

**Plasticity genes in gene x environment interactions:  
expression and inheritance in a  
mouse model of anxiety**

**Dissertation**

an der Fakultät für Biologie  
der Ludwig-Maximilians-Universität München



vorgelegt von

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München, September 2015



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Dissertation eingereicht am: 24. September 2015

Mündliche Prüfung am: 18. März 2016



## Abstract

The past years of research have clearly demonstrated that the interaction of genetic, environmental and epigenetic factors contribute to the individual variation of the vulnerability to stress, anxiety and depression and finally up to the manifestation of psychopathology.

This study focused on the impact of gene x environment interactions on anxiety-related and depression-like behaviors and neurogenesis. Therefore, we used a well-established mouse model, selectively bred for anxiety-related behavior, which represents two extremes: high (HAB) vs. low (LAB) anxiety-related behavior. To shift the two extreme genetic predispositions towards 'normal' behavior, the environment of HAB mice was manipulated in a beneficial way via enriched environment (EE) and the environment of LAB mice in an adverse manner via exposure to chronic mild stress (CMS). EE offered the HAB mice a pleasant, complex environmental setup, which is reflected in reduced anxiety, as assessed in different behavioral tests. In contrast, CMS induced anxiogenic effects and more depression-like behavior in LAB mice. As repeatedly shown for this mouse model, the amygdala, particularly its basolateral nucleus (BLA), is crucially involved in the regulation of anxiety. Thus, we selected the BLA to test the genetic influences of environmental manipulations in a 'for better and for worse manner' by performing a microarray-based gene expression profiling, covering the whole genome, of HAB, HAB-EE, LAB and LAB-CMS animals. Importantly, the same candidate genes that were shown to be differentially expressed between HAB and LAB animals in a microarray analysis of animals 20 generations before were detected, thereby providing strong evidence for a fixed genetic background of the extremes in anxiety-related behavior. In our genome-wide expression assay, we could identify three potential candidate genes, confirmed by qPCR in an independent set of samples, which were differentially expressed in HAB vs. HAB-EE (*Fos*, *Gabrq*) or LAB vs. LAB-CMS (*Cnksr2*) mice. Furthermore, we assessed the environmental impact on genes and possible epigenetic changes in a transgenerational approach. Therefore, we tested if CMS-induced behavioral changes can be transmitted in both males and females until generation F2. A potential transgenerational inheritance could be shown in the group where the parental generation, but not generation F1 and F2, was stressed (CMS-Co-Co). Particularly in female mice, a pronounced anxiogenic, but no pro-depressive effect of

CMS treatment, was observed in each generation (F1, F2). Interestingly, basal plasma corticosterone, a hormone involved in stress response, was significantly reduced for the CMS-Co-Co group in the male F2 generation, suggesting an affected basal neuroendocrine regulation over generations. Earlier studies discovered corticotropin-releasing hormone receptor 1 (*Crrh1*) as a plasticity gene in the environmentally manipulated HAB/LAB mouse model. In the basolateral amygdala, the expression of *Crrh1*, *Cnksr2* (the candidate gene of environmental plasticity described here), as well as other genes associated with the HPA axis in male mice, were correlated with their behavioral performance in the light-dark box (LD) test. A negative correlation between anxiety and the expression of *Crrh1* was detected in the CMS-Co-Co group. These findings are consistent with an at least partial transgenerational inheritance shown for the behavior in the LD test. Finally, to study possible mechanisms of EE-induced anxiolysis, we investigated adult neurogenesis in HAB mice after EE as well as memantine (MM) exposures. MM is a drug used to treat Alzheimer's disease, a possible augmentation therapy of anxiety and known to increase neurogenesis in mice. In our study, both treatments, i.e. EE and MM, significantly decreased anxiety-related behavior of HAB mice and increased the number of newly born neurons. This shows that changes in anxiety-related behavior (environmentally- or pharmacologically-driven) appear to be closely associated with changes in hippocampal neurogenesis, whereas depression-like behavior seems to be unaffected. Taken together, effects of environmental manipulations could be detected in this study on the behavioral, structural and genetic level. The analysis of epigenetic mechanisms in the candidate genes from the microarray analysis, triggered by beneficial or adverse environmental manipulation, as well as of transmitted epigenetic states in the parents and offspring, might shed further light on how environmental effects can shape anxiety-related behavior in a long-lasting manner. Based on such findings, new and complementary treatment strategies have the potential to pave the way to escape from inborn predispositions or unfavorable epigenetically 'fixed' patterns.

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## List of Abbreviations

ACTH	adrenocorticotropin hormone
ADHD	attention deficit hyperactivity disorder
ANOVA	analysis of variance
ATP	adenosintriphosphat
AVP	arginine vasopressin
BLA	basolateral amygdala
BrdU	bromodeoxyuridine
BZ	benzodiazepine
CD-1	ICR (CD-1): outbred mouse strain; Institute of Cancer Research Cesarean derived 1
cDNA	complementary DNA
CeA	central amygdala
Cg	cingulate cortex
CMS	chronic mild stress
CNS	central nervous system
Co	control
CORT	corticosterone
Cp	crossing point
CRH	corticotropin-releasing hormone
cRNA	coding RNA
DAB	3,3'-diaminobenzidine
DCX	doublecortin
DG	dentate gyrus
DMSO	Dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
dsDNA	double-stranded DNA
DSM	Diagnostic and Statistical Manual of Mental Disorders
EE	enriched environment
ELS	early-life stress
EPM	elevated plus-maze
F1	first generation
F2	second generation
FST	forced swim test
GABA	gamma-aminobutyric acid
GABA <sub>A</sub>	gamma-aminobutyric acid A receptor
GAD	generalized anxiety disorder
GCL	granular cell layer
GR	glucocorticoid receptor
GWAS	genome-wide association studies
GxE	gene-environment interaction
HAB	high anxiety-related behavior
HK	housekeeper
HPA	hypothalamus-pituitary-adrenal
i.p.	intraperitoneally
ICD	International Classification of Diseases
IE	immediate early

KWH	Kruskal-Wallis H test
LAB	low anxiety-related behavior
LD	light-dark box
MAPK	mitogen-activated protein kinase
MM	memantine
Mol	molecular layer
MR	mineralcorticoid receptor
mRNA	messenger ribonucleic acid
MWU	Mann-Whitney U test
NAB	normal anxiety-related behavior
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NMDA	N-methyl-D-aspartate
OB	olfactory bulb
OF	open field
P	parental
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFC	prefrontal cortex
PND	postnatal day
PTSD	posttraumatic stress disorder
PVC	polyvinylchloride
PVN	hypothalamic paraventricular nucleus
qPCR	quantitative real-time PCR
rGE	gene-environment correlation
RIA	radioimmunoassay
RIN	RNA integrity number
RNA	ribonucleic acid
RT	reverse transcriptase
SAM	sympathetic-adreno-medullary
SEM	standard error of the mean
SGZ	subgranular zone
SNP	single nucleotide polymorphism
SNRI	serotonin-norepinephrine reuptake inhibitor
SSRI	serotonin reuptake inhibitor
SVZ	subventricular zone
TMT	trimethylthiazoline
TST	tail-suspension test
WHO	World Health Organization

## List of Gene symbols

4921530F17Rik	Riken cDNA 4921530F17 gene
A830006F12Rik	Riken cDNA A830006F12 gene
<i>Abca2</i>	ATP-binding cassette, sub-family A (ABC1), member 2
<i>Arc</i>	activity regulated cytoskeletal-associated protein
<i>Adra2c</i>	adrenergic receptor, alpha 2c
<i>B2mg</i>	Beta-2 microglobulin
<i>Ctsb</i>	cathepsin B
<i>Cnksr2</i>	connector enhancer of kinase suppressor of Ras 2
<i>Crhr1</i>	corticotropin-releasing hormone receptor 1
<i>Crhr2</i>	corticotropin-releasing hormone receptor 2
<i>Egr2</i>	early growth response 2
<i>Egr4</i>	early growth response 4
<i>Enpp5</i>	ectonucleotide pyrophosphatase/phosphodiesterase 5
<i>Fos</i>	FBJ osteosarcoma oncogene
<i>Foxp2</i>	forkhead box P2
<i>Gabrq</i>	gamma-aminobutyric acid (GABA) A receptor, subunit theta
<i>Hmgn3</i>	high mobility group nucleosomal binding domain 3
<i>Junb</i>	Jun-B oncogene
<i>Npas4</i>	neuronal PAS domain protein 4
<i>Nr3c1</i>	nuclear receptor subfamily 3, group C, member 1
<i>Polr2b</i>	polymerase (RNA) II (DNA directed) polypeptide B
<i>Rgs9</i>	regulator of G-protein signaling 9
<i>Rpl13a</i>	ribosomal protein L13a
<i>Pbx3</i>	similar to PBX3a; pre B-cell leukemia transcription factor 3
<i>Slc25a17</i>	solute carrier family 25 (mitochondrial carrier, peroxisomal membrane protein), member 17
<i>Stx3</i>	syntaxin 3
<i>Tacr1</i>	tachykinin receptor 1
<i>Ttbk1</i>	tau tubulin kinase 1
<i>Tmem132d</i>	transmembrane protein 132d
YY1	yin yang 1



## 1 Introduction

### 1.1 Mental disorders – anxiety and depression disorders

Mental disorders such as anxiety and depression impose an increasing burden on health worldwide and especially in western societies.

These multifactorial disorders are described in two major classification systems – the Diagnostic and Statistical Manual of Mental Disorders (DSM-5, American Psychiatric Association, 2014) and the International Classification of Diseases (ICD-10, World Health Organization (WHO)). Anxiety disorders (e.g., generalized anxiety disorder (GAD)), neurodevelopmental disorders (e.g., intellectual disability), depressive disorders (e.g., major depressive disorder), trauma- and stressor related disorders (e.g., posttraumatic stress disorder (PTSD)), sleep-wake disorders (e.g., narcolepsy) or neurocognitive disorders (e.g., Alzheimer's disorder) represent a small part of the about 300 psychiatric disorders listed and used for diagnosis of mental disorders in DSM-5 (2014). According to the WHO, at least 350 million people suffer from depression during their lifetime and almost one million people commit suicide every year as a consequence of their disorder (WHO, 2012). Depression as well as anxiety disorders have a two-fold higher lifetime prevalence in women than in men. These disorders also impact child development and growth since one to two out of ten mothers develop depression after childbirth (Kessler, 2003; Weissman and Olfson, 1995). Moreover, as described in the WHO mental health action plan 2013-2020, mental disorders range from mental and behavioral disorders and have their onset already during childhood or adolescence ([http://www.who.int/mental\\_health/publications/action\\_plan/en/](http://www.who.int/mental_health/publications/action_plan/en/); 09.06.2015).

Depressive and anxiety disorders show a great co-occurrence and very high comorbidity rates, which were detected in patients revealing specific vulnerability patterns (Kessler *et al.*, 2008; Lamers *et al.*, 2011). According to several studies, there is a 60% comorbidity of major depression and anxiety disorders (Landgraf, 2001; Ressler and Nemeroff, 2000). For instance, a patient suffering from both panic disorder and social phobia has a 94% risk of developing depression (Simon and Fischmann, 2005; Stein and Uhde, 1988). Comorbidity should also be taken into account in the treatment of psychiatric disorders. In this study, both anxiety-related behavioral tests as well as depression-like tests were performed to detect changes at both anxiety and depression levels.

Non-pathological anxiety can be divided into two categories. State anxiety reflects acute anxious levels at a particular time, whereas trait anxiety is characterized as a long-term response and state (Clement *et al.*, 2007; Gross and Hen, 2004; Sylvers *et al.*, 2011). In contrast, pathological anxiety has a severe influence on life and is divided into six disorders (DSM-IV; Gross and Hen, 2004).

This categorization changed in the new DSM-5, in which PTSD and obsessive-compulsive disorders were separated and now form new categories. Specific phobia, social phobia, panic disorder, agoraphobia, separation anxiety disorder, selective mutism and GAD are now classified syndromes under anxiety disorders (American Psychiatric Association, 2014).

Psychiatric disorders are complex, overlapping, and might be characterized as domains of disorder-related traits (Kas *et al.*, 2007). It is notable that mental disorders such as anxiety and depression are polygenic (Plomin *et al.*, 2009), in which many different variants are combined together with a small contribution (Sullivan *et al.*, 2012). The biological mechanisms underlying psychiatric illness such as anxiety and depression are currently investigated with huge scientific effort, aiming to assemble the neurobiological basis of anxiety and depression step by step.

## **1.2 Gene x environment interaction**

In the development of psychiatric disorders, besides genetic predisposition (see chapter 1.5), environment and epigenetics have been recognized as important factors for creating a phenotype. The interaction and correlation of genes with the environment contribute to the individual variation of anxiety and stress vulnerability up to the manifestation of psychopathology. Furthermore, in biological, social and medical sciences, environment has an increased relevance for brain function, behavior and physiology (Pryce *et al.*, 2002).

Several studies found various influences on symptoms of anxiety and depression in a gender- (Eaves *et al.*, 1997; Heim *et al.*, 2009) or age-specific manner (Tambs and Moum, 1993) and suggest that genes have an impact on environmental measures and *vice versa* (a relationship between individuals and their environment) (Kendler and Baker, 2007). A distinction between gene-environment interactions (GxE) and gene-environment correlation (rGE) is a crucial point to be considered in studies of gene and environment factors in psychiatric disorders (Nugent *et al.*, 2011). rGE are



genetically conveyed dispositions to particular environmental events, whereas GxE are affected by an individual genetic response towards distinct environmental conditions (Lau and Eley, 2004; Plomin *et al.*, 1977; Rutter and Silberg, 2002). Twin studies support the statement that even if a genetic predisposition exists, there is a considerable amount of population variance for developing different psychiatric disorders such as depression, anxiety or in cognitive development (Davis *et al.*, 2009; Landgraf, 2001). The high heritability of almost all psychiatric disorders as well as their accumulation in families was proven in several family, twin and adoption studies (Kendler, 2013).

Research suggests that positive (beneficial) and negative (detrimental) environments can influence the interaction with genes, which show individual responsiveness to environmental stimuli (Belsky *et al.*, 2009; Belsky and Pluess, 2009; Wolf *et al.*, 2008).

Positive environment studies in animals were performed using the concept of enriched environment (EE), which can mimic positive life experiences in humans. The paradigm of EE arose from the “combination of inanimate and social stimulation”, which is reflected as “complex” environment (Rosenzweig *et al.*, 1978). The main goal was to create a semi-natural environment with higher social interactions, exploratory and motor behavior. EE comprised an enlarged home cage with different biological stimuli for exploring, climbing and hiding opportunities as well as a grouped housing and additional nesting material. More and more studies are conducted to investigate influences of EE on different circuitries (Nithianantharajah and Hannan, 2006). Enrichment can have effects on physiological and behavioral properties such as improved memory and learning abilities (Kempermann *et al.*, 1997; Tang *et al.*, 2001) or increased sensory (visual) capabilities (Sale *et al.*, 2004). A growing body of data support the view “that early developmental mechanisms can set the lifelong tendency of an organism to express anxiety in response to threatening stimuli” (reviewed in Gross and Hen, 2004). During early development, several brain structures and neural circuits associated with anxiety show the highest plasticity such as the prefrontal cortex (PFC), amygdala and hippocampus (Gross and Hen, 2004). Moreover, it is well known that the beneficial EE induces anxiolytic effects, although the molecular mechanisms are not completely understood. A study found that downregulation of corticotropin-releasing hormone receptor 1 (*Crh1*)

mRNA expression in the basolateral amygdala (BLA) (Kühne *et al.*, 2012; Van Pett *et al.*, 2000), a major region involved in anxiety regulation (Davis, 1992), was correlated with the anxiolytic effect of EE (Sztainberg *et al.*, 2010). *Crhr1* was critically implicated in high anxious mice exposed to EE and a decreased expression in the BLA and the central amygdala (CeA) was observed (Sotnikov *et al.*, 2014b). In another study, not only the shift in a beneficial manner, but also adverse effects, regulated *Crhr1* expression in the amygdala (Sotnikov *et al.*, 2014a). Additionally, EE was associated with increased progenitor proliferation and differentiation in the amygdala, which can be involved in the beneficial anxiolytic aspects. In that study, EE increased bromodeoxyuridine (BrdU)-positive (BrdU+) newborn cells and suppressed cell death in the amygdala (Okuda *et al.*, 2009).

Besides amygdala, several different studies demonstrated that the hippocampus, which shows high plasticity, is susceptible to environmental stimuli. The hippocampal neurogenesis is increased in animals exposed to EE (Hosseiny *et al.*, 2014; Kempermann *et al.*, 1997). Moreover, there is growing evidence that neurogenesis is contributing to anxiolytic effects (Revest *et al.*, 2009).

Furthermore, research revealed that stress in early life is specified as moderate-severe misery suffering during childhood or adolescence (Nugent *et al.*, 2011). Early life stress (ELS) as a triggering factor might, in combination with genetic predisposition, lead to psychopathology (Nugent *et al.*, 2011). EE can trigger changes in neural circuitry in the hypothalamus, which in turn can influence the hypothalamus-pituitary-adrenal (HPA) axis activity (Cao *et al.*, 2010). Another important fact of EE has to be mentioned. EE has the potential to reverse negative consequences of ELS (Francis *et al.*, 2002) as well as emotional disturbances in rodent models for schizophrenia, depression and PTSD (Takuma *et al.*, 2011). These findings indicate that stress and the HPA axis play critical roles in changes caused by environmental influences during early life.

### 1.3 Hypothalamus-pituitary-adrenal (HPA) axis

The HPA axis, together with another neuroendocrine system, the sympathetic-adreno-medullary (SAM) system, regulates the function of the homeostatic balance in the body. Homeostasis is a complex dynamic equilibrium, which is permanently influenced and disturbed by intrinsic and/or extrinsic physical and physiological events, denoted as stressors (Charmandari *et al.*, 2005; Chrousos and Gold, 1992). The term 'stressor' is defined as a 'stimulus that threatens homeostasis' followed by the 'stress response', which is responsible to get the organism back to homeostasis (Chrousos, 2009). Koolhaas and colleagues emphasized that "stress should be considered as a cognitive perception of uncontrollability and/or unpredictability that is expressed in a physiological and behavioral response" (Koolhaas *et al.*, 2011). Primarily, the hypothalamus responds to a stressor by activating the production and release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) to hypophysial portal vessels synthesized by parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN). CRH, the main hypothalamic regulator, in turn promotes the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary to systemic circulation by binding to its receptor on pituitary corticotropes. This is synergistically supported by AVP. Moreover, released ACTH travels in the bloodstream to the adrenal gland lying atop the kidney, where it stimulates the synthesis and release of glucocorticoid hormones from the *zona fasciculata*. In humans, cortisol is the most prominent glucocorticoid, whereas in rodents corticosterone (CORT) is the most prominent one (Melmed *et al.*, 2011). Glucocorticoids are separated into a binary receptor system: the mineralocorticoid receptor (MR), which is responsive to low glucocorticoid concentrations and the glucocorticoid receptor (GR), which responds mainly to stress. Subsequently, ACTH and CRH secretion is restricted by glucocorticoids mediating the negative feedback mechanisms, which takes place on the level of pituitary gland, PVN and hippocampus (Charmandari *et al.*, 2005; Jones *et al.*, 1977; Lightman and Conway-Campbell, 2010; Smith and Vale, 2006; Walker *et al.*, 2010; Watts, 2005) (Fig. 1.1). A dysfunction of the stress system, indicated by a "sustained hyperactivity and/or hypoactivity", can cause "psychiatric, endocrine, and inflammatory disorders and/or susceptibility to such disorders" (Chrousos and Gold, 1992; Tichomirowa *et al.*, 2005).

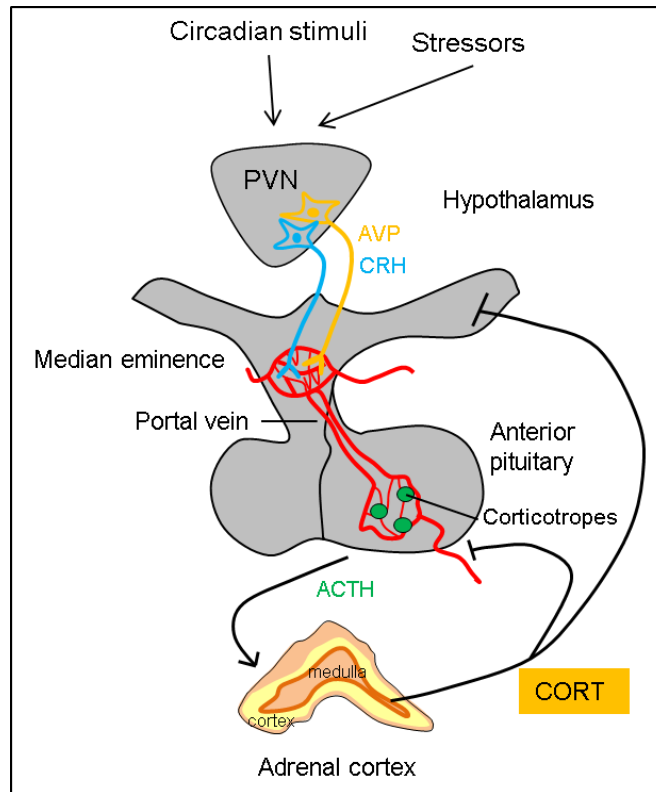


Figure 1.1: **Schematic illustration of different levels of the hypothalamus-pituitary-adrenal (HPA) axis.** The hypothalamus responds to many inputs like circadian stimuli or physical stressors with a secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) to systemic circulation. Thus, the anterior pituitary secretes adrenocorticotropic hormone (ACTH), which induces glucocorticoid release such as corticosterone (CORT) from the adrenal cortex. Subsequently, ACTH and CRH secretion is limited by glucocorticoids mediating the negative feedback mechanism. (Figure adapted from Lightman and Conway-Campbell, 2010)

CRH is one of the major mediators of the effects of stress on the HPA axis and is therefore critically involved in the pathophysiology of stress-related disorders such as depression and anxiety (de Kloet *et al.*, 2005; Holsboer and Ising, 2008; Nemeroff *et al.*, 1984; Refojo *et al.*, 2005; Reul and Holsboer, 2002). It is a 41-amino acid peptide with a 196-amino acid precursor and is widely expressed in the brain. Its biological action is mediated by two G-protein-coupled receptors, CRH-receptor 1 (CRHR1) and CRH-receptor 2 (CRHR2), which are distributed in neocortical, limbic and brainstem regions of the central nervous system (CNS) and on the pituitary corticotropes (Dautzenberg and Hauger, 2002) (reviewed in van Pett *et al.*, 2000). Moreover, twin studies identified a high heritability of HPA axis components and a

high individual variation in its activity, which shows a strongly driven genetic background and environmental influence on the stress system (Mormede *et al.*, 2002; Mormede *et al.*, 2011; Wust *et al.*, 2004). A dysregulation of the HPA axis, for example genetic disturbance of MRs in adult mice, can affect neurogenesis in the hippocampus (Gass *et al.*, 2000).

#### **1.4 Animal models – the HAB/LAB mouse model**

As psychiatric disorders are highly heritable and the number of patients keeps growing constantly, it becomes increasingly important to study the mechanisms behind these disorders. Both, genetic and environmental influences have to be taken into account. The study of GxE in humans shows limitation in monitoring environmental factors and risks, as well as the difficulty of controlling these factors (Heath *et al.*, 2002). Therefore, animal models are the perfect study objects to investigate genes, environments and their interactions on multifactorial disorders (Kas *et al.*, 2007). These models should share endophenotypes, which show behavioral, physiological, neuroendocrine and genetic characteristic symptoms analog to human psychopathology (Bakshi, 2002; Landgraf and Wigger, 2003). It is described that endophenotypes on the one hand represent an instrument to determine the 'downstream' traits or aspects of clinical phenotypes, and the 'upstream' impacts of genes on the other hand (Gottesman and Gould, 2003). For research purposes, three endophenotypic criteria have to be fulfilled by an animal model to count as valid for the respective psychiatric disease. The first one, face validity, describes the similar symptomatology between the model and the human phenotype of the disorder. Similarities in underlying mechanisms and processes of the disorder refer to construct validity, whereas predictive validity has to enable potential therapeutic value for human psychopathology (Landgraf and Wigger, 2003). In addition, 87% alignment of all human gene-coding areas to mouse and rat (Brudno *et al.*, 2004), identical biological pathways (blood pressure, feeding, etc.), and high sensitivity to environmental stimuli, makes rodents as perfect models (Kas *et al.*, 2007; Tecott, 2003).

In this study, an anxiety mouse model was used. Its breeding strategy was successfully applied in the 1980s, where Wistar rats were selectively bred for anxiety-related behavior (Landgraf *et al.*, 2007; Landgraf and Wigger, 2002; Liebsch *et al.*, 1998). Rats are potent model organisms for studying trait anxiety and comorbid depression on the behavioral and neuroendocrine level, but genetic methods are limited. Therefore, this selective breeding approach was applied using outbred Swiss CD-1 mice (Krömer *et al.*, 2005). Two lines were bred for two extremes of anxiety-related behavior: high anxiety-related behavior (HAB) and low anxiety-related behavior (LAB) mice. The key selection criterion was their behavioral performance on the well-established elevated plus-maze (EPM) test for testing anxiety-related behavior (Pellow *et al.*, 1985). In this test, HAB mice were more anxious than LAB mice, independent of gender. The characteristic behavior in HAB mice is that they spend less than 10% of the total test time on the open arms of the EPM test, whereas LAB mice spend more than 50%. In addition, normal anxiety-related behavior (NAB) mice were bred showing intermediate 'normal' behavior on the EPM test. All three breeding lines are depicted in Fig. 1.2 with about 50 generations.

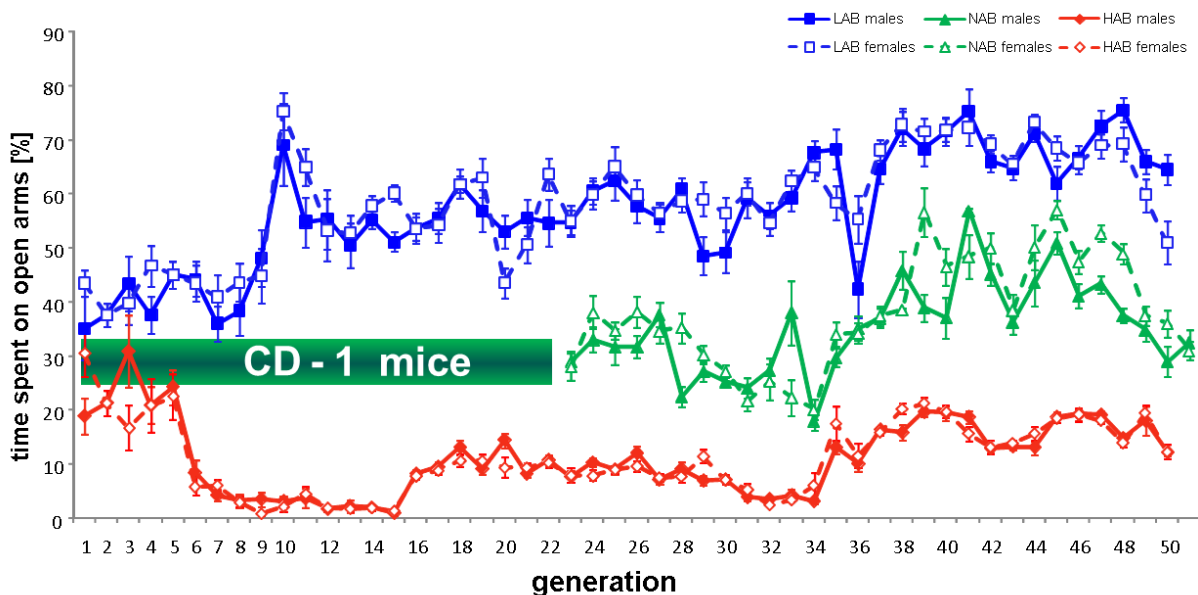


Figure 1.2: **Breeding course of high (HAB), normal (NAB) and low (LAB) anxiety-related behavior mice.** The key selection criterion for breeding is the time spent on the open arms of the elevated plus-maze (EPM) test.

In this validated mouse model of pathological anxiety, several studies in all kinds of directions were conducted to reveal the underlying mechanisms of anxiety and comorbid depression. Starting on the behavioral level, HAB/LAB mice show a constant highly significant difference in their anxiety-related and depression-like behavior in the open-field (OF) test, light-dark box (LD) test, tail-suspension test (TST), forced swim test (FST) (Krömer *et al.*, 2005) or predator odor avoidance (Sotnikov *et al.*, 2011). Independent of the test, HAB mice showed higher measures of anxiety and depression. Further, a higher ultrasonic vocalization was detected in HAB compared to LAB mice (Krömer *et al.*, 2005). Moreover, a higher level of activity and higher locomotor activity was revealed in male and female LAB mice than in HAB and NAB mice in other studies (Krömer *et al.*, 2005; Landgraf *et al.*, 2007). As LAB mice show hyperactivity, active coping styles and additionally cognitive dysfunctions, this mouse line was suggested to be a new model of attention deficit hyperactivity disorder (ADHD)-like symptoms (Yen *et al.*, 2013). On proteomic level, combined with genetics, biomarker candidates were found, which are likely part of metabolic pathways crucial for the phenotype, especially for diseases (Ditzen *et al.*, 2010). Effects of single nucleotide polymorphisms (SNPs) (Kessler *et al.*, 2007) or copy number variants (Brenndörfer *et al.*, 2015) were also investigated in the mouse model for trait anxiety and depression. Genetic differences between HAB and LAB mice are described in chapter 1.5.

Taken together, the results of studies in the HAB/LAB mouse model reveal this animal model as a valid model of anxiety-related and depression-like phenotypes. Based on this evidence, more possible neural and plasticity processes or genetic and epigenetic analyses can be studied to probably optimize future pharmaceutical therapies.

### **1.5 Candidate genes**

As psychiatric disorders are known to be polygenic, the necessity of genome-wide association studies (GWAS) or/and whole transcriptome analysis to detect new risk candidate genes is increasing (Craddock *et al.*, 2008; Czibere *et al.*, 2011). Moreover, as neurobiological pathways are affected by multiple genes, a given gene has merely a small contribution to the variance in the risk for complex disorders (Nugent *et al.*, 2011). As described in Belsky *et al.* (2009) a 'framework of differential

susceptibility' is suggested in which individuals change their 'biological sensitivity to context' (Boyce and Ellis, 2005). A possible candidate gene should have a conserved gene function across related species with a shared correlation between genotype and phenotype (Bunck *et al.*, 2009; Kas *et al.*, 2007). In the studies of GxE, interesting candidate genes, which respond to environmental manipulations are proposed to be 'plasticity genes' rather than 'vulnerability genes' or 'risk alleles' (Belsky *et al.*, 2009). Anxiety disorders are lying at one end of a scale of anxiety continuum, whereas the opposite end would represent a state with extremely low anxiety. Individual states vary throughout their lifetime along this scale with very blurred borders of what is pathological and what is not. Along this continuum, even rigid genetic predispositions to high anxiety can be shifted to a more intermediate level using epigenetic effects and environmental modifications. Genes related to these shifts react from both extremes on adverse factors like stress or beneficial stimuli in a 'for better and for worse manner' according to the 'differential susceptibility hypothesis' ('plasticity hypothesis') (Belsky *et al.*, 2009; Belsky and Pluess, 2009; Pluess and Belsky, 2011).

According to former studies and the well characterized HAB/LAB mouse model, several genes, SNPs and biomarkers were identified, which bear significant linkage to the observed phenotype in this model. To mention a few of them, glyoxalase-I was identified by microarray and proteomic analyses as a protein marker of trait anxiety in several brain regions, with a higher expression in LAB compared to HAB mice (Krömer *et al.*, 2005; Landgraf *et al.*, 2007). Additionally, the same phenotype-dependent difference was found in the protein enolase phosphatase (Ditzen *et al.*, 2006; Ditzen *et al.*, 2010). After quantitative real-time PCR confirmation, the microarray-based gene expression study by Czibere *et al.* (2011) identified 15 differentially expressed candidate genes for the multigenic trait anxiety, e.g., high mobility group nucleosomal binding domain 3 (*Hmgn3*), cathepsin B (*Ctsb*), syntaxin 3 (*Stx3*). Among others, the neuropeptide *Avp* displayed a deficit, which is likely to contribute to the low anxiety of LAB mice. After central release, *Avp* plays a critical role in the regulation of anxiety-related and depression-like behavior and acts as an antidiuretic hormone after peripheral secretion (Bunck *et al.*, 2009; Landgraf *et al.*, 2007). Another candidate gene is the transmembrane protein 132d (*Tmem132d*). In the anxiety mouse model, a higher expression in HAB compared to LAB mice was observed, which was confirmed in human studies. Here, patients with risk genotypes



for panic disorder had higher *Tmem132d* mRNA expression levels in the PFC (Erhardt *et al.*, 2011).

Thus, genetic risk factors (e.g., SNPs) can accumulate over generations to place an individual along the anxiety continuum. In contrast and complementarily, there are epigenetic mechanisms, which allow the individual to adjust along this continuum during its life and even give the option to incorporate 'freshly' acquired traits.

### **1.6 Transgenerational transmission**

Environmental factors can affect gene expression and lead to disease. Moreover, transgenerational implications a novel kind of non-genetic inheritance is a topic of increasing importance in disease etiology (Skinner *et al.*, 2010). In the last years, a growing body of literature proposed that for phenotypic variation in complex traits transgenerational epigenetic effects are strong contributors, but the findings remain controversial (Arai *et al.*, 2009; Heard and Martienssen, 2014; Skinner *et al.*, 2010). Epigenetic transgenerational inheritance has been established in a variety of different species, ranging from plants to humans (Pembrey *et al.*, 2014). As a first study, environmental factors like poisonous and harmful substances mediated transgenerational effects on reproductive disease (Anway *et al.*, 2005) and nutritional abnormalities (high fat diet) (Dunn and Bale, 2011). Transgenerational effects of maternal care, ELS and exposure to stress are also described in several studies (Champagne and Meaney, 2007; Dietz *et al.*, 2011; Gapp *et al.*, 2014; Ward *et al.*, 2013). Epigenetic transgenerational inheritance refers to "the germline (egg or sperm) transmission of epigenetic information between generations in the absence of any environmental exposure" (Skinner, 2011; Skinner, 2014). These effects are independent of inherited changes in the primary DNA sequence (Daxinger and Whitelaw, 2012). In this case, a distinction between 'intergenerational' (parental) or multigenerational exposure and 'truly transgenerational' effects has to be made. The former refers to environmental factors such as hormonal factors, nutritional factors, or stress/toxins, influencing the embryo and its germline *in utero*. By contrast, generations not directly exposed to the triggering event are called true transgenerational effects. Phenotypes can be transmitted for two generations (F2 generation) either through the parental lineage or through the maternal lineage. In the latter case, to establish transgenerational inheritance, the F0 female has to be

pregnant during exposure, which means the *in utero* (F1) as well as the germline of the fetus (the future F2) are affected. Therefore, phenotypic transmission for three generations (F3) is required. In conclusion, a transgenerational effect is considered when the germline is responsible for transmitting epigenetic information to the following generation (reviewed in Skinner, 2014; Stegemann and Buchner, 2015; Heard and Martienssen, 2014) (Fig. 1.3). The mechanisms behind the transfer across generations are epigenetically driven and not mediated by changes in DNA.

