Gene-environment interplay of extreme anxiety-related behavior: implications for corticotropin-releasing hormone receptor 1

Dissertation

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Perfect as the wing of a bird may be, it will never enable the bird to fly if unsupported by the air. Facts are the air of science. Without them a man of science can never rise.

Ivan Pavlov

……..……Dedicated to the ones I love

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List of abbreviations

5-HT	serotonin
5-HTT	serotonin transporter
ADHD	attention deficit hyperactivity disorder
ACTH	adrenocorticotropic hormone
Amy	amygdala
ANOVA	analysis of variance
ATP	adenosine triphosphate
AVP	arginine-vasopressin
BA	butyric acid
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
BLA	basolateral amygdala
Bp	base pairs
BSA	bovine serum albumine
cDNA	complementary DNA
CaRE1	calcium-responsive element 1
CeA	central amygdala
CG	cingulate cortex
ChIP	chromatin immunoprecipitation
CMS	chronic mild stress
CNV	copy number variation
CORT	corticosterone
CpGi	CpG islands
CRE	cAMP responsive element
CRH	corticotropin releasing hormone
Crhr1/2	corticotropin releasing hormone receptor 1/2
CSF	cerebrospinal fluid
DAPI	4',6-Diamidino-2-Phenylindole
Dbh	dopamine beta hydroxylase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNMT	DNA methyltransferase

DSM	Diagnostic and Statistical Manual of mental disorders
dNTP	deoxynucleotide
ECL	electrogenerated chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EE	enriched environment
ELS	early life stress
EMSA	electrophoretic mobility shift assay
EPM	elevated plus-maze
ES	embryonic stem cells
FST	forced swimming test
GxE	gene-environment interaction
Glo1	glyoxalase-1
GR	glucocorticoid receptor
H3K4	lysine in position 4 of H3 histone
H3K9	lysine in position 9 of H3 histone
H3K27	lysine in position 27 of H3 histone
HAB	high anxiety-related behavior
HAC	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HPA	hypothalamic-pituitary-adrenal
IL-10r1	interleukin 10 receptor 1
ISH	*in situ* hybridization
LA	lateral amygdala
LAB	low anxiety-related behavior
LC	locus coeruleus
LDB	light-dark box
LPS	lipopolysaccharides
MeA	medial amygdala
MeCP2	methyl CpG binding protein 2
miRNA	microRNA
mPFC	medial prefrontal cortex
N2a	mouse neuro-2a cell line
NAB	normal anxiety-related behavior

NK1R-A	neurokinin-1-receptor-antagonist
NPC	nasopharyngeal carcinoma cells
Npsr1	neuropeptide C receptor 1
OF	open field test
O/N	over night
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween
PCR	polymerase chain reaction
pCREB	phospho-cAMP responsive element-binding protein
PND	post natal day
PPi	pyrophosphate
PISC	miRNA-induced silencing complex
RT-PCR	reverse transcription polymerase chain reaction
PTSD	posttraumatic stress disorder
PVN	paraventricular nucleus of the hypothalamus
qPCR	quantitative real-time polymerase chain reaction
rGE	gene-environment correlation
RIA	radioimmunoassay
rpm	rotations per minute
SAM	sympatho-adrenomedulary
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
snRNA	small non-coding RNA
SSM	site-specific methylation
SSRI	serotonin specific reuptake inhibitors
TESS	transcription element search system
Tmem132d	transmembrane protein 132d
TMT	trimethylthiazoline
TNF• r1	tumor necrosis factor alpha receptor 1
TSS	translation starting site
TST	tail suspension test
UTR	untranslated region
VPA	valproic acid
YY1	yin yang 1

Abstract

The use of selectively bred mouse models of enhanced fear and/or anxiety-related behavior provides a unique opportunity to identify genetic targets that contribute to pathological anxiety. However, dealing with animal models needs accurate information about their phenotypes. Accordingly, high (HAB), normal (NAB) and low (LAB) anxiety-related behavior mice – a validated model of anxiety disorders - were repeatedly tested in a variety of behavioral paradigms. Whereas most tests to assess anxiety traits are based on fear of novel and open spaces, we took advantage of the inborn fear and associated avoidance of the predator odor (trimethylthiazoline (TMT)) as a measure of anxiety-related behavior. We were able to show that avoidance of TMT reflects the high anxiety phenotype of HAB mice, indicated by the decreased time animals spent in the chamber with TMT compared to NAB and LAB mice. Importantly, this result is not confounded by any deficit of the olfactory system, since mice responded to both the pleasant odor of female urine and the repugnant odor of butyric acid.

To take the influence of environmental stimuli on inborn anxiety further, we next studied the impact of environmental manipulations on the genetically driven phenotype of LAB mice. Therefore, animals were exposed to a series of chronic unpredictable mild stressors (CMS). CMS-treated mice displayed increased anxiety in the TMT-avoidance test, elevated plus-maze (EPM) and light-dark box (LDB). Moreover, these animals were characterized by increased depression-like behavior and a blunted neuroendocrine regulation. Furthermore, TMT-exposure promoted a higher activation of immediate early gene expression, e.g. *c-fos*, in the amygdala, especially in the basolateral nuclei (BLA). *c-Fos* expression pattern correlated with anxiety-related behavior after CMS. Importantly, our electrophysiological studies also indicated a higher activation of amygdala in LAB mice after CMS treatment.

Since corticotropin releasing hormone (CRH) is one of the most important mediators of amygdala activity and is largely involved in the regulation of the anxiety-related behavior, we hypothesized that environmental influences are translated via an altered CRH system. Previous experiments had shown that enriched environment (EE) induced a down-regulation of *Crhr1*. Here, we report that CMS induced higher expression of *Crhr1* in the BLA of LAB mice, in contrast to EE. Thus, these data

indicate, that *Crhr1* expression might be plastic in response to both, beneficial and detrimental, environmental factors.

Thereafter, we studied the role of DNA methylation as a probable mechanism behind the different gene expression. Using pyrosequencing of the bisulfite-converted DNA, one specific CpG site (CpG1) of *Crhr1* was found to be higher methylated after both treatments. In order to evaluate functional importance of this modification, we tested the impact of CpG1 methylation on promoter activity using the luciferase assay and observed that the presence of methylation reduced promoter activity. Moreover, elevated methylation decreased the binding efficiency of the transcription factor Yin Yang 1 (YY1) as indicated by electrophoretic mobility shift assay (EMSA). Furthermore, we analyzed whether a higher expression of *YY1* in the BLA of LAB mice, observed after CMS, contributed to the elevation of *Crhr1*. Indeed, overexpression of YY1 in the neuronal cell culture enhanced both *Crhr1* expression and *Crhr1* promoter activity. Finally, we estimated the effects of combininig CpG1 site-specific methylation with *YY1* overexpression on *Crhr1* promoter activity and tested whether *in vitro* overexpression of *YY1* induced methylation of CpG1.

Altogether, our data suggest that even a rigid genetic predisposition to low anxiety-related behavior could be rescued by environmental modification and provide evidence that the epigenetic regulation of *Crhr1* expression in the BLA is a possible underlying mechanism behind.

1 INTRODUCTION

1.1 What is an anxiety disorder?

Emotions are central states of the brain. Among a variety of them, fear and anxiety are basic and fundamental, with deep roots in the phylogenetic past (Belzung and Philippot, 2007). They are evoked as response to danger or threat and facilitate actions to maintain safety. Whereas anxiety and fear are protective in many settings, in pathological conditions they become excessive and uncontrollable. For instance, during public speaking anxious behavior is typical for almost everyone. However, healthy people could decrease these unpleasant feelings by training their speech in the lecture room before the actual performance, whereas, in case of pathologically highly anxious people “will have a severe and debilitating autonomic, cognitive, and somatic reaction to even the suggestion of giving a public presentation“(Rosen and Schulkin, 1998). Moreover, the subsequent state can be physically destructive, resulting in cardiovascular (Carney et al., 2005; Rozanski et al., 2005), gastrointestinal (Blanchard et al., 2004; Walker et al., 1995), pulmonary (Goodwin et al., 2003; Katon et al., 2007) and other comorbid pathologies (for review see Roy-Byrne et al., 2005). Thus, revealing the fundamentals of anxiety- and fear-related disorders is extremely important not only due to the high prevalence of these diseases within the population and their considerable impact on the quality of life (Alonso et al., 2011; Kessler and Wang, 2008), but also because these unpleasant states could be a risk factor for other common pathologies.

Although excess fear and anxiety are both under the umbrella category of anxiety disorders (American Psychiatric Association, in Sylvers et al., 2011), they differ crucially in the aetiology and articulate various systems of the body (for review see Sylvers et al., 2011). Classically, anxiety is considered to be a generalized response to an unknown threat, whereas fear is focused on a known external danger (Steimer, 2002). Thus, fear-evoked emotions have a clearly identified stressor and are usually based on an innate emotional program (Belzung and Philippot, 2007; Ekman, 1992). In contrast, anxiety lacks this stressor determinism and, therefore, requires more cognitive capacities than fear (Belzung and Philippot, 2007). Another more complex

classification also distinguishes “state anxiety”, occurring at a particular time and place, and “trait anxiety”, characterized as a permanent state of an organism (Clement et al., 2007; Sylvers et al., 2011). However, despite these differences, excessive fear is *the sine qua non* of most of anxiety pathologies (Rosen and Schulkin, 1998), and both fear and anxiety are usually integral and inalienable parts of other conditions.

Pathological anxiety is manifested in various symptoms of anxiety states classified into six major disorders: generalized anxiety disorder, social and simple phobia, panic disorder, post-traumatic stress disorder and obsessive-compulsive disorder (Gross and Hen, 2004). But what turns normal anxiety into a pathological condition? There are three groups of factors that may contribute to the development of unpleasant states: genetic factors, experience during critical stages in the development, and physical or psychological trauma at any age (Rosen and Schulkin, 1998). Since the first group has an innate origin and an exert regulation on the level of gene expression, the second and the third groups have environmental causality and depend on surrounding conditions. Recent literature corroborates that the combination of both genetic and environmental factors is crucial for the development of psychiatric disorders. Thus, the consequences of this gene-environment interplay are detrimental in the genesis of anxiety psychopathology.

1.2 Gene-environment interplay in relation to anxiety disorders

1.2.1 Concepts of gene-environment interplay

It is obvious now that successful attempts to constructively leverage advances of the recent years in understanding the nature of anxiety disorders depend upon our ability to merge genetic and environmental influences in the development of mental diseases (Meaney,2010). The increasing amount of literature (Fig. 1A) suggests two principal mechanisms of gene-environment co-action: gene-environment interaction (GxE) and gene-environment correlation (rGE) (Jaffee and Price, 2012; Rutter and Silberg, 2002). Conceptually, GxE proposes genetically-driven individual sensitivity to a specific environmental event (Kendler and Eaves, 1986), whereas rGE refers to genetic effects in individual differences in liability to exposure to particular environmental circumstances (Jaffee and Price, 2012; Rutter and Silberg, 2002). Practically, it means that rGE strictly differentiates personal genetic background (e.g. presence or absence of risk allele), that in one case leads to the development of a

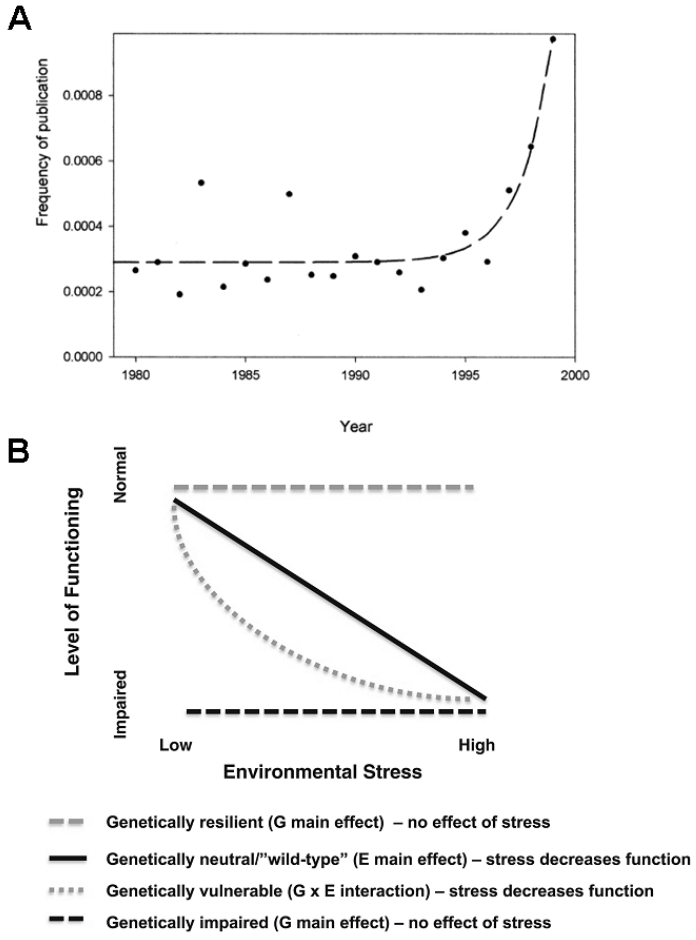

Fig.1. Frequency of publications using the words "environment interaction" or "environmental interactions" and dealing with gene-environment interactions (Clement et al., 2007) (A). Conceptual model of gene-environment interaction proposed by Nugent et al. (2011) (B). High environmental stress degrades functioning (solid line). Genetic differences does not effect functioning under low stress conditions, but impaired when facing intense stress (curved line). Genetically determined resilience (grey dashed line) or impairment (black dashed line) are reflected by normal or impaired functioning, respectively (Nugent et al, 2011).

pathological state (when carrying allele A) and in another case does not (when carrying allele B) during exposure to a novel environment. On the contrary, GxE implies the individual response as a continuum (Fig. 1B), where the genetic background determines the extent of an effect (allele A ensures more aversive effects compared to allele B). Nevertheless, implication of GxE and rGE in dynamic mechanisms of the regulation of behavior involves both, genetic and environmental risks.

1.2.2 Gene-environment interplay from the perspective of genetic background

Genetic factors might account for over 30% of the variability for anxiety-related traits (Gustavsson et al., 1996; Kendler et al., 1995). First-degree relatives have an approximately four- to six-fold increased risk of proband's disorder (Hettema, 2005), whereas global heritability is estimated at the level of 30-50% (Flint, 1999; Parmigiani

et al., 1999). Twin-studies, being even more informative due to a considerably high genetic similarity of participants, have also regarded heritability of anxiety-related disorders in the range of 20-40% (Hattema at al., 2005; Scherrer et al., 2000). However, the genetic nature of the disease is not completely understood. Polymorphisms in the nucleotide sequence of “anxiety” genes have been considered as probable cause of malfunction in most studies. They can be presented as a single nucleotide polymorphism (SNP) - variation of a single nucleotide - or an insertion-deletion polymorphism - a short repeated sequence (usually two to four nucleotides) that occur in stretches of a variable length (“microsatellites”). Many tested candidate genes encode neurotransmitter systems (e.g. serotoninergic, dopaminergic, glutamatergic etc.) and neuropeptides (e.g. corticotrophin releasing hormone (CRH), brain-derived neurotrophic factor (BDNF)).

The best described example analyzes polymorphisms in the promoter of the gene that encodes the serotonin transporter protein (5-HTT), *SLC6A4*. Serotonin (5-HT) is known for its mood and behavior modulating effects and has been implicated in the pathology of anxiety and depression disorders. It was found that *SLC6A4* contains a functional insertion-deletion polymorphism (Capsi et al., 2003; Lesch et al., 1996). These results in the presence of a short allele (consisting of 14 repeats) with a frequency of approximately 45% among European-American ancestry (Gelernter et al., 1998) and a long allele (consisting of 16 repeats). Experiments indicate that the long allele is associated with higher transcription of 5-HTT and increased 5-HT reuptake from the synapse, compared to the short allele (Kenna et al., 2012; Lesch et al., 1996). Moreover, several SNPs have been found in the promoter of the long allele, which, potentially, could decrease its activity to the level of the short allele (Hu et al., 2005; Lipsky et al., 2009). Although these studies support strong genetic influences, gene-environment interaction was found to comprise additional variance. Thus, the presence of the short allele was found to be a risk factor for psychiatric illnesses only for those individuals who experienced a childhood trauma and/or major life stressors (Capsi et al., 2003). In monkeys, as in humans, the short allele is also associated with increased emotional reactivity (Champoux et al., 2002), but decreased 5-HT metabolism is found only in nursery-reared individuals and not in mother-reared ones (Bennett et al., 2002). Thus, environmental influences, especially in early life, can be crucial for upraising of genetic predisposition into pathology.

1.2.3 Gene-environment interplay from the perspective of environmental influences

Twin studies indicate that for trait anxiety, environmental influences account for a substantial proportion (in some cases more than half) of the population variability (Plomin, 2008). Some of the most common human environmental risk factors that could mediate on-set of anxiety disorders are negative family relationships (Emery, 1982), poor social support (e.g. friendship quality) (Kessler et al., 1992) and stressful life events (Kendler et al., 1995). The sensitivity to negative events in early life, such as childhood maltreatment (e.g. sexual/physical abuse, severe neglect) or adverse family environment (e.g. maternal depression, paternal loss, divorce) (Nugent et al., 2011), is especially high, and they significantly increase the risk of development of both “internalizing disorders“ (such as depression and anxiety) (Bhatia and Bhatia, 2007; Mathews et al., 2008) and “externalizing disorders” (like drug and alcohol abuse) (Kaufman et al., 2007; Widom et al., 2007) in adult life. However, the precise investigation of environmental influences in humans has substantial difficulties due to impracticability to control all the variety of possible surroundings and the huge genetic diversity of mankind. Therefore, attempts have been made to model these conditions in animal studies (refer to section 1.4).

One of the most successful approaches refers to the model of early life stress (ELS), that comprises three hours of daily maternal separation during the first days of life (in most studies a period of two weeks after birth). This model tries to mimic (with considerable assumptions) childhood maltreatment and induces strong perturbations in anxiety-related behavior (Kalinichev et al., 2002; McIntoch et al., 1999). In alternative models, animals are exposed to other types of stressors like social defeat (Huhman, 2006; Rygula et al., 2005), chronic restraint stress (Chiba et al., 2012; Zhai et al., 2013) or a combination of different stressors (Bondi et al., 2008; Charkravarty et al., 2013). Moreover, from the perspective of gene-environment interplay, the stress-diathesis theory is of considerable importance. The central point of this theory is the idea that some individuals are disproportionately affected by environmental stressors due to their “vulnerability”. Thus, “diathesis”, in this regard, refers to a genetic predisposition and, therefore, links consequences of a stressful event with genetic risk factors.

However, most studies consider environmental impacts only from the negative end of the spectrum and restricting behavioral outcomes only as negative, whereas the absence of adversity is considered to be the “good” end of the environmental exposure. Thus, such studies fail to measure all the positive environmental impacts and, therefore, largely restrict a range of psychological and behavioral outcomes. In contrast, Kaufman and colleagues (2004) showed that the absence of social support increases the risk of depression in children carrying a short allele of *SLC6A4* (detailed information on polymorphism nature is described in the previous section), whereas positive social support minimizes possible risks. Animal studies also modelled and analyzed influences of beneficial environmental conditions, termed “enriched environment” (EE), on behavioral and emotional parameters. Thus, EE is able to reverse negative consequences of ELS (Cui et al., 2006; Francis et al., 2002) and induce anxiolytic effects in “normal” anxiety animals (Brenes Saenz et al., 2006; Chapillon et al., 1999; Sztainberg et al., 2010).

Assuming bidirectional influences of environmental factors, Belsky and colleagues have reconsidered the stress-diathesis theory (Belsky and Pluess, 2009) and proposed “that the very same individuals who may be most adversely affected by many kinds of stressors may simultaneously reap the most benefit from environmental support and enrichment”. He suggested “plasticity genes” instead of “vulnerability genes”, that are amenable to respond to both beneficial and detrimental environmental influences and accordingly shape the individual phenotype (Belsky et al., 2009).

Altogether, gene-environment interplay constitutes a complex mechanism with a so far unknown precise contribution of genetic and environmental factors in the pathogenesis of specific anxiety disorder. Interestingly, Hicks and colleagues (2009) conducted a comprehensive analysis examining multiple environmental risks and concluded “that in the absence of environmental stressors inherited characteristics will play a greater role in the emergence of internalizing disorders symptoms” and “while environmental stressors have a general effect of increasing the mean-levels and variance of psychopathology, the mechanisms of their influence will differ depending on the nature of the psychopathological condition”.

1.3 Epigenetic mechanism behind gene-environment interplay

As discussed above, gene-environment co-actions play a crucial role in the onset of anxiety disorders, however, merely assuming, that a phenotype is a consequence of this interaction, is oversimplified. Since it is not possible to change an individual's genetic background, more attention is paid to studying environmental influences. Thus, there is a need to have a systemic description of molecular consequences of that interaction in order to predict the outcome in each particular case.

The phenotypic characteristics depend on the expression of genes; however, environments cannot alter gene sequences. This non-DNA sequence-based mechanism of regulation of transcriptional activity is called "epigenetic regulation". Nowadays, a wide use of this term has strayed "epigenetic" from its original rigorous

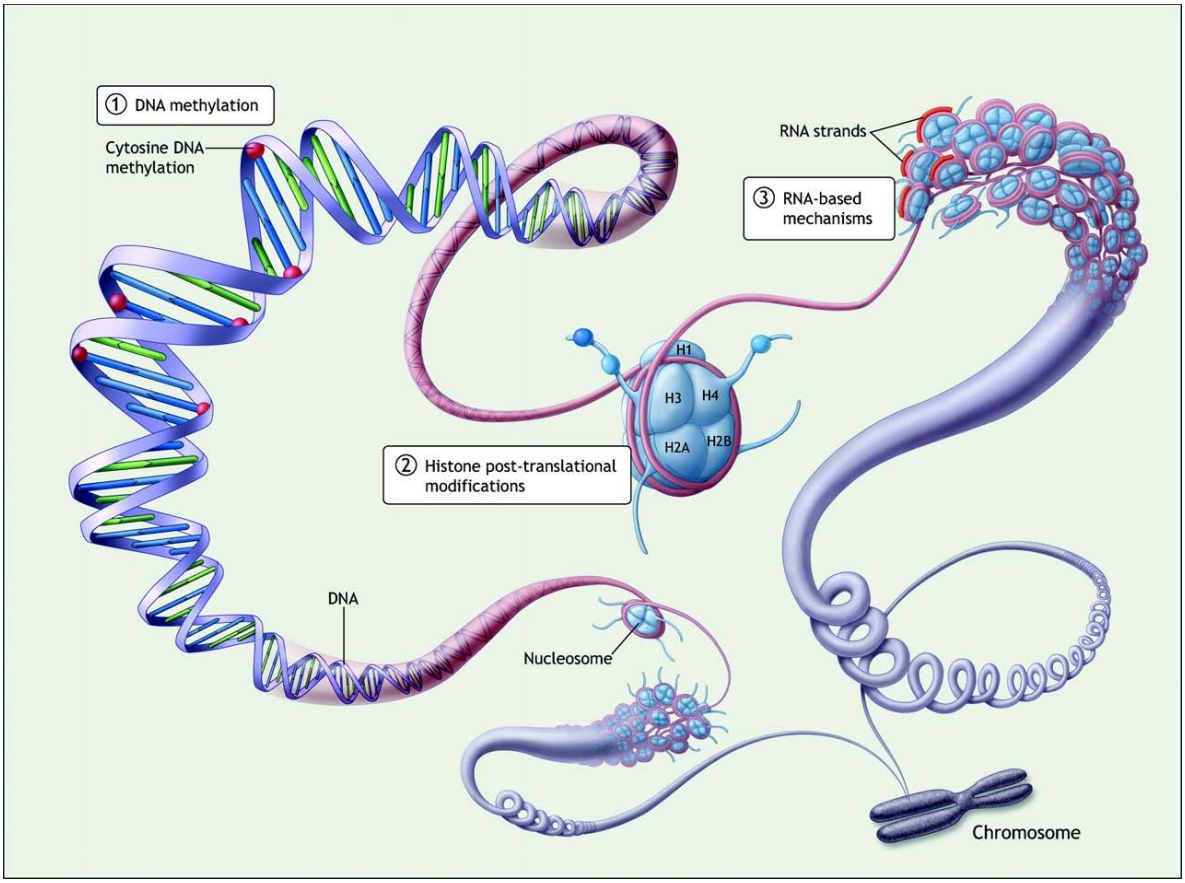

Fig. 2: Epigenetic mechanism of gene regulation. Epigenetic regulation comprise three highly interrelated mechanism: (1) DNA methylation, (2) Posttranslational modifications of the histone amino terminal tails and (3) RNA-based mechanisms. Cartoon adapted from Matouk and Marsden (2008).

use and has brought some confusion in its conceptualization; therefore, here it is restricted only to the regulation of gene transcription without altering underlying sequence. Epigenetic modifications are of great interest because these changes are a possible way to explain how life events can lead to psychiatric disorders. Recent literature assumes three major types of epigenetic regulation: DNA methylation, histone modification and regulation by non-coding RNA.

Almost 65 years have passed since the discovery of DNA methylation, and it took about 30 years to understand the role it plays. DNA methylation is an evolutionary ancient mechanism of regulation of the transcriptional activity, however, it is highly dependant on phylogeny and many non-mammalian species lack it (yeast, fruit fly, most of worms) (for review see Yi, 2012). DNA methylation refers to the structural modification of DNA by the addition of a methyl group to cytosine linked with an adjacent guanine through a phosphodiester bond (CpG). The overall amount of CpGs is relatively low, however, their distribution is non-random and there are DNA regions with a particularly high concentration of CpGs, defined as CpG islands (CpGi) (Takai and Jones, 2003). Most CpGs are methylated, whereas only CpGs of the transcriptionally active genes are less or non-methylated (Jabbarry and Bernardi, 2004). The level of methylation is higher in the brain (0.98 mol percent of methylated CpG) compared to other organs for both humans (Ehrlich et al., 1982) and mice (Tawa et al., 1990). The methylation pattern is established by a class of enzymes called DNA methyltransferases (DNMT). DNMT3a and DNMT3b are responsible for *de novo* methylation, whereas the maintenance of methylation pattern is catalyzed by DNMT1. Remarkably, it was found that postmitotic neurons accumulate exclusively high amount of DNMT1 (Inano, 2000) and expression of *DNMT1* was increased in cortical •-aminobutiric acid (GABA) interneurons of psychotic patients suffering from schizophrenia and bipolar disorder (Veldic, 2004; 2005). Numerous studies indicate that methylation of the promoter is associated with sustained decrease in gene expression, hence, it was suggested that CpG methylation exerts a regulatory role on transcriptional activity. Later it was found that methylated CpGs within promoters are targeted by methyl-CpG-binding domain-containing proteins like methyl CpG binding protein 2 (MeCP2). *MeCP2* is also expressed more abundantly in the brain than in any other tissue (Aber, et al., 2003). Stress-induced regulation of *arginine-vasopressin* (AVP) expression could serve as an example of such epigenetic translation of environmental influences on the molecular level. ELS in mice

decreases methylation of the *Avp* promoter in the PVN that mediates loss of MeCP2 binding. This results in constantly higher expression on *Avp* mRNA, along with the increased basal level of corticosterone (CORT) and stable pro-depressive phenotype (Murgatroyd et al., 2009).

Binding of the MeCP2 complex initiates a cascade of molecular events that promotes transcriptional silencing (e.g. modification of histones). Histones - are proteins that package DNA into a structural unit. Eight histone proteins – two of each H2A, H2B, H3 and H4 – form an octamer, called nucleosome. Approximately 147 nucleotide base pairs (bp) are wrapped around one nucleosome. There are many types of known covalent modifications of histones: methylation, acetylation, phosphorylation, ubiquitination, and others. Modifications are associated with an “open” or “closed” chromatin state and are termed “histone code”. For example, an open chromatin state can be the caused by methylation of lysine in position 4 of H3 histone (H3K4) or of acetylation of lysine in position 9 or 27 of the histone H3 (H3K9 and H3K27 accordingly) (Benevolenskaya, 2007; Koch et al., 2007). Setting epigenetic “marks” allows to regulate DNA accessibility in a very sensitive way. For instance, monomethylation of H3K9 or H3K27 is associated with transcriptional activation, whereas di- or tri-methylation induces transcriptional repression (Barski et al., 2007; Rosenfeld et al., 2009). These dynamic modifications in chromatin structure (occurring within minutes) are carried out by specific enzymes. For example, histone acetylation is mediated by histone acetyltransferases (HACs) that transfer an acetyl group (cleaved from acetyl-coenzyme A) to the lysine residue (Felsenfeld and Groudine, 2003). Reomval of the same is achieved by histone deacetylases (HDACs), enzymes that remove acetyl groups. Interestingly, valproic acid, a widely used drug in psychiatry (mood-stabilizer), was found to be an HDAC inhibitor (Jeong et al., 2003; Laeng, et al., 2004). This suggests a potential role of histone modification in the development of psychopathology. For instance, Hunter et al. (2009) have found that chronic restraint stress significantly decreased the level of H3K9 tri-methylation in the dentate gyrus, whereas the treatment with fluoxetine reversed the decrease.

Recent studies suggest that DNA methylation and histone modifications are occurring coincidently. It was found, that membrane depolarization induced demethylation of *BDNF* promoter and simultaneously shifted from H3K9 di-methylation to H3K4 acetylation that resulted in increased gene expression

(Martinovich et al., 2003). Weaver *et al*. (2004) have demonstrated that rat pups, that received more licking and grooming from their mothers, had lower DNA methylation and higher H3K9 acetylation along a key regulatory site of the glucocorticoid receptor (GR) promoter in the hippocampus. Remarkably, these effects could be reversed by cross-fostering.

Apart from DNA methylation and histone modification, there are other epigenetic mechanism such as DNA hydroxymethylation, nucleosome sliding and repositioning, replacement of histones with other proteins, regulation by non-coding RNA. All of them exert control over gene transcription and, therefore, potentially could play an important role in the development of psychopathology.

1.4 The role of the CRH system in anxiety-related behavior

1.4.1 CRH: discovery and relation to anxiety

The list of candidate genes involved in the development of psychiatric disorders is constantly increasing. These genes are usually part of complex systems like the serotoninergic, dopaminergic, glutamatergic, glucocorticoid, etc. Stress exposure activates the CRH system, which is an ancient and well-studied stress-response system of the body. Its peptides have participated in the regulation of stress-coping behavior for over 550 million years (Chang and Hsu, 2004); however, detailed mechanisms of action are still unknown. Thus, uncovering intrinsic molecular cascades of the CRH system could help to tie together the chain of events constituting the stress response.

In the 1950s, Guillemin and Rosenberg as well as Saffran and Schally independently observed the presence of a factor in hypothalamic extracts (called corticotropin-realising factor (CRF or CRH here)), that stimulated the release of adrenocorticotropic hormone (ACTH). This factor was purified and a 41-aminoacid sequence was identified (Spiess et al., 1981; Vale et al., 1981). Originally, the role of CRH was limited to the regulation of the hypothalamic-pituitary-adrenal (HPA) axis as the mediator of the neuroendocrine response to environmental perturbations (Peterson and Guillemin, 1974; Saffran and Schally, 1974). Produced in the paraventricular nucleus (PVN) of the hypothalamus, CRH is secreted from the median eminence from where it travels into the pituitary to release ACTH, which in turn promotes the release of glucocorticosteroids. However, besides the

hypothalamus, CRH is widely expressed in extrahypothalamic circuits (Swanson et al., 1983), where it acts as neuromodulator.

As such, CRH plays an important role in developing, integrating and coordinating the work of various components of the stress response system (Dunn and Swiergiel, 1999; Koob and Heinrichs, 1999). Accordingly, exposure to acute stress leads to a significant increase of CRH in the brain and to a higher expression of anxiety in rodents and nonhuman primates (Bakshi and Kalin, 2000), which could be blocked by administration of CRH antagonists (Kalin, 1985; Koob and Heinrichs, 1999). Moreover, overexpression (e.g., transgenic mice) or exogenous administration of the peptide, also induces an increase in anxiety and a decrease in locomotion, explorative activity and cognitive functions (Heinrichs et al., 1996; Stanzel-Poore et al., 1994). These results stimulated clinical research to use CRH as a potential biomarker of stress-related disorders. Indeed, several publications reported increased CRH in the cerebrospinal fluid (CSF) of patients suffering from depression (Nemeroff et al., 1984), posttraumatic stress disorder (Baker et al., 1999; Bremner et al., 1997), bipolar and anxiety disorders (Fossey et al., 1996).

1.4.2 CRH receptor 1: implication to anxiety disorders and pharmacological interventions

The action of CRH is mediated by two G-protein coupled receptors thought to have evolved early in chordate evolution: receptor type 1 (CRHR1) and type 2 (CRHR2) (Chang and Hsu, 2004). Both receptors exhibit a different affinity to biological ligands and are distributed unequally throughout the brain (Bashi and Kalin, 2000). Although the role of CRHR2 is still illusive, the effects of CRHR1 on anxiety-related and depression-like behaviors are well studied and repeatedly replicated. Thus, *Crhr1*-knockout mice exhibit decreased anxiety-related behavior compared to wild-type mice in different behavioral paradigms (Contarino et al., 1999; Smith et al., 1998; Timpl et al., 1998). Injection of CRHR1 antagonists in different brain structures results in anxiolytic effects (Heim and Nemeroff, 1999; Kirby et al., 2000). Based on these data, a lot of efforts have been put into the development of CRHR1 antagonist to treat stress-related psychiatric disorders in clinical practice. Although most of the preliminary trials corroborate the efficiency of the tested antagonists (Holsboer and Ising, 2008; Zobel et al., 2000), none of the drugs have passed Phase III (Zorrilla et al., 2013). For instance, promising (from preclinical studies) antagonists ONO-

2333Ms and CP-316311 for treatment of major depression and GSK561679 and GW876008 for treatment of anxiety disorders were discontinued due to a missing proof of concept in double-blind, placebo-controlled trials (Binneman et al., 2008; Zorrilla et al., 2013).

The controversial results of CRHR1 antagonist studies enforced neuroscientists to rethink the complexity of the CRH system in order of more elaborate organization and to consider genetic influences on drug response. Accordingly, one study correlates functional polymorphisms in *Crhr1* with the risk to develop psychopathology following childhood trauma (Bradley et al., 2008; Polanzcyk et al., 2009) and with differential response to antidepressant treatment (Liu, 2007; Licinio, 2004). A recently published study by Refojo et al. (2011) showed two distinct ways of CRHR1 signal transduction, thereby possibly giving one reason why antagonist treatment lacks proof of concept in clinical studies so far. It was found that signal transduction through CRHR1 present in glutamatergic neurons induced anxiogenic effects, whereas activation of CRHR1 in dopaminergic neurons induced anxiolytic effects. Thus, an imbalance in CRHR1-controlled glutamatergic and dopaminergic neuronal pathways could mediate the development of psychiatric disorders and explain poor efficiency of antagonist treatment.

