The AVP Deficit in LAB Mice: Physiological and Behavioral Effects

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Brief Description

The increased incidence of psychiatric disorders, such as anxiety disorders and depression, makes a strengthened search of genetic and environmental causal factors essential. Besides clinical studies, the broad preclinical research identifies continuously involved neuronal circuits, proteins, and genes representing new candidates in the progress of pharmacological research and the development of new therapies.

In this context, an animal model of extremes in trait anxiety, simulating pathologic anxiety, was generated to investigate the neuronal and genetic basis. Thus, CD1 mice selectively and bi-directionally inbred concerning their anxiety-related behavior form two lines, the high (HAB) and the low (LAB) anxiety-related behavior mice. The two lines display, after 24 generations, robust differences in trait anxiety and, additionally, in depression-like behavior, reflecting the clinical comorbidity of anxiety and depression, both of which are potentially based on a few selected genes in the two lines. The peptide arginine-vasopressin (AVP) is one factor found to be differentially expressed between the two mouse lines. In the present manuscript its involvement in the behavioral phenotype is scrutinized.

As the antidiuretic hormone, AVP expressed in the hypothalamic paraventricular nucleus (PVN) and the supraoptic nucleus is well known to regulate peripherally the body water balance. Therefore, the physiological consequence of the differences in *Avp* expression was analyzed, uncovering signs of central diabetes insipidus in LAB mice, an AVP deficit-related disease in humans. Symptoms also seen in LAB mice are increased daily fluid intake and high amounts of highly diluted urine as a result of the inability to secrete enough AVP in the blood circulation.

Besides the antidiuretic function, AVP of the PVN is potentially involved in emotionality-related behaviors and further in the regulation of the hypothalamo-pituitary-adrenocortical axis, the neuroendocrine stress response. Thus, the peripherally observable strong deficit in AVP might also be present in the brain of LAB mice, causing a dysregulation of anxiety-related behavior in these animals. Indeed, the less anxious LAB mice exhibit less releasable AVP in the PVN compared to HAB and “normal” CD1 mice, supporting the role of AVP as a crucial regulatory factor of emotionality

Besides the genetic predisposition, environmental factors, especially maternal and social interactions after birth, display a significant parameter in shaping the genetically given behavioral traits in emotionality. Therefore, we tested the maternal rearing behavior of HAB and LAB dams for differences possibly involved in the development of the two phenotypes. As dams of the two lines differ in their nursing style with LAB mothers showing less arched back nursing, a posture associated with the quality of maternal investment, we cross-fostered pups of the two lines to quantify the maternal influence on the anxiety- and stress-related phenotype of HAB and LAB mice. As we found just slight shifts in some parameters still within the range of the HAB and LAB phenotype, the two breeding lines can be defined as mainly genetically distinct, providing a beneficial tool to identify genes responsible for pathologic alterations in human diseases.

1 Introduction

Anxiety and affective disorders are a growing social and economic burden, not only in the developed countries, but increasingly also in the less developed parts of the world. Thus, lifetime prevalence in Europe and the USA for mood disorders have risen in the past decade from 14.0% to 20.8% and for anxiety disorders from 13.6% to 28.8% with a twofold higher risk in women than in men (Alonso et al., 2004b; Kessler et al., 2005a). Consequences are not only a loss of mental and physical quality of life, but also a high economic burden as a result of a three to four times higher loss of working days of people suffering from psychiatric disorders compared to people without a mental disorder (Alonso et al., 2004c). Moreover, the "Global Burden of Disease Study" of the "World Health Organization" of 1990 ranks unipolar depression as the fourth leading cause of disability-adjusted life-years (Ustun et al., 2004) with a prediction of a change to rank two in the year 2020 (Lopez and Murray, 1998). This resulted in 1990 in total costs of 77.4 billion US-dollars with an increase to 83.1 billion US-dollars in 2000 and, astoundingly, with just one third as direct costs for the treatment of the patients (Greenberg et al., 2003). Despite the high social and economic burden, and finally personal suffering, only half of the patients with a serious disorder and around 25% of mild mental disorder patients receive adequate treatment (Demyttenaere et al., 2004; Kessler et al., 2005b). Reasons may be the still widespread social rejection of mental disorders and partly faulty diagnostics.

Affective disorders including major depression, mania and bipolar (manic-depressive) disorder show a high comorbidity with anxiety disorders, especially with generalized anxiety disorder (33.7%), panic disorder (29.4%), agoraphobia (25.8%), and post-traumatic stress disorder (20.7%) (Alonso et al., 2004a), whereas the risk of depression in patients suffering from an anxiety disorder is higher than the other way round (Hettema et al., 2006). Patients suffering from major depression exhibit not only depressed mood but also irritability, low self esteem, hopelessness, guilt, decreased ability to concentrate, insomnia or hypersomnia, decreased interest in pleasurable stimuli, and finally thoughts of death (Nestler et al., 2002). Manic episodes in contrast are defined by increased activity and talkativeness, agitation, overestimation, increased distractibility, and flight of ideas. Moreover, in 15% of cases the depression leads to suicide (Hegerl and Rupprecht, 2006). In anxiety disorders, patients show increased and extended anxiety or fear over a longer

period. On the one hand, the anxiety can be unspecific as in panic disorder characterized by a sudden feeling of intense terror; on the other hand, it can be related to special subjects or situations as in phobias, leading to avoidance behavior. Both result in symptoms of palpitation, sweating, trembling, shortness of breath, blushing, or chills (Alpers et al., 2006). Initiating events can be severe traumatic experiences, such as loss of a loved person, but also chronic private or work-related stress. The loss of close family-structures, which can alleviate these difficult periods in life, and the anonymity of the modern cities support the increase in psychiatric disorders. Thus, socio-demographic parameters of different studies show that the life- and environmental situations can influence the development of affective and anxiety disorders, as the prevalence of unmarried, unemployed, disabled or people living in large cities is higher than in the other groups (Alonso et al., 2004a, b; Kessler et al., 2005a). But, on the other hand, there is also a high genetic predisposition to develop anxiety and affective disorders, as not everybody exposed to chronic stress or a traumatic event develops symptoms of anxiety and depression and vice versa. In this context, family studies show a three-fold higher risk to develop depression and a three- to five-fold higher risk to develop anxiety disorders in first-degree relatives of patients suffering from these disorders (Lieb, 2005; Merikangas and Low, 2005; Smoller and Finn, 2003). Thus, the genetic risk of developing an anxiety disorder is around 30-40% and for depression 40-50% (Hegerl and Rupprecht, 2006; Nestler et al., 2002), also revealed in broad twin studies (Merikangas and Low, 2005; Smoller and Finn, 2003).

In the last decades the knowledge about a genetic basis and further the association of mental disorders with first candidate genes changed the idea of a miraculous or religious reason to a biological and neuroscientific founded cause of psychiatric diseases and in parallel the way of treatment. Although already the Arabic-Islamic culture kept elaborated facilities to care for their mentally disturbed and similar facilities were also established in Spain in the second part of the medieval times, the history of psychiatry in Europe of the last 5 centuries is singed by the highhanded confinement of mentally disordered people combined with permanent restraint or tranquilization. In the beginning of the 19th century, psychiatry was established as a medical field dissociated from a religious and demonic context, and it gradually embraced a neurological background. Proceedings in the research of neuroanatomy by Franz Nissl, Ramón y Cajal, Camillo Golgi and Korbinian Brodmann together with

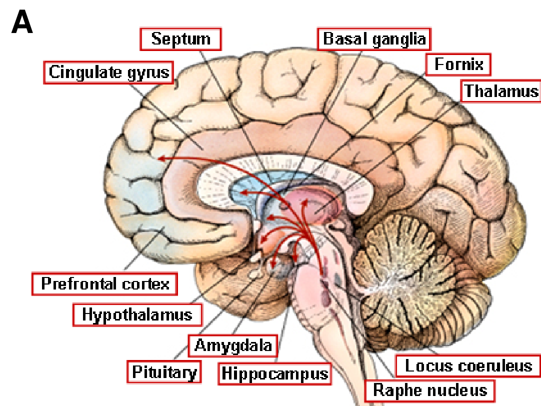
his new system to classify psychiatric diseases allowed Emil Kraepelin to combine biological and clinical methodology and thereby to start a new era in psychiatry in the beginning of the 20th century (Schott and Tölle, 2006). With the introduction of the psychoanalysis by Sigmund Freud in the first half of the 20th century and the development of the first psychotropics in the 1950s, the psychiatry took a big step forward in the treatment of mental disorders (Laux et al., 2000; Schott and Tölle, 2006; Wong and Licinio, 2001). However, despite the progressive and successful development of new and more effective substances in the last 50 years, the delayed therapeutic effectiveness, high side effects of all pharmaceuticals, and finally complete remission in only 50% of all patients demonstrate the small knowledge about the underpinning neurobiological mechanisms (Nestler et al., 2002). Thus, it is necessary to analyze in detail the underlying neuronal circuits to find more specific treatments with less adverse effects.

1.1 Neuroendocrine Background of the Stress Response

Fear is a basic mechanism, which presumably evolved in order to allow an organism to react quickly to threatening situations, to protect the body from injuries, to maintain a physiological homeostasis, and ultimately to save the organism's life. Interfering factors, called stressors, are intrinsic or extrinsic forces disturbing the physiological equilibrium of the body (homeostasis) (Tsigos and Chrousos, 2002). Stressors can be real threats, such as decreased blood pressure due to an injury or increased plasma osmolality after diminished fluid intake mediated by systemic mechanisms such as visceral and somatic pain, humoral inflammatory signals or baro- and osmoreceptors. Further, situations including confrontation with predators or new environments can be realized innately or by learning as life threatening. These predicted stressors are mainly processed by limbic structures (Engelmann et al., 2004). In case of a dangerous situation, the organism reacts with a non-specific startle response followed by a specific fight or flight behavior to diminish or avoid the stressor (Engelmann et al., 2004).

In the presence of a life-threatening stressor, the stress response system answers immediately with an activation of the autonomous nervous system to prepare the body for a fast and active reaction to avoid the stressor. Both the sympathetic and parasympathetic system regulate an increase in blood pressure, heart and

respiratory rate, gluconeogenesis, and lipolysis to provide the body with the required oxygen and nutrients (Charmandari et al., 2005). Thereby, a central role is assumed by the sympathetic-adrenomedullary system (SAS), where finally epinephrine and norepinephrine are released from the adrenal medulla into blood circulation to increase the necessary metabolic activities. Besides its peripheral function norepinephrine is also released centrally from neurons of the locus coeruleus (LC) interacting among others with amygdala and hypothalamus to influence behaviors accompanying increased activation of the autonomic and neuroendocrine stress response, such as increased arousal, alertness, and attention or inhibition of appetite, feeding, and reproductive behavior (Charmandari et al., 2005; Tsigos and Chrousos, 2002).



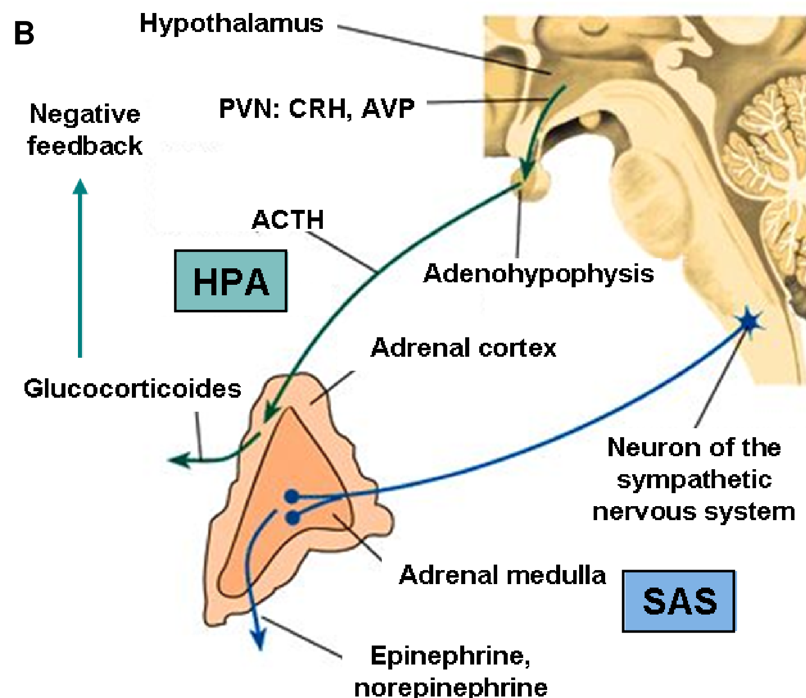


Fig. 1. The stress response. **A**, Overview of the brain areas involved in emotionality and stress response. **B**, HPA axis and SAS effect the release of glucocorticoides and norepinephrine / epinephrine, respectively, to prepare the body for the stress response.

Secondarily, with a higher latency and also during more severe, long-lasting, and predicted stressors, the hypothalamo-pituitary-adrenocortical (HPA) axis is activated by the release of corticotropin-releasing hormone (CRH), a 41-amino-acid peptide, from parvocellular neurons of the hypothalamic paraventricular nucleus (PVN). CRH is secreted from axonal terminals in the *Zona externa* of the median eminence into the hypophysial portal system. Furthermore, the co-segregated nonapeptide arginine-vasopressin (AVP) potentiates the effect of CRH. Both, CRH and AVP activate the

secretion of adrenocorticotropic hormone (ACTH) from the corticotrope cells of the anterior pituitary into blood circulation (Carrasco and Van de Kar, 2003; Engelmann et al., 2004). The two peptides operate at the secretory cells via CRH receptor 1 (CRH R1) and AVP receptor 1b (V1b) thereby activating second messenger pathways resulting both in an increased synthesis of the ACTH precursor (proopiomelanocortin = POMC) gene, and an increased secretion of ACTH itself. Both receptors are composed of seven transmembrane domains and are G-protein-coupled with a following adenylyl cyclase-responsible increase in cAMP activating protein kinase A after binding of CRH and an activation of the phosphatidylinositol pathway leading to increased protein kinase C activity after binding of AVP (Klinke and Silbernagl, 2001). The main target of ACTH is the adrenal cortex, where it stimulates the synthesis and secretion of glucocorticoids from the *Zona fasciculata*. The glucocorticoids cortisol (main glucocorticoid in humans, 95%) and corticosterone (main glucocorticoid in mice, 95%) increase metabolic activities, such as gluconeogenesis and lipolysis to increase the plasma glucose level, inhibit inflammatory and immune responses, and influence paracrinely the synthesis of epinephrine. Finally, they regulate both the basal HPA axis activity and terminate the stress response via a negative feedback-loop to reestablish and maintain the organisms homeostasis (Engelmann et al., 2004). Thereby, corticosterone/cortisol binds on glucocorticoid (GR) and mineralocorticoid (MR) receptors at the level of the pituitary, the hypothalamus, and the hippocampus to influence the release of CRH and ACTH. MRs, exhibiting a higher affinity for glucocorticoids, respond mainly to basal concentrations, maintaining a basal HPA axis activation level, whereas the GRs are activated at higher, stress-related glucocorticoid levels, mediating the negative feedback (Carrasco and Van de Kar, 2003; Charmandari et al., 2005; Klinke and Silbernagl, 2001).

Besides the parvocellular neurons, the PVN contains AVP and oxytocin (OXT) expressing magnocellular neurons projecting to the neurohypophysis (HNS = hypothalamic-neurohypophysial system). There they secrete AVP and OXT directly from axonal terminals into the blood circulation. The secretion of AVP by the HNS is required for the maintenance of a physiological plasma osmolality as it regulates the water reuptake from the primary urine at the level of the renal collection ducts. OXT regulates the contraction of uterus muscles at parturition and is involved in the milk injection reflex. Further, also the magnocellular neurons of the supraoptic nucleus

(SON) contribute to the AVP and OXT secretion of the HNS (Burbach et al., 2001; Swaab, 1998). In case of a stress response, the HNS is also involved in the activation of the HPA axis by the release of AVP and OXT locally from dendrites and somata of magnocellular PVN neurons modulating the activity of parvocellular PVN neurons. Additionally, AVP and OXT influence the secretion of ACTH *en passant* from axons at the level of the median eminence secreted in the portal vessel or from axonal terminals secreted into the general circulation reaching the posterior pituitary by a short portal vessel (Engelmann et al., 2004).

Besides the PVN, activating the HPA axis, the stress system includes several interactions with other brain areas, regulating the autonomic and the endocrine stress response, stress-related behaviors, and cognition. Thus, there is a reciprocal interaction between CRH of the PVN and the central norepinephrine system, at which the PVN receives norepinephrine and epinephrine input from the nucleus of the solitary tract to integrate visceral and somatic sensory information (Herman et al.,

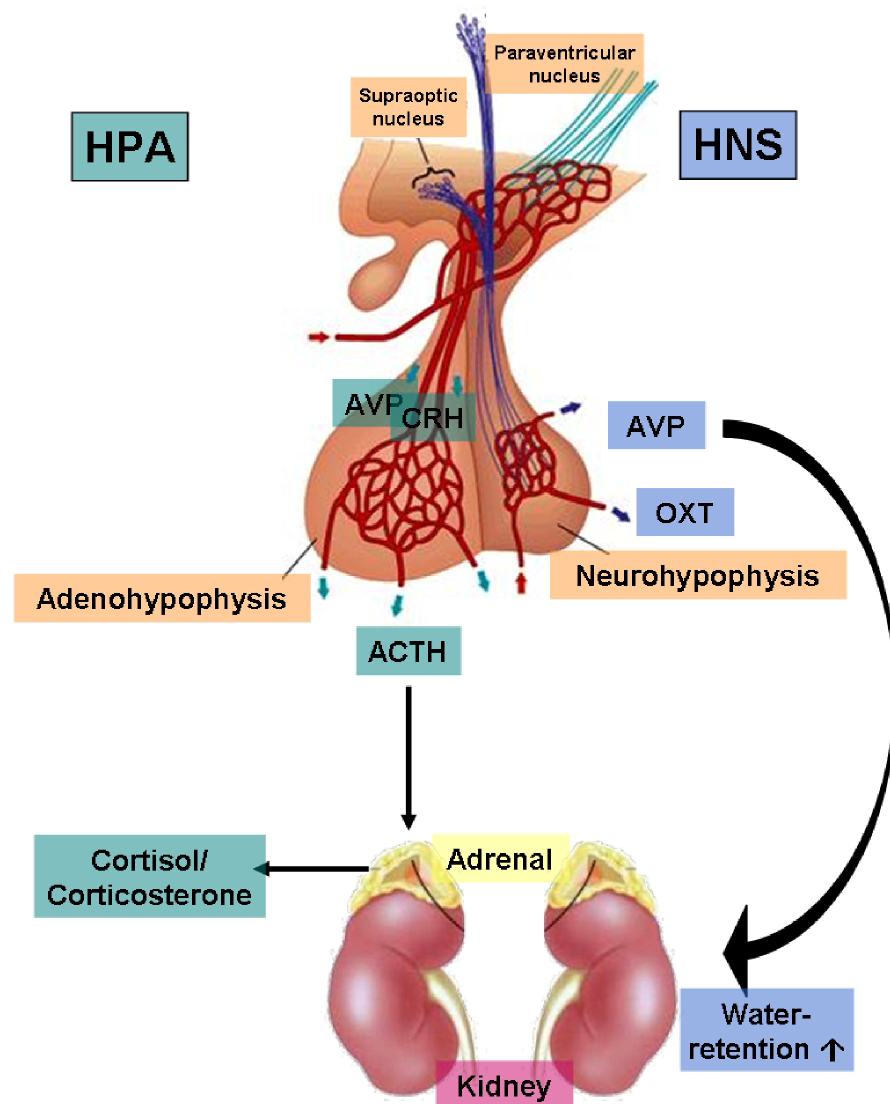


Fig. 2. The two functions of AVP. AVP released in the portal vessel of the adenohypophysis potentiates the effect of CRH in activating the HPA axis. Released into blood circulation from the neurohypophysis AVP activates water retention in the kidney to maintain physiological plasma volume and plasma osmolality.

2003). On the other hand, the PVN holds CRH connections to the LC involved in the autonomic stress response (Tsigos and Chrousos, 2002). Further, the PVN is innervated by limbic areas, receiving excitatory glutamatergic and inhibitory GABAergic projections from the ventral subiculum, the prefrontal cortex, the amygdala, and the lateral septum (LS). Additionally, the GABAergic projections are mainly connected to the PVN via projections of the bed nucleus of the stria terminalis (BNST) and the peri-PVN region. The PVN also receives excitatory serotonergic projections involved in HPA axis activation directly from the raphe nucleus and also indirectly by serotonergic innervations of the hippocampus, the prefrontal cortex, and the amygdala (Herman et al., 2002; Herman et al., 2003). With regard to a reciprocal interaction, the PVN in turn projects directly and indirectly back to these brain regions.

During the last few years, besides the "classic" transmitters glutamate and GABA and the peptides AVP and CRH, other neuropeptides, such as substance P, neuropeptide Y, and galanine in connection with the equivalent receptors, have been shown to play a role in anxiety- and stress-related responses (Holmes et al., 2003)

Finally, when a stressor exceeds a certain threshold in quality and quantity, leading in parallel to a chronic disruption of the homeostasis, the adaptive stress response can change into a maladaptive state (distress) with harmful and drastic consequences, including also alterations on a molecular and genetic level (Charmandari et al., 2005; Engelmann et al., 2004). These maladaptive changes in the stress response are the neuroendocrine basis of psychiatric disorders, such as anxiety and affective disorders.

1.2 Biological Bases and Pharmacological Treatment of Anxiety and Affective Disorders

As anxiety and affective disorders are highly comorbit, it is not astonishing that the underlying central circuits with the appendant nuclei, neurotransmitters and receptors seem to overlap. Nevertheless, the exact interactions and alterations causing mood disorders are still slightly understood.

In consequence of the use of substances, coincidentally found to be effective in the treatment of psychiatric disorders, a main focus in research and treatment are the

catecholamines dopamine and norepinephrine and the amine neurotransmitter serotonin. Synthesized from neurons in the substantia nigra and ventral tegmental area (dopamine), the LC (norepinephrine), and the raphe nuclei (serotonin), the transmitters are released in several brain areas involved in the modulation of various physiological functions and behaviors including attention, sleep-wake cycles, information processing, learning and memory, pain, mood, and anxiety (Bear et al., 2006). The use of psychopharmacological drugs started in 1952 with the discovery of the antipsychotic substance Chlorpromazin, a D_2 receptor antagonist, used as the first neurolepticum. In the same year, the first monoaminoxidase inhibitor (MAO), Iproniazid, was implemented, followed in 1957 by Imipramin, a tricyclic antidepressant (TCA) (Laux et al., 2000; Nestler et al., 2002). Both show an antidepressive effect by increasing the bioactive amount of serotonin and norepinephrine. The MAOs inhibit the degrading enzyme monoamineoxidase, whereas the TCAs block the reuptake of the transmitter into the cell (Göthert et al., 1998). In the 1960s, the discovery of the benzodiazepine-tranquilizers, Chlordiazepoxide and Diazepam, provided sedative and anxiolytic substances multifarious applicable in psychiatric disorders (Laux et al., 2000). Benzodiazepines bind on the α-subunit of the $GABA_A$ receptors, increasing their inhibitory activity (Göthert et al., 1998). In the following years, more specific drugs with fewer adverse effects were tried to develop. The selective serotonin reuptake inhibitors (SSRI), the serotonin-norpeinephrine reuptake inhibitors (SNRI) and, latest, selective norepinephrine reuptake inhibitors (NARI) are expected to be more specific in their effect. Nevertheless, although these substances display higher safety concerning death by overdose and show higher tolerability (Peretti et al., 2000), they also exhibit side effects, like the former substances, including increased appetite, fatigue, dizziness, sleep disturbances and agitation, diarrhea, nausea, and sexual dysfunctions. Especially the long-term side effects diminish patients' quality of life by impairing familial, social, and professional abilities (Cassano and Fava, 2004), leading in 8% of the treated patients to discontinuation of the therapy with antidepressants (Laux et al., 2000), anyhow less than caused by TCAs in the case of SSRIs (Anderson, 2000). Further, they show a delayed therapeutic effectiveness in patients, despite a rapid increase in extracellular serotonin levels upon starting treatment. This points not to a direct effect of the transmitter, but rather to changes in other neuronal circuits by a long-term elevation of serotonin in the brain (Nestler et

al., 2002). Indeed, it has been shown that SSRIs decrease the activity of the HPA axis, rather due to an increased glucocorticoid receptor expression in the hippocampus that strengthens the negative feedback. However, the effectiveness of SSRIs in the treatment of anxiety and affective disorders, paralleled by the influence on the HPA axis activity, brought the HPA axis in the researchers' focus.

In the recent years, clinical examinations and research showed the HPA axis to be involved in the development of anxiety and affective disorders. Thus, patients suffering from anxiety and depressive disorders showed a hyperactive HPA axis possibly due to increased excitatory input of the hyperactive amygdala or a decreased inhibition by the hippocampus. The diminished negative feedback in depressed patients has also been shown by the Dexamethason (DEX)/CRH test. DEX, a synthetic glucocorticoid, binds the GR at the level of the pituitary, and in higher dosages also at the level of the hippocampus, to initiate the negative feedback, inhibiting the activation of the HPA axis (Karssen et al., 2005). As healthy subjects exhibit a total DEX-related suppression of a CRH-initiated plasma ACTH increase, depressed patients show in 60% of all cases an increase in ACTH secretion (Heuser et al., 1994; Ising et al., 2005). Furthermore, 60% of patients in a major depression episode exhibit not only increased cortisol and ACTH levels but also an increase in CRH expression. By contrast, investigations of the HPA axis activity in anxiety disorders gave different results. In patients suffering from panic disorder, basal and stress-induced cortisol levels were reported to be normal or increased depending on the stressor, whereas social and specific phobias induce an increased cortisol secretion after stress exposure. Also, DEX-induced cortisol-nonsuppression was found in some patients suffering from panic disorders and generalized anxiety disorder, whereas analyses of CRH concentrations in the liquor or expression levels showed inconsistent results.

Nevertheless, the involvement of the different transmitters as well as CRH and AVP on the activity of the HPA axis and the etiology of anxiety and affective disorders still deserves further and closer attention. As ethical and moral standards exclude humans from most of the genetic, molecular, histological, and pharmacological studies because of the need of invasive and manipulative techniques, the use of animal models in research is elementary.

1.3 Animal Models of Anxiety and Affective Disorders

To investigate neuronal circuits and their specific alterations causing anxiety and affective disorders, animal models play a fundamental role in research. This includes genetic approaches by manipulating defined targets or phenotype-based studies, simulating behavioral traits of clinical relevance. Both approaches are powerful tools and should finally interact and excite each other.

To simulate anxiety and affective disorders, it is possible to induce anxiety and depression-like behavior by exposing mice or rats to chronic social (Haller et al., 1999; Karolewicz and Paul, 2001), non-social mild stress (Mitra et al., 2005; Willner, 1997, 2005), or to the learned helplessness paradigm (Seligman and Beagley, 1975; Shanks and Anisman, 1993). In contrast to the chronic social stress or the leaned helplessness paradigm, in the chronic non-social stress paradigm, the animals can habituate to the situation, loosing stress-induced behavioral and neuronal changes. However, chronic stress, learned helplessness, or challenging situations, such as behavioral tests, reflect only short-term or momentary states of emotionality, more vulnerable to environmental conditions and the experimental design. In contrast, genetic manipulations or a selectively bred trait is fixed in the animal and therefore more usable to identify involved neuronal circuits and genes and vice versa (Belzung and Griebel, 2001; Lister, 1990)

Concerning genetic manipulations, there is a wide range of well-established knockout and transgenic mice, concerning clinically already established but also newly described transmitter systems. Thus, MAO-A/B-, catechol-O-methyltransferase-, or norepinephrine transporter-knockout, leading to an increase in norepinephrine, serotonin, and dopamine in the brain, reduces anxiety- and depression-like behavior in specific behavioral tests. Further, several serotonin receptor (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C}, 5-HT_{5A}) and a serotonin transporter knockout mice exist displaying an altered anxiety and depression-like phenotype. Also the GABA system is a target for genetic manipulation, affecting several subunits of the GABA receptor. More recently, the HPA axis got in the focus of research, generating knockouts and transgenics of CRH, the CRH receptors, V1a and V1b, and of GR (Muller and Holsboer, 2006). Also other neuropeptide systems, such as substance P and neuropeptide Y, are investigated in this context by manipulating genetically their receptors, NK1 and NPYR (Cryan and Mombereau, 2004; Finn et al., 2003; Holmes, 2001; Urani et al., 2005).

Conventional knockout animals lack the targeted gene product already from early development. As a consequence this can lead to unintentional alterations in gene expression or peptide synthesis, trying to compensate the lack or other severe or lethal developmental dysfunctions. These side effects can distort investigations and cause false positive or negative results. To avoid this, the development of conditional knockouts, where the lack of the gene product is regional and temporal restricted and manageable, provides a successful tool for more precise genetic manipulation (Plomin and Crabbe, 2000).
Nevertheless, though the manipulation of single genes is helpful to trace specific circuits and interactions, it is less effective in discovering broad dependencies and new, yet unnoticed factors. As anxiety and affective disorders are not based on the alteration of a single gene, but on multiple genes of varying but rather small effect size, a more global approach is advantageous. Thus, the investigation and comparison of inbred mouse strains or selected breeding lines provides an opportunity to link different behavioral phenotypes with a genetic background (Belzung and Griebel, 2001). Over the last century, the in research commonly used mouse (BALB, C57, DBA, A/C etc.) and rat (Fischer, Lewis, Wistar etc.) strains were described to be emotionally different. This led to a more focused comparison of the different stains in anxiety- and depression-like, exploratory, and cognitive behavior (Bouwknecht and Paylor, 2002; Brodkin et al., 1998; Carola et al., 2004; Stohr et al., 2000; Trullas and Skolnick, 1993). To reveal the underlying genetic patterns of the different phenotypic characteristics, genetic mappings or linkage studies, such as quantitative trait locus (QTL) mapping, single nucleotide polymorphism screening, and microarray expression analysis, have been undertaken. Also the analysis of F2 segregating mice of two different strains or lines is a helpful approach to identify chromosomal localizations of genes contributing to specific phenotypes like anxiety- or depression-like behavior (Clement et al., 2002; Plomin and Crabbe, 2000).
In comparison to the analysis of inbred strains, the selective breeding of mice or rats according to a specific phenotype leads to the fixation of a stable feature (trait) in these animals, and can isolate alleles that are associated with this trait (Clement et al., 2002; Phillips and Belknap, 2002). As inbreeding of mice for at least 20 generations leads to genetically homozygote animals (Plomin and Crabbe, 2000), the selective inbreeding of two lines according to one phenotypic difference results in homozygous strains dissociating the specific underlying genes. In the past decades,

mice and rats were selected for high and low defecation in the open field (Broadhurst, 1975; DeFries et al., 1978), for their time of acquisition, alcohol sensitivity, exploration, attack-latency, or coping strategy (Bignami, 1965; Clement et al., 2002; Veenema et al., 2003).

