habituation to the FC chamber, and similarly to the novel cage observations (see 4.2.1) HAB mice revealed increased freezing and immobility time due to novel environment, whereas the locomotor activity and the number and time of rearing were significantly decreased compared to NAB animals. These data are in accordance with former phenotyping data (see 4.2.1) which underline the HABs´ suppression of locomotor activity and explorative behavior in various tests, reflecting one dimension of their hyperemotional

status (Henderson et al., 2004). This anxiety-based suppression of explorative behavior in HAB mice is constantly measurable at any experimental day investigated, excluding effects of context-habituation in these animals. This influence of hyperemotionality on behavior is additionally indicated by the HABs´ increased freezing response upon first tone presentation. It is rather likely that these animals, according to their genetic background, reveal a more sensitive or excitable perceptual-response system, which in turn leads to a hyper-interpretation of environmental cues. As it has been suggested before for social memory, this seems to be additionally true for non-social cues, such as novel environment or even a tone. Indeed, exaggerated responses to stimulation have been reported from patients suffering from anxiety disorders underlying hyperexcitability of certain brain structures (Rosen and Schulkin, 1998). Research investigating the neural basis of emotion, including lesion, pharmacological and neurophysiological studies, has identified the amygdala as one important structure in the circuits that underlie the central state of conditioned and unconditioned fear (Chen et al., 2006; Kim and Jung, 2006). The amygdala receives multimodal sensory input associated with danger and sends in turn processed signal to other brain regions to generate defensive behavioral responses (Blanchard and Blanchard, 1972; Kalin et al., 2001; Etkin et al., 2004). Anatomically, the amygdala receives input from diverse brain areas (e.g. thalamus, neocortex, olfactory cortex, hippocampus) and consequently mediates fear responses in a variety of brain regions (e.g. BNST, PAG for freezing, lateral hypothalamus for sympathetic activation (LeDoux, 2000; Kim and Jung, 2006).

Although the role of the amygdala in detecting and responding to threatening stimuli is well recognized, the contribution of each amydala nucleus remains poorly understood. The lateral (LA) and the BLA nuclei are thought to be the primary sites for sensory input and the place for the formation of the US-CS association. The CeA nucleus, in contrast, is the primary site for output (LeDoux, 1995, 2000; Sah et al., 2003; Lanuza et al., 2004; Kim and Jung, 2006). Freezing as one example for defensive behavior occurs upon threatening stimuli or after conditioning, with the LA, BLA, and CeA being required for acquisition and expression of conditioned behavior (Muller et al., 1997; Gale et al., 2004; Chen et al., 2006). Indicated by the Fos-antibody staining, MEMRI or ISH, the amygdala nuclei in HAB mice were constantly highlighted in terms of over-expression of *Avp* or *Crh*, alteration in synaptic neurotransmission or neuronal activation under basal conditions as well as after mild stress exposure (Czisch, Muigg, in preparation; see 4.2.2). Therefore, it is strongly suggested that in particular these brain areas have to be investigated in detail in order to encode the underlying neurotransmitter systems and molecular mechanisms contributing to the

increased hyper-emotional behavioral responses as well as the fast acquisition observed in HAB animals. However, independent of the emotional status, both groups, HAB and NAB, similarly acquire the tone-shock association, indicated by a similar increase in freezing response to repeated tone-shock events.

NAB animals, during extinction training, exhibited a decline in the conditioned behavior, measured by the reduction in freezing, upon repeated tone presentation. This decline in freezing response by repeated presentation of the CS (in absence of US) indicates short-term extinction, which is believed to represent new learning, the CS is no longer associated with the US (Kim and Jung, 2006). However, HAB mice, relative to NAB animals, revealed impaired extinction during extinction training one day after conditioning, indicated by a lack of reduction of the conditioned behavior throughout the CS presentations.

Several hypotheses can be suggested to explain this phenomenon observed in HAB animals. As "resistance to extinction" has been described to be a sensitive measure of the strength of acquisition (Annau and Kamin, 1961; Myers and Davis, 2002), it is conceivable that, due to the increased emotional response together with the fast acquisition in HAB mice, the storage of aversive experience is strengthened, thus consequently impairing extinction.

In the last decades an enormous scientific effort has been undertaken to uncover the neuronal mechanisms underlying the phenomenon of extinction. Lesion, electrophysiological, molecular, and pharmacological studies suggest, among others, the cholinergic, dopaminergic, noradrenergic, serotinergic, opioid, gabaergic, glutamatergic, and vasopressinergic systems, as well as intracellular signaling and protein synthesis as major players involved in extinction processes (Manson, 1983; Myers and Davis, 2002; Bruchey et al., 2006). As the possible mechanisms underlying the phenomena of impaired extinction are currently under investigation, only first hypotheses will be introduced.

The PFC has been proposed as an essential brain area for extinction (Morgan et al., 1993). In a general sense, this proposal makes sense considering the role of the PFC in the inhibitory control of maladaptive behavior. When a CS no longer signals a US, it would be beneficial for an animal to refrain from committing previously adaptive, but now unnecessary behavior (Kim and Jung, 2006). In this regard, the PFC is known to project directly to a number of subcortical brain structures, including amygdala, PAG, and hypothalamus, exerting inhibitory influences (Myers and Davis, 2002; Akirav et al., 2006; Bruchey et al., 2006; Kim and Jung, 2006). Studies on neuronal activation under basal and after mild stress exposure revealed a pattern of hyperexcitability in HAB mice in various brain regions, including the amygdala, PAG, nucleus accumbens, or the LS (as described in the previous

chapters), whereas, in contrast, exclusively the PFC exhibited hypo-activation (Czisch, Muigg, in preparation). According to these data and the previous findings concerning hyper-emotionality and freezing responses during extinction training, it is suggested that an impaired PFC-mediated inhibition of subcortical regions in HAB animals is leading to an impaired control of maladaptive behavior and extinction. Moreover, these regions, mainly the PFC and amygdala, have additionally been implicated in psychiatric patient in association with cognitive alterations (LaBar et al., 1998; Grady and Keightley, 2002; Milad et al., 2006). Therefore, our primary focus is the axis between PFC and subcortical regions, especially the amygdala nuclei, to uncover neurotransmitter systems and molecular underpinnings contributing to the HAB-specific phenotype. Moreover, AVP and CRH have been well described (see previous chapters) to contribute to the animals´ behavior in the HAB/LAB mouse model, and both systems have been broadly discussed in the modulation of learning and extinction (Manson, 1983; Koob et al., 1986; Radulovic et al., 1999; Croiset et al., 2000; Radulovic et al., 2000; Risbrough et al., 2003; Debiec, 2005; Stoppel et al., 2006). Moreover, human studies in depressed patients and in patients suffering from PTSD suggest both neuropeptides to be major players in the inability to erase negative experiences and traumatic events (Manson, 1983; Yehuda, 2001; Grady and Keightley, 2002). Therefore, further studies will focus on the molecular and pharmacological intervention of these systems to rescue the observed phenotypic differences at an emotional and cognitive level. Furthermore, electrophysiological studies have to be investigated to uncover differences in long-term potentiation, neuronal activation and possible neurotransmitter system mediating changes in cognitive abilities. Moreover, a current microarray study performed in HAB, NAB, and LAB mice includes, among other brain areas, the PFC, amygdala nuclei, and the PVN. This broad screening of expression profiles in various brain regions will give rise to many new candidate genes worth to be investigated as possible major contributors underlying cognitive alterations.

To avoid false positve results, several control experiments have to be performed to distinguish between behavioral responses caused by the hyper-emotionality in HAB mice and alterations in extinction. As HAB mice do not only show increased freezing response during exposure to a novel environment as well as to tone presentation without shock experience, it is necessary to study the animals´ behavioral response upon repeated tone presentations on consequent days. These experiments are necessary, as it can not be determined, if the increased freezing response in HAB mice during retention (d6), is

reflecting an impairment of long-term extinction or an exaggerated interpretation of the tone per se in these animals.

Numerous human and animal studies report a successful improvement of extinction upon administration of D-cycl or Cort, by supporting relearning (Walker et al., 2002; Ledgerwood et al., 2005; Bertotto et al., 2006; Cai et al., 2006; Davis et al., 2006; Kim and Jung, 2006; Lee et al., 2006; Yang et al., 2006), whereas others failed to show any effects (Guastella et al., 2007). However, both HAB and NAB mice did not reveal any changes in behavior during habituation or during extinction training or retention in comparison to the respective control group. As previous investigations are primarily focusing on rats as experimental animal, the drug concentrations, number, time and locus of administration are strongly varying between the studies, this issue of rescuing impaired extinction via D-cycl or Cort in HAB/NAB mice has to be investigated in more detail.

Taken together, as we are dealing with animals which highly differ in their emotional status, it is certainly difficult to separate their emotional response from those reflecting alterations in cognition. Although it is quite clear that the brain circuits contributing to both, anxiety and cognition, are rather similar or at least overlapping to a certain amount (Landgraf and Wigger, 2002; Kalueff, 2007), it is important to use adequate tests that clearly separates emotional from cognitive parameters.

Concerning social memory the increased social discrimination ability in HAB animals seem to be advantageous, possibly indicating the animals´ increased need to communicate in a protected environment. In a clinical context however, HAB mice, reflect the situation in psychiatric patients, who are unable to erase traumatic events (Yehuda, 2001).

7   Conclusion and Perspectives

By selective inbreeding, we succeeded in generating two mouse lines that significantly differ in their innate emotionality. This divergence is not only restricted to the anxiety-related behavior measured in the EPM test, used as the selection criterion, but includes additional tests reflecting anxiety in rodents. Moreover, differences in explorative and depression-like behavior have been depicted. This is an intriguing finding as no selection pressure was exerted on other behavioral criteria throughout the years. Similarly to HAB rats, HAB mice revealed comorbid depression-like behavior and, therefore, resemble the clinical situation of high comorbidity between these psychopathologies. Moreover, alterations in locomotor activity and explorative behavior, indicated in several behavioral tasks, have been identified as indicators reflecting various cross-test dimensions of anxiety.
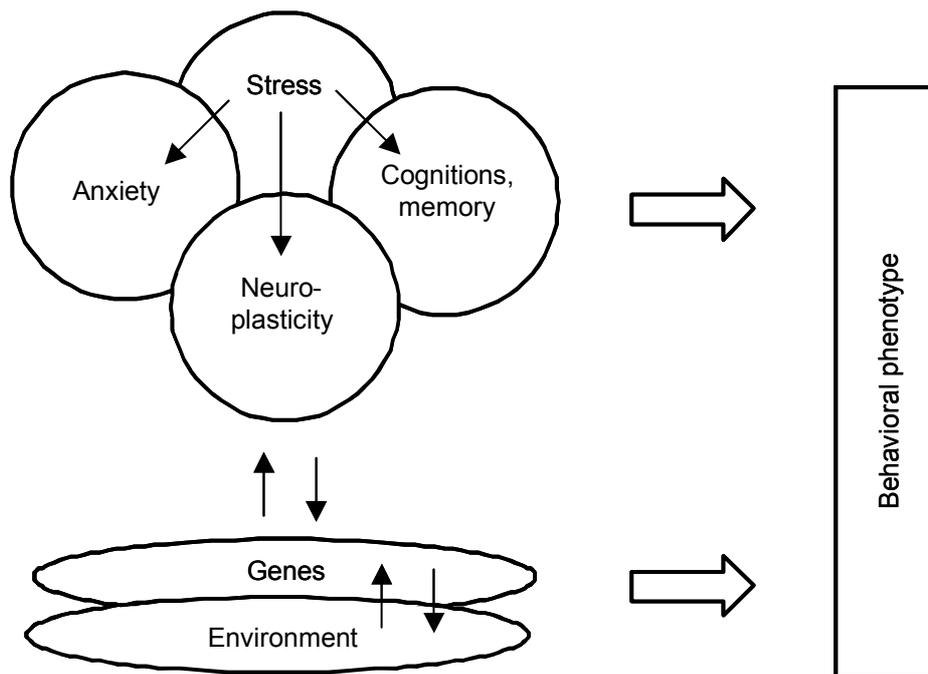
Figure 7.1:   Various criteria and their interplay involved in shaping the behavioral phenotype (Kalueff, 2007).

As clearly demonstrated (Fig. 7.1), the behavioral phenotype is determined by a variety of intermingling criteria, which have in part been scrutinized in several studies in our mouse model.