1.4.3 CRH receptor 1 role in the amygdala

The aforementioned results are in line with studies that have investiged the different roles of the CRH system in isolated brain regions (Bashi and Kalin, 2000). In this

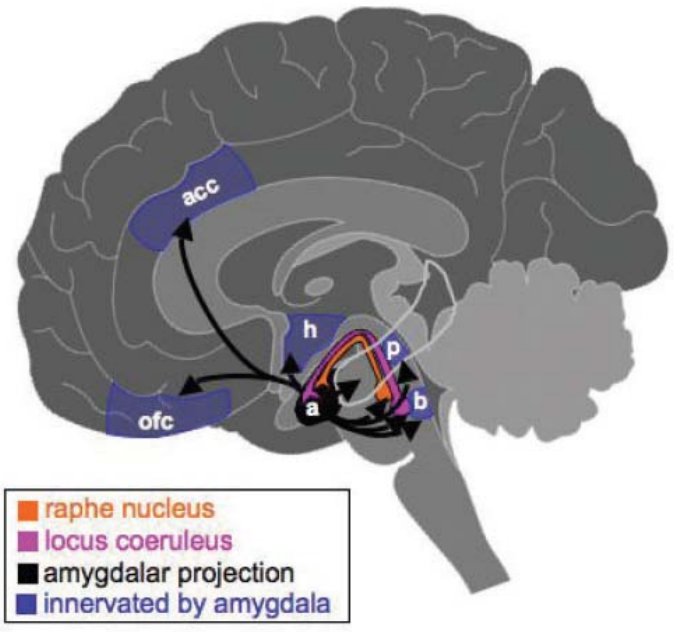

Fig.3. Amygdala-centered anatomy related to anxiety disorders (Stahl and Dana, 2008). The hippocampus is shown as a transparent outline for clarity. acc = anterior cingulate cortex; h = hypothalamus; p = periaqueductal grey; b = parabrachial nucleus; ofc = orbitofrontal cortex. Adopted from Stahl and Wise (2008).

context, the amygdala is of particular interest since it is a major extra-hypothalamic source of CRH-containing neurons and it expresses both receptor types, *Crhr1* and *Crhr2* (Reul and Holsboer, 2002). Human studies indicate that amygdala activation is positively correlated with the degree of trait anxiety (Carlson et al., 2011). Moreover, amygdala activation is increased in patients suffering from posttraumatic stress disorder (PTSD) (Armony et al., 2005), whereas reduced anxiety after antidepressant treatment or cognitive behavioral therapy is associated with reduced activity (Furmark et al., 2002). Animal studies also suggest that changes in amygdala activity can alter state anxiety (Avrabos et al., 2013; Engin and Treit, 2008). Amygdala sends numerous projections to other brain regions like the thalamus, hypothalamus, locus coeruleus and cortex (Fig. 2); therefore, its activation during environmental perturbations could have triggering role in the generalization of the brain response. Animal studies found that the concentration of neurotransmitters significantly increased in the amygdala after restraint stress, footshock or during expression of conditioned fear (Dunn, 1988; Morilak et al., 2005; Yokoyama et al., 2005).

CRH - is one of the most important mediators of amygdalar monoaminergic activity in response to anxiogenic stimuli (Forster, book; Mo et al., 2008). Physiological stress induces *Crh* expression (Makino et al., 1999) and increases the amount of CRH as measured by *in vivo* microdialysis in the amygdala (Mountney et al., 2011). On the other hand, intra-amygdala injection of CRH increased anxiety-related behavior (Heim and Nemeroff, 1999). Results of Refojo et al. (2011) indicate that in the amygdala *Crhr1* is mainly expressed in glutamatergic neurons (Fig. 3) and were found to be associated with the transmission of anxiogenic stimuli. Therefore, the

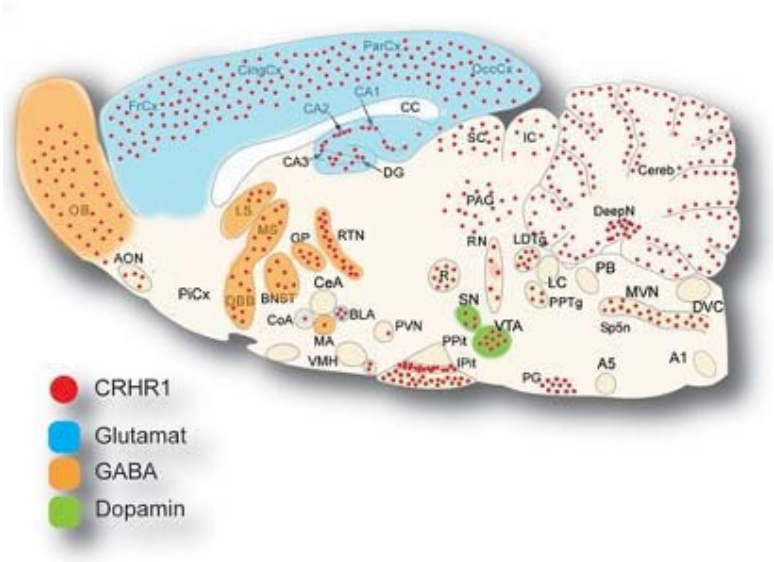

Fig.4. Schematic cross-section of a mouse brain showing the distribution of CRHR1 gene activity and thea ssociated neurotransmitter specificity (Deussing JM, MPI of Psychiatry, Munich, Germany).

above mentioned effects of CRH, could be ascribed to transmission through this receptor. Indeed, intra-amygdala injection of *Crhr1* antisense nucleotides or CRHR1 antagonists prevent CRH- and stress-induced anxiety-related behaviors (Heinrichs et al., 1997; Liebsch et al., 1995; 1999). The local amygdalar CRH receptor activation is postulated to be responsible for the propagation of stressed-induced alterations (Dunn and Berridge, 1990; Sajdyk, et al., 1999). Not only detrimental, but also favourable conditions affect the functioning of the CRH system in the amygdala. Thus, chronic treatment with benzodiazepines (alprazolam), current first-line prescription for treatment of anxiety disorders, reduces expression of *Crhr1* in the basolateral amygdala (BLA) (Skelton, 2000). Sztainberg and colleagues (2010) have shown that anxiolytic effects of EE are mediated by the decrease of *Crhr1* expression in the BLA. This was confirmed by lentiviral deletion of the receptor in the BLA that also induced decreased anxiety-related behavior. Yet, drivers of chronic pathological alterations in this system are still unknown, though extensive studies have been performed in the last decades. For instance, Chen *et. al* (2012) have found that maternal separation is associated with *Crh* promoter demethylation. This epigenetic regulation seems to be tissue specific since the decrease in methylation was found in the PVN, but not in the central amygdala (CeA). Demethylation resulted in higher *Crh* expression in the PVN and consequently altered HPA axis regulation. Another study (Haramati et al., 2011) reported an increased amount of microRNA34c (miRNA34c) after acute and chronic stress, whereas lentiviral overexpression of this miRNA in the CeA had anxiolytic effects. One of the targets of the miRNA34 is *Crhr1*. Binding of the miRNA to 5'-untranslated region of *Crhr1* induced gene silencing, therefore, observed anxiolytic effects of the miRNA34c overexpression could be ascribed to down-regulation of the *Crhr1* in the amygdala. Nonetheless, knowledge about the role of DNA methylation in the regulation of *Crhr1* is still scarce. This is the reason why our study put the central focus thereupon.

1.5 Modeling anxiety disorders in mice

1.5.1 Testing validity of anxiety models

In the beginning of the 20th century, Emil Kraepelin established the classification of numerous psychiatric diseases, based on common patterns of symptoms over time. Later, the American Psychiatric Association introduced the "Diagnostic and Statistical Manual of mental disorders" (DSM), which categorized anxiety as a separate Axis I

Fig. 5. Behavioral tests for evaluation of anxiety-related behavior: open field (A), elevated plus maze (B) and light-dark box (C).

clinical syndrome (American Psychiatric Association, 1994). Such taxonomy, as originally outlined by Kraepelin's criteria, implies that anxiety had distinct etiological origins, symptoms expression, pathophysiological substrates and treatment outcomes (Sufka et al., 2006). Given the large prevalence of anxiety disorders, a lot of efforts have been put on the creation of appropriate animal models in order to study the biological underpinnings of this psychopathology. Thus, animal models must resemble aforementioned description of the disease and thus require to meet three types of validity: face validity, construct validity and predictive validity. Face validity entails that an observed response in humans and an animal model is similar/identical with respect to the behavioral and physiological response. In other words, a model has face validity, if it "looks like" it is going to measure what it is supposed to measure. Predictive validity implies that clinically effective compounds (anxiogenic and anxiolyitic) exert a similar impact on the model, whereas anxiogenic agents, on the contrary, elicited opposite effects, whereas drugs that have no effect in humans should still have no effect on the model. Construct validity means that both animal model and human behavior share similar theoretical rationale.

But how can we model anxiety disorders? The conundrum scientists are facing is that without knowledge of the exact disease mechanism in humans, it is hard to develop a proper model, but without an appropriate model, it is difficult to uncover the mechanism of human diseases (Gerlai, 2006). In an attempt to pursue this question, a wide range of behavioral testing paradigms have been developed to access anxiety-related behavior in animals. Examples include *inter alia* the open field test (OF), the elevated plus maze (EPM), the light dark box (LDB) and the holeboard test. These behavioral paradigms are considered as "models", although they test an acute emotional response (what is described in 1.1 as "state" anxiety) and do not evoke pathology ("trait" anxiety) (Kalueff et al., 2007; Bourin et al., 2007). Most of the

utilized tests are based on the conflict between exploration and avoidance of open, brightly lit spaces.

The OF (Fig. 4A), originally introduced by Calvin S. Hall (1934), consists in placing animals in the unknown arena, surrounded by walls. The Observer counts the time an animal has spent in the periphery (relatively safe environment) and the central zone (unsafe environment), the number of rearing, levels of defecation and urination. Animals with higher anxiety-related behavior are characterized by less time spent in the central zone. Pharmacological treatments with benzodiazepine receptor full antagonist or 5-HT1A receptor antagonist elicit anxiolytic-like effects in this procedure (Prut and Belzung, 2003). However, since different overall activities can mask or generate false positive results, strong locomotor component is the main criticism against this model.

The EPM and LDB tests (Fig. 4B,C), in contrast to the OF, are less dependent on animal's locomotor activity and therefore provide a more sophisticated approach to the evaluation of anxiety-related behavior. The EPM was developed by Pellow and colleagues (1985) to test anxiety-like traits in rats, later it was adopted for mice (Lister, 1987). The EPM is constructed in shape of a "plus" with two opened and two closed arms (enclosed by walls) opposite to each other. Arms are crossed in the middle zone, called the central square (or neutral zone). It is considered, that the more entries an animal does to open arms and the more time it spends there, the less anxious phenotype it has. The rank order preference profile is "closed arms>central square>open arms", meaning that the majority of time subjects spend in a more safe compartment of the closed arms. This tendency is suppressed by both anxiogenic and anxiolytic drugs. Thus, benzodiazepines (Menard and Treit, 1999), GABA- and glutamate-related compounds (Carobrez, 2003) increase the time animals spend in the open arms; on the other hand, benzodiazepine receptor agonist FG 7142 and CGS 8216 decreases this parameter (Pellow and File, 1986). Interestingly, however, treatment with drugs regulating the serotoninergic system provided inconsistent results using this test (Menard and Treit, 1999). Similar to the EPM, the LDB validity has been proven pharmacologically: treatment with benzodiazepines reduced anxiety-related behavior in this paradigm (Costall et al., 1989). The apparatus consists of two inter-connected compartments of different sizes, black (dark) and white (light). A white compartment is usually bigger in size and more brightly illuminated. The main criteria for the estimation of anxiety-related

behavior are the number of entries and the time spent in the light compartment. In contrast to EPM, anxiolytic properties of drugs regulating the serotoninergic system could be evaluated using this test (Costall et al., 1989).

Apart from these paradigms, primarily based on fear of brightly lit, novel and open spaces, many scientists try to develop testing conditions, which would reflect a more natural situation and would provide higher sensitivity. Rodents have a keen sense of smell and operate in a novel environment using olfactory cues. Thus, recently designed testing paradigms involve application of predator odors, like trimethylthiazoline (TMT). TMT is a component of fox faeces and it provides painless, controllable and measurable stimuli. Testing conditions vary from lab to lab, but, generally, an observer estimates approaches or time an individual spent in contact with the odor (Sotnikov et al., 2011; Takahashi et al., 2005). Importantly, treatment with benzodiazepines or serotonin specific reuptake inhibitors (SSRI) increased approach and decreased defensiveness towards predator odor (Dielenberg and McGregor, 2001; McGregor et al., 2002).

1.5.2 The HAB/LAB mouse model

The approaches described above allow to evaluate anxiety-related behavior from different points accurately, but they are restricted by available mouse/rat strains. Although anxiety risk factors are presented in the normal population of rodents it is difficult to separate beneficial from detrimental factors, due to the high genetic heterogeneity. A potentially fruitful approach to resolve this challenge is to apply selective inbreeding to accumulate specific risk factors at a higher penetrance (Sartori et al., 2011).

Thus, several mouse lines with altered anxiety-related behavior were developed using this approach. For instance, Szego and colleagues (2010) have recently established an AX/NAX mouse model on the basis of anticipatory anxiety behavior during the handling procedure. AX mice never volunteered handling and displayed increased anxiety-related behavior in the OF, EPM and LDB. Using two-dimensional gel electrophoresis 82 proteins were found to be differentially regulated between AX and NAX lines, 34 of them were previously identified in other studies of anxiety and depression disorders.

However, the best studied and validated model of anxiety pathology is the HAB/LAB model, established in the Max Planck Institute of Psychiatry in Munich. The key

parameter for breeding is percent time spent on the open arms of the EPM. Originally it was validated for rats, later for mice. Since current work was performed using HAB/LAB mice, only the mouse model is discussed. The high and low anxiety-related behavior (HAB and LAB accordingly) mice were bred for >50 generations from a genetically heterogeneous CD-1 population. This breeding rose two divergent lines, with HAB mice spending <10% and LAB spending >60% of testing time on the open arms of the EPM. This model meets validity criteria mentioned above.

Face validity is confirmed by showing constant and predictive behavioral outcomes in a variety of tests. Thus, HAB/LAB mice exhibit different anxiety-related behavior not only measured in the EPM but also in the OF, LDB (Krömer et al., 2005; Muigg et al., 2009), elevated platform (unpublished results), predator odor avoidance (Sotnikov et al., 2011) and number of ultrasonic vocalizations (Krömer et al., 2005). Clinical studies indicate high comorbidity of anxiety and depression disorders (Hettema et al., 2003; Mineka et al., 1998). Indeed, in HAB/LAB mice this comorbidity is also reported (Krömer et al., 2005). Furthermore, HAB mice display enhanced fear learning in a classical cued paradigm suggesting that trait anxiety is associated with stronger fear memory (Sartori et al., 2011). The same association is observed in patients suffering

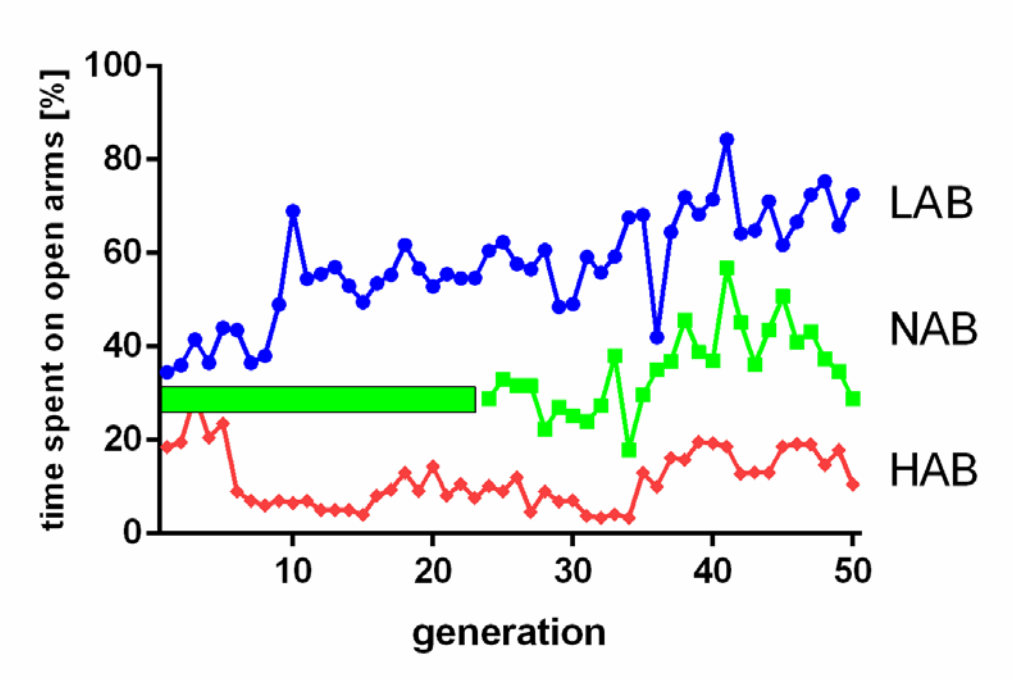

Fig.6: Breeding course if high (HAB), “normal” (NAB) and low (LAB) anxiety-related behavior mice with percent time spent on open arms of the EPM as a key breeding criteria.

from anxiety disorders (Lissek et al., 2005). Importantly, the robustness of the phenotype was evaluated in different laboratories (Sartori et al., 2011). Predictive validity of the model is also successfully confirmed by a variety of pharmacological treatments. Thus, treatment with neurokinin-1-receptor-antagonist (NK1R-A) L-822,429 or selective serotonin reuptake enhancer Tianeptine (both are used in clinics to treat depression and anxiety) decreased anxiety-related behavior of HABs (Sah et al., 2012). Enhanced fear response of HABs is attenuated by treatment with NK1R-A (L-822,429) or selective benzodiazepine agonist L-838,417 (Sartori et al., 2011). Chronic administration of selective serotonin reuptake inhibitor (SSRI) Fluoxetine (prescribed mainly for patients suffering from depression) normalized high depression-like behavior of HABs (Sah et al., 2012).

Genome-wide case-control association analysis in patients with panic disorders revealed *TMEM132D* as a promising candidate gene of this psychiatric pathology (2011). This gene is also higher expressed in the frontal cortex of individuals, who carry a risk phenotype for panic disorder. Remarkably, in HAB/LAB mice *Tmem132d* mRNA is also upregulated in the cingulate cortex, confirming construct validity of the model. Another example is a *glyoxalase-1*. Patients suffering from mood disorders are characterized by higher expression of *glyoxalase-1* mRNA, whereas no difference is reported in the remissive state (Fujimoto et al., 2008). Meanwhile, in HAB/LAB mice the amount of methylglyoxal – a substrate for glyoxalase-1 - is higher in LAB mice compared to HAB, indicating that similar molecular pathways are altered in humans and mice (Krömer et al., 2005; Landgraf et al., 2007; Hambsch et al., 2010). Moreover, recently Yen *et al*. (2013) showed calming effects of treatment with lithium (mood stabilizer to prevent manic episodes in bipolar disorder) and amphetamine (used to treat attention deficit hyperactivity disorder (ADHD)) on locomotor activity of LABs, suggesting that these mice display similar pharmacological response patterns as ADHD patients.

Altogether, these results indicate that HAB/LAB mice are indeed a promising model of anxiety disorders meeting all criteria of validity; therefore, this model can be used to elucidate the genesis of anxiety psychopathologies in humans.

2 Aims of the thesis

The present thesis focuses on the further characterization of anxiety-related behavior in the HAB/LAB mouse model using a novel sensitive testing approach. Therefore, a behavioral test based on predator odor avoidance was established and evaluated. We further aimed to investigate the impact of early life treatment on the inborn anxiety phenotype. Ultimately, the goal was to gain insight into the molecular mechanisms of the gene-environment interplay using both beneficial and detrimental environmental and genetic risk factors. Our previous studies (Markt, 2012; Bauer, 2013) have shown the impact of EE on anxiety-related behavior and expression of *Crhr1* in HAB mice. Here we investigated opposite effects of chronic mild stress (CMS) on behavior and *Crhr1* expression in LAB mice. Finally, our aim was to uncover epigenetic mechanisms behind different *Crhr1* expression patterns after EE and CMS in the basolateral amygdala.

Specific aims:

1. To characterize the anxiety-related behavior of HAB/NAB/LAB based on avoidance of TMT.
2. To study effects of CMS exposure on behavioral and endocrine parameters of LAB mice.
3. To verify the role of the amygdala in increased anxiety-related behavior after CMS using immediate early gene expression.
4. To investigate the influences of CMS on the expression and methylation of CpGi of *Crhr1* in the BLA.
5. To evaluate the impact of CpG1 methylation on promoter activity of *Crhr1*.
6. To examine the role of transcription factor YY1 in the regulation of *Crhr1* expression and its possible contribution to stress-related effects.
7. To test the interaction between *Crhr1* CpG1 methylation and YY1 binding.

3 Material and methods

3.1 Animals and housing conditions

High (HAB), normal (NAB) or low (LAB) anxiety-related behavior mice used for experiments were selectively (in)bred from a CD1 outbred population for >45 generations in the animal facility of the Max Planck Institute of Psychiatry in Munich. The key criterion for the breeding was percent time spent on open arms of the EPM (HAB <15%, NAB 35-45%, LAB >60%). One week prior to experiments, mice were transferred from their respective breeding facility to a room adjacent to the testing room to accomodate to the novel environment. CD1 mice were purchased from Charles River (Sulzfeld, Germany) and were kept also separate from the testing room. All mice were single housed and conditions were adjusted to standard with a room temperature of 23 ± 2°C, relative air humidity of 60 ± 5% and 12/12-hour light-dark cycle with beginning of the light phase at 8 a.m. Mice received food (Altromin 1314 TPF; protein 22.5%, fat 5%, fibre 4.5%, ash 6%) and tap water *ad libitum*.

All animal experiments were approved by the Government of Upper Bavaria and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Government of Upper Bavaria and the European Communities Council Directive of 24 November 1986 (86/609/EEC). All tests were conducted between 9 a.m. and 1 p.m. Only male mice were used for experiments.

3.2 Behavioral phenotyping

3.2.1 TMT-avoidance test

The three-chambered apparatus for TMT-avoidance test is a box consisting of white PVC and divided into three equally sized chambers (30cm each) by two dividers with rectangular gates (Fig. 7). The left and right compartments were used to place a neutral and an odor stimulus. The box was placed inside a fume hood to ensure fast volatilization of the odor after every tested animal. The illumination was adjusted to 45-50lux. When different odors were used in the fume hood, 30min additional interval was added to allow complete elimination of the odor before the next animal was tested. The apparatus was cleaned with water containing a detergent and dried before each trial. All experiments were videotaped and analyzed by Any-maze (Version 4.60, Stoelting Co., Wood Dale, USA).

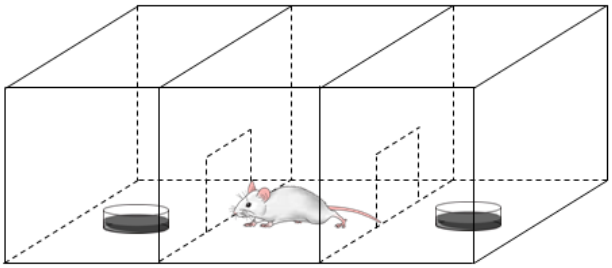

Fig. 7: Three-chambered apparatus used for TMT-avoidance test.

3.2.1.1 Avoidance behavior of CD1 mice

24 male CD1 mice were used for this experiment. TMT-avoidance test took 15min every day. During the first nine days of testing, animals were allowed to habituate inside the apparatus, without a Petri dish or odorant, to measure their initial preference for each chamber. On day 10, animals were divided into three groups, according to the odor they were exposed to: cat odor (*n* = 8), butyric acid (*n* = 8) and TMT (*n* = 8). 35µl of TMT (undiluted, Phero Tech, Delta, British Columbia, Canada) or butyric acid (Sigma-Aldrich, Germany) were pipetted on a filter paper placed in the right chamber, which the mice had preferred most throughout the first nine days. Instead of filter paper, a clean terry cloth 25 cm × 25cm with cat fur odor was placed in the same compartment as TMT and butyric acid. It had been prepared by placing it on the cat's bed overnight and rubbing the cloth on the cat's body before using it for testing the next day, whereas a fresh similar cloth was used in the neutral compartment. To avoid the effect of initial predilection during testing, on days 13 to 14, the odorant was placed in the neutral chamber, leaving the previous odor compartment free of odor.

3.2.1.2 Avoidance behavior of HAB/NAB/LAB mice

In this experiment, only four days of habituation were used to measure the initial preference for one of the outer chambers. After habituation, on day 5, 35µl of TMT was applied in the same compartment as in case of CD1 mice. On days 6 and 7 animals were tested in the three-chambered apparatus without any odor to check that the odor was completely volatilized and had no effects on rodents' behavior anymore. On day 8, 35 µl of female mice urine collected from six to eight females of

the respective line was applied the same way as TMT. Afterwards, during days 9-11, animals were tested without the presence of any odor, similar to days 6 and 7. Finally, on day 12 mice were exposed to 35µl of butyric acid, the same way as it was done for TMT or female urine. Both female urine and butyric acid were used to test for the functionality of the olfactory system in HAB/NAB/LAB mice.

CMS treated and control LAB mice were exposed to TMT the same way. Only habituation time was decreased to one day for better evaluation of stress-induced effects.

3.2.2 Elevated plus-maze test (EPM)

The plus-shaped EPM was made out of dark gray PVC and consisted of two opposing open (30 x 5cm, with light intensity changing gradually from 300lux to 50lux) and two opposing closed arms (30 x 5 x 15cm, with light intensity 10lux) connected by a central platform (5 x 5cm). The EPM was located 40cm above the floor. At the beginning of each 5min trial, the mouse was placed on the central platform facing a closed arm. The apparatus was cleaned with water containing a detergent before each test session and behavior was monitored by a video camera fixed above the EPM. All experiments were analyzed using Any-maze. Entries, latency to first entry in and percent time spent on open arms were recorded as anxiety-related indices.

3.2.3 Light-dark box (LDB)

The LDB comprised a dark (16 x 27 x 27cm) and a light compartment (32 x 27 x 27cm) illuminated with 400lux and <20lux, respectively. During 5min of testing, percent time in the light compartment, entries and latency to enter the light compartment were evaluated as indicators of anxiety-related behavior. Mice with higher levels of anxiety will enter the light compartment with higher latency, less often and spent less time in it.

3.2.4 Home cage activity (HCA)

Since locomotor and exploration activity during behavioral test are strongly affected by stress-related factors like fear of novelty, we measured the animals' activity in the familiar environment, i.e. home cage. The quantification was performed via an automated system (Inframot; TSE, Bad Homburg, Germany). Animals were housed in type 3 Makrolon cages (265 x 150 x420mm) covered with an iron lid with a photo

beam sensor on top. Activity was counted, when the mouse passed the sensor. Eight animals were tracked simultaneously over a period of 96 hours, thus, three dark and light cycles were completely analyzed.

3.2.5 Tail suspension test (TST)

In the TST, the mouse was suspended from a metal frame by fixing the last 2cm of their tail with adhesive tape. After fixation, animals were recorded with a video camera for 6min and videos were later analyzed using a custom Eventlog program. Since antidepressant administration was shown to affect time spent immobile in the TST (Porsolt et al., 1987; Steru et al., 1985), this parameter was used as an indicator of depression-like behavior. Immobility was considered when animals stopped any body movements, except slight head swingings. Moreover, we additionally analyzed latency to the first immobility episode and total number of immobile episodes to get more detailed information about the behavioral readouts. However, the term “depression-like” should be considered with caution, since recent literature suggest “immobility” as a measure reflecting coping strategy in a stressful situation, rather then an emotional state.

3.2.6 Forced swimming test (FST)

FST, similar to TST, exploits stress-induced behavior to characterize depression-like traits. In this test, mice are placed in a 2l glass cylinder (diameter of 135mm and height of 280mm) filled with room temperature water (22.5±1°C) for 6min. Before putting the animal back into its home cage, it was dried with a towel. Behavior was recorded on video tape and analyzed later using Eventlog program. Time spent floating, latency to first floating episode and total number of floating episodes were counted. Floating was considered when animal did not show any movements, except slight balancing movements. In line with previous discussion, there is no concordance in the scientific community whether immobility indeed reflects “depression-like”• behavior or “coping strategy”. Therefore, additionally we performed a stress-independent test to characterize depression-like behavior, the sucrose preference test.

3.2.7 Sucrose preference test

The sucrose consumption test – is a pharmacologically validated paradigm for measuring anhedonic-like behavior, a core symptom of depression (Forbes et al., 1996; Monleon et al., 1995). However, since this work was performed on adolescent

animals, several modifications had to be introduced in order to adapt the model to specific experimental conditions. Liquids consumption from two identical bottles placed on the left and right sides of the cage was analyzed during 10 days. Bottles with water/sucrose were weighed every morning at 8 a.m. to evaluate the amount of consumed liquid and then filled with fresh content. Since body weight could affect the total amount of drunk liquid, the final volume was divided by body weight to measure a relative consumption value. During days 1-4 both bottles only contained a water solution, so that any basal preference of a position/bottle could be evaluated. Similarly, during days 5-6 both bottles were filled with 4% sucrose solution (Sigma-Aldrich) to get animals familiar with a sweet content and to measure the basal position/bottle preference. Finally, during days 7-10, bottles were filled either with water or sucrose solution. The preference was evaluated by dividing the relative consumption of the sweet solution by the relative consumption of water. Unlike other protocols, food and water deprivation were excluded due to ethical reasons.

3.3 Analysis of neuroendocrinological parameters

3.3.1 HPA axis regulation

We analyzed HPA axis regulation via measuring basal and reactive CORT release. Our earlier experiments indicated, that the highest difference in CORT levels between HAB/LAB mice could be found in the afternoon (Sotnikov et al., unpublished). Therefore, we collected blood samples from animals between 3 p.m. and 6 p.m. Blood was taken from the ventral tail vessel in less than two minutes and collected in Microvette® CB300 coated with potassium-EDTA tubes (code: 16444, Sarstedt, Nümbrecht, Germany). Plasma was separated from cellular constituents via centrifugation for 10min at 4000rpm and frozen at -20°C until further analysis. by HPA axis reactivity and feedback regulation was tested via exposure to 6min forced swimming. Blood samples were collected in three time points: directly after stress, 30min and 60min later.

3.3.2 Radioimmunoassay (RIA)

Plasma samples taken at basal state and 60 min after FST were diluted 1:13.5, whereas samples collected directly or 30 min after stress were diluted 1:100. 10µl of plasma was used to determine the concentration of CORT using RIA kit (MP Biomedicals, Solon, Ohio, USA) according to the manufacturer’s instructions. All

samples were measured in duplicates, intra- and inter-assay coefficients were below 10%.

3.3.3 Body and organs weight

Animals were weighed on PND15 and were separated in treatment groups randomly, so that no *a priori* difference could be found between experimental groups. On PND 44 animals were weighed again to estimate treatment effects on individual body weight parameters. After animals were sacrified on day 52, both right and left adrenals and thymus were accurately removed with a scalpel and weighed using an analytical scale. Finally, the weight of the structure (or combined in case of adrenals) was divided by the individual body weight to obtain the relative weight of the structure.

3.4 Chronic mild stress paradigm (CMS)

CMS was applied from PND 15 to PND 44 and comprised array of alternating mild stressors to elicit an anxiogenic and pro-depressive phenotype including maternal separation (PND 15-28), wet bedding, cage tilt, overcrowding, mild footshock, overnight illumination, white noise (adopted from Willner et al., 1987). A detailed description of the stress procedure is presented in Table 1. Food and water deprivation were excluded due to ethical reasons. Unstressed control mice were housed under normal conditions without any manipulations.

3.5 Killing of animals and brain harvesting

Animals were deeply anesthetized with Forene (ABBOTT GmbH, Wiesbaden, Germany) and quickly decapitated. Brains were accurately removed, quick-frozen in 2-methylbutan (CarlRoth, Karlsruhe, Germany) and stored at -80°C until further analysis.

3.6 c-Fos in situ hybridization

The frozen brains were cut into 20µm slices and mounted to Superfrost microscope slides (Menzel, Braunschweig, Germany) in a cryostat (Microm MH50, Microm, Walldorf, Germany), shortly dried and stored at -80°C until further analysis.

c-Fos expression was measured in 5 brain regions: medial prefrontal cortex (mPFC), amygdala (Amy), hippocampus (Hipp), PVN and locus coeruleus (LC). Subdivisions of the amygdala – basolateral (BLA), central (CeA), lateral (LA) and medial (MeA) - and the hippocampus - CA1, CA2, CA3 and the dentate gyrus - were analyzed

Table 1: Comprehensive protocol of the chronic mild stress paradigm.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1	13:30 - 16:30 maternal separation	10:00-13:00 maternal separation	15:00-18:00 maternal. separation	11:00-14:00 maternal separation	13:00 - 16:00 maternal separation	8:00-11:00 maternal separation	16:00-19:00 maternal separation
Week 2	10:00-13:00 maternal separation alone	9:00-12:00 maternal separation without sawdust	12:00-17:00 maternal separation light off	8:00 - 11:00 maternal separation cage tilt 45°	15:00-18:00 maternal separation 16:00 - 17:00 stroboskopic illumination (60 flashes/min)	11:00-14:00 maternal separation 12:00-13:00 white noise 85 dB	16:00 maternal separation foot shocks (0,7 mA 2sec duration, 3 times with period 40 sec)
Week 3	9:00 - 9:30 restraint stress overnight illumination	10:00 - 10:30 restraint stress 17:00 wet bedding	10:00 normal bedding 12:00-19:00 stroboscopic illumination light off 19:00-19:30 restraint stress	12:00-12:30 restraint stress 16:00 overcrowding	9:00 overcrowding end 16:00-16:30 restraint stress 18:00 cage tilt 45°	10:00 cage tilt end 13:00 - 13:30 restraint stress 17:00 without sawdust	9:00 without sawdust end 12:00-12:30 restraint stress 18:00 - 19:00 without sawdust with water on the cage bottom
Week 4	9:30-10:00 restraint stress 14:00 cage changing 18.00-20.00 white noise overnight illumination	8:00 home cage 12:30-13:00 restraint stress 17:00 overcrowding + without sawdust	9:30 -14:30 stroboscopic illumination light off 18:00- 18.30 restraint stress	11:00 - 11:30 restraint stress 17:00 - 18:00 without sawdust with water on the cage bottom	9:30 - 10:00 restraint stress 11:00 - 18:00 cage tilt 45° 19:00 paired housing	9:00 paired housing end 16:00 -16:30 restraint stress 19:30 wet bedding	16:00 - 16:30 restraint stress 18:30 - 21:30 white noise overnight illumination

separately. The mPCF included sections of prelimbic, infralimbic and cingulate cortices.A ribonucleotide probe for *c-fos* was designed to cover the sequence of the murine *c-fos* gene nucleotides 258-738 of the GenBank accession no NM_010234. ISH with ^{35}S-UTP-labelled riboprobe for *c-fos* was performed as described before (Refojo et al., 2011; Schmidt et al., 2007). cRNA were synthesized from PCR products by in vitro transcription with ^{35}S-UTP (PerkinElmer, Rodgau, Germany) using T7 or SP6 RNA Polymerase (Roche). The probes were treated with DNAase I (Roche) and purified (Rneasy Kit, Qiagen). The brain sections were fixed in 4% paraformaldehyde, washed and acetylated in 0.25% acetic anhydride. The slides were dehydrated in ascending concentrations of ethanol, degreased with chloroform and air dried. After adding 100µl of hybridization buffer per slide, containing 3-7x10^{6}cpm of ^{35}S-UTP-labelled riboprobe, the slides were coverslipped and incubated overnight at 55-57°C. The next day, coverslips were removed, the slides were washed and incubated in RNase A solution. Finally, the sections were desalted and dehydrated.

The sections were exposed to radiation-sensitive films (Kodak Biomax MR films, Eastman KodakCo., Rochester, NY, USA) for 5 days. The films were scanned, *c-fos* signal intensity (optical density) was assessed by Image J software (version 1.44p, National Institutes of Health, Bethesda, USA). For each animal, bilateral structures of one slice were calculated, subtracting the background from the value. The background signal was measured in structures not expressing *c-fos*.

3.7 RNA extraction, cDNA preparation and quantitative real-time PCR

The brain areas of interest were identified using the 2nd edition of the Mouse Brain Atlas (Paxinos, 2001). 200µm brain slices were collected on Superfrost microscope slides (Menzel, Braunschweig, Germany) and CG, BLA and LC were punched out using micropunchers (Fig. 8) (Fine Science Tools, Heidelberg, Germany) RNA isolation was performed using the standard protocol for Trizol-chloroform-based RNA precipitation. 200µl of TRI-Reagent (Sigma-Aldrich, Hamburg, Germany) was added to a PCR tube containing brain punches from one region. Punches were solved in the TRI-reagent by pipeting up and down for 2min. When all punches were completely dissolved, 100µl of TRI-Reagent and 30µl of autoclaved bidistilled water were added.