Methodological background of analyzing mice and rats for their emotionality is a wide variety of behavioral test, including conditioned and unconditioned tests. Conflict or conditioned tests are based on the pairing of an aversive stimulus (electric shock) with a positive stimulus, such as food (Geller-Seifer test, Vogel conflict test), or with a neutral stimulus (fear conditioning) to analyze the avoidance behavior. Anxiolytic substances have been shown to reduce the reaction on the conditioned stimulus (File, 1985; Kulkarni and Reddy, 1996). By contrast, unconditioned tests use the conflict between the impulse of the animal to explore new environments and the avoidance of unknown areas (Finn et al., 2004; Kulkarni and Reddy, 1996; Ohl, 2003). Thus, more anxious animals avoid the open, unprotected and lit compartments of the test apparatus, such as the central part of the open field (Harro, 1993) or the holeboard (Ohl, 2003), the open arms of the elevated plus maze (Lister, 1987; Pellow et al., 1985), or the lit part of the dark/light box (Bourin and Hascoet, 2003). Anxiolytic drugs enhance the time the animals spend exploring the aversive compartment. As some drugs, such as bezodiazepines, have a sedative effect in higher dosages, the locomotor activity, basically connected to exploratory behavior, has to be carefully observed to eliminate false positive results. Other anxiety tests use the social context of mice or rats by analyzing latency to and time of social contact or dominant and submissive behavior (File, 1985; Finn et al., 2004). Finally, also mouse and rat pups can be analyzed to their emotionality during development, as the number of ultrasonic vocalization calls, emitted during separation from their mother and the nest, is correlated to anxiety (Branchi et al., 2001; Insel et al., 1986). To investigate depression-like behavior, the coping strategy of the animal in an impasse situation can be investigated. In this context, the animal can be forced to swim (forced swim test) (Porsolt et al., 1977a; Porsolt et al., 1977b) or hang up on its tail (tail suspension test) (Steru et al., 1985; Trullas et al., 1989). In both situation, the activity of the animal points to the coping strategy with displaying high rates of floating or immobility, reflecting a passive coping style and therefore depression-like behavior. Further, the investigation of anhedonia (loss of interest in pleasure), a core symptom of depression in humans, by analyzing the reduced intake of sucrose

solution or sweet food as well as impairments in place preference conditioning or brain stimulation, is a well established paradigm to analyze depression-like behavior in rats and mice (Willner et al., 1992).

Animal models of clinical conditions, whether chronically induced, genetically modified, or selectively bred share the need of validity. Three validation criteria were established in the last decades: Face validity predicts an identical behavioral and physiological response in the animal compared to the response observed in humans. Construct validity relates to the similarity of the psychological and biological factors underlying both the animal model and the human disease. Finally, predictive validity requires the sensitivity of the model to clinical effective drugs (Belzung and Griebel, 2001).

As mice or rats are not miniature versions of humans, we can never entirely create human psychopathologies, like anxiety or depression, in an animal model. Nevertheless, fundamental behavioral and linked neuronal structures are conserved in both, giving the possibility to investigate behavior, the underlying neuronal circuits and genes (Cryan and Holmes, 2005). Anxiety and depressive disorders are very complex syndromes with a high heterogeneity of clinical symptoms. Though, it is not possible to model in mice or rats some of the main symptoms observed in patients, including depressed mood, feeling of worthlessness, and thoughts of death, or even depression or anxiety disorders per se. Nevertheless, animal models are suitable to simulate several single aspects, like anhedonia, concentration problems, weight loss or gain, sleep disturbances, agitation, and hypercortisolism, providing the possibility to close a gap of knowledge in clinical research of psychiatric disorders (Cryan and Mombereau, 2004).

1.4 Arginine-Vasopressin

The nonapeptide AVP, discovered concerning its chemical structure by Du Vigneaud in 1955, is biosynthesized from a prepropeptide (human: 164 amino acids; mus musculus: 168 amino acids), including besides the AVP part a signal peptide with 19 (human) to 23 (mus musculus) amino acids, the carrier protein neurophysin II (NPII) with 93 amino acids, and a glycoprotein of 39 amino acids and unknown function (Burbach et al., 2001; de Bree and Burbach, 1998). The gene encoding the AVP precursor lies on chromosome 20 (human) and 2 (mus musculus) respectively and is

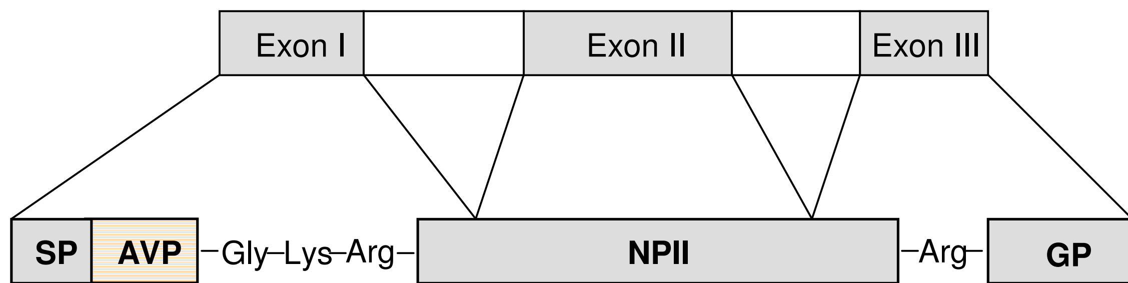


Fig. 3. Structure of the gene and the prepropeptide of AVP. The three exons of the gene encode for a signal peptide (SP), arginine-vasopressin (AVP), neurophysin II (NPII), and a glycoprotein (GP).

composed of 3 exons. Exon 1 encodes the signal peptide, the AVP part, a 3 amino acids linker, and the N-terminal part of NP II. The second exon includes the highly conserved central part of NPII (67 amino acids), whereas the third exon encodes for the C-terminal part of the NPII (17 amino acids), an arginine linker, and the glycoprotein. After cotranslational translocation of the AVP prepropeptide into the endoplasmic reticulum (ER), it is processed on its way across the Golgi apparatus and in the large dense core vesicles transporting it to the axonal terminals. After truncation of the signal peptide in the ER, AVP is bound with its N-terminal domain into the binding pocket of NPII. Both AVP and NPII include several disulphide bridges (AVP: one; NPII: seven) necessary for folding and consequently for forming the AVP-NPII-complex. During the process of the propeptide, the glycoprotein is cleaved in the Golgi apparatus. Following, the AVP-NPII-complex matures to a biologically active AVP by four enzyme-dependent steps.

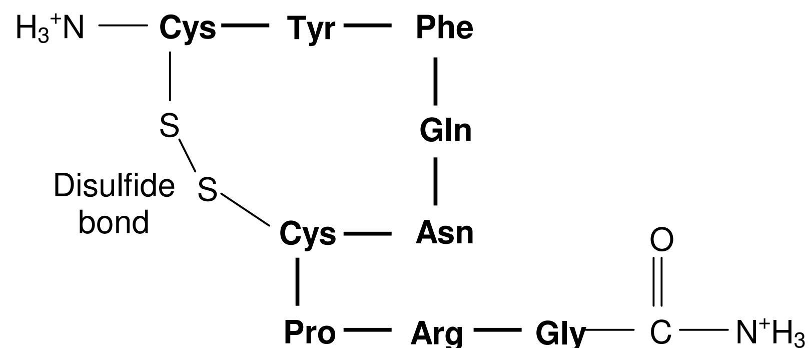


Fig. 4. Structure of AVP. The peptide is composed of 9 amino acids and includes one disulfide bond.

AVP is secreted from axons of magnocellular neurons of the SON and PVN into the systemic circulation at the level of the neurohypophysis (HNS) (Burbach et al., 2001; Landgraf and Neumann, 2004). It acts as an antidiuretic factor to preserve body water balance (Swaab, 1998). Plasma hyperosmolality, detected by the magnocellular cells itself or by osmoresposive cells of the subfornical organ and the organum vasculosum of the stria terminalis, initiates the AVP release into circulation. Likewise, a fall of blood pressure can initiate the AVP release from magnocellular neurons via

norepinephrinergic projections of brain stem centers, receiving information from baro- and chemoreceptors. After release, AVP activates, via binding to the V2 receptor and activation of the adenylylcyclase-cAMP-protein kinase A-pathway, the insertion of aquaporins, mainly aquaporin-2, in the apical membrane of the renal collection ducts of the kidneys. This increase in permeability leads to water retention from the kidney and concentrates the 170l of primary urine to maximal 2l. This highly sensitive mechanism preserves a physiological plasma volume and osmolality (Bourque et al., 1994; Knepper, 1994). Thus, an deficit in plasma AVP (<2pg/ml) and following plasma AVP increase leads to an inability of proper water retention from the kidney, causing hypotonic polyuria (>2l, <800mosmol/kg), inadequate plasma volume and plasma osmolality (>293 mosmol/kg) and high fluid intake (polydipsia) (Robertson et al., 1976; Verbalis, 2003), known as central/neurohypophysial diabetes insipidus (cDI). Besides infectious, inflammatory, trauma- or tumor-induced manipulation of the pituitary, autosomal dominant or recessive mutations of the *Avp* gene cause the AVP deficit (Verbalis, 2003). At present, 56 dominant or recessive single nucleotide polymorphisms (SNP) in the AVP precursor gene are identified to be responsible for cDI (Fig. 5), concerning the signal peptide (5), the AVP part (3), and the NPII domain (48) (Christensen and Rittig, 2006). Mutations cause inefficient cleavage of the signal peptide and/or inadequate folding and binding of the AVP part with the NPII moiety

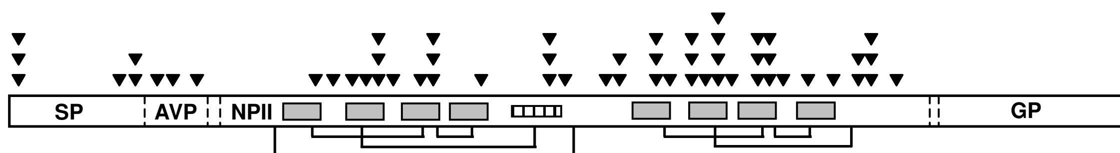


Fig. 5. Localization of the 56 SNPs inducing cDI on the *Avp* precursor gene. Arrowheads represent the SNPs, shaded boxes indicate β-strands, the striped box indicates the α-helix and the brackets show the 7 disulfide bridges. SP, signal peptide; AVP, arginine-vasopressin; NPII, neurophysin II ; GP, glycoprotein. Adopted from Christensen and Rittig, 2006.

(Beuret et al., 1999; Christensen et al., 2004; Ito et al., 1993; Nijenhuis et al., 2001; Nijenhuis et al., 2000). Consequences are retention and accumulations of the mutant AVP-NPII-precursor in the ER (Nijenhuis et al., 1999). The accumulation results in a disrupted processing of the precursor and further in diminished release of AVP from the posterior pituitary. In addition, the accumulated aggregates of the mutated AVP precursor interfere in the ER with the processing of intact AVP precursors (dominant-negative effect) (Ito et al., 1999), as well as with other proteins leading to cell death of the *Avp*-expressing neurons (Hansen et al., 1997; Ito and Jameson, 1997; Nijenhuis et al., 1999). While in the beginning of the disease the normal allele produces

enough AVP, facilitating osmoregulation, its dimerization with the mutant prohormone and the following progressive cell degeneration causes the delayed onset of the disease in childhood and the gradual advancement of cDI (Hansen et al, 1997). Further, from autopsy studies of cDI patients it is known that atrophy occurs in magnocellular neurons in the PVN and SON as well as in the neurohypophysis (Bergeron et al, 1991; Christensen et al, 2004)

Besides the antidiuretic function, AVP was identified to be also co-expressed with CRH in parvocellular neurons of the PVN (Kiss et al., 1984; Sawchenko et al., 1984), potentiating the effect of CRH on the release of ACTH from the anterior pituitary, thereby activating the HPA axis in response to a stressor, as described in 1.1 (Antoni, 1993; Carrasco and Van de Kar, 2003). Parallel to this neuroendocrine function, AVP acts as a neurotransmitter/neuromodulator within the brain (de Wied et al., 1993; Swanson and Sawchenko, 1983). This includes axonal hypothalamic projections to autonomic brainstem centers, such as the LC or the nucleus of the solitary tract, where it modulates the sympathetic and parasympathetic nervous system (Charmandari et al., 2005) or cardiorespiratory adjustments during stress response (Bailey et al., 2006). Further, axonal projections to and somato-dendritical release in hypothalamic and limbic brain areas are involved in social memory (Bielsky et al., 2005; Bielsky and Young, 2004; Landgraf et al., 2003), social bonding (Young et al., 1999; Young and Wang, 2004; Young et al., 1997), aggression (Ferris et al., 2006; Ferris et al., 1997), emotionality, and stress-related behavior (Landgraf and Neumann, 2004; Landgraf et al., 1998; Wotjak et al., 1996b). Also the wide distribution of the V1a and V1b receptors within the brain, including the LS, the amygdala, the BNST, the hippocampus, and the hypothalamus (Barberis and Tribollet, 1996; Hernando et al., 2001) makes the vasopressinergic system likely to be involved in multiple functions. Consequently, AVP got in the focus of research regarding alterations and pathological changes of social behavior and emotionality, the latter including anxiety and affective disorders and the dysregulation of the HPA axis (Gispen-de Wied and Jansen, 2002; Kim and Gorman, 2005; Nestler et al., 2002). Thus, in depressed patients AVP was found to be elevated in plasma (Inder et al., 1997; van Londen et al., 1997), and also the number of *Avp*-expressing neurons in the PVN increases (Purba et al., 1996; Raadsheer et al., 1994), suggesting a role of AVP, besides CRH, in the dysregulation of the HPA axis and in the development of anxiety and affective disorders. Also in rats, chronic or repeated stress enhances

AVP in the external zone of the median eminence (De Goeij et al., 1992; Nakase et al., 1998; Wotjak et al., 1996a), anxiety-related behavior and HPA-axis activity (Landgraf et al., 1998; Liebsch et al., 1998a; Wigger et al., 2004), and leads to an elevation in *V1b* mRNA in the pituitary (Rabadan-Diehl et al., 1995). In the DEX/CRH test, hypoanxious rats, displaying DEX nonsuppression and a greater ACTH and corticosterone release to CRH after DEX treatment, showed a inhibited increase of ACTH and corticosterone after V1a/b receptor antagonist pretreatment (Keck et al., 2002). This confirms a shift of the main contribution in activating the HPA axis under chronic stress from CRH to AVP (Aguilera and Rabadan-Diehl, 2000; Tilders et al., 1993). However, the exact action of AVP regarding ACTH secretion under chronic stress, dysregulation of the HPA axis (Aguilera and Rabadan-Diehl, 2000; Engelmann et al., 2004; Scott and Dinan, 2002), and intra-brain functions in the development of anxiety and affective disorders is poorly understood.

1.5 Pre- and Postnatal Influences on Stress-related Behavior

Besides the genetic predisposition giving rise to the development of anxiety and depression, environmental factors play a role in the etiology of mood disorders. During the prenatal phase, maternal stress-related endocrine and intra-uterine parameters might be crucial, whereas in the postnatal phase the parental rearing behavior as well as social and nonsocial experiences shape the individual's emotionality.

During pregnancy, prolonged periods of stress can alter the fetal environment and thereby influence the development and the physical and mental health of the child (Van den Bergh et al., 2005). In human studies associations were found between chronic stress, resulting in higher levels of cortisol and CRH, and preterm birth, reduced birth weigh, and developmental impairments (Weinstock, 2005). Nevertheless, there is a lack of evidence of an involvement of prenatal stress or stress hormone levels during pregnancy on the fetal brain and their consequences on behavior. In animal studies, chronic stress during pregnancy led to increased maternal and fetal plasma corticosterone levels (Takahashi et al., 1998). Prenatally stressed adult rats displayed a reduced number of hippocampal GR and MR (Barbazanges et al., 1996; Henry et al., 1994) and altered neuronal activation of hippocampus, LC, and PVN (Viltart et al., 2006) in connection with altered HPA axis

activity and negative feedback. Further, increased brain CRH (Cratty et al., 1995; Fujioka et al., 1999) and behavioral alterations, such as hyperanxiety and increased depression-like behavior (Burlet et al., 2005; Fride and Weinstock, 1988; Frye and Wawrzycki, 2003; Patin et al., 2005; Vallee et al., 1997) could be observed in prenatally stressed rats.

After birth, the housing conditions shape the behavior of an animal. Therefore, an "enriched environment" enhances possibilities of activity, sensory stimulation, and learning opportunities, resulting in decreased anxiety-related behavior, altered HPA axis reactivity, and increased learning and memory abilities (Barbelivien et al., 2006; Olsson and Dahlborn, 2002; Welberg et al., 2006).

Secondly, postnatal social interactions shape the behavior of the offspring. They are involved in the development of emotionality, social skills, and character. In animal models, the contact to littermates and to other conspecifics during adolescence influenced anxiety, stress-related behavior, and the underlying mechanisms. Thus, early weaning or isolation after weaning induced more anxious and aggressive behavior (Kikusui et al., 2004) and behavioral, hormonal, and autonomic hyper-reactive stress-responses (Ito et al., 2006; Weiss et al., 2004). Further, it was shown, that the number and the emotional status of cagemates during growth and after weaning influences anxiety-related and depression-like behavior, exploration urge, social abilities as well as BDNF-related hippocampal cell survival with strain and sex differences (Branchi et al., 2006a; Branchi et al., 2006b; Holmes et al., 2005).

The most relevant postnatal non-genetic factor influencing emotionality is the interaction with the parents. Thus, neglect, a distant parental relationship, violence, or stressful familial conditions are associated with the development of anxiety disorders and depression (Canetti et al., 1997; Holmes and Robins, 1988; Parker, 1981), as well as disturbed HPA axis development and responsiveness (Tarullo and Gunnar, 2006). Also in rats and mice, maternal rearing behavior, including the amount of maternal investment and nursing style, shape the animals' behavior (Calatayud and Belzung, 2001; Calatayud et al., 2004). The first days after birth, dams display an active and extensive nursing with bending over the pups with an arched back (arched back nursing) giving the pups the possibility to suckle in a smooth and un-exhausting way. Later, the time invested in maternal care decreases and the nursing periods get shorter. Further, dams switch from arched back nursing to less extensive positions, such as lying on the pups or on the side (Meaney, 2001). During nursing, rat and

mouse dams show intensive licking and grooming of the pups. It was examined, that the number of lickings together with the time spent in arched back nursing influences the development of emotionality-related behavior (Meaney, 2001). Thus, pups receiving lots of lickings and arched back nursing displayed less anxiety-related behavior and less stress-induced corticosterone release as adults (Anisman et al., 1998; Francis et al., 1999b). Further, this is associated with decreased *Crh* mRNA expression levels in the PVN, increased $GABA_A$ receptor subunit mRNA expression in the amygdala and hippocampus, and increased GR mRNA expression in the hippocampus (Caldji et al., 2003; Francis et al., 1999a; Francis et al., 2003; Liu et al., 1997), the latter transferred by DNA acetylation and methylation (Weaver et al., 2005; Weaver et al., 2006). Moreover, pups of both more anxious rat and mouse strains, with their mothers displaying less lickings and arched back nursing, cross-fostered to a high licking and arched back nursing mother, exhibited reduced anxiety (Francis et al., 1999a; Francis et al., 2003; Priebe et al., 2005), underlining the influence of the maternal rearing behavior on genetically determined traits.

Additionally, the maternal rearing behavior is non-genetically transmitted to female offspring, as cross-fostered high licking and low licking females showed the maternal behavior received as pups (Francis et al., 1999a). Moreover, maternally deprived (5 hrs) females, treated with artificial stroking during separation, showed as adults more lickings and arched-back nursing compared to non-treated deprived females (Fleming et al., 2002). Both confirm human studies, revealing the transmission of cold and distant child-parental relationship and childhood violence and abuse across generations (Pears and Capaldi, 2001). Even, more remarkable, mothers who are anxious or depressed show less positive behavior to their babies and have children that are more shy and fearful (Field, 1998; Hirshfeld et al., 1997b, a). An increase in oxytocin receptor binding in the medial preoptic area, the BNST, the LS, the central nucleus of the amygdala, and the ventral medial hypothalamus was shown to be involved in the high licking behavior of the rat dams (Champagne and Meaney, 2006). Further, this behavior was transferred to female offspring by a epigenetic mechanism, including demethylation-induced increase in estrogen receptor expression, affecting oxytocin receptor binding in the medial preoptic area (Champagne et al., 2006).

The need of extensive maternal care, including undisturbed feeding and licking the pups to secure the adequate neonatal development of the HPA axis is also

underlined by studies using maternal deprivation and separation. Thus, pups exhibit a stress-hyporesponsive period from postnatal day 4 to 14 in the rat (Levine, 1994) and from postnatal day 1 to 12 in mice (Schmidt et al., 2003), mainly maintained by maternal behavior. During this stress-hyporesponsive period, pups showed a diminished ACTH and corticosterone reaction on mild stressors, such as 15 min of separation or handling (Schmidt et al., 2003). Nevertheless, stronger stressors, like separating the pups from the mother for 24 hrs, disinhibited the stress-hyporesponsiveness resulting in increased basal corticosterone and ACTH levels as well as an enhanced ACTH and corticosterone stress response (Levine, 1994; Schmidt et al., 2004). Central regulatory factors of the HPA axis are also influenced, including down-regulated basal *Crh*, GR, and MR mRNA expression in the pup (Schmidt et al., 2004).

Further, for rats it was shown that short periods of handling (15 min) have no effect (Huot et al., 2004) or even reduce anxiety-related behavior and stress-induced ACTH release of adult rats, because of an increased maternal investment after reunion (Macri et al., 2004). Thus, dams displayed increased duration of maternal licking and grooming after handling, causing increased Fos expression in the thalamic paraventricular nucleus and the BNST associated with a decrease in *Crh* mRNA expression in the PVN (Fenoglio et al., 2006). In contrast, 180min of maternal separation caused an increase in HPA axis stress-response, increased *Crh* mRNA in the PVN, and decreased cortical GR mRNA expression levels (Huot et al., 2004), whereas the use of foster litters, given to the dams during the 180 min of separation, eliminated the stress-induced HPA axis response and the alterations in CRH and GR expression (Huot et al., 2004), possibly by preventing a separation-induced disruption of maternal behavior (Pryce et al., 2001). Interestingly, Macri et al. (2004) found the same increased compensatory maternal care in 15min handled and 240min separated pups after reunion with following reduced HPA axis and fear response in adult handled and adult separated offspring in comparison to control rats. This reveals a contribution of further factors, besides the amount of maternal care, on HPA axis development and fear response.

Taken together, the interaction of maternal behavior and the development of stress-related neuroendocrine and behavioral parameters, together with the underlying epigenetic mechanisms, are a well-balanced and fragile system.

1.6 Scope of the Thesis

In accordance with the well established rat model (Landgraf and Wigger, 2002; Liebsch et al., 1998a; Liebsch et al., 1998b), we generated a mouse model of trait anxiety allowing besides behavioral, neuroendocrine, and pharmacological studies broad genetic analyses. Therefore, we started in the year 2000 a bi-directional selective breeding of CD1 mice. Taking their anxiety-related behavior on the elevated plus-maze (EPM) as the key selection criterion (Pellow et al, 1985; Lister, 1987), we bred the most and least anxious animals. Thus, we mated mice spending most of the test time on the open arms and mice spending most of the test time on the closed arms, resulting in low anxiety-related behavior (LAB) and high anxiety-related behavior (HAB) mice (Kromer et al., 2005) (Fig. 6B). The animals are now in the 24th generation and show robust behavioral differences on the EPM, indicating trait anxiety (Fig. 6A). Besides the EPM test, the mice were also examined in a variety of other test paradigms for anxiety, including the dark/light box (DaLi), the open field

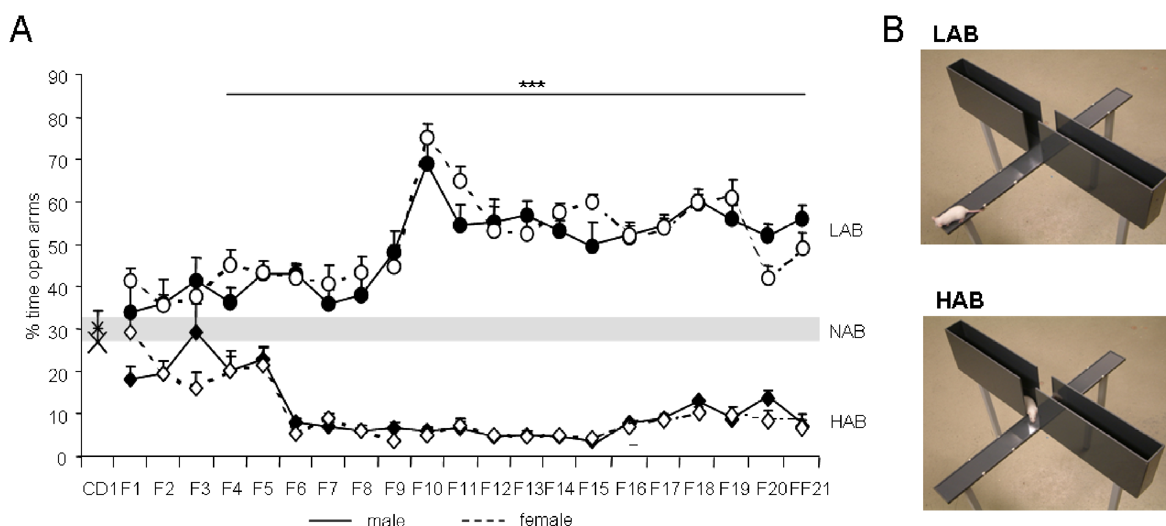


Fig. 6. Breeding progress of the HAB/LAB mouse model. **B**, Unlike non-anxious LAB mice which explore the aversive open arms of the EPM, the anxious HAB mice spend most of the test time in the dim lit closed arms. **A**, EPM data (% time open arms) of the parental male and female CD1 mice (bigger and smaller crosses, respectively) and F1 to F21 generations of male and female HAB and LAB mice. CD1 mice selected as controls independent of their performance (NAB) are shown for comparison (horizontal bar). Independent of gender, HAB and LAB animals differ significantly in their anxiety-related behavior (*** $p<0.001$, F4-F21) with NAB mice displaying a intermediate behavior (n=40-80 per line and generation).

(OF), and the ultrasonic vocalization (USV) test. In consideration of the high clinical comorbidity of depression and anxiety disorders (Alonso et al., 2004a), the two lines were also analyzed in paradigms of depression-like behavior, such as the forced swim test (FS) (Porsolt et al, 1977) and the tail suspension test (TST) (Steru et al, 1985). The results suggest the comorbidity of anxiety-related and depression-like

behavior in our mouse model (Kromer et al., 2005) as shown before in accordingly bred HAB/LAB rats (Landgraf and Wigger, 2002; 2003).

1.6.1 AVP Deficit in LAB Mice

As *Avp* was found to be differently expressed in the PVN of HAB and LAB rats with a higher level in HAB rats associated with higher anxiety-related behavior (Landgraf and Wigger, 2003; Wigger et al., 2004), the analysis of the amount of biological active AVP in the PVN of HAB and LAB mice and as a consequence of the differential availability its physiological function in this model was a major goal of the validation of the HAB/LAB mouse model.

1.6.1.1 Intra-PVN in vivo Microdialysis

We analyzed the amount of releasable AVP in the PVN of HAB and LAB mice by in-vivo microdialysis, a highly beneficial technique to analyze the release of substances, such as neurotransmitters or neuropeptides, into the extracellular fluid of distinct brain areas of freely behaving animals.

1.6.1.2 Examination of the Symptoms of an AVP Deficit

Further, we investigated the physiological consequences of the determined AVP deficit in LAB mice, investigating the daily fluid intake and daily urine osmolality, both known as symptoms of the AVP deficit-induced cDI in humans. Moreover, we challenged the water balance system by a 48-h water deprivation to have a closer look at its total AVP capacity. Additionally, we treated LAB mice with a V_2 receptor agonist to prove the AVP deficit as the origin of the inability in water retention.
To investigate the progress of cDI, we analyzed the daily fluid intake and urine osmolality during development, adulthood and aging. Aged HAB, NAB, and LAB mice were characterized concerning their anxiety-related behavior on the EPM to show stability of the bred trait and additionally concerning a recently found SNP localized in the signal peptide of the AVP precursor gene of LAB mice and possibly related to the AVP deficit. Finally the *Avp* mRNA expression level at different ages was estimated to investigate the cellular consequences of the cDI.