Epigenetics is described as “molecular factors and processes around DNA that are mitotically stable and regulate genome activity independent of DNA” (Skinner, 2011; Skinner *et al.*, 2010). DNA methylation, histone modifications, chromatin structure, and non-coding RNA are counted to epigenetic processes and result in a change of gene expression. DNA methylation is the most likely factor in germline transmission (Skinner, 2014), but all others play critical roles in regulating development (Berger *et al.*, 2009; Rissman and Adli, 2014). Thus, this field of research is relatively new and future studies will be necessary to further evaluate the effects of transgenerational transmission and processes influencing the development of diseases.

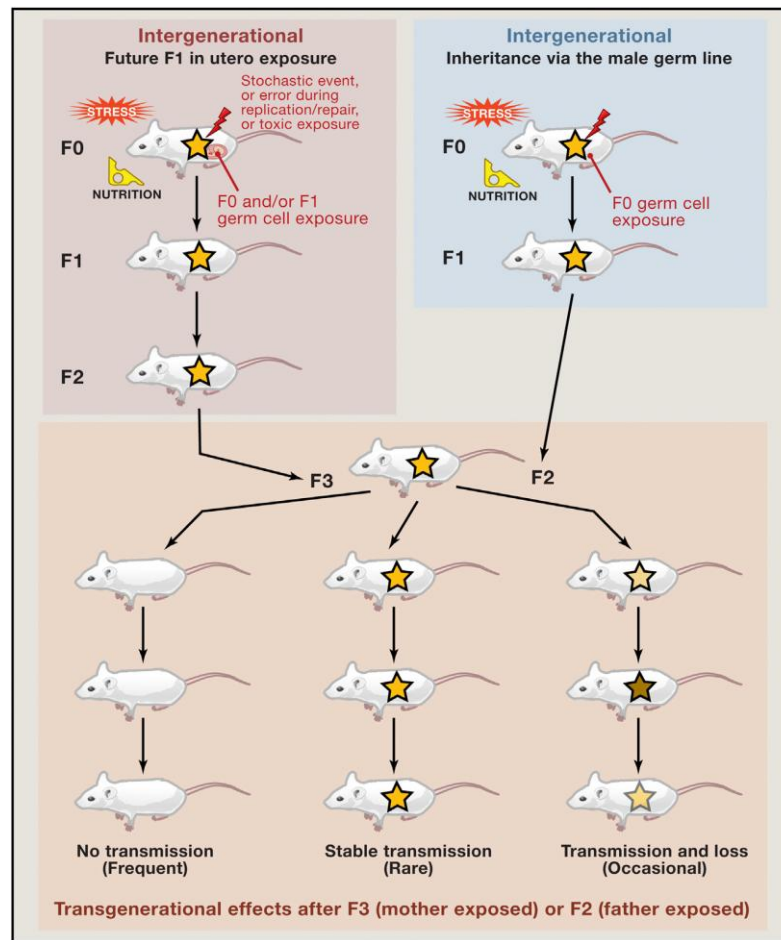


Figure 1.3: **Transgenerational and intergenerational effects.** Environment (toxins, nutrition and stress) can induce epigenetic changes in mammals. To establish transgenerational inheritance through the maternal lineage, exposure on a pregnant female mouse can have an effect on the fetus *in utero* (F1) as well as the germline of the fetus (the future F2). This leads to intergenerational epigenetic inheritance, whereas only F3 individuals can be considered as true transgenerational inheritance. In the case of transmission through the paternal lineage, F0 and its germline (future F1) are exposed (intergenerational). Thus, F2 and following generations are taken as transgenerational inheritance. (Figure adopted from Heard and Martienssen, 2014).

### 1.7 Neurogenesis in psychiatric disorders

During the last years, scientists all over the world have been trying to reveal the mechanisms and the molecular and cellular basis of heterogeneous, multifactorial disorders like anxiety and depression. Neuroimaging studies showed that several brain regions related with stress, cognition, mood and emotion are altered in patients with mood disorders, displaying abnormalities in structure and function (Drevets *et al.*, 2008; Phillips *et al.*, 2003). An interesting circuit involved in these processes is the limbic-cortical-striatal-pallidal-thalamic circuit. In this, orbital and medial prefrontal

cortex, amygdala, hippocampal subiculum, ventromedial striatum, mediodorsal, midline thalamic nuclei and ventral pallidum are connected (Ongür *et al.*, 2003). Besides genetic studies in these brain areas and the importance of amygdala in anxiety disorders, a focus on the well-studied hippocampus in depression gained raising interest. The hippocampus is a brain region, which is connected to the amygdala and PFC (Duman and Monteggia, 2006). It is known to impact on the pathogenesis of depressive disorders, is involved in learning and memory (Jarrard, 1993) and is highly stress sensitive (Lupien *et al.*, 2009). Thus, another important factor in dysfunction of brain regions and neuronal circuits is the regulation of adult neurogenesis (reviewed in Zhao *et al.*, 2008). The neurogenesis is altered during stress, acutely or chronically, which leads to adaptive changes in the hippocampus (Warner-Schmidt and Duman, 2006). In the development of the brain and its functioning, appropriate types of neurons are necessary to be generated in the correct numbers and places, to migrate to their final positions and to set up synaptic connections (Abrous *et al.*, 2005). Neurogenesis is the proliferation and differentiation of adult neural stem cells or progenitors, which is known to continue during adulthood throughout life. Newly born cell proliferation and survival occurs in two specific brain areas: the subventricular zone (SVZ) and the subgranular zone (SGZ). SVZ forms the lining of the lateral ventricles, in which the neurogenic process proceeds in the olfactory bulb (OB), and the SGZ forms part of the dentate gyrus (DG) of the hippocampus area. In the OB, the newborn olfactory neurons mature, differentiate mostly into interneurons, functionally integrate into preexisting neural networks and form synaptic connections, whereas in adult SGZ born neurons become dentate granule cells (Abrous *et al.*, 2005; Braun and Jessberger, 2014; Zhao *et al.*, 2008). In the SGZ, two different types of neural progenitors are specified, which differ in their distinct morphologies and their molecular markers expression (Fig. 1.4). Type 1 cells grow in a radial process through granular cell layer (GCL) into molecular layer (Mol). These radial glia-like neural stem cells can in turn be activated for generating proliferation and transiently amplifying type 2 non-radial cells. This then can cause proliferation of neuroblasts and postmitotic immature neurons (reviewed in Christian *et al.*, 2014). Finally, the neurons mature into DG granular cells over a period of three weeks. When they are incorporated into the circuitry, less than 25% of the newborn neurons survived. These granular neurons are functionally and synaptically integrated and can form synaptic connections for receiving inputs such

as depolarizing responses to GABA or sending synaptic outputs in terms of glutamate (reviewed in Christian *et al.*, 2014).

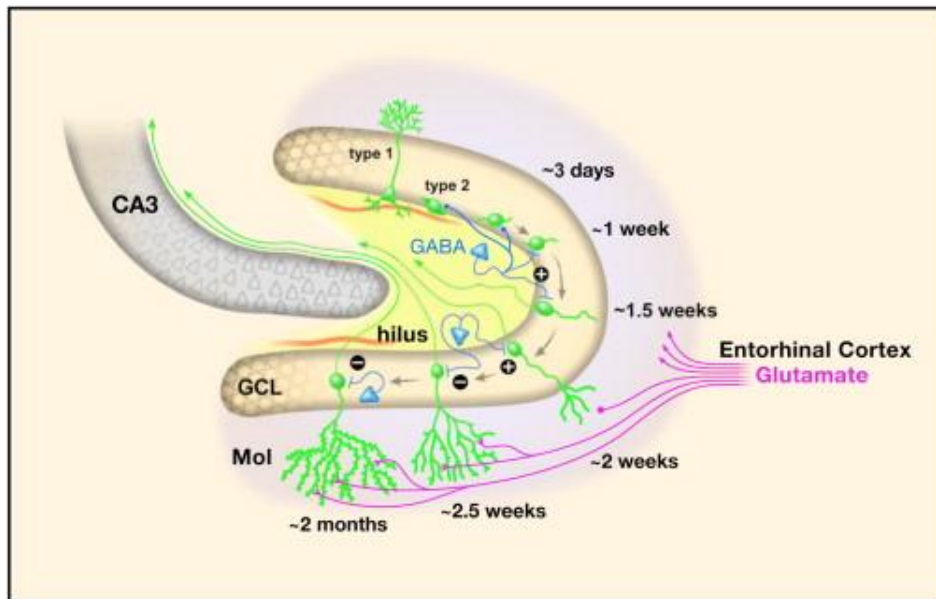


Figure 1.4: **Neurogenesis in the dentate gyrus of the hippocampus.** In the subgranular zone, type 1 and type 2 progenitor cells can be distinguished by their distinct morphologies and their molecular markers expression. Newborn neurons develop through a number of different stages, whereby, a transition from GABA (blue) excitatory to GABA inhibitory and glutamate excitatory inputs arise during the third week after cell birth. GCL, granule cell layer; Mol, molecular layer. (Figure adopted from Zhao *et al.*, 2008).

As already mentioned above, stress is a particularly adverse factor in the regulation of progenitor proliferation and new-neuron survival. Rising evidence indicates that adult hippocampal neurogenesis plays a discriminating role in the suppression of the HPA axis in stress response (Christian *et al.*, 2014). This phenomenon appears in stressed animals and in human patients suffering from major depression (Zhao *et al.*, 2008). Numerous studies reported a reduction of cell proliferation in the SGZ caused by chronic stress (Mirescu and Gould, 2006).

Thus, the DG is a dynamic structure. Dysfunctions in this developing system of adult hippocampal neurogenesis can exist in several brain disorders, which is investigated in animal model studies. Future studies have to focus on various different dynamic up- and down-regulated factors such as endocrine, environmental and pharmacological ones.

## **1.8 Treatment of psychiatric disorders**

Over the past years, treatment of psychiatric disorders started with treating symptoms of each disorder separately. Due to the comorbidity of anxiety and depression, more and more antidepressants have to act on several levels and have to influence multiple networks. A main goal for the development of effective pharmaceutical and/or psychological therapies has to be a reduction of side effects. Research has elucidated that a combination of different treatments including behavioral therapy are most effective. Around 50% of patients suffering from anxiety disorders only respond partly to a chosen pharmacotherapy and need augmentation therapy (Ballenger, 1999; Schwartz *et al.*, 2005). Several different classes of antidepressants, including serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors, are well-established. Anxiolytics such as benzodiazepines (BZ), a SSRI class, are the most widely used pharmaceutical treatments, acting on GABA and function acutely on anxiety (Macaluso *et al.*, 2010). By contrast, most of the available treatments need weeks or months to show a response in patients. As BZs are most effective the first six weeks, a high relapse rate occurs, when a discontinuation is applied. Therefore, a chronic treatment and additional therapies are widely used for an appropriate treatment (reviewed in Ballenger, 1999).

Adult neurogenesis can also be altered by treatment against depression. Several studies revealed that antidepressant drugs increase adult neurogenesis in the DG in contrast to stress, which is reducing cell proliferation (Mirescu and Gould, 2006; Warner-Schmidt and Duman, 2006). This decreasing effect can be reversed or hindered by antidepressants such as fluoxetine, a serotonin-selective reuptake inhibitor. Chronic administration of fluoxetine enhances proliferation and survival of newborn neurons (Encinas *et al.*, 2006). Moreover, several co-regulations of SGZ neurogenesis by antidepressants are existent and under current investigations. For example, the brain-derived neurotrophic factor shows increasing effects on neurogenesis in the survival of newborn neurons as well as anti-depressive effects on behavior in presence or absence of antidepressants (Duman and Monteggia, 2006). Moreover, the administration of insulin-like growth factor 1 is described to increase neurogenesis and cause effects on depression-like and anxiolytic behaviors (Duman and Monteggia, 2006; Malberg *et al.*, 2007).

Furthermore, several trials suggest augmentation therapy as a common use for treating psychiatric disorders if conventional antidepressant anxiolytics fail (Schwartz *et al.*, 2005). New findings in patients propose memantine (MM) as a drug effective for generalized or social anxiety disorders (Schwartz *et al.*, 2012), for depression, ADHD or dementia disorders such as Alzheimer's disease (reviewed in Thomas and Grossberg, 2009). This drug was described to be a possible therapeutic treatment for augmentation therapy of anxiety disorders. MM is a noncompetitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonist and a well-tolerated drug for treatment of Alzheimer's disease (Bassil and Grossberg, 2009). It plays a role in the glutamate-GABA balance, creating less "side effects (weight gain, sexual problems, (e.g., SSRI/SNRI), or addiction (e.g., sedatives))" (Schwartz *et al.*, 2012). Recently, it has been shown to increase neurogenesis in mice (Akers *et al.*, 2014). The rising numbers of patients suffering from anxiety and depression imply to a huge task for future research to develop novel antidepressants agents.

## 1.9 Aims of the thesis

The focus of this thesis was the impact of gene x environment interactions (GxE) on different levels of anxiety-related and depression-like behavior. We took advantage of the extreme genetic predisposition of inborn anxiety of the HAB/LAB mouse model and used two different approaches of early life environmental modifications to induce a bidirectional shift towards 'normal anxiety' – a more beneficial (EE) and an adverse one (CMS).

1. To investigate novel candidate genes, which are differentially expressed between the mouse lines while exposed to diverse environmental conditions, we performed a microarray-based basal gene expression profiling, covering the whole genome. This investigation was performed in the BLA of HAB, LAB, HAB-EE and LAB-CMS animals, a brain area known to be involved in anxiety and depression pathogenesis. Potential plasticity genes were validated via qPCR analysis to verify the involvement in environmental manipulations in the anxiety mouse model.
2. Another important question examined in this work was if anxiogenic effects of adverse environmental experiences in one generation may profoundly impact behavior of subsequent generations. To assess whether transgenerational transmission of CMS-induced behavioral changes occur in both males and females, we analyzed all offspring up to generation F2 in a behavioral test battery. Additionally, basal CORT was measured in generation F2 to evaluate the basal HPA axis activity likely to be related to anxiety.
3. Based on previous studies by Sotnikov *et al.* (2014), *Crhr1* was found to be involved in trait anxiety and showed plasticity in the bidirectional manipulations in the BLA. Therefore, the expression of *Crhr1* and further candidate genes were measured in the CMS transgenerational transmission approach to reveal behavioral and expression correlations over generations.
4. To establish a further link between anxiety predisposition and behavioral shifts on anxiety-related/depression-like behavior, we included a pharmacological approach to influence neurogenesis. Therefore, the effect of beneficial environmental modification (EE) and memantine (MM) as proneurogenic treatment was investigated.



## 2 Materials and Methods

### 2.1 Animals

HAB and LAB mice used in the following studies originated from generations 45 – 53 and were bred in the animal facility of the Max Planck Institute of Psychiatry in Munich. All animals were kept under standard housing conditions (room temperature  $23 \pm 2^\circ\text{C}$ , relative air humidity  $60 \pm 5\%$ , 12:12 h light/dark cycle with lights on at 8 a.m.), with pelleted food (Altromin 1314 TPF; protein 22.5%, fat 5%, fiber 4.5%, ash 6%, Altromin GmbH, Lage, Germany) and drinking water *ad libitum*.

Before any behavioral testing, a five days habituation phase was provided after transferring the mice from their breeding facility to a room next to the testing room. For both environmental manipulations, control groups were housed in Makrolon cages type II (207 x 140 x 265 mm; Bayer MaterialScience, Leverkusen, Germany) including bedding and nesting material (LIE E-001, Abedd Lab and VET Service, Vienna, Austria) either in groups of three (HAB) or single (LAB) of the same sex without any manipulations, except a weekly cage change. The animals used for EE were group-housed (three or up to four) in Makrolon cages type IV (380 x 200 x 590 mm), and the mice used for CMS treatment were kept single-housed in Makrolon cages type II. For the transgenerational approach male and female mice were taken for breeding in Makrolon cages type III (265 x 150 x 420 mm), for the neurogenesis experiment female mice were used only, otherwise all molecular experiments were carried out with male mice.

All animal experiments were carried out according to current regulations for animal experimentation in Germany and Austria and the European Union (European Communities Council Directive 86/609/EEC). The presented work was announced and approved by the appropriate local authority.

## **2.2 Behavioral testing for assessing anxiety-related and depression-like behavior**

All behavioral tests were performed during the light phase between 09:00 a.m. and 01:00 p.m. to assure equally low basal CORT levels due to the circadian rhythm. Evidence was found that circadian rhythms are disturbed and dysregulated in HAB mice, a mouse model for anxiety and comorbid depression (Griesauer *et al.*, 2014). For assessment of anxiety-related behavior, we performed different well-established tests utilizing approach-avoidance conflicts (Bailey and Crawley, 2009; Bourin and Hascoet, 2003; Cryan and Holmes, 2005).

In order to test depression-like behavior in mice, the animals were exposed to a desperate, uncontrollable situation where they were not able to escape or extricate themselves, and their behavior was assessed (Cryan and Holmes, 2005). As a natural strategy the mice have to cope with the situation actively or passively (depression-like) (Lino-de-Oliveira *et al.*, 2002). Two different behavioral reactions can be distinguished, the 'active' characterized as an active attempt to escape from the situation and 'passive coping strategy' in which the situation seems to be accepted (Krömer *et al.*, 2005).

### **2.2.1 Open field (OF) test**

The OF test was used to assess locomotor activity and explorative behavior (Prut and Belzung, 2003). We applied a setting with moderate illumination to measure anxiety-related behavior (Walsh and Cummins, 1976). The apparatus consisted of a grey circular open field PVC arena including a wall of 40 cm height and a field of 60 cm in diameter (see Fig. 2.1). Two different zones were set comprising the inner central zone lit with <50 Lux to create an aversive area and the surrounding outer zone with 15 Lux as a less challenging part. At the beginning of every test session, the mouse was placed into the central inner zone. The OF test lasted 5 min and was videotaped using a computer software (Any-maze 4.50, Stoelting, Illinois, USA). Any-maze tracking software was used to analyze the animal's behavior. The parameters assessed in this test were 'total distance traveled' and 'percentage time spent in the inner zone'. After testing, the animal was returned to its home cage and the maze was cleaned with soapy water, 70% ethanol and dried to leave no odor cues for the subsequent animal.



Figure 2.1: **Open field (OF) test apparatus.** The experimental setup of the OF test consisted of an open field arena surrounded by walls.

### 2.2.2 Elevated plus-maze (EPM) test

The EPM test, a plus-shaped platform, which was elevated 40 cm above the floor, is one of the common behavioral tests for anxiety-related behavior. With this experimental setup the behavior in aversive environment, like illuminated exposed areas as well as the natural exploratory behavior of mice, was analyzed (Lister, 1987). It consisted of two opposing open arms (30 x 5 cm) representing an aversive environment and two opposing closed arms (30 x 5 x 15cm) on the sides reflecting a safe environment connected by a central zone (Fig. 2.2). To set a conflict to either explore the new environment or to avoid the unprotected open arms (Pellow *et al.*, 1985), the open arms were lit by white light of 300 Lux or otherwise the closed were dimly lit with 10 Lux. At the beginning of every test session, the mouse was placed into the central zone of the plus-shape facing a closed arm. Different parameters were automatically measured by the Any-maze software (Any-maze 4.50, Stoelting): time spent on the open arms, the percentage time spent on the open arms (ratio of time spent on the open arms to (total test time – time spent in the neutral zone) in percent), the number of open arm entries, latency to the first open arm entry and the total distance traveled for assessing explorative behavior were videotaped for 5 min and analyzed using the Any-maze software. The apparatus was cleaned with detergent containing water and 70% ethanol before each test to avoid odor irritation.



Figure 2.2: **Elevated plus-maze (EPM) test.** The experimental setup of the EPM test consisted of two opposing brightly lit open arms and two opposing dimly lit closed arms.

### 2.2.3 Light-dark box (LD) test

The LD test was composed of two chambers, a light compartment (32 x 27 x 27 cm) and a dark compartment (16 x 27 x 27 cm) illuminated with 300 Lux and <20 Lux, respectively. The compartments were divided by a wall with a connecting opening (5 x 5 cm) at floor level (Fig. 2.3). The clear separation in light exposure of the two chambers was to create a protected and an aversive environment (Bourin and Hascoet, 2003). To assign anxiety-related behavior, percentage time spent in the light compartment, number of entries and latency to the first entry to the light compartment were measured during 5-min testing. A mouse showing anxious behavior spent significantly more time in the dark, protected compartment than mice treated with anxiolytic drugs (Costall *et al.*, 1989). Each test session started with the mice placed into the dark compartment, and after every mouse the apparatus was cleaned with water containing detergent and 70% ethanol. The test was videotaped by Any-maze software (4.50, Stoelting) and analyzed by a trained person blind to line and treatment using Eventlog 1.0 software (EMCO Software, Reykjavik, Iceland).



Figure 2.3: **Light-dark box (LD) test.** Experimental setup of the LD test consisted of a light and a dark compartment.

#### **2.2.4 Tail-suspension test (TST)**

The TST apparatus was a metal frame on which the mouse was suspended with the last 2 cm of its tail by an adhesive tape (Fig. 2.4). Four mice were tested simultaneously and were recorded with a video camera for 6 min. The videos were later analyzed by a trained person blind to line and treatment using the computer-based Eventlog program (1.0, EMCO Software). This test was used to assess depression-like behavior (Cryan and Mombereau, 2004; Steru *et al.*, 1985), and two different coping styles were distinguished. Moving of mice was defined as active coping (Fig. 2.4 A), whereas immobile phases counted as passive coping (Fig. 2.4 B). Immobility was considered when animals stopped any body movements, except minor head swinging, which was used as an indicator for depression-like behavior.

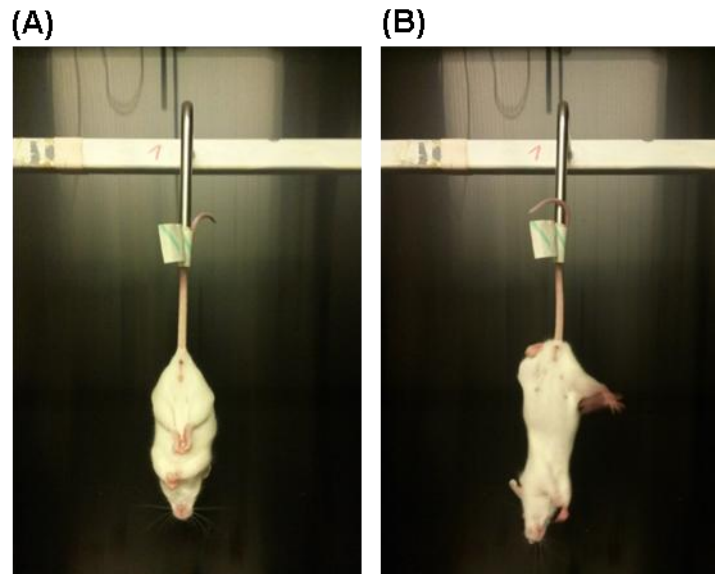


Figure 2.4: **Tail suspension test (TST)**. Experimental setup of the TST consisted of a metal frame on which the mice were suspended by their tail, showing (A) immobile or (B) moving behavior.

### 2.2.5 Forced swim test (FST)

As the second test for measuring depression-like behavior in a highly aversive and inescapable situation, we used the FST, in which the mouse was forced to swim (Porsolt *et al.*, 1977). The FST is the primary and most commonly used test for screening antidepressants. This test creates an aversive situation, which is a strong physical and psychological stressor for the mice (Landgraf *et al.*, 2007). During the 6-min session, the mouse was placed into a 2 l glass cylinder, filled with tap water (room temperature  $22.5 \pm 1^\circ\text{C}$ ) to a level preventing the mouse from escaping the cylinder and touching the bottom with its tail (Fig. 2.5). The mouse was dried with a towel before placing it back into its home cage. Three types of behavior were observed in the FST. Struggling was taken as actively trying to escape the aversive situation with intense, intermittent vertical movement of the two forepaws, while a forward acting swimming movement of all four legs underneath the water surface was counted as swimming. If the mouse was not showing any movement, except for slight balancing movements, it was defined as floating, which corresponds to behavioral despair (Porsolt *et al.*, 1978). The behavior of the mouse was videotaped with a camera and later analyzed using the computer-based Eventlog 1.0 software (EMCO Software). Time spent, latency to first and total numbers of each respective phenotype were scored for statistical analysis.



Figure 2.5: **Forced swim test (FST)**. Experimental setup of the FST consisted of a glass cylinder filled with water.

### 2.3 Environmental manipulations

The extreme genetically fixed predisposition of the anxiety-related behavior mouse model (HAB/LAB) was used as a basis for environmental manipulations in a beneficial (EE) or adverse (CMS) way to test, if the behavior can be changed towards 'normal' behavior. Here, we wanted to reflect the hypothesis 'for better and for worse manner' (Belsky *et al.*, 2009; Belsky and Pluess, 2009), in which both beneficial vs. adverse environmental manipulations have an impact on anxiety-related behavior.

### 2.3.1 Enriched Environment (EE)

The EE design for HAB mice was adapted from Arai *et al.* (2009) and Sotnikov *et al.* (2014) and was used for increasing anxiolytic effects (Markt, 2012).

The paradigm of EE is divided into partial and full enrichment, both lasting 14 days. Partial enrichment started at postnatal day (PND) 15 with a 6 h/day transfer of pups and their respective dam to EE. At PND 28, pups were weaned and transferred to EE permanently in groups of three to four until PND 42 (full enrichment) (Fig. 2.6). EE mice were kept in an enlarged home cage (Makrolon cage type IV) with different biologically relevant stimuli. These included additional nesting material and a  $6 \pm 0.5$  cm thick layer of wood chips (LIE E-001, Abedd Lab & VET Service), retreat options like a plastic inset (22 x 16 x 8 cm) and a tunnel (19.5 x 6 x 6 cm), as well as a wooden ladder and scaffold as climbing possibilities. At the weekly cage change, half of the nesting material was transferred to the new cage to prevent aggressive behavior with remaining olfactory marks (Olsson and Dahlborn, 2002).

After the two weeks period of full enrichment, a behavioral test battery was performed to test the effect of EE. We tested in 48 h test intervals, starting with OF test, followed by EPM and LD tests (described in 2.2.1 – 2.2.3). The EE mice remained in a bigger cage with toys under standard housing conditions for the entire period of testing (see 2.1).

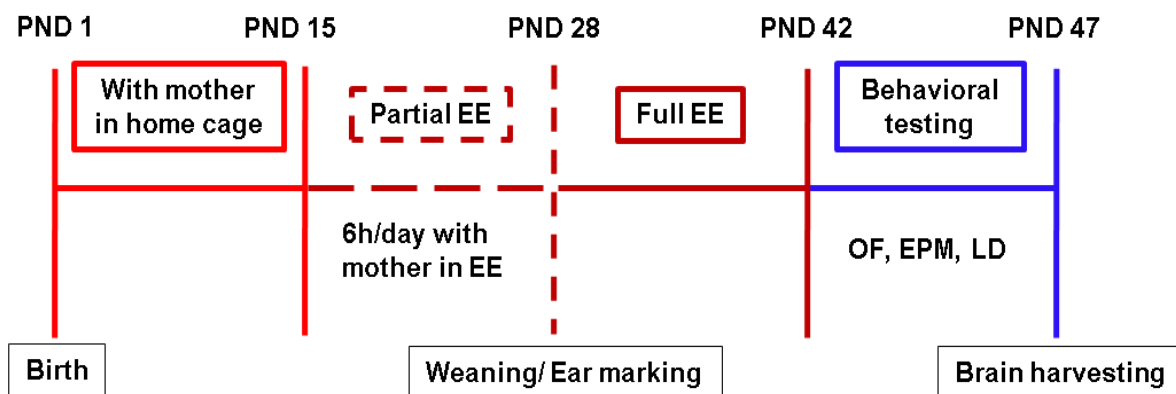


Figure 2.6: **Time course of enriched environment (EE) of HAB mice.** Partial enrichment started at postnatal day (PND) 15 with 6 h per day in EE, and mice remained in full EE from PND 28 followed by a behavioral test battery including open field (OF), elevated plus-maze (EPM) and light-dark box (LD) tests.



### 2.3.2 Chronic mild stress (CMS)

In contrast of creating a beneficial environment in the EE paradigm, for the CMS design we used alternating mild stressors to initiate anxiogenic and pro-depressive effects in LAB mice. The CMS treatment lasted the same period of time as EE and was adapted from Willner *et al.* (1987) and Sotnikov (2013). The mild stressors included from PND 15 to 28 maternal separation (3 h per day; 7 days litterwise, 7 days alone), restraint stress (PND 28 - 42, 30 min per day, see Fig. 2.7), light off during light phase (3 times for 5 h), overnight illumination (twice overnight), damp sawdust (twice overnight), cage tilting (3 times 45°C for 7 h), overcrowding (4 mice per Makrolon cage type II, twice overnight) or paired housing (once overnight), stroboscopic illumination (3 times for 7 h), mild footshock (once 0.7 mA with 2 s duration), white noise (85 dB 3 times for 3 h), damp sawdust (twice overnight), placement to an empty cage with water at the bottom (twice for 1 h) or just in an empty cage (3 times overnight). The stressors were distributed randomly over the four weeks to avoid adaptation, but included not more than two stressors per day. For ethical reasons, food and water deprivation were excluded. Like in EE, behavioral phenotyping was conducted according to the 48 h protocol as described above but without OF test, and for determination of depressive-like behavior, TST and FST followed.

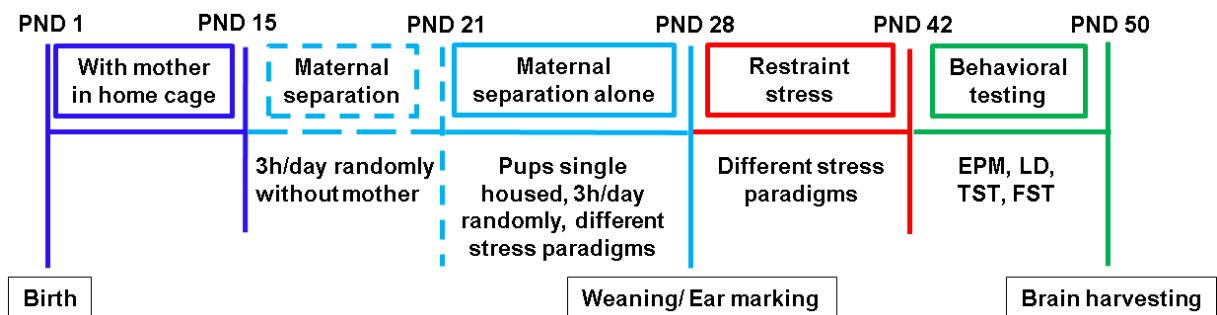


Figure 2.7: **Time course of chronic mild stress (CMS) paradigm of LAB mice.** This stressor paradigm lasting for four weeks, started at postnatal day (PND) 15 with maternal separation adding more stressors, and from PND 28 restraint stress plus one of several mild stressors was conducted. After the different stressors, a behavioral test battery including elevated plus-maze (EPM), light-dark box (LD) tests, tail-suspension test (TST) and forced swim test (FST) was conducted.

## **2.4 Transgenerational inheritance setup of LAB mice**

In this study, we tried to assess whether transgenerational transmission of CMS-induced behavioral changes occur in both males and females. Therefore, we analyzed all offspring up to generation F2 in a behavioral test battery after every CMS manipulation.

As described in 2.3.2, LAB mice were housed until PND 50 and were mated according to their respective behavior. Therefore, two test parameters of anxiety-related behavior were considered: ‘% time spent on the open arms’ in the EPM test and ‘% time spent in the light compartment’ in the LD test. These two parameters served as the key measures of anxiety-related behavior from both tests, as they are least influenced by locomotor activity. Animals performing below and above the respective group means were taken for mating.

For mating, animals were kept together for 14 days in Makrolon cages type III. After determining pregnancy, males and their respective females were separated, and all females with their offspring remained in the Makrolon cages type II until PND 15. After this time point, the chronic mild stress started for all CMS groups with maternal separation, followed by a set of different mild stressors (described in 2.3.2). All non-stressed control (Co) mice were weaned at PND 29 and single-housed in Makrolon cages type II without any manipulation. Breeding was continued until F2. To phenotype each generation a behavioral test battery was used as described in Fig. 2.8 and 2.9. In addition, basal CORT levels were analyzed in F2 mice. Data acquired from males and females were examined separately. The nomenclature used to describe each generation and treatment is illustrated in Fig. 2.8.