Since selective breeding enhances the accumulation of genes relevant for the respective phenotype, several candidates, well described in the regulation of emotionality at the level of the HPA axis, cellular detoxification, and synaptic neurotransmission have been investigated. Our studies highlighted AVP, CRH, Glx1, and *Syt4* as potential mediators contributing to the observed behavioral differences. AVP has been identified to be under-expressed in several brain regions of LAB mice associated with both symptoms of cDI and non-anxious and non-depression-like behaviors. In addition, several genetic polymorphisms have been identified that are likely to play a critical role in the AVP under-expression of these animals. Subsequently, molecular techniques, such as luciferase assays, will help to identify the impact of single as well as combined genetic polymorphisms on the gene expression of AVP. Moreover, it is planned to "rescue" the LAB-specific phenotype by chronic administration of AVP via osmotic minipump implantation.

In contrast to the AVP gene, the highly anxious HAB animals displayed an over-expression of CRH in various brain areas. The significance of CRH over-expression in mediating the HAB-specific phenotype was pharmacologically validated via CRH receptor 1 antagonist administration. Future chronic treatment with this antagonist will test the hypothesis of an even increased anxiolytic effect, highlighting this type of receptor antagonist as a suitable target for clinical studies. As no genetic underpinnings could be identified by sequencing the CRH gene in the HAB/LAB model, further research has to focus on synaptic input, transcription factors and gene interactions to unravel the over-expression in HAB mice.

Additionally, synaptic release, indicated by *Syt4* expression, was found to be altered in both inbred mouse lines in opposite directions, indicating a dysregulation in both extremes of trait anxiety. To assess synaptic neurotransmission in more detail, electrophysiological experiments are currently under investigation to identify specific neurotransmitter systems affected by the alterations in synaptic release. Moreover, the genetic background underlying the variation in presynaptic vesicle fusion has to be examined in more detail. Furthermore, glyoxalase1 (Glx1), a cellular detoxification enzyme, has been identified to be differently expressed at already very early developmental stages in association with the phenotypic divergence. These data allow us to investigate gene expression determined by several polymorphisms identified, shortly after birth. Thus, Glx1 might act as a biomarker, suitable for the early prediction of pathological anxiety (as it can be easily measured in blood samples).

However, given that higher brain functions are polygenic, with single genes having a minor impact on the phenotypic specificity and being hard to detect, it is rather unlikely that only a

few genes are contributing to the hyper- and hypoemotionality observed in these animals. Therefore, the main focus is still on the identification of the orchestra of genes underlying these behavioral extremes. Extensive microarray studies in different brain areas as well as comprehensive proteomics studies will help to approach these targets. Moreover, a currently investigated genome-wide SNP screening performed in a freely-segregating F2 panel will assess polymorphisms differing between HAB and LAB mice and unravel new goals for gene-based analyzes. By using these unbiased approaches, a variety of novel targets, including specific brain regions, neuropeptides, neurotransmitter systems and accompanying receptors will be detected. Additionally, these findings are suited to reveal the primary focus, beside the already identified candidates, which might explain the superior social discrimination abilities as well as the delayed extinction observed in HAB animals.

Given the fact that there is a continuous interaction between the genetic blueprint and the environment, it is necessary to attend this interplay. Indeed, a cross-fostering study revealed differences in maternal behavior in HAB and LAB mothers, but this divergence in rearing styles did not induce any behavioral alterations in HAB and LAB offspring (Keßler, in preparation). However, raising both mouse lines in an enriched environment altered anxiety-related behavior exclusively in HAB mice (Baier, Bunck, Touma, unpublished). Interestingly, although the behavioral phenotypes seem to be strongly genetically determined in these animals, environmental factors are still able to shape the phenotype. This finding emphasizes research on epigenetic factors contributing to this phenomenon of specific interest.

Moreover, as stressors are strongly influencing the behavioral phenotype lifelong, a current study is focusing on stress-vulnerability in the HAB/LAB mouse model by using a chronic social defeat paradigm.

Taken together, the mouse model presented in the current work provides a unique opportunity to investigate anxiety as a multidimensional domain. It covers many clinical core symptoms of anxiety disorders at different levels, including behavior, gene expression, and cognitive alterations. Therefore, it provides a valuable and promising tool to elucidate the neurobiological basis of the continuum from vital to pathological anxiety. Moreover, it might be helpful in the identification of genes and proteins serving as diagnostic and therapeutic targets relevant for both anxiety and cognitive dysregulation.

8   Lists

8.1   List of abbreviations

ACTH	Adrenocorticotropic hormone
am	ante meridiem
ANOVA	Analysis of variance
AVP	Arginine-vasopressin
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
bp	base pairs
cAMP	Cyclic adenosine monophosphate
cDI	Central diabetes insipidus
cDNA	Complementary DNA
CeA	Central amygdala
CNS	Central nervous system
Cort	Corticosterone
Cp	Crossing point
SCN	Suprachiasmatic nucleus
CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin-releasing hormone receptor 1
CRHR2	Corticotropin-releasing hormone receptor 2
CS	Conditioned stimulus
CSF	Cerebrospinal fluid
DaLi	Dark-light box
D-cycl	D-Cycloserine
DMP696	(4-(1,3-Dimethoxyprop-2-ylamine)-2,7-dimethyl-8-(2,4-dichlorophenyl)-pyrazolo[1,5-*a*]-1,3,5-triazine
EPM	Elevated plus-maze
F	First, familiar stimulus animal
F1	Offsprings of reciprocal cross-mated HAB and LAB mice
FC	Cued fear conditioning
FS	Forced swim

GABA	γ-aminobutyric-acid
Glx1	Glyoxalase1
GR	Glucocorticoid receptor
HAB	High anxiety-related behavior
HNS	Hypothalamic-neurohypophyseal system
HPA	Hypothalamo-pituitary-adrenocortical
i.p.	Intraperitoneal
icv	Intracerebroventricular
IEI	Inter-exposure interval
ISH	In situ hybridization
KWH	Kruskal-Wallis *H*-test
LAB	Low anxiety-related behavior
LC	Locus coeruleus
LS	Lateral septum
MAO	Monoamine oxidase
MeA	Medial amygdala
MEMRI	Manganese-enhanced resonance imaging
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MWU	Mann-Whitney U-test
N	Novel stimulus animal
NAB	Normal anxiety-related behavior
NC	Novel cage (behaviour)
NF-1	Nuclear factor 1
NMDA	N-methyl-D-aspartate
NRI	Noradrenaline reuptake inhibitor
OA	Open arm exposure
OXT	Oxytocin
pnd	Postnatal day
PAG	Periaqueductal grey
PFC	Prefrontal cortex
pm	post meridiem
POMC	Proopiomelanocortin
PTSD	Posttraumatic stress disorder

PVN	Paraventricular nucleus
qRTPCR	Quantitative real-time polymerase chain reaction
LA	Lateral amygdala
RIA	Radioimmunoassay
SAM	Sympathetic-adrenomedullary
SCN	Suprachiasmatic nucleus
SD	Social discrimination
SNP	Single nucleotide polymorphism
SNRI	Serotonin and Noradrenaline reuptake inhibitor
SON	Supraoptic nucleus
SR	Social recognition
SSRI	Selective serotonin reuptake inhibitor
SYT4	Synaptotagmin4
TST	Tail-Suspension test
US	Unconditioned stimulus
USV	Ultrasonic vocalization
V1a	AVP receptor 1a
V1b	AVP receptor 1b
WB	Westernblot

8.2   List of figures

8.3   List of tables

9   References

Acher R, Chauvet J (1988) Strucuture, processing and evolution of neurohypophysial hormone-neurophysin precursors. Biochimie 70:1197-1207.

Aerni A, Traber R, Hock C, Roozendaal B, Schelling G, Papassotiropoulos A, Nitsch R, Schnyder U, de Quervain DJ (2004) Low-dose cortisol for symptoms of posttraumatic stress disorder. Am J Psychiatry 161:1488-1490.

Ahmed N (2005) Degradation products of proteins damaged by glycation, oxidation and nitration in clinical type 1 diabetes. Diabetologia 48:1590-1603.

Akirav I, Raizel H, Maroun M (2006) Enhancement of conditioned fear extinction by infusion of the GABA(A) agonist muscimol into the rat prefrontal cortex and amygdala. European Journal of Neuroscience 23:758-764.

Allin JT, Banks EM (1971) Effects of temperature on ultrasound production by infant albino rats. Psychobiology 4:149-156.

Alonso J, Angermeyer MC, Bernert S, Bruffaerts R, Brugha TS, Bryson H, de Girolamo G, Graaf R, Demyttenaere K, Gasquet I, Haro JM, Katz SJ, Kessler RC, Kovess V, Lepine JP, Ormel J, Polidori G, Russo LJ, Vilagut G, Almansa J, Arbabzadeh-Bouchez S, Autonell J, Bernal M, Buist-Bouwman MA, Codony M, Domingo-Salvany A, Ferrer M, Joo SS, Martinez-Alonso M, Matschinger H, Mazzi F, Morgan Z, Morosini P, Palacin C, Romera B, Taub N, Vollebergh WA (2004) Prevalence of mental disorders in Europe: results from the European Study of the Epidemiology of Mental Disorders (ESEMeD) project. Acta Psychiatr Scand Suppl (420):21-27.

Annau Z, Kamin L (1961) The conditioned emotional response as a function of US intensity. J Comp Physiol Psychol 54:428-432.

Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB (1999) The role of corticotropin-releasing factor in depression and anxiety disorders. J Endocrinol 160:1-12.

Arletti R, Bertolini A (1987) Oxytocin acts as an antidepressant in two animal models of depression. Life Sci 41:1725-1730.

Bai F, Li X, Clay M, Lindstrom T, Skolnick P (2001) Intra- and interstrain differences in models of "behavioral despair". Pharmacol Biochem Behav 70:187-192.

Bakshi VP, Smith-Roe S, Newman SM, Grigoriadis DE, Kalin NH (2002) Reduction of stress-induced behavior by antagonism of corticotropin-releasing hormone 2 (CRH2) receptors in lateral septum or CRH1 receptors in amygdala. J Neurosci 22:2926-2935.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee KF (2000) Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nat Genet 24:410-414.

Bale TL, Davis AM, Auger AP, Dorsa DM, McCarthy MM (2001) CNS region-specific oxytocin receptor expression: importance in regulation of anxiety and sex behavior. J Neurosci 21:2546-2552.

Bale TL (2006) Stress sensitivity and the development of affective disorders. Horm Behav 50:529-533.

Bandelow B, Späth C, Alvarez Tichauer G, Brooks A, Hajak G, Rüther E (2002) Early traumatic life events, attitudes, family history, and birth risk factors in patients with panic disorders. Compr Psychiatry 43:269-278.

Barad M (2006) Is extinction of fear erasure or inhibition? Why both, of course. Learn Mem 13:216-23.

Barberis C, Tribollet E (1996) Vasopressin and oxytocin receptors in the central nervous system. Crit Rev Neurobiol 10:119-154.

Bargmann W, Scharrer E (1951) The site of the origin of the hormones of the pituitary. Am Sci 39:255-259.

Bertotto M, Bustos S, Molina VA, Martijena I (2006) Influence of ethanol withdrawal on fear memory: effect of D-cycloserine. Neuroscience 142:979-990.

Bhattacharya SK, Bhattacharya A, Chakrabarti A (1998) Anxiogenic activity of intraventricularly administered arginine vasopressin in the rat. Biogenic Amines 14:367-385.

Bielsky IF, Young LJ (2004) Oxytocin, vasopressin, and social recognition in mammals. Peptides 25:1565-1574.

Bielsky IF, Hu SB, Szegda KL, Westphal H, Young LJ (2004) Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. Neuropsychopharmacology 29:483-493.

Bielsky IF, Hu SB, Ren X, Terwilliger EF, Young LJ (2005) The V1a vasopressin receptor is necessary and sufficient for normal social recognition: a gene replacement study. Neuron 47:503-513.

Birnbaumer M (2000) Vasopressin receptors. Trends Endocrinol Metab 11:406-410.

Blanchard D, Blanchard R (1972) Innate and conditioned reactions to threat in rats with amygdaloid lesions. J Comp Physiol Psychol 81:281-290.

Blumberg MS, Johnson ED, Middlemis-Brown JE (2005) Inhibition of ultrasonic vocalizations by beta-adrenoceptor agonists. Dev Psychobiol 47:66-76.

Bosch OJ, Meddle SL, Beiderbeck DI, Douglas AJ, Neumann ID (2005) Brain oxytocin correlates with maternal aggression: link to anxiety. J Neurosci 25:6807-6815.

Bosch OJ, Kromer SA, Neumann ID (2006) Prenatal stress: opposite effects on anxiety and hypothalamic expression of vasopressin and corticotropin-releasing hormone in rats selectively bred for high and low anxiety. Eur J Neurosci 23:541-551.