1.6.1.3 Viral-Vector-induced Increase in Avp mRNA Expression in the PVN of LAB Mice

To manipulate the expression of AVP in the PVN of LAB mice, we increased the expression level by transferring the wild-type *Avp* gene into PVN neurons via a viral vector. The use of adeno-associated viral (AAV) vectors, based on a nonpathogenic and replication-defective virus due to the deletion of more than 90% of the viral genes, is a successful method for long-term gene expression and phenotype manipulation in animals (Kaplitt et al., 1994; Lo et al., 1999). Therefore, after manipulation, we analyzed anxiety-related, depression-like behavior, and HPA axis stress-reactivity as well as daily fluid intake and urine osmolality. Finally, the *Avp* mRNA expression level in the PVN was examined to validate the method.

1.6.2 Postnatal Maternal Influence on the HAB/LAB-Phenotype

The different anxiety-related behavior of the two lines is supposed to be the result of a genetic variation forced by selective bi-directional inbreeding concerning one parameter on the EPM. To exclude non-genetic postnatal maternal factors influencing the behavioral phenotype of HAB and LAB mice, we investigated the maternal rearing behavior of HAB and LAB mice and afterwards its influence on the behavioral phenotype of the two lines.

1.6.2.1 Maternal Behavior of HAB/LAB Mice

As inbreeding can alter, besides the selected trait, also other behaviors of an animal, we wanted to know, if there are differences in the maternal behavior of HAB and LAB dams. Thus, we investigated the time dams of the two lines spent in caring for the pups and on their detailed nursing style.

1.6.2.2 Cross-Fostering HAB/LAB Mice

To analyze a possible influence of the maternal rearing style on the behavioral phenotype of the two breeding lines, we cross-fostered pups of HAB and LAB mice directly after birth. On postnatal day 5, we analyzed anxiety-related USV in the pups to detect possible short-time influences. As adults, the mice were investigated concerning anxiety-related and depression-like behaviors, as well as in exploration and locomotor activity. Finally, we examined the *Avp* mRNA expression levels in the PVN.

2 Material and Methods

2.1 Animals

We used male and female inbred HAB and LAB mice, male and female offspring of reciprocal cross-mated HAB and LAB mice (CM) bred in our own facility, and adult male CD1 mice (Charles River, Sulzfeld, Germany). Mice were kept in the animal facility of the Max Planck Institute, Munich, in groups of two to five animals in type 2-macrolone cages (25.5 x 19.5 x 13.8cm) in a 12h-light/dark cycle (with lights on at 6a.m.), with room temperature of 23 ± 2℃, humidity of 60%, and tap water and food (Nr. 1314, Altromin GmbH, Germany) *ad libitum*. Experiments were performed between 8a.m. and 1p.m.

At the age of 7 weeks all animals were tested on the EPM for 5 min selecting animals for breeding and experiments (according to (Kromer et al., 2005).

2.2 Projects

2.2.1 AVP Deficit in LAB Mice

2.2.1.1 In vivo Microdialysis

Surgery. For implantation of the microdialysis probes (U-shaped, Spectra/Por hollow dialysis membrane, outer diameter 0.25mm, length 1.5mm), isoflurane (Curamed Pharma, Germany) anaesthetized mice were fixed in a stereotaxic frame (Type 516000, TSE GmbH, Germany). After uncovering the calvaria the microdialysis probe was inserted into the brain by a small hole in the skull and positioned at the right PVN (0.05mm caudal to the bregma, 0.09mm lateral and 0.52mm ventral with an angle of 10° (Paxinos and Franklin, 2001). Afterwards, the probe was fixed by two screws (M1*3, stainless steel, Schrauben Preisinger, Germany) and two-

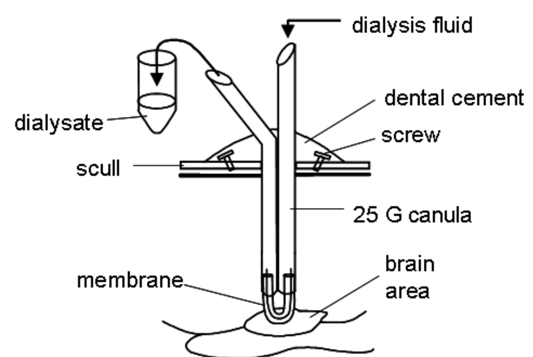


Fig. 7. Microdialysis probe. The probe is fixed on the skull by screws and cement with the menbrane touching the relevant brain area.

component adhesive (Twinlock Cement, Heraeus Kulzer, Germany) on the scull and the wound medicated with iodine (Fig. 7).

Procedure. The experiment took place 48h after surgery between 8a.m. and 1p.m. Four animals, single-housed in special Plexiglas cages (40cm x 23cm x 36cm), were done in parallel. For the experiment the probe's inflow was connected by PSE tubing (inner diameter 0.38mm, PE 20, Karman & Droll, Germany) to a syringe (2.5ml, Hamilton, Bonaduz AG, Switzerland) driven by a pump (E540220, TSE GmbH, Germany). At the beginning probes were perfused at a rate of 50µl/30min with sterile isotonic Ringer´s solution (Braun Melsungen AG, Germany) for 2h to establish an equilibrium between inside and outside the dialysis membrane. After two basal dialysates (sample 1 and 2), two additional samples were collected, one during hypertonic stimulation with 0.5M NaCl solution (sample 3) and one afterwards during perfusion with isotonic Ringer´s solution (sample 4). Samples were stored immediately on dry ice until analyzing by radioimmunoassay.

Histological verification of probe localization. After the experiment, mice were sacrificed, brains removed, snap-frozen in dry ice-chilled N-methylbutane (Roth GmbH, Germany), and stored at –20°C till sectioning with a cryostat (HM 500 O, Microm, Germany). For verification of the probe positioning in the right PVN, 25µm coronar sections were stained with Cresyl violet. Only successfully implanted mice with the probe placed within or adjacent to the PVN were used for data analysis (Fig. 8 A, B).

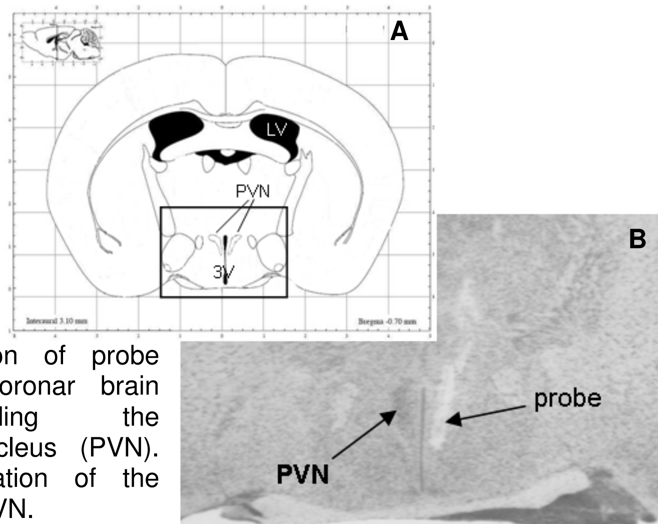


Fig. 8. Verification of probe localization. **A**, Coronar brain section including the paranentricular nucleus (PVN). **B**, Valide localization of the probe in the right PVN.

2.2.1.2 Examination of the Symptoms of an AVP Deficit

24-h fluid intake and urine osmolality. Animals were single-housed after weaning. On PND 21 (week 3), 35 (week 5), 54 (week 7), 140 (week 20), and 350 (week 50) urine osmolality and 24-h fluid intake was measured. The amount of drunken water was calculated as difference of the bottle weight before and after 24h. Urine osmolality

was measured via freezing point depression with an osmometer (Vogel, Germany) in samples of 10µl urine dissolved in 40µl aqua dest. At the different time points, mice were sacrificed, brains removed, snap-frozen in dry ice-chilled N-methylbutane (Roth GmbH, Germany), and stored at –20℃ until sectioning 14µm slides of the PVN region with a cryostat (HM 500 O, Microm, Germany) for *Avp* mRNA *in situ* hybridization.

Water deprivation. For the water deprivation test, mice were kept in groups of four animals for 48h without water. Then, deprived animals and animals that were kept under standard conditions were killed by decapitation. Trunk-blood, hypothalami, pituitaries, and urine were collected for further investigation. Blood was collected in 1ml EDTA-coated tubes (KABE Labortechnik, Germany) with 10µl of Trasylol (500000 KIE, Bayer AG, Germany) on ice and centrifuged for 10min at 3500rpm and 4℃ to get plasma. Tissues were collected in 1.5ml-tubes (Eppendorf-Netheler-Hinz GmbH, Germany) on dry ice and stored at –20℃ until further treatment. AVP levels in plasma, hypothalamus and pituitary were measured by radioimmunoassay. Urine and plasma osmolality were taken by freezing point depression.

DDAVP treatment. To normalize the physiological water balance, the V_2 receptor agonist 1-deamino-8-D-arginine-vasopressin (dDAVP) (Sigma-Aldrich, Germany) in a concentration of 100ng/30g body weight (BW) or vehicle (0.9% saline) was injected i.p. in a volume of 0.1ml/10g BW. Injection took place at 6a.m. Animals were kept in groups of two (pooled data) in metabolic-cages (TECNIPLAST GmbH, Germany). Urine was collected in intervals of 6 hours and centrifuged for 2min at 2500rpm to eliminate dirt particles. Urine osmolality was analyzed by freezing point depression.

2.2.1.3 Viral–Vector-mediated Avp Gene Transduction

AAV vectors. The AAV vectors (provided by Junichi Ideno, Japan) of serotype 2 contains, besides the AVP precursor gene cDNA or the ß-galactosidase (*lacZ*) gene (used as control), the human cytomegalovirus (hCMV) promoter, human growth hormone first intron enhancers, and a simian virus 40 polyadenylation signal sequence between inverted terminal repeats of the viral genome.

Surgery. With an age of 10 weeks isoflurane-anesthetized LAB mice were stereotactically fixed and the AAV-*Avp* vector was injected bilaterally into the PVN. Therefore, the calvarium was exposed and a glass-fiber-capillary (Fig. 9) was inserted into the PVN (0.05mm caudal to the bregma, 0.09mm lateral, and 0.52mm

ventral with an angle of 10° (Paxinos and Franklin, 2001) via a little hole in the skull. Via a tubing (PSE, inner diameter 0.4mm, Karman & Droll, Germany) conducted to a syringe (10µl, Hamilton, Bonaduz AG, Switzerland) 0.5µl of the AAV-*Avp* solution (1x10^{10} genome copies/µl) was slowly, over 30 seconds, injected into the PVN on each side. Finally, the wound was closed with a sterile surgical suture (Hauptner & Herberholz, Germany).

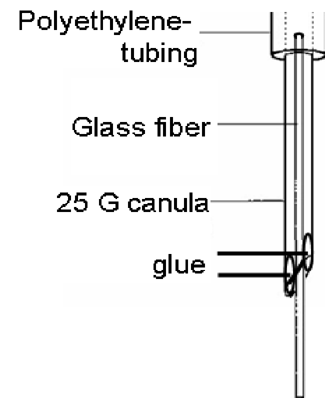


Fig. 9. Glass-fiber-capillary for AAV-*Avp* injection into the PVN.

Procedure. 30 days after surgery the behavior of the animals was analyzed in different test paradigms for anxiety (EPM, DaLi), depression-like behavior (TST, FS), and locomotion and exploration (OF, elevated platform (EPF)), as well as for basal and restrained stress-induced corticosterone concentrations in plasma (stress-reactivity test, SRT) over six weeks with a 3-7-day interval between the tests. 24-h fluid intake and urine osmolality were observed during the whole course of the experiment. 10 weeks after surgery, animals were killed and brains were taken, frozen, and cut in 14µm slides to analyze PVN-*Avp* mRNA-expression by *in situ* hybridization (Fig. 10A). *Avp* mRNA, ^{35}S-labeled in the *in situ* hybridization, was additionally labeled with silver grains. For histological validation of the correct application locus in the PVN region, 1µl black ink was added to the AAV-solution, later visible on the *in situ* hybridization slides and histological incorporeal after Nissel staining with Cresyl violett (Fig. 10B).

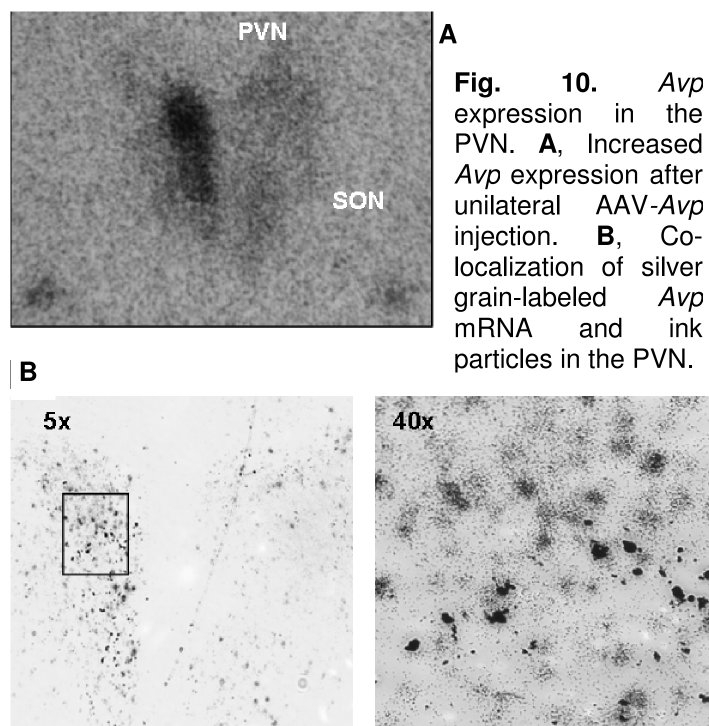


Fig. 10. *Avp* expression in the PVN. **A**, Increased *Avp* expression after unilateral AAV-*Avp* injection. **B**, Co-localization of silver grain-labeled *Avp* mRNA and ink particles in the PVN.

As no behavioral differences between animals with unilateral and bilateral hits, as well as mishits and untreated mice were detectable, following groups were found after histological validation: three mice with a bilateral and three mice with a unilateral hit were merged in the AAV-*Avp* group, one mouse with a bilateral hit and

two mice with a unilateral in the *lacZ* group, and two untreated mice and two *lacZ* mice with mishits in the control group.

2.2.2 Postnatal Maternal Influence on the HAB/LAB-Phenotype

2.2.2.1 Maternal Behavior

Breeding. 20 inbred HAB and 24 inbred LAB females of the 22nd generation were mated with one male animal of the equivalent line. Animals were housed in type 3-macrolone cages, equipped with a red plastic house (ACRE, Tecniplast GmbH, Germany) and nesting material (ABEDD-LAB and VET Service GmbH, Austria). After 2 weeks, pregnancy was controlled and male animals removed from the cages. 5 LAB females did not get pregnant (failed pregnancy). Animals gave birth after 22±2 days. 10 mothers failed to give birth correctly (failed birth, FB) including that they did not attend to clean and suck the pups or even did not notice them after birth. 12 hours after birth, number and gender of the pups were assessed and litters were culled at best to 5 male and 5 female animals per litter. Finally, 5 HAB mothers with their litters were randomly excluded to end up with 12 litters per line (6 cross-fostered and 6 non-fostered; see 2.2.2.2). Animals were housed under standard conditions.

Maternal observation. On PND 2, 4, 8 and 12, maternal behavior was observed 5 times during the day (7-8a.m., 11-12a.m., 3-4p.m., 7-8p.m., 11-12p.m.) at 5min-observation intervals. The following parameters were taken: arched back nursing (Fig. 11A), blanket posture (Fig. 11B), and side posture (together: mother ON); locomotion, eating / drinking / self-grooming, and sleeping (together: mother OFF).

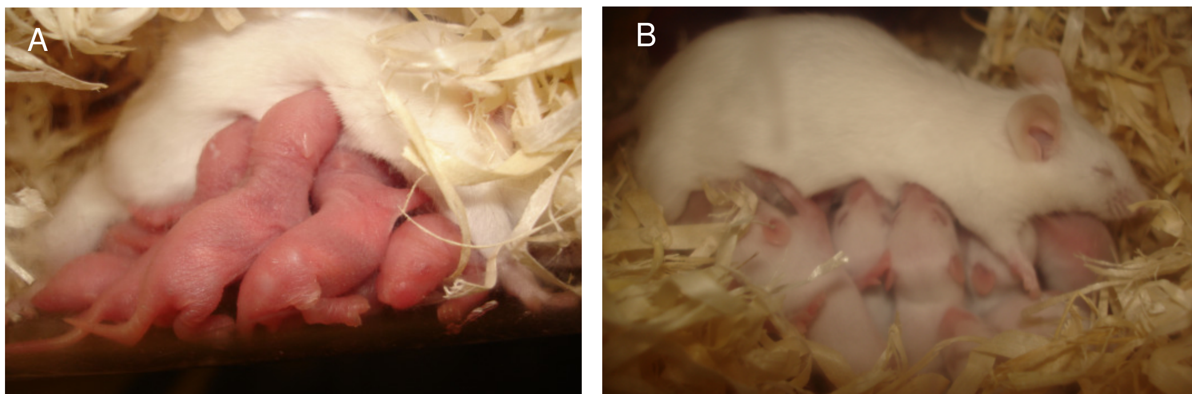


Fig. 11. Maternal nursing styles. **A**, Arched back nursing is characterized by a bent back of the dam while crouching active over the pups. **B**, During blanket posture nursing the dams lay flat on the pups.

2.2.2.2 Cross-Fostering

Animals. 12 hours after birth number and gender of pups of 20 inbred HAB and 24 inbred LAB mothers (see 2.2.2.1) were assessed and the pups fostered to a dam of the other line or put back with their own mother. Finally, 5 non-fostered HAB litters were randomly excluded to end up with 6 cross-fostered and 6 non-fostered litters in each line. After weaning at PND 28, animals were housed in groups of two to five littermates of the same sex in type 2-macrolone cages (25.5 x 19.5 x 13.8cm) under standard conditions.

Procedure. The anxiety-related and depression-like behavior, exploratory and locomotor activity, as well as stress-reactivity-related corticosterone increase in plasma of the adult animals was investigated in different tests, including EPM, TST, OF, and SRT. Tests started with an age of 7 weeks in a 48h-interval. Further, the number of emitted USV calls in 5-day old pups was examined. Additionally, the weight of the pups was assessed on PND 5 (USV), 12, 28 (weaning), and 49 (EPM) to analyze weight gain. Finally, animals were sacrificed, brains removed, frozen, and cut in 14µm slides for *Avp* mRNA *in situ* hybridization.

2.3 Behavioral Tests

2.3.1 USV

On PND 5, pups were separated from their mothers and placed individually on a petri dish (∅ 15cm) in a soundproofed box to analyze the number of ultrasonic vocalization (USV) calls and the locomotion during a 5min-period. The surface of the dish is subdivided in squares of $2cm^2$. Constant temperature was ensured by placing the dish in a water-bath of 20℃. USV calls were detected with a bat detector (Mini 3 bat-detector, Ultra Sound Advice, U.K.) at 70kHz (Fig. 12) and digitally recorded with the “WaveLab Lite” software (Steinberg, USA). In parallel, the pup was observed via a camera and square-crossings were counted. A square crossing is defined as being in one square with half of the body. The dish was cleaned with alcohol between the trails.

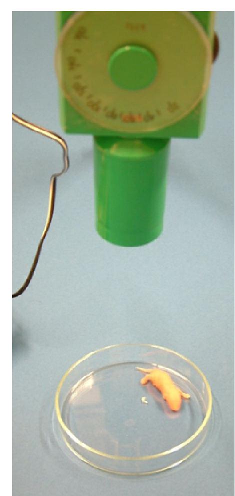


Fig. 12. The bat detector makes the 70 kHz USV calls of the pup hearable.

2.3.2 EPM

The plus-shaped EPM is made of dark gray PVC and consists of two opposing open (30 x 5cm, 300lux) and two opposing closed arms (30 x 5 x 15cm, 10lux) connected by a central platform (5 x 5cm, 90lux). The EPM is located 40cm above the floor and surrounded by a black curtain (Fig. 13).

At the beginning of each 5-min trial, the mouse was placed on the central platform facing a closed arm. The apparatus was cleaned before each test session with water containing a detergent.

Behavior was monitored by a trained observer blind to line or treatment via a video camera fixed above the EPM. Percentage (%) of time spent on the open arms relative to the time spent on all arms, the number of entries into the closed and the open arms, and the latency to the first entry into an open arm were determined using the "plus maze" software (E. Fricke, Germany).

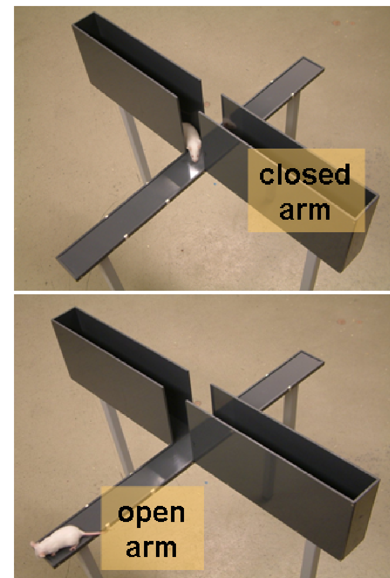


Fig. 13. The amount of time the mouse spend in the closed arms during the test refelects the level of anxiety.

2.3.3 EPF

The EPF is made up of a round platform (∅ 10cm) of gray PVC fixed 40cm above the floor on a wooden stick. After cleaning the platform with water containing a detergent, an animal was placed on the platform and videotaped for 5min. Following parameters were analyzed by a trained observer blind to the treatment or line using the "Eventlog 1.0" software (EMCO): number of head-dippings (defined as an exploratory movement of the head with the snout up to the eyes under the level of the platform), latency to the first head-dip, and number of rearings and grooming (Fig. 14A).

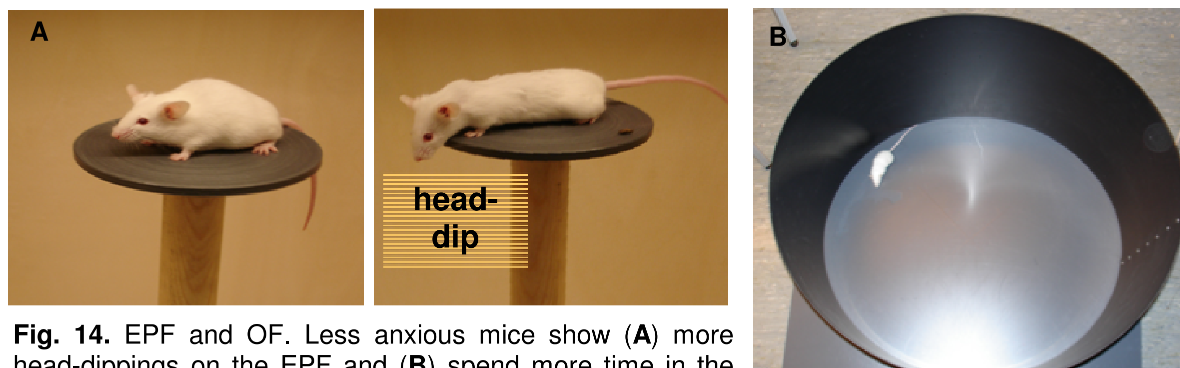


Fig. 14. EPF and OF. Less anxious mice show (**A**) more head-dippings on the EPF and (**B**) spend more time in the inner zone of the OF.

2.3.4 OF

The open field (∅ 60cm) is divided into an inner (∅ 30cm) and an outer area by a line on the floor. The inner zone is additionally divided in four and the outer zone in eight compartments to count line crossings. Animals were placed in the center of the field and observed for 5min. Before each trial, the chamber was cleaned with water containing a detergent. The animals' behavior was videotaped and following parameters were obtained by a trained observer blind to line or treatment using "Eventlog 1.0" software (EMCO): time in the inner / outer zone, number of rearings, and number of line crossings. A mouse was considered to have entered the inner or outer zone when two feet had passed the dividing line (Fig. 14B).

2.3.5 TST

During 6min of TST, the mouse is suspended by the end of its tail to a bar 35cm above the floor (Fig. 15A).

The animals' behavior was videotaped and duration of total immobility was scored by a trained observer blind to line or treatment using "Eventlog 1.0" software (EMCO).

2.3.6 FS

The animal is placed for 6min in a beaker-glass (∅ 11cm) filled with water of 23°C up to 15cm and videotaped (Fig. 15B). Struggling (forepaws brake through the water surface), swimming (leastwise one limb makes swimming-movements), floating (the animal is immobile), and latency to first floating were scored by a trained observer blind to line or treatment using "Eventlog 1.0" software (EMCO).

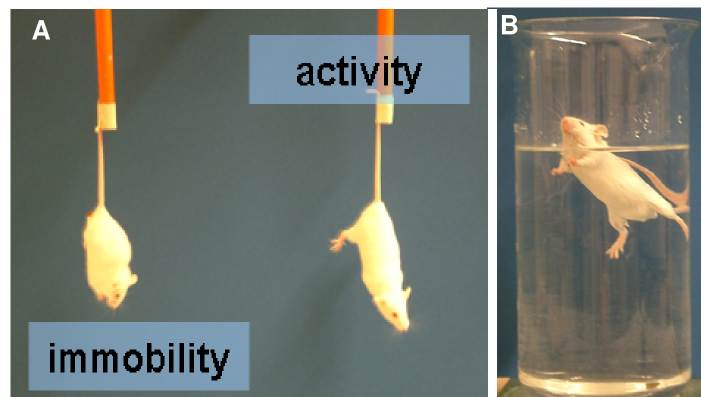


Fig. 15. (**A**) Immobility in the TST and (**B**) floating in the FS reflects depression-like behavior.

2.3.7 SRT

The SRT contained a 15min-restrained stress with a blood collection directly before and after the stressor. For restraint stress the animal was fixed in a 50ml plastic tube (Falkon) fastened on a table by modeling clay to guarantee a stable position (Fig.

16B). Blood was taken from the tail veins into a haematocrit-glass capillary via a little cut (Fig. 16A). After 5min centrifugation at 10000rpm, the plasma was transferred into a 1.5ml tube, and stored at –20°C until further analysis.

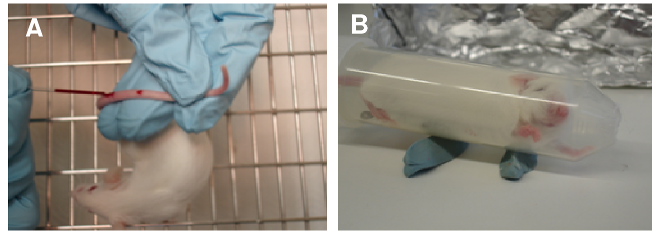


Fig. 16. SRT procedure. **A**, Blood collection from a small cut in the tail vessle. **B**, Mice are restrained for 15min in a 50ml plasice tube.

Initial and stress-induced plasma corticosterone concentration was analyzed using a radioimmunoassay to calculate the increase.

2.4 Analysis

2.4.1 Radioimmunoassays (RIA)

2.4.1.1 Corticosterone-RIA

Plasma corticosterone concentration was analyzed using a commercial available RIA kit (Rat Corticosterone 125I RIA, DRG instruments GmbH, Germany) with modified protocol. After diluting 10µl plasma in 50µl 1* PBS (phosphate buffered saline), 75µl steroid diluent for basal samples and 950µl for stress samples were added, respectively. Corticosterone concentration was determined indirectly by measuring the amount of 125I-radioactive-labelled corticosterone, not bound to an antibody.

2.4.1.2 AVP-RIA

Dialysates and plasma samples were lyophilized and tissue samples homogenized in 1ml buffer. Samples were estimated by sensitive and specific radioimmunoassays (minimal detection limit: 0.03pg/sample; intra- and interassay variations were between 7 and 10%, and 9 and 13%, respectively). Crossreactivities of the antisera with related peptides (including AVP and OXT, respectively) were < 0.7% (Landgraf et al. 1995).

2.4.2 Histochemistry

2.4.2.1 In situ Hybridization

Avp mRNA *in situ* hybridization was conducted according to the protocol of (Wigger et al., 2004). Slides were dehydrated using an alcohol line, including increasing concentrations and chloroform, and finally air-dried. A highly specific 48-base-long oligonucleotide directed against the last 16 amino acids of the glycoprotein that *Avp* mRNA does not share with OXT (5´ gca gaa ggc ccg gcc ggc ccg tcc agc tgc gtg gcg ttg ctc cgg tc; Ivell and Richter, 1984; Villar et al., 1994) was used for hybridization. The oligonucleotides were labeled by using [^{35}S]ATP (NEN DuPont, Germany) and terminal transferase (Tdt, Boehringer, Germany) and purified by tRNA (Sigma, Germany) precipitation. For incubation, the labeled oligonucleotide was add to a hybridization-mix (for details see Wigger et al., 2004) and spread over the slides with a radioactivity of 1 000 000cpm/100µl/slide. Incubation occurred in a humid chamber at 45ºC for 20h. After several washes in saline sodium citrate (SSC), slides were dehydrated and air-dried. Sections were exposed to a radiation-sensitive film (Kodak BioMax, Eastman Kodak Co., Rochester, NY) for 20h and the following blackening of the film was analyzed by "Optimas 5.22" software (Optimas Corporation, Silver Spring, USA).

For each animal 3 matched sections of the relevant region were measured to define the mean hybridization of the probe.

2.4.2.2 Silver Grain Label

^{35}S-labeled *Avp* mRNA slides were dipped for 4sec in a silver-nitrate emulsion (Kodak NTB2; Kodak, USA), dried over night at room temperature, and stored at 4°C for one week in light-tight boxes. All steps, including the development, have to occur in total darkness. After getting on room-temperature for two hours, the slides were developed in Kodak D19 developer solution (Sigma-Aldrich, Germany) for 3-4min, rinsed in water for 30sec, fixed in Kodak fixer solution (Sigma-Aldrich, Germany) for 5-7min and rinsed again in water for 25sec. After removing the emulsion from the backside of the slides with a razor blade, slides were air-dried.