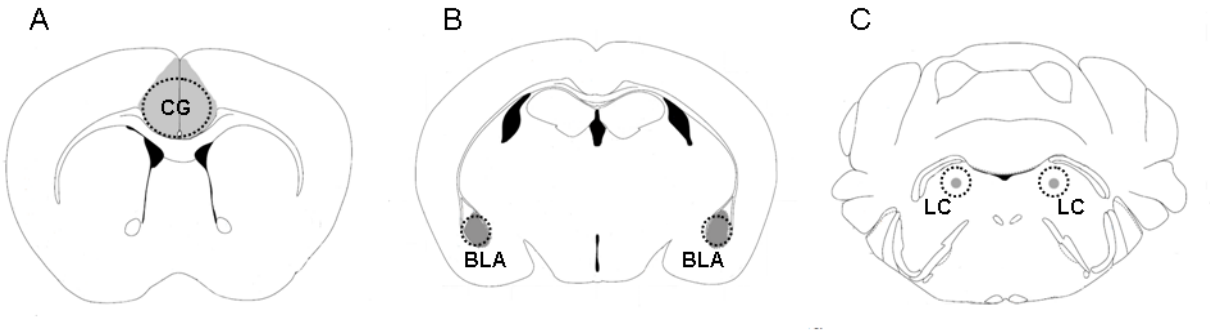

Fig. 8: Dissection of the cingulate cortex (A), basolateral amygdala (B) and locus coeruleus (C) from frozen brain slices by micropunching. Coloured zones highlight the location of the structures, dashed circles illustrate punched out fragments.

To disrupt the secondary structure of RNA, 1µl of linear acrylamide (Ambion, Huntingdon, UK) was used. After addition of 60µl chloroform (Sigma-Aldirch, Hamburg, Germany), samples were vortexed (Vortexer VF2, IKA (R) Labortechnik, Staufen, Germany) for 30s and centrifuged (Centrifuge type Z216MK, Hermle Labortechnik GmbH, Wehingen, Germany) at 13000rpm, 18°C for 5min in order to separate the organic and inorganic phases. The upper aqueous phase, containing the RNA, was transferred to a new 1.5ml Eppendorf tube, while the inter- and the organic phases, containing DNA and proteins, respectively, were discarded. 180µl of isopropanol (Roth, Karlsruhe, Germany) were added to the RNA fraction and briefly mixed. The tubes were stored at -20°C for an O/N precipitation step.

On the next day, samples were centrifuged at 4°C, 13000rpm for 30min. The supernatant was discarded and remaining pellet was washed twice with 500µl ice-cooled 70% ethanol. After each washing, samples were centrifuged for 10min at 13000rpm and the supernatant was discarded both times. The remaining ethanol was removed using a 200µl pipette after a short centrifugation-step at full speed. The RNA was dried at 45°C on a microtube thermo-shaker (PHMT, Grant-bio) until the rest of ethanol got completely evaporated. Precipitated RNA was dissolved in 13µl RNase free water (Qiagen, Hilden, Germany).

RNA concentration, purity and quality were measured on a nanophotometer (Implen, Munich, Germany). Results were considered satisfactory when RNA quantity (A260 >0.1 and <1.0) and quality (A260/A280 >1.7 and <2.0) were in a secure range to ensure a proper measurement and reliable values.

After the adjustment of the concentration to 20ng/µl, 10µl of RNA was used for reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and setup included at least one negative control, containing 10µl of RNase free water instead of a RNA sample. The reaction was performed in a thermal cycler (primus96 advanced, Peqlab, Erlangen, Germany), with the following reaction protocol: initial enzyme activation (10min at 25°C) followed by reverse transcription step (2h at 37°C) and termination (5min at 85°C). Finally, obtained cDNA was diluted 1:5 and was stored at -20°C until further analysis.

cDNA was used to measure gene expression via quantitative real-time PCR (qPCR). A master-mix included 5µl of QuantiFast SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 1µl of autoclaved water and 1µl of each specific forward and reverse primers for the candidate or the housekeeping gene. 8µl of a master mix was pipetted into a LightCycler® Capillary (Roche, Mannheim, Germany). To achieve a total volume of 10µl/reaction, 2µl of cDNA was used. In addition to the samples, which were prepared in duplicates, each run included a qPCR negative control, a RT negative control, as well as a 1:5 and a 1:25 dilution of one of the samples (for standard curve calculations). The qPCR was performed in the LightCycler® 2.0 Real-Time PCR System (Roche), programmed to fit the recommendations of the used QuantiFast SYBR Green PCR Kit (Qiagen): hot start to activate polymerase at 95°C for 5min, amplification with 40 cycles (denaturation at 95°C for 10sec, combined annealing and extension at 60°C for 30sec), melting curve (95°C, 50°C for 10sec and 95°C) and then cooling (42°C for 30 sec). Relative transcript concentrations were calculated using 2(-• • Ct) method (Livak and Schmittgen, 2001).

RNA for analysis of miRNA expression was extracted from the BLA the same way as described above. Reverse transcription was carried out using miScript II RT PCR kit (Qiagen, Hilden, Germany). Master mix was prepared according to manufacturer's instructions and 10µl of extracted RNA and 2µl of RNAase free water were added for a final 20µl reaction mixture. The reverse transcription was performed with the following PCR conditions: reverse transcription (1h at 60°C) and termination (5min at 95°C). The obtained cDNA was diluted 1:20 and was stored at -20°C until further analysis. qPCR was performed using miScript SYBR Green PCR Kit (Qiagen) at following PCR conditions: initial activation step (95°C for 15min) followed by 40 cycles of denaturation (94°C for 15sec), annealing (55°c for 30sec) and extension

(70°C for 30sec). Amount of U6 small non-coding RNA (snRNA) was used as between-samples expression control. Data analysis was performed using 2(-• • Ct) method.

Table 2: List of primers used for mRNA expression of target genes.

Gene	Orientation	Primer sequence (5'• 3')
Crh	forward	GCA GTG CGG GCT CAC CTA CC
Crh	reverse	GGC AGG CAG GAC GAC AGA GC
Crhr1	forward	GCC CCA TGA TCC TGG TCC TGC
Crhr1	reverse	CCA TCG CCG CCA CCT CTT CC
Tmem132d	forward	CAT CCC TTC TTC AGC CAG AG
Tmem132d	reverse	AGT GAG AAC CGC TGA ATG CT
TNF• r1	forward	TGC CAG CCC CCA CCT CTG TT
TNF• r1	reverse	TCG TGC TCG CTC AGC CCC AT
IL-10r1	forward	CCC AGG CCC ACG ATA ACC CC
IL-10r1	reverse	CGG GAT TCC AAG CGG GGC AG
Glo1	forward	CTC TGC CCC AGA GAA CAG TC
Glo1	reverse	TGA TAG AGG CCA CAC AGC AG
Dbh	forward	AGA GAG CCC CTT CCC CTA CCA CAT C
Dbh	reverse	TTT CCG GTC ACT CCA GGC ATC
Npsr1	forward	CTC TTC ACT GAG GTG GGC TC
Npsr1	reverse	CCA GTC CTT CAG TGA ACG TC
YY1	forward	ACC TGG CAT TGA CCT CTC
YY1	reverse	TTA TCC CTG AAC ATC TTT GT
Dicer	forward	CAC GCC TCC TAC CAC TAC AAC A
Dicer	reverse	CCT GGA GAA TGC TGC CGT GGG T
Dnmt1	forward	CCT AGT TCC GTG GCT ACG AGG AGA A
Dnmt1	reverse	TCT CTC TCC TCT GCA GCC GAC TCA
Dnmt3a	forward	CCG CCT CTT CTT TGA GTT C
Dnmt3a	reverse	AGG AAG GTT ACC CCA GAA GTA
Dnmt3b	forward	TTC AGT GAC CAG TCC TCA GAC ACGAA
Dnmt3b	reverse	TCA GAA GGC TGG AGA CCT CCC TCT T
MeCP2	forward	TCT GCT GGA AAG TAT GAT
MeCP2	reverse	AAT CAA TTC TAC TTT AGA GC

Table 3: List of primers used for mRNA expression of housekeeping genes.

Gene	Orientation	Primer sequence (5'• 3')
Rpfl13a	forward	CAC TCT GGA GGA GAA ACG GAA GG
Rpfl13a	reverse	GCA GGC ATG AGG CAA ACA GTC
B2mg	forward	CTA TAT CCT GGC TCA CAC TG
B2mg	reverse	CAT CAT GAT GCT TGA TCA CA
Polr2b	forward	CAA GAC AAG GAT CAT ATC TGA TGG
Polr2b	reverse	AGA GTT TAG ACG ACG CAG GTG

Table 4: List of primers used for miRNA expression studies.

Gene	Application	Primer sequence (5'• 3')
miRNA-34a	target	TGG CAG TGT CTT AGC TGG TTG T
miRNA-34b	target	AGG CAG TGT AAT TAG CTGA TTG T
miRNA-34c	target	AGG CAG TGT AGT TAG CTG ATT GC
U6 snRNA	control	GAT GAC ACG CAA ATT CGT GAA

3.8 DNA extraction, bisulfite conversion and pyrosequencing

DNA isolation from tissue punches or cell culture was performed using the NucleoSpin® Tissue DNA-isolation Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA quantity (A260) and purity (A260/A280) were measured on a nanophotometer (Implen, Munich, Germany) and final concentration was adjusted to 100ng/µl.

DNA samples were bisulfite converted before sequencing using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Treatment of DNA with bisulphite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. The following PCR reaction further converts uracil into adenine, whereas 5-methylcytosine is recognized as usual cytosine. Thus, after bisulphite conversion, the final mixture consists of DNA fragments differ in amount of adenine and cytosine bound with guanin. The quantity of each fragment reflects degree of methylation and could be evaluated by pyrosequencing.

Pyrosequencing (Fig. 9) was performed by Varionostic GmbH (Ulm, Germany) using the Q24 system (Qiagen, Hilden, Germany). The pyrosequencing method is based

on detecting the activity of DNA polymerase with another chemiluminescent enzyme. A reaction for pyrosequencing includes a balanced mixture of four enzymes: the Klenow fragment of the DNA polymerase I, an ATP sulfurylase, the luciferase and an apyrase. The template DNA is immobilized in the pyrosequencing mixture, and unmodified nucleotides (A,C,G or T) are sequentially added to the reaction. When a complementary nucleotide is incorporated into DNA strand, it is quantitatively converted into a bioluminometric signal via releasing of pyrophosphate (PPi). The PP_i is converted into ATP by the ATP sulfurylase using adenosine 5• phosphosulfate as a substrate. The energy, released during reaction, is used by luciferase to oxidize D-luciferin. The product oxyluciferin is produced in an excited state, detected and measured by a charge-coupled device camera. Apyrase degrades unincorporated nucleotides before adding the next nucleotide. This method allows sequencing of a single-stranded DNA and evaluation of polymorphisms in DNA sequence.

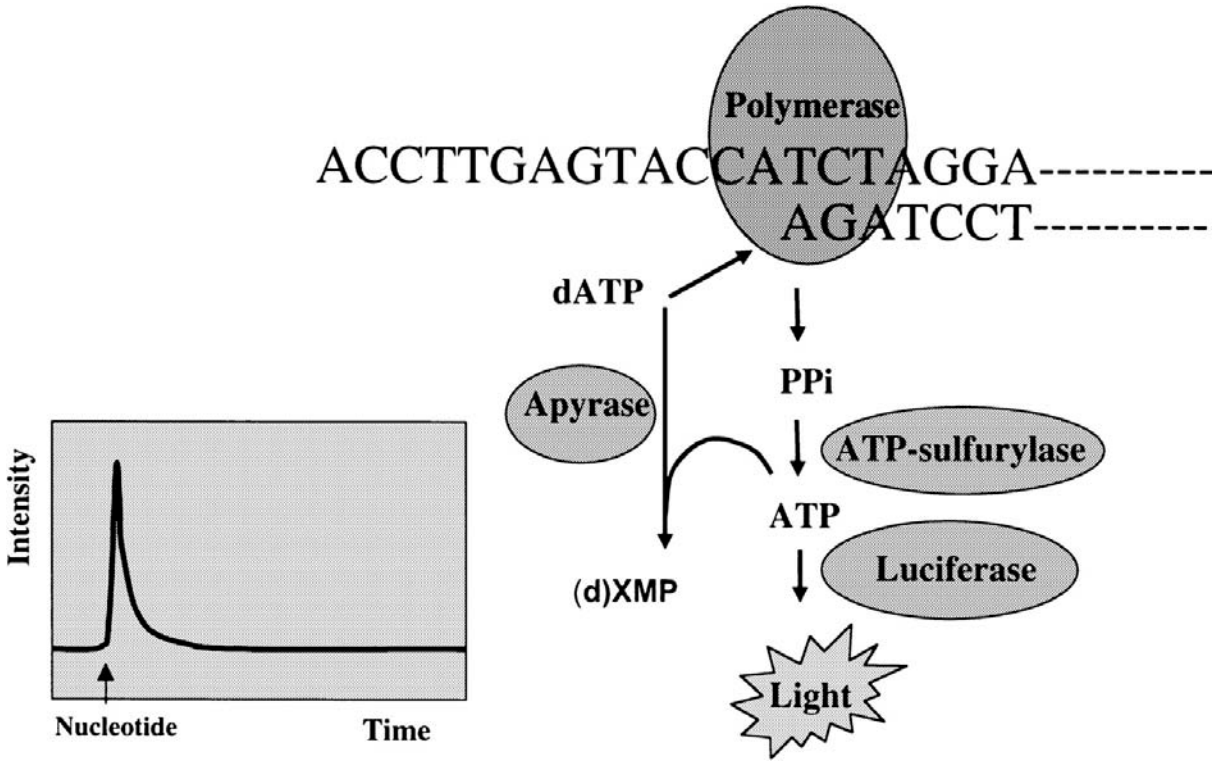

Fig. 9: DNA methylation analysis by pyrosequencing. Adopted from Ronaqhi (2001).

3.9 Construction of promoter-luciferase reporters

To study *Crhr1* promoter properties, several deletion constructs were cloned into the CpG-free luciferase vector (kindly provided by M. Rehli, University Hospital Regensburg, Germany). To this end, genomic DNA of LAB mice was extracted as described above and respective PCR products were generated using Phusion DNA polymerase (New England Biolabs, Frankfurt, Germany). The master-mix consisted of: 10µl of 5X Phusion HF buffer, 1µl of 10mM dNTP mix, 1µl of 5% DMSO, 1µl each respective 2µM forward and reverse primers, 1µl DNA and 0.5µl of Phusion DNA polymerase (2U/µl) and nuclease-free water up to 50µl. PCR reactions were performed at following conditions: polymerase activation (95°C for 5min) followed by 35 cycles of denaturation (95°C for 30sec), annealing (56°c for 60sec) and extension (72°C for 60sec) and final extension period (72°C for 10min). Products were analyzed using gel-electrophoresis and respective bands were cut out and gel-extracted using QIAquick Gel Extraction Kit (Qiagen).

Table 5: List of primers used for construction of *Crhr1* promoter-luciferase reporters.

Primer sequence (5'• 3')	**Orientation**	**Product**
ATC ACT AGT GAG CAG AGG CGA GAG GCA G	forward	290 bp
ATC ACT AGT CCG AGC CCC ACA AGT ACC C	forward	650 bp
ATC ACT AGT GTT CCC GCC GCA GAG CA	forward	880 bp
ATC ACT AGT TGA AGG TGG CGA GAG CTG G	forward	1230 bp
ATC ACT AGT GTA GTG TCC AGA GTT GCC AAG CT	forward	1460 bp
CAG GAC TTT GCT TCA CTG AAC TGT	forward	1790 bp
CAG AAG CTT CCT CGG GCT CGC TCT GTC	reverse	universal

The purified products and the CpG-free luciferase vector were digested with *SpeI* and *HindIII* restriction enzymes (New England Biolabs GmbH, Frankfurt, Germany) and ligated by *T4 DNA ligase* according to manufacturer's instruction. The final products were transformed into chemically competent DH5• bacteria using heat-shock. For this, the ligation mixture was incubated for 10min along with bacteria on ice and then placed on a heating block at 42°C for 45sec and later cooled down. After addition of 500µl of LB medium (20g/l) (Serva electrophoresis GmbH,

Heidelberg, Germany), cells were incubated for 60min at 37% and plated on LB agar (1%) plates.

24 hours afterwards, separate colonies were picked, plasmids were isolated and sequenced. For this, separate colonies were transferred into 10 ml of LB medium and incubated overnight. Plasmid isolation was performed using alkaline lysis with SDS according to the following protocol:

- Bacteria pellet precipitation by centrifugation at 4000rpm with 4°C for 10min.
- Resuspension of the pellet in 200µl of ice-cold Alkaline lysis solution I (50mM glucose, 25mM Tris-Cl (pH 8.0), 10mM EDTA (pH 8.0).
- Addition of 400µl of Alkaline lysis solution II (0.2N NaOH, 1% (w/v) SDS) and mixing.
- Addition of 300µl ice-cold Alkaline lysis solution III (5M potassium acetate, glacial acetic acid and distilled water up to 100 ml) and mixing.
- Separation from cellular content via centrifugation for 5min at 13000rpm.
- Genomic material extraction by mixing 600µl of supernatant with 600µl chloroform:phenol and vigorous vortexing.
- Separation of organic and inorganic phases by centrifugation for 2min at 13000rpm.
- Nucleic acid precipitation with 600µl of isopropanol for 2min at room temperature.
- Nucleic acid precipitation by centrifugation for 5min at 13000rpm.
- Washing of plasmid's pellet with 70% ethanol and consequent centrifugation for 2min at 13000rpm.
- Removal of ethanol beads by heating at 60°C for 5min.

Dried pellets were dissolved in water to a final concentration of 100ng/µl. BigDye Terminator kit v3.1 (Applied Biosystems, California, USA) was used for the sequencing reaction: 2µl sequencing buffer (5X), 0.4µl BigDye reagent, 1µl corresponding forward or reverse primer (2µM) and 2.4µl of plasmid's solution were mixed. Following PCR was carried out: initial denaturation (96°C for 1min) followed by 35 cycles of denaturation (96°C for 10sec), annealing (50°C for 5sec) and

extension (60°C for 4min). The reaction mixture was loaded onto Montage Seq 96 plate (Millipore GmbH, Schwalbach, Germany) and washed twice with 20µl Montage injection solution (Millipore GmbH, Schwalbach, Germany). PCR products were dissolved in 20µl of injection solution and analyzed using capillary electrophoresis on a ABI 3730 DNA analyzer (Life technologies, Darmstadt, Germany) at the Helmholtz Zentrum Institute of Human Genetics (Neuherberg, Germany).

Table 6: List of primers used to verify sequences of *Crhr1* luciferase constructs.

Primer sequence (5'• 3')	**Orientation**	**Product**
GCC CAC TCT ATC TTG ATG AT	forward	485 bp
CCT CCT TCC TAA TTC CCA AC	reverse	
CTT CAG GAC TTT GCT TCA CTG	forward	596 bp
TTC TAA TTC CAC TTC CAG CC	reverse	
CCT GAG AGG TGA AGA TGT TTC	forward	558 bp
CAA TTT AGT GGG GAG GGG AG	reverse	
CCG CTG TCA CCA CTT ATC TT	forward	691 bp
TCG TGT CCC CTC CTC TTT CT	reverse	
TTT TCC CTA GCT GCG GTG GC	forward	572 bp
GTC CTC TCT TAC CTT CAC GA	reverse	

However, since the alkaline lysis with SDS does not remove completely LPS and other endotoxins, for cells culture experiments plasmids were isolated using Plasmid Maxi Prep Kit (Qiagen) according to the manufacturer's protocol. Plasmid sequences were verified by capillary electrophoresis of PCR-amplified products on a ABI 3730 DNA analyzer (Life Technologies, Darmstadt, Germany) at the Max Planck institute of Biochemistry (Martinsried, Munich, Germany).

A plasmid carrying *YY1* cDNA was purchased from the DNA Resource CORE (Clone ID: MmCD00311470). The sequence was analyzed using Platinum Service (DNA Resource CORE) and by capillary electrophoresis of PCR amplified products of the plasmid using ABI 3730 DNA analyzer. The empty plasmid (SPORT6) was obtained by removing the *YY1* cDNA sequence using the restriction enzyme *MluI* (New England Biolabs GmbH, Frankfurt, Germany), subsequent gel purification (QIAquick Gel Extraction Kit, Qiagen) and self-ligation of the empty product.

3.10 *In vitro* methylation of DNA and bisulphite sequencing

Site-specific methylation (SSM) of the *Crhr1* promoter was performed according to Martinowich *et al*. (2003) (Fig. 10). Modified oligonucleotides, carrying either a methylated or unmethylated CpG dinucleotide at the site of interest (CpG1) were synthesized by Sigma Aldrich: 5'-CTC AAG ATG GAG ACC C*GG ACC TGA GAG GTG AA-3' and 3'-TTC ACC TCT CAG GTC C*GG GTC TCC ATC TTG AG-5' (star indicates methylated cytosine). For PCR reaction, each primer was diluted to the

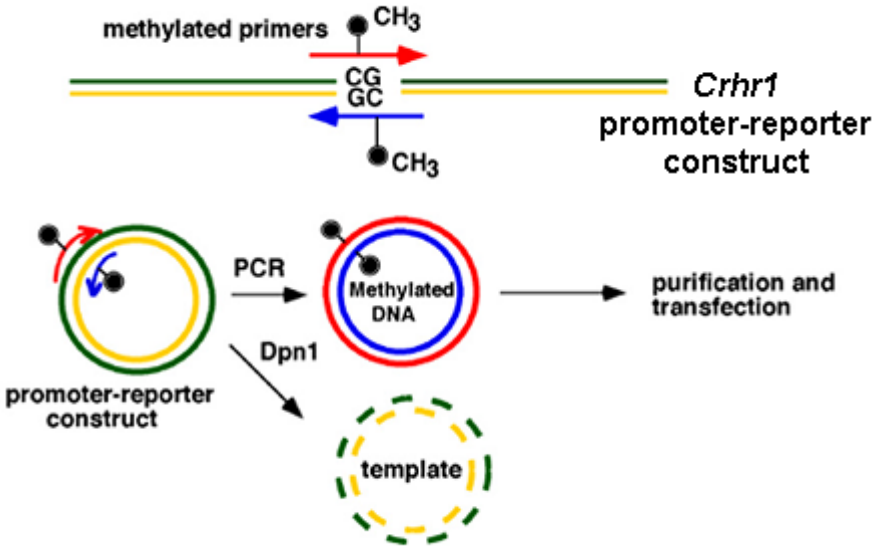

Fig. 10: Schematic overview of the generation of *Crhr1* promoter luciferase reporter construct containing site-specific methylation at CpG1 (adopted from Martinowich et al. (2003)).

concentration of 125ng/µl. The PCR master-mix consisted of: 10µl of 5X Phusion HF buffer, 0.5µl of 25mM dNTP mix, 1µl of each forward and reverse primers, 300ng of plasmid DNA, 1µl of Phusion DNA polymerase (2U/µl) and nuclease-free water up to 50µl. 5µl of PCR product was loaded on agarose gel and respective product size was verified via ethidium bromide staining. The remainig 45µl were incubated with *DpnI* (New England Biolabs GmbH, Frankfurt, Germany) at 37°C overnight. Next day, 1/10th volume of 3M sodium acetate (Sigma Aldrich), 1µl glycogen (10mg/ml) (Carl Roth Gmbh) and 2.5 volumes of 95% ethanol were added to the digested PCR products. The mixture was centrifuged at 13000rpm for 30min at 4°C and the supernatant was decanted afterwards. The pellet was washed additionally twice with 70% ice-cooled ethanol followed by 5min centrifugation at 13000rpm at 4°C. Finally, the pellet was dried at 60° for 5min and resuspended in the ultra pure water.

To assess the impact of a completely methylated *Crhr1* promoter, the construct was incubated overnight with *SssI methylase* (New England Biolabs GmbH, Frankfurt, Germany) according to the following protocol: 5µl of 10X NE buffer, 1µl of 640µM S-adenosylmethionine, 4µl of *SssI methylase*, plasmid DNA 4µg and water up to 50µl. A mock-methylated plasmid was treated in the absence of S-adenosylmethionine. Products were purified using sodium acetate precipitation as decribed above.

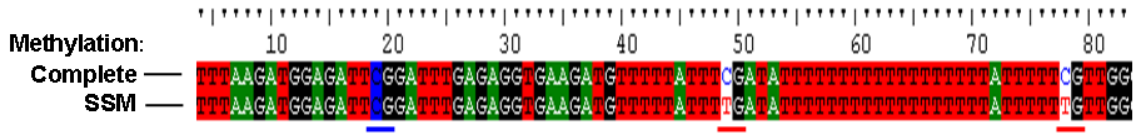

Fig. 11: Schematic illustration of difference between SSM and complete methylation of the *Crhr1* promoter luciferase reporter construct using *SssI* enzyme after bisulphite sequencing.

To check the efficiency of SSM and complete methylation purified products were sequenced. For this, they were treated with sodium bisulphite (EpiTech Bisulfite Kit, Qiagen) and incubated using the following conditions: 5min at 95°C, 25min at 60°C, 5min at 95°C, 85min at 60°C, 5min at 95°C and 175min at 60°C. Then, samples were purified according to the manufacturer's protocol (EpiTech Bisulfite Kit, Qiagen) and sequenced at the Max Planck Institute of Biochemistry (Martinsried, Munich, Germany) using following forward 5'-TGT GGA TTT TGT TTA GTG TGT T-3' and reverse 5'-TAA CTT TCT AAT TCC ACT TCC AA-3' primers (Fig. 11).

3.11 Cell culture, transfection and reporter gene assay

Mouse-neuro-2a cells were cultured in DMEM containing 10% FBS, 1% sodium pyruvate and 1% antibiotic-antimycotic (Life Technologies GmbH, Darmstadt, Germany) under standard conditions (5% CO_2, 37°C). Cells were plated in 96 well plates with a density of 10000 cells per well. The day after seeding, when the cells reached a density of 50 to 70%, they were transfected either with 300ng of a methylated or unmethylated *Crhr1* promoter-reporter construct and with 10ng of pCMV-Gaussia vector as internal control by using Turbofect Transfection reagent (Thermoscientific, Braunschweig, Germany). SV40- pGL3 vector was used as a positive control. The plate was centrifuged for 5min at 280g at room temperature to improve transfection efficiency. The valproic acid (VPA) stimulation was performed 28 hours after transfection by complete exchange of growing medium with a fresh one containing 10nM, 50nM, 100nM, 500nM, 1000nM or 2000nM of VPA (Sigma

Aldrich) or water as a control. To check if YY1 exhibits a regulatory role on *Crhr1* promoter activity, an empty vector (SPORT6) or a vector expressing *YY1* cDNA were co-transfected with plasmids carrying the *Crhr1* promoter-reporter construct and pCMV-Gaussia in a ratio of 14:26:1. The same approach was used to overexpress YY1, when methylated *Crhr1* promoter constructs were transfected. Cells were lysed 40h after transfection to measure firefly and gaussia luciferase activity as described by Schulke et al. (2010). Briefly, supernatant was removed by aspiration and 50µl of passive lysis buffer (100mM KPO4 buffer pH 7.8, 0.2% Triton X-100) was added in each well. Cells were incubated at 37°C for 30min at 800rpm. 20µl of lysate was used for detection of luciferase signal from Firefly and Gaussia using TriStar LB 941 multimode microplate reader (Berthold technologies, Bad Wildbad, Germany). For this, a luminometer injected 50µl of firefly substrate solution (2.5mM $MgCl_2$, 2mM ATP (Sigma Aldrich), 100µl D-Luciferin (P.J.K. Gmbh, Kleinblittersdorf, Germany)) and then 50µl of Gaussia substrate solution (2.2M NaCl, 4.4mM Na_2EDTA, 0.22M KPO_4 buffer pH 5.1, 0.88mg/ml BSA and 6µg/ml Coelenterazin (P.J.K. Gmbh, Kleinblittersdorf, Germany)). Each signal was measured over the period of 10s. Three biological replicates per sample were used. Normalized luciferase activity was obtained by dividing the respective firefly by Renilla activity. The final normalized value represents an average of at least three individual measurements.

3.12 Western blotting

The total YY1 protein was analyzed in pooled amygdala punches from HAB, LAB and stressed LAB mice. For this, whole amygdala was punched from 3 mice and collected in one Eppendorf tube. Total protein fraction was extracted by homogenization of punches in 150µl of homogenization buffer containing freshly prepared protease inhibitor cocktail (Complete ULTRA tablets, Roche Diagnostics Gmbh, Mannheim, Germany). When punches were completely dissolved, 50µl extraction buffer was added. Samples were incubated 15min on ice and then centrifuged 60min at 13000rpm at 4°C. The supernatant containing the protein fraction was stored at -20°C until further analysis.

Protein extraction by fractions was performed to verify that the transfection of a plasmid carrying *YY1* cDNA, indeed, induced a higher amount of YY1 protein in the cytosolic and nuclear fractions. For this, cells were washed several times with PBS and then harvested in 1.5ml Eppendorf tubes. They were lysed in hypotonic buffer

containing a protease inhibitor cocktail (Complete ULTRA tablets, Roche Diagnostics Gmbh, Mannheim, Germany). Cytosolic and nuclear fractions were separated by centrifugation at 6500rpm for 30s. The supernatant containing cytosolic proteins was transferred into a separate tube and stored at -20°C. The nuclear pellet was dissolved in NETN containing a protease inhibitor cocktail (Complete mini, Roche). After centrifugation at 13000rpm for 10min, supernatants with nuclear proteins were saved and stored at -20°C.

Analysis of protein concentration was performed using bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific). For higher reliability, samples were analyzed in triplicates. 200µl of reagent A and 4µl of reagent B were mixed to get a working solution. 10µl of sample and 200µl of working solution were pipetted together and incubated for 30min at 37°C. Samples, as well BSA standards, were measured at 562nm in an enzyme-linked immunosorbent assay plate reader (Dynatech MR7000).

Table 7: Protein extraction buffers.

Buffer	Substance	Concentration
Homogenization buffer	Tris-HCl, pH 7.5	50mM
	NaCl	150mM
	EDTA	3.33mM
Extraction buffer	Tris-HCl, pH 7.5	50mM
	NaCl	150mM
	NP40	2%
	Deoxycolate	2%
Hypotonic buffer	Tris-HCl, pH 7.9	10mM
	KCl	10mM
	EDTA	0.5mM
	NP40	0.1%
	glycerol	10%
NETN buffer	NaCl	100mM
	EDTA	1mM
	NP40	0.5%

After dilution to the same protein concentration, samples were boiled in loading buffer (160mM Tris-HCl (pH 6.8), 5% SDS, 5% • -mercaptoethanol, 40% glycerol, 0.0025% bromophenol blue) for 7min at 95°C. 35µg of proteins was loaded in 10% SDS-polyacrylamide gel and run for 90min at 4°C in electrophoresis buffer (25mM Tris, 192mM glycine). The protein ladder, Page Ruler (Fermentas, Leon-Rot, Germany), was used to control separation efficiency (Fig. 12).

Afterwards, when a good separation was achieved, proteins were transferred onto a nitrocellulose membrane (Protran, Whatman, Dassel, Germany) via side-by-side electrophoresis at 400mA for 60min at 4°C. Next, the membrane was incubated in blocking solution (5% milk in Tris-buffered saline) and cut into pieces. A piece containing proteins of 55-75kDa weight was incubated with anti-YY antibody (sc-1703, Santa Cruz Biotechnology) (diluted 1:500), a piece with 35-55kDa proteins was incubated with anti-• -actin antibody (sc-47778, Santa Cruz Biotechnology) (diluted 1:1000), whereas a piece containing 5-35kDa proteins was incubated with an anti-acetyl-H4 antibody (Millipore) (diluted 1:2000).

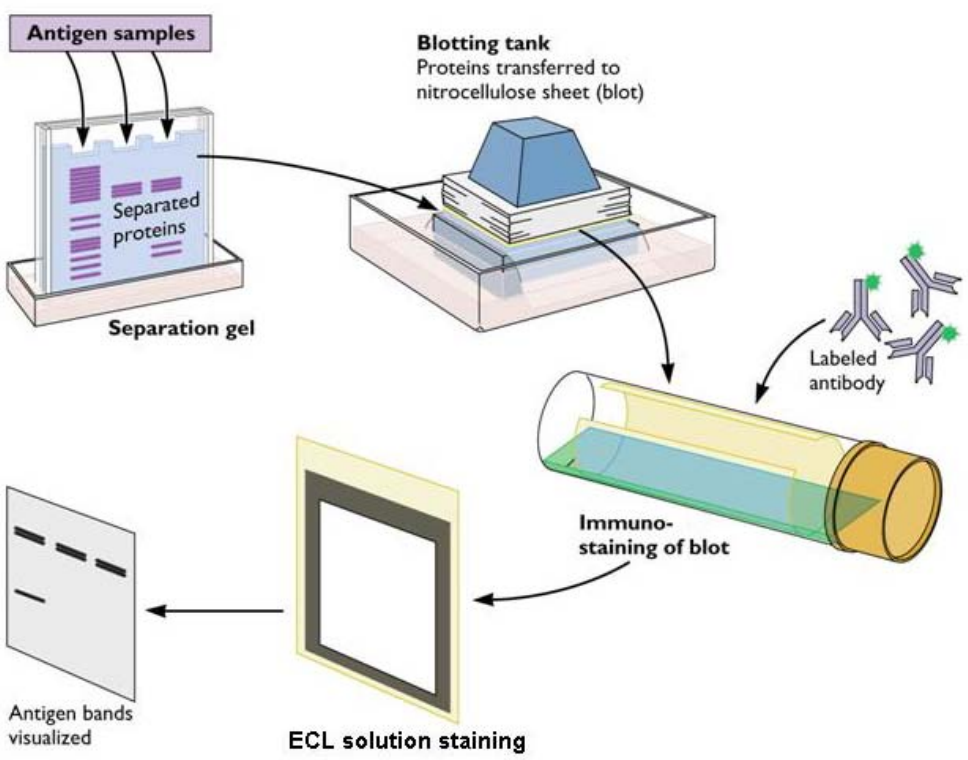

Fig. 12: Schematic illustration of the main steps during Western blot analysis (adopted from http://www.virology.ws/2010/07/07/virology-toolbox-the-western-blot).

After overnight incubation, membranes with proteins were washed several times in washing solution (Tris-buffered saline with 0.1% Tween) and then incubated for two hours with a secondary HRP-conjugated anti-rabbit antibody (New England Biolabs, Frankfurt, Germany). Afterwards, membranes were washed again several times in washing solution to remove unbound antibodies. To visualize bands, membranes were incubated for 2min in chemiluminescence (ECL) solution. ECL was prepared just before analysis by mixing 10ml of solution A (0.1M Tris, 50mg luminol per liter), 1ml of solution B (DMSO, 0.11% para-hydroxycoumarin acid) and 3µl of hydrogen peroxide. Blots were evaluated with the ChemiDoc Imaging System (Bio-Rad).

3.13 Immunofluorescent assays

To visualize an increase of YY1 protein in the transfected neuro-2a cells, *YY1* cDNA was co-transfected with 100ng of GFP-expressing plasmid. Cells were mounted on cover slips (Heinz Herenz, Hamburg, Germany) and 40 hours after transfection were briefly washed in PBS and then incubated in the fixative solution (4% paraformaldehyde in PBS) for 10min at room temperature. Fixative solution was step-wise diluted with PBST to prevent drying-out of cellular membrane. After this, cells were incubated 3min in the permeabilization buffer (0.2% TritonX-100 in PBS), then briefly washed and blocked for 10min in 2% BSA. Incubation with primarily anti-YY1 antibody (sc-1703, Santa Cruz Biotechnology) (diluted 1:100) was performed overnight. On the next day, cover slips were washed 3 times in PBST for 10min and incubated 2h with an anti-rabbit secondary antibody (ALEXA Fluor® 594) (diluted 1:300). Finally, samples were stained 10min in DAPI solution (sc-3598, Santa Cruz Biotechnology) (diluted 1:10000). Cover slides were fixed on Superfrost microscope slides (Menzel, Braunschweig, Germany) in the immuno-mount medium (Shandon, Cheshire, UK) and analyzed by fluorescent microscopy. Images were acquired simultaneously with two acquisition channels using Axio Vision 4.5.