Bramham C, Messaoudi E (2005) BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. Prog Neurobiol 76:99-125.

Branchi I, Santucci D, Puopolo M, Alleva E (2004) Neonatal behaviors associated with ultrasonic vocalizations in mice (mus musculus): A slow-motion analysis. Dev Psychobiol 44:37-44.

Bremner JD, Randall P, Scott TM, Bronen RA, Seibyl JP, Southwick SM, Delaney RC, McCarthy G, Charney DS, Innis RB (1995) MRI-based measurement of hippocampal volume in patients with combat-related posttraumatic stress disorder. Am J Psychiatry 152:973-981.

Briley M, Nutt D (2000) Anxiolytics. Basel, Boston, Berlin: Birkhäuser Verlag.

Brinks V, van de Mark M, de Kloet ER, Oitzl M (2007) Differential MR/GR activation in mice results in emotional states beneficial or impairing for cognition. Neural Plasticity 2007:1-11.

Britton DR, Koob GF, Rivier J, Vale W (1982) Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty. Life Sci 31:363-367.

Bruchey A, Shumake J, Gonzalez-Lima F (2006) Network model of fear extinction and renewal functional pathways. Neuroscience (145(2):423-37

Brudno M, Poliakov A, Salamov A, Cooper GM, Sidow A, Rubin EM, Solovyev V, Batzoglou S, Dubchak I (2004) Automated whole-genome multiple alignment of rat, mouse, and human. Genome Res 14:685-692.

Bruins J, Kovacs GL, Abbes AP, Burbach JP, van den Akker EL, Engel H, Franken AA, de Wied D (2006) Minor disturbances in central nervous system function in familial neurohypophysial diabetes insipidus. Psychoneuroendocrinology 31:80-91.

Brunelli SA, Vinocur DD, Soo-Hoo D, Hofer MA (1997) Five generations of selective breeding for ultrasonic vocalization (USV) responses in N:NIH strain rats. Dev Psychobiol 31:255-265.

Brunelli SA (2005) Selective breeding for an infant phenotype: rat pup ultrasonic vocalization (USV). Behav Genet 35:53-65.

Buijs RM, Hermes MLHJ, Kalsbeek A, van der Woude T, van Heerikhuize JJ (1991) Vasopressin distribution, origin and functions in the central nervous system. In: Vasopressin (Jard S, Jaminson R, eds), pp 149-158: Colloque INSERM/John Libbey Eurotext Ltd.

Bunck M, Czibere L, Horvath C, Graf C, Murgatroyd C, Muigg P, Singewald N, Rafael J, Holsboer F, Spengler D, Landgraf R Allele-specific expression of vasopressin contributes to anxiety-related behavior. Mol Psychiatry submitted.

Burbach JP, Luckman SM, Murphy D, Gainer H (2001) Gene regulation in the magnocellular hypothalamo-neurohypophysial system. Physiol Rev 81:1197-1267.

Burgdorf J, Panksepp J, Brudzynski SM, Kroes R, Moskal JR (2005) Breeding for 50-kHz positive affective vocalization in rats. Behav Genet 35:67-72.

Butler PD, Weiss JM, Stout JC, Nemeroff CB (1990) Corticotropin-releasing factor produces fear-enhancing and behavioral activating effects following infusion into the locus coeruleus. J Neurosci 10:176-183.

Bystritsky A (2006) Treatment-resistance anxiety disorders. Molecular Psychiatry 11(9):805-814.

Cai WH, Blundell J, Han J, Greene RW, Powell CM (2006) Postreactivation glucocorticoids impair recall of established fear memory. J Neurosci 26:9560-9566.

Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002) Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. Neurosci Biobehav Rev 26:321-352.

Cardno A, Gottesman I (2000) Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics. Am J Med Genet 97:12-17.

Carli M, Robbins T, Evenden J, Everitt B (1983) Effects of lesions to ascending noradrenergic neurons on performance on a 5-choice serial reaction time task in rats: implications for theories of dorsal noradrenergic bundle function based on selective attention and arousal. Behav Brain Res 9:361-380.

Carrasco GA, Van de Kar LD (2003) Neuroendocrine pharmacology of stress. Eur J Pharmacol 463:235-272.

Carter CS (2003) Developmental consequences of oxytocin. Physiol Behav 79:383-397.

Carter CS, Pournajafi-Nazarloo H, Kramer KM, Ziegler T, White-Traut R, Bello D, Schwertz D (2007) Oxytocin: behavioral associations and potential as a salivary biomarke. Ann N Y Acad Sci 1098:312-322.

Cassano P, Fava M (2004) Tolerability issues during long-term treatment with anitdepressants. Ann Clin Psychiatry 59:12227-11235.

Chakrabarty A, Roberts MR (2007) Ets-2 and C/EBP-beta are important mediators of ovine trophoblast Kunitz domain protein-1 gene expression in trophoblast. BMC Mol Biol 8:14.

Chapman E (2002) Synaptotagmin: a Ca(2+) sensor that triggers exocytosis. Nat Rev Mol Cell Biol 3:498-508.

Charmandari E, Tsigos C, Chrousos G (2005) Endocrinology of the stress response. Annu Rev Physiol 67:259-284.

Charney D, Bremner JD, Redmond D (1995) Noradrenergic neural substrates for anxiety and fear: clinical associations based on preclinical research. New York: Raven Pres.

Charney D, Deutch A (1996) A Functional Neuroanatomy of Anxiety and Fear: Implication for the Pathology and Treatment of Anxiety Disorders. Critical Reviews in Neurobiology 10:419-446.

Charney DS, Grillon C, Bremner JD (1998) The neurobiological basis of anxiety and fear: circuits, mechanisms, and neurochemical interactions (part 1). The Neuroscientist 4:35-44.

Charney D (2004) Psychological mechanisms of resilience and vulberability. Am J Psychiatry 161:195-216.

Chen F, Wollmer MA, Hoerndli F, Munch G, Kuhla B, Rogaev EI, Tsolaki M, Papassotiropoulos A, Gotz J (2004) Role for glyoxalase I in Alzheimer's disease. Proc Natl Acad Sci U S A 101:7687-7692.

Chen S, Shemyakin A, Wiedenmayer CP (2006) The role of the amygdala and olfaction in unconditioned fear in developing rats. Journal of Neuroscience 26:233-240.

Chen Y, Brunson KL, Muller MB, Cariaga W, Baram TZ (2000) Immunocytochemical distribution of corticotropin-releasing hormone receptor type-1 (CRF(1))-like immunoreactivity in the mouse brain: light microscopy analysis using an antibody directed against the C-terminus. J Comp Neurol 420:305-323.

Christensen J, Rittig S (2006) Familial neurohypophyseal diabetes insipidus - an update. Semin Nephrol 26:209-223.

Cole BJ, Robbins T (1992) Forebrain norepinephrine: role in controlled information processing in the rat. Neuropsychopharmacology 7:129-142.

Cole RL, Sawchenko PE (2002) Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. J Neurosci 22:959-969.

Conner J, Lauterborn J, Yan Q, Gall C, Varon S (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J Neurosci 17:2295-2313.

Conti LH, Costello DG, Martin LA, White MF, Abreu ME (1994) Mouse strain differences in the behavioral effects of corticotropin-releasing factor (CRF) and the CRF antagonist alpha-helical CRF9-41. Pharmacol Biochem Behav 48:497-503.

Cook CJ (2004) Stress induces CRF release in the paraventricular nucleus, and both CRF and GABA release in the amygdala. Physiol Behav 82:751-762.

Cooke B, Hegstrom C, Villeneuve L, Breedlove SM (1998) Sexual differentiation of the vertebrate brain: principles and mechanisms. Front Neuroendocrinol 19:323-362.

Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH, Murray SE, Hill JK, Pantely GA, Hohimer AR, Hatton DC, Phillips TJ, Finn DA, Low MJ, Rittenberg MB, Stenzel P, Stenzel-Poore MP (2000) Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. Nat Genet 24:403-409.

Cratty MS, Ward HE, Johnson EA, Azzaro AJ, Birkle DL (1995) Prenatal stress increases corticotropin-releasing factor (CRF) content and release in rat amygdala minces. Brain Res 675:297-302.

Croiset G, Nijsen MJ, Kamphuis PJ (2000) Role of corticotropin-releasing factor, vasopressin and the autonomic nervous system in learning and memory. Eur J Pharmacol 405:225-234.

Cryan JF, Markou A, Lucki I (2002) Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharmacol Sci 23:238-245.

Cryan JF, Mombereau C (2004) In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. Mol Psychiatry 9:326-357.

Cummings J, Hegarty A (1994) Neurology, psychiatry, and neuropsychiatry. Neurology:209-213.

Cummings J, Mega M (2003) Neuropsychiatry and Behavioral Neuroscience. New York: Oxford University Press.

Dagerlind A, Friberg K, Bean A, Hökfelt T (1992) Sensitive mRNA detection using unfixed tissue: combined radioactive and non-radioactive in situ hybridization. Histochemistry 98:39-49.

D'Amato FR, Scalera E, Sarli C, Moles A (2005) Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? Behav Genet 35:103-112.

Dantzer R, Bluthe RM, Koob GF, Le Moal M (1987) Modulation of social memory in male rats by neurohypophyseal peptides. Psychopharmacology (Berl) 91:363-368.

Dantzer R, Koob GF, Bluthe RM, Le Moal M (1988) Septal vasopressin modulates social memory in male rats. Brain Res 457:143-147.

Dantzer R, Tazi A, Bluthe RM (1990) Cerebral lateralization of olfactory-mediated affective processes in rats. Behav Brain Res 40:53-60.

Dautzenberg F, Hauger R (2002) The CRF peptide family and their receptors: yet more partners discovered. Trends Pharmacol Sci 23:71-77.

Davis M, Ressler K, Rothbaum BO, Richardson R (2006) Effects of D-cycloserine on extinction: translation from preclinical to clinical work. Biol Psychiatry 60:369-375.

de Kloet ER, Reul J, Sutanto W (1990) Corticosteroids and the brain. Journal of Steroid Biochemistry and Molecular Biology 37:387-394.

de Kloet ER, Joels M, Oitzl M, Sutanto W (1991) Implication of brain corticosteroid receptor diversity for the adaptation syndrome concept. Methods Achiev Exp Pathol 14:104-132.

de Kloet ER, Vreugdenhil E, Oitzl MS, Joels M (1998) Brain corticosteroid receptor balance in health and disease. Endocr Rev 19:269-301.

de Kloet ER, Joels M, Holsboer F (2005) Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6:463-475.

De Vries GJ, Buijs RM (1983) The origin of the vasopressinergic and oxytocinergic innervation of the rat brain with special reference to the lateral septum. Brain Res 273:307-317.

Deak T, Nguyen KT, Ehrlich AL, Watkins LR, Spencer RL, Maier SF, Licinio J, Wong ML, Chrousos GP, Webster E, Gold PW (1999) The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. Endocrinology 140:79-86.

Debiec J (2005) Peptides of love and fear: vasopressin and oxytocin modulate the integration of information in the amygdala. Bioessays 27:869-873.

Decker MW (1995) Animal models of cognitive function. Crit Rev Neurobiol 9:321-343.

Deeb S (2004) Molecular genetics of color-vision deficiencies. Vis Neurosci 21:191-196.

Demyttenaere K, Bruffaerts R, Posada-Villa J, Gasquet I, Kovess V, Lepine JP, Angermeyer MC, Bernert S, de Girolamo G, Morosini P, Polidori G, Kikkawa T, Kawakami N, Ono Y, Takeshima T, Uda H, Karam EG, Fayyad JA, Karam AN, Mneimneh ZN, Medina-Mora ME, Borges G, Lara C, de Graaf R, Ormel J, Gureje O, Shen Y, Huang Y, Zhang M, Alonso J, Haro JM, Vilagut G, Bromet EJ, Gluzman S, Webb C, Kessler RC, Merikangas KR, Anthony JC, Von Korff MR, Wang PS, Brugha TS, Aguilar-Gaxiola S, Lee S, Heeringa S, Pennell BE, Zaslavsky AM, Ustun TB, Chatterji S (2004) Prevalence, severity, and unmet need for treatment of mental disorders in the World Health Organization World Mental Health Surveys. Jama 291:2581-2590.

Dichter GS, Brunelli SA, Hofer MA (1996) Elevated plus-maze behavior in adult offspring of selectively bred rats. Physiol Behav 60:299-304.

Diorio D, Viau V, Meaney MJ (1993) The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J Neurosci 13:3839-3847.