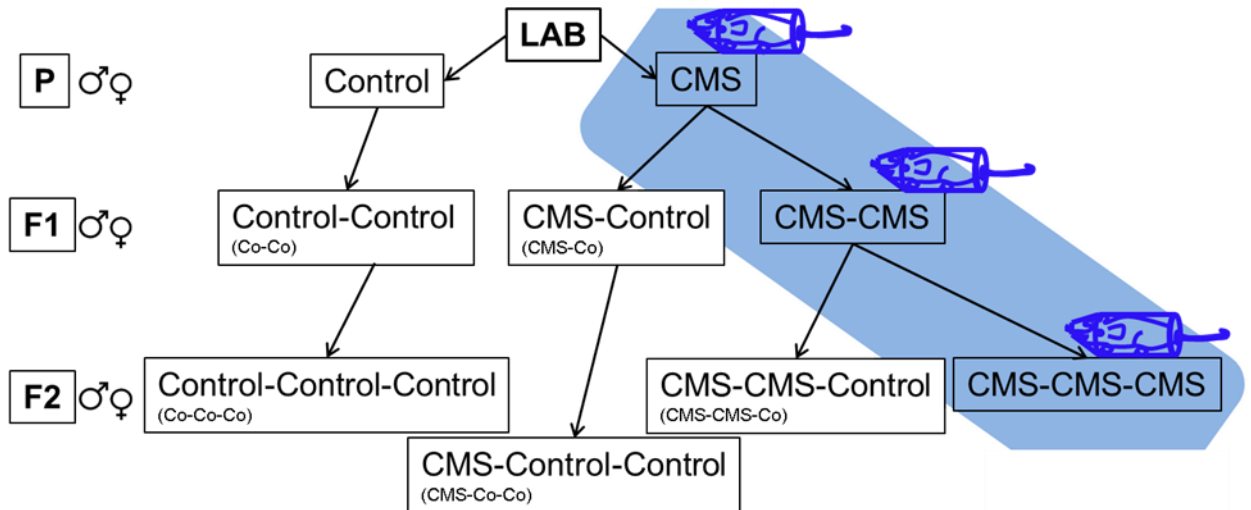


Figure 2.8: **Transgenerational breeding from parental (P) generation until F2 generation showing the possible transgenerational inheritance in LAB mice.** All groups of generations are shown including their abbreviations. Co, control; CMS, chronic mild stress.

Control (Co, Co-Co, Co-Co-Co): animals were always single-housed under standard conditions in Makrolon cages type II without any disturbance apart from a weekly change of cage.

CMS-Control (CMS-Co): parental (P) generation was exposed to different stressors and offspring were raised as control mice.

CMS-CMS-Control (CMS-CMS-Co): P and F1 generation received CMS and offspring were raised as control mice.

CMS, CMS-CMS, CMS-CMS-CMS: all three generations underwent the CMS treatment.

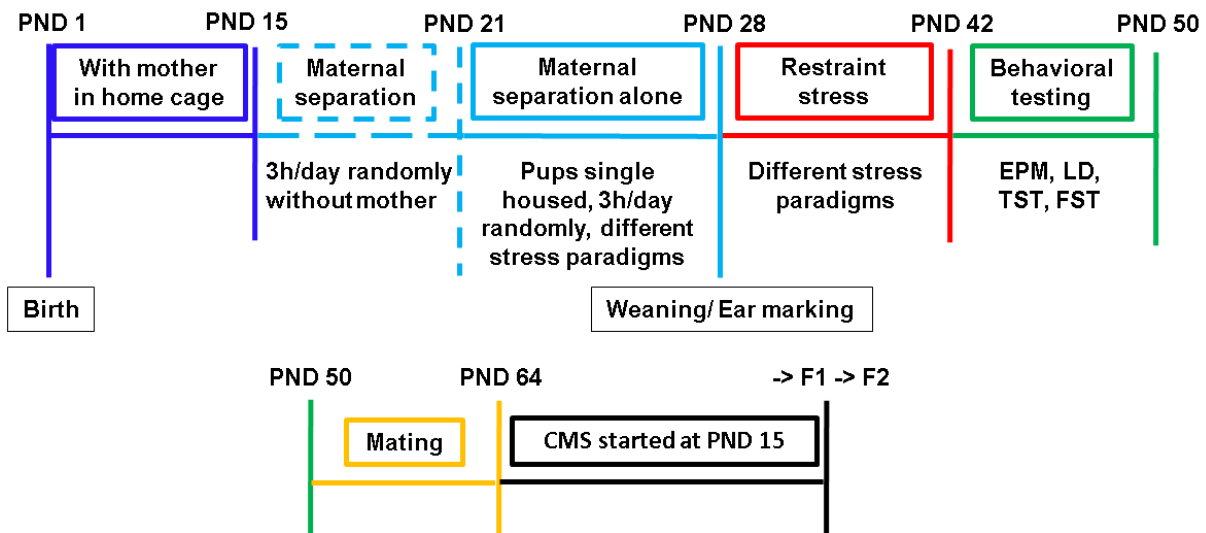


Figure 2.9: **Time course of the transgenerational setup of the chronic mild stress (CMS) paradigm of LAB mice.** As described in 2.3.2, additionally, mating and treatment time of the next two generations are included. This stressor paradigm started at postnatal day (PND) 15 with maternal separation, and from PND 28 restraint stress plus one of several mild stressors was conducted. After the different stressors, a behavioral test battery including elevated plus-maze (EPM), light-dark box (LD) tests, tail-suspension test (TST) and forced swim test (FST) was conducted.

## 2.5 HPA axis

### 2.5.1 Basal corticosterone sampling

For the possible transgenerational transmission of stress, we wanted to compare the basal CORT levels between the four groups (Co-Co-Co, CMS-Co-Co, CMS-CMS-Co, CMS-CMS-CMS) of males and females, respectively, in generation F2. HPA reactivity and feedback regulation was not tested, as FST, a strong stressor, was used as a test for assessing depression-like behavior. Thus, we did not want to apply a second strong stressor to all animals.

Therefore, 24 h after the CMS treatment of F2 and before all behavioral tests were performed blood samples from animals between 09:00 a.m. and 11:30 a.m. were collected. Basal blood samples were collected from the ventral tail vessel within timeframes of no longer than 2 min in Microvette CB300 coated with potassium-EDTA tubes (Sarstedt, Nümbrecht, Germany) and were centrifuged for 10 min at 4000 rpm (4°C) to separate plasma and cellular components. The upper plasma phase was transferred into a clean 1.5 ml Safelock tube (Sarstedt) and stored at

-20°C for further analysis by radioimmunoassay. If the 2-min limit was exceeded, the basal stress level could not be ensured and thus, those results were excluded.

### **2.5.2 Radioimmunoassay (RIA)**

Basal CORT concentration was measured using a commercial RIA kit (DRG Instruments GmbH, Marburg, Germany) by following the manufacturer's protocol with slight modifications. Therefore, 10 µl of blood plasma were taken and diluted 1:13.5 with the Kit Diluent. All samples were measured in duplicate, intra- and inter-assay coefficients were below 10%. For further calculations, mean values from double detections were taken and samples that deviated more than 10% were excluded from statistical analysis. To avoid intra-assay variations, pooled samples of the initial CORT concentration were run at the beginning and at the end of the assay. For calculation of the CORT concentration, a standard curve with known CORT concentrations was used. Radioactivity was measured in a gamma counter (Wallac Wizard 1470 automatic gamma counter, Perkin Elmer Life Science, Rodgau, Germany).

### **2.6 Brain harvesting**

For brain harvesting, all mice were decapitated after Forene (Abbott GmbH, Wiesbaden, Germany) anesthesia under baseline conditions, and brains were carefully removed and quick-frozen in dry ice-cooled 2-methylbutan (Carl Roth GmbH, Karlsruhe, Germany). Brains were stored at -80°C until further use.

### **2.7 Tissue dissection**

Frozen brains were embedded in tissue freezing medium (Jung, Nussloch, Germany) and sectioned in a cryostat (Microm MH50, Microm, Walldorf, Germany) from rostral to caudal. Slices were collected starting at the level of the corpus callosum (Bregma 3.08 mm) until ventral hippocampus was unfolded (Bregma -3.08 mm). The 200 µm slices were fixed on Superfrost microscope slides (Menzel GmbH, Braunschweig, Germany) and were stored at -80°C until micropunching.

To assess gene expression, different target brain regions were determined according to the Mouse Brain Atlas 2<sup>nd</sup> edition (Paxinos and Franklin, 2001). For micropunching procedure (Palkovits, 1973), cingulate cortex (Cg), hypothalamic paraventricular nucleus (PVN) and basolateral amygdala (BLA) (see Fig. 2.10) were punched out with micropunchers with a diameter of 0.5 or 1.0 mm (Fine Science Tools GmbH, Heidelberg, Germany). Depending on the region, two to six punches were collected in 1.5 ml RNase free Safelock tubes (Eppendorf, Hamburg, Germany).

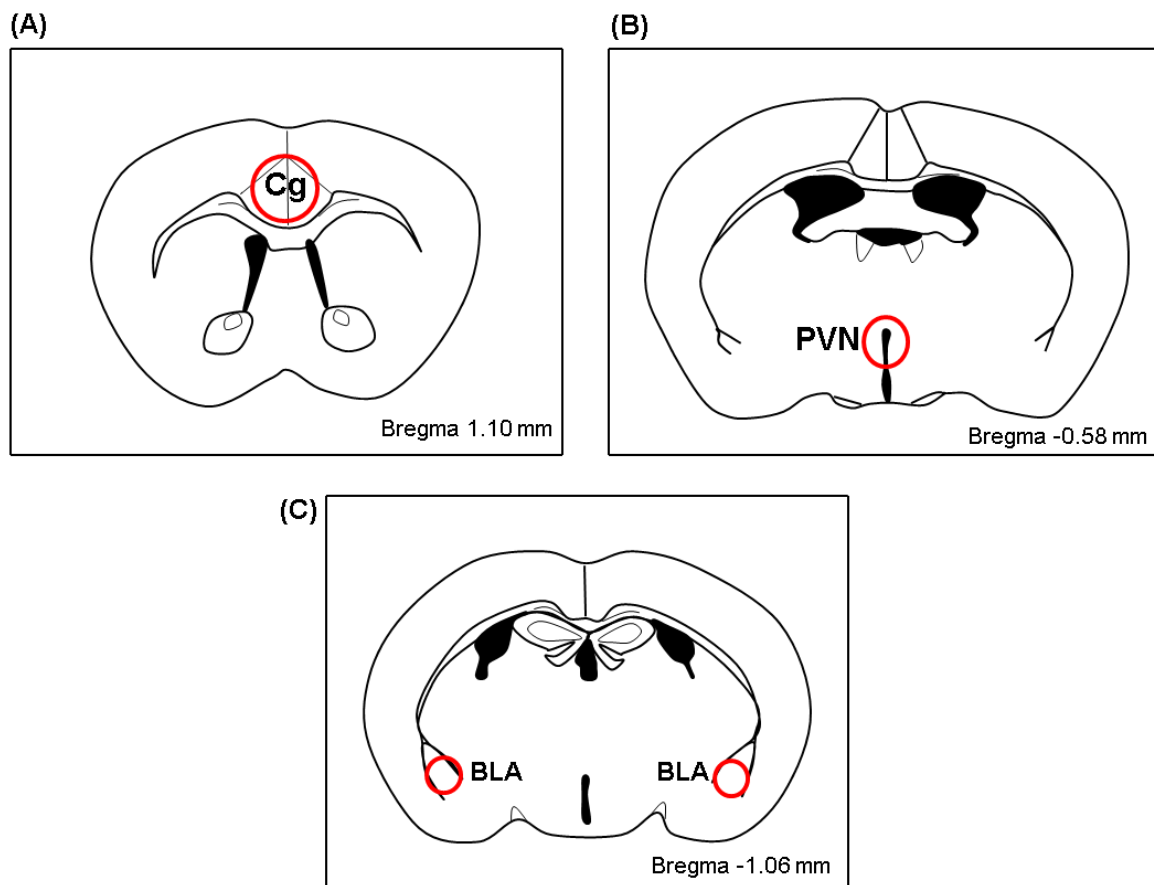


Figure 2.10: **Target brain regions acquired by micropunching.** Red circles indicate the location of micropunched areas (A) cingulate cortex (Cg), (B) hypothalamic paraventricular nucleus (PVN) and (C) basolateral amygdala (BLA). (Figures adapted from Mouse Brain Atlas 2<sup>nd</sup> edition, Paxinos and Franklin, 2001).

## **2.8 Molecular methods**

### **2.8.1 Total RNA isolation**

All RNA-based methods were conducted under a fume hood, surfaces and equipment were treated with 70% ethanol followed by RNase Zap (Ambion, Austin, USA) before and after usage. For all steps, presterilized 1.5 ml Safelock tubes and RNase free filter tips (Sarstedt) were used to avoid RNA degradation by contamination with RNases.

### **2.8.2 Isolation from BLA for microarray analysis**

Total RNA was isolated out of BLA micropunches for microarray analysis according to a TRIzol/Chloroform standard protocol. First, 300 µl pre-cooled (4°C) TRIzol/Tri Reagent (Sigma-Aldrich, Taufkirchen b. München, Germany) and 30 µl bidistilled water (Aqua ad iniectabilia, Braun, Melsungen, Germany) were added to each tube containing brain punches from one animal, and the tissue was briefly homogenized by up and down pipetting using a 200 µl pipette. After adding 1 µl linear acrylamide (5 mg/ml, Ambion) and 60 µl chloroform (Carl Roth GmbH), the samples were vortexed (Vortexer VF2, Janke & Kunkel GmbH, IKA®-Labortechnik, Staufen, Germany) for about 30 s and centrifuged for 5 min at 18°C and 13000 rpm (Centrifuge type Z216MK, Hermle Labortechnik GmbH, Wehingen, Germany). The centrifugation was necessary to separate the aqueous from the organic phase, and 140 µl of the upper aqueous phase containing RNA were transferred into a clean 1.5 ml Safelock tube. The inter- and lower organic phase were discarded. To precipitate RNA, 180 µl of pre-cooled isopropanol (Carl Roth GmbH) were added to the aqueous phase, and samples were incubated at -20°C overnight.

On the next day, purification steps followed starting with centrifuging the samples for 30 min at 4°C and 13000 rpm. The supernatant was removed and the samples were washed with 500 µl pre-cooled (4°C) 70% ethanol two times each with a following centrifugation step at 4°C and 13000 rpm for 10 min and discarding the supernatant. After the two washing steps, the samples were shortly centrifuged at full speed and remaining liquid was removed with a 20 µl pipette. The pellet was dried in a thermoshaker (Grant Instruments Ltd, Cambridgeshire, England) at 45-50°C for 5 min with open lids without overdrying. In order to redissolve the pellet, 15 µl of

ddH<sub>2</sub>O were added by pipetting up and down 10 to 20 times. In a last procedural step, samples were briefly heated (1-2 min) to 95°C in the thermoshaker and spun down shortly at full speed. For the microarray, RNA quality and concentration were measured by using RNA NanoChips and the Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). To assess the RNA quality, the RNA integrity number (RIN factor) was measured and RIN factors >7.15 were taken as satisfactory results (Kiewe *et al.*, 2009). Data was translated into gel-like images (bands) and electropherograms (peaks). All microarray probes were checked before and after amplification. The isolated RNA was stored at -80°C until further use.

### **2.8.3 Isolation from brain tissue micropunches for other analysis**

For further qPCR studies (see 2.8.5), a combined RNA isolation from TRIzol/Chloroform protocol and RNeasy® Plus Micro Kit (Qiagen GmbH, Hilden, Germany) was used. Therefore, 500 µl pre-cooled (4°C) TRIzol/Tri Reagent were added to each micropunching tube and homogenized using autoclaved micropipettes and a 200 µl pipet afterwards to solve all by up and down pipetting. After 5 min incubation on ice, 100 µl chloroform were added, samples were vortexed for 30 s and incubated for 3 min at room temperature. Centrifugation for 15 min at 4°C and 13000 rpm followed, then the upper aqueous phase (200 µl) was pipetted into a fresh 1.5 ml RNase free Safelock tube and mixed 1:1 with 70% ethanol. Afterwards, the mix was transferred to a RNeasy MinElute spin column and centrifuged for 20 s at 23°C at maximum speed. After discarding the flow-through, 700 µl RW1 buffer were added, centrifuged for 20 s under the same conditions and flow-through was discarded again. The next step was the same, just adding 500 µl RPE buffer. After another step with 500 µl RPE buffer and centrifugation for 2 min for washing the spin column membrane, centrifugation followed for 1 min as a drying step. The columns were placed into new 1.5 ml collection tubes, and 2 times 10 µl RNase free H<sub>2</sub>O were added directly to the center of the spin column membrane and centrifuged for 1 min at full speed, respectively, for eluting RNA. Before the RNA was stored at -20°C until cDNA synthesis, RNA concentration was measured. For all qPCR studies, RNA concentration was detected by an Implen NanoPhotometer (Implen, Munich, Germany). Given that just low RNA yields were measured, lidfactor 10 was used.



Results were considered as reliable and in a secure range, when RNA purity absorption ratio A<sub>260</sub>/280 nm was in the range of 1.7 up to 2.1.

#### **2.8.4 Reverse transcription**

Reverse transcription is the process of reversely transcribing RNA into its complementary DNA (cDNA) by using a reverse transcriptase (RT). Before carrying out the cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA), the RNA samples' concentration was adjusted to 100 ng/μl. Following manufacturer's instructions, a master mix (10 μl) consisting of 2 μl 10x RT Buffer, 0.8 μl 25x dNTP Mix (100 mM), 2 μl 10x RT Random Primers, 1 μl Multiscribe™ Reverse Transcriptase and 4.2 μl RNase free H<sub>2</sub>O was prepared and mixed with 10 μl of RNA sample on ice. Every round of RT-PCR, additionally, had a negative RNase free H<sub>2</sub>O control instead of a RNA sample. The cDNA synthesis was performed in a thermal cycler (primus96 advanced, Peqlab Biotechnologie GmbH, Erlangen, Germany) with a 4-step reaction program (initial enzyme activation: 25°C 10 min; reverse transcription: 37°C 120 min; termination: 85°C 5 min; cooling 4°C *ad infinitum*). After dilution of obtained cDNA 1:5 with RNase free H<sub>2</sub>O, cDNA was stored at -20°C until further use.

#### **2.8.5 Quantitative real-time PCR (qPCR)**

This method is a combination of amplification and quantification of mRNA to analyze and determine gene expression levels of specific genes of interest (Higuchi *et al.*, 1993). For this purpose, a specific fluorescent reporter dye (QuantiFast SYBR Green PCR Kit, Qiagen GmbH) binding to double stranded DNA was used. The corresponding fluorescence increases in "real-time". At the end of the amplification process, a melting curve was generated to control the purity of the amplified PCR products. The qPCR was conducted in a 384-well plate in the Roche LightCycler® 480 SW 1.5.1 (Roche Diagnostics, Mannheim, Germany). Each qPCR setup included sample duplicates, a negative control (RNase free H<sub>2</sub>O) and the RT negative control (see 2.8.5). Also a pooled standard dilution series (1:1, 1:5; 1:25, 1:50) for every gene was prepared as a control for primer functionality and for calculating the efficiency. According to the QuantiFast SYBR Green Kit protocol, a master mix (total

8 µl) containing 5 µl 2x QuantiFast SYBR Green PCR Master Mix, 1 µl RNase free H<sub>2</sub>O, 1 µl of the specific forward primer and reverse primer plus 2 µl cDNA (1:5) was prepared and was loaded on each of the 384-wells. After centrifugation of the plate shortly at full speed, the cycling of the LightCycler program was performed in the Roche LightCycler® 480 SW 1.5.1 under following conditions of qPCR run: initial preincubation phase (95°C, 5 min), amplification of 40 cycles in which after each cycle fluorescence was assessed (denaturation at 95°C 10 s; combined annealing and extension at 60°C 30 s, 72°C 10 s), melting curve (95°C 5 s, 50°C 10 s) and cooling (40°C 30 s). The melting curve was generated to control for primer specificity. Data analysis was done with the LightCyclerSoftware 4.0 (Roche Diagnostics, Mannheim, Germany). The absolute quantification fit points method was used to calculate the respective crossing point (C<sub>p</sub>) values, which was done by standardizing thresholds and noise bands of housekeeping (HK) and target genes to equal levels. According to the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001), the mean C<sub>p</sub>s of the HK genes were used as references to normalize the C<sub>p</sub>s of candidate genes. HKs are necessary as reference genes for every brain region, thus they are involved in basal cellular processes, constitutively expressed and should not be capable of being influenced by environment. In this study, the mean of two different HKs (*B2mg*, *Rpl13a*, *Polr2b*) was used, and the relative fold expression was normalized to the mean value of one analyzed group (for microarray LAB or HAB).

## 2.9 Selection of candidate genes and primer design

qPCR primers were designed using the Primer blast tool of the NCBI database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and were based on the sequences provided by the Ensemble database ([www.ensembl.org](http://www.ensembl.org)). To guarantee the amplification of cDNA only, primers were designed to be intron-spanning, if possible, and oligonucleotides were purchased from Sigma-Aldrich (Table 2.1).

Table 2.1: List of all primer oligonucleotides for quantitative real-time PCR with orientation and chromosomal location of the respective gene.

Gene	Chromosome	Primer Orientation	Sequence 5' --> 3'
<b>4921530F17Rik</b>	Y	forward reverse	CCT GAG TCA CAT TCT GCC CA CCA CCA AGG ATG TTG GTG AAT C
<b>A830006F12Rik</b>	1	forward reverse	AGC ACC ACC GTT AAC CTC AG CAG CAG AGG TGA CCA ATC CA
<b>Adra2c</b>	5	forward reverse	ACT GGT CGG TGA CGC AAG CG GCC GGC GGT AGA ACG AGA CG
<b>Arc</b>	15	forward reverse	AGC CTA CAG AGC CAG GAG AA GGT GAT GCC CTT TCC AGA CA
<b>B2mg</b>	2	forward reverse	CTA TAT CCT GGC TCA CAC TG CAT CAT GAT GCT TGA TCA CA
<b>Cnksr2</b>	X	forward reverse	GGT GAG CAA ATG GTC TCC GA TAG CAG CTG GTC TCC ACT GA
<b>Crh</b>	3	forward reverse	GCA GTG CGG GCT CAC CTA CC GGC AGG CAG GAC GAC AGA GC
<b>Crhr1</b>	11	forward reverse	GCC CCA TGA TCC TGG TCC TGC CCA TCG CCG CCA CCT CTT CC
<b>Egr2</b>	10	forward reverse	CCT CGT CGG TGA CCA TCT TC TCG GAT ACG GGA GAT CCA GG
<b>Egr4</b>	6	forward reverse	CTC TCC AAG CCC ACC GAA G AAG CCC AGC TCA AGA AGT CG
<b>Fos</b>	12	forward reverse	GGC TCT CCT GTC AAC ACA CA CTG GTG GAG ATG GCT GTC AC
<b>Foxp2</b>	6	forward reverse	GCA ACA ACA TCT GCT CAG CC CTC CAT GCT TGA TGC CGT TG
<b>Gabrq</b>	X	forward reverse	CAC TTC GAG CTC TCC TCC AG ACC ACA GCT TCAT TTG CAC AG
<b>Hmgn3</b>	9	forward reverse	AGG TGC TAA GGG GAA GAA GG GTC CCG AGA GGT ACG TGA AA
<b>Junb</b>	8	forward reverse	CCC GGA TGT GCA CGA AAA TG GTC GTG TAG AGA CAG GCT GC
<b>Npas4</b>	19	forward reverse	CAC TCG CAA GGG TGT CTT CT AAT CCA GGT AGT GCT GCC AC
<b>Nr3c1 (GR)</b>	18	forward reverse	CAA GGG TCT GGA GAG AGG ACA A TAC AGC TTC CAC ACG TCA GC
<b>Pbx3</b>	2	forward reverse	GTC ACA GAA TGA AAC CGG CG GTC TCA TTA GCT GGG GGT CG
<b>Polr2b</b>	5	forward reverse	CAA GAC AAG GAT CAT ATC TGA TGG AGA GTT TAG ACG ACG CAG GTG

<b><i>Rgs9</i> exon 1-2</b>	11	forward reverse	GGG TCC AGA ATG ACG ATC CG TCT GCA TGT CCT TCA CCA GG
<b><i>Rgs9</i> exon 16-17</b>	11	forward reverse	GAA GTA CGG CGA TCA GTC CA TGT CCA TGG TTT TGC CGT CT
<b><i>Rpl13a</i></b>	7	forward reverse	CAC TCT GGA GGA GAA ACG GAA GG GCA GGC ATG AGG CAA ACA GTC
<b><i>Tacr1</i></b>	6	forward reverse	GGT AGG GAT TAC ACT GTG GGC TGG CGA AGG TAC ACA CAA CC
<b><i>YY1</i></b>	12	forward reverse	ACC TGG CAT TGA CCT CTC TTA TCC CTG AAC ATC TTT GT

## 2.10 RNA amplification for microarray

For array analysis, extracted total RNA from HAB, LAB, HAB-EE and LAB-CMS mice (6 per group) was processed strictly according to the instructions of the Illumina® TotalPrep™-96 RNA Amplification Kit (part number AMIL1791, Ambion). The protocol started with reverse transcription of 180 ng total RNA to synthesize first strand cDNA using T7 Oligo(dT) primers followed by a second strand cDNA synthesis to convert the single-stranded cDNA into a double-stranded DNA (dsDNA) template for transcription. First strand cDNA synthesis incubation was carried out at 42°C and second strand cDNA synthesis at 16°C in a thermal cycler (GeneAmp PCR System 9700, PE Applied Biosystems, Foster City, California, USA). After purification of the dsDNA transcription templates, in vitro transcription to synthesize cRNA was done to generate multiple copies of biotinylated cRNA from the double-stranded cDNA templates. Therefore, incubation at 37°C was performed for 14 h in a 37°C incubation chamber followed by cRNA purification using a PHMT Grantbio thermo-shaker (Keison, Essex, UK). Biotin-labeled cRNA yield and quality was assessed by the Agilent 2100 Bioanalyzer (see 2.8.2).

### 2.11 Array hybridization

For gene expression profiling of cRNA, Illumina gene expression beadchip array (MouseWG-6 v2.0\_11278593 Expression BeadChip; Illumina, San Diego, USA) was used. This screening method allowed the identification of about 46.000 individual transcripts. Six samples were loaded per microarray slide (24 in total). Material and reagents were provided by Illumina, and hybridization was performed strictly following the manufacturer's protocol. In short, starting amount for hybridization was 1500 ng/ $\mu$ l in 10  $\mu$ l for each sample and was mixed with hybridization buffer. After loading each sample onto the designated array field, the slides were put into a hybridization oven provided by Illumina and incubated for 16.5 h. BeadChips were washed several times with different reagents, then signal was developed with streptavidin-Cy3, washed again and dried by centrifugation. A BeadStation scanner (Illumina) was used for detecting fluorescence based hybridization signals and BeadStudio software (Version 2010.1.0.18378) for analyzing data. Statistical analysis is described under 2.13.1.

### 2.12 Neurogenesis experiment

To examine the relationship between anxiety-related behavior and neurogenesis, we investigated whether a proneurogenic substance (such as memantine) (Akers *et al.*, 2014) would modulate the anxiety-related/depression-like behavior in HAB mice in the same way as EE would do. Therefore, standard-housed female HAB mice were treated with memantine hydrochloride (MM, Merck Chemicals GmbH, Schwalbach am Taunus, Germany) dissolved in 0.9% NaCl (Fresenius GmbH, Bad Homburg, Germany) containing 10% DMSO (Sigma-Aldrich) and injected 25 mg/kg (intraperitoneally (i.p.), once per day) to increase neurogenesis in mice (Akers *et al.*, 2014). For control treatment, 0.9% NaCl containing 10% of DMSO (saline, 25 mg/kg, i.p., once per day) was administered to HABs in both standard and enriched environment (EE). For labelling newly born cells to measure neurogenesis, mice were treated with bromodeoxyuridine (BrdU, Sigma-Aldrich) before behavioral phenotyping (Sah *et al.*, 2012).

### 2.12.1 Experimental setup

Before partial enrichment started at PND 15, the groups were assigned to standard or enriched housing with 20 female pups per treatment. At PND 28, all pups were weighed and divided into three groups. Mice were separated into standard-housed HAB, HAB-MM and enriched-housed HAB-EE groups (7-8 mice per group). The injections started at PND 33, with one injection every second day, for six days. Each mouse received an i.p. injection each of either DMSO or MM, 25 mg/kg/day for all, depending on the assigned group. Weights were recorded before the first, third and fifth injection to calculate the dose. At PND 44 and 45, all animals received a BrdU injection (50 mg/kg/day) once per day. Six days later, behavioral phenotyping started with the OF test followed by the LD test 48 h later to determine anxiety-related behavior (described in 2.2.1 and 2.2.3, respectively). As a depression-like behavioral test, FST was chosen and followed after one day of resting (described in 2.2.5). All mice were deeply anesthetized with an overdose of sodium pentobarbital (Sigma-Aldrich) and transcardially perfused two hours after the onset of the FST, before immunohistochemistry was performed. The experimental time course is shown in Fig. 2.11.

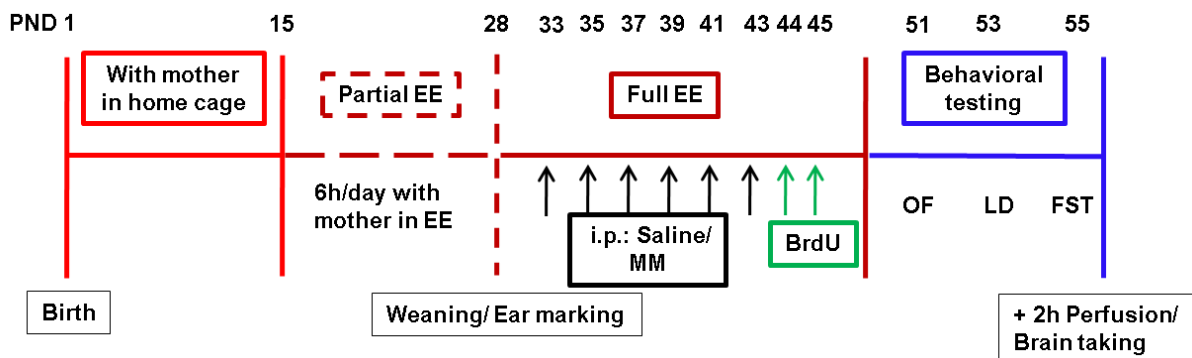


Figure 2.11: **Time course of the neurogenesis experimental setup.** Partial enrichment started at postnatal day (PND) 15 with 6 h per day in EE, and mice remained in full EE from PND 28 followed by a behavioral test battery, including open field (OF), elevated plus-maze (EPM) tests and forced swim test (FST). During the time of full EE, mice received intraperitoneally (i.p.) injections of either saline or memantine (MM) and 5-bromo-2'-deoxyuridine (BrdU).

### 2.12.2 Immunohistochemistry

For Immunohistochemistry, animals were deeply anesthetized with an overdose of sodium pentobarbital (200 mg/kg, Sigma-Aldrich) two hours after the onset of the FST. The animals were then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffered solution (PBS, pH = 7.4). Brains were removed and postfixed in 4% paraformaldehyde in PBS at 4°C overnight. A Vibratome (Ted-Pella, Redding, California, USA) was used to cut coronal sections (50 µm) and were collected in 0.2 M PBS. According to previously described protocols (Sah *et al.*, 2012), free-floating coronal sections were processed for BrdU and doublecortin (DCX) immunodetection. DCX-positive (DCX+) cells are an indication for immature neurons. They were incubated in one of the following primary antibodies: rat anti-BrdU (1/350, AbD Serotec, Puchheim, Germany), goat anti-DCX C18 (1/250, Santa Cruz Biotechnology, Santa Cruz, California, USA). Incubation in a corresponding biotinylated goat anti-rat secondary antibody (1/200, Vector Laboratories, Burlingame, California, USA) or rabbit anti-goat secondary antibody (1/200, Vector Laboratories) followed subsequently. Avidin-biotin-horseradish peroxidase procedure (VECTASTAIN Elite ABC Kit, Vector Laboratories) with 3,3'-diaminobenzidine (DAB) as chromogen was used to visualize these antigen-antibody complexes.

### 2.12.3 Quantification of cells

One-in-eight series of sections of each brain were stained with DAB. Immunoreactive cells were counted using a computer-assisted image analysis system (Nikon E-800 microscope, CCD video camera, Optronics MicroFire, Goleta, CA, USA; Stereo Investigator Software, MicroBrightField Europe e.K., Magdeburg, Germany) throughout the rostrocaudal extent of the GCL of the DG (Sah *et al.*, 2012), using a 20 x or 40 x objective. If the brown-black DAB-stained nucleus was unambiguously darker than background staining, which included all cells from low to high intensities of staining, a cell was considered as BrdU labeled. The lighting of the microscope was optimized for the best visibility of BrdU cells to be analyzed and kept constant for all sections. Statistical analysis is described in 2.13.2.

## 2.13 Statistical methods

### 2.13.1 Statistical analysis of the microarray experiment

For statistical analysis of the microarray experiment, Illumina BeadStudio software (Version 2010.1.0.18378) was used to analyze the raw fluorescence data or signal intensities (BeadSummary Data). This reflects the degree of hybridization of cRNA to beads with the corresponding oligonucleotide probe sequences. Further data processing was done using R 'beadarray' package described by Dunning *et al.* (2007). Array probes that were not different from background fluorescence levels in more than two samples were removed. Data was normalized using the 'vsn' function in R followed by analysis in the Qlucore Omics Explorer. For differential expression analyses, normalized data was subjected to a t-test. For clustering all animals, the function 'hclust' was used. Quality control of microarray data was based on visual inspection of scan images, data distributions, internal Illumina controls, pairwise scatter plots and statistical outlier detection of samples. One HAB-EE sample was detected as outlier and was excluded from all further analysis.

To correct for multiple testing, obtained p-values were applied to the Benjamini-Hochberg false discovery rate (FDR) approach and produced q-values, using characteristics of the p-value distribution (Benjamini and Hochberg, 1995). HAB vs. LAB list was filtered according to both, a >2fold difference in expression and the respective q-values ( $q < 0.05$ ), to get the strongest regulated genes and for the comparisons HAB vs. HAB-EE and LAB vs. LAB-CMS >1.3 fold and >1.15 fold regulation ( $p < 0.05$ ) were applied (Fig. 2.12).