3.14 Electrophoretic mobility shift assay (EMSA)

EMSA probes were prepared by annealing complementary oligonucleotides listed in table 7. The oligos were diluted to 20µg/ml in annealing buffer (10mM $MgCl_2$, 50mM NaCl, 20mM Tris) and 10µl of each, forward and reverse, were mixed together. The mixture was heated to 85°C for 10min and then slowly cooled down to 30°C. Annealed oligos were phosphorylated by incubation at 37°C for 30min with *T4*

polynucleotide kinase (New England Biolabs, Frankfurt, Germany) according to the manufacturer's protocol.

Table 8: List of primers used to analyze YY1 binding to the DNA in EMSA.

Primer sequence (5'• 3')	**Orientation**	**Binding position (from TSS)**
CTA GTT GAA CCT CCT GGA TGG CTG CAG GGC GGG AA CCA	forward	755-760 bp
AGC TTG GTT CCC GCC CTG CAG CCA TCC AGG AGG TTC AA	reverse	
CTA GTC TGT GTA CTC AAG ATG GAG ACC CGG ACC TGA GA	forward	1353-1358 bp
AGC TTC TCA GGT CCG GGT CTC CAT CTT GAG TAC ACA GA	reverse	
CTA GTC AGA GTT GCC AAG CTC TGC TAC ATT TTA AAA TAA	forward	1529-1534 bp
AGC TTT ATT TTA AAA TGT AGC AGA GCT TGG CAA CTC TGA	reverse	
CTA GTC CCA CCC ACC TGG GCG CCA TCT TTA ATG AAA GA	forward	Positive control (Kim et al., 2009)
AGC TTC TTT CAT TAA AGA TGG CGC CCA GGT GGG TGG GA	reverse	

Annealed oligonucleotides and phosphorylated oligonucleotides were cloned in a pCpG-free vector between a *SpeI* and *HindIII* sites as described earlier and subsequently amplified in DH5• E. Coli bacteria. The plasmid was digested with *SpeI* and *HindIII*, the cloned fragment was purified by polyacrylamide gel electrophoresis. For this, the ligation mixture was loaded to a 10% polyacrylamide gel and run at 80V for 2h. After separation, ligated products were visualized by ethidium bromide staining and cut out under UV-light. Gels were smashed in small pieces and incubated in elution buffer (0.5M ammonium acetate, 1mM EDTA pH 8.0) for 14 hours at 37°C. Then, products were purified using sodium acetated precipitation, as described earlier.

Purified double-stranded probes for EMSA were end-labelled with ^{32}P-dCTP (PerkinElmer) using a Klenow fragment (New England Biolabs, Frankfurt, Germany).

For this, 4µl of oligos were incubated with 2µl of 10X Klenow buffer, 1µl Klenow polymerase, 4µl of 5mM dATP and 5µl of 10µCi/µl ^{32}P-dCTP. After 30min, 4µl of 5mM dCTP was added and the mixture was incubated additionally for 5min.

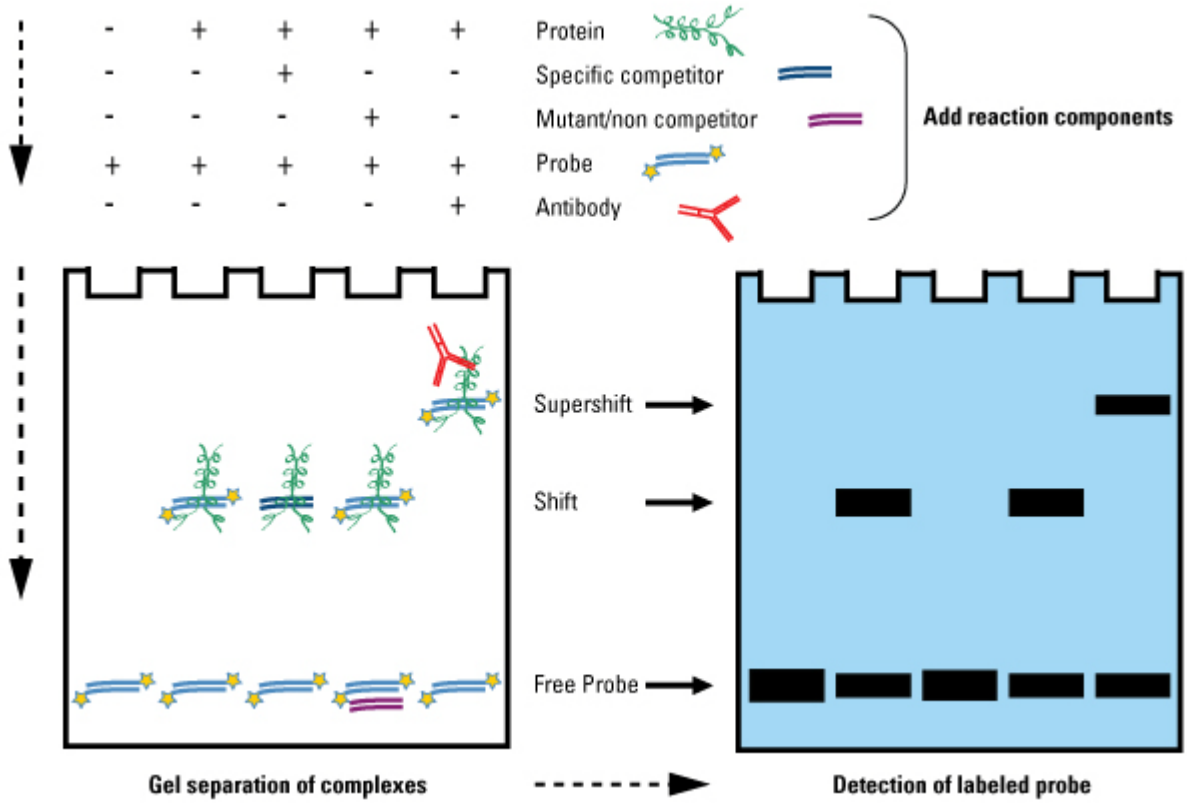

Fig. 13: Working principle of EMSA (adopted from Thermo Scientific http://www.piercenet.com/method/gel-shift-assays-emsa).

Excess radioactivity was removed by purification using bio-spin columns (Bio-Rad Laboratories). Radioactivity of the labelled fragments was measured via liquid scintillation counting. For this, 1µl of labelled oligo was added to 2ml of a liquid scintillation mixture (PPO 100g/l and POPOP 1.25g/l in toluene) and scintillation was analyzed by a liquid scintillation counter. All prepared fragments were diluted to 20000cpm with water.

For the EMSA reaction, nuclear extracts from N2a cells were incubated for 5min in 5X binding buffer (5mM MgCl2, 2.5mM EDTA, 250mM NaCl, 50mM Tris-HCl (pH 7.5), 20% glycerol, 1mM DTT, 2µg poly(dI:dC)/poly(dI:dC) in the final volume of 19µl. 1µl of labelled oligos was added to the EMSA mixture and incubated for an additional 25min at room temperature. The samples were electrophoresed on a 6% non-denaturing polyacrylamide gel in 0.5 TBE buffer (89mM Tris pH 7.6, 89mM boric acid, 2mM EDTA) for 90min at 4°C. Antibodies used in the shift assay (YY1 (sc-

1703X, Santa Cruz Biotechnology), GR (sc-1004X, Santa Cruz Biotechnology) and competitors (excess of unlabelled oligonucleotides) were added to binding buffer containing the nuclear extract 30min before ^{32}P–labelled oligonucleotides. To evaluate the impact of CpG1 methylation on YY1 binding affinity, a plasmid with an incorporated promoter construct for EMSA was incubated with *SssI* methylase (New England Biolabs, Frankfurt, Germany) overnight, whereas a mock-methylated plasmid was treated in the absence of S-adenosylmethionine. To circumvent the presence of non-methylated CpG1, the methylated probe was digested with the methylation-sensitive *HpaII* restriction enzyme (New England Biolabs, Frankfurt, Germany). Gels were finally transferred to the gel drying films (Gel Drying Kit, Promega) that were soaked in drying mixture (40% methanol, 10% glycerol and 7.5% acetic acid) for 5min, and left overnight until complete drying up. Dried gels were exposed to radiation-sensitive films (Kodak Biomax MR films, Eastman KodakCo., Rochester, NY, USA) for 2 days. Films were scanned and optical density was assessed using Image J software (version 1.44p, National Institute of Health, USA).

3.15 Chronic valproic acid (VPA) treatment

To check if VPA induces any changes in behavior of LAB mice, animals were treated during 4 weeks with VPA through drinking water. VPA (Sigma Aldrich, Hamburg, Germany) was dissolved in a 1% ethanol solution to a final concentration 500µg/ml. Starting from PND28, animals were single housed and arranged in vehicle and VPA treated groups. Bottles were weighed and liquids were exchanged with a fresh solution every second day. Liquid consumption per gram of body was assessed via comparison of difference between initial weight and the weight two days later, divided by body weight. Body weight was analyzed every fourth day. From PND58, mice were consequently tested in the EPM, LDB, TST and FST. Two days after the last behavioral test, animals were killed and brains were harvested.

3.16 Statistical analysis

Statistical analysis was performed using PASW Statistics 18. Comparison of behavioral results with normal distribution, evaluated by Shapiro-Wilcoxon test, was performed using one, two or three way analysis of variance (ANOVA). For multiple comparisons, Tukey *post-hoc* was applied. Non-normally distributed data were processed by Mann-Whitney-U test with Kruskal-Wallis ANOVA for multiple comparisons.

To identify avoidance behavior in the TMT-avoidance experiments with CD1 mice (section 3.2.1.1.), the mean time an individual mouse spent in the chamber with odor was compared with the average time spent in the respective compartment during the habituation period, i.e. the values of days 10–12 were compared with those of days 1–9 in the same chamber, and the values from the opposite chamber during days 1–9 were used as a basal reference to test for significance during days 13–14. ANOVA with the within-subject factors (time spent in the odor chamber × day) and between-subject factors (groups: TMT, cat odor, butyric acid) was followed by Tukey *post-hoc* test. To present behavioral response to odor exposure of HAB/NAB/LAB mice (section 3.2.1.2.), –• T index was introduced. -• T reflects changes in the time spent in the chamber during odor exposure in comparison to the time spent in the chamber during habituation with zero indicating an absence of any effect and positive values reflecting the extent of odor avoidance.

Expression data of *c-fos* in brain structures and qPCRs were analyzed using the Mann-Whitney-U test. All reporter gene assays were performed in triplicates (technical replicates) and were replicated independently at least three times (biological replicates). Results are presented as means + S.E.M. Differences were considered statistically significant when $p<0.05$. Comparative illustration data were created using GraphPad Prism 6.

4 RESULTS

4.1 Evaluation of anxiety-related behavior using the TMT-avoidance test

4.1.1 Effects of TMT, cat fur and butyric acid on avoidance behavior of CD1 mice

TMT induced strongest avoidance behavior and highest HPA axis activation during the first presentation and lowest habituation during repeated exposures in the three-chambered test.

We took advantage of the three-chambered test to evaluate avoidance behavior of mice by using predator odor exposure. During the habituation period, we observed no significant difference between treated groups in time spent in the odor chamber (cat odor $F(8,63) = 1.61$, $p = 0.14$, butyric acid $F(8,63) = 1.22$, $p = 0.30$, TMT $F(8,63) = 0.93$, $p = 0.50$) (Fig. 14).

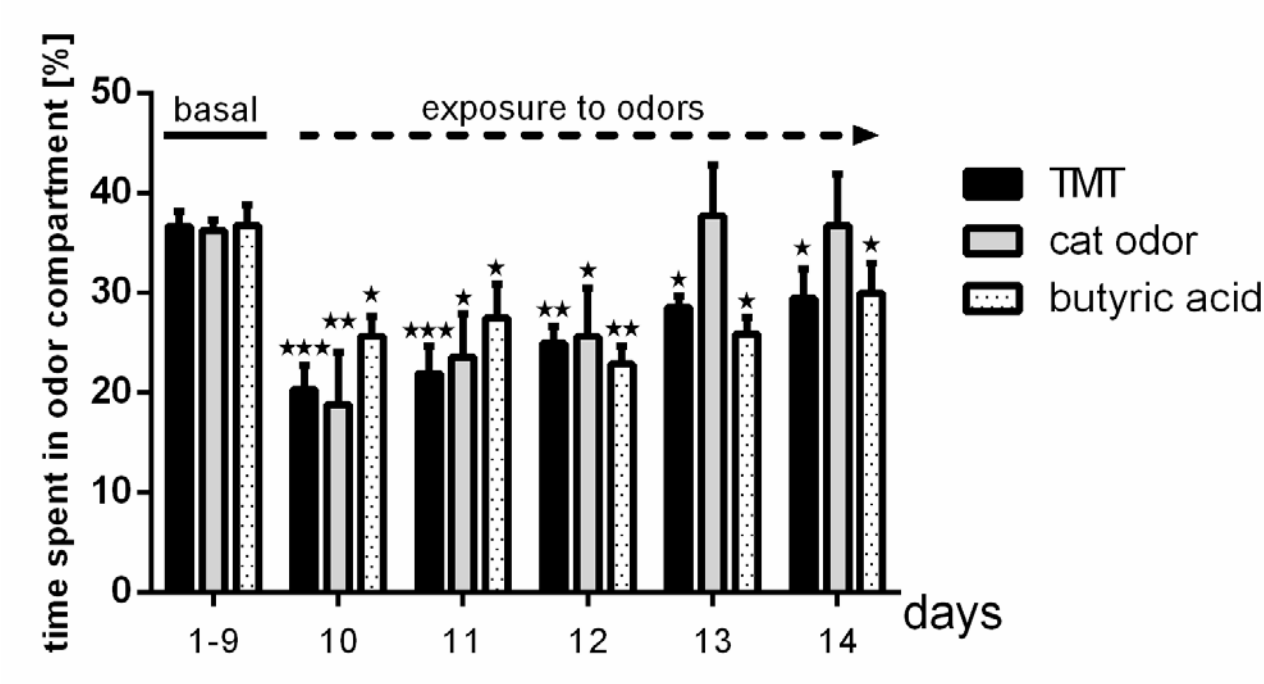

Fig. 14: Behavioral response of CD-1 mice to different odors. Mice avoided TMT, cat odor and butyric acid when exposed for the first time. However, different habituation was observed during repeated exposures the next 4 days. Bars represent means + SEM, n (TMT) = 8, n (cat odor) = 8, n (butyric acid) = 8, ★ $p<0.05$, ★★ $p<0.01$, ★★★ $p<0.001$ compared to habituation period (days 1–9).

When behavior during the habituation period (average days 1-9) was compared with behavior during exposure days (10-14), ANOVA indicated a significant within-group effect of time spent in the chamber for TMT ($F(5,42) = 6.66$, $p < 0.001$), cat fur ($F(5,42) = 3.96$, $p < 0.01$) and butyric acid ($F(5,42) = 3.95$, $p < 0.01$) (Fig. 14). No significant difference was found between treated groups on the first day of odor exposure, i.e. day 10 ($F(2,21) = 0.95$, $p = 0.40$). A Tukey *post-hoc* test indicated a significant reduction for time spent in the odor chamber compared to the habituation period, with the highest reduction in the TMT treated group ($p < 0.001$) and the lowest in the butyric acid group ($p < 0.05$), cat odor exposed mice exhibited intermediate avoidance ($p < 0.01$). Exposures during the next days revealed a gradual increase in time spent in the odor compartment that was observed for all groups. This habituation was strongest in the cat fur group (Fig. 14).

Analysis of blood CORT level after odor exposure indicated a significant increase for TMT treated mice ($F(1,14) = 42.9$, $p < 0.001$), whereas no difference was observed in the two other groups (cat fur $F(1,14) = 2.94$, $p = 0.11$ and butyric acid $F(1,14) = 0.18$, $p = 0.75$) (Fig.15).

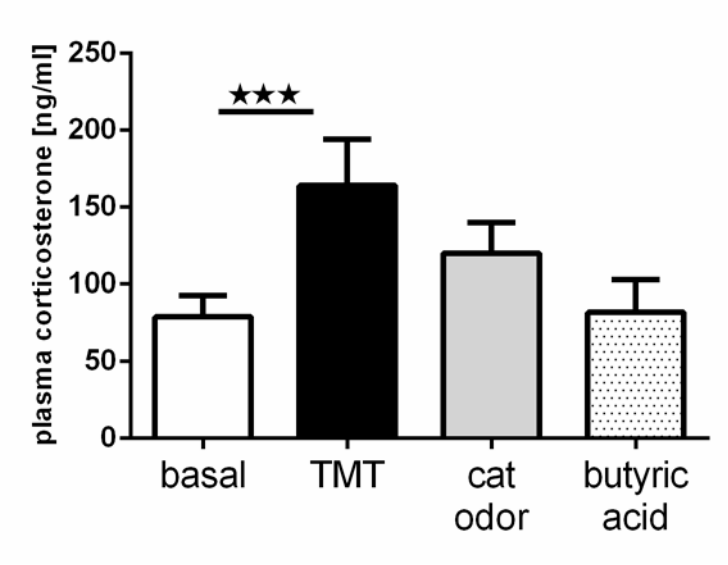

Fig. 15: The plasma corticosterone (CORT) levels in CD-1 mice after exposure to TMT, cat odor or butyric acid. Significant increase in CORT was observed only in the TMT exposed group, whereas no impact of other odors on HPA axis reactivity was detected. Bars represent means + SEM, n (TMT) = 8, n (cat odor) = 8, n (butyric acid) = 8, ★★★ $p<0.001$ relative to basal level.

4.1.2 TMT-avoidance in HAB/NAB/LAB mice

TMT induced high avoidance behavior in HAB mice, but not in LAB. The absence of an olfactory deficit is corroborated by the fact that LABs recognized the pleasant odor of female urine and the unpleasant smell of butyric acid.

Similar to CD1 mice, no difference in behavior during the habituation period (days 1-4) was observed by ANOVA within (LAB $F(3,28) = 0.71$, $p = 0.55$, NAB $F(3,28) = 0.36$, $p = 0.80$, HAB $F(3,28) = 1.88$, $p = 0.15$) or between ($F(2,21) = 1.80$, $p = 0.19$) the HAB/NAB/LAB groups. When exposed to TMT on day 5, HAB and NAB significantly reduced the time spent in the chamber with the odor, as indicated by higher −• T ($F(1,14) = 41.1$, $p < 0.001$ and $F(1,14) = 18.4$, $p < 0.01$, respectively), whereas we observed no significant effect on LAB mice ($F(1,14) = 1.91$, $p = 0.19$) (Fig. 16). Moreover, a Tukey *post-hoc* test revealed a significant difference in

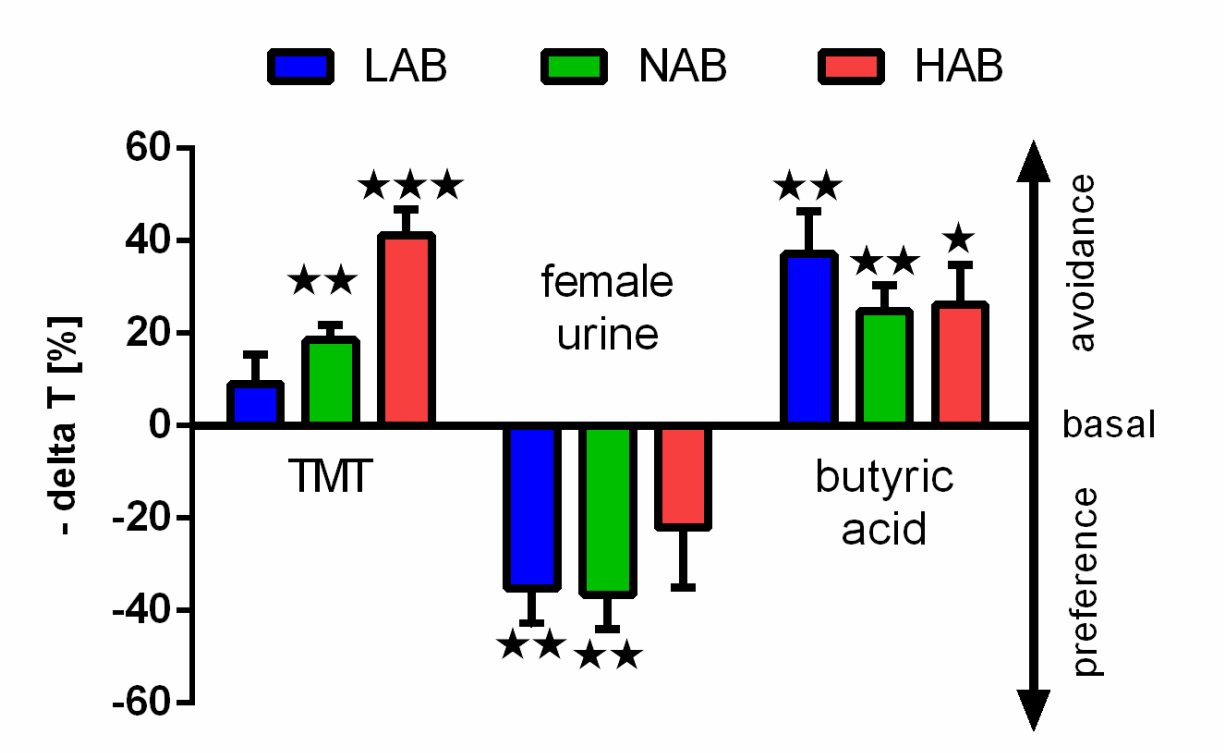

Fig. 16: Behavioral response of HAB/LAB/NAB mice to different odors. HAB and NAB mice, but not LAB, significantly avoided TMT. Female urine attracted LAB and NAB mice, but not HAB. All animals avoided butyric acid significantly. Bars represent means + SEM, n (LAB) = 8, n (NAB) = 8, n (HAB) = 8, ★ $p<0.05$, ★★ $p<0.01$, ★★★ $p<0.001$ relative to basal level.

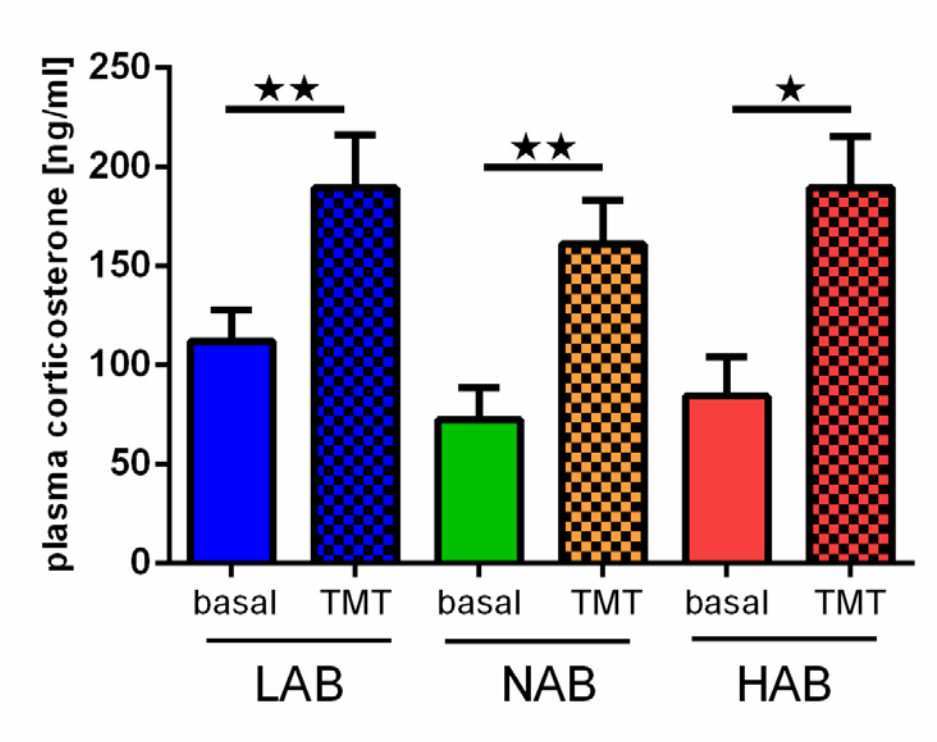

Fig. 17: HPA axis response on TMT exposure. TMT induced similar increase of CORT in all three lines. Bars represent means + SEM, n (LAB) = 8, n (NAB) = 8, n (HAB) = 8, ★ $p<0.05$, ★★ $p<0.01$ relative to basal level.

avoidance behavior between HAB *vs.* LAB mice ($p < 0.001$) and HAB *vs.* NAB mice ($p < 0.05$).

When exposed to female urine on day 8, NAB and LAB mice exhibited a significant preference for this chamber ($F(1,14) = 18.7$, $p < 0.01$ and $F(1,14) = 17.0$, $p < 0.01$, respectively). However, no effect was observed for HAB mice ($F(1,14) = 2.30$, $p = 0.15$) (Fig. 11). Furthermore, exposure to butyric acid on day 12 was followed by avoidance behavior throughout all groups, as reflected by positive −• T values (higher avoidance) (LAB $F(1,14) = 17.4$, $p < 0.01$, NAB $F(1,14) = 16.6$, $p < 0.01$, HAB $F(3,28) = 8.07$, $p < 0.05$) (Fig. 11). Importantly, no difference was found when behavior was assessed between TMT and female urine exposures (days 6-7 LAB $F(2,21) = 0.18$, $p = 0.82$, NAB $F(2,21) = 0.10$, $p = 0.90$, HAB $F(2,21) = 2.60$, $p = 0.10$) as well as female urine and butyric acid exposures (days 9-11 LAB $F(3,28) = 1.80$, $p = 0.17$, NAB $F(3,28) = 1.57$, $p = 0.22$, HAB $F(3,28) = 1.10$, $p = 0.37$). Finally, ANOVA revealed a similar impact of TMT-exposure on CORT levels of all three lines (LAB $F(1,14) = 11.2$, $p < 0.01$, NAB $F(1,14) = 11.0$, $p = 0.01$, HAB $F(1,14) = 6.60$, $p < 0.05$) (Fig. 17).

4.2 Effects of CMS on phenotypic characteristics of LAB mice

4.2.1 Changes in anxiety-related behavior

A significant increase of anxiety-related behavior was observed in CMS-treated mice as evaluated by EPM, LDB and the TMT-avoidance test. Home cage activity assessed locomotion to be independent of anxiety-related behavior and showed no significant difference between experimental groups.

EPM, LDB and the TMT-avoidance test were performed to evaluate the effects of CMS on different aspects of inborn anxiety-related behavior of LAB mice. All results originate from multiple experiments with at least 7 animals per treatment group. The most significant outcomes are illustrated and include the respective values of HAB mice to indicate the direction and effect size.

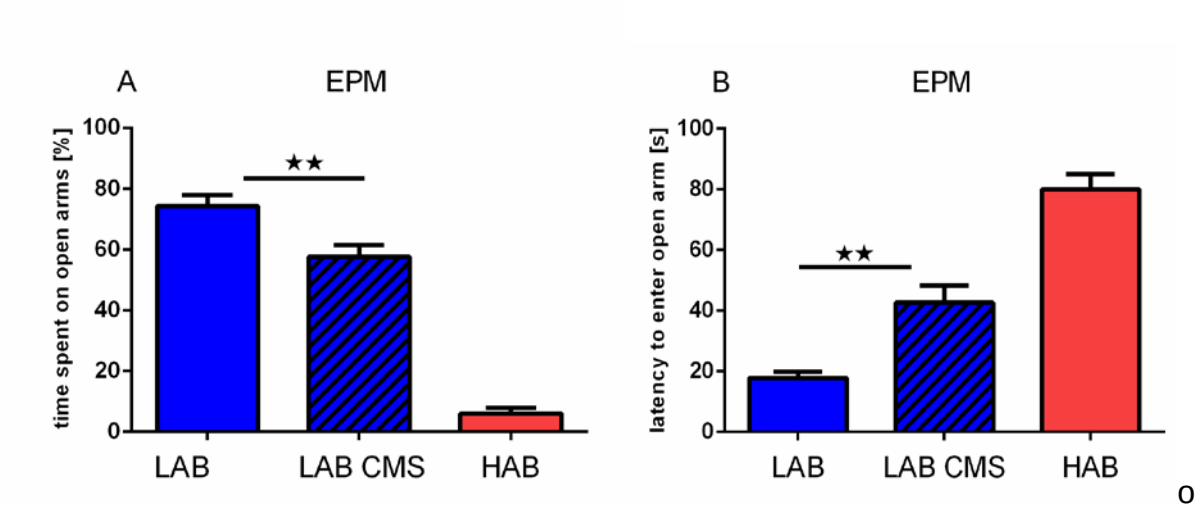

Fig. 18: Effect of CMS on anxiety-related behavior in the EPM. CMS induced an increase in anxiety-related behavior in the EPM, indicated by lower "time spent on the open arms" (A) and higher 'latency to first entry in the open arm' (B). Bars represent means + SEM, n (LAB) = 13, n (LAB CMS) = 12, ★★ $p<0.01$.

Stressed mice exhibited significantly higher anxiety-related behavior in the EPM as indicated by less time spent on the open arms ($F(1,24)=10.0$, $p<0.01$), higher latency to first open arm entry ($F(1,24)=15.0$, $p<0.01$) and less open arm entries ($F(1,24)=4.13$, $p<0.05$) (Fig. 18).

No difference was found in the number of entries to the closed arms ($F(1,24)=1.14$, $p>0.05$) and in the total distance travelled ($F(1,24)=2.24$, $p>0.05$), indicative of an absence of changes in locomotor activity. However, multiple testing of stressed mice suggest a possible impact of CMS on locomotor activity (data not shown) as a

consequence of increased immobility time. This suggests a higher neophobia in the CMS-treated LAB mice. Detailed results of the EPM test are summarized in Table 9.

Table 9: Detailed results of CMS effects on the anxiety-related behavior in the EPM.

Parameter measured	LAB	LAB CMS	p-value
time spent on open arms [%]	74.3 ± 3.7	57.7 ± 3.6	0.004
latency to entre open arm [s]	17.9 ± 2.0	42.7 ± 5.6	0.001
open arms entries [n]	7.0 ± 1.4	4.3 ± 0.3	0.049
closed arms entries [n]	7.1 ± 0.9	6.1 ± 1.0	0.245
total distance travelled [m]	10.7±1.0	9.2 ± 0.5	0.148
time spent immobile [s]	61.0 ± 5.5	67.0 ± 4.4	0.437

Results of the LDB corroborate changes observed in the EPM, an anxiogenic effect of CMS. Stressed LAB mice spent less time in the light compartment ($F(1,24)=10.4$, $p<0.01$), had higher latency time to enter the light compartment ($F(1,24)=4.75$, $p<0.05$) and entered less frequently into the light compartment ($F(1,24)=4.52$, $p<0.05$) (Fig. 19).

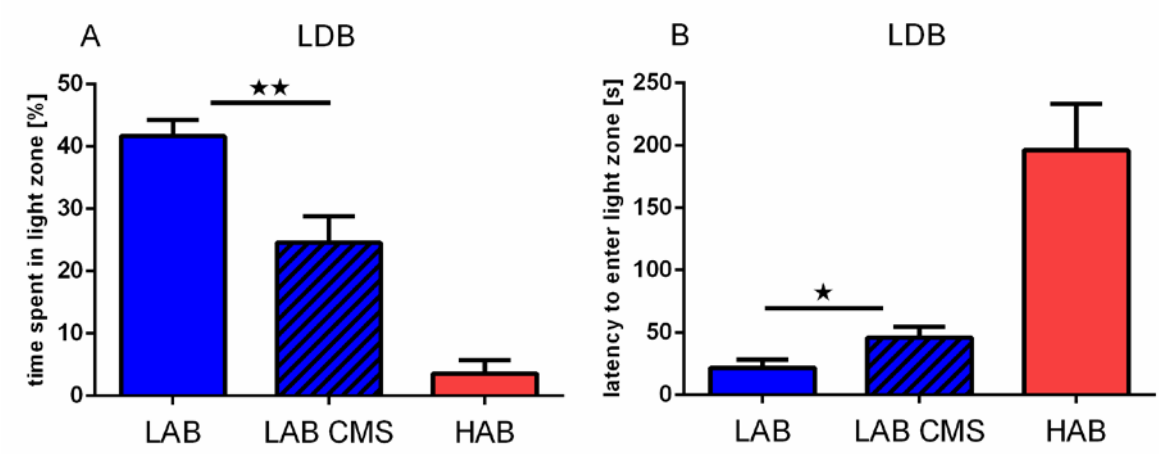

Fig. 19: Chronic stress exposure effects on anxiety-related behavior in the LDB. CMS induced a decrease in time spent in the light compartment (A) and an increase in latency time to first enter the light compartement (B) indicating an anxiogenic effect of CMS. Bars represent means + SEM, n (LAB) = 13, n (LAB CMS) = 12, ★ $p<0.05$, ★★ $p<0.01$.

Rearing frequency is indicative of exploratory activity in the LDB (Bourin and Hascoet, 2003). Fewer rearings were observed for the CMS-exposed group compared to control ($F(1,24)=4.61$, $p<0.05$), which indicates that stressed mice interprete the novel environment of the LDB as more unsafe. Similarly to the EPM, no difference in locomotor activity ($F(1,24)=0.78$, $p>0.05$) and time spent immobile

(F(1,24)=2.31, p>0.05) should be consider with care, since these parameters can vary due to unknown reasons. Detailed results of the LDB test are summarized in Table 10.

Table 10: Detailed results of CMS effects on anxiety-related behavior in the LDB.

Parameter measured	LAB	LAB CMS	p-value
time spent in light compartment [%]	41.6 ± 2.6	24.6 ± 4.3	0.005
latency to enter light compartment [s]	21.5 ± 7.0	46.0 ± 8.7	0.045
entries in light compartment [n]	8.6 ± 1.5	5.5 ± 0.9	0.048
total rearings [n]	24.6 ± 4.6	14.8 ± 2.5	0.048
total distance travelled [m]	14.5 ± 0.9	13.6 ± 1.0	0.148
time spent immobile [s]	22.6 ± 4.2	30.2 ± 3.0	0.437

The anxiogenic effects of CMS that we have observed in the EPM and LDB are further corroborated by a higher avoidance of the predator odor in the three-chambered test and a higher CORT release 15min after odor exposure. -• T index was significantly higher for stressed mice (F(1,13)=4.75, p<0.05), which indicates a higher avoidance of the chamber harboring the predator odor (Fig 20).

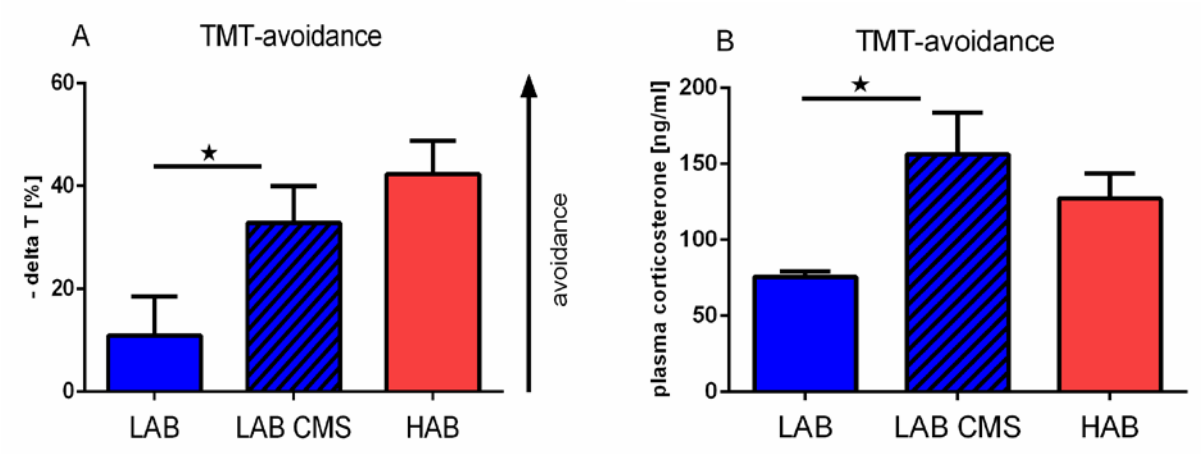

Fig. 20: Effects of CMS on behavior of LAB mice in the TMT-avoidance test. CMS increased TMT avoidance (A) and CORT release after odor exposure (B). Bars represent means + SEM, n (LAB) = 7, n (LAB CMS) = 7, ★ p<0.05.