Ditzen C, Jastorff AM, Kessler MS, Bunck M, Teplytska L, Erhardt A, Kromer SA, Varadarajulu J, Targosz BS, Sayan-Ayata EF, Holsboer F, Landgraf R, Turck CW (2006) Protein biomarkers in a mouse model of extremes in trait anxiety. Mol Cell Proteomics.

Dluzen DE, Kreutzberg JD (1993) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) disrupts social memory/recognition processes in the male mouse. Brain Res 609:98-102.

Dluzen DE, Muraoka S, Landgraf R (1998a) Olfactory bulb norepinephrine depletion abolishes vasopressin and oxytocin preservation of social recognition responses in rats. Neurosci Lett 254:161-164.

Dluzen DE, Muraoka S, Engelmann M, Landgraf R (1998b) The effects of infusion of arginine vasopressin, oxytocin, or their antagonists into the olfactory bulb upon social recognition responses in male rats. Peptides 19:999-1005.

Dluzen DE, Muraoka S, Engelmann M, Ebner K, Landgraf R (2000) Oxytocin induces preservation of social recognition in male rats by activating alpha-adrenoceptors of the olfactory bulb. Eur J Neurosci 12:760-766.

Do-Rego JC, Suaudeau C, Chapouthier G, Costentin J (2002) Mouse lines differing in sensitivity to beta-CCM differ in tasks used for testing antidepressants. Pharmacol Biochem Behav 72:411-416.

Drouin J, Sun Y, Chamberland M, Gauthier Y, De Lean A, Nemer M, Schmidt T (1993) Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. Embo J 12:145-156.

Ducottet C, Belzung C (2004) Behaviour in the elevated plus-maze predicts coping after subchronic mild stress in mice. Physiol Behav 81:417-426.

Duman RS, Monteggia L (2006) A neurotrophic model for stress-related mood disorders. Biol Psychiatry 59:1116-1127.

Dunn AJ, Berridge CW (1990) Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Res Brain Res Rev 15:71-100.

Eckart K, Jahn O, Radulovic J, Radulovic M, Blank T, Stiedl O, Brauns O, Tezval H, Zeyda T, Spiess J (2002) Pharmacology and biology of corticotropin-releasing factor (CRF) receptors. Receptors Channels 8:163-177.

Egashira N, Tanoue A, Matsuda T, Koushi E, Harada S, Takano Y, Tsujimoto G, Mishima K, Iwasaki K, Fujiwara M (2007) Impaired social interaction and reduced anxiety-related behavior in vasopressin V1a receptor knockout mice. Behav Brain Res 178:123-127.

El Yacoubi M, Vaugeois JM (2007) Genetic rodent models of depression. Current Opinion in Pharmacology 7:3-7.

Engelmann M, Landgraf R (1994) Microdialysis administration of vasopressin into the septum improves social recognition in Brattleboro rats. Physiol Behav 55:145-149.

Engelmann M, Wotjak CT, Landgraf R (1995) Social discrimination procedure: an alternative method to investigate juvenile recognition abilities in rats. Physiol Behav 58:315-321.

Engelmann M, Landgraf R, Wotjak CT (2004) The hypothalamic-neurohypophysial system regulates the hypothalamic-pituitary-adrenal axis under stress: an old concept revisited. Front Neuroendocrinol 25:132-149.

Engelmann M, Ludwig M (2004) The activity of the hypothalamo-neurohypophysial system in response to acute stressor exposure: neuroendocrine and electrophysiological observations. Stress 7:91-96.

Etkin A, Klemenhagen KC, Dudman JT, Rogan MT, Hen R, Kandel ER, Hirsch J (2004) Individual differences in trait anxiety predict the response of the basolateral amygdala to unconsciously processed fearful faces. Neuron 44:1043-1055.

Fava M, Kendler KS (2000) Major depressive disorder. Neuron 28:335-341.

Ferguson G, Thomas D, Elferink L, Herschman HR (1999) Synthesis degradation, and subcellular localization of synaptotagmin IV, a neuronal immediate early gene product. J Neurochem 72:1821-1831.

Ferguson G, Chen X, Korenberg J, Herschman HR (2000a) The human synaptotagmin IV gene defines an evolutionary break point between syntenic mouse and human chromosome regions but retain ligand inducibility and tissue specifity. J Biol Chem 275:36920-36926.

Ferguson GD, Herschman HR, Storm DR (2004) Reduced anxiety and depression-like behavior in synaptotagmin IV (-/-) mice. Neuropharmacology 47:604-611.

Ferguson JN, Young LJ, Hearn EF, Matzuk MM, Insel TR, Winslow JT (2000b) Social amnesia in mice lacking the oxytocin gene. Nat Genet 25:284-288.

Ferguson JN, Young LJ, Insel TR (2002) The neuroendocrine basis of social recognition. Front Neuroendocrinol 23:200-224.

Fields RL, House SB, Gainer H (2003) Regulatory domains in the intergenic region of the oxytocin and vasopressin genes that control their hypothalamus-specific expression in vitro. J Neurosci 23:7801-7809.

File SE (2001) Factors controlling measures of anxiety and responses to novelty in the mouse. Behav Brain Res 125:151-157.

Finn CT, Smoller JW (2001) The genetics of panic disorders. Curr Psychiatry Rep 3:13-137.

Finn DA, Rutledge-Gorman MT, Crabbe JC (2003) Genetic animal models of anxiety. Neurogenetics 4:109-135.

Förstl H, Hautzinger M, Roth G (2006) Neurobiologie psychischer Störungen. Heidelberg: Springer-Verlag.

Forth W, Henschler D, Rummel W, Starke K (1998) Allgemeine und spezielle Pharmakologie, 7th Edition. Heidelberg, Berlin, Oxford: Spektrum Akademischer Verlag.

Frank E, Landgraf R (2008) The vasopressin system - from antidiuresis to psychopathology. European Journal of Pharmacology 583(2-3):226-42.

Friske JE, Gammie SC (2005) Environmental enrichment alters plus maze, but not maternal defense performance in mice. Physiol Behav 85:187-194.

Fyer A (1998) Current approches to etiology and pathophysiology of specific phobiy. Biol Psychiatry 44:1295-1304.

Gale G, Anagnostaras SG, Godsil B, Mitchell S, Nozawa T, Sage J, Wiltgen B, Fanselow MS (2004) Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. Journal of Neuroscience 24:3810-3815.

Gass P, Reichardt HM, Strekalova T, Henn F, Troche F (2001) Mice with targeted mutations of glucocorticoid and mineralocorticoid receptors: modells for depression and anxiety? Physiol Behav 73:811-825.

Gold PW, Goodwin FK, Reus VI (1978) Vasopressin in affective illness. Lancet 1:1233-1236.

Gordon JA, Hen R (2004) Genetic approaches to the study of anxiety. Annu Rev Neurosci 27:193-222.

Gorman J, Kent J, Sullivan G, Coplan J (2000) Neuroanatomical hypothesis of panic disorder, revised. Am J Psychiatry 157:493-505.

Gottlicher M, Heck S, Herrlich P (1998) Transcriptional cross-talk, the second mode of steroid hormone receptor action. J Mol Med 76:480-489.

Grady CL, Keightley ML (2002) Studies of altered social cognition in neuropsychiatric disorders using functional neuroimaging. Can J Psychiatry 47:327-336.

Gray TS (1993) Amygdaloid CRF pathways. Role in autonomic, neuroendocrine, and behavioral responses to stress. Ann N Y Acad Sci 697:53-60.

Gregory S, Sekhon M, Schein J, Zhao S, Bentley D (2002) A physical map of the mouse genome. Nature 418:743-750.

Griebel G, Perrault G, Sanger DJ (1998) Characterization of the behavioral profile of the non-peptide CRF receptor antagonist CP-154,526 in anxiety models in rodents. Comparison with diazepam and buspirone. Psychopharmacology (Berl) 138:55-66.

Griebel G (1999) Is there a future for neuropeptide receptor ligands in the treatment of anxiety disorders? Pharmacol Ther 82:1-61.

Griebel G, Simiand J, Serradeil-Le Gal C, Wagnon J, Pascal M, Scatton B, Maffrand JP, Soubrie P (2002) Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. Proc Natl Acad Sci U S A 99:6370-6375.

Griebel G, Gal CS (2005) Non-peptide vasopressin V1b receptor antagonists. In: Handbook of Stress and the Brain (Steckler T, Kalin NH, Reul JMHM, eds), pp 409-421: Elsevier.

Guan X, Blank J, Dluzen D (1993) Depletion of olfactory bulb norepinephrine by 6-OHDA disrupts chemical cue but not social recognition responses in male rats. Brain Res 622:51-57.

Guan X, Dluzen D (1994) Age-related changes of social memory/recognition in male Fischer 344 rats. Behav Brain Res 61:87-90.

Guastella A, Dadds MR, Lovibond P, Mitchell P, Richardson R (2007) A randomized controlled trial of the effect of D-cycloserine on exposure therapy for spider fear. Journal of Psychiatric Research 41:466-471.

Habib K, Weld K, Rice K, Pushkas J, Champoux M, Listwak S, Webster E, Atkinson A, Schulkin J, Contoreggi C, Chrousos G, McCann SM, Suomi S, Higley J, Gold P (2000) Oral administration of corticosteron-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. Proc Natl Acad Sci U S A 97:6079-6084.

Hallbeck M, Hermanson O, Blomqvist A (1999) Distribution of preprovasopressin mRNA in the rat central nervous system. J Comp Neurol 411:181-200.

Hascoet M, Bourin M, Dhonnchadha BA (2001) The mouse light-dark paradigm: a review. Prog Neuropsychopharmacol Biol Psychiatry 25:141-166.

Heim C, Nemeroff CB (2001) The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol Psychiatry 49:1023-1039.

Heinrichs SC, Menzaghi F, Pich EM, Baldwin HA, Rassnick S, Britton KT, Koob GF (1994) Anti-stress action of a corticotropin-releasing factor antagonist on behavioral reactivity to stressors of varying type and intensity. Neuropsychopharmacology 11:179-186.

Heinrichs SC, Lapsansky J, Lovenberg TW, De Souza EB, Chalmers DT (1997) Corticotropin-releasing factor CRF1, but not CRF2, receptors mediate anxiogenic-like behavior. Regul Pept 71:15-21.

Henderson ND, Turri MG, DeFries JC, Flint J (2004) QTL analysis of multiple behavioral measures of anxiety in mice. Behav Genet 34:267-293.

Henniger MS, Ohl F, Holter SM, Weissenbacher P, Toschi N, Lorscher P, Wigger A, Spanagel R, Landgraf R (2000) Unconditioned anxiety and social behaviour in two rat lines selectively bred for high and low anxiety-related behaviour. Behav Brain Res 111:153-163.

Herman JP, Cullinan WE (1997) Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci 20:78-84.

Herman JP, Cullinan WE, Ziegler DR, Tasker JG (2002) Role of the paraventricular nucleus microenvironment in stress integration. Eur J Neurosci 16:381-385.

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE (2003) Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24:151-180.

Hernando F, Schoots O, Lolait SJ, Burbach JP (2001) Immunohistochemical localization of the vasopressin V1b receptor in the rat brain and pituitary gland: anatomical support for its involvement in the central effects of vasopressin. Endocrinology 142:1659-1668.

Hetzenauer A, Sinnegger-Brauns MJ, Striessnig J, Singewald N (2006) Brain activation pattern induced by stimulation of L-type Ca2+-channels: contribution of Ca(V)1.3 and Ca(V)1.2 isoforms. Neuroscience 139:1005-1015.

Holmes A, Heilig M, Rupniak NM, Steckler T, Griebel G (2003) Neuropeptide systems as novel therapeutic targets for depression and anxiety disorders. Trends Pharmacol Sci 24:580-588.

Holsboer F (1989) Psychiatric implications of altered limbic-hypothalamic-pituitary-adrenocortical activity. Eur Arch Psychiatry Neurol Sci 238:302-322.

Holsboer F, Barden N (1996) Antidepressants and hypothalamic-pituitary-adrenocortical regulation. Endocr Rev 17:187-205.

Holsboer F (1999) The rationale for corticotropin-releasing hormone receptor (CRH-R) antagonists to treat depression and anxiety. J Psychiatr Res 33:181-214.

Horch H, Kruttgen A, Portbury S, Katz L (1999) Destabilization of cortical dendrites and spines by BDNF. Neuron 23:353-364.

Hovatta I, Tennant RS, Helton R, Marr RA, Singer O, Redwine JM, Ellison JA, Schadt EE, Verma IM, Lockhart DJ, Barlow C (2005) Glyoxalase 1 and glutathione reductase 1 regulate anxiety in mice. Nature 438:662-666.