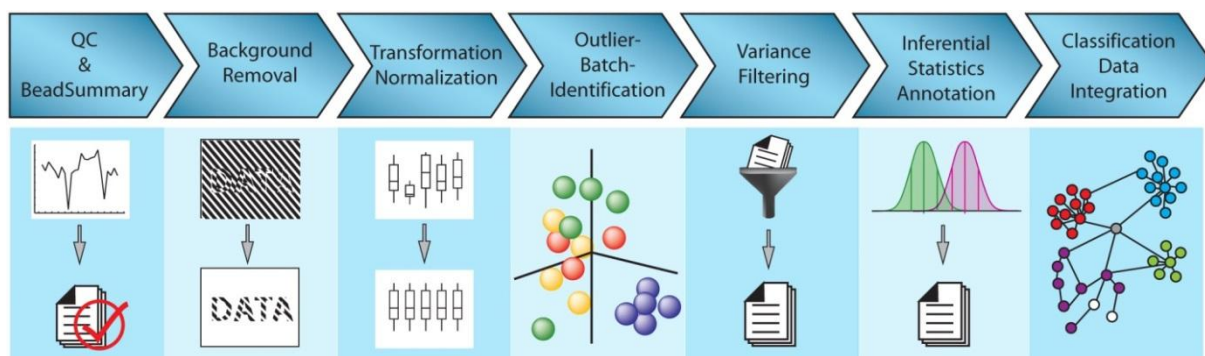


Figure 2.12: **Microarray data analysis workflow.** Statistical steps for analysis of microarray data.



### 2.13.2 Statistical analysis of gene expression, neurogenesis and behavioral data

Data were analyzed by the statistic program SPSS 18.0 (SPSS Inc., Chicago, IL, USA), and comparative illustrations of data were created with GraphPad Prism 5. Normal distribution was evaluated with the Shapiro-Wilcoxon test and variance homogeneity was checked by Levene's test of variance of the data. Since normal distribution was not always required, non-parametric independent comparisons were mostly used for statistical analyses. If the data was normally distributed, unpaired t-test or one-way analysis of variance (ANOVA) was applied followed by the appropriate *post-hoc* test (Bonferroni or Tukey) to correct for multiple comparisons. For independent comparison of two samples, the Mann-Whitney U test (MWU) was performed, and for analysis of more than two samples the Kruskal-Wallis H test (KWH) was applied. If the KWH test was significant, pairwise comparisons were performed with the MWU test followed by *post-hoc* Bonferroni correction. Data with a probability of error lower than 5% were accepted as significant. All data are shown as mean + standard error of the mean (SEM). In Table 2.2, the different significance levels are presented.

Table 2.2: List of the defined significance levels.

<b>n.s.</b> (non-significant)	<b>T</b> (Trend)	<b>*</b> (significant)	<b>**</b>	<b>***</b>
<b>p&gt;0.1</b>	p<0.1	p<0.05	p<0.01	p<0.001

### **2.13.3 Cluster analyses**

The DAVID Bioinformatics tool (version 6.7) is a database for annotation, visualization and integrated discovery and was used for cluster analysis of large sets of gene and protein lists. Therefore, this tool generated systematically biological annotation clusters of differentially expressed genes of the microarray for functional enrichment (<http://david.abcc.ncifcrf.gov/>). Submitting the lists of regulated genes to DAVID bioinformatics tool was done by following a given protocol (Huang *et al.*, 2009). Enrichment scores  $\geq 1.3$  of detected clusters were examined as functional clusters of the differentially expressed genes having a significant biological impact. Every cluster was corrected for multiple testing implemented by Benjamini correction.

## 3 Results

### 3.1 Microarray analysis

Before the microarray-based gene expression profiling for comparing the HAB vs. LAB, HAB vs. HAB-EE and LAB vs. LAB-CMS transcriptomes was performed, mice had to be behaviorally phenotyped (see 3.1.1, 3.1.2). Gene expression results are shown in 3.1.3.

#### 3.1.1 Behavioral tests of microarray animals

The behavior of animals used for the microarray experiment was assessed in the EPM and LD tests. Around 20 male mice per group (HAB, LAB) and treatment were tested. Out of these, six mice per group were selected randomly.

For the EPM test, just in HAB compared to HAB-EE mice a significant difference was observed using the non-parametric MWU test in the percentage time spent on the open arms ( $U=182.5$ ,  $p=0.046$ , mean  $\pm$  SEM: HAB:  $7.17 \pm 1.56$ , HAB-EE:  $11.89 \pm 1.80$ ) indicating an anxiolytic effect of EE. In LAB vs. LAB-CMS no significant difference was found in their percentage time spent on the open arms ( $U=199$ ,  $p=0.403$ , mean  $\pm$  SEM: LAB:  $62.67 \pm 3.38$ , LAB-CMS:  $67.51 \pm 3.68$ ) (Fig. 3.1 A). Six mice per group were chosen for further microarray analysis. In this case, HAB-EE mice spent significantly more time on the open arms of the EPM test ( $U=1.0$ ,  $p=0.006$ , mean  $\pm$  SEM: HAB:  $1.45 \pm 1.20$ , HAB-EE:  $13.72 \pm 2.21$ ). Again, no difference was found for LAB compared to LAB-CMS mice in the percentage time spent on the open arms of the EPM test ( $U=13.0$ ,  $p=0.423$ , mean  $\pm$  SEM: LAB:  $70.07 \pm 4.52$ , LAB-CMS:  $60.57 \pm 6.65$ ) (Fig. 3.1 B).

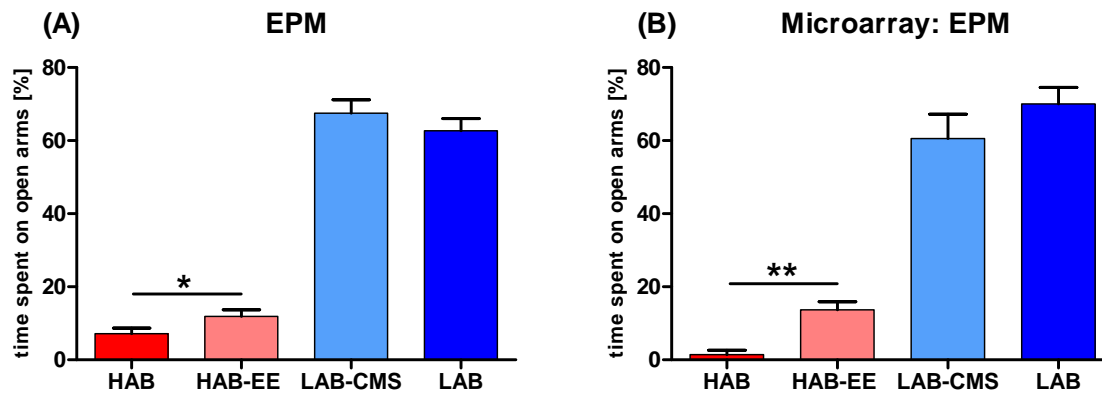


Figure 3.1: **Effect of enriched environment (EE) and chronic mild stress (CMS) on anxiety-related behavior in the elevated plus-maze (EPM) test.** The comparison is shown (A) for all tested mice (N (HAB) = 24, N (HAB-EE) = 23, N (LAB-CMS) = 18, N (LAB) = 26) and (B) for mice selected for microarray analysis (N = 6 per group). Data are shown as mean + SEM, (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

Results in the LD test corroborate changes observed in the EPM test, an anxiolytic effect of EE. HAB-EE mice spent significantly more time in percentage in the light compartment compared to HAB mice ( $U=129.5$ ,  $p=0.001$ , mean  $\pm$  SEM: HAB:  $2.46 \pm 1.20$ , HAB-EE:  $12.35 \pm 2.50$ ). LAB-CMS mice had a significantly decreased percentage time spent in the light compartment ( $U=131.5$ ,  $p=0.014$ , mean  $\pm$  SEM: LAB:  $43.76 \pm 2.98$ , LAB-CMS:  $33.54 \pm 1.98$ ) (Fig. 3.2 A). Almost the same results are shown for the animals used in the microarray analysis. HAB-EE mice spent in percentage more time in the light compartment showing a less anxious phenotype compared to HAB mice ( $U=5.5$ ,  $p=0.037$ , mean  $\pm$  SEM: HAB:  $1.00 \pm 0.68$ , HAB-EE:  $8.83 \pm 5.65$ ). For LAB compared to LAB-CMS mice a trend was observed showing that stressed LAB mice spent less time in the light compartment ( $U=7$ ,  $p=0.078$ , mean  $\pm$  SEM: LAB:  $49.35 \pm 5.20$ , LAB-CMS:  $36.47 \pm 3.79$ ) (Fig. 3.2 B).

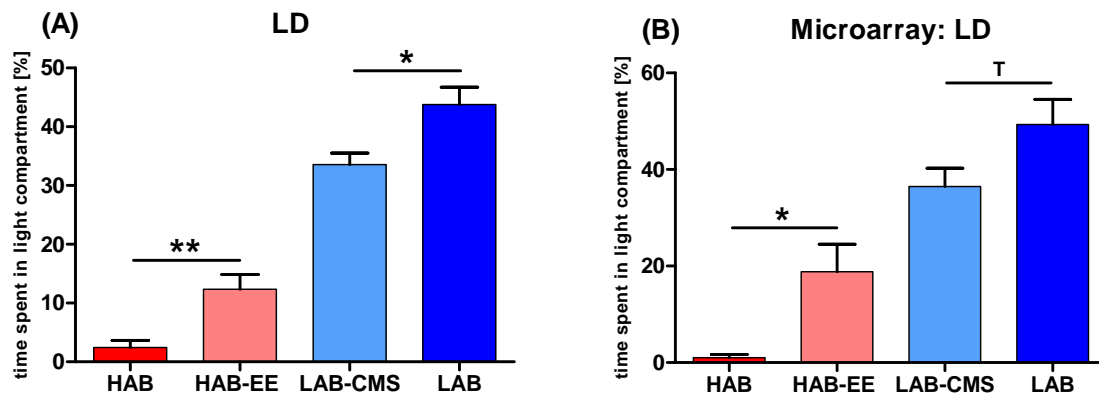


Figure 3.2: **Effect of enriched environment (EE) and chronic mild stress (CMS) on anxiety-related behavior in the light-dark box (LD) test.** The comparison is shown (A) for all tested mice (N (HAB) = 24, N (HAB-EE) = 23, N (LAB-CMS) = 18, N (LAB) = 26) and (B) for mice selected for microarray analysis (N = 6 per group). Data are shown as mean + SEM, (T  $p < 0.1$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ ).

### 3.1.2 Behavioral phenotyping for qPCR validation

For validation of differentially expressed candidate genes in the microarray-based profiling, qPCR analysis was done. For this analysis, mice were taken from an independent batch of animals with HAB mice housed either in standard or in enriched environment as well as unstressed or stressed LAB mice. In this case, again male mice were taken. At the beginning, more mice were tested in three different anxiety tests to reveal the possible effects of the EE manipulation in HAB mice. For qPCR analysis, eight mice per group were taken according to their behavior. To assess anxiety-related behavior, always more than one behavioral test should be performed to converge verification of a phenotype (face validity) (Cryan *et al.*, 2002). Behavioral data for OF, EPM and LD tests for HAB and HAB-EE comparison is shown in Fig. 3.3 until Fig. 3.5.

In the OF test, no significant difference was observed for the parameter 'total distance traveled'. In all mice, no difference in locomotion ( $U=162$ ,  $p=0.266$ , mean  $\pm$  SEM: HAB:  $7.84 \pm 1.74$  m, HAB-EE:  $8.27 \pm 1.05$  m, Fig. 3.3 A) and in percentage time spent in the inner zone ( $U=183$ ,  $p=0.443$ , mean  $\pm$  SEM: HAB:  $0.37 \pm 0.23$ , HAB-EE:  $0.57 \pm 0.26$ ) was detected. The eight selected mice also showed no significant difference in their locomotion ( $U=18$ ,  $p=0.141$ , mean  $\pm$  SEM: HAB:  $4.87 \pm 1.45$  m, HAB-EE:  $8.86 \pm 1.65$  m) and in their percentage time spent in the inner zone ( $U=30$ ,  $p=0.783$ , mean  $\pm$  SEM: HAB:  $0.10 \pm 0.08$ , HAB-EE:  $0.58 \pm 0.40$ ).

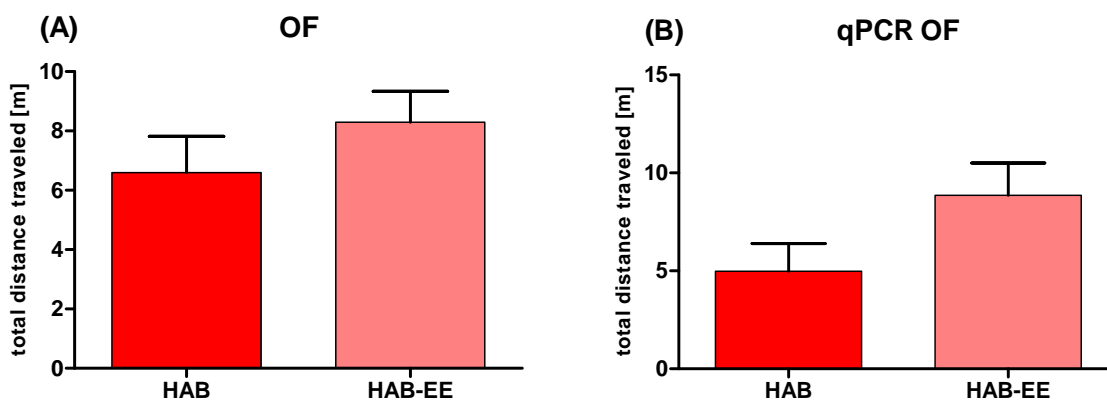


Figure 3.3: **No effect of enriched environment (EE) on locomotion in the open field (OF) test.** The comparison is shown (A) for all tested mice ( $N$  (HAB) = 17,  $N$  (HAB-EE) = 24) and (B) for mice selected for qPCR validation ( $N$  = 8 per group). Data are shown as mean + SEM.

EE-treated mice exhibited significantly lower anxiety-related behavior in the EPM test as indicated by a higher percentage of time spent on the open arms ( $U=65$ ,  $p<0.001$ , mean  $\pm$  SEM: HAB:  $3.07 \pm 1.06$ , HAB-EE:  $10.35 \pm 1.48$ ). The same significant difference was shown for animals chosen for qPCR analysis ( $U=0$ ,  $p<0.001$ , mean  $\pm$  SEM: HAB:  $0.03 \pm 0.03$ , HAB-EE:  $11.27 \pm 2.34$ ) (Fig. 3.4).

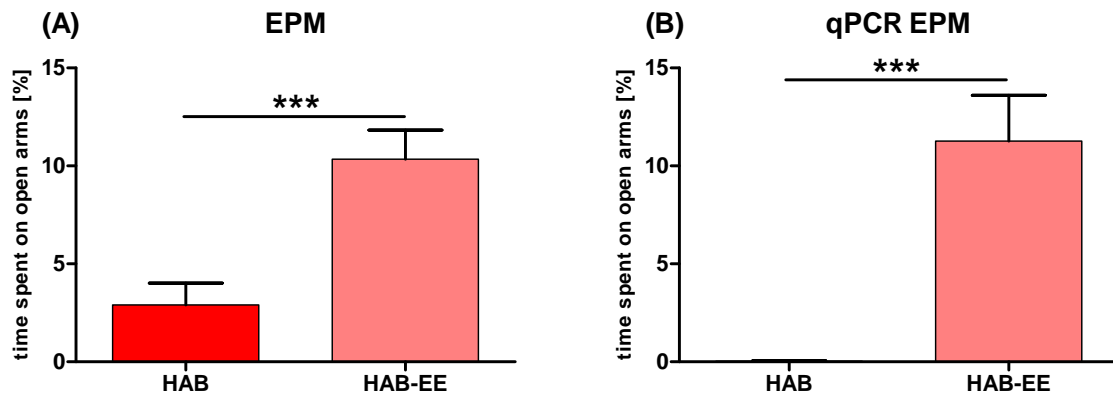


Figure 3.4: **Effect of enriched environment (EE) on anxiety-related behavior in the elevated plus-maze (EPM) test.** The comparison is shown (A) for all tested mice (N (HAB) = 17, N (HAB-EE) = 24) and (B) for mice selected for qPCR validation (N = 8 per group). Data are shown as mean + SEM, (\*\*\*)  $p < 0.001$ .

The third test for assessing anxiety-related behavior was the LD test. EE induced a significant increase in their percentage of time spent in the light compartment of the LD test (U=16.0,  $p < 0.001$ , mean  $\pm$  SEM: HAB:  $1.29 \pm 0.57$ , HAB-EE:  $9.25 \pm 1.42$ , Fig. 3.5 A; U=0,  $p < 0.001$ , mean  $\pm$  SEM: HAB:  $0 \pm 0$ , HAB-EE:  $9.53 \pm 2.41$ , Fig. 3.5 B).

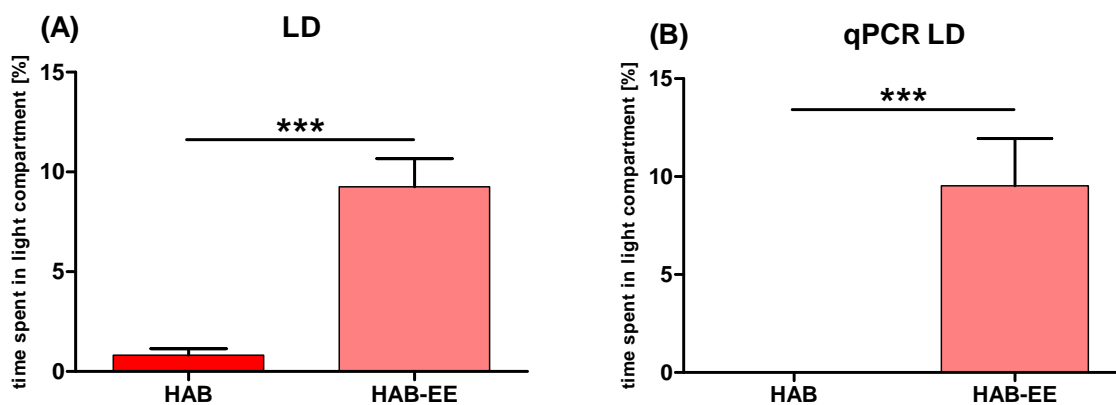


Figure 3.5: **Effect of enriched environment (EE) on anxiety-related behavior in the light-dark box (LD) test.** The comparison is shown (A) for all tested mice (N (HAB) = 17, N (HAB-EE) = 24) and (B) for mice selected for qPCR validation (N = 8 per group). Data are shown as mean + SEM, (\*\*\*)  $p < 0.001$ .

For these animals, no depression-like tests were conducted, because previous experiments only showed a weak effect of EE in males as assessed in TST and FST (Markt, 2012).

Male LAB and LAB-CMS mice for qPCR validation were selected from the parental generation of the transgenerational approach (N=7), and behavioral data are shown in 3.2.2. For these mice, EPM and LD tests were used as anxiety-related tests and TST and FST for assessing depression-like behavior.

### **3.1.3 Microarray detected differentially expressed candidate genes**

Using microarray-based gene expression profiling of BLA tissue from HAB, LAB, HAB-EE and LAB-CMS animals, covering the whole genome, three lists with candidate genes were generated (see 3.1.3.3 – 3.1.3.5).

#### **3.1.3.1 'Hclust' function**

To test how the animals used in this study were related to each other and if there is a distinct separation of the two selective breeding lines, we performed a hierarchical clustering. The 'hclust' function of the 'R' statistical software package showed that HAB and LAB mice are hierarchically different, which was based on the expression distances of all samples. Due to missing quality, one HAB-EE sample was detected as an outlier and was excluded from further analysis (Fig. 3.6).



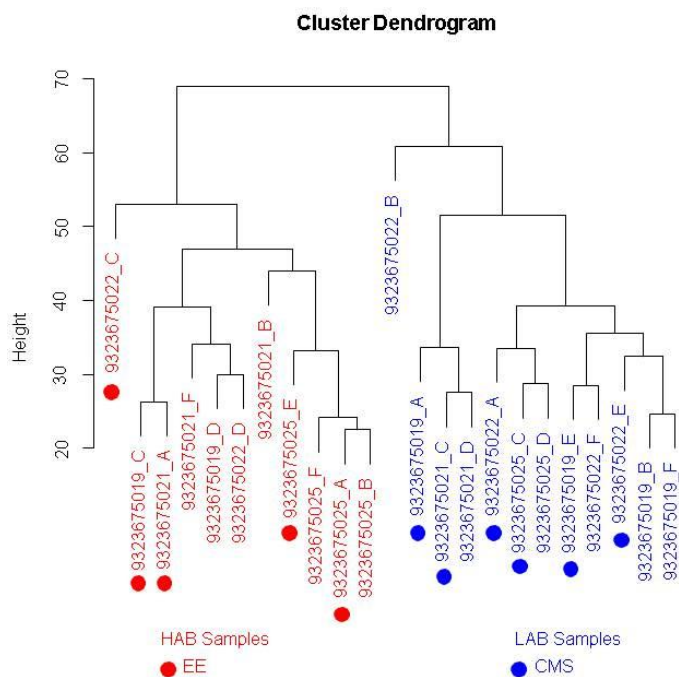


Figure 3.6: **Cluster Dendrogram of all 23 samples shown in hierarchical clustering.** The numbers indicate the microarray identifiers. Red are HAB samples, blue LAB samples, and the dots show the treatments, enriched environment (EE) or chronic mild stress (CMS), (N (HAB/LAB/LAB-CMS) = 6, N (HAB-EE) = 5).

### 3.1.3.2 Cluster analysis of microarray candidate genes

To classify significantly differentially expressed candidate genes of the microarray study, annotational clustering was performed. Therefore, the DAVID Bioinformatics Database for functional annotation clustering was used and was done for three lists: HAB vs. LAB, HAB vs. HAB-EE, LAB vs. LAB-CMS. The investigated gene clusters were named based on the shared/associated functions of the genes they contained. (<http://david.abcc.ncifcrf.gov/>).

For the functional clustering of HAB vs. LAB, five significantly enriched gene clusters were identified (Table 3.1). In the first cluster, 22 genes were found to contribute to different functions in the mitochondrion. Envelope or membrane associated genes are clustered under it. The second cluster contained 21 genes, which are all associated with nucleotide, ATP or nucleoside binding. Nine genes were attributed to the third enriched cluster, acting as cytoplasmic or membrane-bound vesicles. The fourth annotational cluster comprised eight genes involved in various functions of the

immune system. The fifth enriched cluster included five genes, all of them respond to various environment stimuli such as light, radiation or abiotic changes.

Table 3.1: **Significantly enriched gene clusters of 138 genes differentially expressed between HAB vs. LAB in the basolateral amygdala.** Clusters were detected using the functional annotation tool of the DAVID Bioinformatics Database. Genes are sorted in alphabetical order contained in the particular clusters, genes in bold letters are detected in both microarray experiments (Czibere *et al.*, 2011), and were validated in the qPCR follow-up experiment. Genes listed in the respective gene cluster are sorted in alphabetical order. (No. = gene cluster number; % indicates the proportion of genes contributing to the respective cluster among the 138 differentially expressed genes).

No.	Functional association	Enrichment score	Genes	%
1	Mitochondrion	1.8	<i>1300010F03Rik</i> , <i>4930455C21Rik</i> , <b><i>Abca2</i></b> , <i>Aldh3a2</i> , <i>Cox6a2</i> , <b><i>Ctsb</i></b> , <i>Cttnbp2</i> , <i>Cyb5</i> , <i>Dut</i> , <i>Fxc1</i> , <i>Hsp90ab1</i> , <i>Kcnh1</i> , <i>Mff</i> , <i>Mipep</i> , <i>Mosc2</i> , <i>Mrps27</i> , <i>Mtif2</i> , <i>Ndufa13</i> , <i>Slc25a3</i> , <b><i>Slc25a17</i></b> , <i>Slc25a18</i> , <i>Syne1</i>	17.1
2	Nucleotide/ ATP binding	1.7	<i>1300010F03Rik</i> , <b><i>Abca2</i></b> , <i>Atp8a1</i> , <i>Cbwd1</i> , <i>Ddr1</i> , <i>Dgkq</i> , <i>Ehd3</i> , <i>Gnaq</i> , <i>Gtpbp4</i> , <i>Hsp90ab1</i> , <i>Kras</i> , <i>LOC100044756</i> , <i>Mkks</i> , <i>Mtif2</i> , <i>Ndufa13</i> , <i>Nek3</i> , <i>Pip4k2a</i> , <i>Rnps1</i> , <b><i>Ttbk1</i></b> , <i>Ttl</i> , <i>Ube2l</i>	18.0
3	Vesicles	1.6	<i>2400003C14Rik</i> , <b><i>Abca2</i></b> , <i>Ahcy</i> , <i>Atp8a1</i> , <i>Ccdc88a</i> , <b><i>Ctsb</i></b> , <i>Cttnbp2</i> , <i>Ehd3</i> , <i>Hsp90ab1</i>	8.1
4	Immune response	1.5	<i>Cxadr</i> , <i>Fcrls</i> , <i>Fstl5</i> , H2-T10, H2-T23, H2-Q5, <i>LOC100044190</i> , <i>LOC100047788</i>	2.7
5	Stimulus response	1.4	<i>Apbb1</i> , <i>Ercc5</i> , <i>Kras</i> , <i>Mkks</i> , <i>Sdf4</i>	3.6

The submitted list of genes differentially expressed between HAB vs. HAB-EE showed five significant clusters fulfilling enrichment scores  $\geq 1.3$  (Table 3.2). The first cluster counted eight genes, all parts of lipoproteins or the palmitate associated pathway. The second functional cluster consisted of eight genes, which are anchored to the cell membrane via the gpi-anchor or are acting with lipoproteins. All 14 genes of the third cluster interact with the plasma membrane or receptor complexes. Seven genes were assorted to the third cluster, as they are involved in functions related to the postsynaptic membrane, cell junction and cytoskeleton. The last significant cluster contained six genes connected to protein activity or protein binding.

Table 3.2: **Significantly enriched gene clusters of 78 genes differentially expressed between HAB vs. HAB-EE in the basolateral amygdala.** Clusters were detected using the functional annotation tool of the DAVID Bioinformatics Database. Genes are sorted in alphabetical order contained in the particular clusters, genes in bold letters are detected in both microarray experiments (Czibere *et al.*, 2011), and underlined bold written genes are validated in the qPCR follow-up experiment. Genes listed in the respective gene cluster are sorted in alphabetical order. (No. = gene cluster number; % indicates the proportion of genes contributing to the respective cluster among the 78 differentially expressed genes).

No.	Functional association	Enrichment score	Genes	%
1	Lipoprotein/ palmitate associated pathway	1.8	<i>Cdh13, Dlg4, Efna5, Lpl, Lypd1, Sstr2, Tacr1, Wnt5a</i>	11.4
2	Lipoprotein/ gpi-anchor	1.6	<i>Cdh13, Dlg4, Efna5, Lpl, Lypd1, Sstr2, Tacr1, Wnt5a</i>	11.4
3	Plasma membrane/ receptor complex	1.5	<i>Arc, Cd83, Cdh13, Dlg4, Dok3, Ecel1, Evpl, <b>Gabrg</b>, Glra2, Itga11, Itgb11, Kctd6, Rgs9, Synpo</i>	20.0
4	Postsynaptic membrane	1.4	<i>Arc, Dlg4, Evpl, <b>Gabrg</b>, Glra2, Mid2, Synpo</i>	7.1
5	Protein activity	1.3	<i>Cdh13, <b>Fos</b>, Foxp2, Gpd1, Junb, Npas4</i>	8.6

For LAB vs. LAB-CMS, all detected functional clusters in 67 genes had enrichment scores < 1 and failed to be significant according to the criteria set.

### 3.1.3.3 Identification of differentially expressed genes between HAB vs. LAB

The first list revealed 138 differentially regulated genes between HAB and LAB mice in the BLA ( $q < 0.05$ ; 2fold). We compared these potential candidates with a microarray-based gene expression profiling done 20 generations ago in whole brain tissue (Czibere *et al.*, 2011) and, remarkably, the same candidate genes were coming up to be differentially expressed. These genes were also investigated in the first microarray and the follow-up qPCR experiment. Therefore, we only focused on these overlapping genes shown in Fig. 3.7. Two genes (*Enpp5*, *Stx3*) were oppositely regulated compared to the first conducted microarray experiment. *Stx3* proved to be regulated in the opposite way in the qPCR analysis from Czibere *et al.* (2011). The largest expression difference is shown in *Ctsb*, and it is significantly higher expressed in LAB compared to HAB mice ( $p = 4.52 \times 10^{-7}$ ). Detailed results of these genes detected in the microarray from this thesis are shown in Table 3.3.

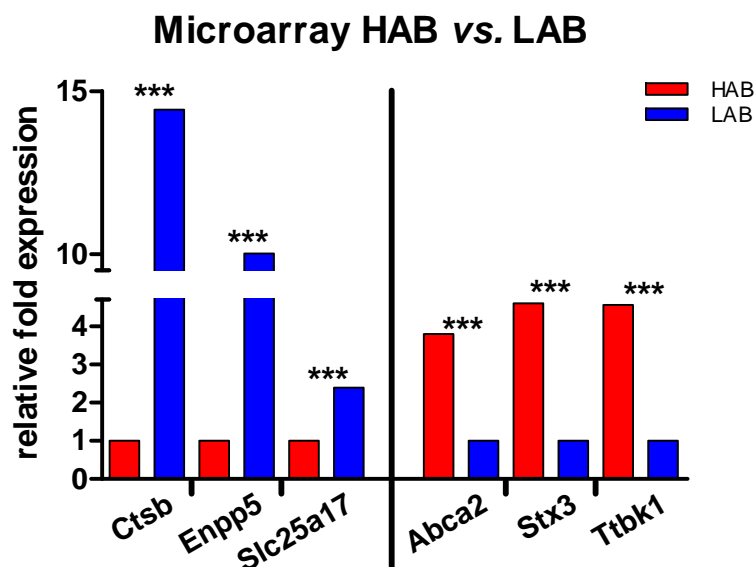


Figure 3.7: Gene expression profiles for HAB vs. LAB mice in the basolateral amygdala as detected by both microarray analysis and at least one confirmation by qPCR. Data are shown as mean value, (N (HAB, LAB, LAB-CMS) = 6, N (HAB-EE) = 5; \*\*\*  $p < 0.001$ ).

Table 3.3: **Differentially expressed genes detected in both microarray experiments and validated at least once with qPCR analysis in whole brain tissue and the basolateral amygdala.** Fold changes of the microarray (MA) marked with # indicate an up-regulation in HAB vs. LAB mice, (N (HAB, LAB, LAB-CMS) = 6, N (HAB-EE) = 5; \*\*\* p<0.001).

Gene	Gene name	Fold change MA	p-value	significance
<b><i>Ctsb</i></b>	cathepsin B	14.433	4.52E-07	***
<b><i>Enpp5</i></b>	ectonucleotide pyrophosphatase/ phosphodiesterase 5	10.024	2.21E-05	***
<b><i>Slc25a17</i></b>	solute carrier family 25 (mitochondrial carrier, peroxisomal membrane protein), member 17	2.391	1.63E-06	***
<b><i>Abca2</i></b>	ATP-binding cassette, sub-family A (ABC1), member 2	3.800#	1.44E-07	***
<b><i>Stx3</i></b>	syntaxin 3	4.601#	1.68E-07	***
<b><i>Ttbk1</i></b>	tau tubulin kinase 1	4.563#	6.02E-06	***

### 3.1.3.4 Identification of differentially expressed genes between HAB vs. HAB-EE

The second list detected 78 differentially expressed genes between HAB and HAB-EE (p<0.05, 1.3 fold). In order to confirm differential expression, selected genes were chosen for follow-up qPCR experiments. 14 genes were selected based on their adjusted p-values in the microarray or their connection to anxiety or psychiatric diseases (Fig. 3.8). Two genes were confirmed by qPCR (*Fos*, *Gabraq*), with *Fos* showing a significantly different regulation in the opposite way as expected. For the other twelve selected genes, no different regulation was confirmed by qPCR. *Hmgn3* was added to analyze as an interesting gene, which was shown to be differentially expressed by Chekmareva *et al.* (2014).

Detailed results of the selected genes detected in the new microarray and chosen for qPCR analysis are shown in Table 3.4. Therefore, ddCT values were standardized by normalizing to the HAB ddCT group mean. For *Rgs9*, two different primer pairs/assays were designed (*Rgs9 exon 1-2*, *Rgs9 exon 16-17*), given that two

transcript variants in the sequence were not overlapping. For all qPCR analyses, the non-parametric MWU test was performed.

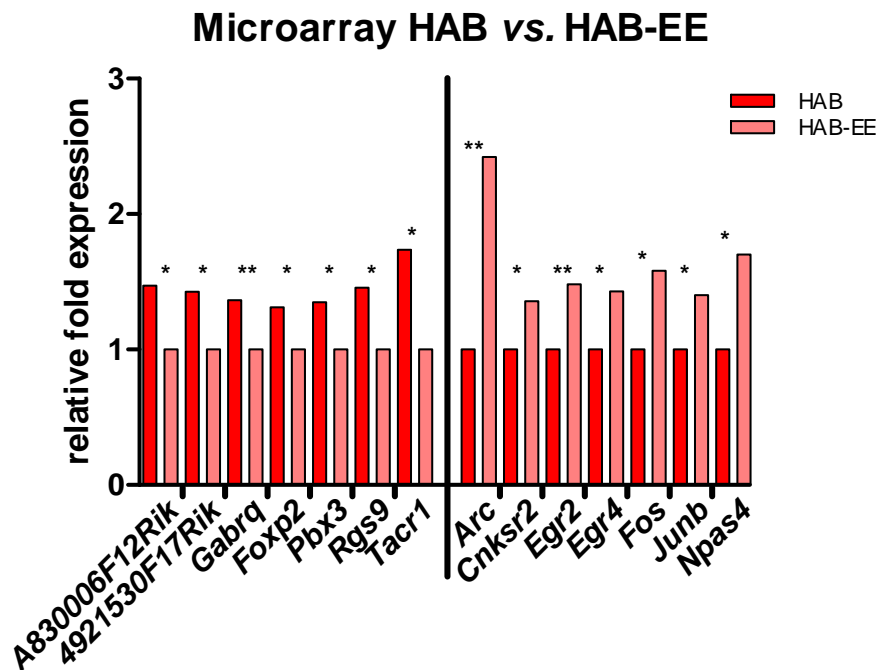


Figure 3.8: **Selected gene expression profiles of 14 genes for HAB vs. HAB-EE mice in the basolateral amygdala from the microarray analysis.** These were used for qPCR analysis only. Data are shown as mean value, (N (HAB) = 6, N (HAB-EE) = 5, \*  $p < 0.05$ , \*\*  $p < 0.01$ ).

Detailed results of the microarray experiment and the follow-up qPCR analysis for all validated genes are shown in Table 3.4.