2.4.3 PCR and Restriction Fragment Length Polymorphism (RFLP) Analysis

Total DNA of HAB, LAB, NAB, and CM mice were isolated from tail tissue using the NucleoSpin kit (Machery-Nagel, Düren, Germany). A fragment of 196bp including the

Avp gene signal peptide coding sequence was amplified by PCR using 5' gtt agc agc cac gtt gtc 3' as forward and 5' ctc ttg ggc agt tct gga ag 3' as reverse primers (MWG Biotech, Ebersberg, Germany), and applying a standard PCR protocol with Taq polymerase. The PCR was carried out under the following conditions: initial denaturation at 94℃ for 4min; 35 identical cycles of denaturation (94℃ for 1min), annealing (57℃ for 1min) and extension (72℃ for 1min); and a final extension of 10min at 72℃. Then 8.5µl of the PCR product were restriction digested with 1µl NEBuffer2 and 0.5µl BstNI (New England Biolabs, Frankfurt am Main, Germany) at 37℃ over night. This digestion would cut the wild type sequence, but would not cut the sequence with an allele carrying the mutation (cytosine replaced by thymine). The products of the digest were loaded on a 2% agarose gel that was run at 50V for 20min then at 70V for 70min.

2.4.4 Statistics

The data presented as means ± SEM were statistically analyzed using SPSS 12.0. Data of two independent groups were compared with the Mann-Whitney U-test (MWU-test). Data of more than two independent groups were calculated with the Kruskal-Wallis *H*-test and in case of significant variation with a *post hoc* comparison using multiple MWU-tests with sequential Bonferroni correction to adjust for multiple comparisons. Data of dependent groups were compared by the Friedman-test followed by the Wilcoxon-test. Significance was accepted with $p < 0.05$.

3 Results

3.1 AVP Deficit in LAB mice

3.1.1 Intra-PVN Release of AVP

Microdialysis (Fig. 17) revealed no difference in the release of AVP under basal conditions (samples 1-3) in HAB and LAB mice, but a significant increase in both groups after hypertonic stimulation ($p<0.001$, sample 4 vs. 3). In contrast to basal conditions, after hypertonic stimulation the amount of AVP released in the PVN of HAB animals is significantly higher than in LAB animals ($p<0.05$, sample 4).

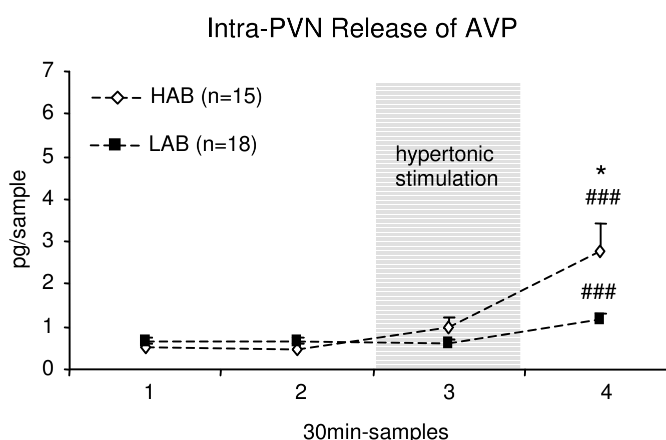


Fig. 17. Intra-PVN release of AVP in HAB and LAB mice under basal conditions and after hypertonic stimulation. * p<0.05 vs. LAB sample 4, ### p<0.001 vs. sample 3.

3.1.2 Symptoms of an AVP Deficit

3.1.2.1 Fluid Intake and Urine Osmolality

Adult mice of the three lines displayed significant differences in fluid intake ($p<0.001$) and urine osmolality ($p<0.05$). Whereas LAB mice were found to drink daily a 2-fold higher amount of water than HAB and NAB mice ($p<0.001$, Fig. 18A), only compared

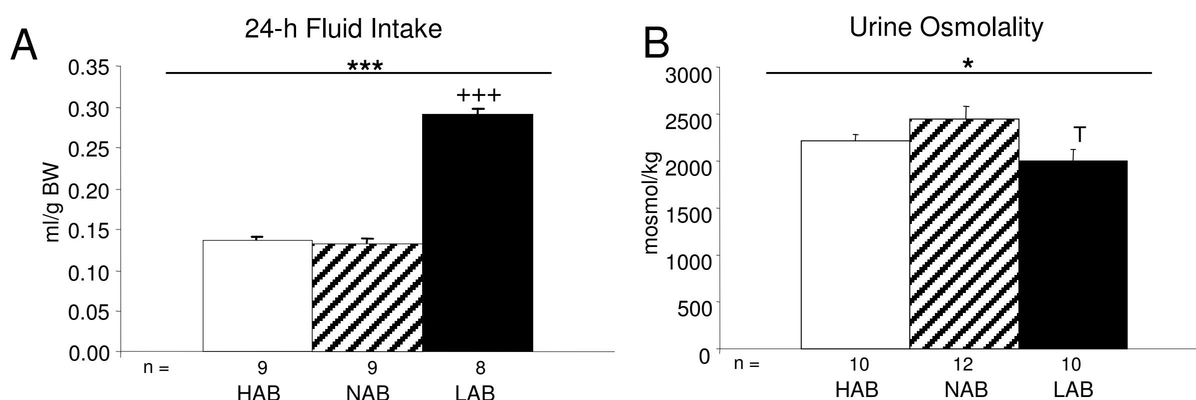


Fig. 18. 24-h fluid intake and urine osmolality of adult 12-14 week old HAB, NAB, and LAB mice. **A**, LAB mice drunk significantly more than HAB and NAB mice. **B**, Urine of LAB mice was significantly lower concentrated than the urine of NAB mice. *** p<0.001, * p<0.05; +++ p < 0.001 vs. HAB and NAB, T p=0.063 vs. NAB.

to NAB mice they displayed a lower urine concentration (p<0.05, Fig. 18B).

3.1.2.2 Water Deprivation

After 48-h water deprivation, we found a significantly increased plasma osmolality (Fig. 19A) in all three lines (HAB: p<0.001; NAB: p<0.01; LAB: p<0.05), being significantly stronger increased in LAB compared to NAB mice (p<0.05). Further, we found higher AVP plasma levels after water deprivation in HAB and NAB mice (p<0.05) compared to basal levels, which does not account for LAB mice (Fig. 19B). Consequently urine concentration (Fig. 19C) increased in all three lines (HAB: p<0.05; NAB: p<0.01; LAB: p<0.01) but less intense in LAB mice compared to HAB mice (p<0.05). There are no differences in the amount of AVP in the hypothalami of HAB, LAB, and NAB mice under basal conditions and after water deprivation (Fig. 20A). The pituitaries exhibited no basal differences in AVP content but a stronger depletion in LAB animals after water deprivation compared to HAB and NAB mice (Fig. 20B, C).

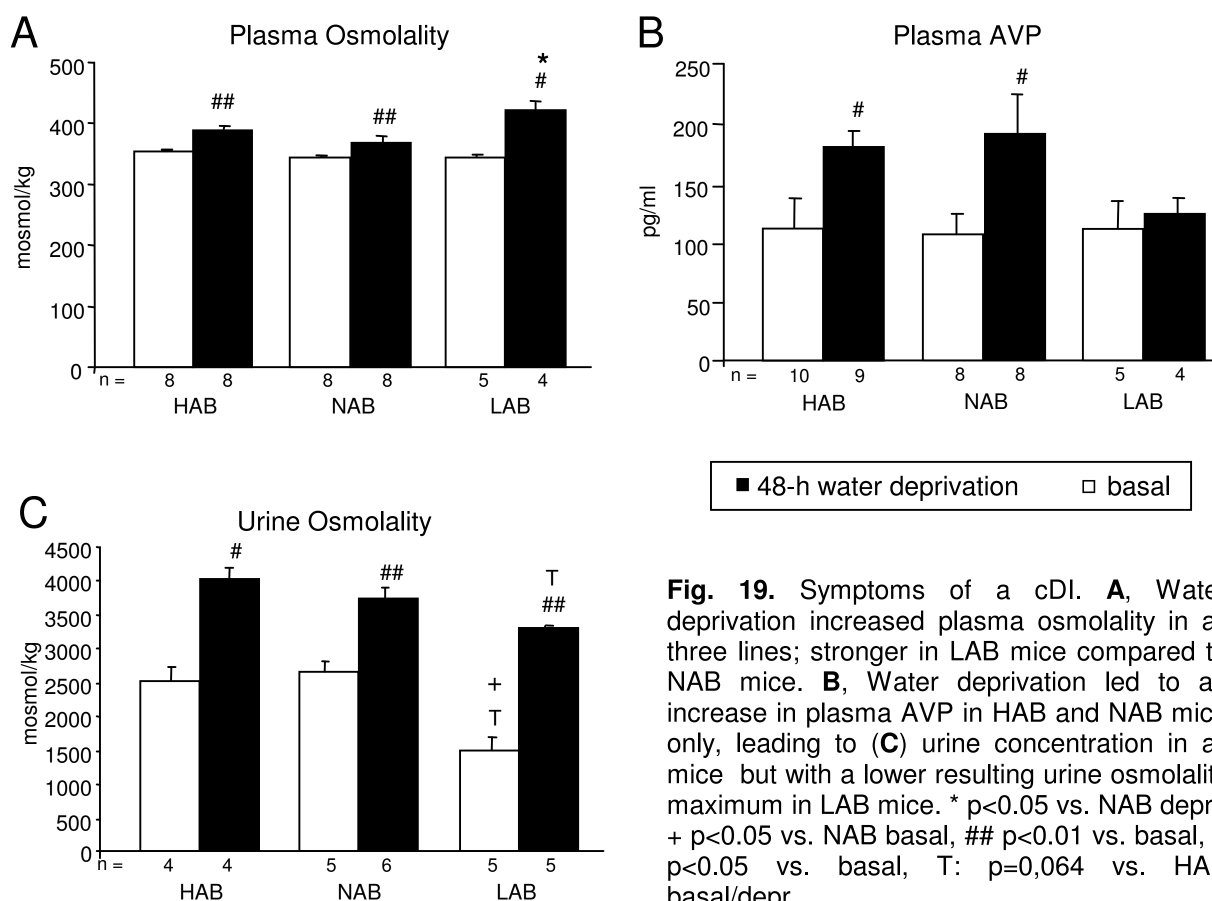


Fig. 19. Symptoms of a cDI. **A**, Water deprivation increased plasma osmolality in all three lines; stronger in LAB mice compared to NAB mice. **B**, Water deprivation led to an increase in plasma AVP in HAB and NAB mice only, leading to (**C**) urine concentration in all mice but with a lower resulting urine osmolality maximum in LAB mice. * p<0.05 vs. NAB depr., + p<0.05 vs. NAB basal, ## p<0.01 vs. basal, # p<0.05 vs. basal, T: p=0,064 vs. HAB basal/depr.

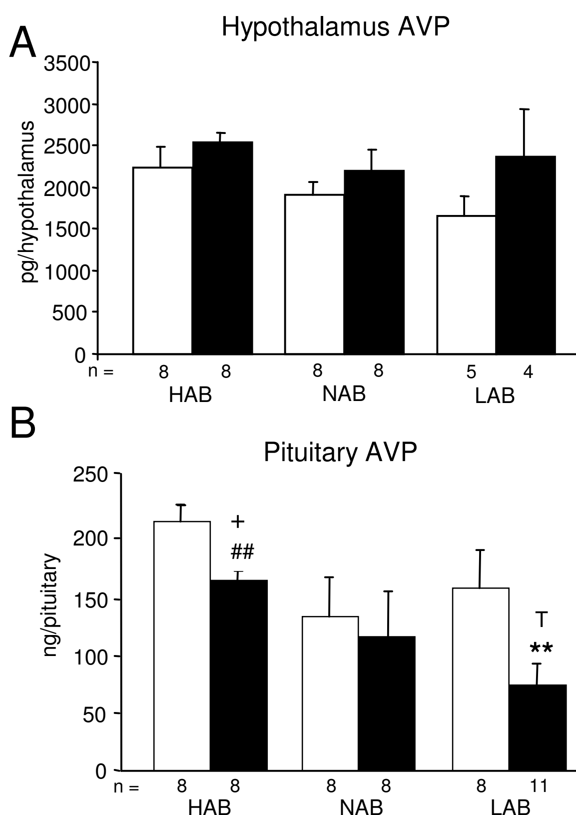


Fig. 20. AVP concentration and depletion in the hypothalamus and pituitary of HAB, NAB, and LAB mice under basal conditions and after 48h of water deprivation. **A**, There were no differences in the amount of AVP in the hypothalamus. **B,** After water deprivation, the AVP concentration in the pituitary of LABs and NABs was lower than in HAB mice. **C**, this was due to a stronger percentage decrease in LABs. ** p<0.01 vs. HAB depr., + p<0.05 vs. NAB depr., ## p<0.01 vs. basal, T p<0.1 vs. basal.

3.1.2.3 DDAVP Treatment

After treatment with vehicle LAB mice displayed a significant lower urine osmolality than HAB mice (p<0.05). Treatment with dDAVP (Fig. 21) led to a significant increase in urine osmolality in HAB, NAB, and LAB mice (p<0.05), but with a significant lower osmolality in LAB animals comparable to basal HAB and NAB levels (p<0.05).

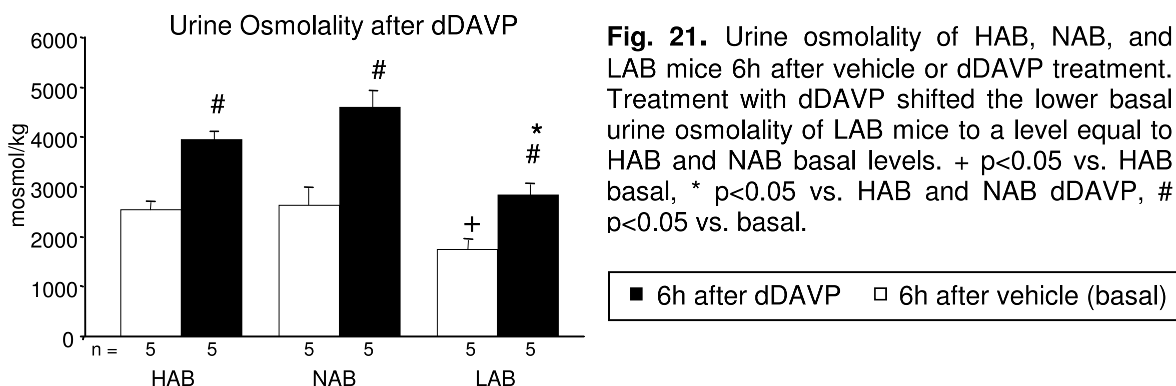


Fig. 21. Urine osmolality of HAB, NAB, and LAB mice 6h after vehicle or dDAVP treatment. Treatment with dDAVP shifted the lower basal urine osmolality of LAB mice to a level equal to HAB and NAB basal levels. + p<0.05 vs. HAB basal, * p<0.05 vs. HAB and NAB dDAVP, # p<0.05 vs. basal.

3.1.2.4 Progress of cDI Symptoms

From the 5th week on, HAB, NAB, LAB, and CM mice showed significant differences in 24h-fluid intake (week 5: p<0.01; weeks 7, 20, and 50: p<0.001, Fig. 22A) and urine osmolality (weeks 5, 7, 50: p<0.01; week 20: p<0.001, Fig. 22B). In more detail, at weeks 5, 7, 20, and 50 LAB mice drunk significant more than HAB mice (p 0.001).

Additionally, at week 7 water intake was higher than in NABs (p<0.001) and at weeks 20 and 50 also higher than in NABs (p<0.001) and CM mice (p<0.01). Further, at week 20 HAB mice drunk significantly less than NABs (p<0.05) and CMs (p<0.001) and at week 50 less than CM (p<0.05) mice. Similar was found in urine osmolality with significant differences between LAB and HAB mice at week 5 (p<0.01), between LABs, NABs (p<0.05), and CMs (p<0.05), respectively, at week 7 and between LAB and the three other lines at weeks 20 (p<0.001) and 50 (p<0.01 vs. HAB, p<0.05 vs. NAB, p<0.001 vs. CM).

The progress in fluid intake in LAB mice was also shown by an increase over the time (p < 0.001) with a significant higher fluid intake at week 5 compared to week 3 (p<0.01), at week 20 relative to weeks 3, 5, and 7 (p<0.001) and at week 50 compared to weeks 3 and 7 (p<0.01). Also in urine osmolality we found a progressive decrease over time with LAB mice at weeks 20 (p<0.05) and 50 (p<0.001) displaying lower urine osmolalities than LABs at weeks 3 and 7.

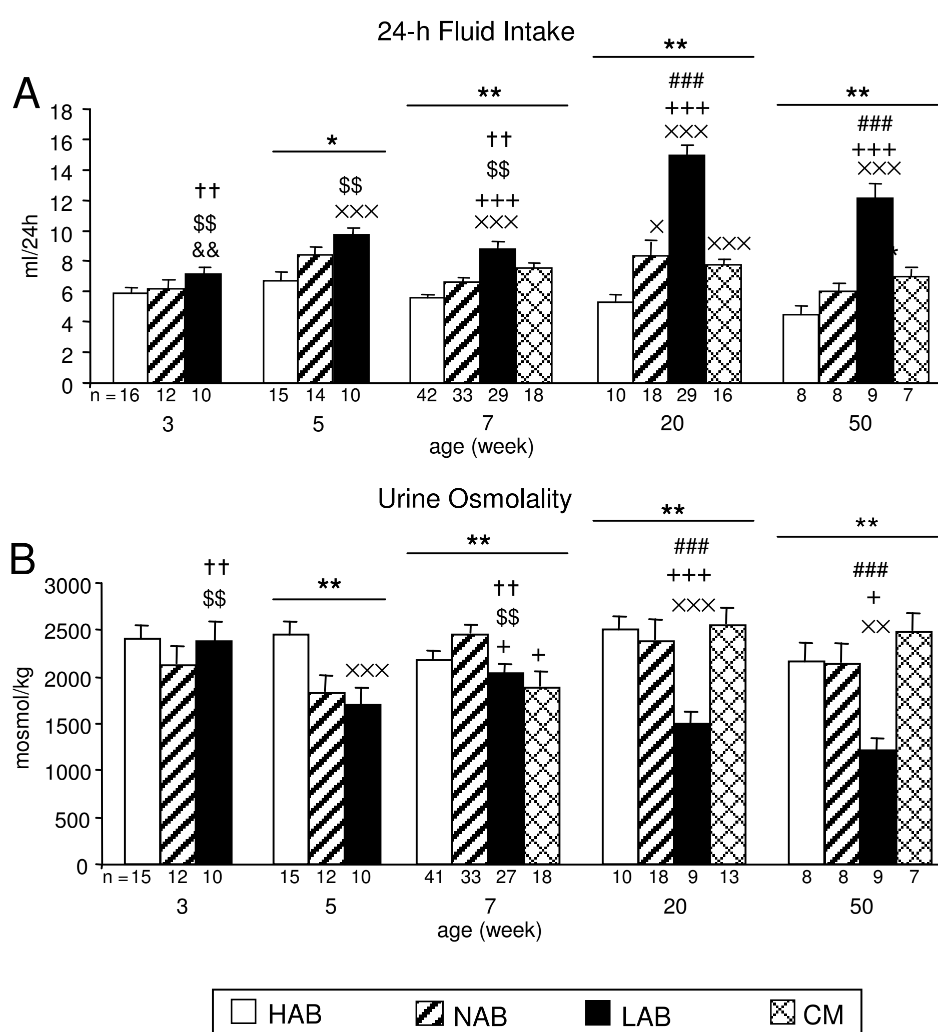


Fig. 22. Signs of cDI. **A**, 24-h fluid intake of HAB, NAB, LAB, and CM mice from week 3 to week 50. LAB drunk significantly more than the other lines. At week 20 and 50, HAB drunk less than NAB and CM. **B**, Urine osmolality of HAB, NAB, LAB, and CM mice from week 3 to week 50. Urine osmolality of LAB mice wasless compared to the other lines. At week 20 and 50, HAB drunk less than NAB and CM. ** p<0.01, * p<0.05 (same age); ××× p<0.001 vs. HAB, ×× p<0.01 vs. HAB, × p<0.05 vs. HAB, +++ p<0.001 vs. NAB, + p<0.05 vs. NAB, ### p<0.001 vs. CM, p< 0.01 vs LAB50, $$ p<0.01 vs. LAB20, && p<0.01 vs. LAB5.

3.1.2.5 EPM Behavior of aged HAB, NAB, and LAB Mice

In the EPM test HAB, NAB, and LAB mice at an age of 7 and 50 weeks, respectively, (Fig. 23) showed significant differences in percentage of time spent on the open arms ($p < 0.001$). *Post hoc* comparisons between the three lines confirmed the differences between HABs, NABs, and LABs at an age of 7 weeks (HAB vs. LAB/NAB: $p<0.01$; NAB vs. LAB: $p<0.05$) and an age of 50 weeks (LAB vs. HAB/NAB: $p<0.01$; HAB vs. NAB: $p<0.05$). There are no differences in percentage of time spent on the open arms for HABs, NABs, and LABs between the two time points.

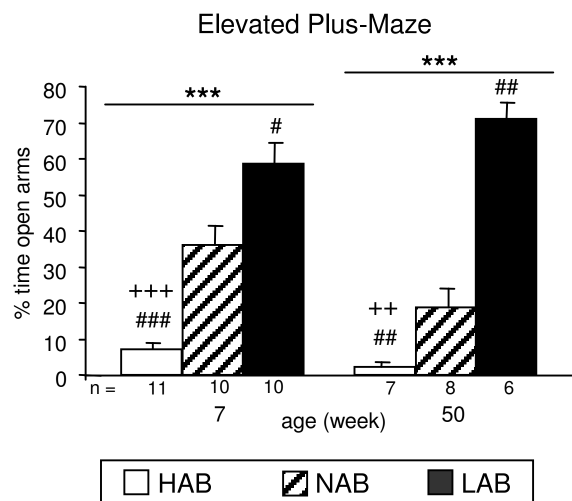


Fig. 23. Anxiety-related behavior on the EPM of 7 and 50 week old HAB, NAB, and LAB mice. HAB animals of both ages spend significantly more and LAB animals of both ages spend significantly less time on the open arm of the EPM than NAB mice. *** p<0.001 (same age); +++ p<0.001 vs. LAB, ++ p<0.01 vs. LAB, ### p<0.001 vs. NAB, ## p<.0.1 vs. NAB, # p<0.05 vs. NAB.

3.1.2.6 RFLP Analysis in the AVP Precursor Gene

The restriction enzyme cuts the wild-type sequence producing two small fragments of 112 and 84bp (HAB allele), whereas the sequence carrying the mutation is not affected by the enzyme resulting in one fragment of 196bp visible on the gel (Fig. 24).

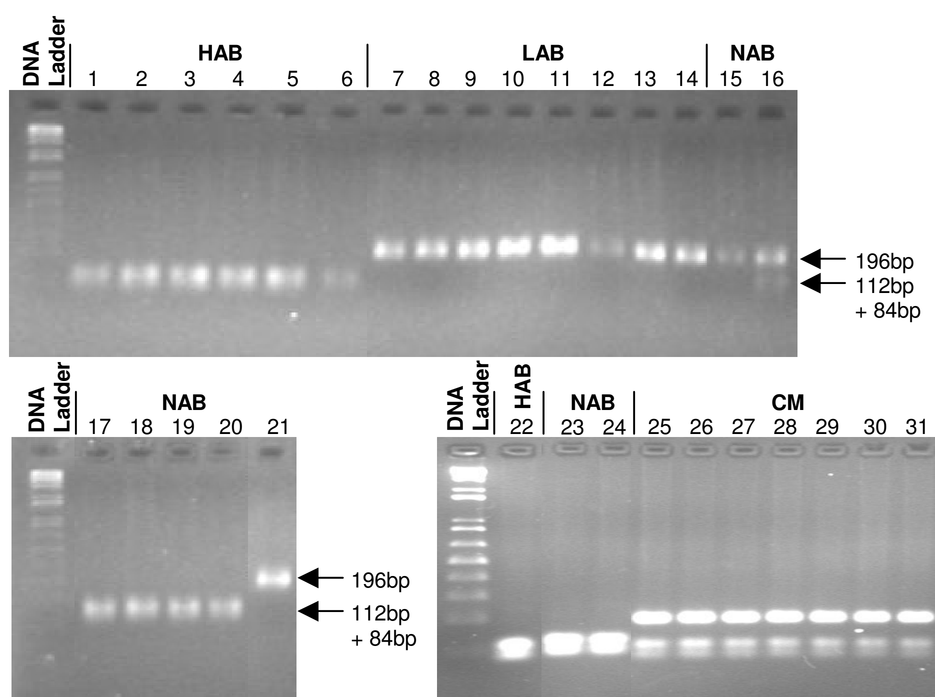


Fig. 24. RFLP analysis showed two fragments (112 and 84bp) for HABs (line 1-6, 22) and one fragment of 196bp for LABs (line 7-14) mice, 3 fragments for heterozygous CM mice (line 25-31), and all possibilities for NAB mice (line 17-20, 23, 24: homozygous for the wild-type allele, line 15, 21 homozygous for the mutant allele and line 16 heterozygous).

HAB, LAB, NAB, and CM mice analyzed for their *Avp* sequence had all the expected alleles. LAB mice were homozygous for the C(+40)T mutation, HAB mice were homozygous for the wild-type allele, NAB animals were both homozygous for the wild type or the mutant allele as well as heterozygous, and CM mice were heterozygous.

3.1.2.7 Avp mRNA Expression in the PVN

In situ hybridization of *Avp* mRNA in the PVN showed a significant difference concerning both relative intensity (Fig. 25A) and area (Fig. 25B) of labeled *Avp* mRNA between the lines. At PND 5, weeks 3 and 7 ($p<0.01/0.05$), with a lower *Avp* mRNA label in LAB mice disappearing at week 20, and an increase of labeled area in LABs over the time ($p<0.05$) shown by significant differences between PND 5 and weeks 7 and 20 ($p<0.05$), respectively.

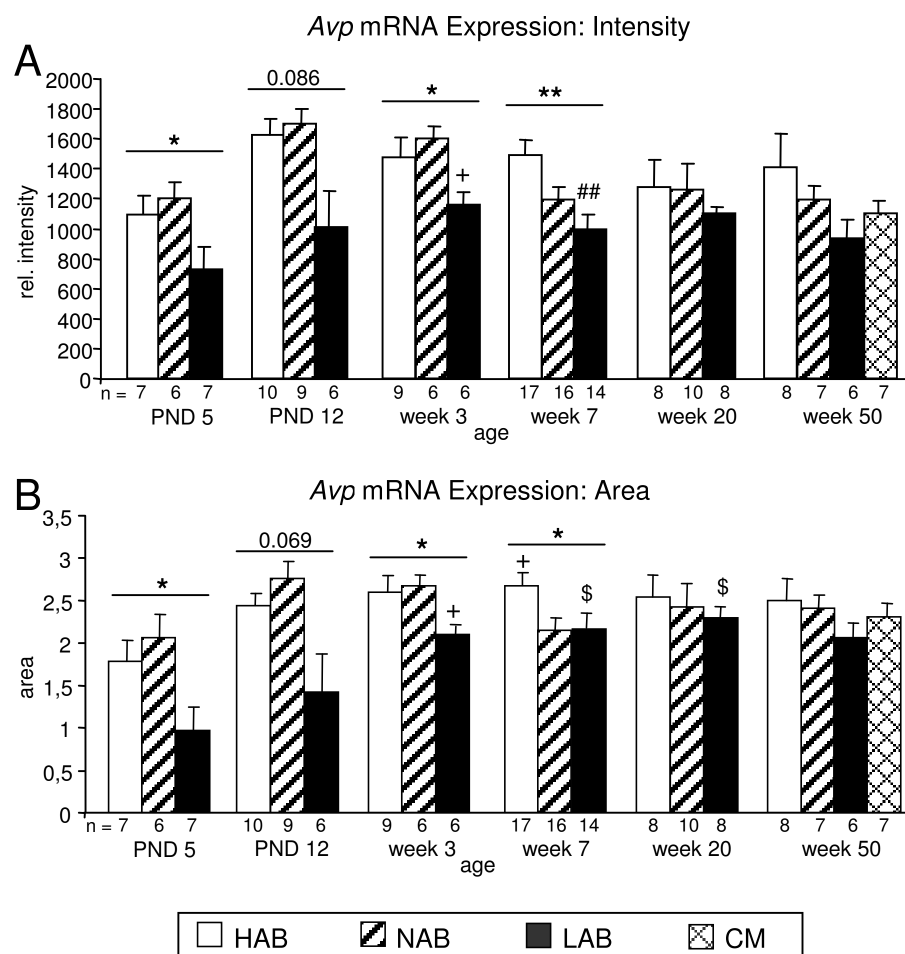


Fig. 25. *Avp* mRNA *in situ* hybridization in PVN neurons of HAB, NAB, LAB, and CM mice. **A**, Relative intensity of *Avp* mRNA label. **B**, Area of *Avp* mRNA label. LAB mice showed a decreased *Avp* mRNA-labeled area at PND 5, week 3 and 7 compared to HAB and NAB mice, respectively, with an increase from PND 5 to later timepoints. ** $p<0.01$, * $p<0.05$ (same age); ## $p<0.001$ vs. HAB, + $p<0.05$ vs. NAB, & $p<0.05$ vs. LAB5.

with the close collaboration of Mirjam Bunck

3.1.3 Viral-Vector-induced Increase in *Avp* mRNA Expression in the PVN of LAB Mice

3.1.3.1 Behavior

In the EPM test, animals showed significant differences in the latency to the first entry onto the open arms (p<0.01) with AAV-*Avp* treated mice displaying an increased latency (p<0.05 vs. AAV-*lacZ*; p<0.01 vs. Control) but no differences in the time spent on the open arms (Fig. 26A). Additionally, the three groups displayed a significant difference in total (p<0.05) and a trend in open (p<0.061) arm entries with AAV-*Avp* treated mice having less total entries compared to AAV-*lacZ* treated and Control mice (p<0.05). This is due to a decreased number of entries onto the open arms compared to control mice (p<0.05), but no differences onto the closed arms (Fig. 26B).