Insel TR, Young LJ (2001) The neurobiology of attachment. Nat Rev Neurosci 2:129-136.

Ising M, Kunzel HE, Binder EB, Nickel T, Modell S, Holsboer F (2005) The combined dexamethasone/CRH test as a potential surrogate marker in depression. Prog Neuropsychopharmacol Biol Psychiatry 29:1085-1093.

Ising M, Holsboer F (2006) Genetics of stress response and stress-related disorders. Dialogues Clin Neurosci 8:433-444.

Ito M, Jameson JL (1997) Molecular basis of autosomal dominant neurohypophyseal diabetes insipidus. Cellular toxicity caused by accumulation of mutant vasopressin precursors within the endoplasmic reticulum. J Clin Invest 99:1897-1905.

Ito M, Yu R, Jameson JL (1999) Mutant vasopressin precursors that cause autosomal dominant neurohypophyseal diabetes insipidus retain dimerization and impair the secretion of wild-type proteins. J Biol Chem 274:9029.9037.

Ivell R, Richter D (1984) Structure and comparison of the oxytocin and vasopressin genes from rat. Proc Natl Acad Sci U S A 81:2006-2010.

Ji C, Chen Y, Centrella M, McCarthy TL (1999) Activation of the insulin-like growth factor-binding protein-5 promoter in osteoblasts by cooperative E box, CCAAT enhancer-binding protein, and nuclear factor-1 deoxyribonucleic acid-binding sequences. Endocrinology 140:4564-4572.

Jing X, Ratty AK, Murphy D (1998) Ontogeny of the vasopressin and oxytocin RNAs in the mouse hypothalamus. Neurosci Res 30:343-349.

Kafitz K, Rose C, Thoenen H, Konnerth A (1999) Neurotrophin-evoked rapid excitation through TrkB receptors. Nature (London) 401:918-921.

Kalin NH, Shelton SE, Davidson RJ, Kelley AE (2001) The primate amygdala mediates acute fear but not the behavioral and physiological components of anxious temperament. J Neurosci 21:2067-2074.

Kalsbeek A, Buijs RM, Engelmann M, Wotjak CT, Landgraf R (1995) In vivo measurement of a diurnal variation in vasopressin release in the rat suprachiasmatic nucleus. Brain Res 682:75-82.

Kalueff AV (2007) Neurobiology of memory and anxiety: from genes to behavior. Neural Plasticity 2007:1-12.

Kalueff AV, Wheaton M, Murphy D (2007) What´s wrong with my mouse model? Advantages and strategies in animal modeling of anxiety and depression. Behavioral Brain Research 179:1-18.

Karege F, Vaudan G, Schwald M, Perroud N, La Harpe R (2005) Neurotrophin levels in postmortem brains of suicide victims and the effects of antemortem diagnosis and psychotropic drugs. Mol Brain Res 136:29-37.

Kato T, Iwamoto K, Kakiuchi C, Kuratomi G, Okazaki Y (2005) Genetic and epigenetic difference causing discordance between monozygotic twins as a clue to molecular basis of mental disorders. Mol Psychiatry 10:622-630.

Keck ME, Welt T, Wigger A, Renner U, Engelmann M, Holsboer F, Landgraf R (2001) The anxiolytic effect of the CRH(1) receptor antagonist R121919 depends on innate emotionality in rats. Eur J Neurosci 13:373-380.

Keck ME, Wigger A, Welt T, Muller MB, Gesing A, Reul JM, Holsboer F, Landgraf R, Neumann ID (2002) Vasopressin mediates the response of the combined dexamethasone/CRH test in hyper-anxious rats: implications for pathogenesis of affective disorders. Neuropsychopharmacology 26:94-105.

Keck ME, Welt T, Muller MB, Landgraf R, Holsboer F (2003a) The high-affinity non-peptide CRH1 receptor antagonist R121919 attenuates stress-induced alterations in plasma oxytocin, prolactin, and testosterone secretion in rats. Pharmacopsychiatry 36:27-31.

Keck ME, Welt T, Muller MB, Uhr M, Ohl F, Wigger A, Toschi N, Holsboer F, Landgraf R (2003b) Reduction of hypothalamic vasopressinergic hyperdrive contributes to clinically relevant behavioral and neuroendocrine effects of chronic paroxetine treatment in a psychopathological rat model. Neuropsychopharmacology 28:235-243.

Kendler KS, Neale MC, Kessler R, Heath AC, Eaves LJ (1993) Panic disorders in women: a population-based twin study. Psychol Med 23:397-406.

Kendler KS (2005) Toward a philosophical structure for psychiatry. Am J Psychiatry 162:433-440.

Keßler M, Murgatroyd C, Bunck M, Czibere L, Frank E, Jacob W, Horvath C, Muigg P, Holsboer F, Singewald N, Spengler D, Landgraf R (2007) Diabetes insipidus and, partially, low anxiety-related behavior are linked to a SNP-associated vasopressin deficit in LAB mice. European Journal of Neuroscience 26(10):2857-64.

Kessler M, Chiu W, Demler O, Merikangas K, Walters E (2005a) Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry 51(1):617-627.

Kessler M, Demler O, Frank R, Olfson M, Pincus H, Walters E, al. e (2005b) Prevalence and treatment of mental disorders, 1990 to 2003. N Engl J Med:2515-2523.

Kessler R, McGonagle KA, Zhao S, Nelson CB, Hughes M, S E, al. e (1994) Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. Arch Gen Psychiatry:8-19.

Kessler RC, Keller MB, Wittchen HU (2001) The epidemiology of generalized anxiety disorder. Psychiatr Clin North Am 24:19-39.

Kim J, Schrier R (1998) Vasopressin processing defects in the Brattleboro rat: implications for hereditary central diabetes insipidus in humans? Proc Assoc Am Physicians 110:380-386.

Kim JJ, Jung MW (2006) Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. Neurosci Biobehav Rev 30:188-202.

Klinke R, Silbernagel S (2000) Lehrbuch der Physiologie. Stuttgart: Thieme.

Koob G (1999) Corticotropin-releasing factor, norepinehrine, and stress. Biol Psychiatry 46:1167-1180.

Koob GF, Dantzer R, Bluthe RM, Lebrun C, Bloom FE, Le Moal M (1986) Central injections of arginine vasopressin prolong extinction of active avoidance. Peptides 7:213-218.

Kovacs KJ (1998) c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem Int 33:287-297.

Kraus RJ, Shadley L, Mertz JE (2001) Nuclear factor 1 family members mediate repression of the BK virus late promoter. Virology 287:89-104.

Kromer SA, Kessler MS, Milfay D, Birg IN, Bunck M, Czibere L, Panhuysen M, Putz B, Deussing JM, Holsboer F, Landgraf R, Turck CW (2005) Identification of glyoxalase-I as a protein marker in a mouse model of extremes in trait anxiety. J Neurosci 25:4375-4384.

Kugaya A, Fujita M, Innis RB (2000) Applications of SPECT imaging of dopaminergic neurotransmission in neuropsychiatric disorders. Ann Nucl Med 14:1-9.

LaBaer J (2005) So, you want to look for biomarkers (introduction to the special biomarkers issue). J Proteome Res 4:1053-1059.

LaBar KS, Gatenby JC, Gore JC, LeDoux JE, Phelps EA (1998) Human amygdala activation during conditioned fear acquisition and extinction: a mixed-trial fMRI study. Neuron 20:937-945.

Land H, Schutz G, Schmale H, Richter D (1982) Nucleotide sequence of cloned cDNA encoding bovine arginine vasopressin-neurophysin II precursor. Nature 295(5847): 299-303.

Landgraf R, Gerstberger R, Montkowski A, Probst JC, Wotjak CT, Holsboer F, Engelmann M (1995) V1 vasopressin receptor antisense oligodeoxynucleotide into septum reduces vasopressin binding, social discrimination abilities, and anxiety-related behavior in rats. J Neurosci 15:4250-4258.

Landgraf R (2001) Neuropeptides and anxiety-related behavior. Endocr J 48:517-533.

Landgraf R, Wigger A (2002) High vs low anxiety-related behavior rats: an animal model of extremes in trait anxiety. Behav Genet 32:301-314.

Landgraf R (2003) HAB/LAB rats: an animal model of extremes in trait anxiety and depression. Clinical neuroscience research 3:239-244.

Landgraf R, Wigger A (2003) Born to be anxious: neuroendocrine and genetic correlates of trait anxiety in HAB rats. Stress 6:111-119.

Landgraf R, Frank E, Aldag JM, Neumann ID, Sharer CA, Ren X, Terwilliger EF, Niwa M, Wigger A, Young LJ (2003) Viral vector-mediated gene transfer of the vole V1a vasopressin receptor in the rat septum: improved social discrimination and active social behaviour. Eur J Neurosci 18:403-411.

Landgraf R, Neumann ID (2004) Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. Front Neuroendocrinol 25:150-176.

Landgraf R (2006) The involvement of the vasopressin system in stress-related disorders. CNS Neurol Disord Drug Targets 5:167-179.

Landgraf R, Kessler MS, Bunck M, Murgatroyd C, Spengler D, Zimbelmann M, Nussbaumer M, Czibere L, Turck CW, Singewald N, Rujescu D, Frank E (2007) Candidate genes of anxiety-related behavior in HAB/LAB rats and mice: focus on vasopressin and glyoxalase-I. Neurosci Biobehav Rev 31:89-102.

Lanuza E, Nader K, LeDoux J (2004) Unconditioned stimulus pathways to the amydgala: effects of posterior thalamic and cortical lesions on fear conditioning. Neuroscience 125:305-315.

Lavicky J, Dunn AJ (1993) Corticotropin-releasing factor stimulates catecholamine release in hypothalamus and prefrontal cortex in freely moving rats as assessed by microdialysis. J Neurochem 60:602-612.

Le Moal M, Dantzer R, Michaud B, Koop G (1987) Centrally injected arginine vasopressin (AVP) facilitates social memory in rats. Neurosc Lett 77:353-359.

Ledgerwood L, Richardson R, Cranney J (2005) D-cycloserine facilitates extinction of learned fear: effects on reacquisition and generalized extinction. Biol Psychiatry 57:841-847.

LeDoux JE (1995) Emotion: clues from the brain. Annu Rev Psychol 46:209-235.

LeDoux JE (2000) Emotion circuits in the brain. Annu Rev Neurosci 23:155-184.

Lee JL, Milton AL, Everitt BJ (2006) Reconsolidation and extinction of conditioned fear: inhibition and potentiation. J Neurosci 26:10051-10056.

Leon A, Portera L, Weissman M (1995) The social costs of anxiety disorders. Br J Psychiatry Suppl (27):19-22.

Levine J, Cole DP, Chengappa KN, Gershon S (2001) Anxiety disorders and major depression, together or apart. Depress Anxiety 14:94-104.

Lieb R, Isensee B, Hofler M, Pfister H, Wittchen HU (2002) Parental major depression and the risk of depression and other mental disorders in offspring: a prospective-longitudinal community study. Arch Gen Psychiatry 59:365-374.

Lieb R, Becker E, Altamura C (2005) The epidemiology of generalized anxiety disorder in Europe. Eur Neuropsychopharmacol 15:445-452.

Liebsch G, Landgraf R, Gerstberger R, Probst JC, Wotjak CT, Engelmann M, Holsboer F, Montkowski A (1995) Chronic infusion of a CRH1 receptor antisense oligodeoxynucleotide into the central nucleus of the amygdala reduced anxiety-related behavior in socially defeated rats. Regul Pept 59:229-239.

Liebsch G, Montkowski A, Holsboer F, Landgraf R (1997) Behavioral profiles of two Wistar rats selectively bred for high and low anxiety-related behavior. Behavioral Brain Research 94:301-310.

Liebsch G, Linthorst AC, Neumann ID, Reul JM, Holsboer F, Landgraf R (1998) Behavioral, physiological, and neuroendocrine stress responses and differential sensitivity to diazepam in two Wistar rat lines selectively bred for high- and low-anxiety-related behavior. Neuropsychopharmacology 19:381-396.

Liebsch G, Landgraf R, Engelmann M, Lorscher P, Holsboer F (1999) Differential behavioural effects of chronic infusion of CRH 1 and CRH 2 receptor antisense oligonucleotides into the rat brain. J Psychiatr Res 33:153-163.

Lightman SL, Insel TR, Ingram CD (2002) New genomic avenues in behavioural neuroendocrinology. Eur J Neurosci 16:369-372.

Lister RG (1987) The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92:180-185.

Lister RG (1990) Ethologically-based animal models of anxiety disorders. Pharmacol Ther 46:321-340.