The CMS group also showed less entries to the chamber with TMT (F(1,13)=20.2, p<0.001) and had a higher latency to enter the chamber with odor (F(1,13)=10.8, p<0.01). Although locomotor activity differed significantly (F(1,13)=14.5, p<0.01), this

change is likely to be caused by higher immobility of stressed mice ($F(1,13)=10.8$, $p<0.01$) possibly indicating increased neophobia.

Table 11: Detailed results of the TMT-avoidance test.

Parameter measured	LAB	LAB CMS	p-value
− T index [%]	10.9 ± 7.5	32.8 ± 7.1	0.048
plasma corticosterone [ng/ml]	75.6 ± 3.7	156.5 ± 27.1	0.016
entries in TMT compartment [n]	26.5 ± 2.0	14.5 ± 1.8	0.000
latency to enter TMT compartment [s]	9.4 ± 3.5	36.0 ± 10.0	0.005
total distance travelled [m]	105.0 ± 8.8	68.0 ± 4.0	0.002
time spent immobile [s]	51.1 ± 10.8	106.1 ± 12.8	0.005

Moreover, TMT exposure induced a higher release of CORT in the CMS-treated mice ($F(1,13)=7.60$, $p<0.05$), indicating either increased stress-reactivity or a more aversive interpretation of the presented cue (Fig. 20). Detailed results of the TMT-avoidance test are summarized in Table 11.

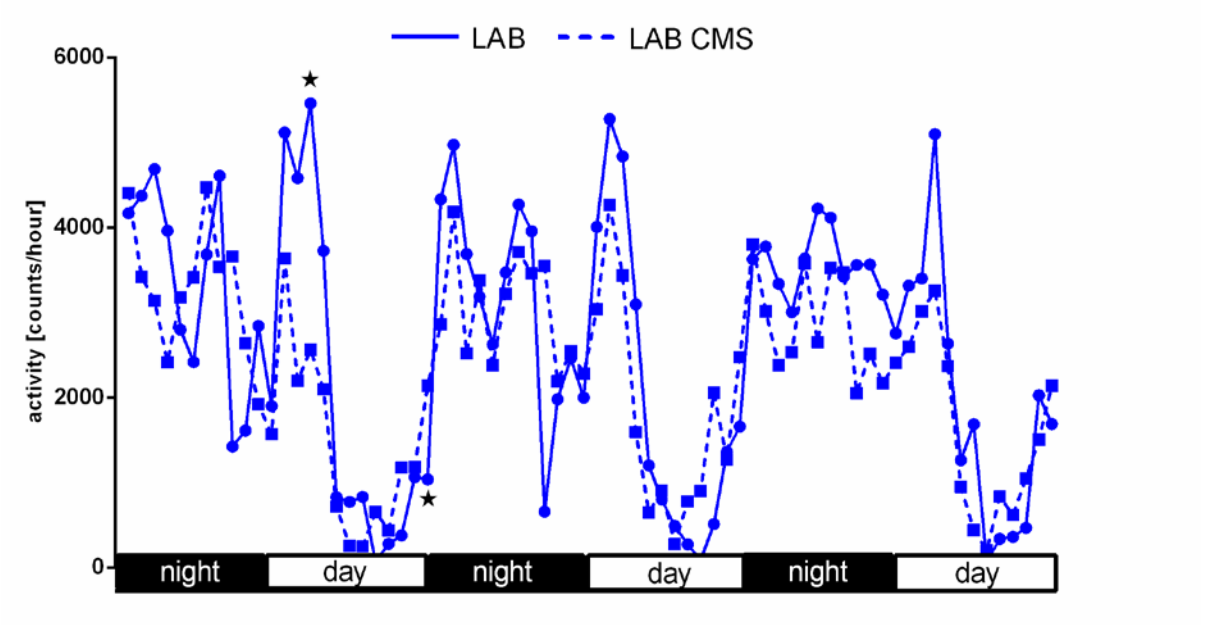

Fig. 21: Effects of CMS on home cage activity. Chronic stress exposure had almost no impact on locomotor activity in the home cage. n (LAB) = 6, n (LAB CMS) = 6, ★ $p<0.05$.

As discussed earlier, CMS can affect locomotor activity in behavioral tests (e.g. TMT-avoidance test) and thus, interfere or mask precise estimation of anxiety-related behavior. Therefore, we evaluated locomotor activity of stressed and unstressed LAB

mice in a more natural-like situation, i.e. home cage activity by using a computer-based system. No overall difference was found by a repeated measures ANOVA ($F(1,11)=0.00$, $p=0.989$) indicating that an observed difference in locomotion during behavioral testings can be ascribed to the increased neophobia of stressed mice and as such further corroborating anxiogenic effects of the CMS (Fig. 21).

4.2.2 Changes in depression-like behavior

CMS treatment increased depression-like behavior in the TST and FST. Moreover, chronic stress induced anhedonic behavior in the sucrose preference test.

Both, TST and FST, found pro-depressive effects of the CMS. Thus, stressed LAB mice spent more time immobile in the TST ($F(1,22)=6.94$, $p<0.05$) and floated significantly more in the FST ($F(1,22)=4.63$, $p<0.05$) (Fig. 22).

Moreover, latency to first immobility and floating episode were decreased in the CMS treated group ($F(1,22)=6.52$, $p<0.05$ and $F(1,22)=10.9$, $p<0.01$, respectevely), contrary to the number of floating episodes, which was increased ($F(1,22)=7.60$, $p<0.05$). Detailed results of the TST and FST are summarized in Table 12.

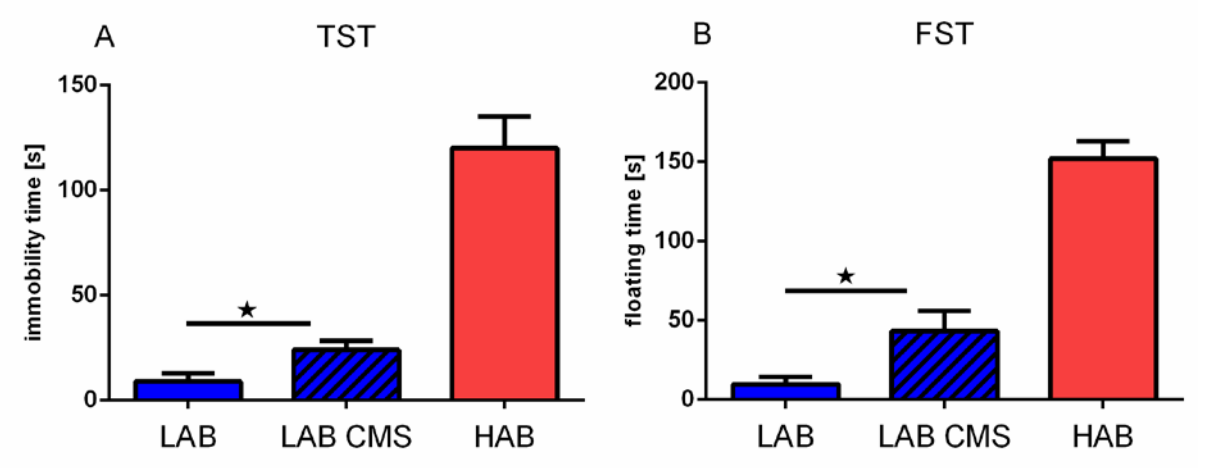

Fig. 22: Effects of CMS on depression-like behavior. CMS increased immobility in the TST (A) and floating time in the FST (B). Bars represent means + SEM, n (LAB) = 11, n (LAB CMS) = 12, ★ $p<0.05$.

Table 12: Detailed parameters measured in the TST and FST to highlight changes in depression-like behavior after CMS exposure.

	Parameter measured	LAB	LAB CMS	p-value
TST	immobility [s]	9.0 ± 3.7	23.9 ± 4.3	0.011
	latency to first immobility [s]	262.5 ± 21.9	177.9 ± 24.8	0.014
	number of immobile episodes [n]	4.8 ± 1.2	6.7 ± 1.0	0.269
FST	floating time [s]	9.5 ± 4.8	43.4 ± 12.7	0.048
	latency to first floating [s]	273.0 ± 41.0	133.4 ± 21.0	0.005
	number of floating episodes [n]	4.3 ± 2.0	12.3 ± 2.0	0.015

To characterize depression-like behavior using a stress-independent approach, we performed a sucrose preference test. Analysis of bottle preference found, that animals preferred to drink from a particular bottle (left or right), though both bottles contained identical solutions (either water or sucrose in both bottles) ($F(1,31)=9.12$, $p<0.01$ for water and $F(1,31)=8.20$, $p<0.01$ for sucrose) (Fig. 23).

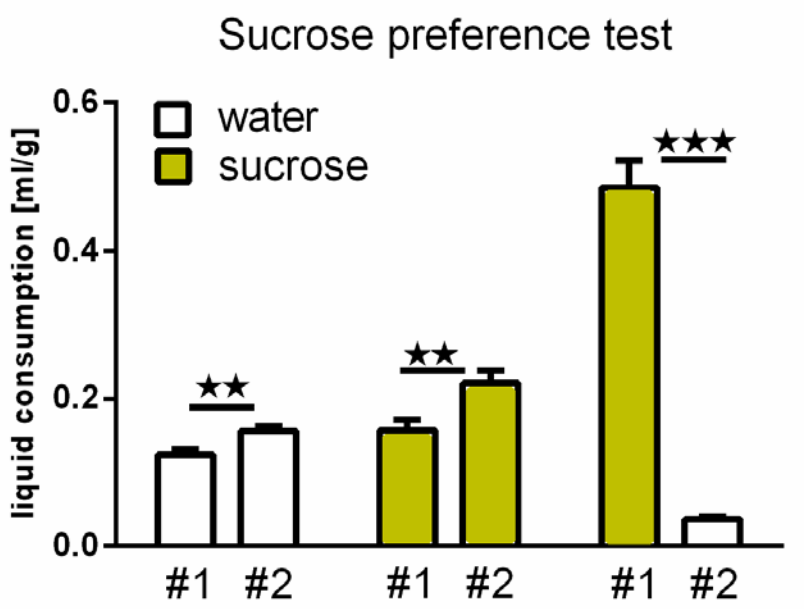

Fig. 23: Results of sucrose preference test. Mice preferred particular bottle position (#1 or #2) when two identical solutions (w (water) or s (sucrose)) were presented. Consumption of the sweet solution was much higher and independent of position. Bars represent means + SEM, n (#1) = 9, n (#2) = 9, ★★ $p<0.01$, ★★★ $p<0.001$.

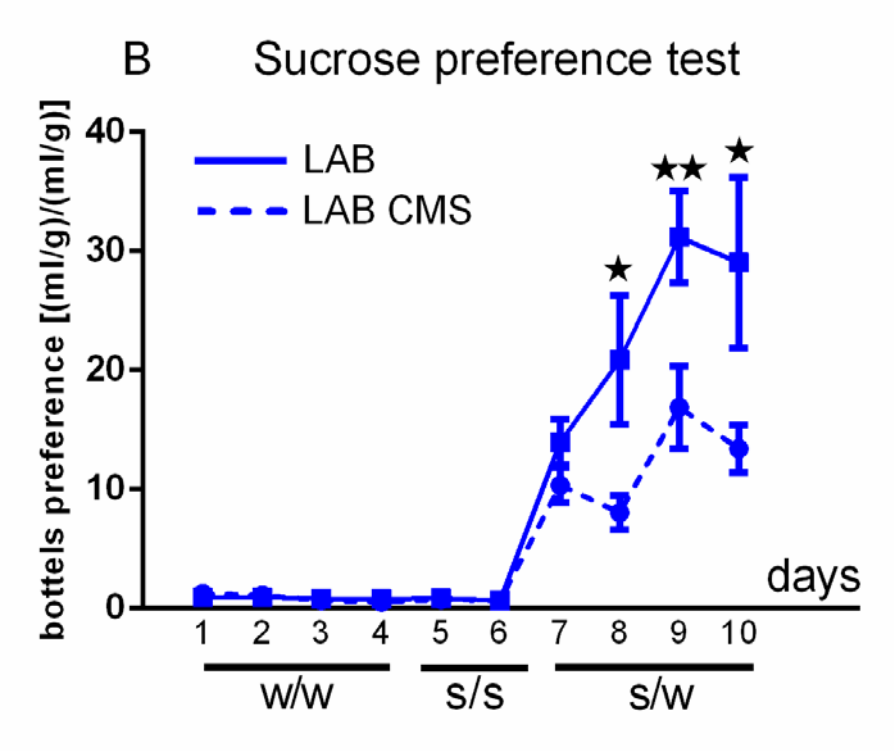

Fig. 24: Results of sucrose preference test. CMS treated animals exhibited higher anhedonic behavior, indicated by less preference of sucrose solution (B). Bars represent mean + SEM, n (LAB) = 9, n (LAB CMS) = 9, ★ $p<0.05$, ★★ $p<0.01$.

To grant a more sensitive discrimination of preference-avoidance between water and sucrose, the less prefered position was used for placing the sucrose solution, since it was expected that mice would consume the sweet solution more likely. Indeed, despite of initial bottle aversion, mice consumed mainly sucrose solution ($F(1,31)=146$, $p<0.001$), with control mice preferring sucrose solution more than CMS-treated (MANOVA, Tukey *post-hoc* test, ($F(1,13)=6.16$, $p<0.05$ for day 8; ($F(1,13)= 12.6$, $p<0.01$ for day 9; ($F(1,13)=5.85$, $p<0.05$ for day 10) (Fig. 24). These results point towards a higher anhedonic behavior of stressed mice compared to controls.

4.2.3 Changes in neuroendocrine parameters

CMS induced delayed HPA axis reactivity and a stronger negative feedback. Systemic effects of stress were indicated by a profound loss of body weight and hypertrophy of adrenals.

Neuroendocrinological parameters provide the most unbiased approach for the evaluation of stress-induced effects on different systems of a body. Alterations in

CORT secretion during stress exposure is a validated parameter of a dysregulated HPA axis.

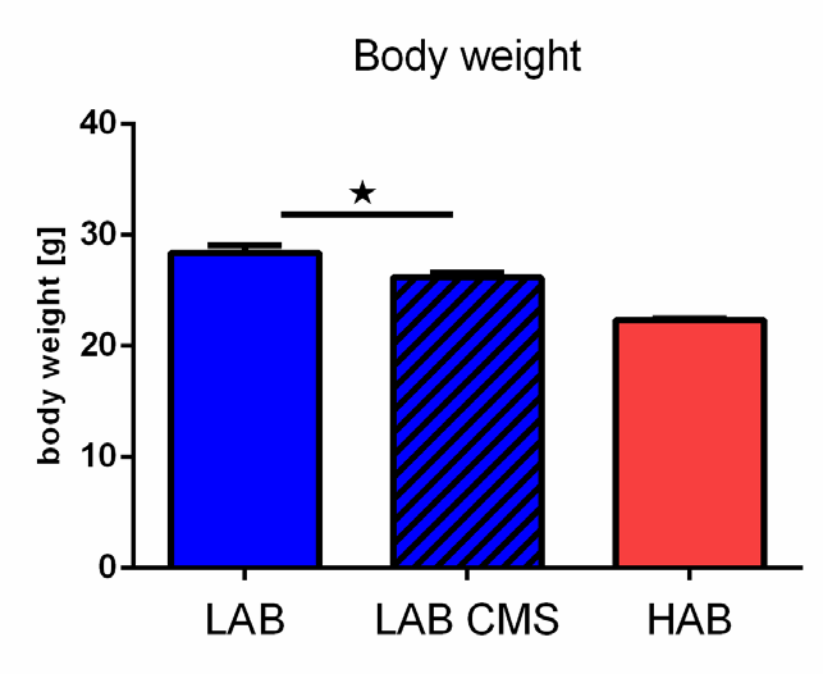

Fig. 25: Effects of stress on body weight. CMS induced loss of body weight in LAB mice Bars represent means + SEM, n (LAB) = 11, n (LAB CMS) = 9, ★ p<0.05.

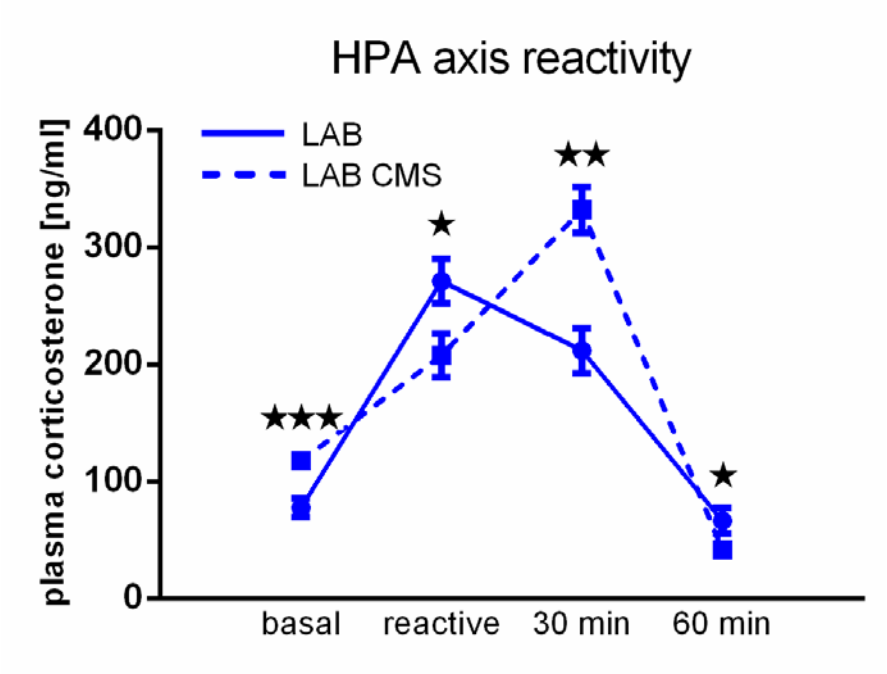

Fig. 26: Effects of stress on HPA axis regulation. Significantly higher basal CORT, delayed HPA reactivity and stronger feedback regulation were observed in stressed animals (B). Bars represent means + SEM, n (LAB) = 7-11, n (LAB CMS) = 7-9, ★ p<0.05, ★★ p<0.01, ★★★ p<0.001.

We found that stressed mice had decreased body weight F(1,28)=6.80, p<0.05) (Fig. 25), possibly as a result of chronically elevated plasma corticosterone in CMS-exposed LAB mice (F(1,28)=16.9, p<0.001) (Fig. 26). In order to evaluet HPA axis reactivity, we measured stress-induced increase in blood CORT. We observed lower CORT realease in stressed animals directly after exposure to forced swimming (F(1,14)=5.41, p<0.001), however, 30min later, the level of CORT was significantly higher in the CMS group compared to controls (F(1,14)=18.6, p<0.01) (Fig. 26). Moreover, not only reactivity, but also feedback regulation was found to be altered. Thus, one hour after forced swimming, we observed significantly higher suppression of the HPA axis in CMS-treated mice indicated by lower plasma CORT (F(1,14)=4.65, p<0.05) (Fig. 26).

Numerous reports suggested that chronic stress induces adrenal hypertrophy and thymus dysplasia. We also found that CMS-exposed mice had a higher mass of adrenals per gram of body weight (F(1,26)=5.41, p<0.05) (Fig. 27A). Although we have not observed difference in the mass of thymus per gram of body weight (F(1,26)=0.32, p>0.05) (Fig. 27B), this can be due to the rather small size of this structure in mice.

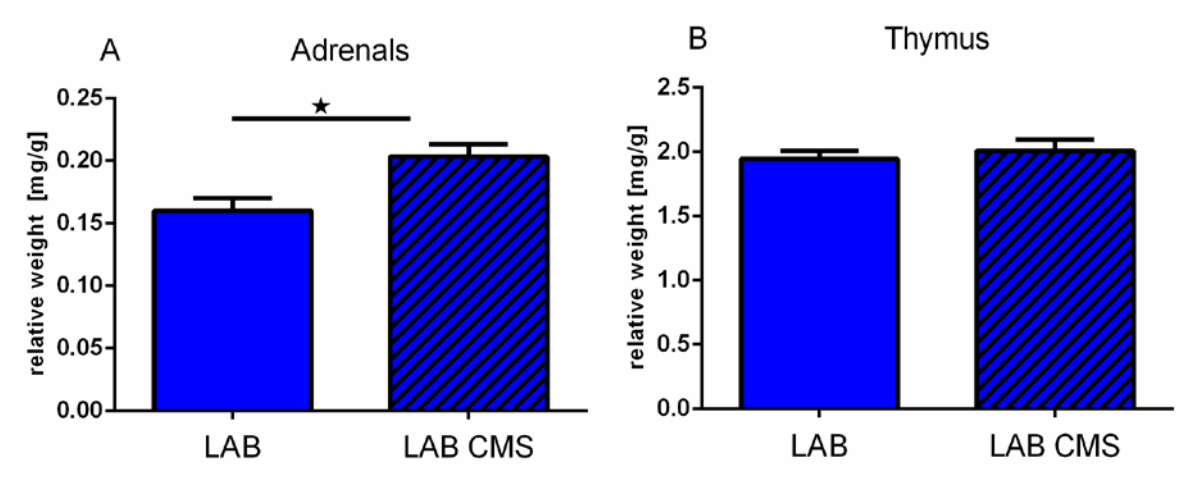

Fig. 27: Effects of CMS on relative weight of the adrenals and thymus. Chronic stress resulted in hypertrophy of adrenals (A), but did not affect weight of thymus (B). Bars represent means + SEM, n (LAB) = 14, n (LAB CMS) = 13, ★ p<0.05.

4.3 Effect of CMS on *c-fos* expression after TMT exposure

CMS treatment induced higher activation of *c-fos* expression in the PVN, CeA and BLA after exposure to TMT.

The TMT-avoidance test provided reliable estimation of changes of anxiety-related behavior after CMS. Exposure to TMT induced a significant activation of immediate early gene expression, which could be used to map brain activity. This approach allowed us to identify brain regions involved in the observed avoidance behavior and to analyze how their activity is modulated by chronic stress exposure. We found higher *c-fos* expression in the mPFC, PVN, Amy, and LC (for all, $p<0.01$) and lower in the CA1 region of the Hipp ($p<0.01$) of HAB mice compared to LAB mice (Table 13). Stressed animals increased *c-fos* expression after TMT exposure only in the Amy and in the PVN ($p<0.05$ both) (Fig. 28).

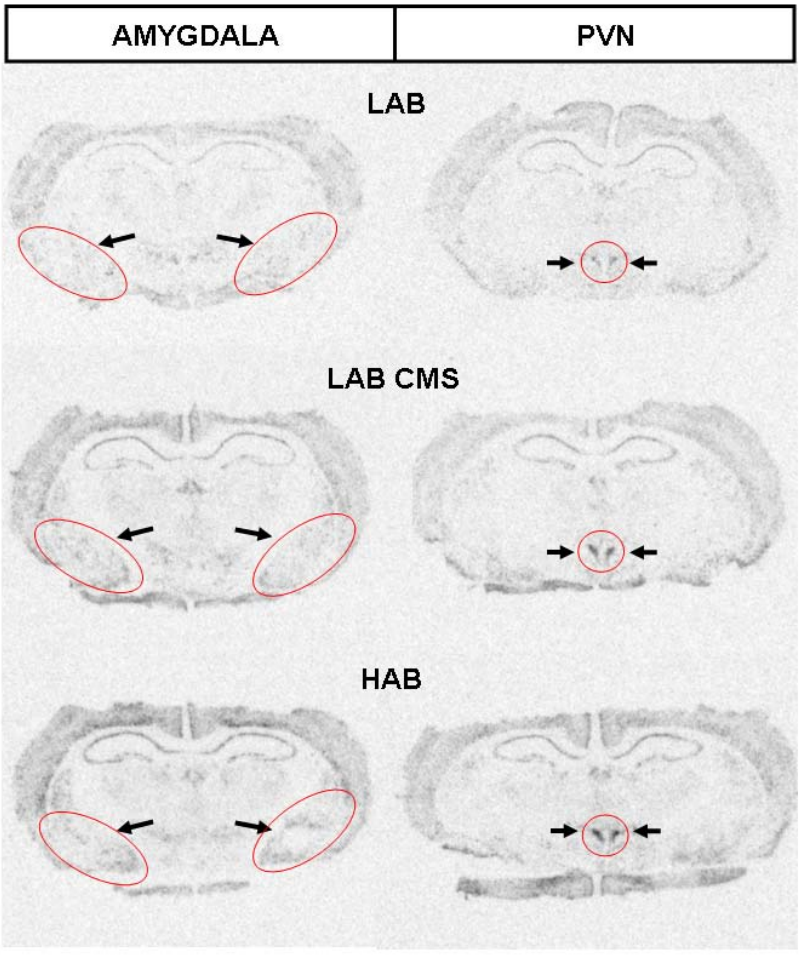

Fig. 28: *c-Fos* expression in the Amy and PVN of LAB, CMS-exposed LAB and HAB mice.

Higher activity of PVN in stressed LAB and HAB mice might be explained by a higher HPA axis activation in these animals (see section 4.2.3.), thus we studied the amygdalar expression of *c-fos* in more detail. We observed an insignificant increase in early gene expression in the lateral and medial parts ($p>0.05$), whereas the expression in the basolateral and central nuclei of Amy was significantly higher (for both $p<0.05$) (Table 13).

Table 13: *c-Fos* expression in different brain region of LAB, CMS-exposed LAB and HAB mice. Values depicted as means ± SEM. n (LAB) = 7, n (LAB CMS) = 7, n (HAB) = 7, * $p<0.05$ and ** $p<0.01$ for LAB *vs.* HAB, # $p<0.05$ for LAB *vs.* LAB CMS.

Brain region	LAB	CMS LAB	HAB
mPFC	32.1 ± 3.8	25.8 ± 1.8	51.6 ± 2.7 **
PVN	16.0 ± 1.4	32.0 ± 4.5 #	48.0 ± 4.7 **
Amygdala			
Basolateral	5.4 ± 0.7	8.2 ± 0.7 #	11.4 ± 1.3 *
Lateral	4.6 ± 1.1	6.5 ± 1.0	4.6 ± 1.3
Central	1.1 ± 0.8	4.0 ± 0.4 #	5.8 ± 0.8 *
Medial	14.3 ± 1.3	15.9 ± 1.1	18.9 ± 2.6
Hippocampus			
CA1	26.5 ± 1.9	25.2 ± 2.7	20.2 ± 1.2 **
CA2	23.3 ± 2.5	18.9 ± 2.4	21.9 ± 1.5
CA3	15.4 ± 0.7	12.0 ± 0.8	13.0 ± 0.6
Dentate gyrus	10.9 ± 1.5	11.0 ± 2.1	9.9 ± 1.3
Locus coeruleus	23.2 ± 2.1	20.5 ± 3.6	27.6 ± 1.6 **

4.4 Effect of CMS on gene expression in the BLA

Several genes were found to be differently expressed after CMS treatment. However, only *Crhr1* expression met the criteria of a "plasticity gene".

Based on literature data and earlier studies (Czibere, 2008; Markt, 2012), we analyzed expression of several candidate genes in three different brain regions known to be associated with anxiety-related behavior (Table 14). This work was performed in close collaboration with Dr. Patrick Oliver Markt (2012). Using qPCR we

identified significant differences in the expression of several genes after chronic stress exposure, results are summarized in Table 15.

Table 14: Following genes were analyzed in three brain regions of LAB and LAB-CMS mice.

Gene name	Gene symbol	Brain region
transmembrane protein 132d	*Tmem132d*	CG
glyoxalase-1	*Glo1*	CG
corticotropin realising hormone	*Crh*	BLA
corticotropin realising hormone receptor 1	*Crhr1*	BLA
Interleukin-10 receptor 1	*IL-10r1*	BLA
tumor necrosis factor alpha receptor 1	*TNF• r1*	BLA
dopamine beta hydroxylase	*Dbh*	LC
neuropeptide S receptor 1	*Npsr1*	LC

Table 15: Differences in mRNA expression. Expression of eight candidate genes after CMS was evaluated using qPCR. Transcriptional activity of three of them was changed after chronic stress exposure. Arrows indicates increase•( ), decrease (•) or no changes (•) in gene expression after CMS exposure. n (LAB) = 7, n (LAB CMS) = 7.

Gene symbol	CMS effects	p-value
Tmem132d	•	0.035
Glo1	•	1.000
Crh	•	0.110
Crhr1	•	0.025
IL-10r1	•	0.406
TNF• r1	•	1.000
Dbh	•	0.873
Npsr1	•	0.035

Out of three genes, which were affected by CMS treatment, we selected *Crhr1* for further analysis, since only this gene met our criteria of a “plasticity gene of anxiety-related behavior“: (i) expression differences between HAB and LAB mice, (ii) CMS-induced change in gene expression reflecting a shift in anxiety-related behavior, (iii) vulnerability to epigenetic regulation.

Here we found that *Tmem132d*, *Npsr1* and *Crhr1* were differently expressed after CMS exposure (Table 7), moreover these genes were differently expressed between HAB and LAB mice (Erhardt, 2011; Markt, 2012; Naik, 2013) and, therefore, meet the above mentioned criteria.

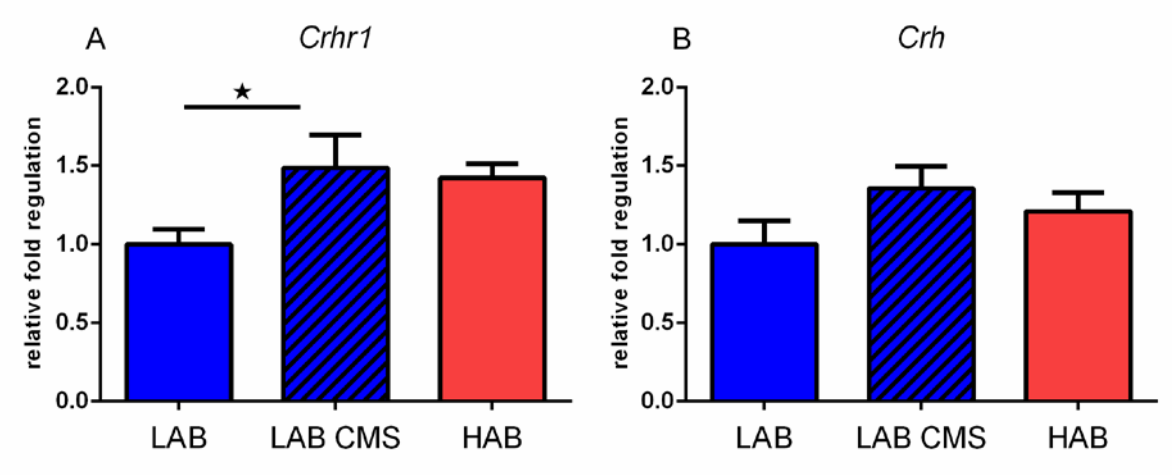

Fig. 29: Expression of *Crh* and *Crhr1* after CMS exposure. Whereas significant differences were observed in *Crhr1* expression, no changes were found in the amount of mRNA of its ligand, CRH. Bars represent mean + SEM, n (LAB) = 7, n (LAB CMS) = 7, ★ $p<0.05$.

However, higher *Tmem132d* expression in the CG was found to be associated with higher anxiety-related behavior of HAB mice (Erhardt, 2011), whereas we observed that higher anxiety-related behavior of stressed animals was associated with lower expression of *Tmem132d.* This disrepancy between behavior and gene expression changes excludes *Tmem132d* from this list of "candidate genes". Furthermore, an independent study found *Npsr1* gene to be non-plastic (Naik, 2013). Thus, only *Crhr1* met all criteria described above and, therefore, epigenetic mechanisms behind its different expression were studied in further experiments. Importantly, no changes were observed in the expression of *Crh* ($p>0.1$) (Fig. 29), thereby minimizing possible masking effects of the ligand.

4.5 Effect of CMS on *Crhr1* DNA methylation in the BLA

Chronic stress exposure increased CpG1 methylation. Methylation has a regulatory role on the *Crhr1* promoter activity, estimated using *Crhr1* promoter-reporter constructs with site-specific methylation.

We performed *in silico* analysis (CpG island searcher, Version 10/29/04) and found a CpG island (CpGi) in the *Crhr1* gene (Fig. 30), making this gene susceptible for DNA

methylation. Moreover, we analyzed the expression of enzymes responsible for DNA methylation and proteins involved in silencing complex formation with

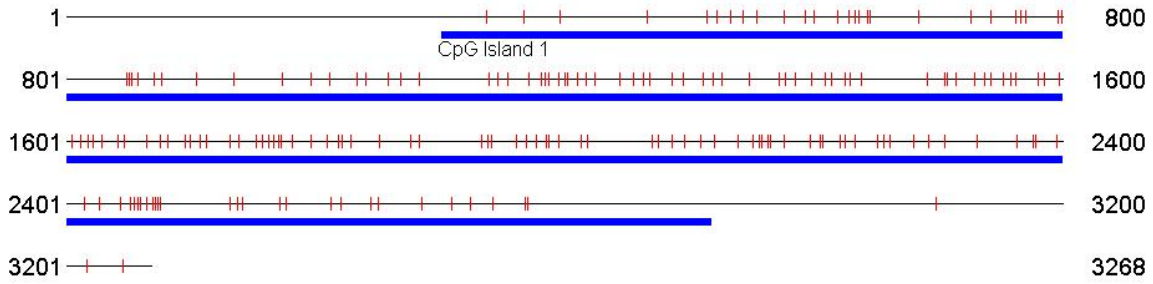

Fig. 30: *In silico* prediction of the 2796 bp CpG island in the *Crhr1* gene.

Table 16: Expression of enzymes responsible for DNA methylation and MeCP2, a protein recognising methylated cytosines of CpGs.

Gene name	Gene symbol	CMS effects	p-value
DNA methyltransferase 1	*Dnmt1*	•	0.022
DNA methyltransferase 3a	*Dnmt3a*	•	0.025
DNA methyltransferase 3b	*Dnmt3b*	•	0.886
methyl CpG binding protein 2	*MeCP2*	•	0.198

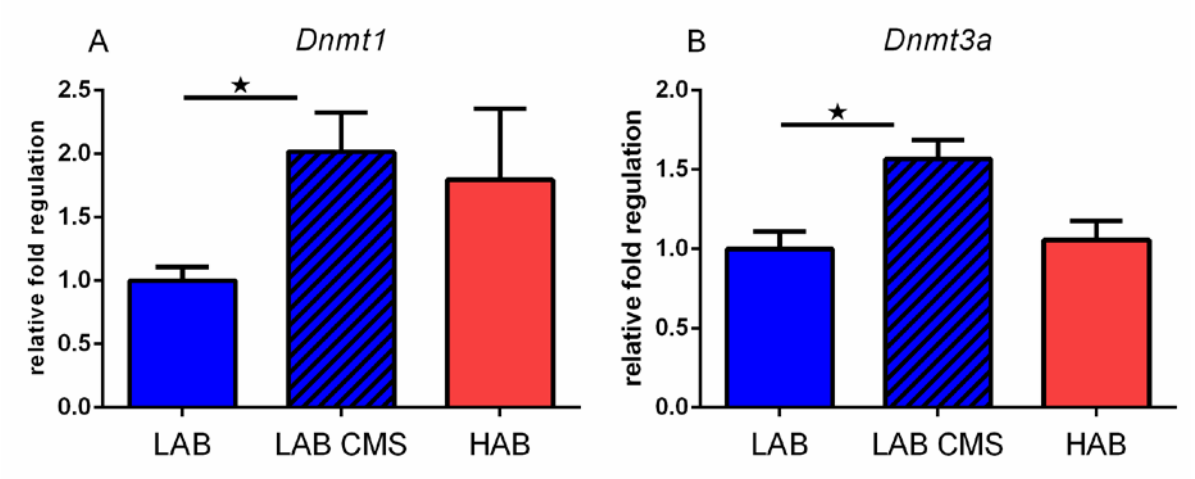

Fig 31: Effect of chronic stress exposure on the expression of *DNMT1* and *DNMT3a*. CMS induced the expression of enzymes involved in DNA methylation *Dnmt1* (A) and *Dnmt3a* (B). Bars represent means + SEM, n (LAB) = 7, n (LAB CMS) = 7, ★ $p<0.05$.

methylated DNA. Thus, expression of enzymes responsible for the maintenance of methylation, *Dnmt1,* and for *de novo* methylation, *Dnmt3a*, were significantly increased in stressed mice ($p<0.05$ for both) (Fig. 31), whereas no difference was found in the expression of *Dnmt3b* and *MeCP2* ($p>0.1$ both) (Table 16). Altogether, these data suggest that CMS could induce changes in methylation of *Crhr1* CpGi that could drive different *Crhr1* expression. Thus, using pyrosequencing of bisulfite-converted DNA, we analyzed the methylation of all 186 single CpG sites of the CpGi. No significant difference was found in total methylation between LAB, LAB-CMS and HAB mice using a Kruskal-Wallis ANOVA ($H(2, N=542) = 1.61$, $p=0.43$) (Fig. 32A).