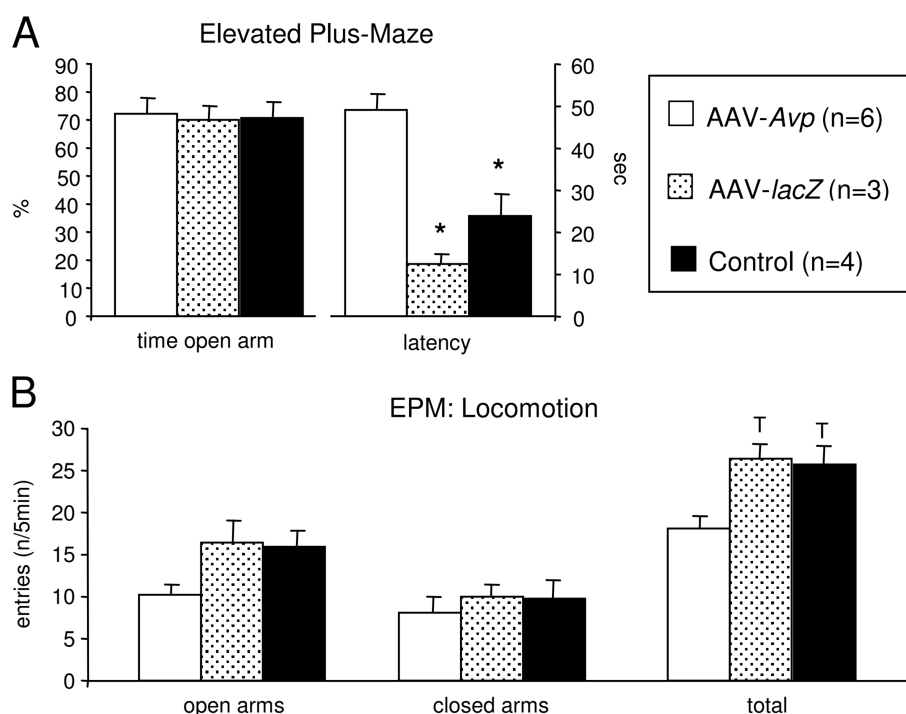


Fig. 26. EPM behavior after AAV-*Avp* treatment. AAV-*Avp* treated mice showed (**A**) a increased latency to the first open arm entry and (**B**) decreased locomotion due to less open arm entries. * p<0.05 vs. AVP, T p<0.075 vs. AVP.

In further tests, AAV-*Avp* treated animals showed no significant behavioral differences in the DaLi (Fig. 27A), on the EPF (Fig. 27B), in the FS test (Fig. 27C), in TST (Fig. 27D), and in the OF (Fig. 27E, F) compared to AAV-*lacZ* treated and Control animals. Also the SRT revealed no differences in initial, stress values, and the increase of plasma corticosterone between the three treatment groups (Fig. 27G).

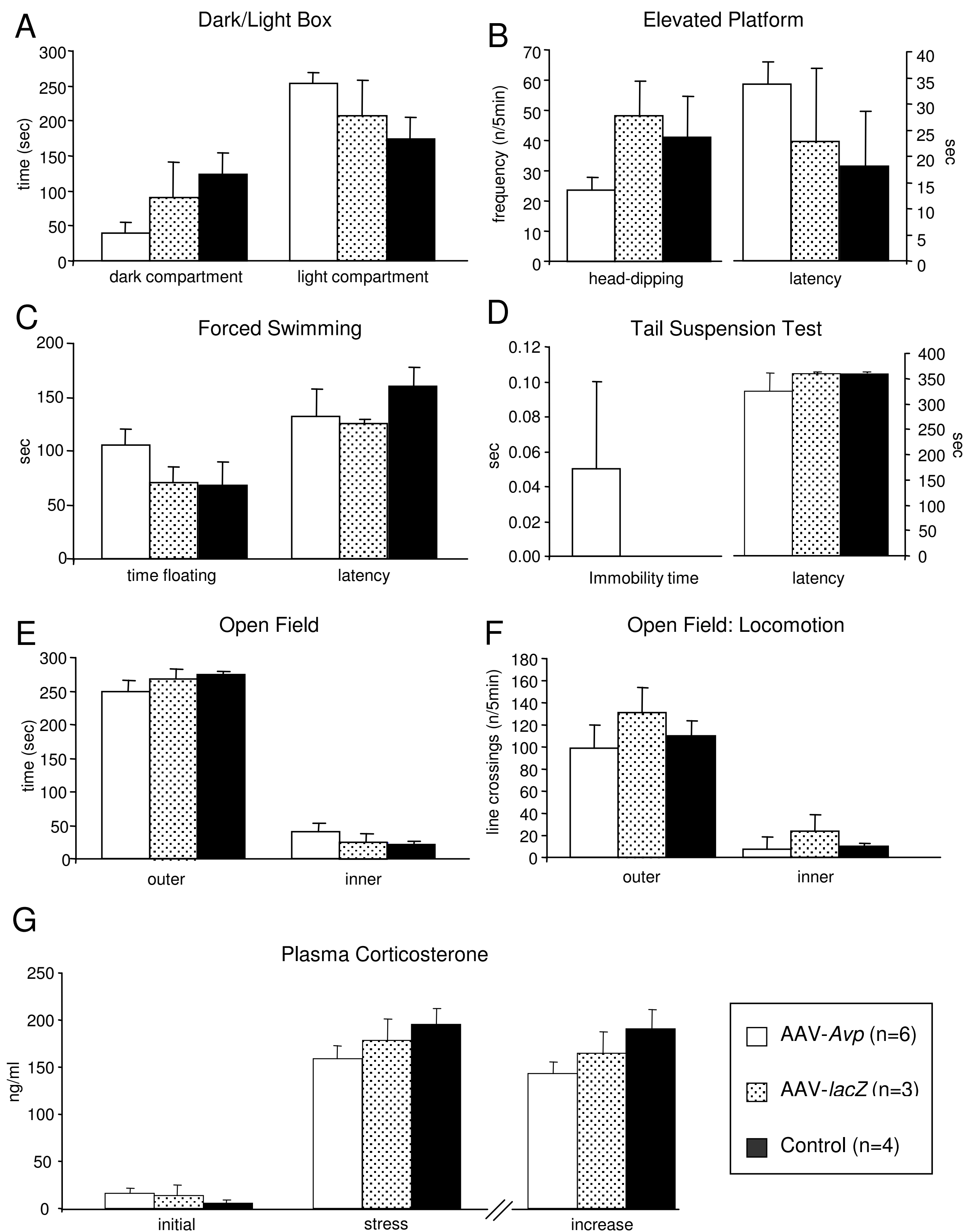


Fig. 27. Behavioral analysis after AAV-*Avp* treatment. AAV-*Avp* treated mice displayed no behavioral differences (**A**) in the DaLi, (**B**) on the EPF, (**C**) in the FS test, (**D**) in the TST, and (**E**, **F**) in the OF compared to AAV-*lacZ* treated and Control animals. **G**, AAV-*Avp* treated mice showed no different initial or stress-induced maximal values and increase of plasma corticosterone concentrations after restrained stress.

3.1.3.2 Fluid Intake and Urine Osmolality

Over the time course of the experiment, from day 10 to day 65 after surgery, AAV-*Avp* treated mice exhibited a significant difference in 24-h fluid intake (p<0.001, Fig. 28A) and urine osmolality (p<0.05, Fig. 28B) due to daily fluctuations, but with no noticeable decrease or increase over time. This is confirmed by no differences in fluid intake and urine osmolality found between the three treatment groups within single time points.

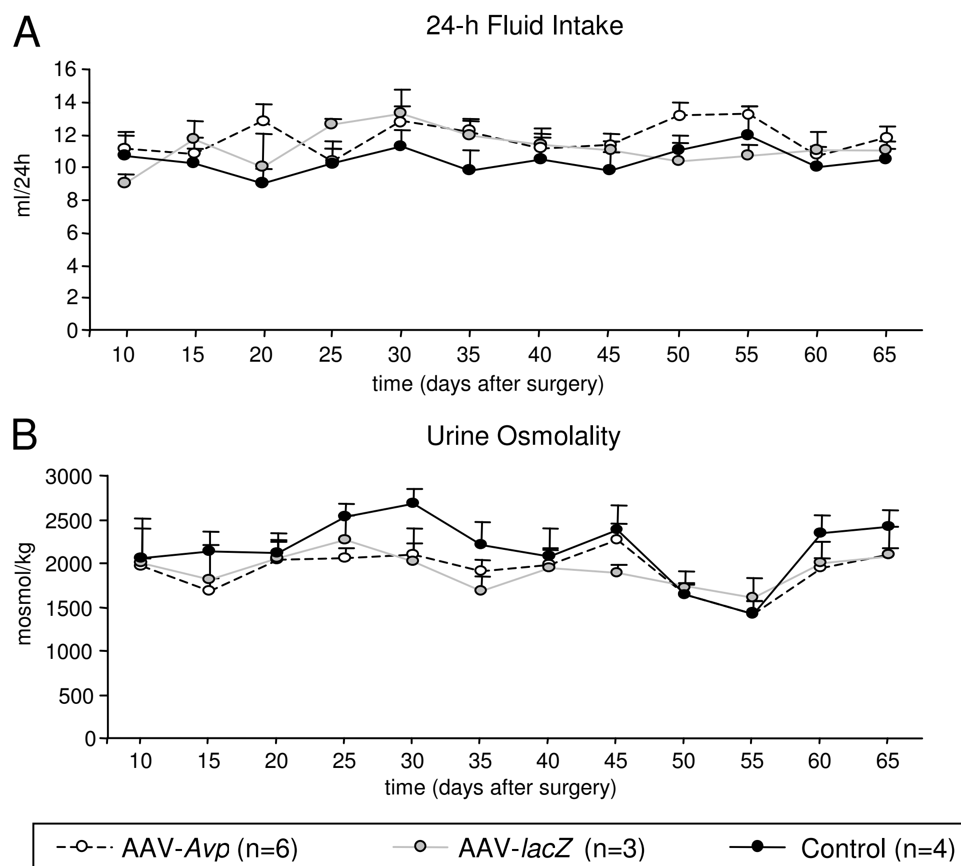


Fig. 28. AAV-*Avp* influence on daily fluid intake and urine osmolality. (**A**) Fluid intake and (**B**) urine osmolality revealed no difference between the three groups on each time point. Over the time course, fluid intake and urine osmlality showed a significant difference over the time for the AAV-*Avp* group but with unspecific fluctuations.

3.1.3.3 Avp mRNA Expression in the PVN

Statistical analysis of *Avp* mRNA *in situ* hybridization revealed just a tangential difference in *Avp* mRNA expression in the PVN (p=0.093) with AAV-*Avp* treated mice displaying higher levels of *Avp* mRNA expression compared to animals of the AAV-*lacZ* treated group and Controls (Fig. 29).

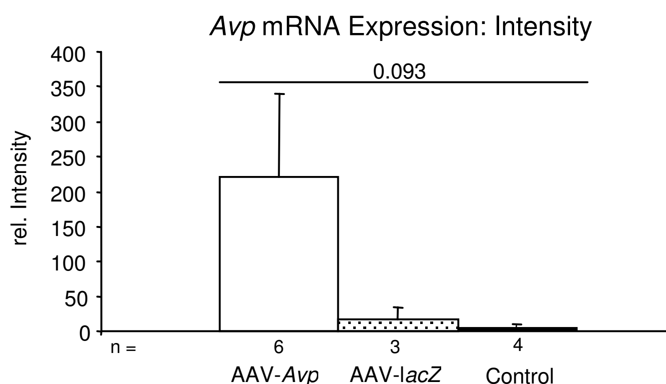


Fig. 29. *Avp* mRNA expression in the PVN after AAV-*Avp* treatment. AAV-*Avp* treated LAB mice expressed more *Avp* mRNA than AAV-*lacZ* treated or Control mice.

3.2 Postnatal Maternal Influence on the HAB/LAB-Phenotype

3.2.1 Maternal Behavior of HAB and LAB Mice

3.2.1.1 Reproductive Success

All of the HAB and 79.1% of the LAB females got pregnant. 15% HAB and 36.8% LAB dams lost their pups directly during or after birth due to a lack of correct maternal behavior including licking and nursing. This resulted in a rate of total reproduction failures of 15% in HAB mothers and 50% in LAB mothers (Fig. 30).

		mated dams	failed pregnancy	dams gave birth	failed birth	mated dams	total failures
HAB	n	20	0	20	3	20	3
	%	100	0	100	15	100	15
LAB	n	24	5	19	7	24	12
	%	100	20.8	100	36.8	100	50

Fig. 30. Breeding success in HAB and LAB mice. All HAB females got pregnant, but 3 failed to give birth correctly. 5 LAB females failed to get pregnant and 7 lost their pups during birth. This resulted in a total failure rate of 15% in HAB and 50% in LAB mice.

3.2.1.2 Time Course

In the following, data of HABs and LABs are further split in Control and Cross group, because of the experimental design (see: 2.2.2.1, 2.2.2.2). On PND 2 and 4 dams of the four groups exhibited great differences in maternal investment (Mother ON: PND 2 $p<0.05$, PND 4 $p<0.05$, Fig. 31A; Mother OFF: PND 2 $p<0.05$, PND 4 $p<0.05$, Fig. 31B). This was attributed to differences in arched back nursing, still existent on PND 8 ($p<0.01$, Fig. 31C). Though, Bonferroni correction due to multiple testing resulted in no significant differences in between the four treatment groups. A biologically justified comparison of HAB and LAB dams within a treatment group, neglecting the Bonferroni correction (used in all following comparisons), led to significant higher levels of arched back nursing in HAB dams of both groups compared to the equivalent LAB dams (PND 2 $p<0.05$, PND 4 $p<0.01$, PND 8 $p<0.01$ (vs. Control), Fig. 31C). All four groups showed a decline in arched back nursing over the course of time ($p<0.001/0.01$, Fig. 31C). Blanked posture nursing increases over the time ($p<0.05/0.01$), except in LAB Cross mothers, with a strong difference between the lines ($p<0.001$) in both treatment groups on PND 12 ($p<0.001$; Fig. 31E).

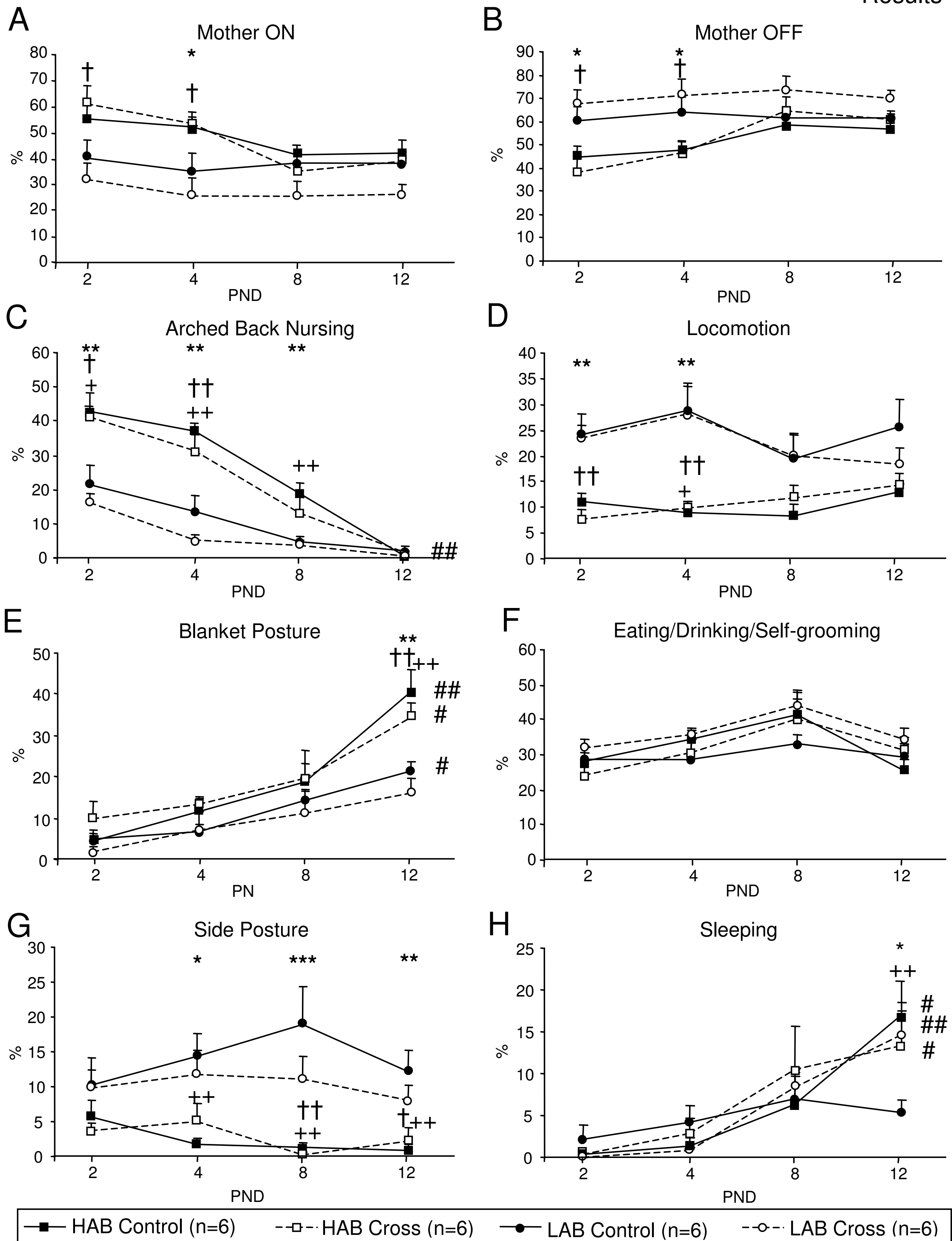


Fig. 31. Time Course**. A, B**, On PND 2 and 4 dams of the two lines exhibited differences in the maternal rearing style due to differences in (**C**) arched back nursing also seen on PND 8 with higher levels in HAB dams in both treatment- groups, but with an decline over the time course in all groups. **E**, Blanked posture nursing increases except in LAB cross mothers, with finally HAB mothers exhibiting higher levels on PND 12. **G**, LAB dams displayed increased levels of side posture compared to HAB mothers on PND 4, 8, and 12. **D**, outside the nest, LAB mothers showed a increased locomotor activity compared to HAB mothers on PND 2 and 4. **F**, Both lines show no differences in active self-directed behavior (eating/drinking/self-grooming) and (**H**) a parallel increase in sleeping over the observation time except of LAB control dams. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (same time); ++ $p < 0.01$, + $p < 0.05$ vs. LAB Control, †† $p < 0.01$, † $p < 0.05$ vs. LAB Cross, ## $p < 0.01$, # $p < 0.05$ (same line).

Side posture nursing revealed no difference on PND 2, but on PND 4 ($p<0.05$) with a difference between HAB and LAB in the Control group ($p<0.01$), and on PND 8 and PND 12 ($p<0.01$) with differences between HAB and LAB dams in both treatment groups ($p<0.05/0.01$, Fig. 31G). Further, the four groups showed differences in locomotor activity on PND 2 and 4 ($p<0.01$) with LAB mothers exhibiting higher locomotion than HAB mothers of the same group on PND 2 ($p=0.065$ (Control), $p<0.01$ (Cross)) and 4 ($p<0.01$, Fig. 31D). The difference diminished on PND 8 and 12. The four groups displayed no differences in active self-directed behaviors (eating/drinking/self-grooming, Fig. 31F) and sleeping (Fig. 31H) over the observation time except on PND 12 in the latter ($p<0.05$) due to LAB Control dams sleeping significantly less than HAB control and LAB Cross dams ($p<0.01$). This was also seen by an increase in sleeping in HAB Controls and crosses and LAB Cross mothers ($p<0.05/0.01$), which was not seen in LAB Controls.

3.2.1.3 PND 2

The main differences in maternal investment between the lines were at 3-4p.m. (Mother ON: $p<0.05$, Mother OFF: $p<0.01$, Fig. 32A, B). In particular, arched back nursing ($p<0.01$) and blanked posture ($p<0.05$) were different, with HAB mothers of both treatment groups showing more arched back nursing ($p<0.05/0.01$, Fig. 32C) and HAB Cross displaying more blanked posture nursing ($p<0.05$, Fig. 32E) compared to the equivalent LAB group. Also at 11-12p.m., arched back nursing was significantly different between the lines ($p<0.05$, Fig. 32C). Concerning side posture nursing, the groups exhibited no differences (Fig. 32G). Further, the lines showed differences in locomotion and active self-directed behavior (eating/drinking/self-grooming), significant at 7-8a.m. and 3-4 p.m. ($p<0.05$). In the former, HAB Crosses and HAB Controls, respectively, exhibiting lower locomotor activity than the equivalent LAB group ($p<0.05$; Fig. 32D). In the latter, significant difference were displayed at 3-4p.m. ($p<0.05$; Fig. 32F). Concerning sleeping, the groups exhibited no differences (Fig. 32H).

3.2.1.4 PND 4

The main intraday differences in nursing behavior were also on PND4 at 3-4p.m. ($p<0.05$, Fig. 33A), but with more spacious and pronounced alterations concerning arched back nursing ($p<0.05/0.01$, Fig. 33C) and side posture nursing ($p<0.01$, Fig. 33G). Thus, LAB mothers showed less arched back nursing at 11-12a.m. ($p<0.01$),

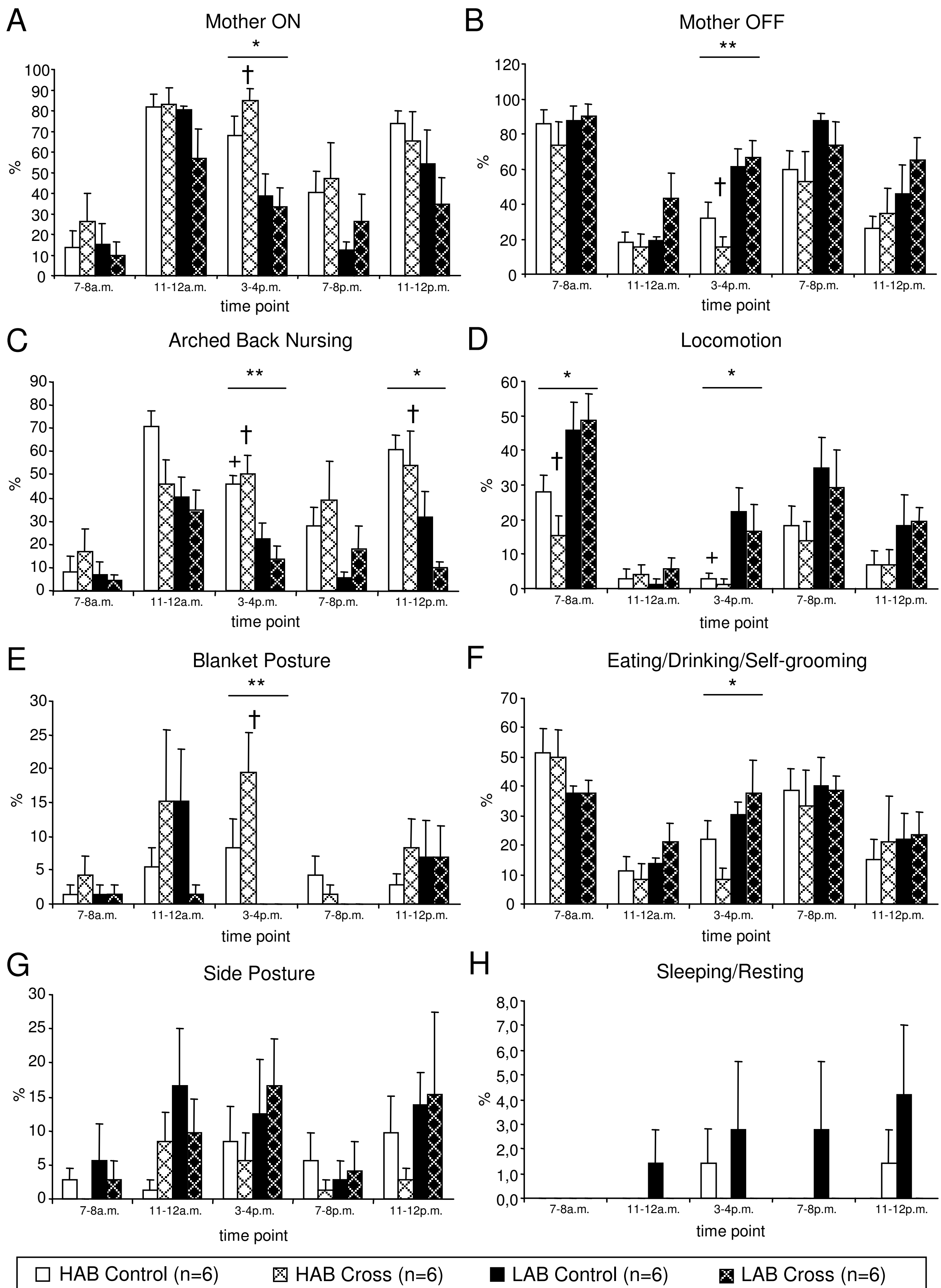


Fig. 32. PND 2. **A, B,** On PND 2 the main intraday differences in maternal care were at 3-4p.m. At that time HAB dams showed high investment in nursing, mainly (**C**) ached back and blacked posture. **G**, The four groups exhibited no difference in side posture nursing. Outside the nest, LAB dams displayed (**D**) high locomotor activity and (**F**) active self-directed behavior (eating/drinking, grooming) with the most obvious differences at 3-4 p.m.. ** p < 0.01, * p < 0.05 (same time piont); + p < 0.05 vs. LAB Control, †† p < 0.01, † p < 0.05 vs. LAB Cross.

3-4p.m. ($p<0.01$), and 11-12p.m. ($p<0.01$) and higher levels of side posture nursing at 11-12a.m. ($p<0.01$) than HAB mothers. Although Mother OFF revealed no significant differences between the lines (Fig. 33B), locomotion showed differences, significant at 7-8a.m. ($p<0.01$) and 3-4p.m. ($p<0.05$, Fig. 34D) with HAB mothers exhibiting lower levels ($p<0.05/0.01$).

3.2.1.5 PND 8

Mother ON and mother OFF revealed significant differences at 11-12a.m. ($p<0.05$, Fig. 34A, B), with no differences between the lines but between LAB Control and LAB Cross dams ($p<0.05$). Arched back nursing was less shown at PND 8 except at 11-12a.m. and 3-4p.m., revealing differences between the lines ($p<0.05/0.01$) with HAB mothers displaying higher levels compared to LAB mothers ($p<0.05$, Fig. 34C). The lines showed no differences in blanked posture (Fig. 34E), but in side posture nursing ($p<0.01$) with LAB mothers still exhibit higher levels than HAB mothers ($p<0.01$, Fig. 34G). Outside the nest, the mice displayed differences in locomotion (Fig. 31D) and active self-directed behavior (eating/drinking/self-grooming Fig. 34F), significant ($p< 0.05$) at 11-12p.m. and at 11-12a.m., respectively.

3.2.1.6 PND 12

HAB and LAB dams showed no differences in time spent inside or outside the nest (Fig. 325A, B). They performed less arched back nursing during the day with no difference between the lines (Fig. 35C), but more blanked posture nursing, especially in HAB mothers at 11-12a.m. ($p<0.05$, Fig. 35E), and side posture nursing, especially in LAB mothers at 11-12a.m. and 3-4p.m. ($p<0.05$, Fig. 35G). Outside the nest differences in locomotion are less distinct, but with differences at 3-4p.m. ($p<0.05$, Fig. 35D). The difference in sleeping is attributed to very low sleeping in LAB Control mothers at 7-8a.m. ($p<0.05$, Fig. 35H).

