Cross-talk of genetically and environmentally modulated epigenetic factors in the development of anxiety-related behavior: In-depth analyses of candidate genes

Dissertation

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"It is more important to know what sort of person has a disease than to know what sort of disease a person has. "

Hippocrates

To my parents and brother

Abstract

Here, I studied the role of two candidate genes *i.e.* neuropeptide S receptor 1 (Npsr1) and transmembrane protein 132D (Tmem132d), in two psychopathological animal models of anxiety-related behavior because of recent studies showing importance of these candidates in limbic areas and the frontal cortex of panic disorder patients, respectively. The two animal models are rat (r) and mouse (m), high anxiety-related behavior (rHAB/mHAB) and low anxiety-related behavior (rLAB/mLAB).

To understand the anxiolytic role of neuropeptide S (NPS), basal *Npsr1* mRNA expression was studied in limbic brain regions of HAB *vs*. LAB rodents, i.e. the paraventricular nucleus of hypothalamus (PVN) and amygdala, because these regions have been implicated in anxiety and fear attenuating responses of NPS and also due to differences in long-term activity based on cytochrome c oxidase activity in mHAB *vs*. mLAB. There was significantly lower basal *Npsr1* mRNA expression in the basolateral amygdala of mHAB and also in the PVN of rHAB compared to corresponding LABs. To study the genetic underpinnings underlying this differential expression, *Npsr1* DNA sequencing was carried out, which revealed several polymorphisms including single-nucleotide polymorphisms (SNP), insertions and deletions. By using dual reporter (luciferase) assays, I could show that the SNPs in the whole HAB promoter construct cause a significant decrease in promoter activity, thus confirming our *in vivo* findings in both rats and mice. Interestingly, however, when the promoter constructs were shortened to 500 bp relative to translational (ATG) start site, there was a two-fold higher HAB promoter activity, which could be attributed to the introduction of a polymorphism with putative binding site for the glucocorticoid receptor (GR) transcription factor. The higher HAB promoter activity was suppressed by dexamethasone (a GR activator), thus suggesting the presence of a polymorphism that favors GR binding. These findings are analogous to the higher HAB specific allele expression in cross-mated F1 offspring, which allows us to study the HAB *vs*. LAB alleles in the same cellular environment, irrespective of any epigenetic or other environmentally mediated factors that might modulate or interact with *cis*-acting factors. In addition, there was no difference in *Npsr1* mRNA expression in the basolateral amygdala of mHAB and mLAB subjected to environmental enrichment (EE) and unpredictable chronic mild stress (UCMS), respectively. Thus it is a non-plastic gene as it does not respond to

environmental challenges faced by the susceptible animal models.

Similarly, for *Tmem132d*, using dual luciferase assays, two SNPs in the mHAB promoter region were shown to cause an increase in its corresponding promoter activity, and there was no difference in DNA methylation in the mHAB *vs.* mLAB *Tmem132d* promoter region, which explains the observed higher *Tmem132d* mRNA expression in the anterior cingulate cortex of mHAB. However, mHABs subjected to EE had higher *Tmem132d* mRNA expression, while mLAB undergoing UCMS had corresponding lower gene expression. To study the *cis*-trans interaction, we also subjected cross-mated F1 offspring to EE or UCMS and found that both groups have higher mLAB allelic expression, which could be attributed to differences in DNA methylation.

Finally, I could show that there was no difference in DNA methylation in the basal mHAB *vs.* mLAB *Tmem132d* promoter and that two SNPs in the mHAB promoter were sufficient to cause a higher corresponding promoter activity, which explains the *in vivo* findings observed in the anterior cingulate cortex. Furthermore, F1 offspring subjected to EE or UCMS had a significantly lower mHAB-specific allele expression which was negatively correlated with DNA methylation, in the *Tmem132d* promoter region, thus this suggests cross-talk between genetic and environmentally mediated epigenetic factors.

In summary, the data suggests a strong evolutionary conserved role of the NPS system considering the similar findings in rats and mice. However, *Npsr1* is a non-plastic gene as it is not amenable to the different environmental manipulations applied to the animals. On the other hand, the plastic gene *Tmem132d,* is differentially expressed, thus making the animals more susceptible to environmental influences. Here, it could be revealed, that SNPs in the mHAB *Tmem132d* promoter cause higher promoter activity and that environmental manipulation can modulate the gene's corresponding expression through DNA methylation.