Littleton J, Serano T, Rubin G, Ganetzky B, Chapman E (1999) Synaptic function modulated by changes in the ratio of synaptotagmin I and IV. Nature 400:757-760.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Lodish H, Berk A, Zipursky S, Matsudaira P, Baltimore D, Darnell J (2001) Molecular Cell Biology, 4th Edition. New York: W.H. Freeman and Company.

Lu XH, Li LJ, Li CQ, Luo XG (2000) [Potential anxiolytic role of c-fos antisense oligonucleotide in social-defeated rats]. Hunan Yi Ke Da Xue Xue Bao 25:535-538.

Ludwig M, Bull PM, Tobin VA, Sabatier N, Landgraf R, Dayanithi G, Leng G (2005) Regulation of activity-dependent dendritic vasopressin release from rat supraoptic neurones. J Physiol 564:515-522.

Maciag C, Dent G, Gilligan P, He L, Dowling K, Ko T, Levine S, Smith M (2002) Effects of the non-peptide CRF antagonist (DMP696)on the behavioral and endocrine sequelae of maternal separation. Neuropsychopharmacology 26:574-582.

Makino S, Hashimoto K, Gold PW (2002) Multiple feedback mechanisms activating corticotropin-releasing hormone system in the brain during stress. Pharmacol Biochem Behav 73:147-158.

Mansbach RS, Brooks EN, Chen YL (1997) Antidepressant-like effects of CP-154,526, a selective CRF1 receptor antagonist. Eur J Pharmacol 323:21-26.

Manson S (1983) The neurochemistry and pharmacology of extinction behavior. Neuroscience and biobehavioral reviews 7:325- 347.

Maren S (2005a) Building and burying fear memories in the brain. Neuroscientist 11:89-99.

Maren S (2005b) Central and basolateral amygdala neurons crash the aversive conditioning party: Theoretical comment on Rorick-Kehn and Steinmetz (2005). Behav Neurosci 119:1406-1410.

Martin JB (2002) The integration of neurology, psychiatry, and neuroscience in the 21st century. Am J Psychiatry 159:695-704.

Martinowich K, Manji H, Lu B (2007) New insights into BDNF function in depression and anxiety. Nat Neurosci 10:1089-1093.

McAllister A, Katz L, Lo D (1997) Opposing role for endogenous BDNF and NT-3 in regulating cortical dendritic growth. Neuron 18:767-778.

McEwen BS, Stellar E (1993) Stress and the individual. Mechanisms leading to disease. Arch Intern Med 153:2093-2101.

McKinney WT, Jr., Bunney WE, Jr. (1969) Animal model of depression. I. Review of evidence: implications for research. Arch Gen Psychiatry 21:240-248.

McKinney WT (2001) Overview of the past contributions of animal models and their changing place in psychiatry. Semin Clin Neuropsychiatry 6:68-78.

McLellan A (1994) The glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. Clin Sci 87:21-29.

McNaughton N (1997) Cognitive dysfunction resulting from hippocampal hyperactivity--a possible cause of anxiety disorder? Pharmacol Biochem Behav 56:603-611.

Menzaghi F, Heinrichs S, Merlo Pich E, Tilders FJH, Koob GF (1992) Attenuation of endocrine and behavioral responses to social conflict stress in rats by microinjection of cytotoxic antibody to corticotropin releasing factor and Ricin A chain toxin within paraventricular nuclei. Soc Neurosc Abstr 18:535.

Meynen G, Unmehopa UA, Heerikhuize JJ, Hofman MA, Swaab DF, Hoogendijk WJ (2006) Increased Arginine Vasopressin mRNA Expression in the Human Hypothalamus in Depression: A Preliminary Report. Biol Psychiatry 60 (8):892-5.

Milad MR, Rauch SL, Pitman RK, Quirk GJ (2006) Fear extinction in rats: Implications for human brain imaging and anxiety disorders. Biol Psychiatry 73:61-71.

Mirnics K, Middleton FA, Lewis D, Levitt P (2001) Analysis of complex brain disorders with gene expression microarrays: schizophrenia as a disease of the synapse. Trends Neurosci 24:479-486.

Mlynarik M, Zelena D, Bagdy G, Makara GB, Jezova D (2007) Signs of attenuated depression-like behavior in vasopressin deficient Brattleboro rats. Horm Behav 51:395-405.

Möller H, Müller W, Rüther E (2002) Moderne Antidepressiva. Stuttgart, New York: Georg Thieme Verlag.

Moller HJ (2004) [Therapy resistance to antidepressants. Definition, prevalence, predictors, and interventional possibilities]. Nervenarzt 75:499-515; quiz 516-497.

Montkowski A, Barden N, Wotjak C, Stec I, Ganster J, Meaney M, Engelmann M, Reul JM, Landgraf R, Holsboer F (1995) Long-term antidepressant treatment reduces behavioural deficits in transgenic mice with impaired glucocorticoid receptor function. J Neuroendocrinol 7:841-845.

Morgan C, Grillon C, Southwick SM, Davis M, Charney D (1995) Fear-potentiated startle in posttraumatic stress disorder. Biol Psychiatry 38:378-385.

Morgan M, Romanski L, LeDous J (1993) Extinction of emotional learning: contribution of the medial prefrontal cortex. Neurosci Lett 63:109-113.

Muller J, Corodimas K, Fridel Z, LeDoux J (1997) Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol in fusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli. Behav Neurosci 111:683-691.

Muller MB, Landgraf R, Keck ME (2000) Vasopressin, major depression, and hypothalamic-pituitary-adrenocortical desensitization. Biol Psychiatry 48:330-333.

Muller MB, Keck ME, Binder EB, Kresse AE, Hagemeyer TP, Landgraf R, Holsboer F, Uhr M (2003) ABCB1 (MDR1)-type P-glycoproteins at the blood-brain barrier modulate the activity of the hypothalamic-pituitary-adrenocortical system: implications for affective disorder. Neuropsychopharmacology 28:1991-1999.

Munck A, Guyre PM, Holbrook NJ (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr Rev 5:25-44.

Murakami S, Imbe H, Morikawa Y, Kubo C, Senba E (2005) Chronic stress, as well as acute stress, reduces BDNF mRNA expression in the rat hippocampus but less robustly. Neuroscience Research 53:129-139.

Murgatroyd C, Wigger A, Frank E, Singewald N, Bunck M, Holsboer F, Landgraf R, Spengler D (2004) Impaired repression at a vasopressin promoter polymorphism underlies overexpression of vasopressin in a rat model of trait anxiety. J Neurosci 24:7762-7770.

Myers K, Davis M (2002) Behavioral and neural analyis of extinction. Neuron 36:567-584.

Nair A, Vaidya V (2006) Cyclic AMP response element binding protein and brain-derived neurotrophic factor: Molecules that modulate our mood? J Biochem 31:423-434.

Nemeroff CB (1996) The corticotropin-releasing factor (CRF) hypothesis of depression: new findings and new directions. Mol Psychiatry 1:336-342.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002a) Neurobiology of depression. Neuron 34:13-25.

Nestler EJ, Gould E, Manji H, Buncan M, Duman RS, Greshenfeld HK, Hen R, Koester S, Lederhendler I, Meaney M, Robbins T, Winsky L, Zalcman S (2002b) Preclinical models: status of basic research in depression. Biol Psychiatry 52:503-528.

Neumann ID, Torner L, Wigger A (2000a) Brain oxytocin: differential inhibition of neuroendocrine stress responses and anxiety-related behaviour in virgin, pregnant and lactating rats. Neuroscience 95:567-575.

Neumann ID, Kromer SA, Toschi N, Ebner K (2000b) Brain oxytocin inhibits the (re)activity of the hypothalamo-pituitary-adrenal axis in male rats: involvement of hypothalamic and limbic brain regions. Regul Pept 96:31-38.

Nielsen DM, Carey GJ, Gold LH (2004) Antidepressant-like activity of corticotropin-releasing factor type-1 receptor antagonists in mice. Eur J Pharmacol 499:135-146.

Ohl F (2005) Animal models of anxiety. Handb Exp Pharmacol (169):35-69.

Ohl F, Toschi N, Wigger A, Henniger MS, Landgraf R (2001) Dimensions of emotionality in a rat model of innate anxiety. Behav Neurosci 115:429-436.

Ohl F, Roedel A, Binder E, F H (2003) Impact of high and low anxiety on cognitive performance in a modified hole board test in C57BL/6 and DBA/2 mice. Eur J Neurosci 17:128-136.

Oitzl M, van Haarst AD, de Kloet ER (1997a) Behavioral and neuroendocrine responses controlled by the concerted action of central mineralocorticoid (MRS) and glucocorticoid receptors (GRS). Psychoneuroendocrinology 22:87-93.

Oitzl M, de Kloet ER, Joels M, Schmid W, Cole T (1997b) Spatial learning deficits in mice with a targeted glucocorticoid gene disruption. Eur J Neurosci 9:2284-2296.

Oitzl M, Fluttert M, Sutanto W, de Kloet ER (1998) Continuos blockade of brain glucocorticoid receptors facilitates spatial learning and memory in rats. Eur J Neurosci 10:3759-3766.

Okuyama S, Chaki S, Kawashima N, Suzuki Y, Ogawa S, Nakazato A, Kumagai T, Okubo T, Tomisawa K (1999) Receptor binding, behavioral, and electrophysiological profiles of nonpeptide corticotropin-releasing factor subtype 1 receptor antagonists CRA1000 and CRA1001. J Pharmacol Exp Ther 289:926-935.

Ostrowski NL, Lolait SJ, Young WS, 3rd (1994) Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. Endocrinology 135:1511-1528.

Overmier JB, Seligman ME (1967) Effects of inescapable shock upon subsequent escape and avoidance responding. J Comp Physiol Psychol 63:28-33.

Owens MJ, Nemeroff CB (1991) Physiology and pharmacology of corticotropin-releasing factor. Pharmacol Rev 43:425-473.

Pariante CM, Miller AH (2001) Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. Biol Psychiatry 49:391-404.

Patel P, Lopez J, Lyons DM, Burke S, Wallace M, Schatzberg AF (2000) Glucocorticoid and mineralocorticoid receptor mRNA expression in squirrel monkey brain. Journal of Psychiatric Research 34:383-392.

Paul ES, Harding EJ, Mendl M (2005) Measuring emotional processes in animals: the utility of a cognitive approach. Neurosci Biobehav Rev 29:469-491.

Paxinos G, Franklin K (2001) The mouse brain in stereotaxic coordinates, 2nd Edition. San Diego, San Francisco, New York, Boston, London, Sydney, Tokyo: Academic Press.

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149-167.

Phelps EA, LeDoux JE (2005) Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron 48:175-187.

Pinsonneault J, Sadee W (2003) Pharmacogenomics of multigenic diseases: Sex-specific differences in diseases and treatment outcome. AAPS PharmSci 5:E29.

Plomin R, Crabbe J (2000) DNA. Psychol Bull 126:806-828.

Plotsky P, Zurich K, Mathys C, Sanchez M, Thrivikraman KV, F H, Wigger A, Landgraf R (2000) Region-specific alterations in corticotropin-releasing factor (CRF) mRNA distribution and CRF-R2 binding in rats bred for high anxiety-related behavior (HAB). Soc Neurosc Abstr 26:206-207.

Popik P, Vetulani J, Bisaga A, van Ree JM (1991) Recognition cue in the rat´s social memory paradigm. J Basic Clin Physiol Pharmacol 2:315-327.

Popik P, Vetulani J, van Ree JM (1992) Low doses of oxytocin facilitate social recognition in rats. Psychopharmacology 106:71-74.

Porsolt RD, Le Pichon M, Jalfre M (1977) Depression: a new animal model sensitive to antidepressant treatments. Nature 266:730-732.

Porsolt RD, Bertin A, Jalfre M (1978) "Behavioural despair" in rats and mice: strain differences and the effects of imipramine. Eur J Pharmacol 51:291-294.

Price BH, Adams RD, Coyle JT (2000) Neurology and psychiatry: closing the great divide. Neurology 54:8-14.

Price ML, Lucki I (2001) Regulation of serotonin release in the lateral septum and striatum by corticotropin-releasing factor. J Neurosci 21:2833-2841.

Purba JS, Hoogendijk WJ, Hofman MA, Swaab DF (1996) Increased number of vasopressin- and oxytocin-expressing neurons in the paraventricular nucleus of the hypothalamus in depression. Arch Gen Psychiatry 53:137-143.

Raadsheer FC, Hoogendijk WJ, Stam FC, Tilders FJ, Swaab DF (1994) Increased numbers of corticotropin-releasing hormone expressing neurons in the hypothalamic paraventricular nucleus of depressed patients. Neuroendocrinology 60:436-444.