Table 3.4: **Differentially expressed genes detected in the microarray experiment and their validation by qPCR analysis in the basolateral amygdala.** Fold changes of the microarray (MA) marked with # indicate an up-regulation in HAB vs. HAB-EE mice. Statistically significant results are indicated by bold letters, (n.s. = not significant, n.a. = not available, MWU = Mann-Whitney U test), (MA: N (HAB) = 6; N (HAB-EE) = 5; qPCR: N (HAB, HAB-EE) = 8; n.s. p>0.1, \* p<0.05).

Gene	Gene name	Fold change MA	p-value MWU qPCR	significance	HAB-EE mean $\pm$ SEM	HAB mean $\pm$ SEM
<b>Arc</b>	activity regulated cytoskeletal-associated protein	2.42	0.208	n.s.	0.81 $\pm$ 0.11	1.00 $\pm$ 0.11
<b>Cnksr2</b>	connector enhancer of kinase suppressor of Ras 2	1.36	0.401	n.s.	1.40 $\pm$ 0.34	1.00 $\pm$ 0.11
<b>Egr2</b>	early growth response 2	1.48	0.141	n.s.	0.76 $\pm$ 0.11	1.00 $\pm$ 0.10
<b>Egr4</b>	early growth response 4	1.43	0.916	n.s.	0.96 $\pm$ 0.10	1.00 $\pm$ 0.13
<b>Fos</b>	FBJ osteosarcoma oncogene	1.58	<b>0.036</b>	*	<b>0.55 <math>\pm</math> 0.09</b>	<b>1.00 <math>\pm</math> 0.15</b>
<b>Foxp2</b>	forkhead box P2	1.31#	0.462	n.s.	0.80 $\pm$ 0.27	1.00 $\pm$ 0.30
<b>Gabrq</b>	gamma-aminobutyric acid (GABA) A receptor, subunit theta	1.36#	<b>0.012</b>	*	<b>0.56 <math>\pm</math> 0.17</b>	<b>1.00 <math>\pm</math> 0.15</b>
<b>Hmgn3</b>	high mobility group nucleosomal binding domain 3	n.a.	0.674	n.s.	0.93 $\pm$ 0.14	1.00 $\pm$ 0.13
<b>Junb</b>	Jun-B oncogene	1.40	0.248	n.s.	0.88 $\pm$ 0.11	1.00 $\pm$ 0.11
<b>4921530 F17Rik</b>	Riken cDNA 4921530F17 gene	1.43#	0.834	n.s.	1.02 $\pm$ 0.22	1.00 $\pm$ 0.18
<b>Npas4</b>	neuronal PAS domain protein 4	1.70	0.916	n.s.	0.86 $\pm$ 0.16	1.00 $\pm$ 0.23

<b><i>Pbx3</i></b>	similar to PBX3a; pre B-cell leukemia transcription factor 3	1.35#	0.208	n.s.	0.82 ± 0.22	1.00 ± 0.24
<b><i>Rgs9</i> 1st assay</b>	regulator of G-protein signaling 9	1.74#	0.817	n.s.	1.29 ± 0.50	1.00 ± 0.27
<b><i>Rgs9</i> 2nd assay</b>			0.355	n.s.	1.11 ± 0.58	1.00 ± 0.21
<b><i>Tacr1</i></b>	tachykinin receptor 1	1.46#	0.529	n.s.	0.81 ± 0.18	1.00 ± 0.22
<b><i>A830006</i> <i>F12Rik</i></b>	Riken cDNA A830006F12 gene	1.47#	0.753	n.s.	1.07 ± 0.19	1.00 ± 0.11

### 3.1.3.5 Identification of differentially expressed genes between LAB vs. LAB-CMS

The comparison between LAB and LAB-CMS revealed 67 genes, which showed significant expression difference ( $p < 0.05$ , 1.15 fold). Three genes (*Cnksr2*, *Foxp2*, *Adra2c*) were chosen for qPCR analysis. *Cnksr2* and *Foxp2* (Fig. 3.9) were also present in the comparison between HAB and HAB-EE as potential candidates for plasticity. *Hmgn3* was again added as an interesting gene to analyze. Additionally, *Fos* and *Gabrq* were analyzed due to the fact that they were detected as differentially expressed in HAB vs. HAB-EE in qPCR described before (3.1.3.4). In qPCR analyses, ddCT values were standardized by normalizing to the LAB group mean (Table 3.5). For all qPCR analyses, MWU test was performed.



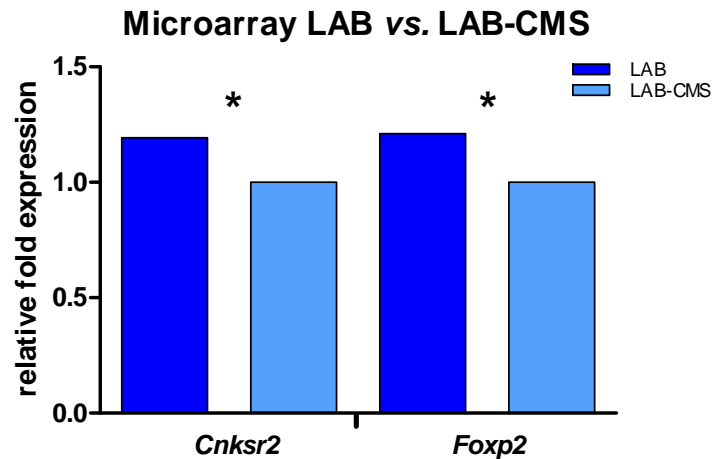


Figure 3.9: Selected gene expression profiles of two genes for LAB vs. LAB-CMS mice in the basolateral amygdala from the microarray analysis. These genes were used for subsequent qPCR analysis. Data are shown as mean value, (N (LAB, LAB-CMS) = 6; \* p<0.05).

Table 3.5: Differentially expressed genes detected in the microarray experiment and their validation by qPCR analysis in the basolateral amygdala. All fold changes of the microarray (MA) indicate an up-regulation in LAB vs. LAB-CMS mice. Statistically significant results are indicated by bold letters, (n.s. = not significant, n.a. = not available, MWU = Mann-Whitney U test), (MA: N (LAB, LAB-CMS) = 6; qPCR: N (LAB, LAB-CMS) = 7; n.s. p>0.1, \* p<0.05).

Gene	Gene name	Fold change MA	p-value MWU qPCR	significance	LAB-CMS mean $\pm$ SEM	LAB mean $\pm$ SEM
<i>Adra2c</i>	adrenergic receptor, alpha 2c	1.159	0.898	n.s.	1.08 $\pm$ 0.26	1.00 $\pm$ 0.14
<i>Cnksr2</i>	connector enhancer of kinase suppressor of Ras 2	1.193	<b>0.018</b>	<b>*</b>	<b>0.79 <math>\pm</math> 0.04</b>	<b>1.00 <math>\pm</math> 0.06</b>
<i>Fos</i>	FBJ osteosarcoma oncogene	n.a.	0.225	n.s.	1.33 $\pm$ 0.16	1.00 $\pm$ 0.14
<i>Foxp2</i>	forkhead box P2	1.211	0.406	n.s.	1.15 $\pm$ 0.23	1.00 $\pm$ 0.39
<i>Gabrq</i>	gamma-aminobutyric acid (GABA) A receptor, subunit theta	n.a.	0.482	n.s.	0.56 $\pm$ 0.06	1.00 $\pm$ 0.31

<b><i>Hmgn3</i></b>	high mobility group nucleosomal binding domain 3	n.a.	0.277	n.s.	0.85 ± 0.10	1.00 ± 0.09
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A summary of the three candidate genes of plasticity, which were differentially regulated between HAB and HAB-EE or LAB and LAB-CMS mice in the analyzed BLA, is shown in Fig. 3.10. Since *Foxp2* was detected in both comparisons in the microarray experiment, results are shown in the summary as well.

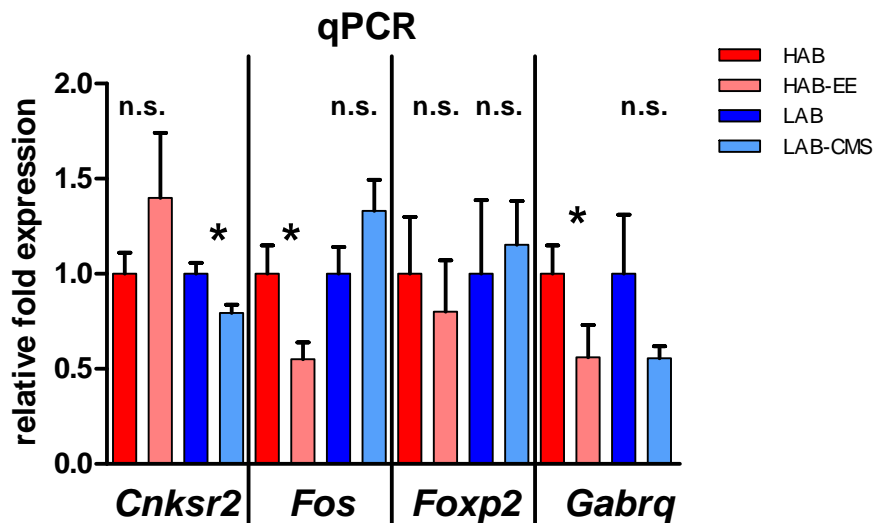


Figure 3.10: **Summary of gene expression profiles of four interesting candidate genes of plasticity.** Gene expression is shown for HAB vs. HAB-EE and for LAB vs. LAB-CMS mice in the basolateral amygdala. Data are shown as mean + SEM, (N (HAB, HAB-EE) = 8, N (LAB, LAB-CMS) = 7; n.s. p>0.1, \* p<0.05).

### 3.1.3.6 Identification of differentially expressed genes in the cingulate cortex

Additionally, selected genes of the microarray were analyzed by qPCR analysis in another important region known to be associated with anxiety-related behavior, the cingulate cortex (Cg) (Shin and Liberzon, 2010) (Table 3.6 and 3.7).

There, only *Fos* was found to be differentially regulated between HAB and HAB-EE with a higher expression in HAB mice, as already seen in the BLA.

Table 3.6: Analyzed genes in qPCR in the cingulate cortex between HAB vs. HAB-EE. Statistically significant result for *Fos* is indicated by bold letters, (n.s. = not significant, MWU = Mann-Whitney U test), (N (HAB, HAB-EE) = 8; n.s.  $p > 0.1$ , \*  $p < 0.05$ ).

Gene	p-value MWU qPCR	significance	HAB-EE mean $\pm$ SEM	HAB mean $\pm$ SEM
<i>Arc</i>	0.674	n.s.	0.78 $\pm$ 0.09	1.00 $\pm$ 0.19
<i>Cnksr2</i>	0.248	n.s.	0.81 $\pm$ 0.10	1.00 $\pm$ 0.15
<i>Fos</i>	<b>0.016</b>	<b>*</b>	<b>0.53 <math>\pm</math> 0.06</b>	<b>1.00 <math>\pm</math> 0.16</b>
<i>Foxp2</i>	0.294	n.s.	0.92 $\pm$ 0.11	1.00 $\pm$ 0.36
<i>Gabrq</i>	0.753	n.s.	0.86 $\pm$ 0.09	1.00 $\pm$ 0.23
<i>Hmgn3</i>	0.753	n.s.	0.92 $\pm$ 0.07	1.00 $\pm$ 0.20

Table 3.7: Analyzed genes in qPCR in the cingulate cortex between LAB vs. LAB-CMS. (n.s. = not significant, MWU = Mann-Whitney U test), (N (LAB, LAB-CMS) = 7; n.s.  $p > 0.1$ ).

Gene	p-value MWU qPCR	significance	LAB-CMS mean $\pm$ SEM	LAB mean $\pm$ SEM
<i>Cnksr2</i>	0.949	n.s.	1.09 $\pm$ 0.23	1.00 $\pm$ 0.13
<i>Fos</i>	0.224	n.s.	1.28 $\pm$ 0.13	1.00 $\pm$ 0.17
<i>Foxp2</i>	0.482	n.s.	1.09 $\pm$ 0.18	1.00 $\pm$ 0.14
<i>Gabrq</i>	0.749	n.s.	1.31 $\pm$ 0.38	1.00 $\pm$ 0.15
<i>Hmgn3</i>	0.225	n.s.	1.09 $\pm$ 0.07	1.00 $\pm$ 0.11

### 3.2 Transgenerational transmission of CMS

In this study, we wanted to investigate transgenerational effects of anxiety-related and depression-like behavior. Therefore, we performed CMS as described earlier in 2.3.2. For validation of the microarray, we took male LAB (Co) and LAB-CMS (CMS) mice from the parental generation. The influence of CMS on the breeding success for the next generation is shown in 3.2.1., followed by behavioral tests: EPM, LD tests for assessing anxiety-related behavior, and TST, FST for determining depression-like behavior for each generation separately.

#### 3.2.1 Breeding

First of all, we examined whether CMS treatment had any influence on the breeding success of the first and second generations. To create the parental generation, LAB mothers were not stressed at all, but at PND 15 the CMS treatment with maternal separation was started. Here, no difference was observed in the breeding, thus the mothers were not stressed during the mating and first two nursing phases. The parental generation had to be mated twice as the breeding was not successful. This was independent of the CMS treatment as no significant difference was observed between CMS-treated and Co parents regarding breeding success (Table 3.8).

Table 3.8: **Breeding success from parental (P) generation of generating generation F1 and F1 to F2.**

	breeding line/ treatment		pairs mated	litters alive	number of pups raised
P	Co	2 matings	16	3	26
	CMS		25	9	79
F1	Co-Co		12	5	29
	CMS-Co		18	10	60
	CMS-CMS		16	8	62

### 3.2.2 Parental (P) generation

The EPM test was performed to measure locomotion and anxiety-related behavior in males and females, and if CMS treatment had an impact on behavior. Male and female data was processed separately. First of all, CMS-treated female mice showed a decrease of their locomotor activity on the EPM test as indicated by total distance traveled ( $U=171$ ,  $p=0.001$ ), whereas in male mice no difference was observed ( $U=224$ ,  $p=0.980$ ) (Fig. 3.11). No significant difference in anxiety-related behavior measured by the parameters 'percentage time spent on the open arms' and 'number of entries to the open arms' were shown for neither male nor female. CMS-treated female mice required significantly more time to the first entry to the open arms compared to standard Co female mice ( $U=231.5$ ,  $p=0.021$ ). In male mice, again, no difference was observed ( $U=211$ ,  $p=0.730$ ). Detailed results of the EPM test are summarized in Table 3.9.

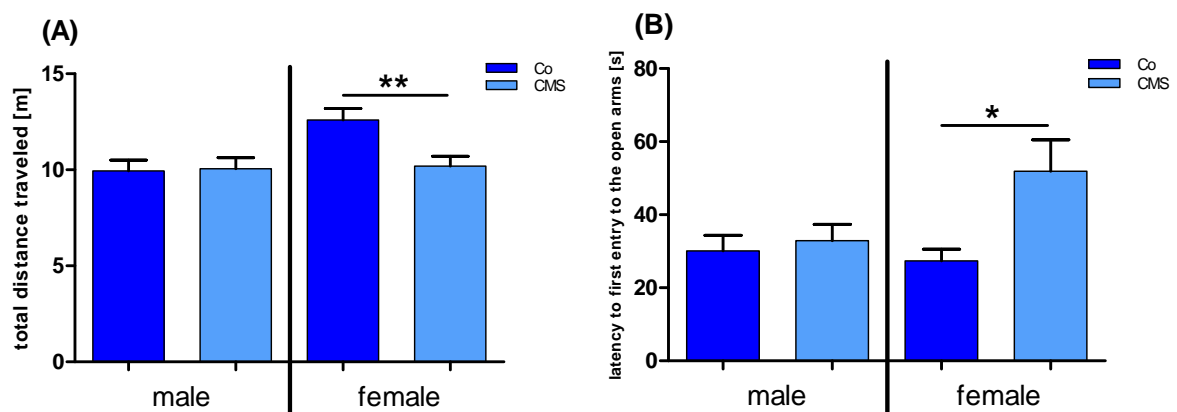


Figure 3.11: **Effect of chronic mild stress (CMS) in the elevated plus-maze (EPM) test for male and female LAB mice.** CMS induced in female mice (A) a decrease of total distance traveled in the EPM test and (B) a higher latency to the first entry to the open arms. Data are shown as mean + SEM, (male: N (Co) = 18, N (CMS) = 25; female: N = 27 per group; \*  $p < 0.05$ , \*\*  $p < 0.01$ ).

Table 3.9: **Behavioral data of the elevated plus-maze test between CMS and Co groups for both sexes.** Statistical significance detected with Mann-Whitney U test (MWU), (n.s.  $p > 0.1$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ ).

		total distance traveled [m]	open arm entries [n]	latency to the first entry to the open arms [s]	time spent on the open arms [%]
Co	male	9.94 ± 0.56	8.00 ± 1.51	30.11 ± 4.27	54.24 ± 3.09
	female	12.60 ± 0.60	6.70 ± 0.61	27.39 ± 3.21	58.00 ± 3.12
CMS	male	10.05 ± 0.58	6.08 ± 0.50	32.90 ± 4.45	57.49 ± 2.87
	female	10.19 ± 0.52	5.93 ± 0.40	51.93 ± 8.58	53.12 ± 3.02
MWU	male	0.980	0.358	0.730	0.313
		n.s.	n.s.	n.s.	n.s.
	female	0.001	0.236	0.021	0.373
		**	n.s.	*	n.s.

In the LD test, a significant difference in anxiety-related behavior was detected for male and female mice. The CMS group spent significantly less percentage in the light compartment (male:  $U=94$ ,  $p=0.002$ , mean ± SEM: Co:  $47.46 \pm 3.30$ , CMS:  $31.53 \pm 2.78$ ; female:  $U=129$ ,  $p < 0.001$ , mean ± SEM: Co:  $49.95 \pm 2.16$ , CMS:  $37.34 \pm 2.58$ ), as well as showed less entries to the light compartment than the Co group for both genders (male:  $t(49)=2.869$ ,  $p=0.007$ , mean ± SEM: Co:  $10.35 \pm 1.13$ , CMS:  $6.72 \pm 0.71$ ; female:  $t(52)=4.525$ ,  $p < 0.001$ , mean ± SEM: Co:  $12.96 \pm 0.98$ , CMS:  $7.63 \pm 0.66$ ), respectively (Fig. 3.12). In the parameter 'latency to the first entry to the light compartment', only in female CMS mice a later entry was shown (female:  $U=169$ ,  $p=0.001$ , mean ± SEM: Co:  $12.91 \pm 4.14$  s, CMS:  $36.99 \pm 11.20$  s). Male mice showed no difference in this parameter (male:  $U=161$ ,  $p=0.187$ , mean ± SEM: Co:  $31.10 \pm 10.74$  s, CMS:  $41.73 \pm 12.21$  s).

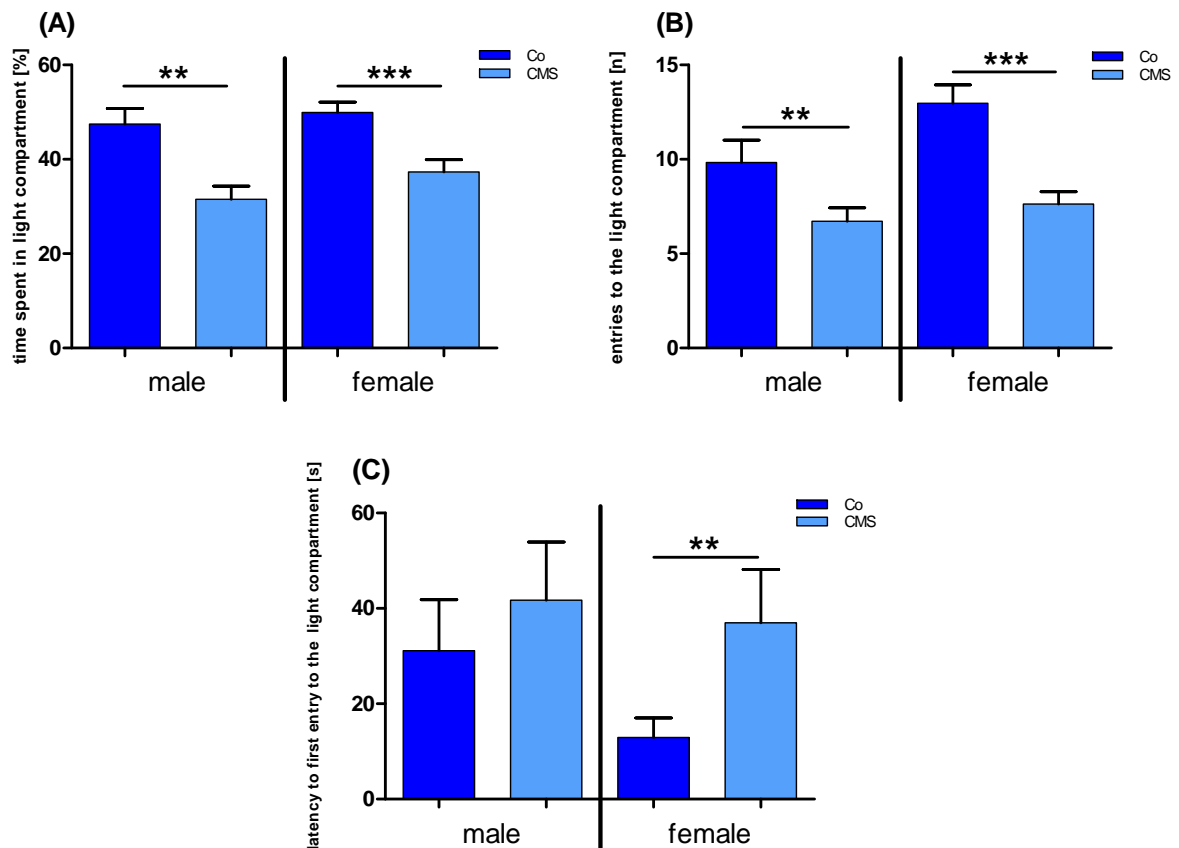


Figure 3.12: **Effect of chronic mild stress (CMS) in the light-dark box test for male and female LAB mice.** CMS induced (A) a decrease of time spent in the light compartment in male and female mice, (B) a lower number of entries to the light compartment in male and female mice, and (C) a later entry to the light compartment in female mice, but not in male mice. Data are shown as mean + SEM, (male: N (Co) = 17, N (CMS) = 25); female: N = 27 per group; \*\* p<0.01, \*\*\* p<0.001).

The TST was one of the two tests used to assess depression-like behavior. CMS-treated female mice spent significantly more time immobile compared to Co mice (female: U=206, p=0.042, mean ± SEM: Co: 65.42 ± 11.64 s, CMS: 105.79 ± 17.53 s) and also showed a trend for more immobile episodes (female: U=218, p=0.069, mean ± SEM: Co: 5.74 ± 0.67, CMS: 6.91 ± 0.57) during the 6-min testing time, which reflects a passive coping style in a stressful situation rather than an emotional state (Fig. 3.13). In male mice, no significant difference in neither time immobile (male: U=160, p=0.587, mean ± SEM: Co: 54.84 ± 9.36 s, CMS: 59.96 ± 8.08 s) nor in the number of immobile episodes (male: U=123, p=0.101, mean ± SEM: Co: 5.00 ± 0.54, CMS: 6.57 ± 0.75) was observed. The parameter 'latency to first immobility' was significantly different in male and female mice and was increased in the CMS

group (male:  $U=78$ ,  $p=0.003$ , mean  $\pm$  SEM: Co:  $30.00 \pm 16.07$  s, CMS:  $81.33 \pm 20.91$  s; female:  $U=171.5$ ,  $p=0.007$ , mean  $\pm$  SEM: Co:  $19.03 \pm 9.03$  s, CMS:  $50.74 \pm 13.18$  s).

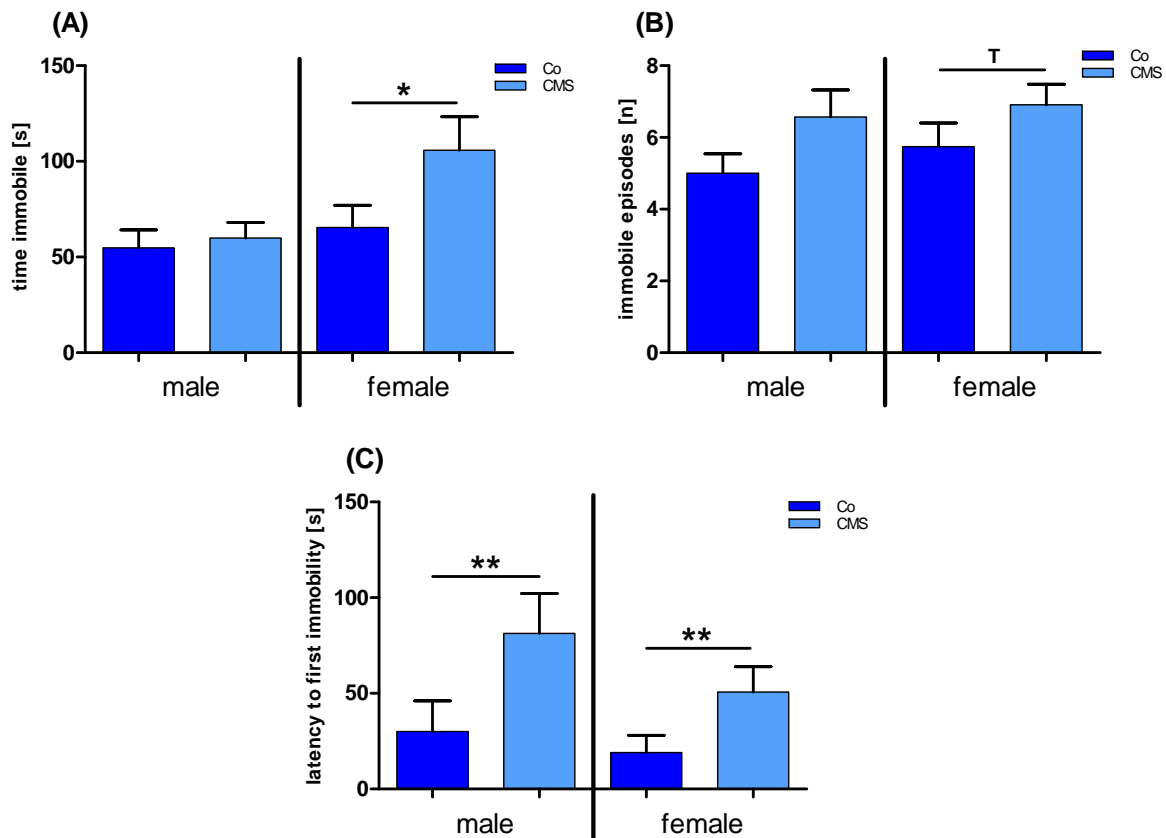


Figure 3.13: **Effect of chronic mild stress (CMS) in the tail-suspension test for male and female LAB mice.** CMS induced (A) an increase of depression-like behavior in female mice, indicated by a higher time immobile and (B) a higher number of immobile episodes in the female group compared to Co mice. No difference was observed in males for both parameters. (C) CMS treatment showed in male and female mice a higher latency to first immobility. Data are shown as mean + SEM, (male: N (Co) = 17, N (CMS) = 21; female: N (Co) = 27, N (CMS) = 23; T  $p < 0.1$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ ).

Furthermore, we tested the CMS- and Co-treated mice in the FST to reveal depression-like behavior. Although, we could find a pro-depressive effect of CMS treatment in the TST for female mice, no significant difference was observed neither in floating time in male and female mice in the FST (male:  $U=174.5$ ,  $p=0.330$ ; female:  $U=240$ ,  $p=0.113$ ), nor in swimming time (male:  $U=210$ ,  $p=0.949$ ; female:  $U=268$ ,  $p=0.291$ ) nor struggling time (male:  $U=170$ ,  $p=0.276$ ; female:  $U=297.5$ ,  $p=0.617$ ) assessing active coping style. Both, CMS-treated male and female mice had a higher number of floating episodes reflected by a trend (male:  $U=143.5$ ,  $p=0.075$ ; female:



U=221, p=0.051), and a decreased latency to the first floating was shown in the female CMS group (male: U=162.5, p=0.200; female: U=236, p=0.097) (Fig. 3.14). Both of these parameters corroborate a pro-depressive effect of CMS as shown in the TST (Table 3.10).

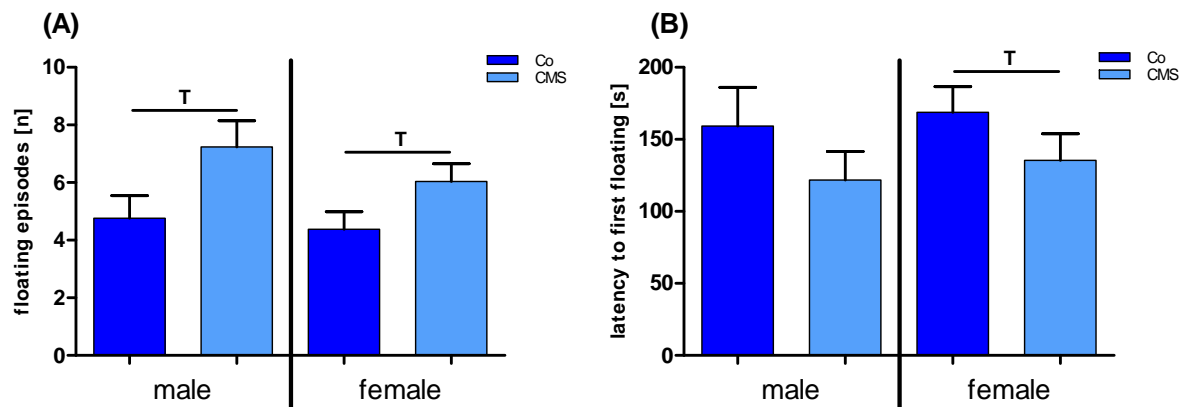


Figure 3.14: **Effect of chronic mild stress (CMS) in the forced swim test for male and female LAB mice.** A trend towards (A) a higher number of floating episodes of CMS mice compared to Co mice in both sexes and (B) a decreased latency to first floating in female, but not in male CMS mice, was observed. Data are shown as mean + SEM, (male: N (Co) = 17, N (CMS) = 25; female: N (Co) = 24, N (CMS) = 27; T p<0.1, \* p<0.05, \*\* p<0.01).

Table 3.10: **Behavioral data of forced swim test between CMS and Co groups for both sexes.** Statistical difference was detected with Mann-Whitney U test (MWU) or unpaired t-test, (n.s. p>0.1, T p<0.1).

		struggling time [s]	swimming time [s]	floating time [s]	floating episodes [n]	latency to first floating [s]
Co	male	70.28 ± 8.37	233.94 ± 11.85	56.98 ± 13.21	4.76 ± 0.78	159.33 ± 26.74
	female	66.60 ± 6.74	240.54 ± 8.33	52.81 ± 10.71	4.38 ± 0.61	168.80 ± 17.78
CMS	male	59.38 ± 6.48	234.54 ± 9.40	68.37 ± 9.77	7.24 ± 0.91	121.72 ± 19.86
	female	63.51 ± 5.55	222.10 ± 13.66	76.54 ± 11.23	6.04 ± 0.61	135.46 ± 18.47
MWU/ t-test	male	0.304 n.s.	0.949 n.s.	0.330 n.s.	0.075 T	0.200 n.s.
	female	0.723 n.s.	0.291 n.s.	0.113 n.s.	0.051 T	0.097 T

Taken together, the CMS paradigm with several unpredictable mild stressors during their early development could change the anxiety-related and depression-like behavior in males and females of the parental generation, which is reflected by an anxiogenic and pro-depressive effect in both sexes. Therefore, CMS represents a reliable basis for further experiments on the influence of environmental manipulation, e.g., the transmission to next generations.

### 3.2.3 F1 generation

To test if CMS-induced changes in behavior can be transmitted from one generation to the next, we applied the CMS paradigm for two subsequent generations. To select the P animals for mating, the behavior in the EPM and LD tests was considered. The F1 generation consisted of three different groups: Co-Co, CMS-Co, CMS-CMS.

In the EPM test, male and female CMS-CMS groups for both sexes showed a significant difference in their total distance traveled compared to the Co-Co group (male:  $F_{(2,34)}=7.156$ ,  $p=0.003$ ; female:  $F_{(2,43)}=16.849$ ,  $p<0.001$ ). Decreased locomotor activity was found for the CMS-CMS group compared to unstressed controls (male: Co-Co vs. CMS-Co:  $p=1.000$ , Co-Co vs. CMS-CMS:  $p=0.016$ , CMS-Co vs. CMS-CMS:  $p=0.005$ ; female: Co-Co vs. CMS-Co:  $p=0.207$ , Co-Co vs. CMS-CMS:  $p=0.008$ , CMS-Co vs. CMS-CMS:  $p<0.001$ , Fig. 3.15). Male CMS-Co mice have a higher number of entries to the open arms of the EPM test compared to CMS-CMS mice, which was not detected in female mice (male:  $F_{(2,34)}=3.941$ ,  $p=0.029$ , *post-hoc*: Co-Co vs. CMS-Co:  $p=0.541$ , Co-Co vs. CMS-CMS:  $p=0.361$ , CMS-Co vs. CMS-CMS:  $p=0.027$ ; female:  $F_{(2,43)}=0.206$ ,  $p=0.814$ , *post-hoc*: Co-Co vs. CMS-Co:  $p=1.000$ , Co-Co vs. CMS-CMS:  $p=1.000$ , CMS-Co vs. CMS-CMS:  $p=1.000$ ). No difference for both sexes was found neither in the parameters 'percentage time spent on the open arms' of the EPM test (male: KWH:  $H(2)=1.251$ ,  $p=0.535$ ; female: KWH:  $H(2)=2.892$ ,  $p=0.235$ ) nor in 'latency to the first open arm entry' (male: KWH:  $H(2)=2.709$ ,  $p=0.258$ ; female: KWH:  $H(2)=1.006$ ,  $p=0.605$ ). Detailed results measured in the EPM test of generation F1 are shown in Table 3.11.