Table of abbreviations and comments

CHI3L1	chitinase 3-like 1
5-HT	5-hydroxytryptamine/serotonin
5-HTT	serotonin transporter
ACTH	Adrenocorticotropin hormone
ADHD	attention-deficit hyperactivity disorder
AEI	Allele expression imbalance
ANOVA	analysis of variance
AP	anterior-posterior
AP-1	activator protein 1
APA	American psychological association
APC	adenomatous polyposis coli
ASD	Acute stress disorder
AVP	arginine-vasopressin
BLAST	Basic local alignment search tool
bp	basepair
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CCAPR	cardioacceleratory peptide receptor
cDNA	complementary DNA
c-fos	cellular proto-oncogene belonging to the immediate early gene family of transcription factors
Cg	Cingulate cortex
CNCS	conserved non-coding sequences
CNV	Copy number variation
COMT	Catechol-o-methyl transferase
CORT	Cortisol / Corticosterone
COX	cytochrome c oxidase
CpG	"—C—phosphate—G—"
Cps	crossing points
CRE	cAMP response element
CRH	Corticotropin-releasing hormone
C-terminal	Carboxyl terminal
CTF	CCAAT box-binding transcription factor
DER	downstream enhancing region
DEX	dexamethasone
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DPAT	8-Hydroxy-N,N-dipropyl-2-aminotetralin
DRD2	dopamine receptor D2
DSM IV-TR	Diagnostic and statistical manual of mental disorders, fourth edition, text revision
Dspp	dentin sialophosphoprotein

DV	dorsal-ventral
EA	European ancestory
EDTA	Ethylenediaminetetraacetic acid
EE/enriched	Environmental enrichment
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ELISA	Enzyme-linked immuno sorbent assay
EMSA	electrophoretic mobility shift assay
ENCODE	Encyclopedia Of DNA Elements
EPF test	elevated platform test
EPM	Elevated plus-maze
eQTL	expression quantitative trait locus
ETAS	Early threat assessment system
FADS2	Fatty acid desaturase 2
FBS	Fetal bovine serum
FKBP5	FK506 binding protein 5
FLAG® tag	DYKDDDDK
fMRI	functional magnetic resonance imaging
FST	Forced swim test
GABA	Gamma-aminobutyric acid
GAD	Generalized anxiety disorder
GPCR	G-protein coupled receptor
GPRA	G protein-coupled receptor for asthma susceptibility
GR	Glucocorticoid receptor
GXE	GeneXEnvironmental
HDS/LDS	high- or low-DPAT-sensitive
HEK 293	human embryonic kidney 293 cells
HES1	hairy and enhancer of split-1
HF	high fidelity
HPA axis	Hypothalamic-pituitary-adrenal axis
ICD-10	10th revision of international statistical classification of diseases and related health problems
icv	intracerebroventricular
Igf2	Insulin-like growth factor 2
Ile	Isoleucine
IQ	Intelligence quotient
K	lysine
kda	kilodalton
KDEL	Lys-Asp-Glu-Leu
'l''	long
LB	Luria Broth
LC area	Locus coeruleus area
LD	linkage disequilibrium
LD box test	Light-Dark box test

LMNA	Lamin A/C
MAO	Monoamine xxidase
MDD	Major depressive disorder
MDR1	multidrug resistance 1
ML	Medial – Lateral
MOLT	mature oligodendrocyte transmembrane
mRNA	messenger ribonucleic acid
MYT1	Myelin transcription factor 1
N	Asparagine
NCAM	neural cell adhesion molecule
NCBI	National Center for Biotechnology Information
NF-1	nuclear factor-1
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NPS	Neuropeptide S
NPSR1	Neuropeptide S receptor 1
NPSR1-A	NPSR1 antagonist
NTC	no template control
N-terminal	Amino terminal
OCD	Obsessive-compulsive disorder
OF test	Open field test
Opti-MEM	Reduced Serum Media is a modification of Eagle's Minimum Essential Media
PCR	polymerase chain reaction
PND	postnatal day
PROMO	prediction of transcription factor binding sites
PTSD	Post-traumatic stress disorder
PVN	paraventricular nucleus
qMSP	Quantitative Methylation specific PCR
qPCR	quantitative PCR
R	arginine
RHA/RLA	Roman high- and low- avoidance rats
rHAB/mHAB	rat / mouse high anxiety-related behavior
Rho	Spearman's rank correlation coefficient
rLAB/mLAB	rat / mouse low anxiety-related behavior
rNAB	rat Normal anxiety-related behavior
rs	reference SNP
S	Serine
's''	short
SD	Social defeat
SDS	*Sodium dodecyl sulfate*
SEM	standard error mean
siRNA	small interfering RNA
SNP	single-nucleotide polymorphism
SNRIs	Serotonin-norepinephrine reuptake inhibitors