Radulovic J, Ruhmann A, Liepold T, Spiess J (1999) Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: differential roles of CRF receptors 1 and 2. J Neurosci 19:5016-5025.

Radulovic J, Fischer A, Katerkamp U, Spiess J (2000) Role of regional neurotransmitter receptors in corticotropin-releasing factor (CRF)-mediated modulation of fear conditioning. Neuropharmacology 39:707-710.

Reijmers LG, van Ree JM, Spruijt BM, Burbach JP, De Wied D (1998) Vasopressin metabolites: a link between vasopressin and memory? Prog Brain Res 119:523-535.

Reppermund S, Zihl J, Lucae S, Horstmann S, Kloiber S, Holsboer F, Ising M (2007) Persistent cognitive impairment in depression: The role of psychopathology and altered hypothalamiy-pituitary-adrenaocortical (HPA) system regulation. Biol Psychiatry 62:400-406.

Reul JM, de Kloet ER (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117:2505-2511.

Reul JM, Holsboer F (2002) Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol 2:23-33.

Richter K, Wolf G, Engelmann M (2005) Social recognition memory requires two stages of protein synthesis in mice. Learn Mem 12:407-413.

Ring RH (2005) The central vasopressinergic system: examining the opportunities for psychiatric drug development. Curr Pharm Des 11:205-225.

Risbrough VB, Hauger RL, Pelleymounter MA, Geyer MA (2003) Role of corticotropin releasing factor (CRF) receptors 1 and 2 in CRF-potentiated acoustic startle in mice. Psychopharmacology (Berl) 170:178-187.

Risbrough VB, Stein M (2006) Role of corticotropin releasing factor in anxiety disorders: A translational research perspective. Hormones and Behavior 50:550-561.

Roozendaal B, Wiersma A, Driscoll P, Koolhaas JM, Bohus B (1992) Vasopressinergic modulation of stress responses in the central amygdala of the Roman high-avoidance and low-avoidance rat. Brain Res 596:35-40.

Rosen JB, Schulkin J (1998) From normal fear to pathological anxiety. Psychol Rev 105:325-350.

Rosenbaum JF, Biederman J, Bolduc E, Hirshfeld D, Faraone SV, Kagan J (1992) Comorbidity of parental anxiety disorders as risk for childhood-onset anxiety in inhibited. Am J Psychiatry 149:475-481.

Rozeboom A, Akil H, Seasholtz AF (2007) Mineralocorticoid receptor overexpression in forebrain decreases anxiety-like behavior and alters the stress response in mice. Proc Natl Acad Sci U S A 104:4688-4693.

Rubin R, Dinan T, Scott L (2001) The neuroendocrinology of affective disorders. New York: Academic Press.

Sadock BJ, Sadock VA (2005) Kaplan & Sadock's comprehensive textbook of psychiatry, 8th edition, 8th Edition. Philadelphia: Lippincott Williams & Wilkins.

Sah P, Faber E, Lopez de Armentia M, Power J (2003) The amygdaloid complex: anatomy and physiology. Physiol Rev 83:803-834.

Salome N, Salchner P, Viltart O, Sequeira H, Wigger A, Landgraf R, Singewald N (2004) Neurobiological correlates of high (HAB) versus low anxiety-related behavior (LAB): differential Fos expression in HAB and LAB rats. Biol Psychiatry 55:715-723.

Salome N, Landgraf R, Viltart O (2006) Confinement to the open arm of the elevated-plus maze as anxiety paradigm: behavioral validation. Behav Neurosci 120:719-723.

Sawchenko PE, Li HY, Ericsson A (2000) Circuits and mechanisms governing hypothalamic responses to stress: a tale of two paradigms. Prog Brain Res 122:61-78.

Scantamburlo G, Hansenne M, Fuchs S, Pitchot W, Maréchal P, Pequeux C, Ansseau M, Legros JJ (2007) Plasma oxytocin levels and anxiety in patients with major depression. Psychoneuroendocrinology 32:407-410.

Schmidt M, Oitzl M, Levine S, de Kloet ER (2002) The HPA system during the postnatal development of CD1 mice and the effects of maternal deprivation. Developmental Brain Research 139:39-49.

Schmidt M, Enthoven L, van der Mark M, Levine S, de Kloet ER, Oitzl MS (2003) The postnatal development of the hypothalamic-pituitary-adrenal axis in the mouse. Int J Dev Neurosci 21:125-132.

Schmidt M, Enthoven L, van Woezik JH, Levine S, de Kloet ER, Oitzl MS (2004) The dynamics of the hypothalamic-pituitary-adrenal axis during maternal deprivation. J Neuroendocrinol 16:52-57.

Schmidt MV, Sterlemann V, Ganea K, Liebl C, Alam S, Harbich D, Greetfeld M, Uhr M, Holsboer F, Muller MB (2007) Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence. Psychoneuroendocrinology 32:417-429.

Schmidt-Kastner R, Wetmore C, Olson L (1996) Comparative study of brain-derived neurotrophic factor messenger RNA and protein at the cellular suggests multiple roles in hippocampus, striatum and cortex. Neuroscience 74:161-183.

Schulz DW, Mansbach RS, Sprouse J, Braselton JP, Collins J, Corman M, Dunaiskis A, Faraci S, Schmidt AW, Seeger T, Seymour P, Tingley FD, 3rd, Winston EN, Chen YL, Heym J (1996) CP-154,526: a potent and selective nonpeptide antagonist of corticotropin releasing factor receptors. Proc Natl Acad Sci U S A 93:10477-10482.

Schwab Y, Mouton J, Chasserot-Golaz S, Marty I, Maulet Y, Jover E (2001) Calcium-dependent translocation of synaptotagmin to the plasma membrane in the dendrites of developing neurones. Mol Brain Res 96:1-13.

Scott LV, Dinan TG (2002) Vasopressin as a target for antidepressant development: an assessment of the available evidence. J Affect Disord 72:113-124.

Selye H (1936) A syndrome produced by diverse nocious agents. Nature 138.

Shabalina SA, Ogurtsov AY, Spiridonov NA (2006) A periodic pattern of mRNA secondary structure created by the genetic code. Nucleic Acids Res 34:2428-2437.

Shettleworth S (1998) Cognition, Evolution and Behavior. Oxford: OUP.

Shimshek D, Bus T, Kim J, Mihaljevic A, Mack V, Seeburg P, Sprengel R, Schaefer A (2005) Enhanced odor discrimination and impaired olfactory memory by spatially controlled switch of AMPA receptors. PLos Biology 3:e354.

Sillaber I, Holsboer F (2004) Animal models of mood disorders. In: Neurobiology of mental illness, 2nd Edition (Charney D, Nestler E, eds). Oxford: Oxford University Press.

Smoller JW, Finn CT (2003) Family, twin, and adoption studies of bipolar disorder. Am J Med Genet C Semin Med Genet 123:48-58.

Spedding M, Neau I, Harsing L (2003) Brain plasticity and pathology in psychiatric disease: sites of action for potential therapy. Curr Opin Pharmacol 3:33-40.

Sprott RL, Ramirez I (1997) Current Inbred and Hybrid Rat and Mouse Models for Gereontological Research. Ilar J 38:104-109.

Steckler T, Holsboer F (1999) Corticotropin-releasing hormone receptor subtypes and emotion. Biol Psychiatry 46:1480-1508.

Steckler T (2005) CRF antagonists as novel treatment strategies for stress-related disorders. In: Handbook of Stress and the Brain (Steckler T, Kalin NH, Reul JMHM, eds), pp 409-421: Elsevier.

Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW (1994) Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. J Neurosci 14:2579-2584.

Steru L, Chermat R, Thierry B, Simon P (1985) The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology (Berl) 85:367-370.

Stokes P, Holtz A (1997) Fluoxetine tenth anniversary update: the progress continues. Clin Ther 19:1135-1250.

Stoppel C, Albrecht A, Pape H, Stork O (2006) Genes and neurons: molecuar insights to fear and anxiety. Genes Brain Behav 5:34-47.

Stratakis CA, Sarlis NJ, Berrettini WH, Badner JA, Chrousos GP, Gershon ES, Detera-Wadleigh SD (1997) Lack of linkage between the corticotropin-releasing hormone (CRH) gene and bipolar affective disorder. Mol Psychiatry 2:483-485.

Strohle A (2003) [The neuroendocrinology of stress and the pathophysiology and therapy of depression and anxiety]. Nervenarzt 74:279-291; quiz 292.

Suaudeau C, Rinaldi D, Lepicard E, Venault P, Crusio WE, Costentin J, Chapouthier G (2000) Divergent levels of anxiety in mice selected for differences in sensitivity to a convulsant agent. Physiol Behav 71:517-523.

Sudhof TC (2002) Synaptotagmins: why so many? J Biol Chem 277:7629-7632.

Sullivan J (2007) Please release me. Neuron 54:493-494.

Swanson LW, Sawchenko PE, Rivier J, Vale WW (1983) Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. Neuroendocrinology 36:165-186.

Szyf M, Weaver IC, Champagne F, Diorio J, Meaney M (2005) Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat. Front Neuroendocrinol 26:139-162.

Tamminga CA (2006) The anatomy of fear extinction. Am J Psychiatry 163:961.

Tanna VL, Wilson AF, Winokur G, Elston RC (1989) Linkage analysis of pure depressive disease. J Psychiatr Res 23:99-107.

Tarantino LM, Bucan M (2000) Dissection of behavior and psychiatric disorders using the mouse as a model. Hum Mol Genet 9:953-965.

Thierry B, Steru L, Chermat R, Simon P (1984) Searching-waiting strategy: a candidate for an evolutionary model of depression? Behav Neural Biol 41:180-189.

Thoenen H (1995) Neurotrophins and neuronal plasticity. Science 270:593-598.

Thor DH, Holloway WR (1982) Social Memory of the Male Laboratory Rat. Journal of Comparative and Physiological Psychology 96:1000-1006.

Thornalley PJ (2003a) Glyoxalase I--structure, function and a critical role in the enzymatic defence against glycation. Biochem Soc Trans 31:1343-1348.

Thornalley PJ (2003b) Quantitative screening of advanced glycation end products in cellular and extracellular proteins by tandem mass spectrometry. Biochem J 375:581-592.

Thornalley PJ (2006) Unease on the role of glyoxalase 1 in high-anxiety-related behaviour. Trends Mol Med 12:195-199.

Tichomirowa M, Keck M, Schneider H, Paez-Pereda M, Renner U, Holsboer F, Stalla G (2001) Endocrine disturbences in depression. J Endocrinol Invest 28:89-99.

Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H (1993) Multiple promoters direct the tissue-specific expression of the rat BDNF gene. Neuron 10:475-489.

Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W (1998) Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat Genet 19:162-166.

Ting J, Kelley B, Sullivan J (2006) Synaptotagmin IV does not alter excitatory fast transmission or fusion pore kinetics in mammalian CNS neurons. Journal of Neuroscience 26:372-380.

Touma C, Bunck M, Stein H, Zeh R, Landgraf R (2006) Mice selected for high or low stress reactivity: A new animal model for affective disorders. Frontiers in Neuroendocrinology 27:56.

Touma C, Bunck M, Glasl L, Nussbaumer M, Palme R, Stein H, Wolferstätter M, Zeh R, Zimbelmann M, Holsboer F, Landgraf R (2007) Mice selected for high versus low stress reactivity: a new animal model for affective disorders. Psychoneuroendocrinology 33(6):839.62.

Trullas R, Skolnick P (1993) Differences in fear motivated behaviors among inbred mouse strains. Psychopharmacology (Berl) 111:323-331.

Tsigos C, Chrousos GP (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53:865-871.

Tsiouris JA (2005) Metabolic depression in hibernation and major depression: an explanatory theory and an animal model of depression. Med Hypotheses 65:829-840.

Usher M, Cohen J, Servan-Schreiber D, Rajkowski J, Aston-Jones G (1999) The role of locus coeruleus in the regulation of cognitive performance. Science 283:549-554.

Ustun TB, Ayuso-Mateos JL, Chatterji S, Mathers C, Murray CJ (2004) Global burden of depressive disorders in the year 2000. Br J Psychiatry 184:386-392.

Vale W, Spiess J, Rivier C, Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213:1394-1397.

Valentino RJ, Foote SL, Page ME (1993) The locus coeruleus as a site for integrating corticotropin-releasing factor and noradrenergic mediation of stress responses. Ann N Y Acad Sci 697:173-188.

Valentino RJ, Commons KG (2005) Peptides that fine-tune the serotonin system. Neuropeptides 39:1-8.