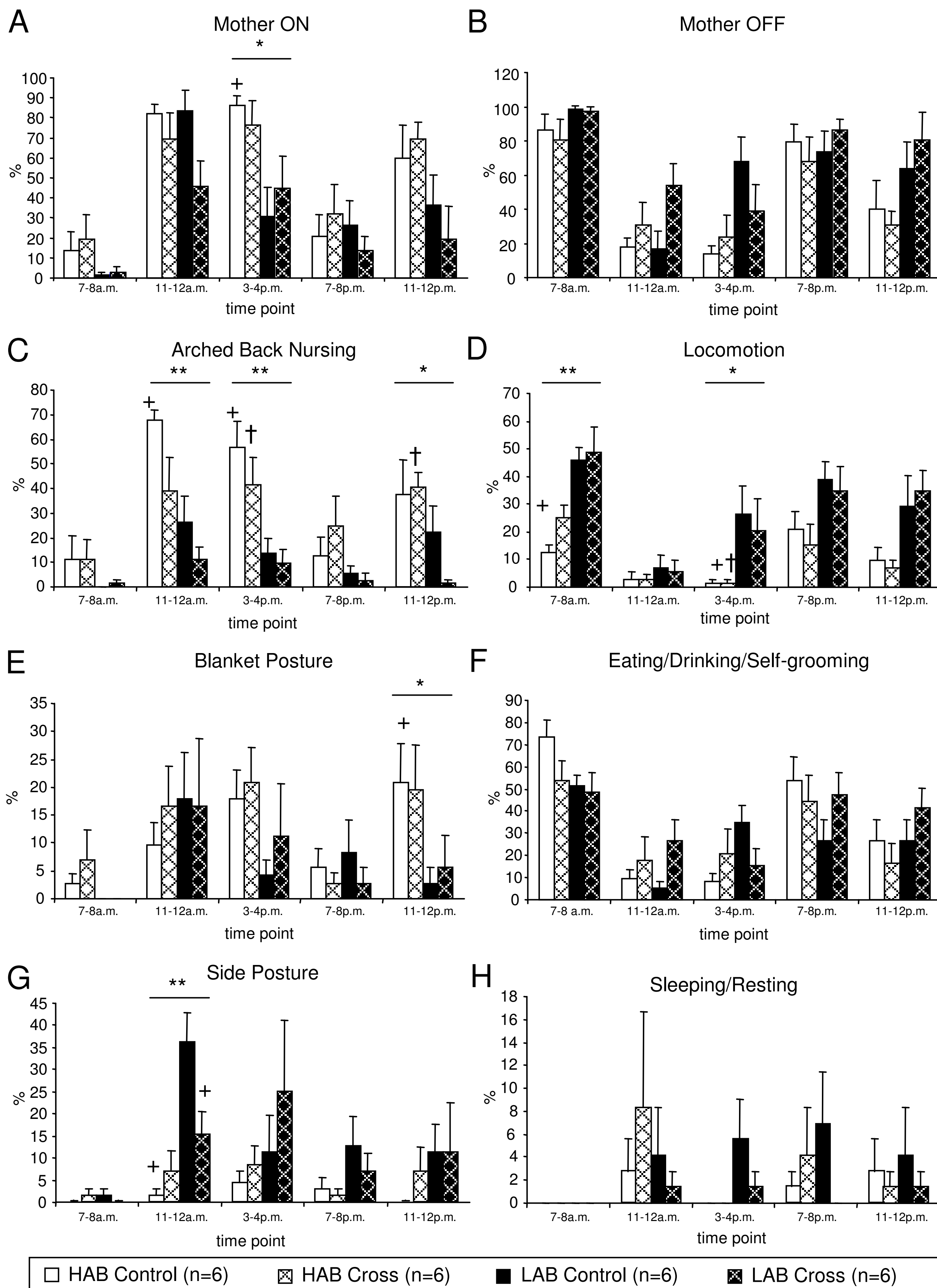


Fig. 33. PND 4. **A**, The main intraday differences in nursing behavior were still at 3-4p.m but with more spacious and pronounced alterations concerning the nursing style over the day. **C**, Thus, LAB mothers showed less arched back nursing at 11-12a.m., 3-4 p.m. and 11-12p.m. and (**G**) higher levels of side posture nursing at 11-12a.m. than HAB mothers. **B**, Although Mother OFF revealed no significant differences between the lines, (**D**) LAB mothers exhibited still differences in locomotion over the day, significant at 7-8a.m. and 3-4p.m. ** $p < 0.01$, * $p < 0.05$ (same time point); ++ $p < 0.01$, + $p < 0.05$ vs. LAB Control, †† $p < 0.01$, † $p < 0.05$ vs. LAB Cross.

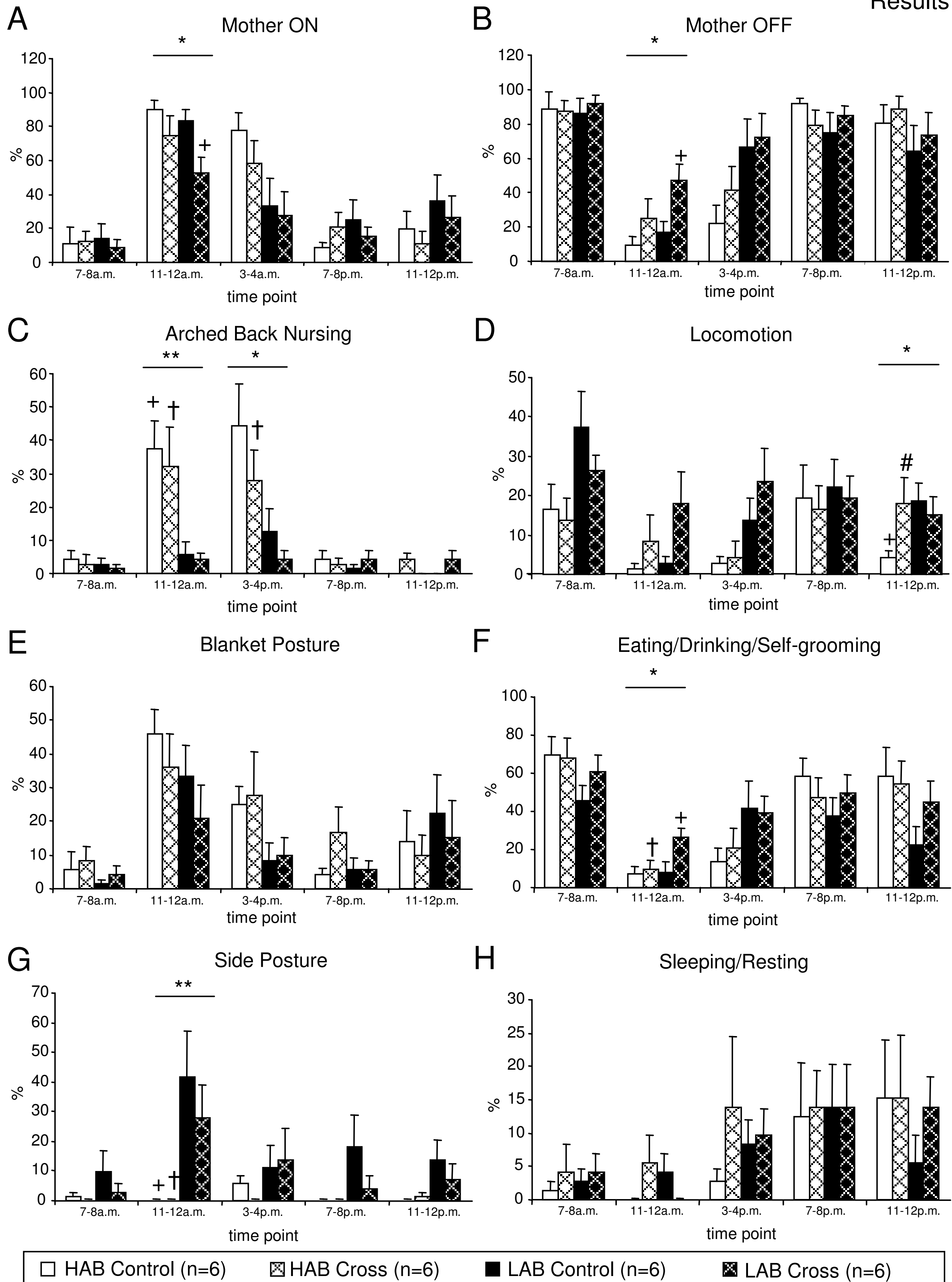


Fig. 34. PND 8. **A, B**, Mother ON and mother OFF revealed differences at 11-12a.m., with no differences between the lines but between LAB Control and LAB Cross dams. **C**, Arched back nursing was less pronounced shown at PND 8 except at 11-12a.m. and 3-4p.m. At this time point HAB mothers displayed higher levels compared to LAB mothers. **E**, The lines showed no differences in blanked posture, but (**G**) in side posture nursing with LAB mothers still exhibiting higher levels than HAB mothers. Outside the nest, the mice displayed differences in (**D**) locomotion and (**F**) active self-directed behavior (eating/drinking, grooming), significant at 11-12p.m. and at 11-12a.m., respectively. ** $p < 0.01$, * $p < 0.05$ (same time point); ++ $p < 0.01$, + $p < 0.05$ vs. LAB Control, † $p < 0.05$ vs. LAB Cross. # $p < 0.05$ vs. HAB Control.

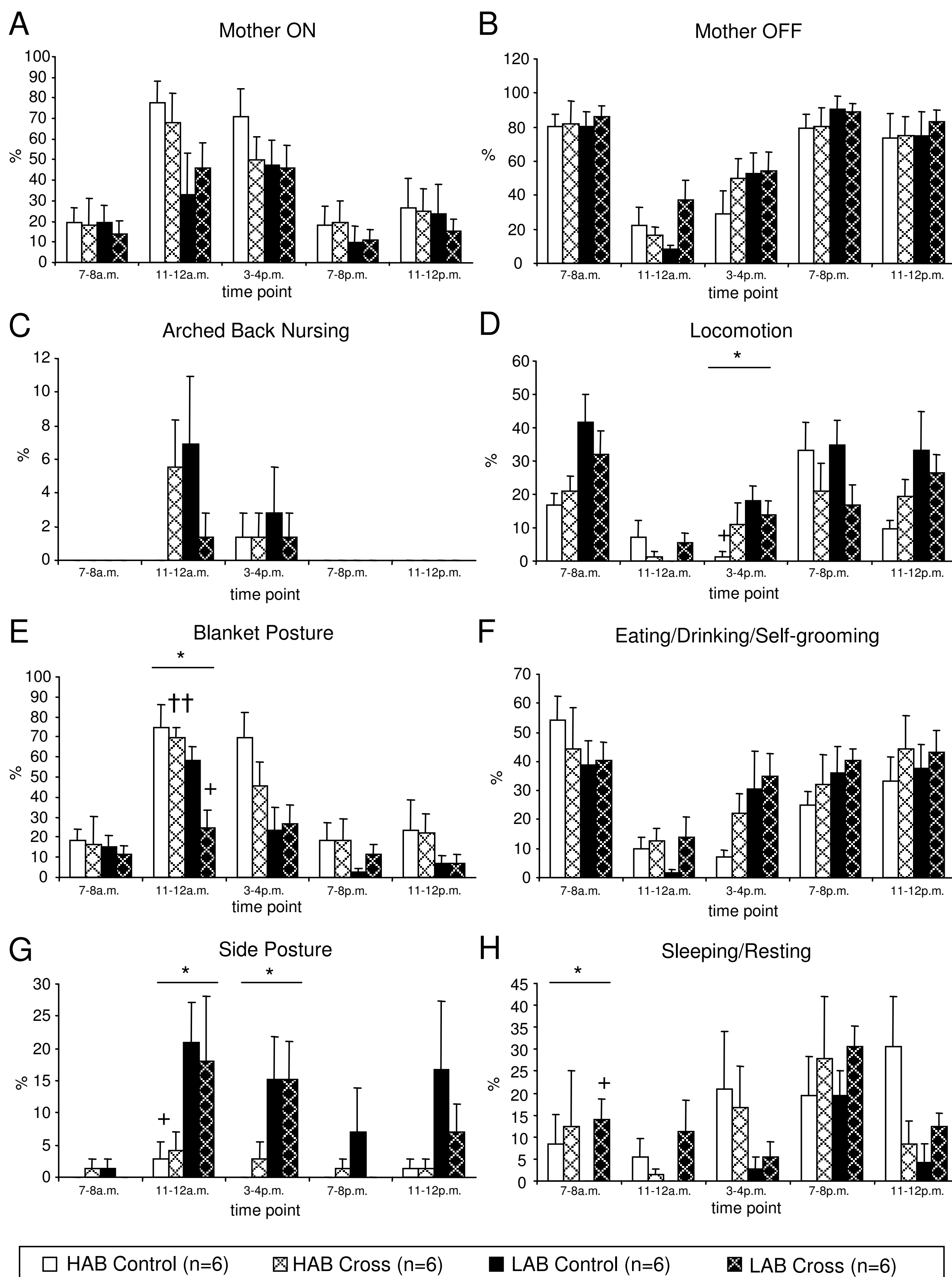


Fig. 35. PND 12. **A, B**, HAB and LAB dams showed no differences in time spent inside or outside the nest. They performed (**C**) less arched back nursing with no difference between the lines, but (**E**) more blanked posture nursing, especially in HAB mothers at 11-12a.m., and (**G**) side posture nursing, especially in LAB mothers at 11-12a.m. and 3-4p.m. **D**, Outside the nest differences in locomotion are less distinct but with differences at 3-4p.m. **H**, The difference in sleeping is attributed to very low sleeping in LAB control mothers. * p < 0.05 (same time point); + p < 0.05 vs. LAB Control, †† p < 0.01 vs. LAB Cross.

3.2.2 Cross-Fostering HAB/LAB Mice

3.2.2.1 Maternal Behavior

In consideration of the data shown above (see 3.2.1.2-3.2.1.6), dams rearing pups of the other line (Cross), in general, displayed no differences in nursing style and maternal investment than mothers rearing their own pups (Control). However, some differences were observed. On PND 4, LAB Cross dams showed less side nursing posture than Control mice at 11-12a.m. ($p<0.05$, Fig. 33G). At PND 8, Mother ON and mother OFF were different between LAB Crosses and LAB Controls at 11-12a.m. ($p<0.05$, Fig 34A, B) with LAB Crosses spending more time outside the nest due to higher levels of active self-directed behavior ($p<0.05$, Fig. 34F). Further, there was a difference between HAB Crosses and HAB Controls at 11-12p.m. concerning locomotion ($p<0.05$, Fig. 34D). At PND 12, LAB Cross mothers showed less blanked posture nursing at 11-12a-m. ($p<0.05$, Fig. 35E) and more sleeping at 7-8a.m. ($p<0.05$, Fig. 35H) compared to LAB Controls.

3.2.2.2 Litter Size

The mean number of born pups assessed 12h after birth, before culling, differed between HABs and LABs with a higher number in HAB litters ($p<0.05$). The equalized litter size after culling was kept also when divided in control and cross-fostered litters. Also after weaning, there are no differences in litter size between HAB and LAB, control and cross-fostered groups. Thus, litters showed no loss of pups from 12h after birth to weaning (Fig. 36).

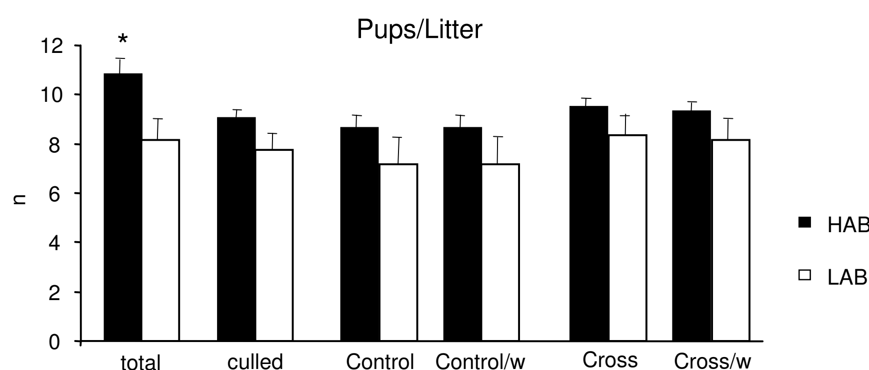


Fig. 36. Litter size. 12h after birth litter size is different between HABs and LABs. After culling, there is no difference in the number of pups with no relevant loss till weaning. * $p<0.05$ vs. LAB; w= weaning.

3.2.2.3 Weight Gain

Male (Fig. 37A) and female (Fig. 37B) pups of all four groups showed a progressive weight gain from PND 5 to PND 49 ($p< 0.001$). At PND 5, pups of both genders of the

LAB control group were heavier (p< 0.001 vs. HAB Control, HAB Cross, LAB Cross) and LAB cross-fostered pups were lighter than the other pups (p<0.001 vs. LAB Control, HAB Cross, p<0.01 vs. HAB Control). The same was found for male pups of the LAB control group at PND 12 (p< 0.001 vs. LAB cross, p<0.01 vs. HAB control,

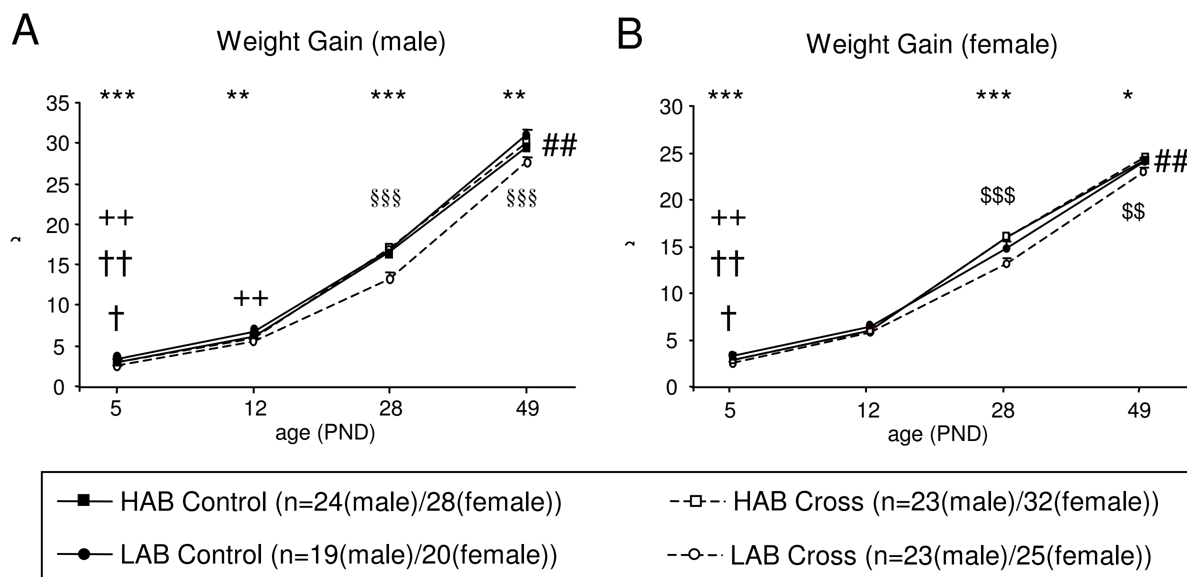


Fig. 37. Weight Gain. **A**, Male and (**B**) female pups of all groups showed a progressive increase in weight. Cross-fostered LAB pups of both genders were lighter than HAB pups and on PND 5 also lighter than LAB Control pups. . *** p<0.001, ** p<0.01, * p<0.05 (same age); ### p<0.001 (same line) in HAB Control, HAB Cross, LAB Control, LAB Cross; +++ p<0.001 LAB Control vs. HAB; ††† p<0.001 LAB Cross vs. LAB Control, HAB Cross; †† p<0.01 LAB Cross vs. HAB Control; §§§ p<0.01 LAB Cross vs. LAB Control, HAB; $$$ p<0.001 LAB Cross vs. HAB; $$ p<0.01 LAB Cross vs. HAB.

p<0.05 vs. HAB Cross), but not for cross-fostered LAB pups and LAB females. On PND 28 and 49, male LAB pups of the control group weighed more than the LAB pups of the cross-fostered litters (p<0.001), but not more than the pups of the HAB litters. Again, cross-fostered mal LAB pups were lighter than HAB pups of both treatments on PND 28 (p<0.001) and on PND 49 (p<0.05 vs. HAB Control, p<0.001 vs. HAB Cross). Among the females, pups of the LAB cross-fostered litters were lighter than the pups of both HAB groups on PND 28 (p< 0.001 vs. HAB Control, HAB Cross) and on PND 49 (p<0.01 vs. HAB Control, HAB Cross).

3.2.2.4 USV

The analysis of the number of USV calls revealed strong differences between the lines (p<0.001, Fig. 38A). Male and female HAB pups emitted significantly more USV calls than LAB pups (p < 0.001). Also the USV-associated locomotion displayed significant differences between the lines (p<0.001, Fig. 38B), with male and female

HAB pups showing higher locomotor activity than LAB pups (p<0.001). Cross-fostering treatment had no influence on USV and locomotion.

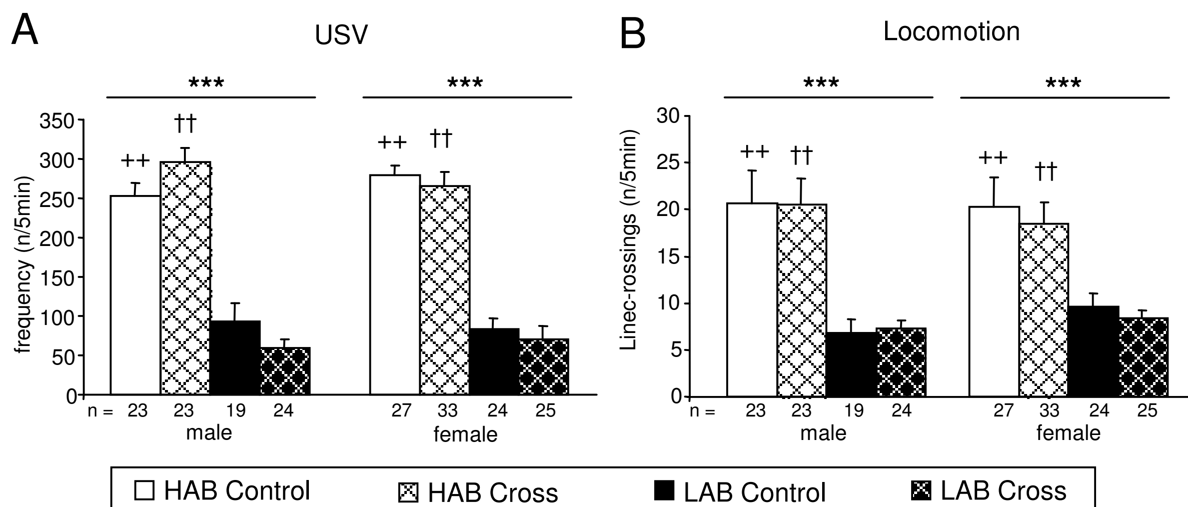


Fig. 38. Ultrasonic Vocalization and Locomotion on PND 5. **A**, 5 day old HAB pups displayed an anxiety-related increase in the number of USV calls compared to LAB pups. **B**, The HAB pups showed also an increase in the USV call connected locomotion. Cross-fostering had no significant influence on USV and locomotion. *** p < 0.001 within gender; ++ p < 0.001vs. LAB Control, †† p < 0.01vs. LAB Cross.

3.2.2.5 EPM

Male and female HAB and LAB mice showed the well established differences (p<0.001, Fig. 39A, B) with LAB mice spending significantly more percentage of time on the open arms independent of treatment (p<0.01, Fig. 39A) and exhibiting more percentage of entries into the open arms (p<0.01, Fig. 39B). Remarkably, male and female cross-fostered HAB mice showed a slight but significant increase in percentage of time spent on the open arms (p<0.05, Fig. 39A). Also the latency to the first entry onto the open arms revealed differences (p<0.001), but reaching significance only in male LAB Control animals (p<0.01, Fig. 39C).

3.2.2.6 TST

Male and female HAB and LAB mice displayed the already known differences (p<0.001, Fig. 40A, B), with LAB mice spending significantly less time immobile (p<0.01, Fig. 40A) and showing a higher latency to the first immobility (p<0.01, Fig. 40B). Cross-fostered mice exhibited no change in immobility time, whereas cross-fostered LABs showed a decrease in latency for both genders, but significant only in females (p<0.05, Fig. 40B).

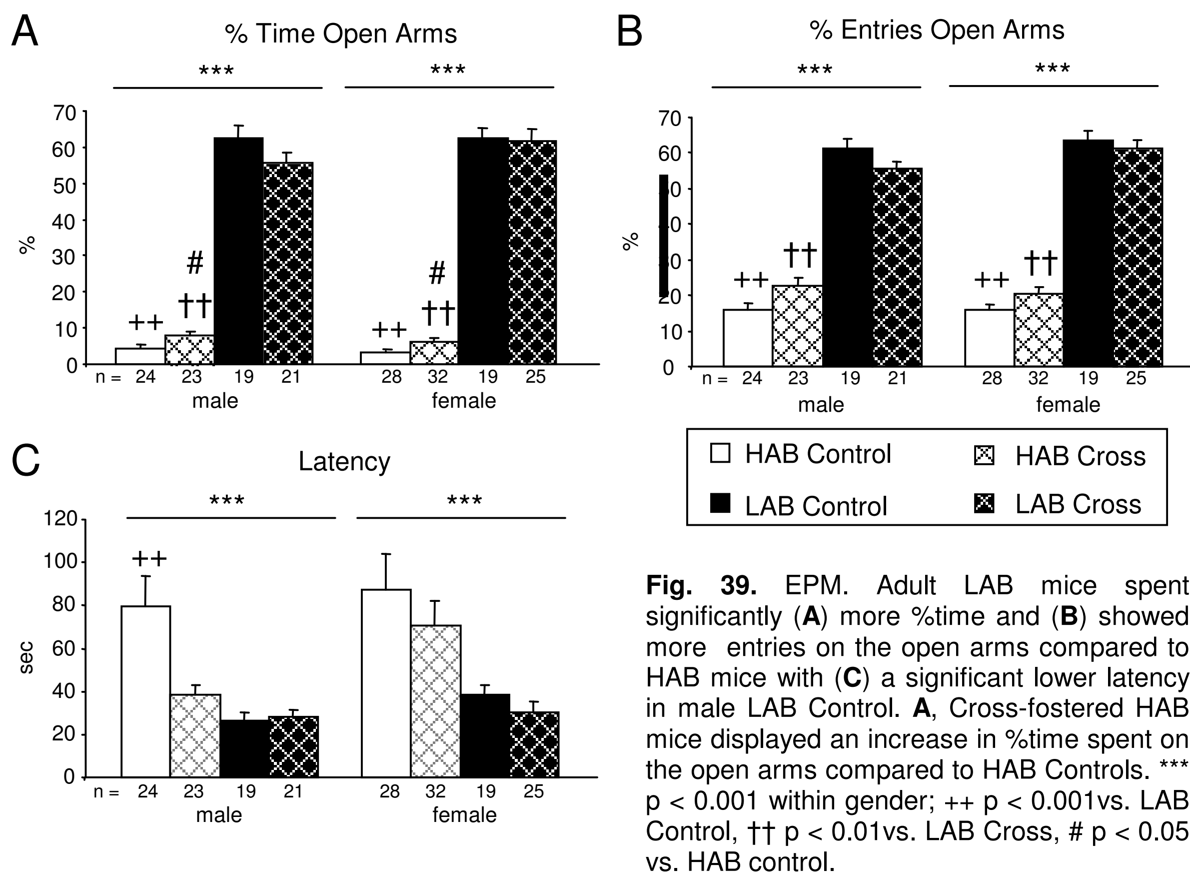


Fig. 39. EPM. Adult LAB mice spent significantly (**A**) more %time and (**B**) showed more entries on the open arms compared to HAB mice with (**C**) a significant lower latency in male LAB Control. **A**, Cross-fostered HAB mice displayed an increase in %time spent on the open arms compared to HAB Controls. *** p < 0.001 within gender; ++ p < 0.001vs. LAB Control, †† p < 0.01vs. LAB Cross, # p < 0.05 vs. HAB control.

3.2.2.7 OF

HAB and LAB mice of both sexes displayed no differences in time spent in the inner or outer zone of the OF (Fig. 41A, B). Analysis of the locomotion in the OF revealed differences between the groups (p<0.001, Fig. 41C) with male and female LAB mice, independent of treatment, displaying more line-crossings than the equivalent HAB mice (p<0.001). Further, the lines showed differences in exploratory behavior (p<0.001, Fig. 41D) as male and female LAB mice exhibited significantly more rearings in the outer zone than HAB mice (p<0.001) with a slight but not significant decrease in cross-fostered LABs compared to LAB control mice.

3.2.2.8 SRT

15min of restraint stress led to a greater corticosterone increase in LAB males than in HAB males (p<0.001, Fig. 42C) with no influence of cross-fostering. Accordingly, male LAB mice exhibited higher corticosterone levels after stress than male HAB mice (p<0.001, Fig. 37A). Females of both lines displayed the same increase and the same post-stress levels (Fig. 42B, C) with no cross-fostering-induced alterations.

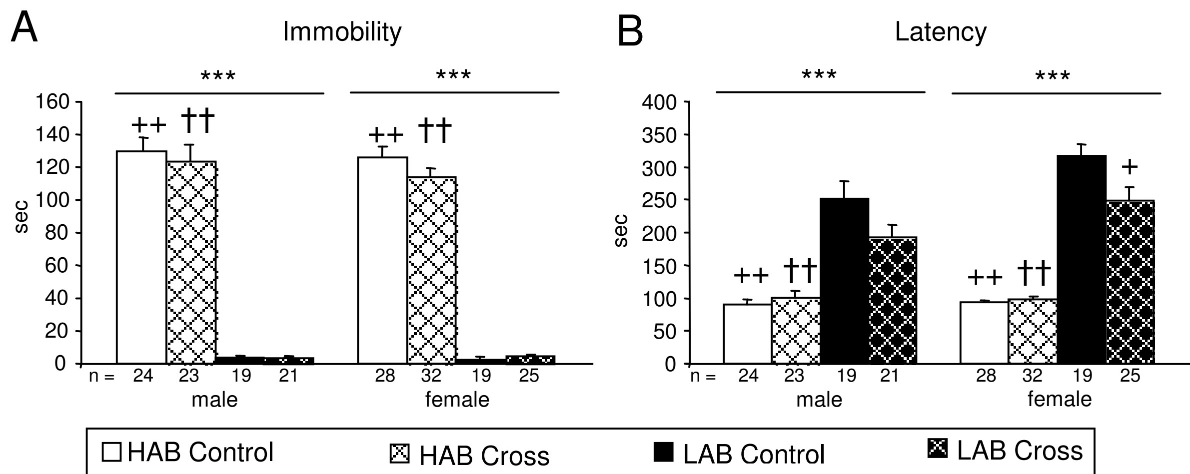


Fig. 40. TST. LAB mice showed a strong decrease in (**A**) immobility time with (**B**) an increase in latency compared to HAB mice. Cross-fostering reduced the latency in LAB females (**B**). *** p < 0.001 within gender; ++ p < 0.001, + p < 0.05 vs. LAB Control, †† p < 0.01 vs. LAB Cross.