SP1	Specificity Protein 1
SSRIs	Selective serotonin reuptake inhibitors
TAFI	thrombin-activable fibrinolysis inhibitor
TBP	TATA binding protein
TBS	Tris-buffered saline
TESS	Transcription Element Search System
TFIIB	Transcription factor IIB
TMEM132D	Transmembrane protein 132D
TPH2	Tryptophan hydroxylase 2
TST	Tail suspension test
UCMS/stressed	unpredictable chronic mild stress
USV	Ultrasonic vocalization
UTRs	untranslated regions
veh	vehicle
VRR1	vasopressin receptor-related receptor 1
WHO	World health organization
Xist	X-inactive specific transcript
Y2H	yeast two hybrid system
YY1	Yin-Yang 1
ZNF219	zinc finger protein 219

Please note: rat, mouse and human gene symbols are written in italicized minor letters with the first letter in capital, also if it refers to the respective mRNA (*e.g.*, *Npsr1*). The rat, mouse and human peptides or proteins are held in capital non-italicized letters (*e.g.*, NPSR1). The non-italicized and minor letters can refer either to gene or protein (*e.g.*; Npsr1).

1.0 Introduction

1.1 Emotional behavior and its theories

Human behavior is a complex product of brain activity, governed by a combination of two mutually interacting factors i.e. heredity and environment (Bear *et al.*, 2006). The life experiences of an individual and the variation in its genetic makeup makes the brain differentially susceptible to modification by subsequent experiences. The sum of inherited and experimental variations leads to physico-chemical changes in the brain, which give rise to a myriad of human behavior. Emotions are a ubiquitous and universal characteristic of human behavior (Ekman & Friesen, 1971; Fessler, 1999; Haselton & Ketelaar, 2006) defined as "a collection of psychological states that include subjective experience, expressive behavior (*e.g.*, facial, bodily, verbal), and peripheral physiological responses (*e.g.*, heart rate, respiration)" (Gross & Barrett, 2011). There is also consensus that emotions are the core part of any psychological model of human mind, however, the rest is subject to debate (Gross & Barrett, 2011).

In the nineteenth century, several theories were proposed to elucidate the nature and origin of emotions. The **James-Lange theory**, proposed independently by William James and Carl Lange, states that emotions are experienced in response to physiological changes in our body (Cannon, 1927). In the presence of a threatening stimulus, for instance, our sensory systems send information to the brain about a prevailing situation, and then the brain sends a processed signal to the body, leading to a change in heart rate, muscular tone, perspiration and dryness of mouth, among others. The sensory system subsequently reacts to the changes elicited by the brain and these changes constitute the emotions. James and Lange concluded that the physiological changes are the emotions and if they were removed there would be no emotions. However, the James-Lange theory was soon criticized by Walter Cannon who studied Sherrington's work and demonstrated that a transection of the spinal cord in animals eliminated body sensations below the cut level, but it did not eliminate emotions (Cannon, 1927). Cannon's work was supplemented by Philip Bard and the subsequent **Cannon-Bard theory** of emotion stated that the physiological responses are caused by emotional states and can even occur in absence of emotions. Another observation by Cannon that disproved the James-Lange theory was that there is no reliable correlation between the emotional experience and the physiological states of the body. He demonstrated that the same visceral responses such as changes in heart rate, pupil dilation and sweating can be associated with experience of fear or anger and can also be observed during fever, feeling cold or breathlessness. Thus, the physiological responses are too general to be linked to a specific emotional state of an individual (Cannon, 1927).