Van de Kar LD, Blair ML (1999) Forebrain pathways mediating stress-induced hormone secretion. Front Neuroendocrinol 20:1-48.

van Gaalen MM, Stenzel-Poore MP, Holsboer F, Steckler T (2002) Effects of transgenic overproduction of CRH on anxiety-like behaviour. Eur J Neurosci 15:2007-2015.

van Londen L, Goekoop JG, van Kempen GM, Frankhuijzen-Sierevogel AC, Wiegant VM, van der Velde EA, De Wied D (1997) Plasma levels of arginine vasopressin elevated in patients with major depression. Neuropsychopharmacology 17:284-292.

van Pett K, Viau V, Bittencourt J, Chan R, Li H, Arias C, Prins G, Perrin M, Vale W, Sawchenko P (2000) Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J Comp Neurol 428:191-212.

van Wimersma Greidanus TB, Maigret C (1996) The role of limbic vasopressin and oxytocin in social recognition. Brain Res 713:153-159.

Vaugeois JM, Passera G, Zuccaro F, Costentin J (1997) Individual differences in response to imipramine in the mouse tail suspension test. Psychopharmacology (Berl) 134:387-391.

Verbalis JG (2003) Diabetes insipidus. Rev Endocr Metab Disord 4:177-185.

Vician L, Lim I, Ferguson G, Tocco G, Baudry M, Herschman HR (1995) Synaptotagmin IV is an immediate early gene induced by depolarization in PC12 cells and in brain. Proc Natl Acad Sci U S A 92:2164-2168.

Vigneaud VD, Lawler HC, Popenoe EA (1953) Enzymic cleavage of glyeinamid from vasopressin and a proposed structure for this pressor-antidiuretic hormone of the posterior pituitary. Journal of the American Chemical Society 75:4880–4881.

Villar MJ, Ceccatelli S, Ronnqvist M, Hokfelt T (1994) Nitric oxide synthase increases in hypothalamic magnocellular neurons after salt loading in the rat. An immunohistochemical and in situ hybridization study. Brain Res 644:273-281.

Walker DL, Ressler KJ, Lu KT, Davis M (2002) Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats. J Neurosci 22:2343-2351.

Wang C, Grishanin R, Earles C, Chang P, Martin T, Chapman E, JAckson M (2001) Synaptotagmin modulation of fusion pore kinetics in regulated exocytosis of dense-core vesicles. Science 294:1111-1115.

Wang Z, Young LJ, De Vries GJ, Insel TR (1998) Voles and vasopressin: a review of molecular, cellular, and behavioral studies of pair bonding and paternal behaviors. Prog Brain Res 119:483-499.

Waterston R, Lindblad-Toh K, Birney E, Rogers J, Abril J (2002) Initial sequenzing and comparative analysis of the mouse genome. Nature 420:520-562.

Watson PJ, Andrews PW (2002) Toward a revised evolutionary adaptationist analysis of depression: the social navigation hypothesis. J Affect Disord 72:1-14.

Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ (2004) Epigenetic programming by maternal behavior. Nat Neurosci 7:847-854.

Weaver IC, Champagne FA, Brown SE, Dymov S, Sharma S, Meaney MJ, Szyf M (2005) Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. J Neurosci 25:11045-11054.

Wersinger SR, Ginns EI, O'Carroll AM, Lolait SJ, Young WS, 3rd (2002) Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. Mol Psychiatry 7:975-984.

Wigger A, Loerscher P, Weissenbacher P, Holsboer F, Landgraf R (2001) Cross-fostering and cross-breeding of HAB and LAB rats: a genetic rat model of anxiety. Behav Genet 31:371-382.

Wigger A, Sanchez MM, Mathys KC, Ebner K, Frank E, Liu D, Kresse A, Neumann ID, Holsboer F, Plotsky PM, Landgraf R (2004) Alterations in central neuropeptide expression, release, and receptor binding in rats bred for high anxiety: critical role of vasopressin. Neuropsychopharmacology 29:1-14.

Windle RJ, Shanks N, Lightman SL, Ingram CD (1997) Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats. Endocrinology 138:2829-2834.

Winslow JT, Insel TR (2004) Neuroendocrine basis of social recognition. Curr Opin Neurobiol 14:248-253.

Wotjak CT, Kubota M, Liebsch G, Montkowski A, Holsboer F, Neumann I, Landgraf R (1996) Release of vasopressin within the rat paraventricular nucleus in response to emotional stress: a novel mechanism of regulating adrenocorticotropic hormone secretion? J Neurosci 16:7725-7732.

Yang YL, Chao PK, Lu KT (2006) Systemic and intra-amygdala administration of glucocorticoid agonist and antagonist modulate extinction of conditioned fear. Neuropsychopharmacology 31:912-924.

Yehuda R (2001) Biology of posttraumatic stress disorder. J Clin Psychiatry 62 Suppl 17:41-46.

Yodofsky S, Hales E (2002) Neuropsychiatry and the Future of Psychiatry and Neurology. American Journal of Psychiatry 159:1261-1264.

Yoshikawa T, Watanabe A, Ishitsuka Y, Nakaya A, Nakatani N (2002) Identification of multiple genetic loci linked to the propensity for "behavioral despair" in mice. Genome Res 12:357-366.

Young LJ (2002) The neurobiology of social recognition, approach, and avoidance. Biol Psychiatry 51:18-26.

Zingg HH (1996) Vasopressin and oxytocin receptors. Baillieres Clin Endocrinol Metab 10:75-96.

Zobel AW, Yassouridis A, Frieboes RM, Holsboer F (1999) Prediction of medium-term outcome by cortisol response to the combined dexamethasone-CRH test in patients with remitted depression. Am J Psychiatry 156:949-951.

Zobel AW, Nickel T, Kunzel HE, Ackl N, Sonntag A, Ising M, Holsboer F (2000) Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. J Psychiatr Res 34:171-181.

10   Acknowledgements

First of all, I thank Prof. Landgraf who provided me the opportunity to do my PhD thesis in his research group at the Max Planck Institute of Psychiatry. Moreover, from my diploma thesis on, he taught me to independently create and develop my own scientific ideas and skills. I thank him for his trust in my abilities.

I want to thank Prof. Boyan for his kind willingness to take over the second evaluation of my dissertation.

Dr. Alexandra Wigger was not only the person who awoke my interest in this research field, supervised me during my diploma thesis, but, despite her absence from the lab, she scientifically and psychologically supported me during my whole PhD thesis. Therefore, I sincerely thank her.

I thank Dr. Mathias Schmidt, not only for his scientific support during our developmental and CRH studies, but also for his unconfined assistance, discussions, and positive feedback of my experiments and results.

Dr. Carsten Wotjak kindly supervised me in the field of learning tasks, during the preparation of my talks, and provided me the opportunity to participate in scientifically challenging discussions. Thank you very much.

Prof. Singewald and his research group always warmly welcomed me during our collaboration projects in Innsbruck. Therefore I want to thank him and his group. I especially want to thank Simone Sartori and Patrick Muigg who offered me the chance to broaden my scientific experience in a warm and pleasant atmosphere.

I thank Prof. Holsboer for providing me the DMP696, which enabled me to successfully perform my CRHR1 study.

Moreover, I want to thank Daniela Harbich for the nice time during the many ISHs that we performed together and Cornelia Graf for her assistance during the qRTPCR analyzes.

Furthermore, I thank all the present and past group members who accompanied and supported me during the last years. In particular, I owe my special thanks to Dr. Elisabeth Frank and Ludwig Czibere for their permanent scientific assistance, their psychological encouragement as well as for the possibility to work in a friendly and nice atmosphere.

Auch möchte ich mich herzlich bei Marina Zimbelmann und Markus Nussbaumer bedanken, deren Unterstützung bei der Anfertigung der Gehirnschnitte und bei der Durchführung vieler Verhaltenstests unentbehrlich war. Mein weiterer Dank gilt den Tierpflegern (insbesondere

Albin Varga), dem Bibliotheksteam, dem technischen Dienst und der mechanischen Werkstatt, durch deren Hilfe mir jeder Arbeitstag erleichtert wurde.

Mein tiefster Dank gilt jedoch meinen Eltern, die mich immer und in allem was ich tat und tue, unterstützt und bestärkt haben. Durch ihre liebevolle Art und Zuneigung haben sie mir ermöglicht, mein Studium, wie auch diese Dissertation, problemlos und mit einem Lächeln auf dem Gesicht, zu bewältigen.

Auch meinen beiden Schwestern, Isabelle und Melanie, möchte ich herzlich für ihre Unterstützung danken.

Ein ganz besonderer Dank gilt Heinz, der mit mir seit Jahren durch „dick und dünn" (durch Studium und Dissertation) geht und mich in Zuneigung und Liebe stets in allem unterstützt hat.

Zu guter Letzt möchte ich meinen Freunden, Vanessa, Anne, Marlene, Florian und Ludwig danken. Sie waren immer für mich da, hatten stets ein offenes Ohr für meine Sorgen und haben mir gezeigt, dass man alles schaffen kann, wenn man gute Freunde hat.

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12   Publications

Bunck M, Schmidt MV, Harbich D, Czibere L, and Landgraf R.
The role of CRH in shaping the phenotype of mice bred for anxiety-related behavior (in preparation). #

Bunck M*, Czibere L*, Horvath C, Graf C, Murgatroyd C, Muigg P, Singewald N, Rafae JI, Deussing JM, Holsboer F, Spengler D and Landgraf R.
A hypomorphic vasopressin allele prevents anxiety- and depression-related behavior. Molecular Psychiatry (2008, submitted). *authors contributed equally #

Muigg P, Scheiber S, Salchner S, Bunck M, Landgraf R, Singewald N.
Differential stress-induced neuronal activation pattern in mouse lines selectively bred for high, normal and low anxiety
Genes, Brain and Behavior (2008, submitted).

Touma C, Bunck M, Glasl L, Nussbaumer M, Palme R, Stein H, Wolferstätter M, Zeh R, Zimbelmann M, Holsboer F, and Landgraf R.
Mice selected for high versus low stress reactivity: a new animal model for accective disorders.
Psychneuroendocrinology Jul; 33(6): 839-62.

Keßler MS, Murgatroyd C, Bunck M, Czibere L, Frank E, Jacob W, Horvath C, Muigg P, Holsboer F, Singewald N, Spengler D, Landgraf R.
Diabetes insipidus and, partially, low anxiety-related behaviour are linked to a SNP-associated vasopressin deficit in LAB mice.
Eur J Neurosci. 2007 Nov; 26(10): 2857-64. #

Marsch R, Foeller E, Rammes G, Bunck M, Kössl M, Holsboer F, Zieglgänsberger W, Landgraf R, Lutz B, Wotjak CT.
Reduced anxiety, conditioned fear, and hippocampal long-term potentiation in transient receptor potential vanilloid type 1 receptor-deficient mice.
J Neurosci. 2007 Jan 24; 27(4): 832-9.

Landgraf R, Kessler MS, <u>Bunck M</u>, Murgatroyd C, Spengler D, Zimbelmann M, Nussbaumer M, Czibere L, Turck CW, Singewald N, Rujescu D, Frank E.
Candidate genes of anxiety-related behavior in HAB/LAB rats and mice: focus on vasopressin and glyoxalase-I.
Neurosci Biobehav Rev. 2007; 31(1): 89-102. Epub 2006 Aug 28. Review. #

Ditzen C, Jastorff AM, Kessler MS, <u>Bunck M</u>, Teplytska L, Erhardt A, Krömer SA, Varadarajulu J, Targosz BS, Sayan-Ayata EF, Holsboer F, Landgraf R, Turck CW.
Protein biomarkers in a mouse model of extremes in trait anxiety.
Mol Cell Proteomics. 2006 Oct; 5(10): 1914-20. Epub 2006 Jun 14

Krömer SA, Kessler MS, Milfay D, Birg IN, <u>Bunck M</u>, Czibere L, Panhuysen M, Pütz B, Deussing JM, Holsboer F, Landgraf R, Turck CW.
Identification of glyoxalase-I as a protein marker in a mouse model of extremes in trait anxiety.
J Neurosci. 2005 Apr 27; 25(17): 4375-84.

Murgatroyd C, Wigger A, Frank E, Singewald N, <u>Bunck M</u>, Holsboer F, Landgraf R, Spengler D.
Impaired repression at a vasopressin promoter polymorphism underlies overexpression of vasopressin in a rat model of trait anxiety.
J Neurosci. 2004 Sep 1; 24(35): 7762-70.

parts of the thesis have been published in these articles.

13 Assertion/Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet.

Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die vorliegende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommision vor.

München, den 15.01.2008