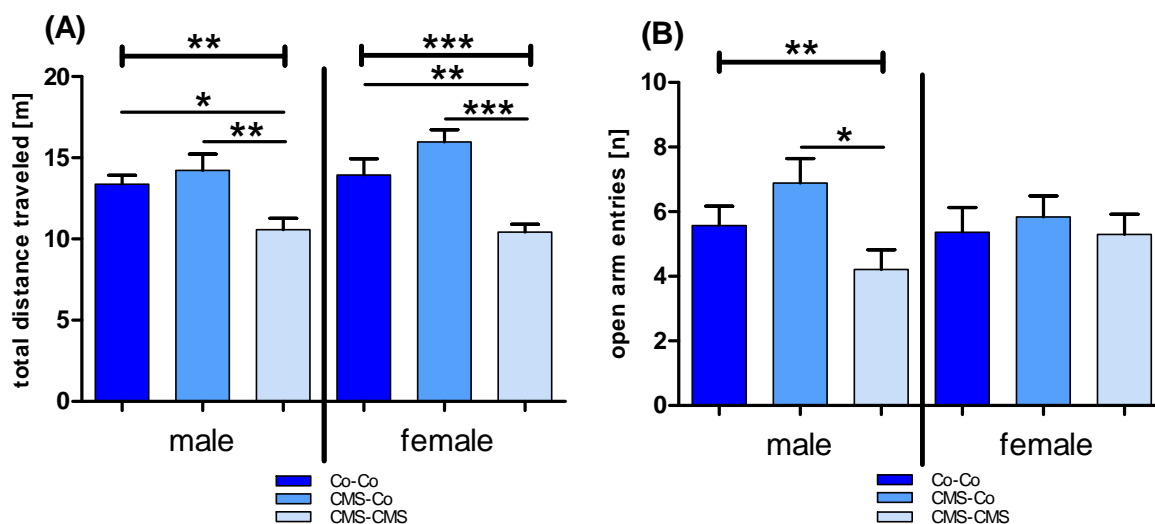


Figure 3.15: **Effect of chronic mild stress (CMS) in the elevated plus-maze test for male and female mice of generation F1.** (A) Both sexes of CMS-CMS mice had a lower total distance traveled in the EPM test compared to Co-Co and CMS-Co groups, and in (B) male mice a lower number of open arm entries was observed. Data are shown as mean + SEM, (male: N (Co-Co) = 14, N (CMS-Co) = 9, N (CMS-CMS) = 14; female: N (Co-Co) = 11, N (CMS-Co) = 18, N (CMS-CMS) = 17; \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001).

Table 3.11: **Behavioral data of the elevated plus-maze test in F1 mice for both sexes.**

Statistical difference was detected with one-way ANOVA or Kruskal-Wallis H test (KWH) followed by an appropriate *post-hoc* test, (n.s. p > 0.1, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001).

		total distance traveled [m]	open arm entries [n]	latency to the first entry to the open arms [s]	time spent on the open arms [%]
Co-Co	male	13.38 ± 0.55	5.57 ± 0.60	33.21 ± 6.83	61.41 ± 3.50
	female	13.94 ± 1.01	5.36 ± 0.77	38.02 ± 4.22	52.51 ± 4.15
CMS-Co	male	14.22 ± 1.00	6.89 ± 0.75	29.02 ± 2.66	48.89 ± 5.18
	female	15.97 ± 0.75	5.83 ± 0.66	37.17 ± 6.81	59.25 ± 3.15
CMS-CMS	male	10.58 ± 0.69	4.21 ± 0.60	46.07 ± 13.54	61.47 ± 5.47
	female	10.42 ± 0.48	5.29 ± 0.62	44.61 ± 7.33	55.75 ± 4.77
ANOVA/KWH	male	0.003 **	0.036 *	0.605 n.s.	0.235 n.s.
	female	<0.001 ***	0.858 n.s.	0.258 n.s.	0.535 n.s.
Co-Co vs. CMS-Co	male	1.000	0.422		
	female	0.207			
Co-Co vs. CMS-CMS	male	0.016	0.154		
	female	0.008			
CMS-Co vs. CMS-CMS	male	0.005	0.039		
	female	<0.001			

The second test for assessing anxiety-related behavior was the LD test. Here, the test corroborated the findings of the EPM test in the parameter ‘number of entries to the light compartment’, but in this case in females and not in males (male: KWH:  $H(2)=2.746$ ,  $p=0.253$ , Co-Co vs. CMS-Co:  $p=0.704$ , Co-Co vs. CMS-CMS:  $p=0.253$ , CMS-Co vs. CMS-CMS:  $p=0.407$ ; female: KWH:  $H(2)=7.943$ ,  $p=0.019$ , Co-Co vs. CMS-Co:  $p=0.257$ , Co-Co vs. CMS-CMS:  $p=0.386$ , CMS-Co vs. CMS-CMS:  $p=0.014$ ). Furthermore, a significant difference in the latency to the first entry to the light compartment was observed in male, but not in female mice (male: KWH:  $H(2)=6.884$ ,  $p=0.032$ ; female: KWH:  $H(2)=2.192$ ,  $p=0.334$ ). The significance could not withstand Bonferroni correction and resulted in just a trend (male: Co-Co vs. CMS-Co:  $p=0.298$ , Co-Co vs. CMS-CMS:  $p=0.073$ , CMS-Co vs. CMS-CMS:  $p=0.089$ ; female: Co-Co vs. CMS-Co:  $p=0.291$ , Co-Co vs. CMS-CMS:  $p=0.126$ , CMS-Co vs. CMS-CMS:  $p=0.792$ ) (Fig. 3.16).

No difference for either sex was found in their percentage time spent in the light compartment of the LD test (male:  $F_{(2,34)}=0.496$ ,  $p=0.613$ ; female:  $F_{(2,43)}=0.955$ ,  $p=0.393$ ) (Table 3.12).

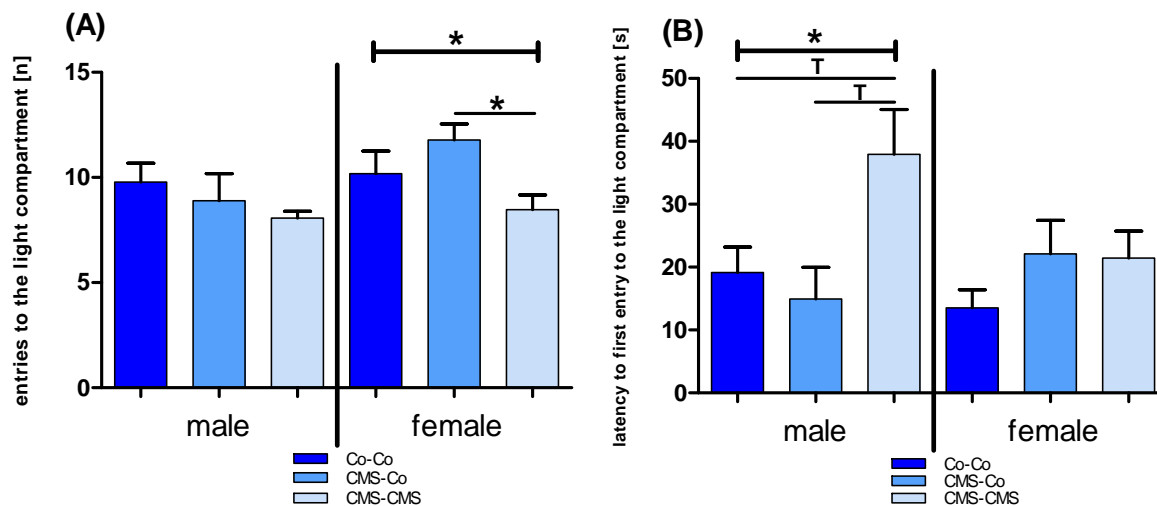


Figure 3.16: **Effect of chronic mild stress (CMS) in the light-dark box test in male and female mice of generation F1.** (A) Female CMS-CMS mice had a lower number of entries to the light compartment compared to CMS-Co group, and (B) male CMS-CMS group showed a higher latency to the first entry to the light compartment. Data are shown as mean + SEM, (male: N (Co-Co) = 14, N (CMS-Co) = 9, N (CMS-CMS) = 14; female: N (Co-Co) = 11, N (CMS-Co) = 18, N (CMS-CMS) = 17; T  $p < 0.1$ , \*  $p < 0.05$ ).

Table 3.12: **Behavioral data of the light-dark box test in F1 mice for both sexes.** Statistical difference was detected with one-way ANOVA or Kruskal-Wallis H test (KWH) followed by an appropriate *post-hoc* test, (n.s.  $p > 0.1$ , \*  $p < 0.05$ ).

		entries to the light compartment [n]	latency to the first entry to the light compartment [s]	time spent in the light compartment [%]
Co-Co	male	9.79 ± 0.90	19.14 ± 4.04	38.09 ± 3.07
	female	10.18 ± 1.08	13.51 ± 2.87	40.78 ± 2.98
CMS-Co	male	8.89 ± 1.30	14.96 ± 5.02	41.73 ± 4.93
	female	11.78 ± 0.76	22.08 ± 5.35	39.24 ± 2.87
CMS-CMS	male	8.07 ± 0.32	37.96 ± 7.11	42.11 ± 2.39
	female	8.47 ± 0.70	21.42 ± 4.32	44.18 ± 2.31
ANOVA/KWH	male	0.253 n.s.	0.032 *	0.613 n.s.
	female	0.019 *	0.334 n.s.	0.393 n.s.
Co-Co vs. CMS-Co	male		0.298	
	female	0.257		
Co-Co vs. CMS-CMS	male		0.073	
	female	0.193		
CMS-Co vs. CMS-CMS	male		0.089	
	female	0.014		

To characterize depression-like behavior, we used the TST. It corroborated the findings in the EPM test, in which stressed mice showed decreased locomotor activity in the TST. In more detail, male CMS-CMS mice spent significantly more time immobile compared to Co-Co and CMS-Co groups, whereas in female mice no significant difference was found (male: KWH:  $H(2)=10.998$ ,  $p=0.004$ , Co-Co vs. CMS-Co:  $p=0.488$ , Co-Co vs. CMS-CMS:  $p=0.010$ , CMS-Co vs. CMS-CMS:  $p=0.020$ ; female: KWH:  $H(2)=1.268$ ,  $p=0.531$ ) (Fig. 3.17). The parameters 'number of immobile episodes' (male: KWH:  $H(2)=4.239$ ,  $p=0.120$ ; female: KWH:  $H(2)=0.317$ ,  $p=0.854$ ) and 'latency to first immobility' were not significantly different (male: KWH:  $H(2)=2.933$ ,  $p=0.231$ ; female: KWH:  $H(2)=0.410$ ,  $p=0.815$ ) (Table 3.13).

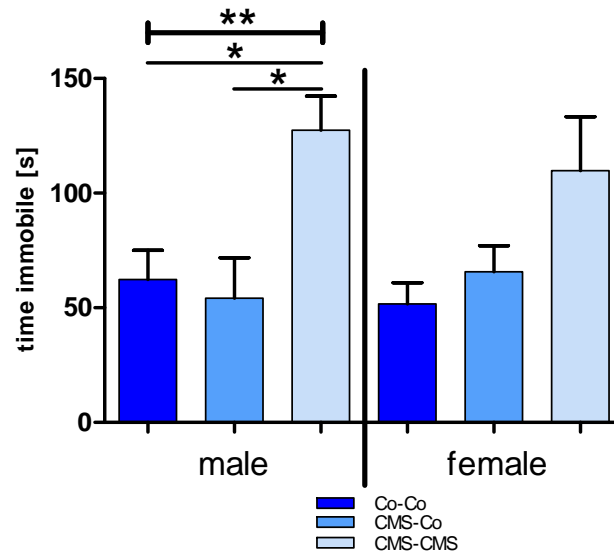


Figure 3.17: **Effect of chronic mild stress (CMS) in the tail-suspension test in male and female mice of generation F1.** Male CMS-CMS mice spent more time immobile compared to Co-Co and CMS-Co group. Data are shown as mean + SEM, (male: N (Co-Co) = 14, N (CMS-Co) = 9, N (CMS-CMS) = 14; female: N (Co-Co) = 10, N (CMS-Co) = 17, N (CMS-CMS) = 17; \*  $p < 0.05$ , \*\*  $p < 0.01$ ).

Table 3.13: **Behavioral data of the tail-suspension test in F1 mice for both sexes.** Statistical difference was detected with Kruskal-Wallis H test (KWH) followed by an appropriate *post-hoc* test, (n.s.  $p > 0.1$ , \*\*  $p < 0.01$ ).

		time immobile [s]	immobile episodes [n]	latency to first immobility [s]
Co-Co	male	62.36 ± 12.68	6.57 ± 0.64	66.65 ± 25.73
	female	51.66 ± 9.33	6.80 ± 1.12	54.84 ± 24.56
CMS-Co	male	54.16 ± 17.71	5.22 ± 1.19	49.37 ± 28.81
	female	65.73 ± 11.35	7.29 ± 0.73	36.71 ± 12.09
CMS-CMS	male	127.47 ± 14.86	8.21 ± 0.70	12.29 ± 5.07
	female	109.83 ± 23.48	7.24 ± 1.11	51.44 ± 22.10
KWH	male	0.004 **	0.120 n.s.	0.231 n.s.
	female	0.531 n.s.	0.854 n.s.	0.815 n.s.
Co-Co vs. CMS-Co	male female	0.488		
Co-Co vs. CMS-CMS	male female	0.010		
CMS-Co vs. CMS-CMS	male female	0.020		

In the FST, only a trend in male F1 mice was found for the parameters 'time struggling' (male:  $F_{(2,33)}=2.729$ ,  $p=0.080$ ) and 'time swimming' (male:  $F_{(2,33)}=2.991$ ,  $p=0.064$ ). For female mice, a trend was revealed only in time struggling (female:  $F_{(2,40)}=2.467$ ,  $p=0.098$ ). No significant difference was observed in the FST neither for time floating, nor number of floating episodes nor the latency to first floating in both sexes (Table 3.14).

Table 3.14: **Behavioral phenotyping in the forced swim test for F1 mice for both sexes.** Statistical difference was detected with one-way ANOVA or Kruskal-Wallis H test (KWH) followed by an appropriate *post-hoc* test, (n.s.  $p>0.1$ , T  $p<0.1$ ).

		time struggling [s]	time swimming [s]	time floating [s]	floating episodes [n]	latency to first floating [s]
Co-Co	male	78.90 ± 9.69	271.74 ± 9.04	10.91 ± 3.15	3.29 ± 0.81	249.89 ± 27.81
	female	59.95 ± 6.74	289.55 ± 8.81	11.65 ± 5.27	2.82 ± 1.13	263.55 ± 35.29
CMS-Co	male	59.51 ± 6.42	274.38 ± 12.00	27.71 ± 9.90	4.00 ± 1.38	216.06 ± 29.65
	female	81.89 ± 7.51	259.96 ± 11.25	19.17 ± 6.81	3.27 ± 0.67	194.27 ± 20.68
CMS-CMS	male	95.49 ± 10.76	240.16 ± 12.22	26.22 ± 8.00	6.00 ± 1.68	193.04 ± 23.86
	female	66.49 ± 6.37	264.90 ± 7.96	30.76 ± 8.44	6.29 ± 1.52	194.93 ± 23.26
ANOVA/ KWH	male	0.080 T	0.064 T	0.225 n.s.	0.515 n.s.	0.279 n.s.
	female	0.098 T	0.109 n.s.	0.185 n.s.	0.177 n.s.	0.218 n.s.
Co-Co vs. CMS-Co	male	0.662	1.000			
	female	0.128				
Co-Co vs. CMS-CMS	male	0.657	0.119			
	female	1.000				
CMS-Co vs. CMS-CMS	male	0.080	0.168			
	female	0.322				

### 3.2.4 F2 generation

In the second generation (F2) treated with CMS, the CMS-CMS-CMS male and female mice exhibited significantly lower locomotor activity in the EPM test as indicated by less total distance traveled (male:  $F_{(2,70)}=3.669$ ,  $p=0.016$ ; female:  $F_{(2,54)}=7.779$ ,  $p<0.001$ ) (Fig. 3.18 A). Furthermore, the male and female CMS-CMS-CMS group revealed a significantly lower number of open arm entries (male: KWH:  $H(3)=10.227$ ,  $p=0.017$ ; female: KWH:  $H(3)=19.917$ ,  $p<0.001$ ) and a significantly increased latency to the first entry to the open arms (male: KWH:  $H(3)=14.382$ ,  $p=0.002$ ; female: KWH:  $H(3)=13.610$ ,  $p=0.003$ ), indicative for more anxiety-related behavior. A conspicuous feature is shown in female Co-Co-Co mice in the number of open arm entries, in which the Co-Co-Co compared to CMS-CMS-CMS group also showed a low number of open arm entries. Moreover, significance of female Co-Co-Co vs. CMS-CMS-CMS and CMS-CMS-Co vs. CMS-CMS-CMS in latency to the first entry to the open arms did not survive *post-hoc* testing. Although the key criterion for anxiety-related behavior measured in the EPM test is the percentage time spent on the open arms, in F2 generation no significant difference was detected in this parameter (male: KWH:  $H(3)=2.910$ ,  $p=0.406$ ; female: KWH:  $H(3)=3.505$ ,  $p=0.320$ ) (Table 3.15). This showed that even if a difference was observed in the parental generation, no difference has to be found in the second generation, and more than one test for assessing anxiety-related behavior has to be performed.



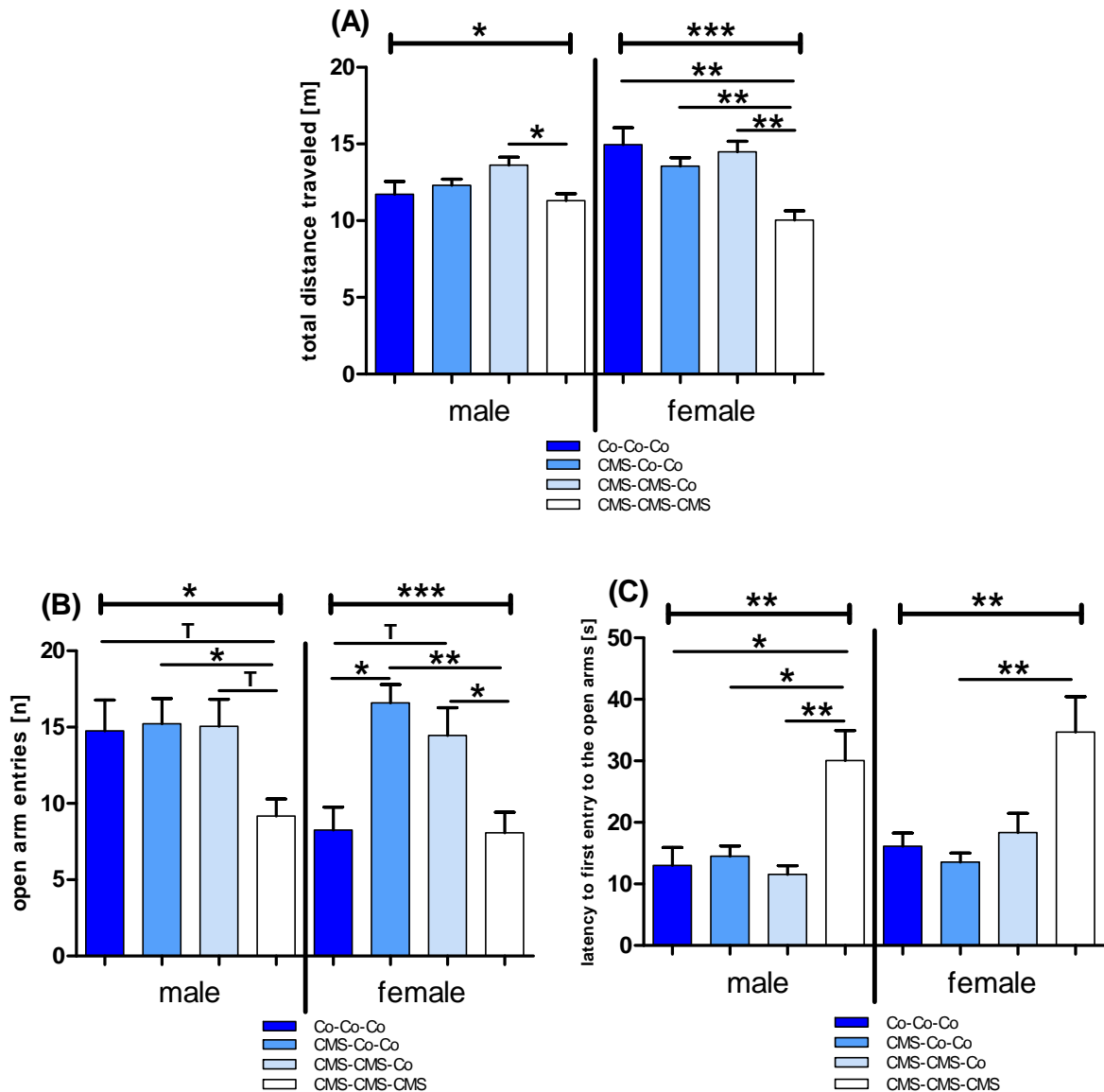


Figure 3.18: **Effect of chronic mild stress (CMS) in the elevated plus-maze test in male and female mice of generation F2.** (A) Female CMS-CMS-CMS mice showed a significantly lower locomotive behavior compared to all other groups, male CMS-CMS-CMS showed a difference compared to CMS-CMS-Co group. (B) CMS-CMS-CMS of both males and females revealed a significantly lower number of open arm entries, also shown in female Co-Co-Co group. (C) Significantly higher latency to the first entry to the open arms was observed in CMS-CMS-CMS group. Data are shown as mean + SEM, (male: N (Co-Co-Co) = 12, N (CMS-Co-Co) = 26, N (CMS-CMS-Co) = 18, N (CMS-CMS-CMS) = 18; female: N (Co-Co-Co) = 8, N (CMS-Co-Co) = 27, N (CMS-CMS-Co) = 11, N (CMS-CMS-CMS) = 12; T p < 0.1, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001).

Table 3.15: **Behavioral phenotyping in the elevated plus-maze test for F2 mice for both sexes.** Statistical difference was detected with one-way ANOVA or Kruskal-Wallis H test (KWH) followed by an appropriate *post-hoc* test, (n.s.  $p > 0.1$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

		total distance traveled [m]	open arm entries [n]	latency to the first entry to the open arms [s]	time spent on the open arms [%]
Co-Co-Co	male	11.73 ± 0.83	14.75 ± 2.03	13.01 ± 2.92	50.86 ± 4.28
	female	14.96 ± 1.11	8.25 ± 1.51	16.15 ± 2.13	61.30 ± 6.03
CMS-Co-Co	male	12.30 ± 0.40	15.23 ± 1.65	14.51 ± 1.69	50.96 ± 2.67
	female	13.55 ± 0.56	16.59 ± 1.21	13.57 ± 1.45	52.00 ± 3.25
CMS-CMS-Co	male	13.63 ± 0.52	15.06 ± 1.78	11.58 ± 1.39	54.98 ± 4.07
	female	14.50 ± 0.68	14.45 ± 1.82	18.35 ± 3.13	52.17 ± 3.25
CMS-CMS-CMS	male	11.31 ± 0.45	9.17 ± 1.13	30.07 ± 4.89	57.69 ± 4.31
	female	10.04 ± 0.60	8.08 ± 1.33	34.69 ± 5.74	54.41 ± 6.42
ANOVA/KWH	male	0.016 *	0.017 *	0.002 **	0.406 n.s.
	female	<0.001 ***	<0.001 ***	0.003 **	0.320 n.s.
Co-Co-Co vs. CMS-Co-Co	male	1.000	1.000	0.583	
	female	1.000	0.011	0.578	
Co-Co-Co vs. CMS-CMS-Co	male	0.140	1.000	0.949	
	female	1.000	0.061	0.804	
Co-Co-Co vs. CMS-CMS-CMS	male	1.000	0.088	0.009	
	female	0.001	0.983	0.154	
CMS-Co-Co vs. CMS-CMS-Co	male	0.319	1.000	0.163	
	female	1.000	0.574	0.403	
CMS-Co-Co vs. CMS-CMS-CMS	male	0.881	0.026	0.004	
	female	0.002	0.002	0.003	
CMS-CMS-Co vs. CMS-CMS-CMS	male	0.014	0.053	0.001	
	female	0.001	0.045	0.169	

Furthermore, in the LD test, used as another test for anxiety-related behavior, male and female mice exhibited a significant difference in several parameters. In males of the CMS-CMS-CMS group, a trend compared to the CMS-Co-Co group was observed in the parameter 'percentage time spent in the light compartment' ( $F_{(3,70)}=2.433$ ,  $p=0.047$ , *post-hoc*: CMS-Co-Co vs. CMS-CMS-CMS:  $p=0.078$ ), which was not shown for all the other group comparisons. For female mice, in the same parameter, the CMS-Co-Co group showed a trend to the Co-Co-Co group and the CMS-CMS-CMS group and a significant difference to the CMS-CMS-Co group ( $F_{(3,54)}=7.489$ ,  $p=0.003$ ; Co-Co-Co vs. CMS-Co-Co:  $p=0.086$ , CMS-Co-Co vs. CMS-CMS-Co:  $p=0.007$ , CMS-Co-Co vs. CMS-CMS-CMS:  $p=0.096$ , others:  $p>0.1$ ). Moreover, a difference was found in males concerning the latency to the first entry to the light compartment (KWH:  $H(3)=8.847$ ,  $p=0.031$ ), but none in the number of entries to the light compartment (KWH:  $H(3)=3.715$ ,  $p=0.294$ ). In females the opposite was the case. A significant difference was detected in the number of entries to the light compartment (KWH:  $H(3)=19.082$ ,  $p<0.001$ ), but none for latency to the first entry to the light compartment (KWH:  $H(3)=3.150$ ,  $p=0.369$ ) (Fig. 3.19 and Table 3.16).

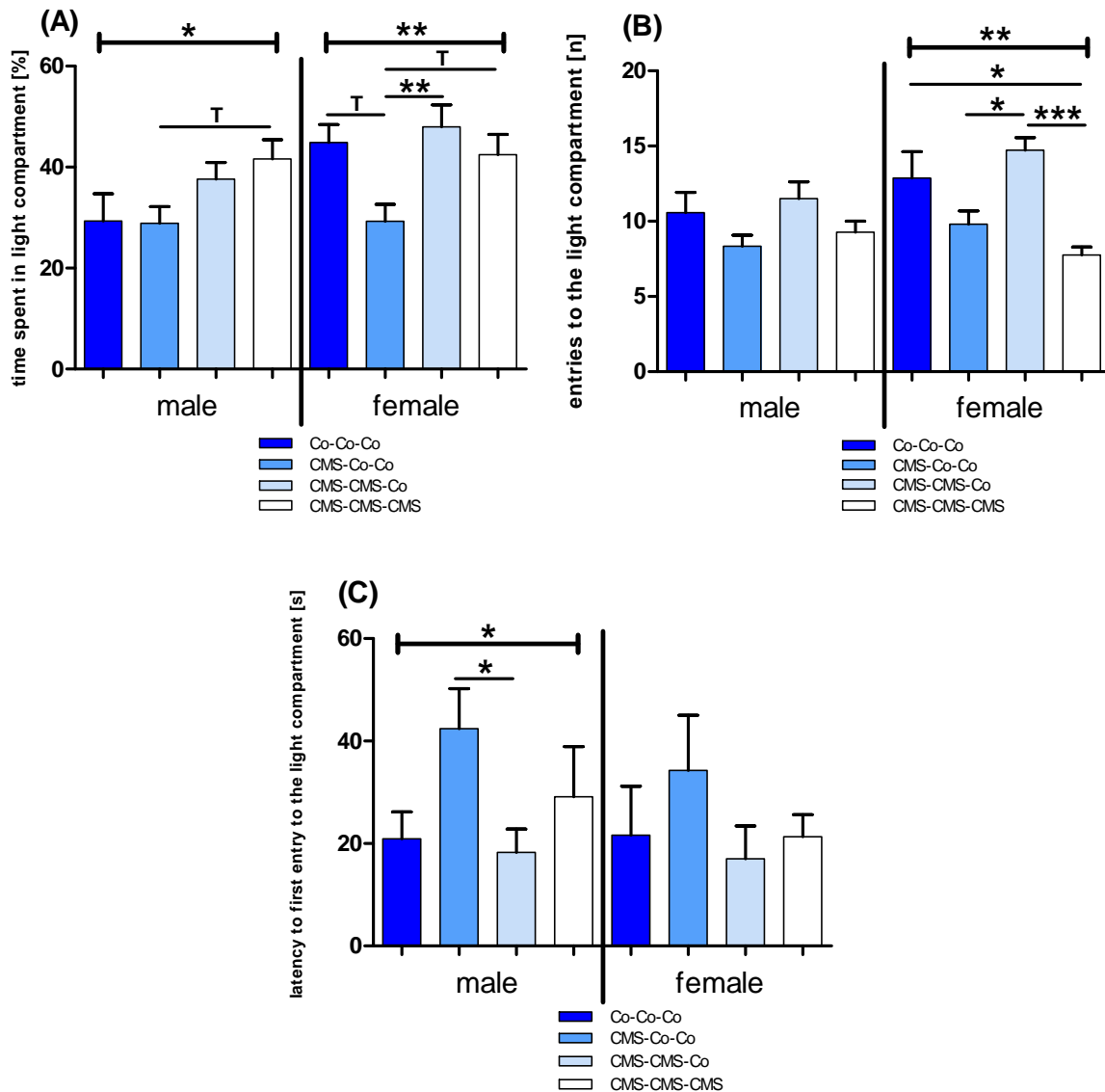


Figure 3.19: **Effect of chronic mild stress (CMS) in the light-dark box test in male and female mice of generation F2.** (A) CMS-CMS-CMS mice showed a trend for higher percentage time spent in the light compartment compared to CMS-Co-Co group, and (B) a significant difference in the number of entries to the light compartment was found in female mice in CMS-CMS-CMS and CMS-Co-Co compared to Co-Co-Co and CMS-CMS-Co groups, but not for males. (C) Male CMS-Co-Co had a significantly increased latency to the first entry to the light compartment compared to CMS-CMS-Co, which was not found in female mice. Data are shown as mean + SEM, (male: N (Co-Co-Co) = 12, N (CMS-Co-Co) = 26, N (CMS-CMS-Co) = 18, N (CMS-CMS-CMS) = 18; female: N (Co-Co-Co) = 8, N (CMS-Co-Co) = 27, N (CMS-CMS-Co) = 11, N (CMS-CMS-CMS) = 12; T  $p < 0.1$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

Table 3.16: **Behavioral phenotyping in the light-dark box test for F2 mice for both sexes.** Statistical difference was detected with one-way ANOVA or Kruskal-Wallis H test (KWH) followed by an appropriate *post-hoc* test, (n.s.  $p > 0.1$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

		entries to the light compartment [n]	latency to the first entry to the light compartment [s]	time spent in the light compartment [%]
Co-Co-Co	male	10.58 ± 1.33	20.91 ± 5.26	29.32 ± 5.39
	female	12.88 ± 1.75	21.63 ± 9.57	44.88 ± 3.56
CMS-Co-Co	male	8.35 ± 0.73	42.45 ± 7.78	28.86 ± 3.31
	female	9.81 ± 0.87	34.29 ± 10.76	29.27 ± 3.33
CMS-CMS-Co	male	11.50 ± 1.13	18.26 ± 4.55	37.64 ± 3.29
	female	14.73 ± 0.83	17.00 ± 6.40	47.96 ± 4.35
CMS-CMS-CMS	male	9.28 ± 0.74	29.15 ± 9.75	41.61 ± 3.80
	female	7.75 ± 0.54	21.31 ± 4.34	42.49 ± 3.99
ANOVA/KWH	male	0.294 n.s.	0.031 *	0.047 *
	female	<0.001 ***	0.369 n.s.	0.003 **
Co-Co-Co vs. CMS-Co-Co	male		0.343	1.000
	female	0.311		0.086
Co-Co-Co vs. CMS-CMS-Co	male		0.794	1.000
	female	0.280		1.000
Co-Co-Co vs. CMS-CMS-CMS	male		0.849	0.283
	female	0.025		1.000
CMS-Co-Co vs. CMS-CMS-Co	male		0.038	0.504
	female	0.010		0.007
CMS-Co-Co vs. CMS-CMS-CMS	male		0.309	0.078
	female	0.221		0.096
CMS-CMS-Co vs. CMS-CMS-CMS	male		0.980	1.000
	female	<0.001		1.000

In F2 mice, no pro-depressive effect was found in the TST. None of the three parameters ‘time immobile’, ‘number of immobile episodes’ nor ‘latency to first immobility’, assessing depression-like behavior, were significantly different (Table 3.17).

Table 3.17: **Behavioral phenotyping in the tail-suspension test for F2 mice for both sexes.** No significant differences were observed, (male: N (Co-Co-Co) = 12, N (CMS-Co-Co) = 24, N (CMS-CMS-Co) = 18, N (CMS-CMS-CMS) = 18; female: N (Co-Co-Co) = 8, N (CMS-Co-Co) = 27, N (CMS-CMS-Co) = 11, N (CMS-CMS-CMS) = 12; n.s.  $p > 0.1$ ).

		time immobile [s]	immobile episodes [n]	latency to first immobility [s]
Co-Co-Co	male	61.94 ± 13.67	6.83 ± 0.93	27.18 ± 16.53
	female	75.21 ± 16.05	7.13 ± 0.77	39.16 ± 23.77
CMS-Co-Co	male	61.38 ± 10.43	6.50 ± 0.79	22.16 ± 10.70
	female	54.57 ± 7.35	5.07 ± 0.49	68.79 ± 22.73
CMS-CMS-Co	male	46.68 ± 6.96	6.39 ± 0.96	24.96 ± 12.44
	female	58.81 ± 16.53	6.09 ± 0.97	9.63 ± 7.81
CMS-CMS-CMS	male	72.10 ± 13.37	7.00 ± 0.71	24.81 ± 12.62
	female	75.86 ± 19.52	6.67 ± 0.89	38.63 ± 27.54
KWH	male	0.518	0.791	0.662
	female	0.675	0.213	0.174
		n.s.	n.s.	n.s.

In the FST of F2 mice, no change in depression-like and stress-coping behaviors in any of the four groups, neither male nor female were seen during the 6-min test paradigm. No significant difference was observed in the parameters 'struggling time', 'swimming time' nor 'floating time' neither in the 'number of floating episodes' nor 'latency to first floating' (Table 3.18).