Darwin's theory of emotion (Darwin, 1896), which was published thirteen years after "On the Origin of Species", stated that emotions such as fear evolved because of their adaptive nature for the survival of the species, and facial expression such as happiness or angriness are innate characteristics that evolved in an individual to convey the friendly or hostile nature to others, thus helping the survival of the species. **Schachter and Singer's two factor theory** of emotion (Schachter & Singer, 1962) states that emotions are a mix of physiological arousal and cognition, thus during physiological arousal, people search for cues in their immediate environment and label them either as pleasant or harmful *e.g.*, when a person sees an approaching snake, physiological arousal is accompanied by cognitive interpretation, which labels the incidence as dangerous.

1.2 Evolution of the emotional brain

Single celled organisms such as *Amoeba* or *Paramecia* have sensing mechanisms that assist them in avoiding toxic substances and guide them towards food nutrients (Macnab & Koshland, 1972). These motile organisms for instance, *Paramecia* have whip-like flagella attached to its cell membrane that aid in their forward movement through the surrounding medium. In addition, there are several receptors spread over the cell membrane that respond to different external cues to control the action of the flagella (Macnab & Koshland, 1972) and subsequently the behavior of the organism (Stein *et al.*, 2009). The advent of marine invertebrates such as sea hare (*Aplysia*) in the Cambrian period, 500 million years ago, was accompanied by organization of group of cells into specialized organs with specific function. The *Aplysia* with a relatively simple nervous system other than detecting signals can represent contingencies between signal and event, learn and also remember (Stein *et al.*, 2009). For example, chemical stimuli like shrimp juice normally ignored by the *Aplysia* when associated repeatedly with an electric shock could be made into a signal for escape response (Kandel, 1983). When the shrimp juice was presented second time, the *Aplysia* responded with additional defensive response such as gill withdrawal and release of protective ink clouds. Thus an innocuous event becomes a signal eliciting anticipatory state where response to threat is excessive, and this may illustrate a simple form of anticipatory anxiety (Stein *et al.*, 2009).

All the vertebrates share a common basic brain plan consisting of three broad areas namely; hindbrain, midbrain and forebrain with evolutionary conserved basic circuits. The basal ganglia are primitive brain structures that control complex species specific behavior such as courtship, aggression and grooming in reptiles and lower animals (Flannelly *et al.*, 2007; Maclean, 1990). The basal ganglia represent an evolutionary early threat assessment system (ETAS) due to its participation in aggressive territorial displays in reptiles and primates (Maclean, 1990). Later the

limbic system, chiefly the amygdala evolved in early mammals for making emotional decisions regarding potential threats long before consciousness (Flannelly *et al.*, 2007).

In addition, prefrontal cortex is also important for emotional decision making, modulation of autonomic nervous system, reasoning and cognitive processing. These three brain regions *i.e.*, basal ganglia, limbic system and prefrontal cortex has been proposed as an ETAS and there might be different ETAS for diverse potential threats such as height or predator attack (Flannelly *et al.*, 2007).

Thus similar to various components of the immune system, which protects our body against different types of pathogenic invasions (Marks & Nesse, 1994), there are several subtypes of emotion which protect us against any kind of threat. These emotions are adaptations shaped by natural selection (Darwin, 1896; Marks & Nesse, 1994). Emotional behavior often includes anxiety and fear, where anxiety can be defined as an emotional anticipation to uncertain aversive cues, while fear is a definite and directed response against threats (*e.g.* an approaching bear) to facilitate appropriate defensive behaviors to reduce harm or injury (Landgraf, 2003).