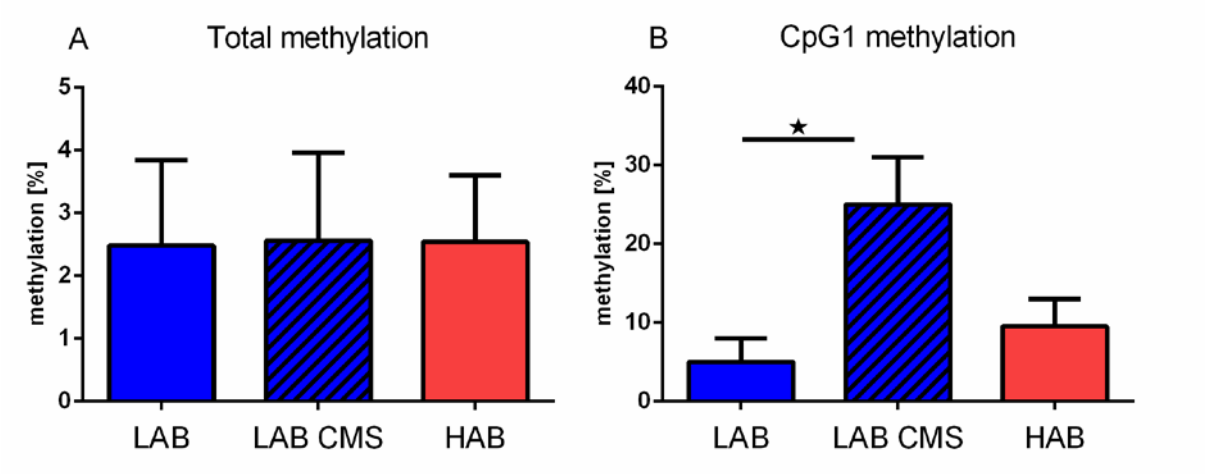

Fig 32: *Crhr1* CpGi methylation. No difference was found in total methylation of *Crhr1* between LAB, LAB-CMS and HAB mice (A). Stress induced a significant increase in methylation of CpG1 (B). Bars represent means + SEM, n (LAB) = 5, n (LAB CMS) = 5, ★ $p<0.05$.

The average methylation did not differ significantly ($p>0.05$) and was at the level of 2.48% for LAB, 2.56% for LAB CMS and 2.54% for HAB mice. Such overall low levels of methylation (<5%) indicate active transcription of the gene. The only significant difference found was the methylation of the first CpG site of the CpGi (CpG1). A higher level of methylation ($p<0.05$) was observed in chronically stressed mice compared to control animals (Fig. 32B).

We next tested the functional impact of CpG1 methylation on *Crhr1* promoter activity. To this end, the LAB promoter was cloned in the CpG-free luciferase-expressing vector. We observed a significant reduction of the luciferase signal in both cases

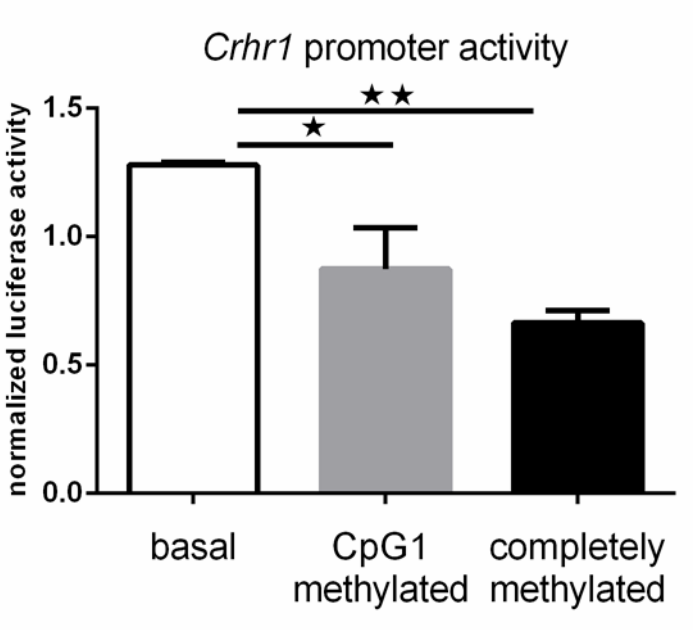

Fig 33: Impact of methylation on the *Crhr1* promoter activity. Significant reduction of *Crhr1* promoter activity was observed for complete and site-specific (CpG1) methylation, estimated using a reporter gene assay. Bars represent mean + SEM, n (biological replicates) = 4, ★ $p<0.05$.

when all CpG dinucleotides were methylated ($p<0.01$) and also when only CpG1 was site-specifically methylated ($p<0.05$) (Fig. 33). This indicates the importance of CpG1 methylation for the control of *Crhr1* expression. Moreover we performed *in silico* analysis based on the available database (Ensemble genome browser) on whole genome bisulfite sequencing of embryonic stem cells (ES) and nasopharyngeal carcinoma cells (NPC) and found CpG1 to be one of the differently methylated CpGs, thereby corroborating the importance of the CpG1 for epigenetic control.

However, the impact of the CpG1 methylation on the promoter activity and CMS-induced increase of CpG1 methylation were in contrast to observed changes in *Crhr1* gene expression. Therefore, we analyzed other possible mechanism that, together with CpG1 methylation, can regulate *Crhr1* expression. We performed computational prediction of transcription binding sites using Transcription Element Search System (TESS: TRANSFAC 7.0 Public (2005) Database and JASPAR Database) around CpG1 and identified a recognition sequence for the binding of transcription factor YY1 (Fig. 34). Further experiments elucidated the role of YY1 in methylation-sensitive regulation of the *Crhr1* promoter activity.

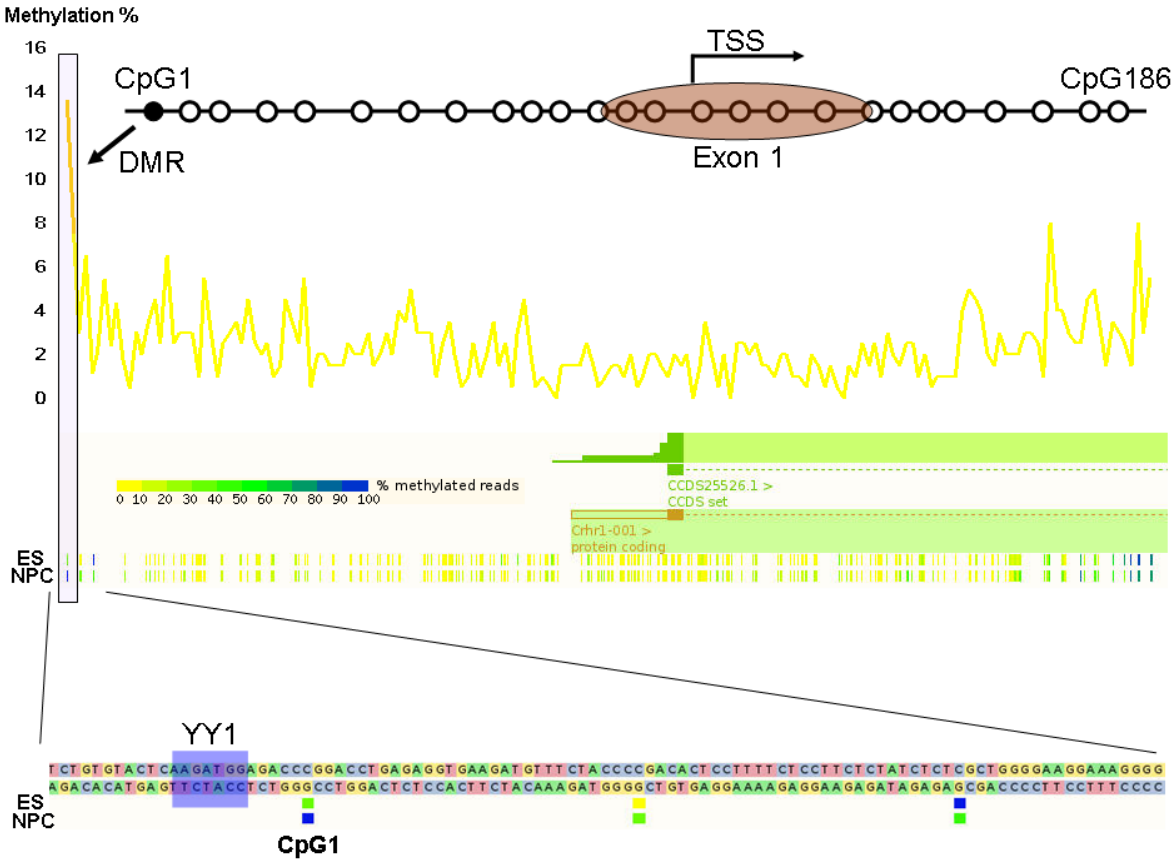

Fig 34: Illustration of differences in *Crhr1* CpGi methylation status between mouse embryonic stems cells (ES), nasopharyngeal carcinoma cells (NPC) and pyrosequencing data of *Crhr1* from BLA of LAB mice. Transcription factor YY1 was predicted to bind close to the CpG1.

4.6 Role of YY1 in the methylation-sensitive regulation of *Crhr1*

4.6.1 *YY1* expression after CMS and its role in the regulation of the *Crhr1* promoter

Higher *YY1* expression was found in the BLA of stressed LAB mice. *In vitro* assays indicated that higher *YY1* expression could induce *Crhr1* expression via enhancement of the promoter activity.

First of all, we studied the effects of chronic stress exposure on the expression of *YY1* in the BLA of LAB mice. We found that CMS-exposure led to increased amounts of *YY1* mRNA ($p<0.05$), however no difference was found in *YY1* between HAB and LAB mice ($p>0.05$) (Fig. 35A). On the protein level, we observed an insignificant

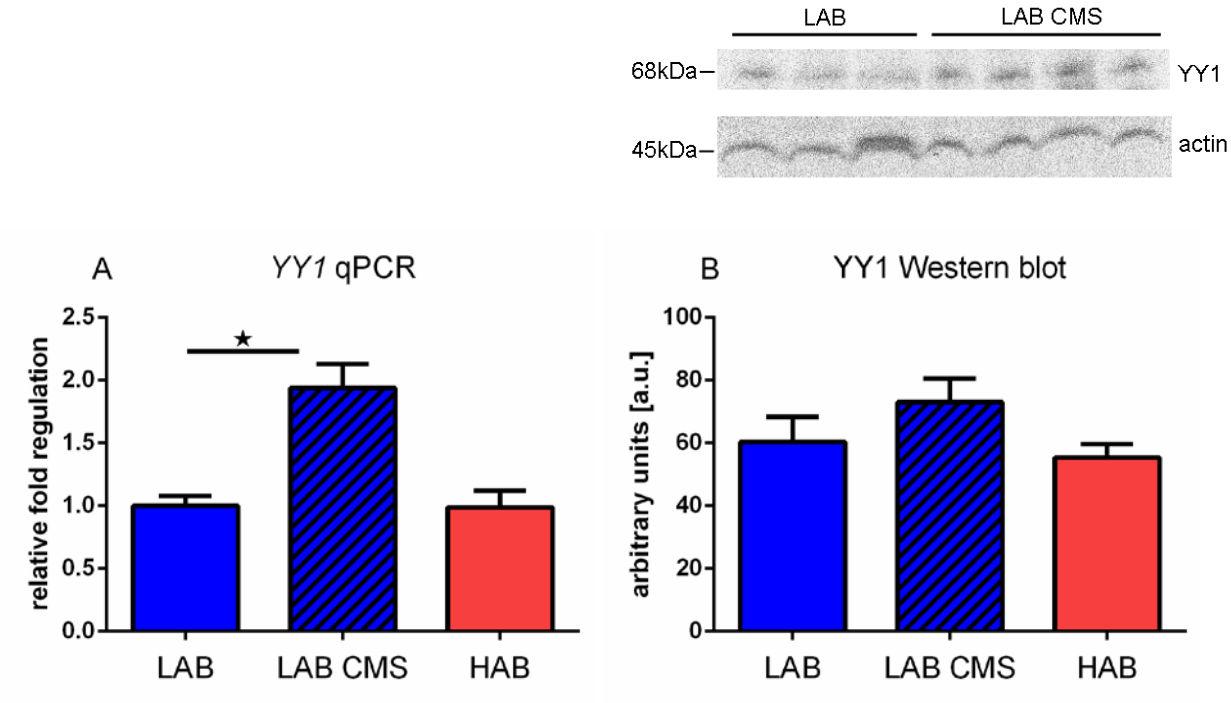

Fig 35: Effects of stress on *YY1* expression and protein. CMS induced an increase in *YY1* expression in the BLA (A), however no difference was observed in the amount of YY1 analysed in pooled whole amygdala extracts (B). Bars represent means + SEM, n (LAB) = 7, n (LAB CMS) = 7, n (HAB) = 7 for A and n (LAB) = 3, n (LAB CMS) = 4, n (HAB) = 4 for B, ★ $p<0.05$.

increase ($p>0.05$) in the amount of YY1 after stress (Fig. 35B). However, since proteins were extracted from pooled whole amygdala punches, this result should be considered with caution.

Literature data suggests that YY1 can act as both, a transcriptional activator and a silencer. TESS predicted three binding positions for YY1 in the *Crhr1* promoter: -755bp to -760bp, -1353bp to -1358bp and -1529bp to -1534bp upstream of the translation starting site (TSS). To get an overall impression about possible impacts of of transcription factor binding on *Crhr1* promoter activity, we generated deletion constructs and analyzed them in reporter gene assays. We observed that the longer size of the promoter always induced higher luciferase activity, thus predicting YY1 as an enhancer of the *Crhr1* promoter (Fig. 36).

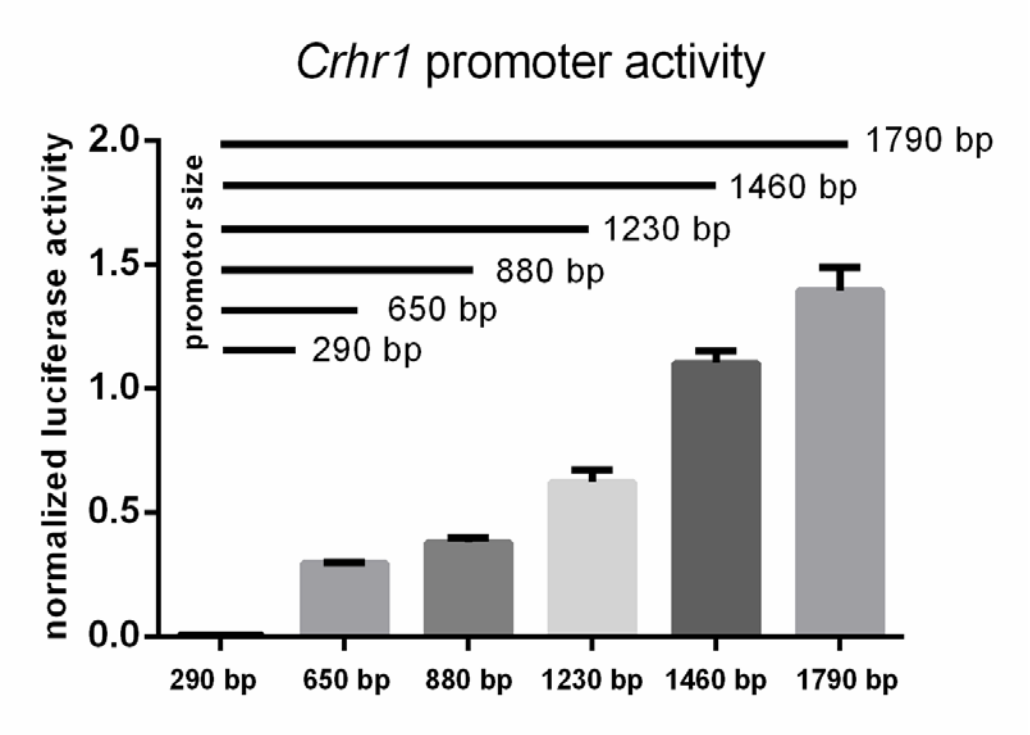

Fig 36: *Crhr1* promoter deletion constructs expressed different activity in the reporter gene assay depending on their size: bigger size induced stronger luciferase signal. Bars represent means + SEM, n (biological replicates) = 3.

Based on this finding, we were interested to know if YY1, indeed, induced an increase in *Crhr1* promoter activity. To this end, we purchased a plasmid carrying *YY1* cDNA under the control of the CMV-promoter and, first of all, analysed if transfection of N2a cells with this plasmid induced accumulation of YY1. Our Western blot analysis (Fig. 37A), indeed, revealed a significant increase of YY1 compared to ubiquitous expression in both cytosolic (Fig. 37B) and nuclear fractions ($p<0.001$) (Fig. 37C). Moreover, to further visualize that difference, we compared the amount of YY1 in cells transfected with *YY1* cDNA or mock transfected. For this, we performed immunohistochemistry staining using anti-YY1 antibody and a fluorescent secondary antibody. Similar to the Western blot, a significantly higher amount of YY1 was found in cDNA transfected cells ($p<0.001$) (Fig. 38), indicating functionality of the selected approach.

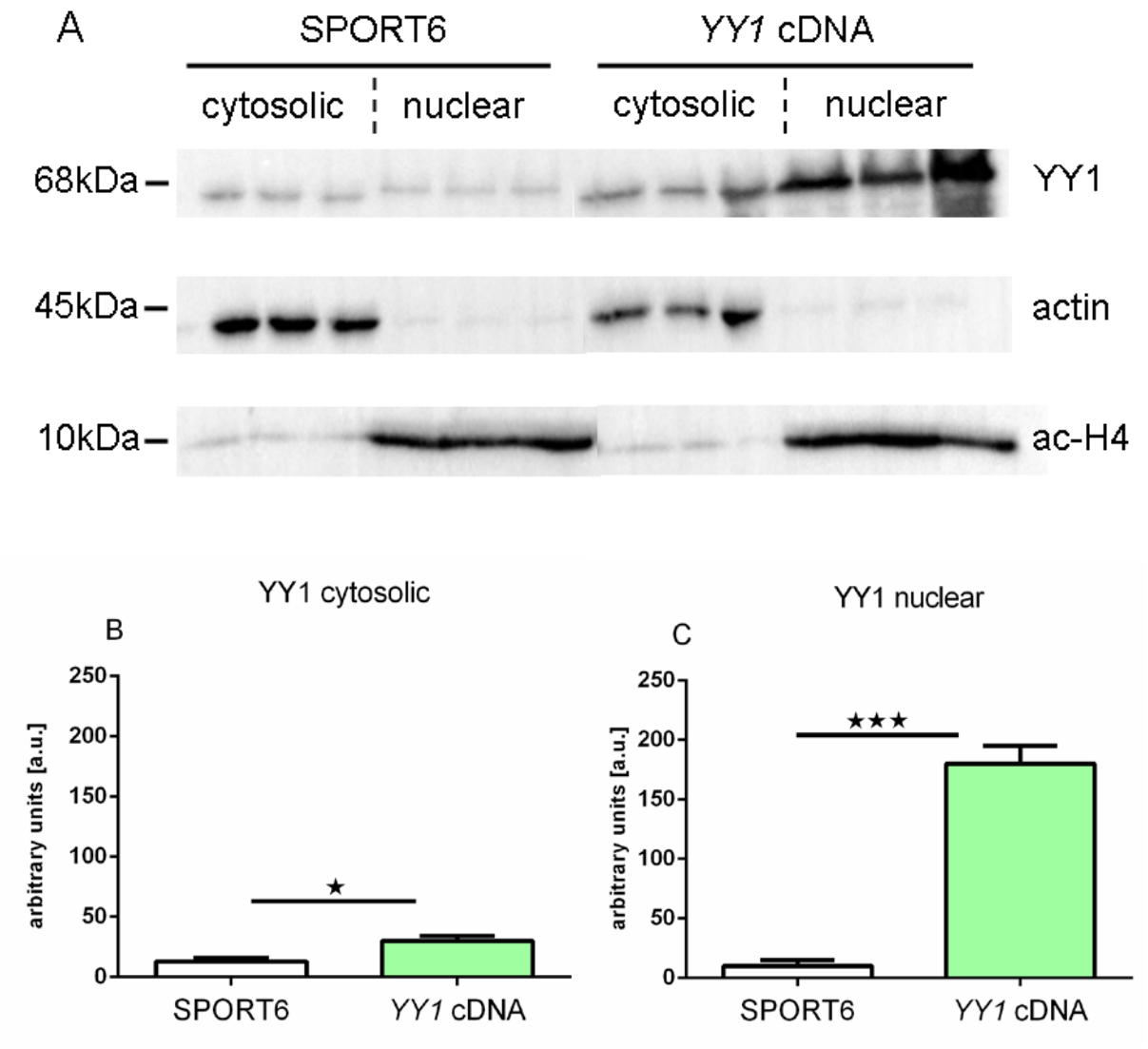

Fig 37: Amount of YY1 in the N2a cells after transfection with *YY1* cDNA. Western blot result (A) showed that *YY1* cDNA transfection in comparison to mock transfection (SPORT6) induced a significant increase in YY1 in the cytosolic (B) and nuclear (C) fractions of N2a cells. Bars represent means + SEM, n (SPORT6) = 6, n (YY1) = 6, ★ $p<0.05$, ★★★ $p<0.001$.

To study the interaction between YY1 and *Crhr1*, we measured the activity of the *Crhr1* promoter during YY1 overexpression. Therefore, a 1790bp construct containing all three predicted sites was co-transfected with *YY1* cDNA plasmid or with empty plasmid (SPORT6) as negative control. Luciferase activity measured three days later was almost 2.5-times higher in cells overexpressing YY1 ($p>0.01$), therefore, confirming an enhancing role of YY1 for *Crhr1* promoter activity (Fig. 39A).

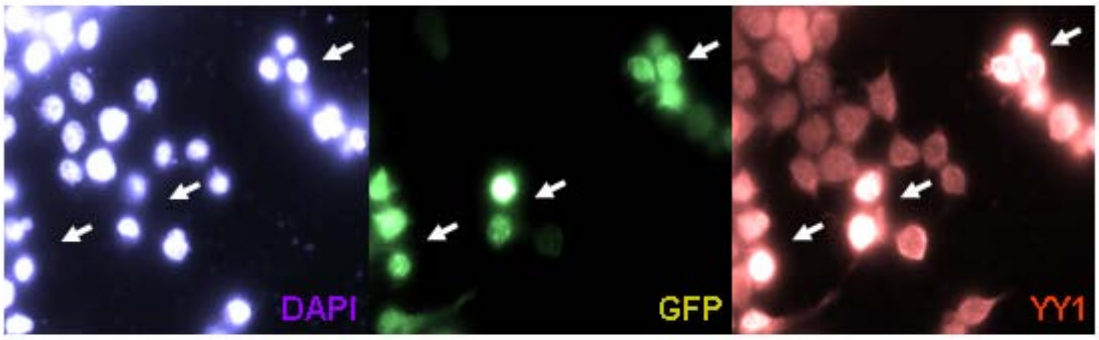

Fig 38: Immunofluorescent staining of cells transfected with *YY1* cDNA. Only *YY1* cDNA transfected cells (GFP marker) increased amount of nuclear (DAPI staining) YY1 (anti-YY1 staining) compared to ubiquitous expression. Arrows indicate the observed effect.

To show that YY1 induced enhanced *Crhr1* expression not only at promoter level, but also at the level of mRNA, we measured *Crhr1* expression in YY1 overexpressing and mock-transfected cells. Indeed, YY1 induced an almost 2-fold increase in *Crhr1* mRNA ($p<0.05$) (Fig. 39B).

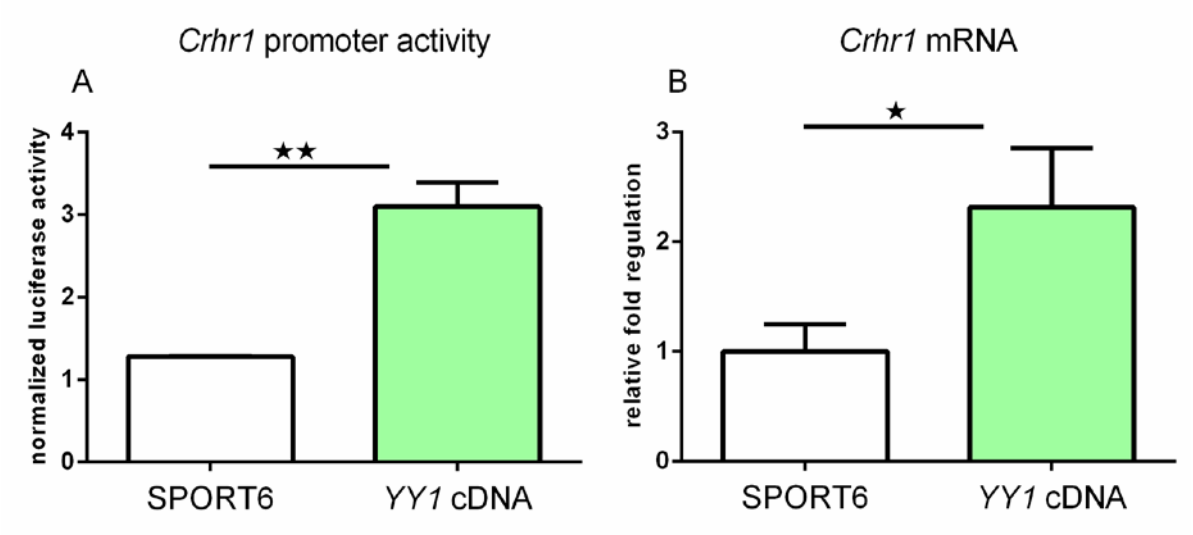

Fig 39: Effects of YY1 overexpression on *Crhr1* expression. YY1 overexpression enhanced *Crhr1* promoter activity in the luciferase assay (A) and induced higher *Crhr1* mRNA expression in the N2a cells (B). Bars represent means + SEM, n (SPORT6) = 7, n (YY1) = 7, ★ $p<0.05$, ★★ $p<0.01$.

4.6.2 Selective binding of YY1 to the recognition sequence close to CpG1

EMSA revealed that YY1 binds specifically to the recognition sequence close to CpG1.

We showed that YY1 exerted a regulatory role on the *Crhr1* promoter, however, it was not clear if the observed increase in *Crhr1* promoter activity was a direct effect of YY1 binding to the predicted recognition sequences or a result of indirect regulation through other transcription factors interacting with YY1. Moreover, we did not know if CpG1 or the other two predicted sequences act as the regulatory binding site for YY1.

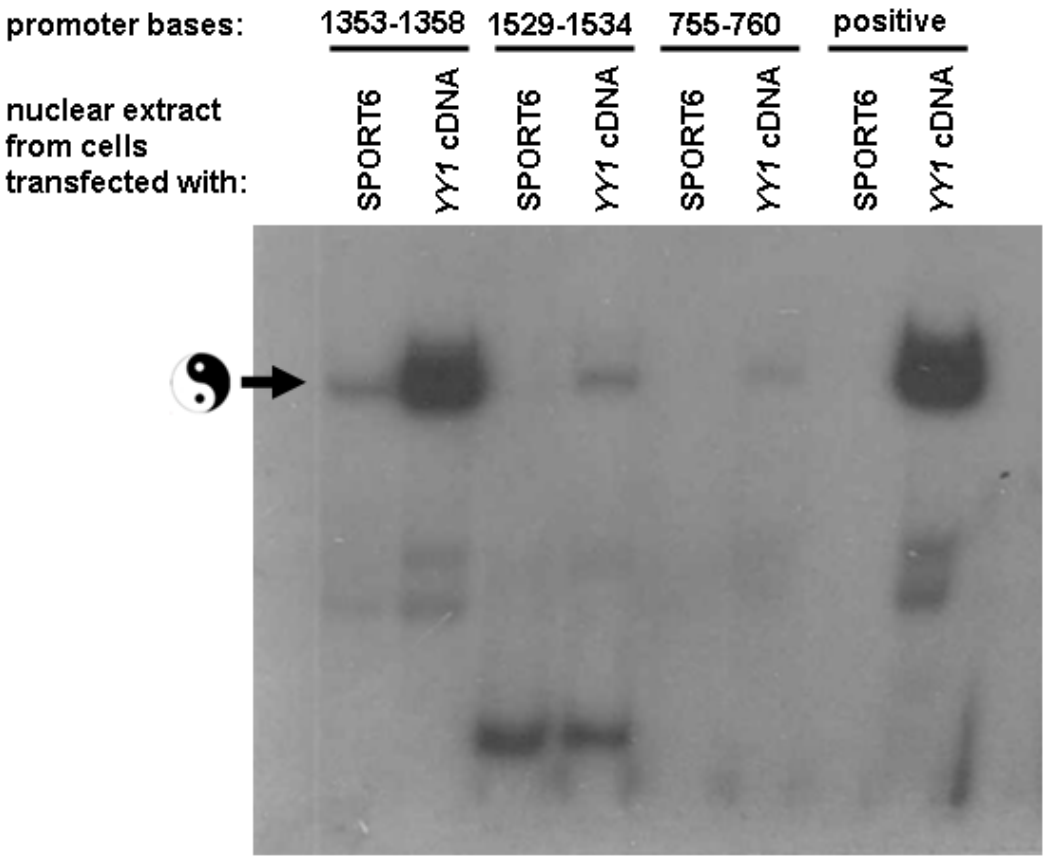

Fig 40: EMSA. YY1 bound preferably to the recognition site close to CpG1 (1353-1358), whereas binding to other positions (1529-1534 and 755-760) was not observed under basal conditions. Positive control corroborated correct identification of binding position.

To answer these questions, we performed EMSA, which showed that under basal conditions only the binding site close to CpG1 could interact with YY1, whereas the other two binding sites exhibited only very weak binding affinity, when YY1 was artificially overexpressed (Fig.40).

The intensity of YY1 binding was comparable to literature data, observed for binding of YY1 to the *Peg3* promoter (positive control was cloned based on Kim et al., 2003). Furthermore, we verified the specificity of binding to the predicted position: (i) increased amount of input proteins induced a higher band intensity, (ii) incubation with specific YY1-antibody, but not with unspecific antibody, prevented band formation, (iii) addition of unlabled probe in excess (competitor)

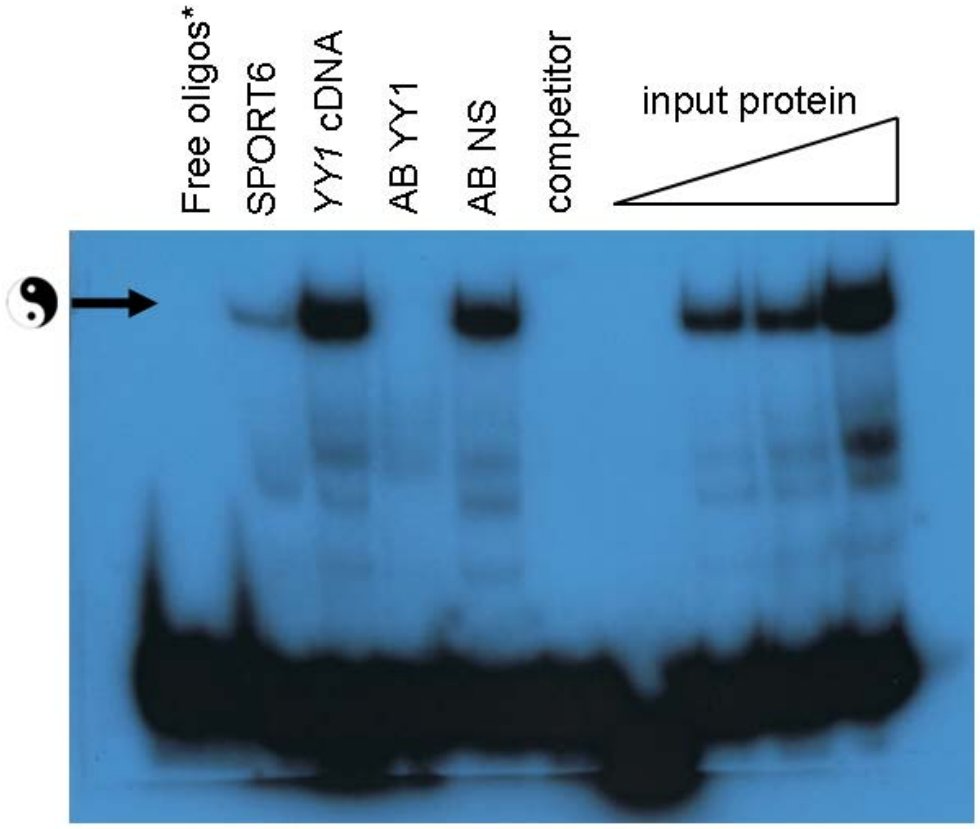

Fig 41: EMSA. YY1 bound specifically to the recognition site: increased band intensity was observed when higher amount of proteins was loaded (input protein); incubation with anti-YY1 specific antibodies (AB YY1) prevented band formation, whereas non-specific antibody had no effect (AB NS); addition of unlabeled probe in excess (competitor) altered band formation.

abolished band formation. Therefore, our experiments support the hypothesis that direct binding of YY1 to the recognition site close to CpG1 causes the observed effects of YY1 on *Crhr1* promoter activity (Fig. 41).

4.6.3 YY1 binds to *Crhr1* in a methylation-sensitive manner, but does not induce increase in methylation of CpG1

The methylation of CpG1 affected binding affinity of YY1 to DNA. Methylation-sensitive binding altered the increase of *Crhr1* promoter activity caused by YY1 overexpression.

To get an idea about the interaction between the CpG1 methylation and binding of the transcription factor YY1, we performed additionial EMSA. First of all, we tested binding of YY1 to the P^{32}-labled oligonucleotide probes with either methylated or mock methylated CpG1 during EMSA. We did not observe a complete prevention of binding of YY1 (Fig. 42A), but affinity of the transcription factor to DNA was reduced significantly ($p<0.05$) (Fig. 42B).