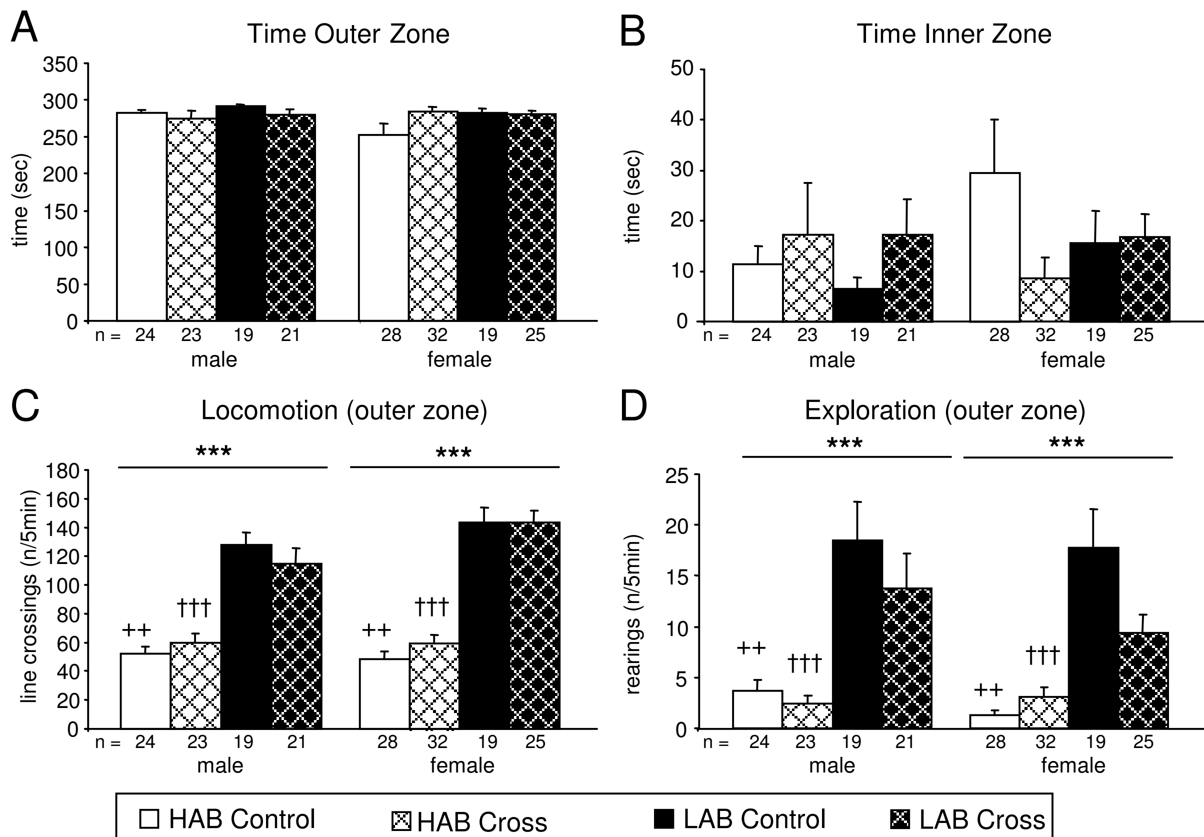


Fig. 41. OF. There were no differences in time spent (**A**) in the inner or (**B**) outer zone of the OF. LAB mice displayed significantly higher locomotor (**C**) and exploratory (**D**) activity in the outer zone. Cross-fostering led to slight but not significant alterations in exploratory behavior. *** p < 0.001 within gender; ++ p < 0.001 vs. LAB Control, ††† p < 0.001 vs. LAB Cross.

3.2.2.9 Avp mRNA Expression.

In situ hybridization of *Avp* mRNA in the PVN revealed significant differences between the groups in relative intensity (p<0.001, Fig. 43A) and area (p<0.001, Fig.

43B). Thus, LAB mice of both treatment groups expressed less *Avp* mRNA than HAB animals. The cross-fostering had no influence on the *Avp* mRNA expression levels.

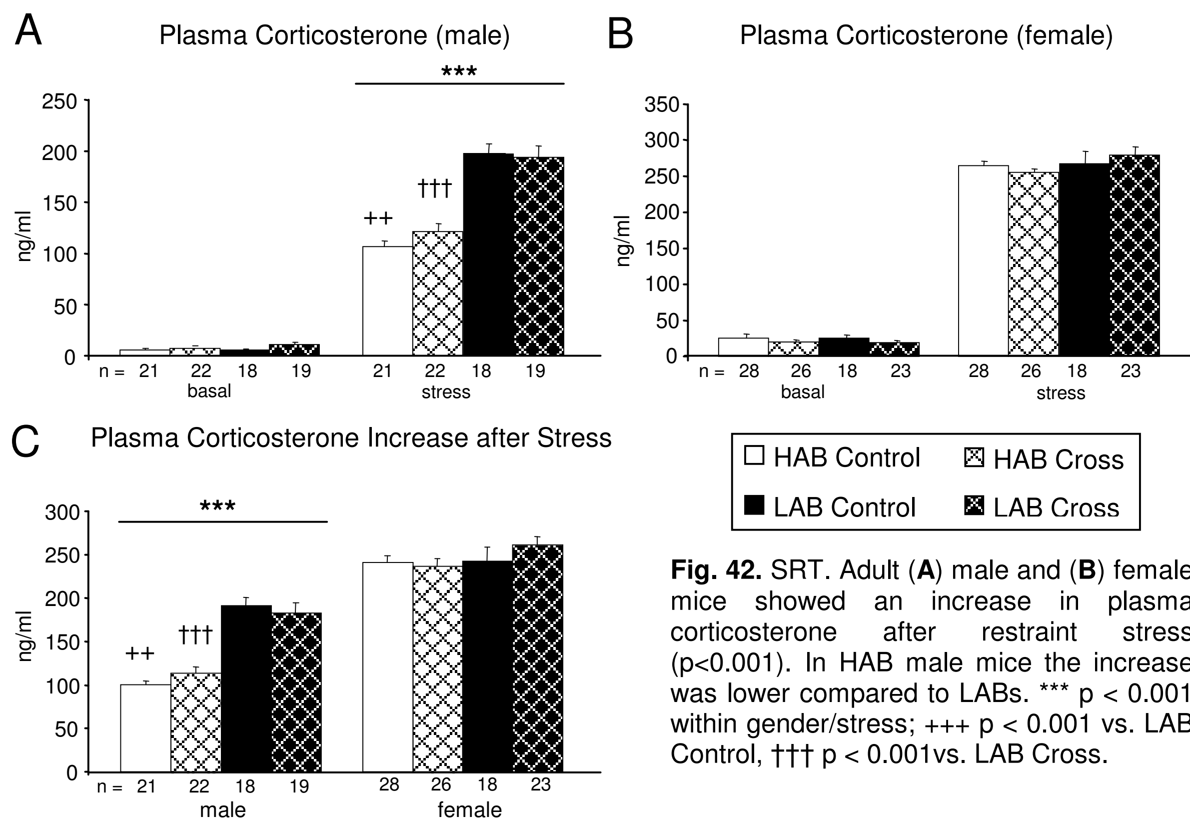


Fig. 42. SRT. Adult (**A**) male and (**B**) female mice showed an increase in plasma corticosterone after restraint stress (p<0.001). In HAB male mice the increase was lower compared to LABs. *** p < 0.001 within gender/stress; +++ p < 0.001 vs. LAB Control, ††† p < 0.001vs. LAB Cross.

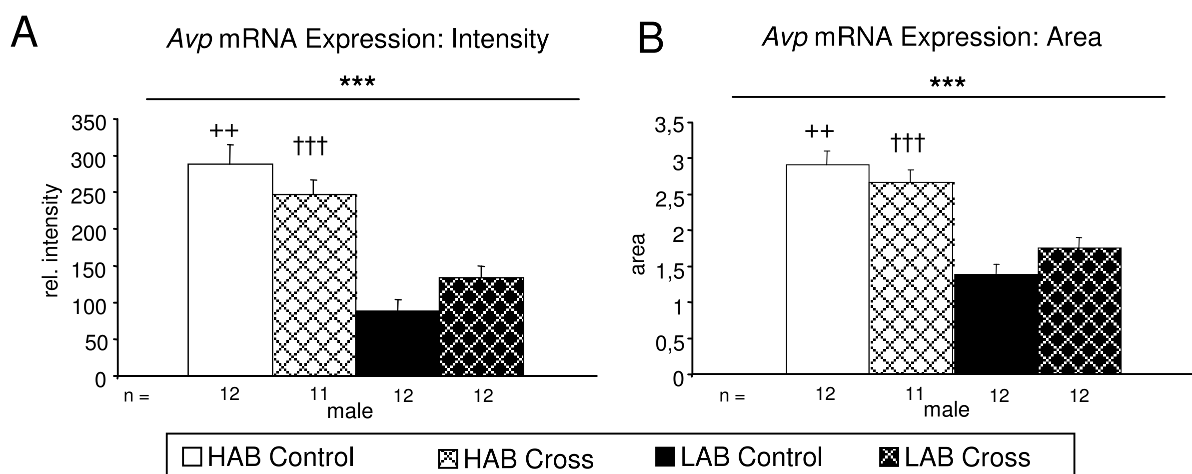


Fig. 43. *Avp* mRNA ISH. LAB mice exhibited a lower (**A**) relative intensity and (**B**) area of *Avp* mRNA expression in the PVN. *** p < 0.001 within gender; +++ p < 0.001 vs. LAB Control, ††† p < 0.001vs. LAB Cross.

4 Discussion

LAB mice displayed, besides their reduced anxiety, symptoms of cDI, revealing a strong deficit of releasable AVP, which was proved in the PVN by microdialysis. This AVP deficit is likely based on a SNP in the signal peptide of the AVP precursor gene. An increase of wild-type *Avp* mRNA expression in the PVN of LAB mice resulted in increased anxiety-related behavior, confirming a partial influence of AVP on trait anxiety. Furthermore, the difference in maternal behavior of HAB and LAB dams had no influence on anxiety-related and depression-like behavior, shown in a cross-fostering study, confirming the genetic basis of the bred phenotype.

4.1 AVP Deficit in LAB Mice

Regarding the observed results, it is very likely that the clear-cut deficit of AVP in the PVN of LAB mice is the key factor in both trait anxiety and cDI.

The microdialysis study revealed a strong deficit in the releasable amount of bioactive AVP in LAB mice compared to the more anxious HAB mice. Since AVP is not only centrally released from somata and dendrites of the PVN neurons or synaptically at the level of the median eminence, but also as antidiuretic hormone into the systemic circulation, the deficit in AVP affects both behavior and the water balance system of LABs. This is reflected by an increased daily fluid intake and a decreased urine osmolality of these mice. Water deprivation for 48h pointed the problem out in a diminished increase of plasma AVP resulting in an inadequate urine concentration and a stronger increase in plasma osmolality in LABs compared to NABs. The treatment with dDAVP, a V2-specific potent AVP analog, increased the urine osmolality to a level typical of HAB and NAB mice. Analyzing the progressive development of the symptoms of cDI revealed a constant increase in fluid intake during adolescence and a robustly elevated level in adulthood accompanied by a steady decrease in urine osmolality. The intensity of anxiety and non-anxiety in HAB and LAB mice, respectively, is not different in aged animals compared to the levels measured in adult, seven week old mice. Analyzed concerning a SNP in the signal peptide of the AVP precursor gene, aged HABs mice were identified as homozygous for the wild-type allele, LABs as homozygous for the mutant allele, CMs as heterozygous, and NABs as carrier of all three variations. Furthermore, *in situ*

hybridization of *Avp* mRNA in the PVN revealed a decreased expression level in LABs. This was present only in adolescence and at the beginning of the adulthood, disappearing during aging.

The existence of the deficit in AVP is reflected by the physiological consequences emerging in LAB animals. A lack of antidiuretic acting AVP leads to a noticeable insufficiency in maintaining a homeostasis in body water and consequently in plasma osmolality and plasma volume (Robertson et al., 1976), known from patients suffering from DI (Verbalis, 2003). Visible signs are an increased water intake and consequently an increase in the amount of secondary urine, reflected by a low urine osmolality, and in fact also displayed by LAB mice. The severe consequences are more obvious after water deprivation, an approach also used in patients. These challenging conditions uncover the inability to increase plasma AVP resulting in a harmful increase in plasma osmolality (Verbalis, 2003). The LAB mice's inability to increase plasma AVP under conditions of water deprivation probably results from a reduced refill-rate of the neurohypophysial synapses with AVP-containing neurosecretory vesicles. This reduction is observable by the depletion of the pituitary reflected by the stronger decrease in the amount of AVP in the pituitary of LAB mice after water deprivation compared to NAB and HAB mice. The treatment with dDAVP, an AVP analog used in clinical diagnostics and therapy of patients (Rado et al., 1976; Vavra et al., 1968; Verbalis, 2003), increased the urine osmolality to a level typical of HAB and NAB mice. This excludes a defect in the V2 receptor-mediated insertion of aquaporines and confirms a deficit in AVP as the underlying ligant of the observed symptoms. A identified cytosine to thymine nucleotide transition (C40T) in the AVP precursor gene, exchanging alanine to valine in the third position of the signal peptide (A(-21)V) in LAB mice (Murgatroyd, unpubl., Fig. 44), is very likely involved in this AVP deficit.

At present, 56 dominant and one recessive SNP in the AVP precursor gene have been identified as the cause of cDI in humans. Most of the mutations, including nucleotide substitutions or deletions, are localized in the NPII part and some in the region, which encodes for AVP. The mutations change the stable three-dimensional structure of the protein by inducing substitutions or deletions of amino acids, involved in disulfide bridges or secondary structures, like β-sheets and a α-helix, (e.g. NPII: G14R, G17V, E47del, S57G, G65V, C73G, C85G; AVP: Y2H, F3del, P7L) or by forming a truncated propeptide (e.g. C61stop, C67stop, C79stop, E87stop) lacking

the glycopeptide and some amino acids of the NPII moiety (Christensen and Rittig, 2006; de Bree and Burbach, 1998; Ito and Jameson, 1997; Ito et al., 1999; Nagasaki et al., 1995; Nijenhuis et al., 1999, 2000; 2001; Rittig et al., 2002; Willcutts et al., 1999) These structural alterations lead to an incorrect folding of the peptides and inadequate binding of vasopressin to its carrier protein, whereby the misfolded

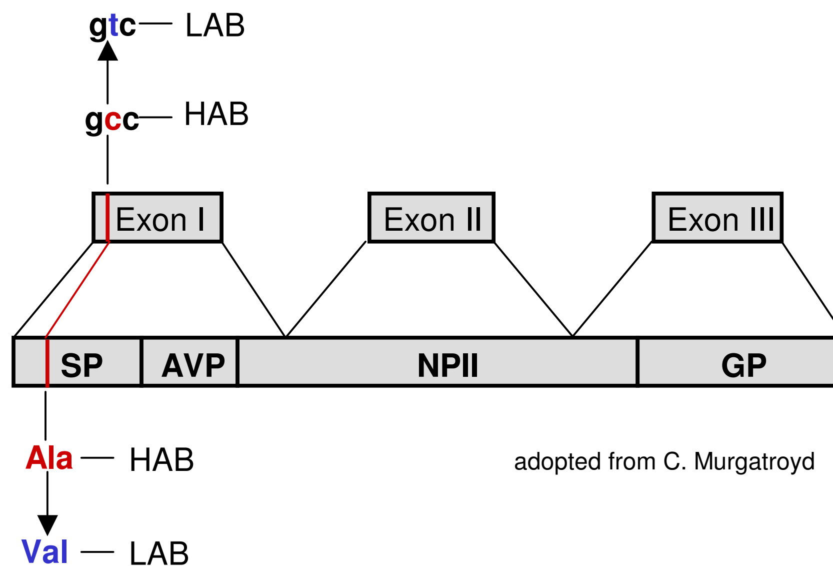


Fig. 44. A SNP in the first exon of the AVP precursor gene causes a cytosin to thymine nucleotide transition at the second position of a triplet leads to an amino acid exchange at the 3rd position of the signal paptide from alanine to valine in LAB mice. SP, signal peptid. NPII, neurophysin II, GP, glycoprotein.

propeptide accumulates in the ER. Accumulation results in an impairment of further processing, axonal transport, and, finally, AVP secretion. The accumulated mutant protein in the ER hinders not only the processing of the wild-type AVP propeptide (dominant-negative effect) but also the processing of other essential proteins, finally resulting in cell death (Ito and Jameson, 1997; Ito et al., 1999). Both cause the delayed onset and progressive course of the disease. Mutations identified in the signal peptide affect the N-terminal and the C-terminal part. One is the very common A(-1)T mutation in the COOH-terminus (Christensen and Rittig, 2006). An involvement of this amino acid in cleavage of the signal peptide was suggested by the finding of a 23 kDa molecule in an in vitro study consistent with an uncleaved prepropeptide unable for proper folding (Ito and Jameson, 1997; Ito et al., 1993; Siggaard et al., 1999). The mutation leads to a cDI with delayed onset and progressive development (Kawakami et al., 1998; McLeod et al., 1993; Siggaard et al., 1999). This pattern is caused by the impaired transport of the misfolded propeptide and the advanced decrease of cell viability (Ito and Jameson, 1997; Ito et al., 1999) provoked by the cytotoxicity of the unfolded propeptide accumulated in the ER as mentioned above. A SNP in the same codon which leads to a substitution of valine for alanine in the same position (A(-1)V) was found in two families with similar

cDI symptoms (Repaske et al., 1997). In contrast, a mouse model carrying the A(-1)T mutation displays no symptoms of cDI (Russell et al., 2003), underlining that the individual severity of this disease is influenced not only genetically but also by other unknown factors.

Two other SNPs identified in the signal peptide change the N-terminal part. The substitution or deletion of one nucleotide (A225G, G227A, G227del) leads to the deletion of the first four amino acids (M1P2D3T4del) (Beuret et al., 1999; Rutishauser et al., 1996). Since methionine forms the translation initiation codon (ATG), this translation is now inhibited. Nevertheless, the translation of the protein takes place, because the fifth amino acid displays an alternative onset for translation. Translation of this protein results in a prepropeptide with a truncated signal peptide lacking a hydrophilic N-terminal segment. Changing or deletion of the N-terminal part leads to a different positioning of the signal peptide within the translocation machinery and reduces the possibility for cleavage from the propeptide (Nilsson et al., 1994). Misfolding and accumulation of the AVP precursor in the ER is the consequence (Beuret et al., 1999).

Comparable processes may occur with the mutant AVP prepropeptide in LAB mice. The exchange of the amino acid alanine to valine may alter the structure of the N-terminal part of the signal peptide, because an additional side chain (methyl group) provided by valine can alter the hydrophobicity. As mentioned above, the structural alteration of the N-terminal part of the signal peptide can inhibit the adequate cleavage of the signal peptide, interfering with the subsequent processing of the peptide. Expected consequences would be an accumulation of the peptide in the ER and a diminished transport of the precursor, resulting in low levels of bioactive and secretable AVP in a progressive manner. The lack of progressive decline in *Avp* mRNA-labeled PVN neurons in aged LABs, possibly reflecting continuous cell death, points to a slightly reduced processing and a partial accumulation of the mutant AVP precursor in the ER. Moreover, the release of a glycosylated uncleaved propeptide with reduced function is possible, similar to the A(-1)T mutation. A complete lack of AVP was shown to be lethal, seen in mice homozygous for the C67stop mutation (Russell et al., 2003). Therefore, the hypothesis that a correlation exists between reduced AVP release and reduced antidiuretic activity fits with the observed phenomena in LAB mice, homozygous for the C40T SNP in the signal peptide. This process was also found for the P7L mutation, causing the only known recessive form

of cDI. Here, the mutant propeptide is not retained in the ER, but it seems to diminish the processing to AVP and NPII (Christensen et al., 2004), leading to the secretion of a Leu-AVP with sufficient endocrine activity, but with decreased binding affinity to the V2 receptor (Willcutts et al., 1999).

A moderate release of a peptide with reduced efficiency would also explain the equal basal AVP plasma levels between HAB, NAB, and LAB mice, in contrast to the elevated fluid intake and decreased urine osmolality in LABs compared to HABs and NABs, as the AVP-RIA is not able to differ between the mature AVP and the unprocessed precursor. Therefore, it is necessary to investigate the precise cellular processes (i.e. quantification of the accumulation of the precursor in the ER and analysis of the diminished processing during axonal transport and of the amount and quality of the released peptide) to evaluate the impact of the C40T SNP on the AVP deficit in LAB mice. In contrast to the non-decrease of *Avp* mRNA labeled cells in the PVN, LAB mice showed an increase in fluid intake and decrease in urine osmolality while aging, pointing to the progressive development of the disease. The magnocellular neurons, responsible for osmolality-dependent AVP release, are located in the PVN and SON with the major part in the latter (Lucassen et al., 1995; Swaab, 1998; Swanson and Sawchenko, 1983). Thus, the main source of the increase in cDI symptoms can be a low, but progressive, cell loss in the SON, not detectable in the PVN by *in situ* hybridization, as the magnocellular part of the PVN is considerably smaller than its parvocellular part (Lucassen et al., 1995; Swaab, 1998; Swanson and Sawchenko, 1983). Moreover, accumulation of the prepropeptide and its cytotoxic effect in the parvocellular neurons is reduced, since the rate of AVP biosynthesis is much lower in parvocellular than in magnocellular neurons (Burbach et al., 2001). Equally, a case report of a 72-year-old man suffering from excessive thirst and urination refers from a loss of magnocellular SON and PVN but not parvocellular neurons in the PVN (Bergeron et al., 1991), supporting the difference in AVP synthesis in the two cell types. Investigations of the SON, including *Avp* mRNA expression levels and the amount of releasable AVP, as well as cell culture studies revealing the effect of the SNP on AVP processing are necessary to find out the exact molecular and cellular background of the cDI symptoms in LAB mice.

Besides the retention of the mutant prohormone in the ER, the extent of degradation of misfolded proteins from the ER can also influence the viability of the cell (Repaske et al., 1997). Since both problems occur to different degrees among the different

mutations, the degree of cDI in patients varies heavily among the known mutation of the AVP-encoding gene (Christensen et al., 2004; Ito and Jameson, 1997; Nijenhuis et al., 2001). However, it is not known whether there is a direct correlation among retention of the mutant prohormone in the ER, degenerative processes, and the severity of cDI, or if additional factors are involved. In fact, transgenic animals carrying the same mutations in the *Avp* gene causing cDI show different degrees and symptoms of the disease. Thus, transgenic mice heterozygous for the same mutation had progressive symptoms, similar to humans, with highly increased water intake and urine output and decreased urine osmolality. They also exhibit a massive loss of magnocellular neurons, in line with the results of several in vitro studies (Russell et al., 2003). In contrast, rats transgenic for the C67stop mutation show slight symptoms of cDI. They exhibit the accumulation of the mutant protein in the ER, but no degenerative processes or death of the affected magnocellular neurons (Davies and Murphy, 2002; Si-Hoe et al., 2000). It follows, that additional factors beside the SNP may form the observed cDI symptoms in LAB mice, affecting the progressive increase in water intake and decrease in urine osmolality without the discussed cellular background.

Besides the release of antidiuretic-acting AVP at the level of the neurohypophysis, AVP involved in the regulation of HPA axis activity and emotionality-related behavior is released synaptically and somato-dendritically from magnocellular and parvocellular neurons of the PVN. Analysis of anxiety-related behavior on the EPM exhibited the same differences in aged animals of the three breeding lines as with an age of 7 weeks, in contrast to the slightly progressive development of the symptoms of cDI. Thus, besides the acute effect of an AVP deficit in emotional-challenging situations, the constant deficit of centrally released AVP likely influences trait anxiety robustly from birth on, or even prenatal. Since AVP was shown to be expressed in the diencephalon already on embryonic day 13.5 and in a region corresponding to the PVN on day 14.5 (Jing et al., 1998), the deficit may shape essential projections and functions of anxiety-related brain regions already during development. Furthermore, since the AVP that is involved in emotionality-related behavior originates mainly from the PVN, not the SON (Swaab, 1998; Swanson and Sawchenko, 1983), the influence of a possible progressive accumulation of the mutant precursor and the consequential cell death, if present at all, seems to play just a subordinate role.

The influence of AVP of PVN neurons is additionally supported by the partial increase in anxiety-related behavior on the EPM in AAV-*Avp* treated LAB mice. In contrast, no significant differences in anxiety-reflecting behaviors in the DaLi and the OF were found. Also depression-like behavior in the TST and the FS test, exploratory activity on the EPF, and locomotor activity in the OF, as well as parameters characterizing the reactivity of the HPA axis displayed no differences in AAV-*Avp* treated compared to AAV-*lacZ* treated or control mice. Furthermore, daily fluid intake and urine osmolality analyzed over several weeks after surgery were not influenced by the treatment with AAV-*Avp* compared to mice treated with AAV-*lacZ* and untreated controls. Nevertheless, *in situ* hybridization of *Avp* mRNA in the PVN showed a strong, but not significant, increase in *Avp* mRNA in AAV-*Avp* treated animals in contrast to the two other treatment groups.

The increase of *Avp* mRNA expression in the PVN by the transduction of the *Avp* gene via a viral vector is accompanied by an increase in the latency to the first open arm entry and a tendentious decrease in total entries. The decrease in the number of total entries resulted from a decrease in open arm entries, but not from a general decrease in locomotor activity, reflected by equal numbers of closed arm entries in all groups. Anyhow, the increase of *Avp* mRNA expression in the PVN was not able to alter the main parameter representing anxiety-related behavior on the EPM, percentage of time spend on the open arms. This is possibly attributed to the difficulty of an artificial increase in gene expression to influence fundamentally and by selective inbreeding already during development manifested circuits. Furthermore, together with the complexity of the anxiety-generating circuits, including several brain regions and additional neurotransmitters/peptides, the manipulation of one parameter is hardly expected to invert an inborn trait. This limited impact of the *Avp* expression increase in PVN neurons by the transduction of the gene via a viral vector is also reflected by the absent modification of anxiety-related and depression-like behavior as well as exploratory and locomotor activity in the additional test paradigms. Furthermore, the increase in *Avp* expression in the PVN had no influence on the water retention in the kidney, demonstrated by an unchanged urine osmolality and fluid intake in AAV-*Avp* treated mice compared to mice of the AAV-*lacZ* treated group and controls. This again reflects the lower involvement of the PVN in contrast to the SON in antidiuretic functions. Finally, although the technique of viral vector-induced gene transduction and long-term expression in the mammalian brain was

successfully used in several approaches using rats, mice, and primates for phenotypic correction (Kaplitt et al., 1994; Landgraf et al., 2003; Malik et al., 2005; Muramatsu et al., 2002; Shen et al., 2000), the right composition of the vector regarding viral elements, serotypes, promoters, transcriptional control elements, and the right injection dose is fundamental for a successful gene transduction (Hermening et al., 2006; Lo et al., 1999; Millecamps et al., 1999; Shevtsova et al., 2005). Thus, although Ideno et al. (2003) achieved an increase in AVP-positive cells and a normalization of the cDI phenotype in Brattleboro rats after *Avp* gene transduction in the SON using the present vector, there is no guaranty for successful transduction and expression of the AAV-*Avp* gene in PVN neurons of LAB mice. So, for the hCMV promoter a very low expression in neuronal cells and a strong expression in non-neuronal cells was shown in comparison to the neuron-specific *synapsin-1* gene (syn) promoter (Kugler et al., 2003). In contrast, hCMV-mediated gene transduction was successfully accomplished in rats and primates, increasing the expression of dopamine-synthesis-involved enzymes in striatal neurons, demonstrated by dual immunofluorescence staining (Muramatsu et al., 2002; Shen et al., 2000).

The lack of dual immunofluorescence staining in the present experiment, proving the neuronal localization of AVP in addition to *in situ* hybridization, which shows the increased expression of *Avp* mRNA in the PVN, is caused by the technical difficulty to use both methods in parallel on sections of the same brain. A consequential increase in the number of animals was not possible due to the limited number of animals produced by selective inbreeding. Nevertheless, the validity that *in situ* hybridization shows a viral-vector induced increase in *Avp* gene expression was demonstrated by a study using a lentiviral vector based expression of the *Avp* gene from a hCMV promoter in the SON of Brattleboro rats, presenting *in situ* hybridization and immunohistochemistry with comparable results. Additionally, besides the possibly low expression levels using the hCMV promoter, also the stability of gene expression with the hCMV is not as persistent as with the syn promoter. Even the use of transgene expression enhancing elements, such as the woodchuck hepatitis virus post-transcriptional regulatory element, was not able to increase and prolong expression to the same level found with the use of vectors including the syn promoter (Glover et al., 2003; Hermening et al., 2006). In any case, an adequate gene expression up to 3 months was shown for the hCMV promoter (Kaplitt et al., 1994),

sufficient for the present study and partially reflected by the strong increase in *Avp* mRNA expression in the PVN of LAB mice 10 weeks after treatment.

Taken together, the AVP deficit in the non-anxious LAB mice, shown by a strongly decreased amount of releasable AVP in the PVN and by dramatic physiological consequences, is probably based on a SNP in the signal peptide of the AVP precursor gene, at least in part. The manipulation of the LAB phenotype by the increase of *Avp* gene expression in the PVN documents the partial involvement of the AVP deficit in their reduced trait anxiety. In rats, the crucial involvement of AVP in emotionality-related behavior (Landgraf, 2001) and in the function of anxiety-related and stress response-related brain regions, like amygdala, BNST, LC, and PVN was repeatedly described (De Goeij et al., 1992; Engelmann et al., 2004; Landgraf et al., 1998; Nakase et al., 1998; Wotjak et al., 1998; Wotjak et al., 2001). Further, in the last years increased AVP plasma concentration and increased *Avp* expression in the PVN and SON were found in depressed patients (Inder et al., 1997; Meynen et al., 2006; Purba et al., 1996; Raadsheer et al., 1994; van Londen et al., 1997). This points to an association of AVP and depression and confirm a crucial involvement of AVP expressed in PVN and SON neurons in the hyperactivity of the HPA axis under chronic stress (Aguilera and Rabadan-Diehl, 2000; Tilders et al., 1993). Moreover, an increased AVP plasma concentration associated with increased plasma corticosterone levels in patients with anxious-retarded depression point to a crucial involvement of AVP in depression with comorbid anxiety (de Winter et al., 2003; Goekoop et al., 2006; Meynen et al., 2006). Unfortunately there is no study about the *Avp* expression levels in PVN and SON neurons in these patients, which would uncover in more detail the underlying neuronal circuits. A strong association of AVP in PVN neurons and trait anxiety was already shown in the HAB/LAB rat model (Landgraf, 2003; Wigger et al., 2004). In rats, a SNP in the promoter region led to a increased *Avp* expression in PVN neurons of HAB rats (Murgatroyd et al., 2004). In LAB mice, the impact of the SNP on the AVP deficit and the functional influence of AVP on the differences in anxiety-related and comorbit depression-like behavior in the HAB/LAB mouse model is a main goal of further investigations. Moreover, since the SNP in the signal peptide cannot directly cause the reduced *Avp* transcription in the PVN and also SNP-induced cytotoxicity is negligible in PVN neurons, intensive screenings for additional SNPs in transcription regulating sites in the promoter region of the AVP precursor gene of LAB mice were performed.