1.3 Prevalence and categorization of anxiety disorders

Anxiety disorders are among the most common psychiatric illnesses with a lifetime prevalence of about 30 % (Alonso *et al.*, 2011; Kessler & Wang, 2008) and they are highly comorbid with major depressive disorders (MDD), with 50-60% of individuals reporting more than one episodes of certain anxiety disorders (Kaufman & Charney, 2000). There are two major nosological systems available for characterization of mental disorders: 1. Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision (DSM IV-TR) and 10th revision of International Statistical Classification of Diseases and Related Health Problems (ICD-10). The DSM IV-TR categorizes anxiety disorders into different classes such as generalized anxiety disorder (GAD), social anxiety disorder, specific phobia, panic disorder with and without agoraphobia, obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD), anxiety secondary to medical condition, acute stress disorder (ASD), and substance-induced anxiety disorder (APA, 2000; Rowney *et al.*, 2010). According to the ICD-10, anxiety disorder is included under a broader category of "neurotic, stress-related, and somatoform disorders". The ICD-10 is mostly used to track the prevalence of health problems worldwide, while the DSM IV-TR is more commonly used by psychiatrist and psychologists (Adornetto *et al.*, 2012; WHO, 1993).

1.4 Treatment of anxiety disorders

The currently available drugs for psychiatric disorders were developed by serendipity in the 1950s by clinical observations of at least three drug cases. First, a drug called reserpine given to

patients with high blood pressure caused psychotic symptoms in 20% of the cases. Second, anti-tuberculosis drugs such as isoniazid and iproniazid were found to have significant elevation of mood in patients. A closer look at these two clinical observations revealed, that reserpine irreversibly blocked the vesicular monoamine transporter, thus, loading of catecholamines and serotonin into synaptic vesicles was affected, and unprotected neurotransmitters were degraded by monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT) (Kirsch, 2010). While, the anti-tuberculosis drugs were found to inhibit the MAO, thereby increasing the availability of these neurotransmitters in the synaptic cleft between neurons. Another study had earlier shown that a tricyclic antidepressant, imipramine inhibits the reuptake of released serotonin and norepinephrine, thus promoting their action in the neuronal synaptic cleft (Kuhn, 1958). All these observations gave birth to the **monoamine hypothesis** of mood disorders which states that depression is a result of decreased activity or deficit of monoamines, such as dopamine, serotonin, and norepinephrine in the brain. Despite these being anti-depressant drugs, they are often prescribed off-label for many types of anxiety disorders, thus indicating that they probably share common neural substrates. Treatment of anxiety disorders often involves a combination of drugs and psychotherapy, such as cognitive behavioral therapy. Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, paroxetine or the more recent serotonin-norepinephrine reuptake inhibitors (SNRIs), such as venlafaxine are the first line of treatment for most types of anxiety disorders (Dunlop & Davis, 2008). For non-responders, benzodiazepines, such as diazepam, which mediate their effects through the inhibitory GABAergic neurotransmission, are prescribed along with antidepressants (Dunlop & Davis, 2008; Luscher *et al.*, 2011). However, many of these drugs either do not work on a large number of patients, have various side effects or the patients relapse soon. Thus there has been a marked shift in focus towards the development of non-GABAergic drug targets and a more endophenotype-based research approach (Cryan & Slattery, 2007; Hasler *et al.*, 2006). Anxiety and depression are thought to have similar genetic origins (Grillon *et al.*, 2005; Kendler, 1996) and often coexist in clinical situations (Aina & Susman, 2006; Stahl, 1997). Furthermore, twin studies have suggested a strong heritable component for anxiety and comorbid depression (Gillespie *et al.*, 2004; Lamb *et al.*, 2010). These results, suggest that mood disorders run in families, and thus genes predispose certain individuals to these illnesses. An approximately 30-40% of genetic factors are estimated to contribute towards trait anxiety (Hettema *et al.*, 2001).

1.5 Predisposing factors of anxiety

The insufficient monoamine hypothesis along with the above observations and subsequent epidemiological studies (Kendler *et al.*, 1995; Magalhaes *et al.*, 2010) showed that stress is a

major contributing factor in the development of anxiety and depressive disorder, and this highlighted the role of hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis activity provides endophenotypes for both anxiety (Kallen *et al.*, 2008) and depression (Vreeburg *et al.*, 2009). It has been consistently found to be hyperactive in these two psychiatric conditions and it does interact with the monoamine system (Magalhaes *et al.*, 2010; Massart *et al.*, 2012). During stress exposure, corticotropin-releasing hormone (CRH) is released from the hypothalamus into the hypophyseal portal blood which then travels to the anterior lobe of pituitary causing release of adrenocorticotropin hormone (ACTH). This in turn, acts on the adrenal glands to cause the release of cortisol or corticosterone (CORT) in the blood. Subsequently, this exerts various responses throughout the body (Bear *et al.*, 2006).