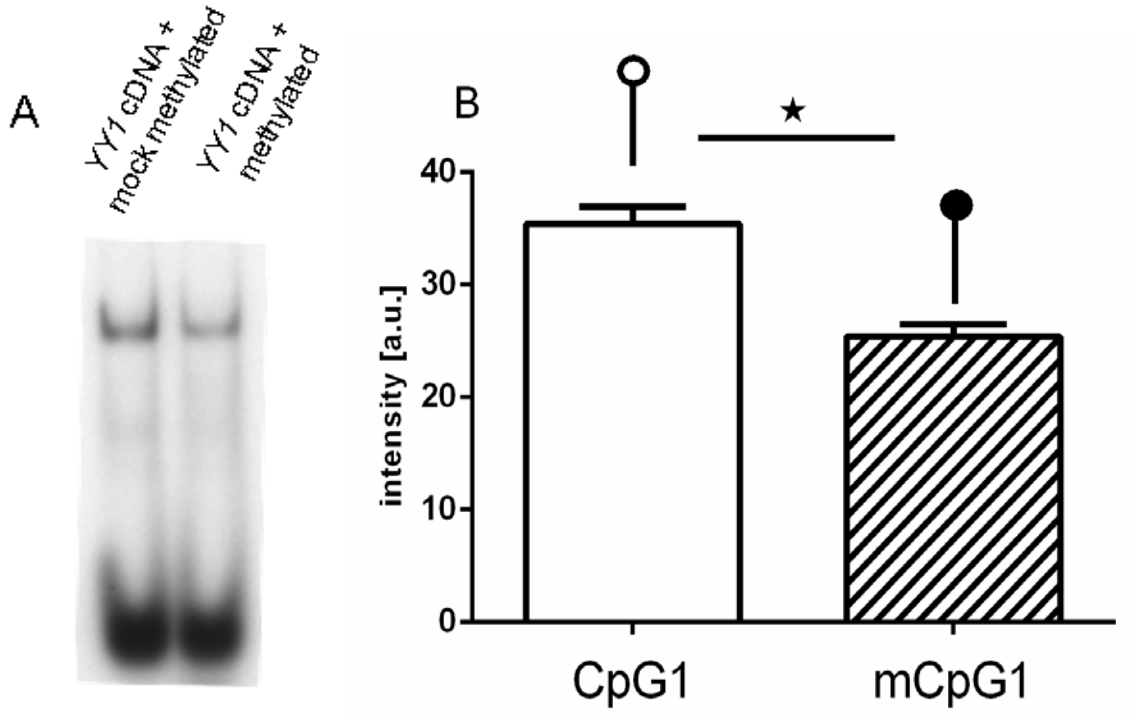

Fig 42: Methylation sensitive binding of YY1. Lower binding affinity of YY1 to the probe with methylated CpG1 (mCpG1) (A), band intensity was evaluated to quantify the difference (B). Bars represent means + SEM, n (biological replicates) = 3, ★ $p<0.05$.

We evaluated the role of CpG1 methylation on promoter activity by using a luciferase assay after YY1 overexpression. YY1 induced significantly lower activation of *Crhr1* promoter when CpG1 was methylated in comparison to a non-methylated state. However, activity of the promoter after YY1 overexpression - even in the presence of methylated CpG1 - was still higher compared to samples transfected with an empty vector ($p<0.05$) (Fig. 43). Moreover, methylation of all CpGs of the *Crhr1* promoter using *SssI* enzyme induced a similar reduction ($p<0.05$) as methylation of merely CpG1 site (Fig. 43), thereby corroborating the critical role of CpG1 in the YY1 methylation-sensitive induction of promoter activity.

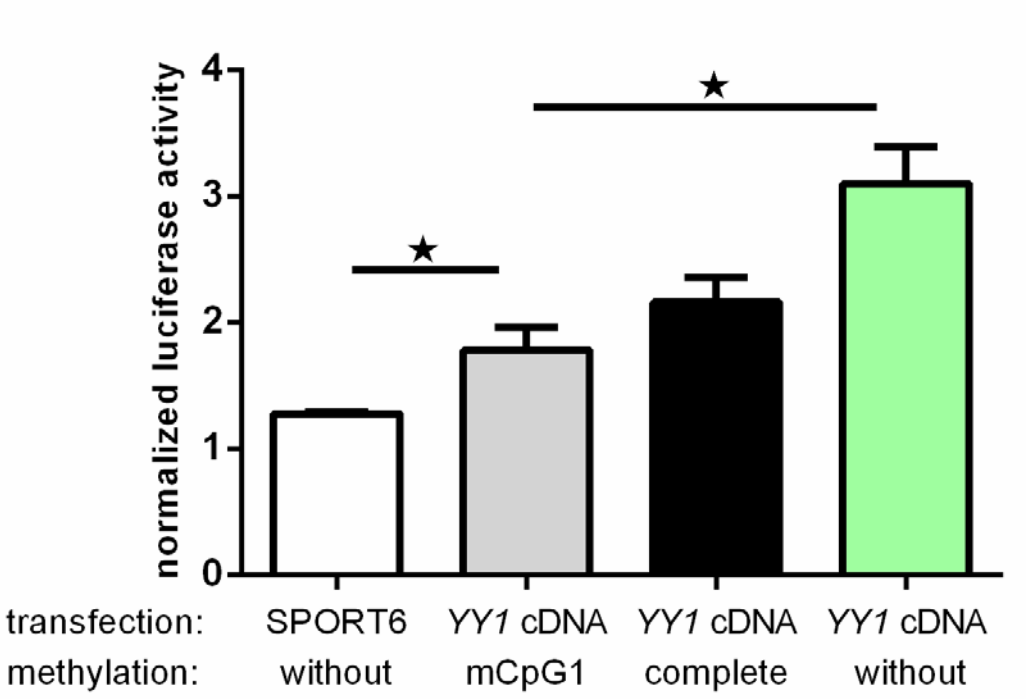

Fig 43: Methylation-sensitive regulation of the *Crhr1* promoter activity by YY1. YY1 induced significantly lower activation of the *Crhr1* promoter when the CpG1 site was methylated (mCpG1), however complete methylation induced a similar reduction in YY1-mediated promoter activation. Bars represent means + SEM, n (biological replicates) = 3-6, ★ $p<0.05$.

Although we showed the importance of CpG1 methylation for the regulation of *Crhr1*, the origin of setting this epigenetic mark is unknown. Thus, a study from Kim *et al*. (2009) suggested that YY1 can participate in *de novo* methylation. Therefore, we

analyzed CpG1 methylation after YY1 overexpression in N2a cells and have not found any difference between mock and YY1 cDNA transfected cells (p>0.05) (Fig. 44).

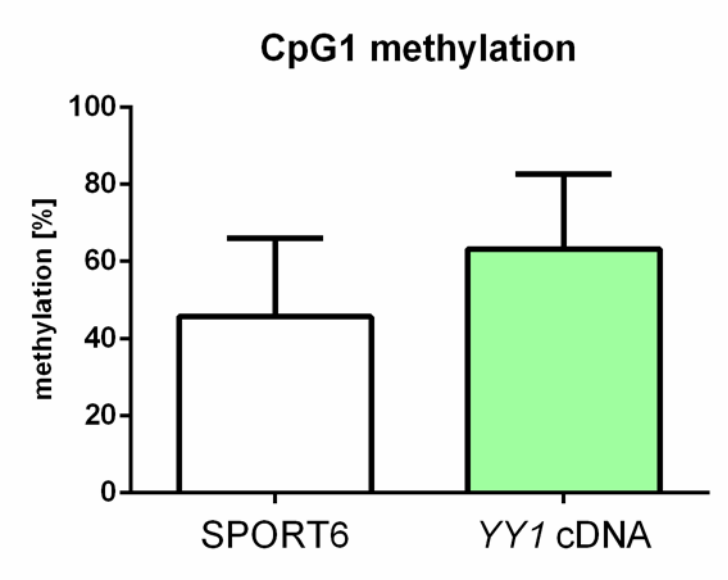

Fig 44: Role of YY1 in CpG1 methylation. YY1 overexpression had no impact on CpG1 methylation. Bars represent means + SEM, n (SPORT6) = 6, n (YY1 cDNA) = 6.

4.7 Possible epigenetic mechanisms contributing to different basal *Crhr1* expression in the BLA of HAB/LAB mice

4.7.1 Absence of genetic variability in *Crhr1* gene between HAB and LAB mice

No SNPs and CNVs were found in the *Crhr1* gene, thereby minimizing the contribution of genetic factors in regulation of *Crhr1*.

Above, we presented possible mechanisms contributing to altered *Crhr1* expression after chronic stress exposure; however, the driving force behind the different basal expression of *Crhr1* between HAB and LAB mice is still unknown. Sequencing of the *Crhr1* promoter did not reveal any SNPs that could contribute to different *Crhr1* regulation (Markt, 2012). We also analyzed possible differences in CNV of the *Crhr1* gene between HAB and LAB mice and did not find any variation (p>0.05) (Fig. 45) in this locus. By doing so, we minimized the contribution of genetic factors and studied further epigenetic mechanisms behind the differential *Crhr1* regulation.

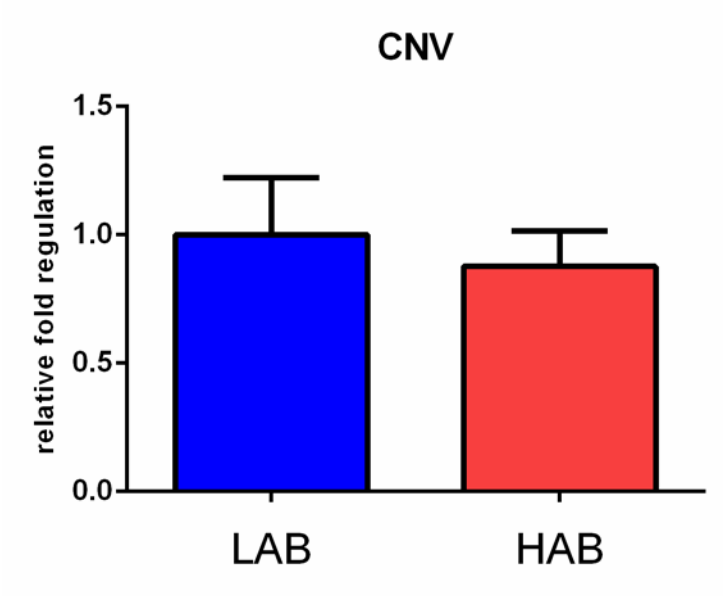

Fig 45: No difference was observed in CNV of *Crhr1* between HAB/LAB mice. Bars represent means + SEM, n (LAB) = 7, n (HAB) = 7.

4.7.2 Possible role of miRNA34 family

miRNA34 were reported to regulate *Crhr1* expression. We observed increased amount of *miRNA34a* in the BLA of LAB mice that could drive lower *Crhr1* expression.

Literature data suggests (Chen et al., 2011; Haramati et al., 2011) a critical role of the miRNA34 family in the regulation of *Crhr1* and *YY1* expression. We analyzed the expression of three miRNAs of this family between HAB and LAB mice: miR34a, miR34b, miR34c. Significantly higher expression was found in the expression of *miRNA34a* in the BLA of LAB compared to HAB mice ($p<0.05$), however, no difference was observed in the amount of *miRNA34b* and *miRNA34c* ($p>0.05$ both) (Fig. 46). Moreover, we analyzed expression of *Dicer*, an enzyme important for miRNAs maturation and formation of the silencing complex. In contrast to *miR34a*, we found significantly lower amounts of *Dicer* mRNA in LAB mice (Fig. 46). Thus, our data suggest that basal *Crhr1* expression difference between HAB and LAB mice might be strongly affected by different amount of *miRNA34a*.

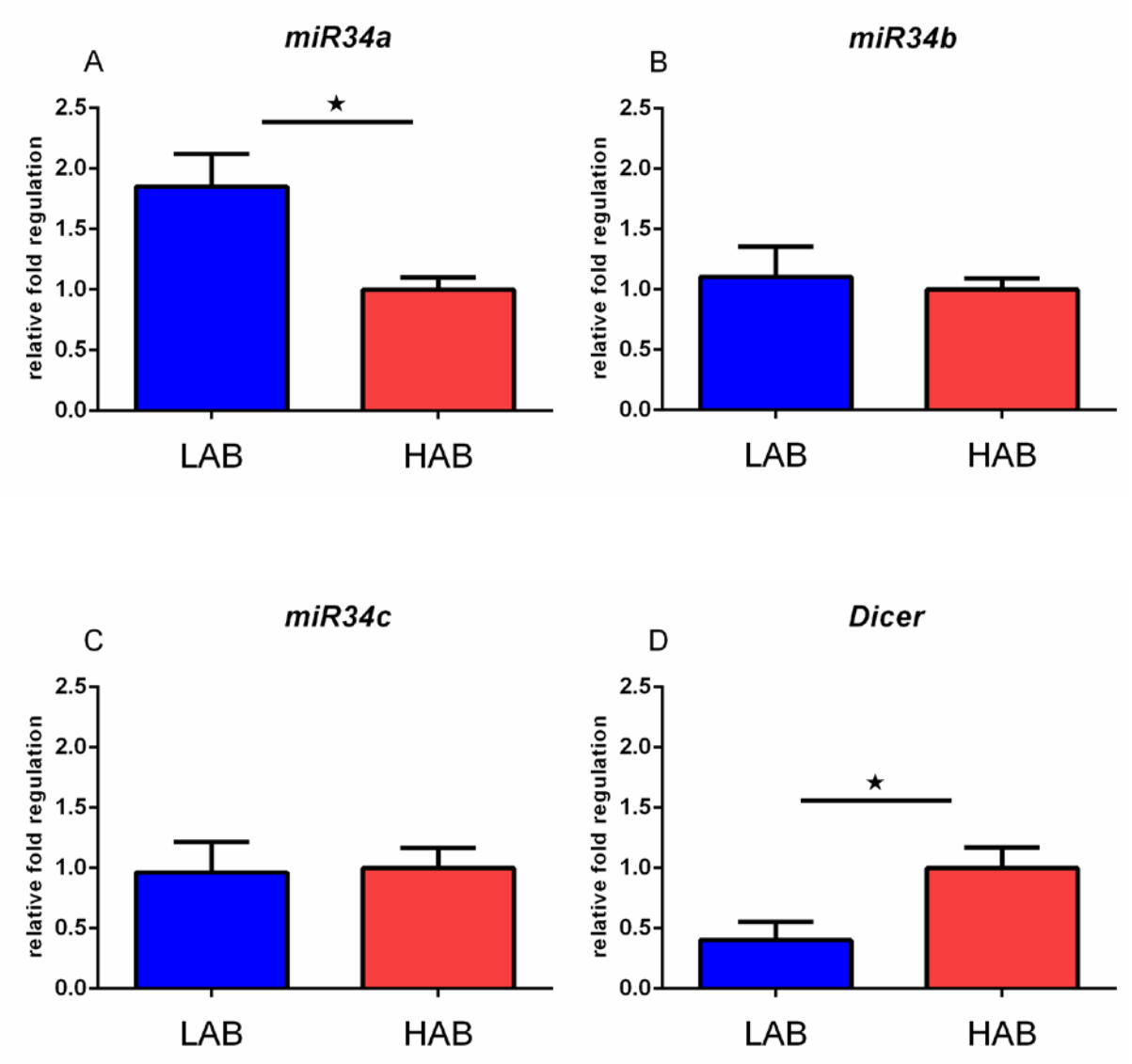

Fig 46: Expression of members of the miRNA34 family. Significantly higher expression of *miRNA34a* was found in the BLA of LAB compared to HAB mice (A), whereas no difference was found in expression of *miRNA34b* and *miRNA34b* (B,C). Amount of *Dicer* mRNA was lower in LAB mice compared to HAB (D). Bars represent means + SEM, n (LAB) = 7, n (HAB) = 7, ★ $p<0.05$.

4.7.3 Possible role of histone modifications

HDACi valproic acid increased *Crhr1* promoter activity and *Crhr1* mRNA expression *in vitro*, but did not have any impact on anxiety-related and depression-like behavior.

We did not observe any differences in the methylation of *Crhr1* at basal level. Therefore, we studied another non-sequence based epigenetic regulation – histone modifications. We tested if *Crhr1* promoter activity is changed upon treatment with

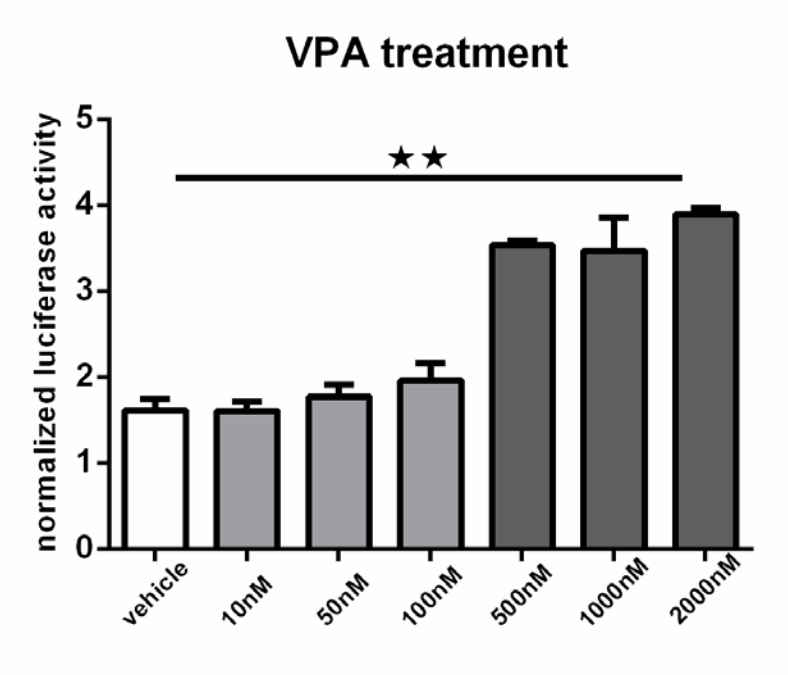

Fig 47: Effects of VPA on *Crhr1* promoter activity. VPA treatment induced a significant increase in *Crhr1* promoter activity when used at concentrations above 500nM (A). Bars represent means + SEM, n (vehicle) = 6, n (VPA) = 6, ★ $p<0.05$**,** ★★ $p<0.01$.

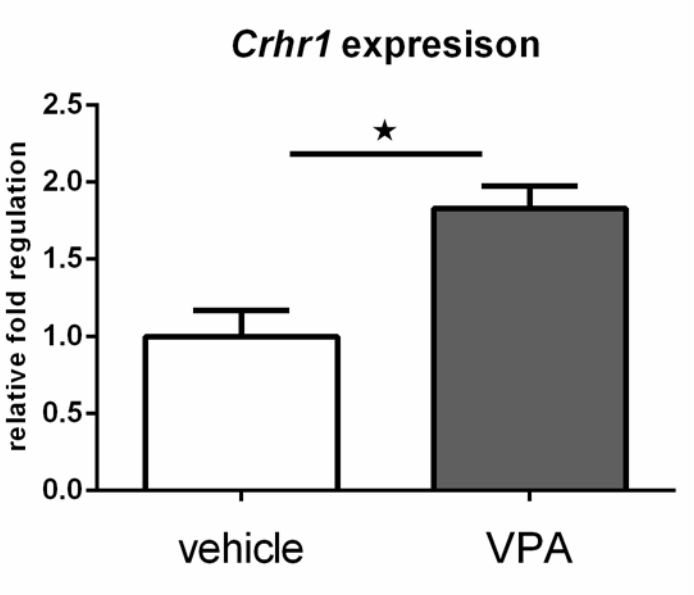

Fig 48: Effects of VPA on *Crhr1* expression *in vitro*. 24 hour incubation in the DMEM medium with 1000nM VPA induced higher *Crhr1* in N2a cells. Bars represent means + SEM, n (vehicle) = 6, n (VPA) = 6, ★ $p<0.05$.

HDACi valproic acid. Indeed, we observed a significant increase in luciferase activity when transfected cells were treated with 500nM or higher concentrations of VPA (for

all p<0.05) (Fig. 47). Furthermore, an increase in promoter activity after VPA treatment was also followed by an increase in *Crhr1* mRNA (p<0.05) (Fig. 48), corroborating a strong impact of HDACi on *Crhr1* expression.

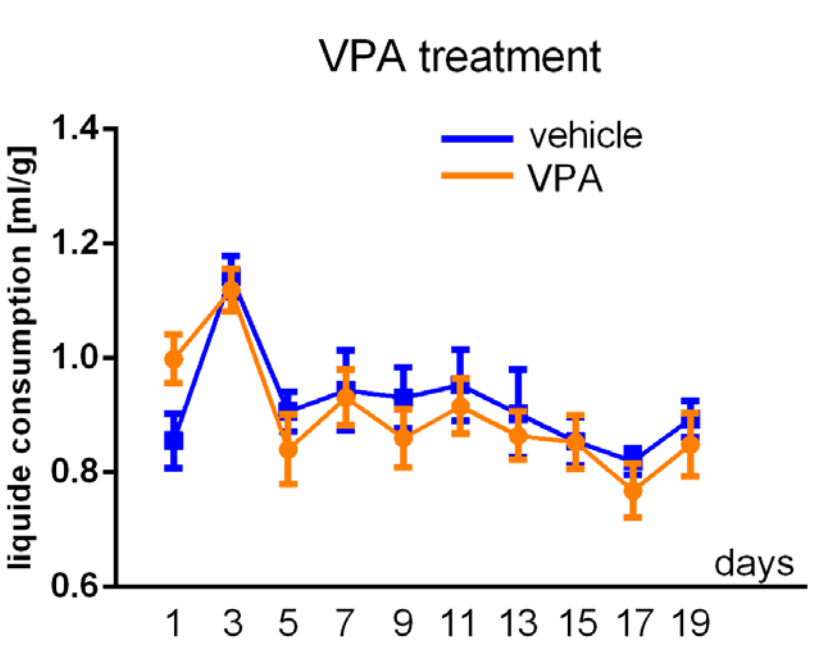

Fig 49: Liquid consumption. No difference was found in liquid consumption between VPA and vehicle treated groups, indicating no aversity to VPA solution consumption. Points represent means ± SEM, n (vehicle) = 6, n (VPA) = 10.

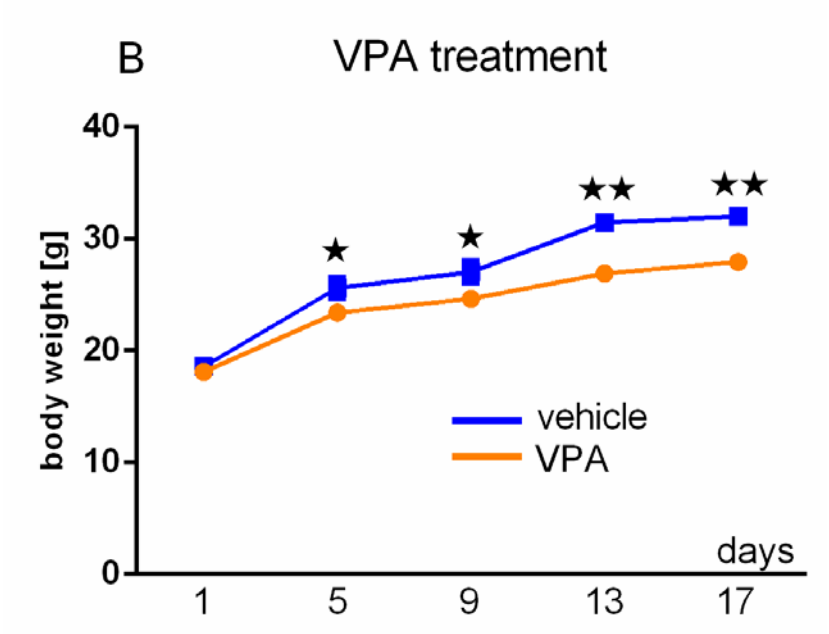

Fig 50: Body weight. VPA treatment significantly decreased body weight of animals. Points represent means ± SEM, n (vehicle) = 6, n (VPA) = 10, ★ p<0.05**,** ★★ p<0.01.

To evaluate the *in vivo* impact of HDACi, we treated LAB mice chronically with VPA via drinking water. Kruskal-Wallis ANOVA revealed no significant differences between VPA and vehicle treated groups ($p>0.05$ for all analyzed data) (Fig. 49), however, VPA administration induced a significant reduction of body weight (day 5 $p<0.05$, day 9 $p<0.05$, day 13 $p<0.01$, day 17 $p<0.01$) (Fig. 50).

We analized anxiety-related and depression-like behavior of mice from vehicle and VPA treatment groups using EPM, LDB, TST and FST. One-way ANOVA revealed no impact of treatment on any of measured paramenters. Results are summarized in Table 17.

Table 17: Detailed results of VPA treatment on anxiety-related and depression-like behavior. No difference was found in any measured parameters in the EPM, LDB, TST and FST.

	Parameter measured	LAB vehicle	LAB VPA	p-value
EPM	time spent on open arms [%]	72.9 ± 3.8	73.3 ± 4.4	0.948
	latency to enter open arm [s]	8.7 ± 1.6	8.6 ± 1.9	0.992
	open arms entries [n]	8.5 ± 1.0	8.0 ± 1.4	0.761
	total distance travelled [m]	14.6 ± 1.4	13.4 ± 0.7	0.488
LDB	time spent in light compartment [%]	43.1 ± 2.9	50.0 ± 5.0	0.079
	latency to enter light compartment [s]	49.3 ± 15.5	31.5 ± 3.9	0.346
	entries in light compartment [n]	8.4 ± 0.7	8.1 ± 1.0	0.782
	total distance travelled [m]	17.0 ± 1.3	15.4 ± 1.6	0.148
TST	immobility time [s]	19.5 ± 7.1	22.1 ± 8.0	0.965
	latency to first immobility [s]	271.3 ± 15.8	285.7 ± 15.0	0.457
	number of immobile episodes [n]	4.3 ± 1.5	5.7 ± 1.2	0.459
FST	floating time [s]	14.7 ± 6.3	8.9 ± 3.9	0.539
	latency to first floating [s]	206.9 ± 43.4	235.9 ± 45.0	0.719
	number of floating episodes [n]	4.8 ± 1.5	3.6 ± 0.9	0.844

5 DISCUSSION

5.1 TMT avoidance as a novel approach to evaluate anxiety-related behavior

Animal models of psychiatric disorders are a valuable and, so far, irreplaceable tool to investigate molecular mechanisms underlying psychopathology. However, it is illusory to create a certain behavioral phenotype that would precisely reflect a human disorder. Whereas a complex clinical picture can hardly be proven to exist in animals, certain endophenotypes could be successfully modelled in mice. Multiple tests were developed to assess anxiety-related behavior in rodents. Most of them are primarily based on an approach/avoidance conflict to novel, open, brightly lit spaces and their innate exploratory drive. However, mice are living in an olfactory determined world and, therefore, here we established a predator odor avoidance test to measure anxiety-related behavior in the HAB/LAB model.

First experiments on CD1 mice indicated that TMT, but not cat odor or butyric acid, elicited consistent avoidance behavior and provoked a dramatic activation of the HPA axis. TMT - a volatile component of fox feces - warns mice about a potentially dangerous area in natural environments. Therefore, TMT avoidance is an emotional anticipation of an aversive situation (predator encounter) and as such is close to trait anxiety (Landgraf, 2003). Moreover, only TMT induced a significant neuroendocrine response, corroborating aversive interpretation of the presented cue.

When HAB, NAB and LAB mice were exposed to TMT, a significant difference in odor avoidance was observed. HAB mice avoided to visit the chamber with the predator odor significantly more often than LAB mice, with NAB showing intermediate avoidance behavior. This is in line with our previous results, indicating stronger emotional anticipation of HAB mice of a variety of stimuli like air puff and light (Landgraf, 2007; Muigg, 2009). Importantly, the difference in response to TMT was not due to a deficit of the olfactory system, as LAB and NAB mice did not or only moderately avoid TMT, all lines still responded to both the pleasant odor of female urine and the repugnant odor of butyric acid. Interestingly, TMT exposure altered HPA axis to the same level in all three lines. Potentially, an application of a lower dose of TMT could reveal individual neuroendocrine reactivity in the respective lines.

Thus, here we extended differences reported earlier regarding anxiety-related behavior of HAB/LAB mice already evaluated by traditional behavioral paradigms (OF, EPM, LDB) by including another and more natural sensory modality - olfaction. Moreover, TMT provided the most accurate evaluation of change of anxiety-related behavior in LAB mice after chronic mild stress exposure, described in the next section.

5.2 Chronic mild stress increased anxiety-related and depression-like behaviors in LAB mice

Major life stressors are a main risk factor for the development of anxiety and depression in humans. Therefore, a CMS paradigm was introduced by Paul Willner in the late 1980s to model similar environmental influences in rodents. In essence, CMS consists of repeated exposure to an array of variable and unpredictable, mild stressors over a sustained period of time. The duration of the stress procedure differs from laboratory to laboratory, but usually is in the range of two to eight weeks. Although, considerable difficulties with the establishment of the relevant CMS model were reported (Willner, 1997; Porsolt and Papp, 1998), recent literature corroborates CMS effects on anxiety-related (D'Aquila et al., 1994; Griebel et al., 2002; Rössler et al., 2000) and depression-like (Griebel et al., 2002; Strekalova et al., 2004; Tannebaum et al., 2002) behavior, body weight (Isingrini et al., 2010; Sterennburh et al., 2011), cognitive functions (Cuadrado-Tejedor et al., 2011; Parihar et al., 2011), sleep (Cheeta et al., 1997; Henningsen et al., 2009; Li et al., 2008; Moreau et al., 1995;), locomotor activity (D'Aquila et al., 2000; Gorka et al., 1996; Katz, 1982) and sexual behavior (Brotto et al., 2001; D'Aquila et al., 1994; Gronli et al., 2005). The validity of the model is supported by the fact that chronic treatment with clinically-effective antidepressants reversed stress-induced effects (Willner, 1987; 2005). A number of studies indicated considerable differences in sensitivity to CMS between different strains of mice (Ducottet and Belzung, 2004; Griffiths et al., 1992; Pothion et al., 2004). The basis of this difference might lie in a genetic susceptibility to stressful events. However, it is not known to what extent genetic factors contribute to individual stress-reactivity. Thererfore, we tested here if CMS exposure can alter the genetically determined low anxiety-related behavior of LAB mice.

Indeed, we showed that 4 weeks of stress induced profound effects on behavioral and neuroendocrine characteristics of LAB animals. CMS-exposed mice exhibited

higher anxiety-related behavior in a variety of paradigms: stressed LAB mice spent less time on the open arms of the EPM, in the light compartment of the LDB and in the chamber harboring TMT odor. Interestingly, although all these tests revealed a significant increase in anxiety-related behavior after CMS, EPM estimated a treatment effect on the level of 21% (reduction in time spent on the open arms), whereas for LDB it was 41% (reduction in time spent in the light zone) and for TMT-avoidance 67% (reduction in time spent in the chamber with TMT). The higher efficiency of the TMT-avoidance test, underlining the necessity of the first part of this thesis, might be explained by the nature of a stressor, since a predator odor is a naturally occurring encounter involving the most sensitive sense of mice, olfaction, and thus, yield the most accurate representation of stress responses. On the other hand, a smaller treatment effect in the EPM might be due to increased robustness of this trait, as it was used for selective breeding. Moreover, Ducottet and Belzung (2004) reported that anxiety-related behavior in the EPM could only explain 31% of the CMS-induced effects.

Considering a complex comorbidity between anxiety and depression disorders (Stein and Heimberg, 2004; Yerevanian et al., 2001) and a pivotal role of stress factors in the genesis of depression (Paykel, 1994; Kessler, 1997), we also evaluated the effect of CMS on depression-like behavior of LAB mice. In both, the forced swimming and tail suspension tests, stressed mice exhibited higher depression-like traits, indicated by 62.5% and 78% increase in time spent immobile compared to controls, respectively. Moreover, we performed a stress-independent evaluation of depression-like behavior using a sucrose preference test. The lower preference of a bottle with sweet solution found in CMS exposed mice indicated higher anhedonic behavior in the stressed animals, one of the core symptoms of depression. Thus, our data suggest that CMS had a strong impact on depression-like behavior, which is in line with other studies that showed profound effects of CMS on parameters reflecting depression-like behavior, *inter alia* increase in anhedonic behavior (Willner et al., 1987; 1992; Katz, 1982), higher alcohol preference (D'Aquila et al., 1994; Smith et al., 1996), decreased sexual behavior (Brotto et al., 2001; D'Aquila et al., 1994; Gronli et al., 2005), decreased aggression (Gambarana et al., 2001; Ossowska et al., 2004; Pardon et al., 2000) and grooming (D'Aquila, et al., 2000; Santarelli et al., 2003).

Stress can alter homeostasis of certain body systems, whereas prolonged and maladaptive stressful conditions can change the functional tone of these systems and lead to dysregulation. Stress activates the sympatho-adrenomedulary (SAM) and HPA systems, therefore primary disturbance could be found in the components of these systems. Clinical data corroborate, that patients suffering from depression are characterized by elevated serum glucocorticoids (Holsboer, 2001; Krishnan and Nestler, 2008; Steckler et al., 1999), whereas some anxiety psychopathologies, like posttraumatic stress disorder (PTSD), are characterized by a hypersensitive feedback regulation of the HPA axis and, subsequently, a lower level of blood glucocorticoids (Bremner et al., 2007; Meewisse et al., 2007; Yehuda, 2009). Moreover, structural changes in the adrenal gland - one of the most important stress-responsive organs activated by both SAM and HPA systems - were reported in depressed (Amsterdam et al., 1987; Carroll et al., 1976; Nemeroff et al., 1992) and suicide (Dorovini-Zis and Zis, 1987; Dumser et al., 1998; Szigethy et al., 1994) patients. As a consequence of chronic stress exposure, an altered neuroendocrine regulation and eating behavior can lead to changes in the body weight. Thus, clinical studies indicate possible consequences of stress-related disorders on gain (Anderson et al., 2006; Pagoto et al., 2012; Petry et al., 2008; Pagoto et al., 2012) or loss (Andreasson et al., 2007; Bulik et al., 2007; Ohseik and Williams, 2011) of body weight. Consequently, the described features were analyzed in LAB mice exposed to CMS. Stressed animals exhibited elevated basal CORT levels, whereas in response to stress a remarkable shift from normal HPA axis reactivity was observed. More precisely, forced swimming induced higher CORT secretion 30 min after the test, however, stressed animals were characterized by stronger feedback regulation as indicated by a lower blood CORT level 60 min after swimming. Moreover, a significant body weight loss of CMS exposed mice was observed, whereas adrenal gland weight was increased, once again indicating systemic effects of stress exposure on the body.

Altogether, these data corroborate that the utilized CMS model induced changes on both behavioral and neuroendocrine levels, thereby recapitulating some key endophenotypes of anxious patients.

5.3 TMT exposure reveals a critical role of the basolateral amygdala for the anxiety shift after CMS

c-Fos expression is a widely used marker for mapping brain activity in response to external stimuli (Hughes and Dragunow, 1995; Sagar et al., 1988). Thus, earlier Muigg and colleagues (2009) found that HAB/NAB/LAB mice showed different patterns of *c-fos* expression after unavoidable exposure to the open arms of the EPM. Here, we used *c-fos* expression to identify brain regions that are primarily involved in changes of anxiety traits of LAB mice after CMS. Since TMT avoidance provided the most reliable estimation of anxiety-related behavior after CMS, we analyzed *c-fos* expression after odor exposure. We also compared the outcome with similar of HAB mice after TMT exposure, as an indicator of extremely high anxiety response.

Our data supports and extends earlier findings (Muigg et al., 2009), which indicate that stress induces stronger *c-fos* activation in most of the brain regions of HAB compared to LAB mice. Higher gene expression was found in the mPFC, PVN and Amy of HAB mice compared to LAB after odor exposure, whereas no difference was observed in the LC or DG. Lower expression of *c-fos* in HAB mice was found only in the CA1 region of the Hipp. When stressed LAB mice were exposed to TMT, increased *c-fos* compared to non-stressed animals was observed in the PVN and Amy, but not in the mPFC or any other brain region. These results indicate that changes in PVN and Amy activitied after CMS exposure reflect a shift in anxiety-related behavior along the anxiety continuum from LAB towards HAB. However, the PVN is known to participate in HPA axis activation and, therefore, could be primarily involved in the endocrine response to the odor rather than in the modulation of anxiety-related behavior. Indeed, a significant difference in blood corticosterone was found between the groups. TMT exposure induced a higher HPA axis response in HAB and stressed LAB mice compared to control LAB mice. Thus, higher *c-fos* expression in the PVN of HAB and stressed LAB mice could be addressed to higher HPA axis response after TMT exposure. Moreover, the finding that Amy activity reflects changes in anxiety after CMS was further supported by a recent electrophysiological study. Avrabos and colleagues (2013) used voltage-sensitive dye imaging to observe a lower signal propagation through the amygdala of LAB mice compared to HAB, whereas chronic stress exposure increased this measure in LAB.