Table 3.18: **Behavioral phenotyping in the forced swim test for F2 mice for both sexes.** No significant differences were observed, (male: N (Co-Co-Co) = 11, N (CMS-Co-Co) = 24, N (CMS-CMS-Co) = 18, N (CMS-CMS-CMS) = 18; female: N (Co-Co-Co) = 8, N (CMS-Co-Co) = 27, N (CMS-CMS-Co) = 11, N (CMS-CMS-CMS) = 12; n.s.  $p < 0.1$ ).

		struggling time [s]	swimming time [s]	floating time [s]	floating episodes [n]	latency to first floating [s]
Co-Co-Co	male	65.75 ± 8.77	280.49 ± 9.50	15.34 ± 4.56	4.36 ± 0.81	143.87 ± 28.44
	female	52.81 ± 13.73	293.45 ± 15.36	15.41 ± 3.37	3.63 ± 1.19	135.01 ± 31.33
CMS-Co-Co	male	68.77 ± 6.52	275.97 ± 6.01	17.34 ± 3.92	4.79 ± 0.74	177.16 ± 20.47
	female	67.29 ± 6.20	264.7 ± 6.20	29.58 ± 6.60	4.26 ± 0.55	186.39 ± 19.98
CMS-CMS-Co	male	61.20 ± 6.59	272.55 ± 8.95	28.13 ± 7.06	5.65 ± 1.07	156.76 ± 23.24
	female	56.79 ± 9.23	268.62 ± 13.63	36.52 ± 12.37	6.36 ± 1.44	172.57 ± 35.34
CMS-CMS-CMS	male	81.38 ± 7.42	250.26 ± 9.98	30.20 ± 8.75	5.06 ± 0.99	196.49 ± 27.95
	female	62.57 ± 9.95	254.07 ± 17.07	45.63 ± 16.00	5.17 ± 1.13	177.44 ± 33.35
KWH	male	0.154	0.137	0.577	0.819	0.433
	female	0.652	0.529	0.856	0.453	0.704
		n.s.	n.s.	n.s.	n.s.	n.s.

### 3.2.5 Basal CORT in generation F2

As described earlier by Sotnikov *et al.* (2013), there were significantly higher basal CORT levels in stressed mice compared to non-stressed mice, delayed HPA reactivity and stronger feedback regulation.

Here, basal CORT of male and female mice of generation F2 was measured in blood plasma by means of radioimmunoassay. In male mice, the basal plasma CORT was significantly different between all four groups, which was reflected by a trend in female mice (KWH: male:  $H(3)=27.828$ ,  $p<0.001$ , female:  $H(3)=7.250$ ,  $p=0.064$ ; mean  $\pm$  SEM: male: Co-Co-Co:  $20.29 \pm 9.28$  ng/ml, CMS-Co-Co:  $12.56 \pm 7.00$  ng/ml, CMS-CMS-Co:  $4.83 \pm 1.09$  ng/ml, CMS-CMS-CMS:  $48.85 \pm 7.05$  ng/ml, female: Co-Co-Co:  $20.05 \pm 5.93$  ng/ml, CMS-Co-Co:  $32.80 \pm 3.20$  ng/ml, CMS-CMS-Co:  $28.58 \pm 7.47$  ng/ml, CMS-CMS-CMS:  $55.45 \pm 12.00$  ng/ml) (Fig. 3.20). In the group of all three stressed generations (CMS-CMS-CMS) of male mice compared to control (Co-Co-Co) group, a significantly increased CORT level was found (Co-Co-Co vs. CMS-CMS-CMS:  $U=30$ ,  $p=0.007$ ; CMS-Co-Co vs. CMS-CMS-CMS:  $U=25$ ,  $p<0.001$ ; CMS-CMS-Co vs. CMS-CMS-CMS:  $U=7$ ,  $p<0.001$ ), whereas for females a difference was observed ( $U=16$ ,  $p=0.033$ ), which did not survive *post-hoc* test. In male mice, a trend was observed between Co-Co-Co and CMS-Co-Co, as well as a significant difference was shown compared to CMS-CMS-Co (Co-Co-Co vs. CMS-Co-Co:  $U=64$ ,  $p=0.068$ ; Co-Co-Co vs. CMS-CMS-Co:  $U=36$ ,  $p=0.023$ ), but between CMS-Co-Co and CMS-CMS-Co no difference was found ( $U=103$ ,  $p=0.382$ ). Basal CORT levels are already different, indicating that CMS is inducing different HPA axis regulation.



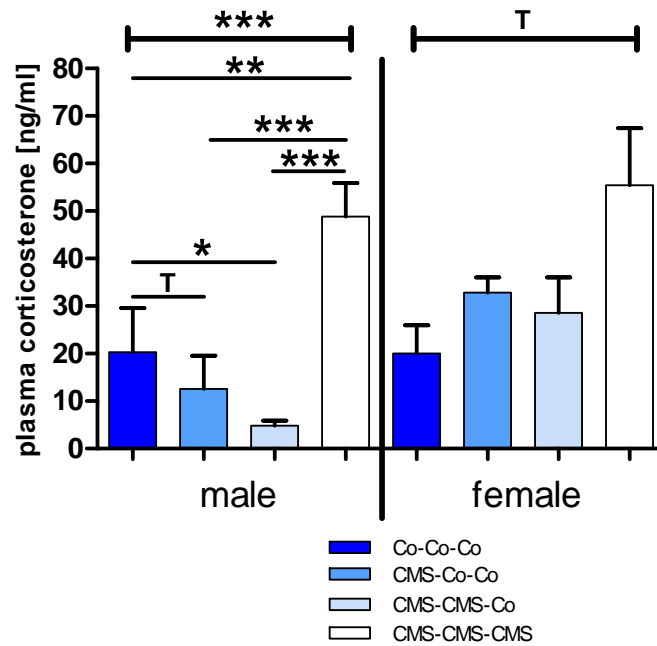


Figure 3.20: **Effect of chronic mild stress (CMS) on basal plasma corticosterone level in male and female mice of generation F2.** A significant difference was found in males, but not in females. Data are shown as mean + SEM, (male: N (Co-Co-Co) = 13, N (CMS-Co-Co) = 18, N (CMS-CMS-Co) = 14, N (CMS-CMS-CMS) = 15; female: N (Co-Co-Co) = 8, N (CMS-Co-Co) = 26, N (CMS-CMS-Co) = 10, N (CMS-CMS-CMS) = 10; T  $p < 0.1$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

### 3.2.6 Correlation analysis of candidate genes

In order to determine the degree of correlation between the behavior on the LD test and the differential gene expression levels of all three generations of the transgenerational approach, we selected male mice according to their behavior from low to high anxiety between 0% and 60% time spent in the light compartment of the LD test. This parameter was used as an indicator of anxiety and showed robust differences after CMS manipulation. Several genes (*Crhr1*, *Crh*, *GR*, *YY1*, *Cnksr2*), known to be involved in stress and showing differences in earlier studies (Sotnikov *et al.*, 2014a), were measured by qPCR in both the BLA and PVN. The correlation was calculated to specify its correlation coefficient  $r$ , and the relative expression of the gene of interest was plotted against the parameter 'percentage time spent in the light compartment'. The determined correlation coefficients constituted a significant effect in the CMS-Co-Co group for *Crhr1* expression in the BLA ( $r = -0.6970$ ,  $p = 0.031$ ;

Fig. 3.21). This suggests that more anxious mice have a higher expression of *Crhr1* in the BLA. No other correlation was detected.

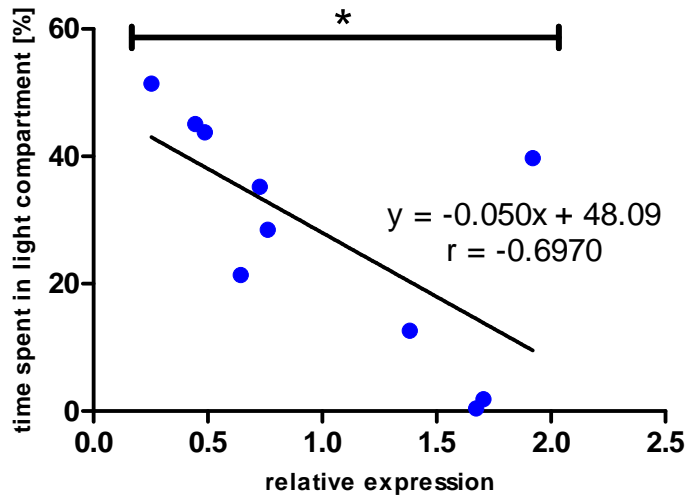


Figure 3.21: Correlation in the CMS-Co-Co group of the relative expression of *Crhr1* in the basolateral amygdala with the percentage time spent in the light compartment. Data are shown as mean + SEM, (N (CMS-Co-Co) = 10; \*  $p < 0.05$ ,  $0.5 < r < 0.7$ : moderate correlation).

### 3.3 Effects of EE or memantine (MM) on anxiety-related/depression-like behavior and neurogenesis

#### 3.3.1 Behavioral tests

First of all, to assess the changes in neurogenesis, we performed behavioral testing to investigate the effect of environmental manipulation combined with a pharmacological approach for potential increase of neurogenesis.

EE is described in chapter 3.1.2 to affect anxiety-related behavior in HAB mice, which we could confirm independently of pharmacological treatment, since HAB and HAB-EE received identical injections of saline. A behavioral test battery was performed comprising OF, LD tests and FST to assess behavioral changes. EE as well as MM treatment induced no effect on locomotion in the OF test indicated by the parameter 'total distance traveled' ( $F_{(2,18)}=1.399$ ,  $p=0.272$ , mean  $\pm$  SEM: HAB:  $9.51 \pm 2.28$  m, HAB-MM:  $16.89 \pm 3.81$  m, HAB-EE:  $12.72 \pm 2.67$  m; Fig. 3.22 A). In the LD test, a significant increase in the percentage time spent in the light compartment was observed in HAB-MM and HAB-EE mice compared to HAB mice ( $F_{(2,18)}=22.806$ ,  $p < 0.001$ , HAB vs. HAB-MM:  $p=0.001$ , HAB vs. HAB-EE:  $p < 0.001$ , mean  $\pm$  SEM:

HAB:  $10.20 \pm 2.64$ , HAB-MM:  $31.83 \pm 2.03$ , HAB-EE:  $37.93 \pm 3.50$ ; Fig. 3.22 B). Between the groups HAB-MM and HAB-EE no difference was detected in the percentage time spent in the light compartment (HAB-MM vs. HAB-EE:  $p=0.407$ ). Furthermore, no significant effect on the floating time in the FST was observed, indicating no effect on depression-like behavior of EE or MM treatment ( $F_{(2,18)}=0.611$ ,  $p=0.554$ , mean  $\pm$  SEM: HAB:  $88.71 \pm 16.74$  s, HAB-MM:  $114.18 \pm 29.12$  s, HAB-EE:  $85.23 \pm 14.04$  s; Fig. 3.22 C).

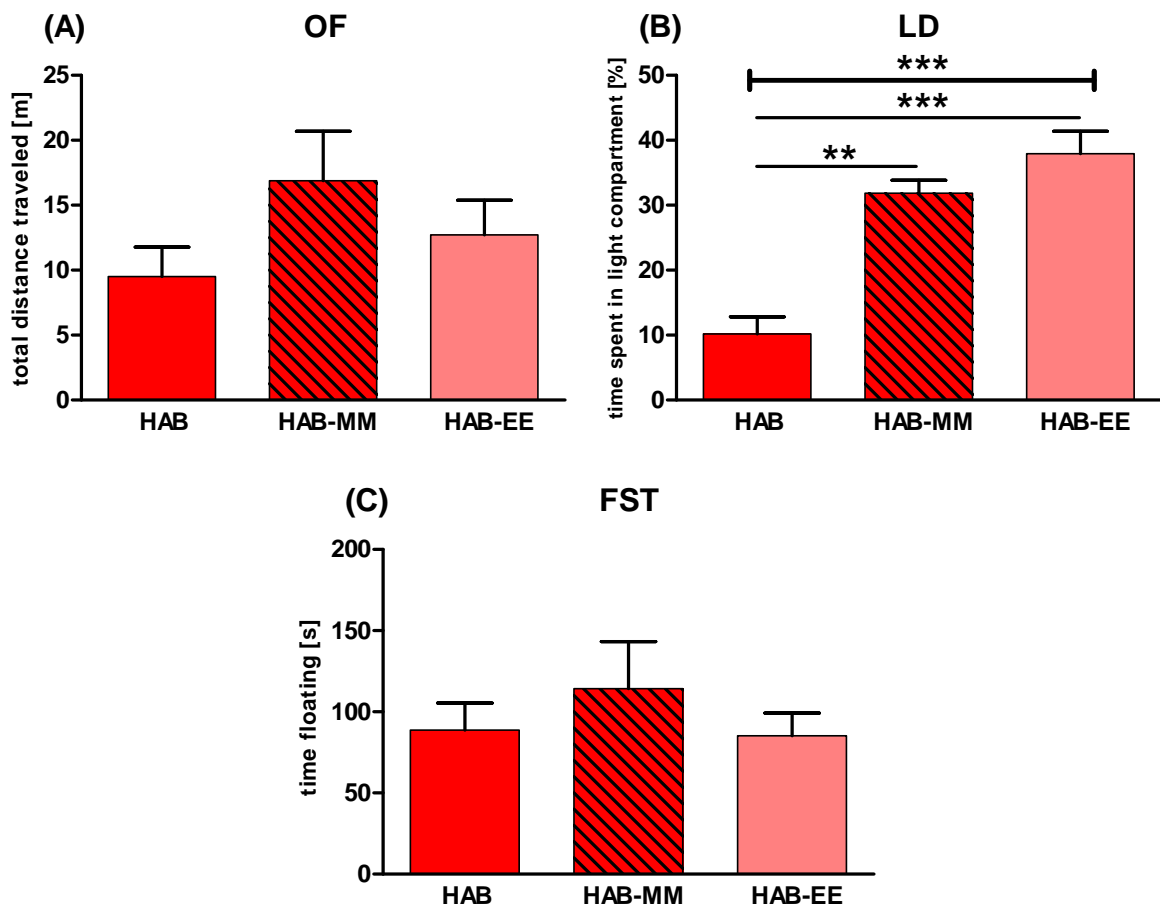


Figure 3.22: **Effects of enriched environment (EE) and injection of memantine (MM) in HAB mice in different behavioral tests.** Three main parameters measured in the performed behavioral tests are shown: (A) total distance traveled in the open field (OF) test, (B) percentage time spent in the light compartment in the light-dark box (LD) test, and (C) time floating in the forced swim test (FST). Data are shown as mean + SEM, (N (HAB) = 7, N (HAB-MM) = 5, N (HAB-EE) = 9; \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

### 3.3.2 Neurogenesis

We wanted to investigate these two manipulations (described in chapter 2.3.1 and 3.3.1) on neurogenesis in HAB mice. Both treatments, environmental manipulation as well as a pharmacological treatment with MM showed an increase in the number of BrdU+ cells surviving in the DG ( $F_{(2,18)}=5.635$ ,  $p=0.013$ , mean  $\pm$  SEM: HAB:  $11.96 \pm 0.74$ , HAB-MM:  $15.27 \pm 0.53$ , HAB-EE:  $15.44 \pm 0.90$ ; Fig. 3.23 A, 3.24). As shown earlier (Sah, 2012), EE showed a significant influence on the survival of newly born cells in the DG in comparison to standard-housed HAB mice, here treated with saline (HAB vs. HAB-EE:  $p=0.015$ ). Additionally, injection of MM in the standard-housed HAB (HAB-MM) mice revealed an increase in the survival of newly born cells compared to HAB mice (HAB vs. HAB-MM:  $p=0.049$ ). No difference between the number of BrdU+ cells between HAB-MM and HAB-EE group was observed (HAB-MM vs. HAB-EE:  $p=0.998$ ).

Furthermore, DCX was used as a marker of the immature neurons. Similarly to BrdU+ cells, a higher number of DCX+ cells were observed in HAB-MM and HAB-EE groups compared to the group treated with saline, indicating a higher number of immature neurons in the DG of the hippocampus ( $F_{(2,18)}=7.932$ ,  $p=0.003$ , mean  $\pm$  SEM: HAB:  $155.23 \pm 4.55$ , HAB-MM:  $200.42 \pm 14.11$ , HAB-EE:  $200.41 \pm 9.10$ ; HAB vs. HAB-MM:  $p=0.015$ , HAB vs. HAB-EE:  $p=0.005$ ; Fig. 3.23 B, 3.25 ). No difference was found in the number of DCX+ cells between HAB-MM and HAB-EE (HAB-MM vs. HAB-EE:  $p=1.000$ ). Both findings indicate a higher rate of neurogenesis in both HAB-MM and HAB-EE mice compared to HAB controls. Images of neurogenesis in the DG are shown for BrdU+ cells in Fig. 3.24 and for DCX+ cells in Fig. 3.25.

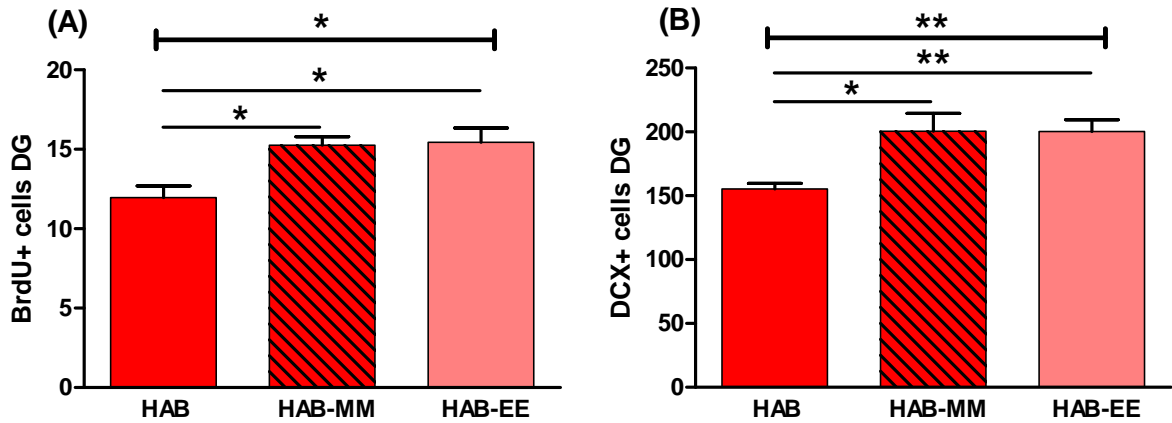


Figure 3.23: **Effects of enriched environment (EE) and memantine (MM) injection on neurogenesis in HAB mice in the dentate gyrus (DG) of the hippocampus.** Effects are shown of (A) survival of newly born cells indicated by number of BrdU+ cells and (B) immature neurons indicated by the number of DCX+ cells. Data are shown as mean + SEM, (N (HAB) = 7, N (HAB-MM) = 5, N (HAB-EE) = 9; \* p<0.05, \*\* p<0.01). BrdU, bromodeoxyuridine; DCX, doublecortin.

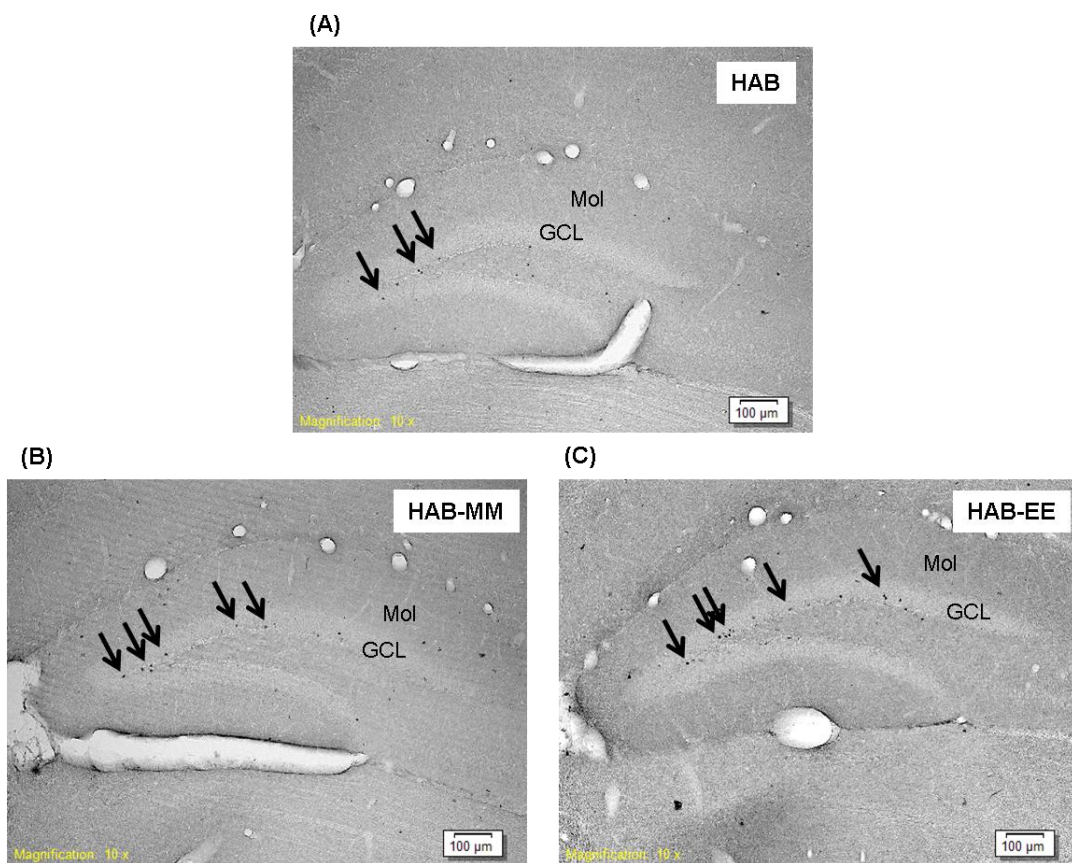


Figure 3.24: **Bromodeoxyuridine (BrdU) images of neurogenesis in the dentate gyrus (DG) of the hippocampus.** BrdU+ cells in (A) HAB, (B) HAB-MM and (C) HAB-EE mice in the subgranular and granular cell layer (GCL) of the DG (indicated by black arrows). Scale bar 100 μm. Mol, molecular layer.

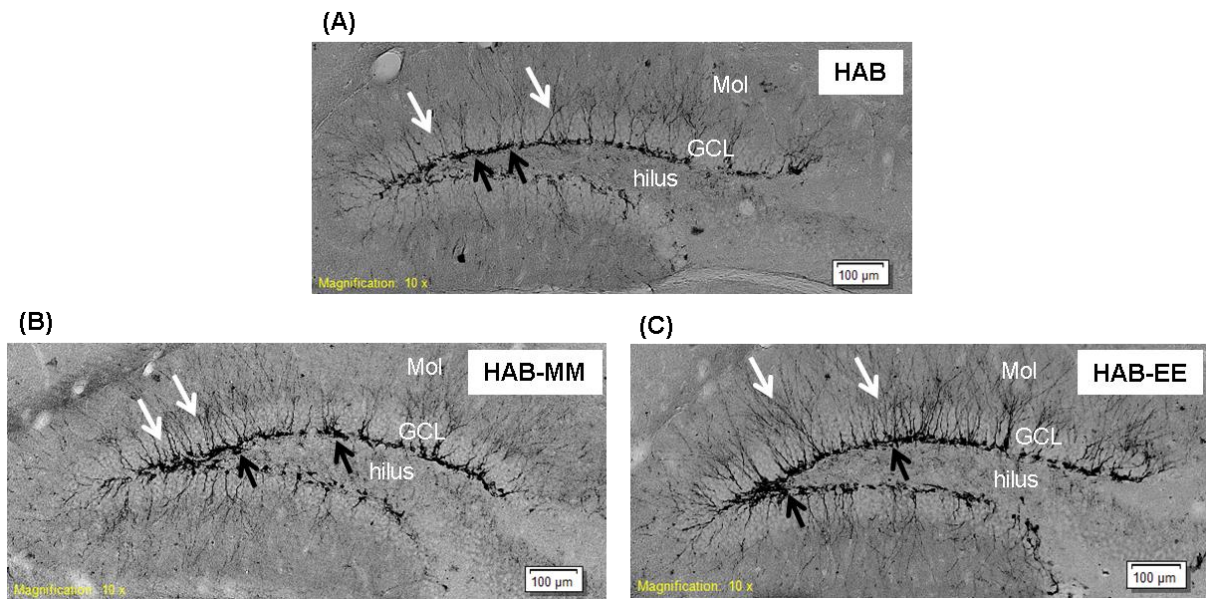


Figure 3.25: **Doublecortin (DCX) images of neurogenesis in the dentate gyrus (DG) of the hippocampus.** DCX+ cells in (A) HAB, (B) HAB-MM and (C) HAB-EE mice represent cell bodies in the subgranular and granular layer of the DG (indicated by black arrows) and the dendrites projecting into the granular cell layer (GCL) and the molecular layer (Mol) (indicated by white arrows) of the DG. Scale bar 100 μm.

## 4 Discussion

In this study, we took advantage of the selective breeding of the HAB/LAB anxiety mouse model, where HAB mice show high and LAB mice low anxiety-related and comorbid depression-like behaviors. This selective, bidirectional breeding was established to conserve genetic components that lead to these two extremes in anxiety-related behavior. We here were able to converge both extremes towards normal behavior using environmental manipulations. In this context, it seems that gene x environment interaction (GxE) plays a crucial role for shaping anxiety.

EE was applied to reduce anxiety of HAB mice, whereas CMS was used to induce an anxiogenic effect in LAB mice. Both environmental manipulations were done as described previously (Markt, 2012; Sotnikov, 2013). In this study, new batches of both manipulations were created from the 45<sup>th</sup> HAB/LAB generation of our breeding. Indeed, EE demonstrated robust anxiolytic effects in HAB mice, which were shown in two of three anxiety-related behavioral tests. No difference was observed in locomotion and explorative behavior of the OF test (Fig. 3.3). The EE-exposed HAB mice exhibited less anxious behavior verified in the validated EPM (Fig. 3.4) and LD (Fig. 3.5) behavioral tests.

On the other hand, in LAB mice CMS treatment induced an anxiogenic effect in the EPM test for female mice (Fig. 3.11) and in the LD test for both sexes (Fig. 3.12). For LAB mice, also a shift in depression-like behavior was observed. Both sexes exhibited higher depression-like behavior after exposure to CMS compared to normal LAB controls, which were highly active (Fig. 3.13, 3.14). In the following, different approaches were used to reveal effects of environmental manipulation on multiple levels, e.g., behavior, gene regulation and neurogenesis.

### 4.1 Microarray studies

To assess the level of gene regulation, we first identified new candidate genes that showed plasticity of gene expression upon environmental manipulations. Thus, after inducing a behavioral change we performed microarray-based gene expression profiling to compare the HAB, HAB-EE, LAB and LAB-CMS transcriptomes in the BLA.

The BLA has been suggested to function as an integration center between other nuclei of the amygdala to react properly to stressors and to mediate phenotypic plasticity (Campeau and Davis, 1995). This structure was chosen for analysis since it was described to be involved in the regulation of anxiety-related behavior (Felix-Ortiz *et al.*, 2013; Wang *et al.*, 2011) and, importantly, to play a critical role in behavioral response to environmental manipulations (Sotnikov *et al.*, 2014a). Furthermore, studies of Sotnikov *et al.* (2014b) found differences in the amygdala response to predator odor exposure between HAB and LAB mice. These findings were supported by an electrophysiological study, where a lower signal propagation was found through the amygdala of LAB compared to HAB mice utilizing voltage-sensitive dye imaging (Avrastos *et al.*, 2013). Moreover, in clinics, functional neuroimaging showed an increased activity of the amygdala in PTSD and phobia patients (Etkin and Wager, 2007).

In this study, using a combination of beneficial and adverse environmental manipulations and whole genome gene expression profiling, we were able to identify novel candidate genes in the BLA potentially involved in the rescue of inborn anxiety-related behavior of HAB and LAB mice.

Altogether, 138 candidate genes were differentially regulated in HAB vs. LAB, 78 genes in HAB vs. HAB-EE and 67 genes in LAB vs. LAB-CMS, according to the microarray study.

First of all, for HAB vs. LAB mice, a highly significant difference in the regulation of genes was observed. The microarray conducted in generation 45 between HAB and LAB animals confirmed the same six candidate genes as detected already more than 20 generations ago, irrespective of gender, in various brain regions (Czibere *et al.*, 2011) (Fig. 4.1). Thus, this underlines the strongly fixed genetic background of the respective phenotype and the robustness of the trait under basal conditions. This clear separation of the two mouse lines bred for anxiety-related behavior is presented in the created cluster dendrogram showing the relationship with 'hclust' function of R (see Fig. 3.6). Out of the 138 highly significant genes, we were focusing on the six genes, which were observed to be differentially expressed in microarray experiments twice as well as at least once validated by qPCR analysis (Fig. 3.7, Table 3.3). *Ctsb*, *Enpp5* and *Slc25a17* were higher expressed in LAB compared to HAB mice. In



contrast, *Abca2*, *Stx3* and *Ttbk1* had a higher expression in HAB mice. Compared to the previous microarray (Czibere *et al.*, 2011), *Enpp5* showed contradictory results.

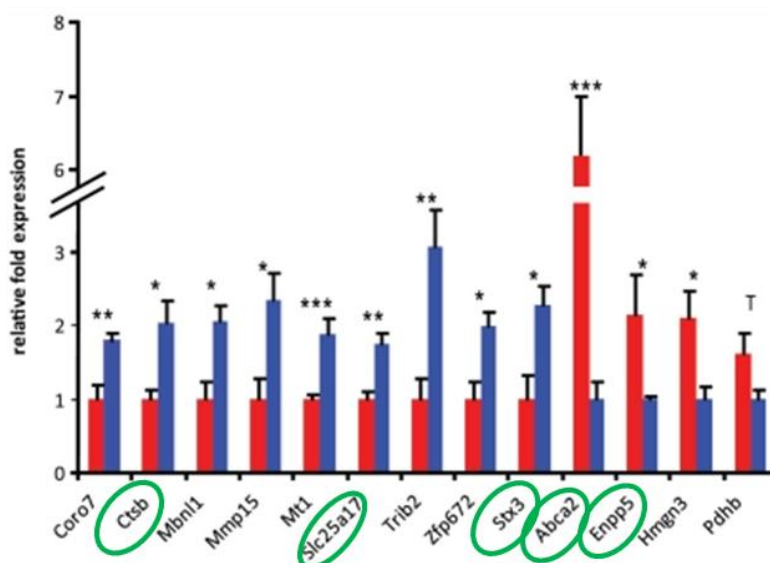


Figure 4.1: **Gene expression profiles confirmed by qPCR of HAB vs. LAB mice in multiple brain regions of the HAB/LAB mouse model.** Data are presented as mean + SEM, (N = 6-10 per group, T p<0.1, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001). (Figure adapted from Czibere *et al.*, 2011).

Using annotational cluster analysis (<http://david.abcc.ncifcrf.gov/>) (see Table 3.1), the first cluster contained genes contributing to different functions in the mitochondrion, so-called mitochondrially active genes. Mitochondria are involved in several different functions, like energy metabolism and are an integral part of various cell signaling cascades (McBride *et al.*, 2006). *Abca2*, *Ctsb* and *Slc25a17*, three of our identified candidate genes, differentially regulated between HABs and LABs, can be classified in this cluster. Moreover, *Ctsb* and *Abca2* appeared in one more cluster together associated with cytoplasmic or membrane-bounded vesicles. Furthermore, *Abca2* was found in another cluster arrangement together with *Ttbk1* in association to nucleotide and ATP binding.

*Ctsb* was the highlight of the previous transcriptome analysis (Czibere *et al.*, 2011) (Fig. 4.1) and showed in the current study the most significant regulation in LAB compared to HAB mice (see Fig. 3.7). This can be explained in part by the fact that about 90 variations in the sequence of the *Ctsb* gene were identified varying between HAB/LAB mice (Czibere *et al.*, 2011). In that study, *Ctsb* knock-out mice were

behavioral tested, and an effect of *Ctsb* deficiency on depression-like behavior in males and females was detected.

Recently, a link between administration of a substance 'poloxamer 407' on serum lipids profiles including *Ctsb* and anxiety levels was discovered with an increase of these cysteine proteases in liver and heart tissues. The higher activity of lipids resulted in an increase of anxiety behavior in the EPM test (Korolenko *et al.*, 2013). In this case, it was explained to appear as a therapeutic target for atherosclerosis in a mouse model of hyperlipidemia and atherosclerosis.

For *Abca2*, an ATP-binding cassette (ABC) transporter, in both microarray studies a deficiency in LAB compared to HAB mice was found (Fig. 4.1, 3.7). *Abca2* was suggested to be a therapeutic target in cancer and nervous system disorders such as in early onset Alzheimer's disease or myelin-related disorders (Mack *et al.*, 2008). In that review, the contribution of *Abca2* as "a mediator of intracellular sterol transport" to human diseases was analyzed and described. Furthermore, a Japanese research group used a knockout mouse line for *Abca2* showing increased environmental stress vulnerability and decreased locomotor capabilities (Sakai *et al.*, 2007).

An interesting gene involved in neurometabolism is *Slc25a17*, which was described to function as a peroxisomal ATP transporter (Agrimi *et al.*, 2012; Visser *et al.*, 2002). *Ttbk1* is not well-described, however, in the context of anxiety. This gene is found to have implications in the pathological phosphorylation of tau in Alzheimer's disease (Lund *et al.*, 2013).

Furthermore, an impact on metabolism is known for *Stx3* (Darios and Davletov, 2006) and *Enpp5*. *Stx3* is described to be important as a plasma membrane protein required for neurite growth and neural development (Darios and Davletov, 2006). The latter gene is counted as one of several ENPP enzymes functioning as significant players in various pathological conditions, as well as key regulators of crucial physiological signaling pathways such as purine or pyrimidine signaling regulation (Masse *et al.*, 2010).

Thus, all these differentially expressed genes indicate robust basic differences between HAB and LAB, most likely also affecting neuronal function finally changing and shaping the respective phenotypic differences.

For the HAB vs. HAB-EE and LAB vs. LAB-CMS, three out of 14 possible plasticity genes detected in the microarray study could be confirmed in subsequent qPCR analyses. Since, *Fos* showed a contradictory regulation between microarray (Fig. 3.8) and qPCR analyses (Fig. 3.10), the reason for this discrepancy can be manifold and should be addressed in further studies. *Foxp2* and *Cnksr2* were detected in the microarray analysis as differentially regulated, but *Foxp2* could not be validated by qPCR (Fig. 3.10).