4.2 Postnatal Maternal Influence on the HAB/LAB-Phenotype

The analysis of maternal investment and rearing style revealed slight behavioral differences between HAB and LAB mothers with a minor impact on the formation of anxiety-related behavior of the two breeding lines affirming the fixed genetic manifestation of trait anxiety and comorbid depression in HAB and LAB mice accomplished by selective breeding.

In contrast to HAB females, LAB females showed a higher rate of failed pregnancies and births resulting in a reproductive success of only 50% in the LAB line compared to 85% in the HAB line. It remains unclear why only the LAB line is affected by the well-known “inbreeding depression”, as the possible main source of reproductive failures. Inbreeding depression is the reduction of fitness and fertility by the increased accumulation of deleterious recessive alleles (Swindell and Bouzat, 2006) which is caused by an increase in the level of homozygosity due to consanguineous mating. This process can be diminished by natural selection purging deleterious genes out. Under artificial laboratory conditions the impact of natural selection, like predator stress, nutrition competitors, or foraging, is less effective enhancing the accumulation of deleterious genes by inbreeding. Furthermore, also environmental conditions shape the intensity of inbreeding depression by a varying influence on the expression levels of the different deleterious recessive alleles (Armbruster and Reed, 2005). This might cause the strong difference between HAB and LAB dams concerning reproductive success, assuming that the individually accumulated deleterious recessive genes in the HAB and LAB line, underlying inbreeding depression, are differently influenced by the environmental conditions.

On days 2 and 4 after delivery, LAB and HAB mothers spent different amounts of time on maternal care with HAB mothers spending more time inside the nest than LAB mothers. Outside the nest, we found no differences in self-directed behavior, such as eating, drinking, and self-grooming, but on a higher locomotor activity of LAB mothers. Furthermore, the maternal rearing style varied between the dams of the two lines with HAB mothers showing twice as much arched back nursing than the LAB mothers on PND 2 and 4, and the latter preferring the side posture. Over the time course of observation, dams of all groups showed a progressive decrease in arched back nursing and an increase in blanked posture, while the difference in side posture nursing was constant. On closer examination of the four postnatal days, the

examined mothers spent nearly the whole day inside the nest on PND 2, but over the observation period nursing was predominantly performed during the resting phase (11-12a.m. and 3-4p.m.). Consistently, the lower amount of arched back nursing of LAB mothers on PND 2, 4, and 8 was observable on all five daily time points investigated, but most prominently and consistently at 3-4p.m. Interestingly, the difference in locomotor activity on PND 2 and 4, was also significant at time point 3-4p.m. On PND 4, 8, and 12 the strong preference in side posture nursing of LAB dams was mainly observable at 11-12a.m., but with increasing rates also at 3-4 p.m. and 11-12p.m. Cross-fostering of the pups induced no alterations in the time spent on maternal care of the type of rearing style. Thus, there are only a few trivial differences in maternal behavior between mothers with cross-fostered pups and mothers with their own pups.

The difference in maternal investment on PND 2 and 4 were mainly attributed to the absence of LAB mothers from the nest at 3-4p.m. On the first 4 days after birth, HAB mothers spent nearly the whole day inside the nest nursing the pups. HAB dams were outside the nest for eating, drinking, and self-grooming only for the hour after the light was turned on (7-8a.m.) and at PND 4 also for the hour after the light was turned off (7-8p.m.). In contrast, LAB mothers were, in addition to the hours after lights on and lights off, also outside the nest at 3-4p.m. and on PND 4 at 11-12p.m. Furthermore, during the time outside the nest, LAB dams spent time not only on self-directed behavior, but they spent the main portion of their time on locomotor activity. These differences can be ascribed to a shifted circadian rhythm and an increased locomotor activity of the LAB line, as has already been shown for male LAB mice in a homecage-activity study (Singewald et al., unpubl.). This experiment showed, besides the increased activity over the dark phase, an altered resting-activity rhythm of LAB mice compared to HAB mice. Thus, HAB mice showed nearly no activity over the 12 hours of lights on, whereas LAB mice rested only for the 2 hours from 12a.m. to 2p.m. This reflects the strong differences in locomotor activity and maternal investment at 3-4p.m. between HAB and LAB dams. At PND 8 and 12, HAB mothers reduced their time inside the nest to the core period of the light phase (11-12a.m. and 3-4p.m.), comparable with LABs. The amount of time outside the nest all mothers spent more and more with resting.

The resting-activity rhythm, which includes locomotor activity, sleep-wake behavior, feeding and drinking, body temperature, and corticosterone/cortisol levels, is primarily

affected by the rhythmic activity of the suprachiasmatic nucleus (SCN), the biological clock (Kalsbeek and Buijs, 2002). 10% to 30% of the SCN neurons are vasopressinergic, playing a main role in the connections of the SCN (Kalsbeek et al., 2002). Besides projections to hypothalamic neurons regulating endocrine systems by the release of CRH, thyrotropin-releasing hormone (TRH), and gonadotropin-releasing hormone (GnRH), SCN neurons project directly and indirectly via PVN neurons to autonomic neurons in the brain stem and the spinal cord. These autonomic centers, such as the dorsal motor nucleus of the vagus of the intermediolateral columns, influence sympathetic and parasympathetic activity. Lastly, the SCN holds projections to other hypothalamic areas, such as the subparaventricular nucleus (sPVN) and the posterior hypothalamic area (PHA) (Kalsbeek and Buijs, 2002). The sympathetic-parasympathetic system in the periphery and the sPVN and the PHA via cortical projections within the brain are mainly involved in the sleep-wake cycle and the rest-activity rhythm (Abrahamson and Moore, 2006; Buijs and Kalsbeek, 2001). Interestingly, the SCN projections to the autonomic neurons are segregated into sympathetic- and parasympathetic-projecting neurons. These projections are vasopressinergic with GABA or glutamate as co-transmitter, pointing to an opposite vasopressinergic regulation of the two branches, which depend on the co-transmitter (Buijs et al., 2003). Provided that the AVP deficit is caused by the SNP in the signal peptide of the AVP precursor gene, this deficit is not restricted to the PVN, but observable in all other vasopressinergic neurons. Thus, the reduced amount of AVP found in the PVN is likely also present in the vasopressinergic neurons of the SCN disrupting the balance of the sympathetic-parasympathetic system and causing the alteration of the normal rest-activity rhythm and the elevated locomotor activity of LAB male and female mice. This hypothesis is supported by the finding that tyrosine hydroxilase activity is increased in the adrenals of male LAB mice (Touma, unpubl.), reflecting an increased amount of synthesized norepinephrine and enhanced sympathetic activity. Vasopressinergic projections of the SCN are also crucially involved in the regulation of the estrous cycle, reproductive behavior, pregnancy, and nursing. The SCN projects here in the form of a daily signal to estrogen-sensitive GnRH neurons in the MPOA, where every 4-5 days, during proestrus, this signal together with a positive estrogen feedback initiates the release of GnRH in the median eminence, resulting in a surge of luteinizing hormone (LH) from the posterior pituitary into systemic circulation (Barbacka-Surowiak et al., 2003;

Kriegsfeld and Silver, 2006; Palm et al., 1999; Palm et al., 2001b). LH in combination with the follicle-stimulating hormone stimulates the proliferation of follicular cells, inducing an increased progesterone concentration and ovulation, and the luteinization of the follicles. In parallel to the ovulation, progesterone and estrogen coordinate mating behavior via MPOA neurons to facilitate receptivity and pregnancy (Barbacka-Surowiak et al., 2003; Kriegsfeld and Silver, 2006).

In this context, it is not astonishing that female *Clock* mutant mice carrying a 51 amino acid deletion in the transcriptional-activation domain of the CLOCK protein display a disrupted estrous cycle, strong inhibition of the LH surge, low estradiol and progesterone levels, increased fetal reabsorbtion, and a higher incidence of failed pregnancies (Miller et al., 2004). In more detail, core clock genes and clock-controlled genes induce the circadian rhythmicity of the SCN. When CLOCK in a heterodimeric complex with BMAL1, it enhances the transcription of the *Period* (Per) and *Cryptochrome* (*Cyr*) genes by binding to the E-box (CACGTG) domain in the promoter region of these genes. The accumulation in the cytoplasm of PER and CRY proteins over the day leads to the negative feedback on transcription of *Clock* and *Bmal1*. Besides the *Per* and *Cyr* genes, the *Avp* gene also includes the E-box in its promoter region, and is regulated by the CLOCK:BMAL1 complex (Kriegsfeld and Silver, 2006; Munoz et al., 2002). Furthermore, *Avp* mRNA expression in the SCN is reduced in the female *Clock* mutant mice compared to wild-type mice (Miller et al., 2006), pointing to an involvement of AVP in the circadian coordination of the estrous cycle. In addition to the inhibited LH surge, low progesterone and estrogen levels, and pregnancy failures, female *Clock* mutant mice display a loss of daily rhythmicity in plasma prolactin (PRL) levels (Hoshino et al., 2006). As PRL stimulates milk production and secretion from the mammary glands and centrally via PRL receptors in the MPOA, together with estrogen and oxytocin, maternal behavior (Bridges et al., 1990; Lucas et al., 1998; Pedersen et al., 1994; Sheehan and Numan, 2002), it is not astonishing that these dams showed also a loss of daily rhythmicity in nursing behavior and decreased milk secretion, which results in decreased weight gain and reduced survival of pups. Furthermore, *Clock* mutant dams build low nests without a ridge formation compared to wild-type mothers (Dolatshad et al., 2006; Hoshino et al., 2006). Again, vasopressinergic SCN projections were shown to be involved in the dopaminergic inhibition of PRL release from the adenohypophysis regulating, besides the stimulatory effect of suckling, the daily PRL surge (Palm et al., 2001a).

This involvement of AVP in the regulation of the estrous cycle, pregnancy, and maternal behavior points to a relevant connection of the AVP deficit and the different reproductive success and maternal behavior of LAB mothers.
Again, the reduced amount of AVP found in the PVN is likely also present in the vasopressinergic neurons of the SCN. This might cause a lower activation signal of the SCN, affecting the intensity of the LH and PRL surge and the following mating behavior, pregnancy success, and maternal behavior. The reduced number of pregnant females, successful deliveries, and born pups in the LAB line indicates an AVP deficit, a diminished LH surge, and a less effective fertilization and pregnancy. Furthermore, as mentioned above, LAB mothers do not only show different rhythmicity in nursing, but also differences in nursing style. Thus, LAB mothers spent less time in arched back nursing, the most effective posture for suckling. Moreover, while HAB mothers showed arched back nursing, LAB dams displayed less but sufficient side posture nursing, apparently enough to ensure the growth of the pups. This was reflected by no meaningful differences in the survival rate and weight gain of HAB and LAB pups, also after cross-fostering. Thus, although the AVP deficit might disturb the circadian rhythmicity of PRL release, the influence on milk ejection seems to be minor, possibly due to the stimulatory effect of suckling (Arey et al., 1991; Febo et al., 2005). On the other hand, the disturbed circadian signal at the level of the MPOA at birth and postpartum might influence maternal care including nursing and pup retrieval. The connection between estrogen receptor expression and estrogen-dependent oxytocin receptor binding, as well as PRL receptor expression and PRL in the MPOA, and maternal behavior was already shown in the high/low LG-ABN rats (Champagne et al., 2006). In addition, an increased hippocampal GR expression, due to DNA methylation and enhanced transcription factor binding in the GR-promoter region, and decreased stress reactivity and anxiety was also associated with high amounts of pup licking and arched back nursing in these rats (Szyf et al., 2005). These data suggest a strong epigenetic influence of maternal care on anxiety-related behavior with a non-genetic transmission. However, there is a lack in evidence on a neuronal or molecular level for the connection of maternal behavior with the hippocampal alteration in GR expression. Remarkably, in contrast to the LG-ABN rats, in the HAB/LAB mouse model the less anxious mice spent less time in maternal care and displayed less arched back nursing. This might be due to different or additionally involved, possibly vasopressinergic, neuronal circuits in the HAB/LAB

mice, as AVP seems not be involved in the phenotype of the high/low LG-ABN rats. Thus, instead of the established circuit, including the MPOA, the ventral tegmental area, the nucleus accumbens, and the ventral pallidum, that generates maternal behavior under the influence of oxytocin and estrogen (Numan et al., 2005; Stack et al., 2002) and is altered in the LG-ABN rats, in the LAB mice other regions with vasopressinergic projections such as the hippocampus, LS, BNST, amygdala, or caudal periaqueductal gray (cPAG) might be involved (Hallbeck et al., 1999). An effect of these regions on maternal behavior was already shown. Thus, hippocampal and septal lesions lead to uncoordinated nest building and pup retrieval (Fleischer and Slotnick, 1978; Kimble et al., 1967; Terlecki and Sainsbury, 1978), whereas the amygdala and the BNST are involved together with the main olfactory bulb in the olfactory recognition of the offspring (Fleming et al., 1980; Levy et al., 2004), and the cPAG is involved in the performance of the arched back nursing position (kyphosis) in rats (Lonstein and Stern, 1998). These regions were also determined to express an increased number of c-Fos positive cells in lactating rats (Lonstein et al., 1998) and in rats that were given hormonal stimuli at pregnancy termination (Sheehan and Numan, 2002).

In contrast, the role of AVP in maternal behavior is poorly understood, as there are few studies showing an increase in AVP immunoreactivity in the hypothalamus during pregnancy and postpartum (Caba et al., 1996) or the induction of maternal behavior after intracerebroventricular AVP injection (Pedersen et al., 1982). A likely possibility is the involvement of AVP in maternal behavior via an influence on mother-pup recognition. AVP was identified to be mainly involved in social recognition throughout species including pair bonding in voles (Young and Wang, 2004) and dominant-subordinate behavior in hamsters (Ferris et al., 2006). Olfactory stimuli, the main signals of recognition-relevant information, are projected from the olfactory bulbs via the amygdala and the BNST to the LS and the MPOA (Ferguson et al., 2002). This pathway includes vasopressinergic projections into the LS shown to be relevant in social recognition (Bielsky et al., 2005). Remarkably, the results of a previous social recognition/discrimination study discovered strong deficits in the social memory of male LAB mice (Bunck, unpubl.), supporting the hypothesis of a crucial impact of the AVP deficit on social recognition in male and female LAB mice.

In summary, a vasopressinergic deficit affecting the amygdala, the LS, and the MPOA, as well as the BNST and the cPAG, can be an additional factor generating

the disturbed maternal behavior of female LAB mice displaying insufficient nest-building, pup-retrieval, and pup recognition after birth, leading to higher levels of death or infanticide (personal observations), besides the above mentioned deficiencies in reproductive behavior, pregnancy, and arched back nursing related to an altered circadian rhythm and an AVP deficit in the SCN. Additional, extensive studies have to prove the deficit of AVP in these brain areas in LAB mice and its involvement in the disturbed reproductive and maternal behavior.

In the second part of the study, the influence of maternal behavior on the divergent emotionality of the HAB and LAB lines were investigated by cross-fostering the offspring. Although the litter size was differing between the lines directly after birth, after culling the number of pups and pup survival were equal until weaning. Furthermore, this is confirmed by the progressive weight gain of the offspring in all groups with no meaningful differences. Both of these findings indicate a sufficient food supply, resulting in an equal physiological growth of all groups.

Cross-fostering HAB and LAB pups induced no alteration in the number of emitted USV calls and the amount of associated locomotion at PND 5. Male and female HAB mice of both treatment groups showed a higher amount of USV calls and line crossings compared to LAB pups. In adulthood, cross-fostered HAB mice of both genders displayed a slight, but significant, increase in the percentage of time spent on the open arms of the EPM in comparison to the control groups. All other anxiety-related parameters on the EPM showed no alteration by cross-fostering. Therefore, the well-established difference in percentage of time spent on the open arms between HAB and LAB mice was completely reproduced. Also in the TST, the characteristic differences between the different lines were reproduced. Except for a slight reduction of the latency to the first immobility in cross-fostered female LABs, HAB and LAB mice displayed no changes in depression-like behavior after cross-fostering. In the OF, LAB mice displayed increased locomotor activity and exploratory activity with no alterations by cross-fostering. The SRT revealed a stronger increase in plasma corticosterone after 15min of restraint stress in male LAB mice compared to HAB mice but again no difference between the two treatments. *In situ* hybridization of *Avp* mRNA expression showed a higher intensity and larger area of labeled *Avp* mRNA in the PVN of HAB mice of both genders. Again, cross-fostering had no effect in the expression level of *Avp*.

The analysis of anxiety-related, depression-like, and stress-related behaviors in adults revealed just a slight decrease in anxiety-related behavior in HAB mice. Nevertheless, this increase in percentage of time spent on the open arms on the EPM is just a fractional amount of the time LAB mice spent on the open arms. Thus, the increase is measurable, but with no consequence on the classification of these mice in the inborn HAB phenotype. In addition, there are no alterations observed in the other anxiety-related parameters, such as latency or percentage of entries on the open arms. Also the analysis of the USV calls at PND 5 revealed no influence of the cross-fostering. However, one can expect an increasing influence of the maternal behavior on the phenotype, with no alterations after birth but in adulthood. Also in depression-like behavior, the cross-fostering caused no changes in the phenotype, except for a slight decrease in the latency to the first immobility in LAB females, who showed no difference in their absolute immobility time. The reactivity of the HPA axis to a stressor showed a stronger increase in plasma corticosterone concentration in male LABs compared to HAB mice but not in female mice. Females displayed the known higher initial and stress corticosterone levels likely due to the stimulatory effect of estrogen on AVP and CRH synthesis in the parvocellular neurons of the PVN (Kalsbeek et al., 2002). No differences were found between cross-fostered and control animals. Finally, locomotor and exploratory activity were different between the lines, with LAB mice exhibiting a higher locomotor activity and showing more rearings in the OF, reflecting an increased exploratory drive likely based on the non-anxious phenotype of LAB mice but possibly further influenced by their hyperactivity. Again, cross-fostering of the offspring induced no change in the inborn characteristics. The expression of *Avp* as a possible molecular background revealed the already shown difference between HAB and LAB in both cross-fostered mice and the control group.

Cross-fostering was used in several rat and mice lines that demonstrated differences in emotionality and related physiology in order to estimate the postnatal maternal influence on the phenotype. In spontaneously hypertensive rats (SHR), cross-fostered postpartum to normotensive Wistar-Kyoto (WKY) dams, the blood pressure was reduced but the higher exploratory drive in the OF remained the same (Cierpial and McCarty, 1991; Cierpial et al., 1989). Also in the high (HR) and low responder (LR) rats, based on their exploratory locomotion in the open filed, cross-fostering had a minor effect on the phenotype of the LR line (Stead et al., 2006), while high and low shuttle box avoidance rats displayed no phenotypic alterations after cross-fostering

(Ohta et al., 1998). A maternal influence in form of nursing and licking is assumed to have an impact on the future phenotype of the pups of the SHR/WKY rats and the HR/LR lines, as it is already mentioned for high and low LG-ABN rats and the mouse strains BALB/c and C57BL/6 (Francis et al., 1999a; Priebe et al., 2005). Interestingly the phenotypic alterations after cross-fostering were mostly related to just one of the breeding lines, pointing to a combination of the genetic background of the pup and the nursing behavior of the dams necessary to modulate the emotional phenotype of the offspring. Thereby the mother-infant interaction, including a feedback loop, might be of special importance. Besides olfactory interaction, the intensity and amount of the USV calls influence the extent of maternal care (Smotherman et al., 1974). Thus, with respect to the differences in emitted USV calls between HAB and LAB mice, the interaction of LAB dams with HAB pups might be different in comparison to the interaction of LAB dams with their biological offspring, leading to enhanced pup licking of the LAB dam, which is stimulated by the higher number of USV calls of HAB pups. This intensified mother-pup interaction might cause the slight decrease in anxiety-like behavior observed in HAB mice. In contrast, the mother-pup interaction of HAB dams and LAB pups might be less disturbed by the low number of USV calls from the LAB pups because of the normal or enhanced maternal investment given by HAB mothers, which does not result in an alteration of the anxiety-related behavior of cross-fostered LAB pups. Further, it is unexplained if this enhanced mother-pup interaction of LAB mothers could also be reflected by altered arched back nursing or time spent inside the nest, or if the more intensive care occurs during nursing without prolonging the time inside the nest or changing the nursing style. Additionally, more detailed studies of postpartum maternal behavior, including pup-licking, are necessary to analyze the mother-pup interaction of HAB and LAB mice and the impact on anxiety-related behavior.

In consideration of these data, the anxiety-related and depression-like phenotype in HAB and LAB mice, induced by selective inbreeding, is neglectably influenced by maternal rearing behavior and is therefore predominately genetically based. Remarkably, already in the HAB/LAB rat model cross-fostering had no influence on the inborn phenotypes (Wigger et al., 2001), confirming the effect of the used breeding strategy to induce genetic alterations that are functionally linked to trait anxiety. Furthermore, the differences in emotionality and in maternal care are just marginally functionally intermingled, but are probably causally related due to a

pleiotropic effect of the *Avp* gene, as both can be traced back on a genetically determined central AVP deficit. In addition, regarding the multigenic character of complex traits like maternal behavior and anxiety, AVP cannot be the sole factor forming theses traits. Thus, broad genetic screenings and expression studies are necessary to find additional factors involved in the strong behavioral differences in HAB and LAB mice.

5 Conclusion and Perspectives

Selective breeding for a particular phenotype is a valuable and successful tool to generate valid animal models of affective disorders. Therefore, breeding criterions are based on clinically observed symptoms, such as anhedonia, decreased activity, cognitive disturbances, enhanced anxiety and startle, HPA axis hyper-reactivity, and altered social behaviors. Since complex traits are multigenic, breeding for a specific behavior enhances the accumulation of genes according to this behavior, providing a valuable tool for genetic screenings. Once a gene is identified to be involved in the phenotype, the functional relevance can be evaluated by selective manipulation to definitively provide a new target for clinical research and new drug development. Despite the advantages of these animal models, such as their ability to inclose several underlying factors with their functional interaction and pathological impact, this can also be of disadvantage, since the investigation of different functionally intermingled genes and neuronal circuits and the dissection of single underlying elements is complicated. On the other hand, since most genes are pleiotropic, the modification of one gene can change several traits interfering with the trait of interest. In case of the HAB/LAB mouse model, selected for extremes in trait anxiety based on their behavior on the EPM, AVP is differently expressed at least shown for the PVN. A SNP in the signal peptide of the AVP precursor gene is likely to be involved in the AVP deficit in LAB mice.

The presented alterations in body water regulation and changes in maternal behavior confirm that a genetically induced general AVP deficit is influencing several additional AVP-associated brain areas in their functions including the SON, SCN, BNST, amygdala, MPOA, and LS. Still, the molecular and cellular effects of the SNP on peptide processing and cell viability have to be analyzed to prove the functional impact of the mutation on the AVP release. Furthermore, besides the SNP in the signal peptide, which influences the processing of the peptide, additional SNPs are present in transcription factor binding sites of the promoter region of the AVP precursor gene in LAB mice (Czibere, unpubl.), and may possibly decrease the *Avp* transcription. This might be restricted to the anxiety-related *Avp* expression in the PVN by PVN specific transcription factors binding at these binding sites, pronouncing the AVP deficit in this context compared to the other mentioned pathways. Thus, detailed genetic studies also have to elucidate the transcriptional activity of the AVP

precursor gene in parvocellular and magnocellular PVN neurons and other brain areas in LAB mice. Moreover, in the HAB line, a possible increase of transcriptional activity of the AVP precursor gene has to be investigated, since SNPs were also identified in this line in the promoter region. In any case, additionally observed behavioral alterations in the LAB line, such as increased locomotor activity and altered activity patterns, deficits in social and spatial memory (Bunck, unpubl.), and disturbed inter-male interaction and increased aggression (Frank & Keßler, unpubl.) support the concept of an overall influence of the genetically based AVP deficit. In the future, extensive behavioral, neuroendocrine, histochemical, and pharmacological studies of the HAB, NAB, and the LAB line will provide the possibility to decode the neuronal circuits underlying the diverse altered behavioral traits and their interactions and predominantly their influence on anxiety.

With respect on the multigenic character of complex traits, such as anxiety, and in consideration of the results of the AAV study, indicating only a partial involvement of AVP in the development of anxiety, an involvement of additional genes in the extremes of trait anxiety of the HAB and LAB lines are likely. Extensive microarray studies in different brain areas and a genome screen (Czibere, unpubl.) will uncover further candidates involved in the HAB/LAB phenotypes and in anxiety-related behavior. In histochemistry studies CRH was found to be higher expressed in the PVN of HAB mice compared to NAB and LAB mice and the treatment of HAB mice with a CRH R1 antagonist revealed an involvement of CRH in the HAB phenotype (Bunck, unpubl.). Furthermore, recent microdialysis studies showed elevated serotonin levels in the PVN of LAB mice under basal conditions and a decreased serotonin stress response in HAB mice compared to NAB mice (Margich, unpubl.). Moreover, c-Fos studies uncovered increased neuronal activity after different stressors in several brain areas, such as the medial and lateral amygdala, BNST, LC, PAG, MPOA, LS, nucleus accumbens, and several hypothalamic nuclei, in HABs compared to NABs (Muigg & Nguyen, unpubl.). Some of these areas were already identified in extensive c-Fos studies in HAB/LAB rats to be involved in anxiety-related behavior (Frank et al., 2006; Salome et al., 2004; Singewald, 2006). Increased tyrosine hydroxilase activity found in the LC of HAB mice points to an involvement of increased central norepinephrine in the HAB phenotype (Nguyen, unpubl.). These data point to a hyperexcitability in anxiety circuitries and the stress response-involved brain regions in HAB mice compared to NAB and LAB mice associated with an

increased anxiety-related and depression-like behavior of the former. In the future, widespread experiments will be necessary to dissect the functional involvement of the different brain areas, the associated neurotransmitters and peptides, and the underlying genetic factors that induce the different anxiety-related and depression-like behaviors in HAB and LAB mice. The identification of glyoxalase I as a valuable biomarker for trait anxiety (Ditzen et al., 2006; Kromer et al., 2005) was a first success in the extensive analysis of the HAB/LAB mouse model.

Taken together, the two breeding lines represent an animal model to decode and analyze the different elements of anxiety disorders and depression. With respect to the concept of endophenotypes, the HAB line provides a unique opportunity to identify some single aspects of the multigenic pathophysiology of anxiety, each based on a few genes, including neuroendocrine, neurophysiological, cognitive, and psychopathological components. On the other hand, in the LAB line the influence of a single genetic variation on functionally different circuits can be analyzed. Furthermore, these mice give the opportunity to investigate simple interlocking of different behavioral and physiological functions by one peptide in more complex processes.

Thus, the HAB/LAB mouse model is a valuable and promising tool to understand the physiological and pathological mechanisms of anxiety and stress-related behaviors.

6 List of Abbreviations

AAV	adeno-associated virus
ACTH	adrenocorticotropic hormone
AVP	arginine-vasopressin
BNST	bed nucleus of the stria terminalis
cDI	central diabetes insipidus
CRH	corticotropin-releasing hormone
CRH R1	CRH receptor 1
DaLi	dark-light box
dDAVP	1-deamino-8-D-arginine-vasopressin
DEX	dexamethason
EPF	elevated platform
EPM	elevated plus maze
ER	endoplasmic reticulum
FS	forced swim
GABA	gamma-aminoutyric-acid
GnRH	gonadotropin-releasing hormone
GP	glycoprotein
GR	glucocorticoid receptor
HAB	high anxiety-related behavior
hCMV	human cytomegalovirus
HNS	hypothalamo-neurohypophysial-system
HPA-axis	hypothalam-pituitary-adrenocortical-axis
LAB	low anxiety-related behavior
lacZ	ß-galactosidase gene
LC	locus coeruleus
LH	luteinizing hormone

LS	lateral septum
MAO	monoaminoxidase inhibitor
MPOA	medial preoptic area
MR	mineralocorticoid receptor
NAB	normal anxiety-related behavior
NPII	neurophysin II
OF	open field
OXT	oxytocin
PAG	periaqueductal gray
PND	postnatal day
PVN	paraventricular nucleus
POMC	proopiomelanocortin
PRL	prolactin
RIA	radioimmunoassay
SAS	sympathetic-adrenomedullary-system
SCN	suprachiasmatic nucleus
SNP	single nucleotide polymorphism
SON	suprachiasmatic nucleus
SP	signal peptide
SRT	stress reactivity test
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TRH	thyrotropin-releasing hormone
TST	tail suspension test
USV	ultrasonic vocalization
V1a/b	ACP receptor 1a and 1b

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9 Curriculum vitae

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10 Publications

- Landgraf R., Keßler M.S., Bunck M., Murgatroyd C., Spengler D., Zimbelmann M., Nußbaumer M., Czibere L., Singewald N., Rujescu D. and Frank E., 2006. Candidate genes of anxiety-related behavior in HAB/LAB rats and mice: focus on vasopressin and glyoxalase-I. Neurosci. Biobehav., epub ahead of print.

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