Furthermore, since the Amy has a complex, but well defined structure with specific features of each nucleus, we analyzed which part(s) of the Amy contributed to the observed differences. We found that after TMT exposure LA and MeA exhibited insignificant increases in *c-fos* expression between control and CMS treated LAB mice, whereas significantly higher expression was observed in the CeA and BLA of stressed animals. This is in line with clinical studies indicating that patients suffering from generalized anxiety disorder may have abnormal activity generated in CeA and BLA (Etkin et al., 2009). Moreover, recently Tye and colleagues (2011) showed that an activation of BLA-CeA circuit is causally involved in controlling unconditioned anxiety-related behavior. Interestingly, HAB mice housed in EE showed opposite effects to CMS: decreased anxiety-related behavior after EE was followed by decreased *c-fos* expression in the BLA after TMT exposure (Sotnikov et al., submitted). These data are also in line with Nikolaev et al. (2002) reporting decreased c-Fos in the amygdala of EE rats after aversive conditioning. Moreover, other studies also found an important role of the BLA in response to predator odor (Day et al., 2004; Dielenberg et al., 2001; Vazdarjanova, 2001), whereas lesion of this nucleus prevented TMT induced avoidance (Müller and Fendt, 2006). The central role of the BLA in response to stressful stimuli is not surprising, since it is known that this structure has a broad modulatory role and, via sending projections to other brain regions, regulates specific behavioral responses (Campeau and Davis, 1995; LeDoux, 1993; Müller et al., 2003; Tye et al., 2011). Hence, it was proposed that the BLA is a major integrator and relay center for incoming and outgoing information and, thus, necessary for an adequate anxiety response and phenotypic plasticity. However, the molecular mechanisms behind this phenomenon are largely unknown.

CRH is the main mediator of amygdala activity, therefore, we hypothesized that a dysregulation of the amygdalar CRH system could be an underling mechanism of changes in the anxiety-related behavior and amygdala activity after CMS.

5.4 CMS increased expression of *Crhr1* and site-specific methylation of its promoter in the BLA

It was postulated that during stress exposure, CRH is released within the amygdala, and further activation of CRH receptors is a substrate for stress-induced alterations of affective behavior. Within the amygdala, the BLA and CeA have long been known

to regulate affective behavior (Davis, 1992; Hilton and Zbrozina, 1963; Sanders and Shekhar, 1991). However, components of the CRH system are not equally distributed between these structures. Thus, CRH-positive cell bodies were found in the CeA, but not in the BLA (Swanson et al., 1983; Van Pett et al., 2000), in contrast, the *Crhr1* receptor is almost not expressed in the CeA, whereas, in the BLA it is abundantly presented (Kühne et al., 2012; Van Pett et al., 2000). Therefore, it seems that CeA is a likely source of endogenous CRH, activating CRHR1 in the BLA, although both, BLA and CeA, also receive and send CRH projections to the bed nucleus of stria terminalis (BNST) (Shekhar et al., 2005; Swanson et al., 1983; Van Pett et al., 2000). Acute stress increases the amount of CRH in the CeA (Roozendaal et al., 2002) that later invades the BLA (Mountney et al., 2011; Roozendaal et al., 2002). The activation of CRHR1 in the BLA increases the excitability of neurons (Rainnie et al., 1992) and, therefore, it was suggested that an increase in neuronal activity in the BLA may contribute to stress-induced increase in the anxiety state (Rainnie et al., 2004). CRHR1 was found to be important for memory consolidation, since the injection of CRHR1 antagonists in the BLA reduced freezing after contextual fear conditioning (Hubbard et al., 2007). Moreover, chronic stress exposure induced a sustained increase of CRH in the amygdala (Cratty et al., 1995). Consequently, a repeated activation of CRHR1 in the BLA resulted in higher sensitivity of the neurons (Sanders et al., 1995; Saidyk et al., 1999; Saidyk and Gehlert, 2000), potentially via NMDA receptor-mediated calcium flux and activation of the calcium-calmoduling protein kinase II (CaMKII) cascade. Repeated intra-BLA injections of urocortin, a CRH receptors ligand, induced long-lasting changes in anxiety-related behavior that persisted for weeks (Rainnie et al., 2004). Furthermore, repeated stress exposure induced an increase of *Crhr1* in the amygdala, whereas injection of a CRHR1 antagonist prevented stress-induced behavioral changes (Gehlert et al., 2005). Finally, Roger et al. (2013) has recently reported about a strong association between anxiety-related behavior, amygdala activity and *Crhr1* in nonhuman primates.

Our *c-fos* (Sotnikov et al., submitted) and electrophysiological (Avrabos et al., 2013) data indicate a higher amygdala activity in CMS-treated compared to control LAB mice, especially in the CeA and BLA nuclei. Whereas most of the CRH effects are transmitted through CRHR1 in the amygdala, we hypothesized that different *Crhr1* expression in the BLA could be the underling mechanism causing changes in anxiety-related behavior and amygdala activity after CMS. We analyzed the amount

of *Crhr1* mRNA in the BLA of stressed and control LAB mice and indeed observed a higher expression of *Crhr1* in the CMS treated group. Earlier, Markt (2012) showed that there is a higher expression of *Crhr1* in the BLA of HAB compared to LAB mice, whereas the injection of a CRHR1 antagonist into the BLA of HAB animals induced anxiolytic effects. Moreover, decreased *Crhr1* expression and anxiety-related behavior were observed in HAB mice housed in EE. This is in line with Sztainberg et al. (2011) who also found that a decrease in anxiety-related behavior is mediated via lower *Crhr1* expression in the BLA of "normal" anxiety mice after EE or after lentiviral knockdown of *Crhr1* in the BLA. Altogether, our study (Sotnikov, Markt et al., submitted) extends all previous findings on *Crhr1* by showing that this gene exhibits bidirectional plasticity and can be shifted to the better or worse manner depending on the type of environmental manipulation. To our knowledge, this is the first example of such plasticity at molecular level.

We next studied the epigenetic mechanism behind this gene-environment interplay of *Crhr1* and CMS. Our *in silico* analysis identified a 2796bp long CpG island in the *Crhr1* gene, covering 1387bp of the promoter region, exon 1 and parts of intron 1. The presence of a CpG island grants gene regulation via DNA methylation. Moreover, we found, that expression of enzymes involved in maintenance (DNMT1) and *de novo* (DNMT3a) methylation were elevated after CMS exposure. This is in line with Matriscianno and collegues (2013), who reported that prenatal stress induced a long-lasting elevation of DNMT1 and DNMT3a in the frontal cortex and hippocampus.

Using pyrosequencing of bisulfite-treated DNA we were able to identify a differently methylated CpG site in the promoter of *Crhr1*. We found that stress induced an increase in methylation of CpG1 (-1348bp from transcription start site), whereas no difference in methylation was observed in any other CpG. Interestingly and unexpectedly, the same position was also found to be higher methylated in HAB mice housed in EE compared to control HAB and LAB mice (Markt, 2012). Moreover, we used an available data base (Ensemble Genome Browser) of a whole genome bisulfite sequencing to compare methylation of *Crhr1* CpGs of embryonic stem cells (ES) and a nasopharyngeal carcinoma cell line (NPC) and found that CpG1 is one of the differently methylated regions (DMR) between the cells, highlighting the importance of this site in epigenetic control. We further evaluated the functional importance of methylation at this position on promoter activity. We cloned a part of

the promoter carrying CpG1 in the CpG-free luciferase expressing vector and subjected it to either a complete or a site-specific methylation. Both assays confirmed a profound role of methylation on promoter activity, remarkably site-specific methylation (SSM) of CpG1 alone was enough to reduce the reporter gene expression. These data indicate that methylation of CpG1 provides a highly-sensitive biological approach to the regulation of *Crhr1* gene expression.

Whereas recent literature supports that both adding (McGowan et al., 2009) and removing (Murgatroyd et al., 2009) methyl groups are active and reversible processes (Ito et al., 2011) directly involved in the regulation of anxiety-related behavior, there is no consensus whether a complete or a SSM is more important for gene regulation. Recent studies reported that environmental treatments induced gross changes in methylation of stress-related genes, *inter alia GR* (Weather et al., 2004), *BDNF* (Roth et al., 2009), *Crh* (Elliot et al., 2010), *AVP* (Murgatroyd et al., 2009), whereas later studies, on the contrary, reported the importance of site-specific methylation of *GR* (McGowan et al., 2009), *BDNF* (Martinowich et al., 2003), *Crh* (Chen et al., 2012) and *Oxytocin* (Mamrut et al., 2013). However, dramatic changes in methylation of promoters usually occur under pathological conditions like cancer (Bergman and Cedar, 2013; Johnson et al., 2012), whereas SSM, from a biological perspective, provides an elegant and low-cost energy way to adjust gene expression to present needs. Thus, SSM of the *GR* promoter influences binding of the transcription factor NGFI-A (McGowan et al., 2009), methylation of the *Crh* occurs at CpGs around a cAMP responsive element (CRE) and effects binding of the phospho-cAMP responsive element-binding protein (pCREB) (Chen et al., 2012), SSM of CpGs of the *BDNF* regulates the activity of calcium-responsive element 1 (CaRE 1), CRE and upstream stimulatory factor-binding site (E-box) (Martinowich et al., 2003). Based on these data, we performed *in silico* analysis and found a recognition sequence for binding of transcription factor YY1 close to the CpG1. Therefore, we tested the interaction between CpG1 methylation and YY1 binding.

5.5 Binding of the transcription factor YY1 enhanced *Crhr1* promoter activity in a methylation-sensetive manner

The transcription factor YY1 is a multifunctional protein that has a fundamental role in cell proliferation, differentiation and apoptosis (Sui et al., 2004; Vega et al., 2005). *YY1* is highly expressed in the brain tissue and plays an important function in

neuronal development (Donohoe et al., 1999; Morgan et al., 2004), differentiation (Affar et al., 2006; Seo et al., 1996; Yang et al., 1998) and myelination (Berndt et al., 2001; He et al., 2007). There is also evidence on a possible role of YY1 in the development of Alzheimer disease (Rossner et al., 2006; Zambrano et al., 1997) and autism (Benayed et al., 2005; Brune et al., 2008). To date, no information is available about a possible involvement of the transcription factor in the psychopathology of anxiety and/or depression. Here, we measured *YY1* expression and found almost two times higher amounts of this transcription factor in the BLA of CMS treated LAB mice compared to controls, highlighting a possible role of YY1 in stress-related disorders.

YY1 regulates a large number of genes, approximately 10% of the total mammalian gene set (Gordon et al., 2006; Schug et al., 2005). However, what distinguishes this transcription factor from others is its ability not only to initiate, but also to regulate transcriptional activity (Gordon et al., 2006). Moreover, YY1 can simultaneously serve as a transcriptional activator and a silencer. This dual role is well illustrated in the example of autoregulation of expression through its own promoter binding. Thus, when the level of protein is normal, YY1 induces the activation of its own promoter; however, high levels of the protein inverse the mechanism and YY1 becomes a repressor of its own promoter (Kim et al., 2009). Our experiments supported the activatory role of YY1 on *Crhr1* promoter activity. The transfection of N2a cells (neuronal cell culture) with a plasmid carrying *YY1* cDNA induced a significant accumulation of YY1 within the cell nuclei. The increase of transcription factor stimulated a higher *Crhr1* promoter activity and, consequently, a higher *Crhr1* mRNA expression. Current literature suggests three mechanisms driving YY1-induced transcriptional activation: (i) a direct activation via the association with TATA-binding proteins (Nguyen et al., 2004), (ii) an interaction with cellular factors unmasking YY1 activation domain (Thomas and Seto, 1999), (iii) a recruitment of other activating transcription factors (Lee et al., 1995; 1998; Thomas and Seto, 1999). On the other hand, there are also three models explaining YY1-induced transcriptional silencing: (i) presence of overlapping binding sites of other transcription factors that compete with YY1 for occupancy (TF-DNA competition) (Shi et al., 1991), (ii) interference with other transcription factors independent of physical interactions with DNA (TF-TF interaction) (Guo et al., 1995; Galvin and Shi, 1997), (iii) recruitment of corepressors that directly act to facilitate transcriptional repression (TF-corepressor interaction)

(Thomas and Seto, 1999; Weill et al., 2003). Thus, we were interested in knowing if the observed effect of YY1 on promoter activity is indeed a result of YY1 binding to the recognition sequence close to CpG1 (direct activation) or if it is a product of interaction with other transcription factors regulating *Crh1* expression (indirect activation). Our results clearly indicated the direct binding of YY1 to the *Crhr1* promoter via EMSA. Moreover, binding of YY1 to other *in silico* predicted position was not observed under basal conditions, highlighting the importance of the recognition site at CpG1 in the YY1-mediated effects.

Although many studies indicate the involvement of YY1 in epigenetic regulation of transcriptional activity, there is a considerable controversy concerning the methylation-sensitive binding of YY1. Thus, one of the earliest studies, investigating

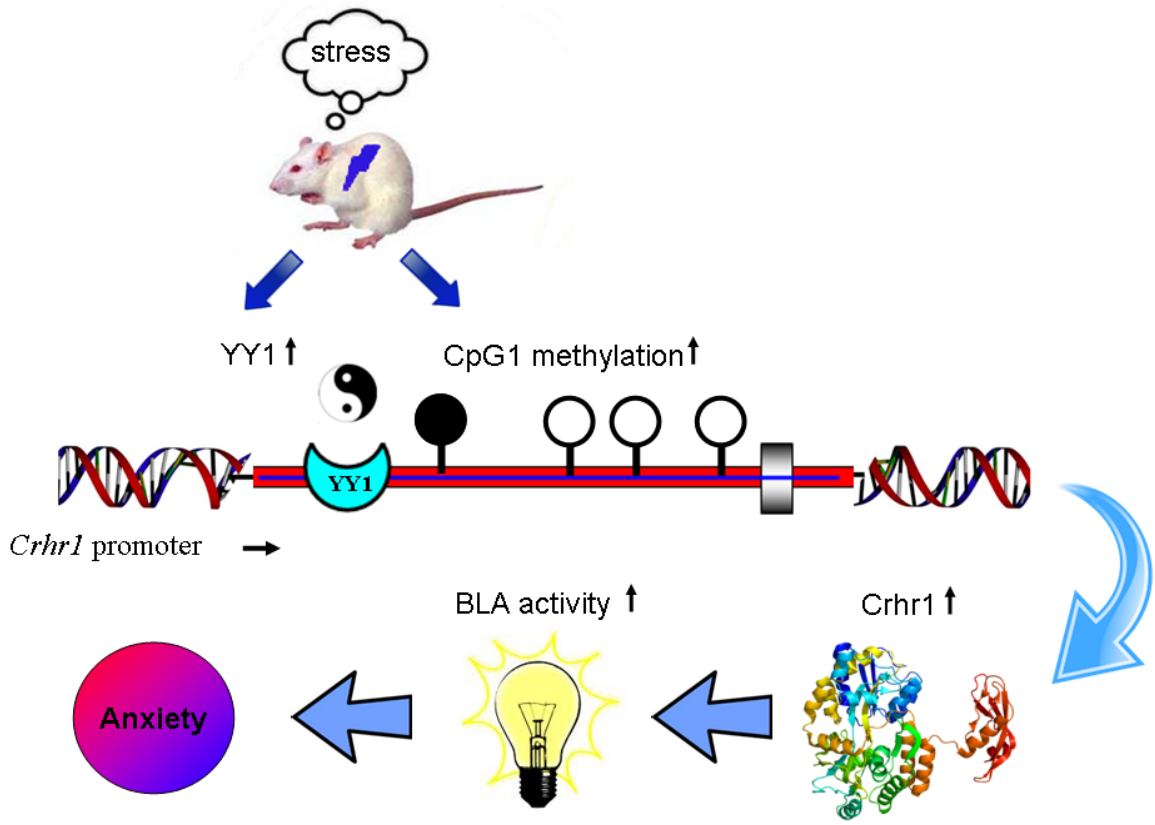

Fig. 51: Hypothesized cascade of events caused by chronic stress exposure. CMS induces an increase in *YY1* expression and CpG1 methylation. This leads to the increase in *Crhr1* expression in the BLA and, consequently, higher activation of the structure during stress exposure. Through the projections of the BLA to other brain structures, increased anxiety-related behavior is the consequence.

methylation-sensitive binding of YY1, reported that, although methylation plays an important role in gene silencing, it does not exert any impact on the binding ability of YY1 (Gaston and Fried, 1995a; 1995b).

However, later studies provided contadicting examples like methylation-sensitive, binding of YY1 (Kim et al., 2003; Sekimata et al., 2011). Our experiments found that methylation of CpG1 did not completely abolish the binding of YY1, but significantly reduced the affinity of the transcription factor to the DNA. On the functional level, YY1 overexpression induced an increase in promoter activity in both conditions, with methylated CpG1 and without, however, a significantly higher induction was observed in the absence of methylation. Importantly, this was a specific feature of the CpG1 methylation, since a complete methylation of the promoter did not induce a stronger reduction of YY-induced promoter activation. Furthermore, Kim *et al.* (2008, 2009) studied the role of YY1 in controlling of imprinting domains of several genes. They found that a high incidence of lethality among conditional YY1-knockout mice could be explained by a higher expression of several imprinted genes during oocyte and blastocyst stages. These changes in expression were a result of loss of DNA methylation at the imprinting control regions. Thus, the authors speculated that YY1 could have an important role in *de novo* DNA methylation. The same effects were observed using N2a cells, where YY1-knockdown resulted in methylation changes of several imprinted genes (Kim et al., 2007). Therefore, since YY1 reduction leads to the loss of methylation, we hypothesized that higher *YY1* expression after CMS could induce CpG1 methylation of the *Crhr1* promoter. Thus, we overexpressed YY1 in the N2a cells and analyzed methylation of CpG1. No effect of elevated transcription factor on CpG1 methylation was observed, suggesting another mechanism of regulation of this epigenetic mark in response to external environmental influences.

Altogether, we showed that a higher expression of YY1 after CMS can lead to increased *Crhr1* expression, whereas the exact role of methylation of CpG1 is obscure; however, it could play an important role in fine-tuning YY1-induced effects. Our results might provide a possible mechanism of environmentally driven *Crhr1* regulation. However, the final cause of different expression of *Crhr1* between HAB and LAB mice is still unknown. Based on the observed role of epigenetic factors in the regulation of *Crhr1*, the next section addresses alternative mechanisms that could contribute to *Crhr1* expression.

5.6 Other possible epigenetic mechanisms contributing to *Crhr1* expression

The impact of SNPs (Czibere, 2008) and CNV (Brenndörfer, 2013) on different gene expressions between HAB and LAB mice was extensively studied during the last decade. However, we have not found any SNPs in the promoter region, nor different CNV between HAB/LAB in this genomic locus, which could regulate *Crhr1* expression. Therefore, based on available literature data, we analyzed possible non-sequence based mechanisms likely contributing to *Crhr1* expression.

A recent study by Haramati et al. (2011) found that acute and chronic stress induced increased miRNA34c (miR34a) - a member of miRNA34 (miR34) family - in the CeA. Lentiviral-mediated overexpression of miRNA within the CeA decreased anxiety-related behavior. Authors suggested that one of the primary targets of miRNAs of this family is *Crhr1*.

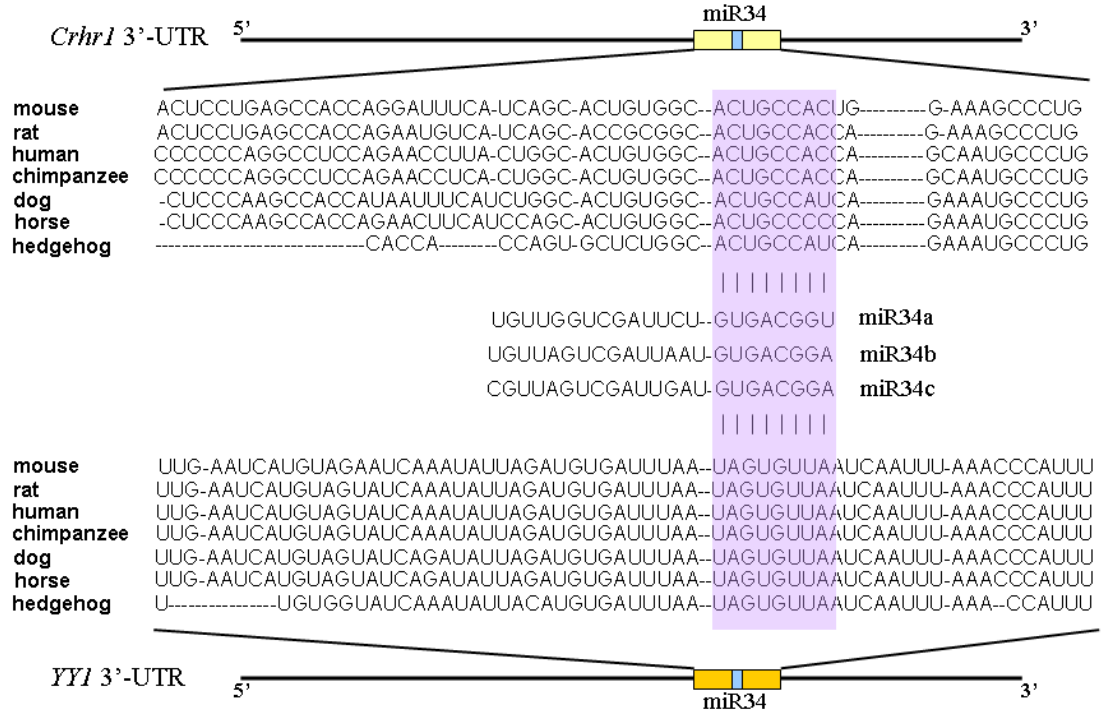

Fig. 52: Evolutionary conserved binding site for miRNA34 family on *Crhr1*-3'UTR and *YY1*-3'UTR found using TargetScan (http://targetscan.org).

Luciferase assays proved that binding of miR34 to the evolutionary conserved seeding site on the 3'UTR of *Crhr1*, indeed, exerted a regulatory role on gene expression and CRH signal transduction. Furthermore, a separate study by Chen and colleagues (2011), using systematic proteome analysis, demonstrated that the expression of *YY1* and its downstream proteins is reduced by miR34a. miRNA directly targets *YY1* through miR34a-binding site within the 3'UTR region. Altogether, these data indicate that miR-34a could be an important player regulating both, *Crhr1* and YY1. We analyzed the expression of miR34 family members within the BLA of HAB and LAB mice and found a significant up-regulation of *miR34a*, whereas no difference was found in expression of *miR34b* and *miR34c*. Thus, a higher expression of *miR34a* could directly regulate *Crhr1* expression in the BLA, explaining a different gene expression. Although, we have not found differences in *YY1* expression in the BLA and the amount of YY1 protein in the complete amygdala between HAB/LAB mice, it is not known how this miRNA is regulated after CMS and what role it plays in stress-induced *YY1* expression. Moreover, the expression of *Dicer*, an enzyme involved in miRNA maturation and miRNA-induced silencing complex (RISC) formation, was down-regulated in LAB compared to HAB, what is opposing the proposed action suggested by Haramati and colleagues (2011), namely anxiogenic effects of *Dicer* ablation.

As it was discussed earlier, binding of YY1 attracts other co-factors, which regulate the coupling of the transcription machinery with DNA together. The HDAC2/1 complex was identified as one of these co-factors (Yang et al., 1996; 1997). Furthermore, some studies suggested that YY1 is critical in regulating histone genes (Eliassen et al., 1998; Last et al., 1999). Thus, using available ChIP-seq data (Ensemble Genome Browser) performed on embryonic stem cells (ES) and nasopharyngeal carcinoma cells (NPC), we analyzed a possible impact of histone modification on *Crhr1* regulation. The comparison of two cell lines revealed that (i) the "histone code" of *Crhr1* differs significantly between the cells, (ii) 2kb (above TSS) of the promoter is the most vulnerable region for histone modification. Thus, we tested the impact of HDACi on promoter activity and *Crhr1* expression. We showed here that treatment with valproic acid (VPA), a well studied HDACi, induced a significant increase in both *Crhr1* promoter activity and mRNA. Interestingly, clinical studies found that VPA can be used as a mood-stabilizer. Our earlier study (Markt, 2012) showed that six injections of valproic acid within two weeks increased anxiety-

related behavior in HAB mice. Therefore, we tested here the effect of VPA on behavior of LAB mice. However, literature data suggest considerable uncertainty regarding the way of administration of VPA due to its pharmacological properties. Based on literature data (Löscher and Nau, 1982) treatment with 500µg/ml solution should consistently maintain brain VPA concentration at 140ng/ml (almost 2 times higher minimal effective dose in the cell culture). In line with a similar study (Löscher

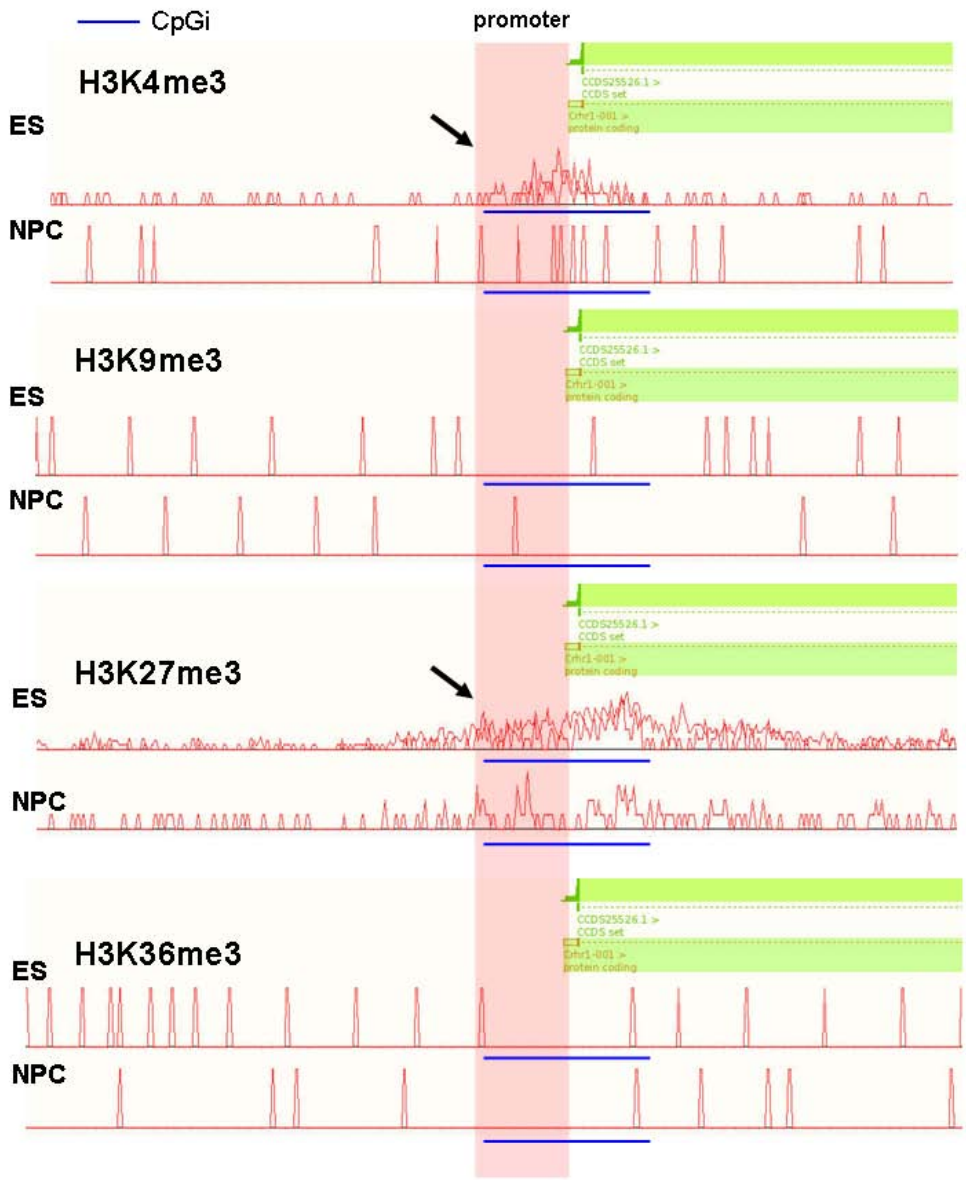

Fig. 53: ChIP-seq data (Ensemble Genome Browser) on embryonic stem cells (ES) and nasopharyngeal carcinoma cells (NPC) suggest 2kb promoter region to be vulnerable for regulation via histone modifications.

and Nau, 1982), chronic treatment with VPA reduced body weight of LAB mice, however, it did not effect liquid consumption per gram of body weight. Behavioral testings after 4 week of administration revealed no impact of treatment on any measured anxiety-related and depression-like parameter. The negative result can be explained by common action of VPA on expression of *Crhr1* as well as other genes in different brain regions, which ablate amygdala-mediated *Crhr1* effects. New studies investigating intra-amygdala injection of VPA could shed light on this question.

Moreover, anxiogenic effects of VPA in HAB mice together with the absence of any effect in LAB suggest that VPA has a specific action (if any) only for a particular anxiety disorder, whereas other pathological conditions could be insensitive to such treatment. In any case, our study suggests the need of identification and strict classification of psychopathologies, which could be treated using VPA.

5.7 Summary and perspectives

This study investigated the anxiety-related phenotype of HAB vs. LAB mice, its possible environmental plasticity and molecular events underlying these behavioral changes of anxiety. First of all, for better characterization of anxiety-related behavior, we established a reliable and sensitive behavioral model based on predator odor avoidance providing the phenotypic basis for subsequent studies. Second, we showed that exposure of LAB mice to an unpleasant environment (CMS) induced changes in behavior, neuroendocrine regulation and pattern of *c-fos* expression in the brain. *Crhr1* was the first identified gene critically involved in the phenotype plasticity of anxiety in HAB and LAB mice. Differential expression of *Crhr1* in the BLA after stress could be mediated via changes in epigenetic regulation of its promoter. Thus, higher methylation of CpG1 in stressed LAB mice might decrease binding of the transcription factor YY1 to the *Crhr1* promoter *in vitro*. This cascade of molecular events is suggested to be behind the environmentally driven changes in *Crhr1* expression. Whereas no changes in the methylation of *Crhr1* CpGi were found between HAB and LAB mice, we tested here a possible contribution of miRNA34 family and histone modifications in the regulation of differential basal expression of *Crhr1*.

Future experiments should focus on studying YY1 binding to the *Crhr1* promoter *in vivo* using chromatin immunoprecipitation (ChIP). Furthermore, a close relationship between CRH and glucocorticoids systems during the stress response suggests a

possible common biological target. Our preliminary data corroborate a regulatory effect of glucocorticoids on *Crhr1* expression and promoter activity, therefore future experiments will be addressed to *in vivo* studies of this system. Particularly, we are interested in investigating the role of GR and *Crhr1* in controlling HPA regulation. These experiments could shed some light on the origin of altered HPA axis of our HAB/LAB mice and in several psychiatric diseases including PTSD and depression.

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8 Curriculum Vitae

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Publications:

2014 **Sotnikov SV**, Markt PO, Malik V, Chekmareva NYu, Naik RN, Sah A, Singewald N, Holsboer F, Czibere, L, Landgraf R. Bidirectional rescue of extreme genetic predispositions to anxiety: impact of CRH receptor 1 as epigenetic plasticity gene in the amygdala. Transl Psychiatry. 2014 Feb 11;4:e359. doi: 10.1038/tp.2013.127.

Sotnikov SV, Chekmareva NYu, Schmid B, Harbich D, MaliK V, Bauer S, Kuehne C, Markt PO, Deussing JM, Schmidt MV, Landgraf R. Effects of predator odor on anxiety-related behavior and immediate early gene expression after enriched environment suggest a critical role of *Crhr1*. Eur J Neurosci. 2014 May 20. doi: 10.1111/ejn.12624.

Sotnikov SV, Wittmann A, Bunck M, Bauer S, Deussing JM, Schmidt MV, Touma C, Landgraf R, Czibere L. Blunted HPA axis reactivity reveals glucocorticoid system dysbalance in a mouse model of extremly high anxiety-related behavior. Psychoneuroendocrinology(2014), http://dx.doi.org/10.1016/j.psyneuen.2014.06.006

Sotnikov SV, Markt PO. Epigenetic regulation of Crhr1: implication for anxiety-related disorders. Receptors and clinical investigation. *In press*.

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2013 Avrabos C, **Sotnikov SV**, Dine J, Markt PO, Holsboer F, Landgraf R, Eder M. Real-time imaging of amygdalar network dynamics in vitro reveals a neurophysiological link to behavior in a mouse model of extremes in trait anxiety. J Neurosci. 2013 Oct 9;33(41):16262-7.

Umryukhin AE, Chekmareva NYu, **Sotnikov SV**, Vetrile LA, Zaharova IA. Microdialysis study of hippocampal glutamate and GABA profile in active and passive rats after immunization by glutamate-BSA conjugate. Nevr. Vestnik Becht. 2013; XLV(1): 27-33.

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2011 **Sotnikov SV**, Markt PO, Umriukhin AE, Landgraf R. Genetic predisposition to anxiety-related behavior predicts predator odor response. Behav Brain Res. 2011 Nov 20;225(1):230-4.

Sudakov KV, Ivanova EA, Koplik EV, Kotov AV, Kravtsov AN, Meshcheriakov AF, Pertsov SS, **Sotnikov SV**, Tolpygo SM, Umriukhin AE, Umriukhin PE. Immune links in the system organization of behavior. Usp Fiziol Nauk. 2011 Jul-Sep;42(3):81-96.

2010 Sudakov SK, **Sotnikov SV**, Chekmareva NY, Kolpakov AA, Chumakova YA, Umryukhin AE. Changes in • -endorphin level in the cingulate cortex in rats after peripheral loperamide and methylnaloxone administration at rest and during emotional stress. Bull Exp Biol Med. 2010 Aug;149(2):167-9.

2009 **Sotnikov SV**, Stepaniuk VL, Umriukhin AE. Influence of exposure to immobilisation stress on blood concentration of TNF alpha and IL-4 in rats active and passive in the open field test. Zh Vyssh Nerv Deiat Im I P Pavlova. 2009 Nov-Dec;59(6):736-42.

2008 Vetrile LA, Evseev VA, Umriukhin AE, Kravtzov AN, **Sotnikov SV**, Zakharova IA. Involvement of autoantibodies to neurotransmitters in mechanisms of stress reaction in rats. Bull Exp Biol Med. 2008 Dec;146(6):687-90.

Conferences participation:

2013 AGNP annual meeting, 19-21 Sep 2013, Munich, Germany (poster).
IBNS annual meeting, 25-30 Jun 2013, Dublin, Ireland (talk & poster).
ADAA annual meeting, 3-7 Apr 2013, La Jolla, USA (poster).
INTERACT, 21-22 Mar 2013, Munich, Germany (talk).

2010 SfN Annual Meeting, 13-17 Nov 2010, San Diego, USA (poster).
Mini-Convention Frontiers in Addiction Research, 13 Nov 2010, San Diego, USA (poster).

2009 International Pirogov Medical Conference, 14-15 Oct 2009, Moscow, Russia (talk).

2008 International congress “Neuroscience for medicine and psychology”, 7-13 Jun, 2008, Crimea, Ukraine (poster).

Scholarships and awards:

2013 Anxiety and Depression Association of America (ADAA), Career development travel award, ADAA annual meeting.
Internation Behavioral Neuroscience Society (IBNS), Travel award, IBNS annual meeting.

2010 International Brain Research Organization (IBRO), Young Career Investigator award, SfN annual meeting.

2009 President's of Russia Scholarship for talented students, Ministry of Education of Russian Federation.

2008 Russian Academy of Medical Sciences (RAMS), Travel award, “Neuroscince for medicine and psychology”, 2008, Crimea, Ukraine.

9 Declaration/ Erklärung

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

München, den ....

(Unterschrift)

Hiermit erkläre ich,

dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist und

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

München, den ....

(Unterschrift)