Interestingly, two validated genes - *Gabrq* and *Cnksr2* - are X-linked. For instance, it is known, that syndromes like ADHD or X-linked intellectual disability are linked to loci on the X-chromosome (Houge *et al.*, 2012; Vaags *et al.*, 2014). In a recent study, the linkage to ADHD was found in the HAB/LAB mouse model, in which the hyperactivity of LAB mice was rescued by amphetamine treatment resulting in a reduced locomotor activity (Yen *et al.*, 2013). Therefore, further studies regarding maternal inheritance might be of great interest.

It is known that the amygdala is highly connected to cortical structures, e.g., the Cg, which appears to be correlated with the formation of anxiety traits (Most *et al.*, 2006; Shin and Liberzon, 2010). Therefore, a range of differentially expressed candidate genes of plasticity were additionally measured in the Cg using qPCR analysis. No significant difference in gene expression was detected (see 3.1.3.6). This indicates that in our model the BLA is more likely to play a significant role in environment-induced plasticity rather than the Cg.

As our focus laid on environmental manipulation and gene-environment interactions, the three differentially expressed candidate genes of plasticity (*Fos*, *Gabrq*, *Cnksr2*) are discussed in detail in the following section.

## ***Fos***

The first investigated gene *Fos* showed a significantly higher expression in HAB compared to HAB-EE mice in qPCR analysis (see Fig. 3.10). It was detected in the microarray experiment, but showed a regulation in the opposite direction. However, the data of the qPCR analysis seems more reliable as it is based on two primers and not only on one probe. *Fos* is an 'immediate early (IE) gene' and acts as a marker of cell activation in earlier stages after an exposure to a stressor/stimulus (Greenberg and Ziff, 1984; Hughes and Dragunow, 1995; Sagar *et al.*, 1988). "It has been

suggested that it acts as a ‘third messenger’ molecule in signal transduction systems, where it would couple short-term intracellular signals elicited by a variety of extracellular stimuli to long-term responses by altering gene expression” (Reddy *et al.*, 1988; Sagar *et al.*, 1988).

A high distribution density was shown all over the whole brain, however, basal expression is relatively low (Sagar *et al.*, 1988) (<http://www.brain-map.org/>, 20.05.2015).

Malik and colleagues (2014) suggested that the transcription factor FOS controls enhancer function reflected by regulation of activity-dependent gene expression. This can occur by binding to similar sites in gene enhancers (Malik *et al.*, 2014). Two phases of gene transcription in reaction to neuronal stimulation are described. First of all, within minutes as early phase after excitation (stimulation) ‘IE genes’ are transcribed (Greenberg and Ziff, 1984). In the later phase, arising over hours, genes, which are responsible for encoding ‘plasticity-related products’, can be turned on.

Several previous studies on HAB/NAB/LAB mice grown up in standard or modified environmental conditions reported different *c-Fos* activity after applying different stressors to map *c-fos* expression. Muigg *et al.* (2009) used unavoidable exposure to the open arms of the EPM test and detected strongly faceted *c-fos* expression phenomena. Another study by Sotnikov *et al.* (2014b) showed an increased *c-fos* expression in several brain regions after trimethylthiazoline (TMT) - a synthetic fox fecal odor - exposure. In this study, authors found a higher basal *c-fos* expression in HAB than in HAB-EE mice only in the hippocampus using *in situ* hybridization. After exposure to TMT, a significantly lower expression in the BLA was observed in the EE-treated mice, which we found here using qPCR already at basal conditions. All this data are consistent with reduced amygdalar *Fos* in EE rats induced by aversive conditioning (Nikolaev *et al.*, 2002). Also higher expression of this gene was observed in the amygdala of HAB compared to LAB mice. Although our data did not show significant differences in the comparison between LAB and LAB-CMS under basal condition (see Fig. 3.8), after exposure to TMT, LAB-CMS mice showed an increase in *c-fos* in the amygdala and PVN (Sotnikov, 2013). Moreover, these results are in line with previous electrophysiological studies (Avrabos *et al.*, 2013). Thus, HAB-EE mice showed low amygdala activity, as it was observed in LAB mice. Inversely, CMS-experienced and HAB mice exhibited increased activity. This is in line with our data, in which HAB-EE mice showed reduced anxiety behavior as well as

lower *Fos* expression in the BLA (Sotnikov *et al.*, 2014b). All these findings describe an 'IE gene', which is expressed relatively low in most CNS areas at basal condition, but increases after exposure. We studied long-term EE modifications and found a higher basal level of *Fos* after several weeks of EE in comparison to standard-housed HAB mice. Hence, constant exposure to external stimuli, like EE, can have an impact on *Fos* expression. Thus, this observed difference in gene expression suggests *Fos* as a candidate gene of plasticity showing an effect in long-term exposure. In addition, constantly increased expression of *Fos* might trigger further mechanisms, like neurogenesis (Sah, 2012), which might alter brain function and will be discussed later.

### ***Gabrq***

Another gene expressed lower in HAB-EE compared to HAB mice (see Fig. 3.10), is a gene coding for the theta subunit of GABA<sub>A</sub> receptors (*Gabrq*). Its sequence has the highest similarity with the  $\beta 1$  subunit. A dysregulated GABA system is implicated in the pathology of anxiety disorders (Nemeroff, 2003; Nutt and Malizia, 2001).

GABA<sub>A</sub> receptors have been shown strongly to be selective, and variations of GABA<sub>A</sub> receptor genes are influencing a component of the bipolar disorder phenotype (Craddock *et al.*, 2010). Several studies identified that GABA, the major inhibitory neurotransmitter in the CNS, has an important role in depression and anxiety (Kalueff and Nutt, 2007). Avrabos and colleagues (2013) detected changes in EE-induced neuronal activity propagation caused by GABA receptor-mediated inhibition.

According to the Allen Institute for Brain Science, *Gabrq* has a high expression in the hypothalamus in an adult mouse brain detected by immunohistochemistry (<http://www.brain-map.org/>, 20.05.2015). In human and monkey brain, *Gabrq* is shown to be distributed in the amygdala, hippocampus and further areas, whereas in the rat brain it is highly expressed in the *striatum* and *locus coeruleus* (Bonnert *et al.*, 1999; Sinkkonen *et al.*, 2000). It is known that the medial hypothalamus receives efferent signals from the CeA to activate the sympathetic nervous system. Ranna and colleagues (2006) found out that  $\theta$  subunits added to  $\alpha 3\beta 1$  GABA<sub>A</sub> receptors can be relevant for new drug targets. *Gabrq* is an X-linked gene and was identified in a study of females with Autism Spectrum Disorder to have a functional variant in this disease (Butler *et al.*, 2015).

Not much is known about the function of this  $\theta$  subunit in mouse brain, since it was detected with different properties/high level of divergence within species (Sinkkonen *et al.*, 2000). The co-assembly of  $\theta$  with  $\alpha\beta\gamma$  revealed an affinity decrease of GABA in recombinant cells and has a benzodiazepine modulatory site (Bonnert *et al.*, 1999). It is described that BZs potentiate the actions of GABA at the GABA<sub>A</sub> receptor to function as an anxiolytic drug (Nemeroff, 2003; Nutt and Malizia, 2001). BZs are modulating the GABA<sub>A</sub> receptor allosterically and are known to be the most used drug treatment for acute anxiety (Macaluso *et al.*, 2010; Singewald *et al.*, 2015).

Acute activation is therefore linked to a directly involvement in anxiety-regulating mechanisms, whereas SSRIs have a delayed effect and require time to create plasticity changes (Olivier *et al.*, 2013). With these changes of the receptor, the affinity for GABA can be increased (Nemeroff, 2003; Ballenger, 1998). In our findings, the HAB mice, which represent the high anxiety phenotype, show higher expression of the  $\theta$  subunit gene. Therefore, a dysfunction in the GABA affinity might exist. EE mice have less of this subtype, which could lead to a higher GABA affinity comparable to the way BZs act (see chapter 1.8). In our cluster analysis, *Gabrq* is represented in two enriched clusters associated with plasma membrane/receptor complex and postsynaptic membrane function (see Table 3.2). This gives a hint that in plasticity receptor complexes and membrane it may have a critical role in modulating anxiety, and minor subtypes have a relevant influence in “physiology and as pharmacological targets” (Ranna *et al.*, 2006). Until now, these findings could guide future approaches for developing new selective compounds for this receptor subtype.

### ***Cnksr2***

In our study, *Cnksr2* was detected in both microarray comparisons (HAB vs. HAB-EE, LAB vs. LAB-CMS; see Fig. 3.8 and 3.9) in the BLA. Not much is known about the functions of *Cnksr2*. The connector enhancer of kinase suppressor of Ras 2 is functioning as an adaptor protein or regulator of the mitogen-activated protein kinase (MAPK) pathways downstream from Ras. It is suggested that CNK2 is responsible for including various regulatory pathways to get an appropriate biological response to external stimuli (Bumeister *et al.*, 2004). This gene product is induced by vitamin D and inhibits apoptosis in certain cancer cells. It may also play a role in ternary

complex assembly of synaptic proteins at the postsynaptic membrane and coupling of signal transduction to membrane/cytoskeletal remodeling (<http://string-db.org/>; 15.06.2015). The gene was found to be highly expressed in different brain regions like amygdala, hippocampus and cerebellum (Houge *et al.*, 2012).

In a study with male patients, the deficiency in *Cnksr2* is a marker for a special disease characterized by attention-deficit/hyperactivity, intellectual and language deficits (Vaags *et al.*, 2014). Earlier studies showed that many similarities to human ADHD appear in LAB mice based on their behavior and their response to pharmacological treatment (Yen *et al.*, 2013). A significant difference was detected in the microarray study and was validated in the LAB vs. LAB-CMS comparison determined by a higher expression in LAB mice (see Fig. 3.10). According to these findings, LAB mice should have an absence or a lower expression of *Cnksr2* gene as they are handled as a potential novel model for the complex disorder ADHD.

The main contribution of MAPKs is acting in the network of signal transduction pathways, in which they are managing major developmental changes or acute responses to hormones (Pearson *et al.*, 2001).

A specific kinase is described to be activated by different stressors and is entailed in various dysfunctions like Alzheimer's disease (Obata *et al.*, 2000). *Cnksr2*, as a possible regulator of the MAPK signaling pathway, showed plasticity for environmental changes. Here, stressed LAB mice exhibited a lower expression and therefore, this gene can be possibly involved in the action of signal transduction in our mouse model. It is also known that *c-fos* (see *Fos*) has an important role in the biological process of stress-activated MAPK cascades (Tanos *et al.*, 2005). *c-fos* is one of the activated genes in cellular reactions in transcription induced by Ras (Zhang and Liu, 2002). As a consequence, the identification of the exact ways of action of MAP kinase might be a promising approach to determine possible mechanisms shaping anxiety in the HAB/LAB mouse model.

In conclusion, the microarray is consistent in detecting differences in gene expression, however, complementary approaches for validation have to be performed. The detailed molecular mechanisms how these genes can be involved in changes of anxiety-related behavior remain largely unknown. In the following section, we tried to assess the critical question if changes in plasticity genes (driven by

environmental modifications) can be transmitted to their offspring, thus possibly affecting behavior in upcoming generations.

#### **4.2 Transgenerational transmission**

In these series of experiments, we investigated if anxiogenic effects of adverse environmental experiences in one generation may profoundly impact behavior of subsequent generations. To assess whether transgenerational transmission of CMS-induced behavioral changes occur in both males and females, we analyzed offspring up to generation F2 in a battery of behavioral tests. Thus, we used two tests to evaluate anxiety-related behavior (EPM, LD tests) as well as two tests to measure depression-like behavior (TST, FST). These tests were chosen since stable and reliable differences were observed for the parental generation after environmental manipulations.

In the parental generation, CMS induced reduced locomotive behavior, anxiogenic and pro-depressive effects, which were observed overall in female mice. In male mice, a difference was shown in only one out of two tests. These slight discrepancies might arise from the fact that different behavioral tests measure different aspects of anxiety-related and depressive-like behavior (Belzung and Griebel, 2001; Sartori *et al.*, 2011). These CMS-induced effects were also seen partially in the F1 generation (CMS-CMS) and F2 generations, where all 3 generations (CMS-CMS-CMS) were exposed to environmental stimuli (see Fig. 2.8). This anxiogenic and pro-depressive effect of CMS corroborates the robustness and reproducibility of our paradigm. As every generation was directly exposed to CMS – parents, embryo (F1) and embryo's gametal cells (F2) – an intergenerational effect could be considered, whereas a transgenerational effect in this groups has to be excluded according to its definition (Daxinger and Whitelaw, 2012; Lightman and Conway-Campbell, 2010; Skinner, 2014). We used this line to verify if the CMS-induced effects were stable across generations, but no additive effect was entailed. A so called multigenerational exposure is shown here, which describes the individual phenotypic behavior of environmental exposure at each generation. This in turn can promote epigenetic programming (Skinner, 2008).

In contrast, in the LD test, one out of four conducted behavioral tests, a possible transgenerational effect in generation F2 was measured (CMS-Co-Co, see Fig. 3.19).



Female mice (CMS-Co-Co) exhibited a significant anxiogenic effect in several parameters of this test (see Fig. 3.19 A and Fig. 3.19 B), whereas for male mice the effect is only seen in the latency to the first entry to the light compartment (see Fig. 3.19 C). Different mechanisms and inheritance could be the reason, thus in our study mothers and fathers were exposed to CMS. At this point, the impact of lifelong maternal or paternal exposures to progeny neurodevelopment is less studied, whereas perturbations on pregnant females are known to influence fetal development (reviewed in Bale *et al.*, 2010).

On the one hand, it is known that a transgenerational effect could be transmitted through the paternal lineage. Several studies suggested a partial contribution of “father’s stressful experiences” to the “individuals’ risk of stress-related disorders” (Dietz and Nestler, 2012). Dias and Ressler revealed that parental olfactory experience was transgenerationally inherited via parental gametes in F2 generation. This was additionally shown by cross-fostering (Dias and Ressler, 2014). Paternal transmission after chronic social defeat stress was reported by another study (Dietz *et al.*, 2011). Modest changes are likely to be transmitted epigenetically from father to its F1 male and female offspring. The transgenerational transmission of stress susceptibility traits in the progeny of CMS mice implies an epigenetic change in the paternal germline (Dietz *et al.*, 2011).

On the other hand, Franklin *et al.* (2010) reported a transgenerational transmission of the negative impact of early stress caused by maternal separation. Authors suggested that changes in DNA methylation altered gene expression in the germline of early-life stressed males. A relation to modifications of promoters, for example of *Crhr2*, was found. Thus, transmission may occur through males or females, offspring can be influenced in a sex-dependent manner. This phenomenon of sex-dependent expression of a trait was also shown in humans (Pembrey *et al.*, 2006; Vige *et al.*, 2008).

Numerous studies highlight the negative effect of stress on breeding success, and that stressors interfere with pregnancy (deCatanzaro and Macniven, 1992; DeSantis and Schmaltz, 1984; Ebensperger, 1998). However, no effect was observed regarding breeding success between CMS and Co groups (see Table 3.8) in our study.

Maternal behavior is supposed to play an important role in transmission across generations (Huot *et al.*, 2004; Schmauss *et al.*, 2014; Siegmund *et al.*, 2009). It has

also been suggested that behavioral deficits can be transmitted through females independently of maternal care as well as not concerned by cross-fostering (Weiss *et al.*, 2011). This is in line with previous studies on maternal behavior and cross-fostering paradigms, which have reported that LAB mice/rats show, in general, less maternal care compared to HAB mice/rats (Kessler *et al.*, 2011; Wigger *et al.*, 2001). These results already indicated that the different anxiety-related behavior in HAB vs. LAB rats and mice was rather defined genetically than postnatally (Landgraf and Wigger, 2002).

Several studies provided evidence that drug treatments altering, e.g., epigenetics, can reverse or prevent inter- and transgenerational effects on future generations (Schmauss *et al.*, 2014; Xiong *et al.*, 2015). Interestingly, although specific traits cannot be expressed clearly by parents, they can be transmitted and expressed by their offspring. This phenomenon shows that mice can function as 'silent' or asymptomatic carriers of certain behavioral changes (Franklin *et al.*, 2010). Similar findings are reported in humans, but the mechanisms behind remain not well understood (Kim *et al.*, 2009; Roseboom *et al.*, 2006).

In addition to assess behavioral changes after stress, basal CORT was measured in generation F2 to evaluate the relation of basal HPA activity to anxiety. It is known that stress and HPA axis are critically involved in environmental induced changes (Mormede *et al.*, 2002). In our study, we observed a significant difference in the basal plasma CORT for male mice between the four groups, which was not shown for female mice (see Fig. 3.20). The highest basal CORT levels were revealed in the group of all three stressed generations. Accordingly, the level was reduced in the Co-Co-Co group as well as more decreased in the group of stressed grandparents (CMS-Co-Co) and parents (CMS-CMS-Co). These findings indicate that CMS has induced different HPA axis regulation already at basal level. Our results are in keeping with a previous study by Sotnikov *et al.* (2013), which reported significantly higher basal CORT levels in stressed compared to non-stressed mice, delayed HPA reactivity and stronger feedback regulation. Reduced CORT levels were observed in all unstressed groups compared to the CMS-treated group in all three generations. Only in male mice, the groups, where the parents (F1) or grandparents (P) experienced one CMS treatment cycle, CORT levels were even lower compared to the Co-Co-Co group. This might hint to an adaptation to stress over the generations.

A previous study investigated the influence of chronic stress during adolescence of male mice before breeding (Rodgers *et al.*, 2013). Authors showed a reduced HPA axis responsiveness as well as changes in sperm microRNA content induced by germ cell epigenetic reprogramming. An additive effect of CMS throughout generations was observable in F2 mice on the level of basal CORT. More recently, Dietz and colleagues found an increased baseline plasma level of CORT in F1 male offspring of chronically defeated fathers (Dietz *et al.*, 2011). Thus, our results in the F2 generation are comparable with the CMS-CMS-CMS group. Like the results described here, previous studies also reported that stress in the parental generation can alter the stress reactivity of their progeny (Bertram *et al.*, 2008; Matthews and Phillips, 2010; Rodgers *et al.*, 2013). Either increased or decreased dysregulation in the stress reactivity system can be indicative of different abilities to respond appropriately (Xiong *et al.*, 2015).

In mice, the postnatal development of the HPA axis is divided into two phases. The first one, lasting from PND 1 until PND 12, is indicated by low basal CORT, a higher expression of *Crh* in the PVN, as well as no reaction to stress in promoted CORT or ACTH levels (Schmidt *et al.*, 2002). The second phase, until PND 16, showed higher CORT levels, a decreased *Crh* expression, a high *GR* expression in the hippocampus and a response to mild stressors (reviewed in Schmidt *et al.*, 2003). Thus, as the different central regulators (*Crh*, *Crhr1*, *GR*) of the HPA axis displayed different expression levels, we were interested in their expression in our transgenerational experiment. Only a correlation between *Crhr1* expression and anxiety-related behavior observed in the parameter 'percentage time spent in the light compartment' of the LD test was found for the male CMS-Co-Co group in the BLA (see Fig. 3.21). Remarkably, this corroborates our transgenerational findings in the LD test. More anxious mice have a higher expression of *Crhr1*. As described earlier by Sotnikov *et al.* (2014b), *Crhr1* was found to be involved in trait anxiety. A higher expression of *Crhr1* mRNA was observed in the CMS-treated group, but not of the ligand CRH, which is in line with our results. Furthermore, this phenomenon was well-discussed in Sotnikov (2013), and it is known that the *Crhr1* is largely expressed in the BLA, whereas *Crh* was found in the CeA (Kühne *et al.*, 2012; Van Pett *et al.*, 2000). Previous studies have reported the link between the dysregulation of the *Crh* system and the development and maintenance of stress-related disorders (de Kloet *et al.*, 2005; Holsboer, 1999; Holsboer and Ising, 2008). The limbic *Crhr1* conveys

anxiety-related behavior and hormonal adaptation to stress (Müller *et al.*, 2003). Furthermore, a study found a bidirectional role of *Crhr1* in anxiety. On the one hand, transmission of anxiogenic stimuli was shown in the amygdala and the hippocampus in glutamatergic neurons, whereas in midbrain dopaminergic neurons the anxiety-related behavior and in the PFC the dopamine release was decreased (Refojo *et al.*, 2011). In addition, Weiss and colleagues (2011) found a decreased *Crhr2* expression in the amygdala and hypothalamus in early life stressed females. Hence, the critical involvement of amygdala in anxiety and stress-like responses is verified.

These findings reveal a strong influence of *Crhr1* in the BLA and in HPA axis regulation, but it might not have a high impact on anxiety-related behavior in one generation. PVN is a key structure in the HPA axis and Yin Yang 1 (YY1) is the transcription factor suggested to play a possible role in stress-related disorders (Sotnikov *et al.*, 2014a). No correlation between expression levels and anxiety-related behavior in the PVN and for YY1 was measured. Accumulating evidence suggests that neuroendocrine responses to stress can be transmitted to subsequent generations, which is also supported by our results.

These experiments highlight that the interaction of environmental and epigenetic factors with genetic predisposition plays an important role in the onset of psychiatric disorders. Environmental manipulations (e.g., stress) together with existing genetic variation can cause a new phenotype, which can be fixed in subsequent generations and can influence pathological phenotypes of future generations. Adaptive responses can arise much faster in reaction to environmental stimuli without waiting “for the occurrence of mutation, which, in the original genetic background, mimics the response well enough to enjoy a selective advantage” (Tost, 2008). Thus, this rapid adaptation is an evolutionary advantage of transgenerational epigenetic inheritance.

As shown so far, shifts in anxiety of both extremes (HAB, LAB) by environmental manipulations (EE, CMS) towards ‘normal behavior’ are connected to distinctive changes in gene expression, as for the proposed candidate genes of plasticity, like *Fos*, *Gabrq* or *Cnksr2*. These shifts along the anxiety continuum can also influence offspring of the treatment-exposed animals. As the described candidate genes are mainly characterized as genes related to neuronal activity and cellular transcriptional

activity, neurogenesis, that is known to play a significant role in disorders concerning anxiety-related phenotypes, might also be affected by the environmental treatments.

### 4.3 Neurogenesis

Changes in anxiety-related behavior appear to be closely associated with changes in hippocampal neurogenesis, whereas modulation of enhanced depression-like behavior seems to be regulated by neurogenesis-independent mechanisms (Sah, 2012; Sah *et al.*, 2012). We investigated a relationship and link between reduced anxiety in HAB mice after EE and neurogenesis, additionally using a pharmacological approach. A recent study showed that MM treatment increases neurogenesis in mice (Akers *et al.*, 2014) and is used as an augmentation therapy for anxiety disorders (Schwartz *et al.*, 2012). Therefore, we were interested if MM could increase neurogenesis and in parallel show anxiolytic effects in our genetically predisposed anxiety mouse model in a similar way as EE treatment does. Thus, various behavioral tests and immunohistochemistry verifications were taken to assess behavioral and neurogenesis effects. Indeed, significant anxiolytic effects of HAB mice treated with MM or EE were shown in the LD test (see Fig. 3.22 B), as well as increased neurogenesis indicated by increased number of BrdU+ cells and DCX+ cells in the DG (see Fig. 3.23). BrdU is a thymidine analogue, which is incorporated into the DNA in dividing cells and can be detected immunohistochemically in their progeny (Kuhn, 1996). BrdU cells give an indication of the number of newly born cells, whereas the number of DCX+ cells shows the number of immature neurons. Our results demonstrate reduced anxiety-related behavior of the EE- and MM-treated mice reflected in the LD test. No differences were observed in locomotion measured in the OF test (see Fig. 3.22 A) nor in depression-like behavior (see Fig. 3.22 C).

Our data are consistent with an earlier study performed by Sah *et al.* (2012) showing an anxiolytic effect of EE and an increase in neurogenesis in HAB mice. Thus, no effect of EE on depression-like behavior was also demonstrated in this case. This suggests that neurogenesis appears to be related to anxiety-related rather than depression-like behavior.

Several studies observed the interplay of EE and increased neurogenesis (Hosseiny *et al.*, 2014; Kempermann *et al.*, 1997), which we could validate in our anxiety mouse model, as well as the alteration of neurogenesis by MM treatment. Experience-dependent neuroanatomical plasticity caused by environmental or by

pharmacological changes is known to have an impact on neurogenesis (Cameron *et al.*, 1995; Cummins *et al.*, 1973; McEwen, 1996). Revest and colleagues (2009) showed that a deficit of hippocampal neurogenesis enhanced anxiety-related behaviors as revealed in a series of behavioral tests using avoidance of threatening situations.

To assess whether EE and MM treatment can complement each other, we performed an EE or pharmacological treatment to HAB mice. Interestingly, treatment with MM decreased anxiety-related behavior observed in the LD test as well as increased neurogenesis indicated by a higher number of newly born and immature neurons. It is suggested that MM, a noncompetitive NMDA glutamate receptor antagonist, is relevant for the glutamate-GABA balance. Both glutamate, an excitatory neurotransmitter and GABA, an inhibitory neurotransmitter, are critically involved in the development of the pathophysiology of anxiety (Cortese and Phan, 2005; Kalueff and Nutt, 2007). MM functions via decreasing glutamate activity, which can be followed by reduced anxiety. Schwartz *et al.* (2012) suggested that in a patient, treated with MM, a lowered level of glutamate is present and therefore, the own GABA system has the power to decrease the symptoms of generalized anxiety disorder. Hippocampal neurogenesis and the expression of GABA<sub>A</sub> receptors are inhibited and reduced by early life stress (Mirescu *et al.*, 2004). Another study found a deficit in adult SGZ neurogenesis and an anxiety-related and depressive-like condition, which is possibly caused by a moderate reduction of GABA<sub>A</sub> receptor function in immature neurons (Earnheart *et al.*, 2007). The balance of glutamate and GABA activity seems to play a crucial role in the development of several mood disorders such as GAD and depression.

In this study, we used i.p. injection as described by Akers *et al.* (2014), since it has likely different pharmacodynamical effects to oral administration. Here, no negative effects on the condition of the mice were observed, although we injected several times i.p. Other studies indicate that high doses of oral MM administration in mice enhance spatial learning and alleviates anxiety (Minkeviciene *et al.*, 2008). This information could be a relevant factor to further pharmacological studies and drug development to establish a compatible treating method. Our data supports the possible augmentation therapy of MM for patients suffering from anxiety or depression (Ferguson and Shingleton, 2007; Schwartz *et al.*, 2012) and for patients being less responsive to usual antidepressant anxiolytics. Therefore, the present

study suggests MM or a pharmacological analog as a potent agent to reduce anxiety, potentially even in clinically relevant settings. The similar results that we could obtain, using the MM and EE approaches on HAB mice, point to a strong common underlying mechanism, as with both treatments, neurogenesis was increased and anxiety-related behavior decreased. Thus, we could demonstrate a direct connection of EE and neurogenesis, and MM and anxiety-related behavior. These are two connections that have been mentioned by studies before separately. However, the underlying processes remain still to be elucidated.





## 5 Conclusion and Perspectives

In summary, our results clearly showed that environmental modifications from both extremes of an anxiety continuum could be shifted in the direction of normal behavior, and, in addition to the previously found *Crhr1* (Sotnikov *et al.*, 2014a), three other genes (*Fos*, *Gabrq*, *Cnksr2*) in the BLA should be considered for the category of candidate genes of plasticity.

In the second part, a transgenerational inheritance effect of CMS treatment could be confirmed based on an investigation of anxiety-related and depression-like behavioral phenotypes of two following generations.

Finally, using a pharmacological approach, we could show that anxiolytic effects of EE or MM could be closely associated with increased hippocampal neurogenesis.

The findings presented in this study implicate that GxE can be observed on different pathways/network levels: neuronal network (neurogenesis), neuropeptide systems (CRH), genetic and transcriptional (*Fos*) levels. These data provide the basis for diverse consecutive experiments. As often discussed, GxE has an important role in the etiology of anxiety disorders, which is reflected in changes of the phenotype, whereas a genetic background comprising all genes and non-coding sequences cannot be altered. Therefore, more studies in the direction of analyzing the involvement of molecular actions and their implications have to be performed to gain a better knowledge about individual reactions on GxE.

First of all, after the confirmation of the genetically stable predisposition of HAB and LAB mice, future experiments should focus on the detailed examination of the differentially expressed candidate genes of plasticity. For *Cnksr2* and *Gabrq*, *in situ* hybridization can be used to detect higher or lower expression levels of the candidate genes. This technique can give information about the distribution of expression patterns in different brain regions after environmental manipulations. Moreover, studies should investigate the long-term changes of *Fos* and the mechanisms behind. Furthermore, the biological information level is limited by mRNA expression analysis. Therefore, it is necessary to look further into cellular processes. The amount of mRNA was measured in this study, but nothing is known about the functionality of the respective proteins. Immunostaining techniques, like Western

blotting, could be used to investigate absence or presence of a protein of interest, its sub-cellular localization and changes in its expression, degradation or its posttranslational modifications, e.g., phosphorylation patterns.

Thus, while the microarray study focused on environment-induced changes, the epigenetic factors contributing to these changes remain largely unknown. Studies on DNA methylation, histone modifications, chromatin structure, and non-coding RNA, which are counted to epigenetic processes, add valuable information to further analyses.

Second, this study suggests a CMS-induced transgenerational transmission to subsequent generations. Since we stressed both sexes and mated them, further analyses should focus on paternal vs. maternal influences to reveal gender effects separately. A method of choice for future studies might be to investigate paternal stress exposure on sperm microRNA content (Rodgers *et al.*, 2013). Furthermore, the correlation/association of *Crhr1* to anxiety-related behavior points to an effect of inheritable epigenetic factors. Therefore, future studies should look closer on epigenetic reprogramming, e.g., DNA methylation, transmission and especially the influence of *Crhr1* and the candidate genes of plasticity (*Fos*, *Gabrq*, *Cnksr2*) investigated in this study.

Third, it will be an issue of future studies to determine the further clinical use of proneurogenic treatment (e.g., MM) in psychiatric disorders. In addition to changes in neurogenesis, the influence of MM on gene expression levels in various brain regions might be a further area of investigation.

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## Curriculum vitae

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03/2015 **Travel award** of the IBRO Pan-European Committee (PERC) and the Federation of European Neuroscience Societies (FENS) to visit the 9<sup>th</sup> World Congress of the International Brain Research Organization (IBRO), Rio de Janeiro, Brazil



## Acknowledgements

An erster Stelle möchte ich meinen aufrichtigen Dank meinem Doktorvater Herrn Prof. Dr. Rainer Landgraf aussprechen, der es mir ermöglicht hat, meine Doktorarbeit in diesem interessanten Themenbereich in seiner Arbeitsgruppe am Max-Planck-Institut für Psychiatrie anfertigen zu dürfen. Ich danke ihm für seine hilfreiche Unterstützung, sein Vertrauen in meine Fähigkeiten und meine Arbeit und für die Möglichkeit mich weiterzuentwickeln.

Ich bedanke mich sehr bei Prof. Dr. Gisela Grupe, die die Mühen der Zweitkorrektur bereitwillig auf sich genommen hat. Danke auch an alle anderen Mitglieder der Prüfungskommission.

Mein herzlichstes Dankeschön gilt meinem Betreuer Dr. Ludwig Czibere. Besonders bedanke ich mich für seine tolle, motivierende, stets optimistische und freundschaftliche Unterstützung, seinen Rat und die Beantwortung aller Fragen. Vielen Dank für die vielen hilfreichen Tipps und die aufopfernde Hilfsbereitschaft auch nach deiner Zeit am MPI. Danke für die tolle Freundschaft!

Desweiteren bedanke ich mich bei unserer Direktorin Dr. Elisabeth Binder, ihrer Arbeitsgruppe und anderen Mitgliedern des MPIs, insbesondere bei Dr. Peter Weber, Susann Sauer und Claudia Kühne, für die praktische Durchführung und statistische Auswertung des Microarrays.

Weiterhin bedanke ich mich bei Prof. Dr. Nicolas Singewald und seiner Arbeitsgruppe aus Innsbruck. I would like to thank Dr. Anupam Sah, my cooperation partner of the neurogenesis part. Without your help it would not have been possible.

Ein großer Dank geht an Silja McIlwrick, meine Büropartnerin. Danke für die tolle Zeit, die anregenden Gespräche, die wir als letzte Doktorandinnen der AG Landgraf/AG Touma verbracht haben, und die gegenseitige Motivation.

Bei Markus Nußbaumer und Marina Zimbelmann möchte ich mich herzlichst für ihre helfenden Hände bedanken, ohne die die Versuche zeitlich manchmal nicht

durchführbar gewesen wären. Insbesondere möchte ich mich bei Dr. Sergey Sotnikov bedanken, der mir als vorletzter Doktorand der Arbeitsgruppe mit Rat und Tat zur Seite stand. Danke für die hilfreichen Ratschläge, die zum Gelingen der Arbeit beigetragen haben.

Weiterhin gilt mein Dank der gesamten ehemaligen Arbeitsgruppe Landgraf, meinen Bürogenossen und anderen Arbeitsgruppen für die erfolgreiche, lehrreiche Zusammenarbeit, ihre Hilfsbereitschaft, die wunderbaren „cake times“ und das tolle Arbeitsklima. Vielen Dank an Dr. Julia Brenndörfer, Dr. Roshan Naik, Dr. Jan-Michael Heinzmann, Dr. Gabriele Mattos, Natalia Chekmareva, Victoria Malik, Isabel Bauer, Tom Schwarzbauer, Alexandra Rechenberg, Tobias Pohl, Dr. Chadi Touma, Dr. Mira Jakovcevski, Stella Iurato, Mary Gazea und allen anderen.

Ich bedanke mich ebenso bei meinem Freundeskreis für seine Unterstützung und seinen Beistand in der ganzen Zeit.

Mein allergrößter Dank gilt meinen geliebten Eltern und meinem lieben Bruder. Ohne eure Liebe, Unterstützung, Aufmunterung, Motivation und den Glauben an mich, wäre ich niemals so weit gekommen. Danke, dass ihr immer für mich da seid.



## Declaration/Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt ist.

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dass ich mich anderweitig einer Doktorprüfung ohne Erfolg **nicht** unterzogen habe.

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