The significant inheritance and the role of stress in mood disorders led to the development of the diathesis-stress model, which suggests that individuals are at risk to develop mental illness because of their genetic makeup or predisposition (diathesis) that makes them vulnerable to particular disorders under adverse conditions (Nemeroff, 1998). In particular, early childhood stress was found to cause increase in both, the number of CRH expressing neurons and also the amount of CRH gene transcripts (Nemeroff, 1998). However, in recent years the role of glia in the pathophysiology of mood disorders has also been appreciated (Coyle & Schwarcz, 2000) (Sibille *et al.*, 2009). The term glia (which means glue in German) was first coined by Rudolf Virchow after observing a sort of connective tissue underlying ependyma of cerebral ventricles in a psychiatric journal nearly 160 years ago (Virchow, 1846). Oligodendrocytes are one of the major types of glial cells, which produce myelin and thus insulate and support the axons of nerve cells in the central nervous system. However, oligodendrocytes have recently been implicated in emotional disorders after several studies showed that their cell numbers and related transcripts dwindle in the prefrontal cortex and amygdala of schizophrenia, major depression and other mood disorders (Aston *et al.*, 2005; Hamidi *et al.*, 2004; Uranova *et al.*, 2004).

1.6 Role of epigenetics in mood and anxiety disorders

Traditional aetiological studies have largely focused on the search for genetic factors; however the role of epigenetic factors, in the regulation of gene expression independent of any changes to the underlying DNA sequence, has also come under focus in the development of mood disorders (Kubota *et al.*, 2012; Tsankova *et al.*, 2007).

The word epigenetics was originally coined by Conrad Waddington in 1942 to describe how genes interact with their products to produce a particular phenotype while studying the development of embryos. However, epigenetics is best defined as, "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird, 2007).

Chromatin which contains histone and non-histone proteins bound to genomic DNA is a dynamic structure which responds to diverse environmental stimuli to regulate access to its DNA. The epigenetic processes that modulate this DNA access include DNA methylation, histone modifications, nucleosome modeling, nuclear dynamics and the interaction of chromatin with noncoding RNAs ("The dynamic epigenome," 2013). DNA methylation in mammals involves transfer of a methyl moiety from S-adenosylmethionine to the 5' position of the cytosine ring, where these cytosine are usually followed by a guanosine, and are termed CpGs. This process is maintained by a family of DNA methyltransferase (DNMTs). An example of this process would be the erasure of DNA methylation in early embryonic stages, which is then reestablished during implantation (Bergman & Cedar, 2013), however studies in model organisms have shown that certain loci in the genome are resistant to this erasure and thus DNA methylation may serve as a marker for transgenerational inheritance (Lane *et al.*, 2003; Xing *et al.*, 2007).

The importance of environment on the physiology, behavior and brain regulation has been recognized to cause stark differences in phenotypic variability (Benaroya-Milshtein *et al.*, 2004; Larsson *et al.*, 2002). To mimic socio-environmental stressors in rodents, unpredictable chronic mild stress (UCMS/stressed), has been shown to cause reduced hippocampal neurogenesis, impairment of HPA axis and these effects were reversible by fluoxetine treatment (Surget *et al.*, 2011). Likewise, environmental enrichment (EE/enriched) which is defined as "a combination of complex inanimate and social stimulation" (Rosenzweig *et al.*, 1978) consists of an environment with toys, tunnels, ladders, running wheel that would lead to social interaction and stimulation of exploratory and motor behavior (van Praag *et al.*, 2000). EE has been shown to cause increase in exploratory behavior and decrease in anxiety along with a reduction of ß-amyloid plaques in a mouse model of Alzheimer's disease (Gortz *et al.*, 2008). EE has also been shown to have reversing effects on the neuronal dysfunction and cognitive decline in rodent models of aging and neurodegenerative disorders (Frick & Benoit, 2010).

There are also several studies showing how environment affects the epigenome of the organism. This is illustrated with increased pup licking, grooming and arched-back nursing by rat mothers, which altered their offspring epigenome at the glucocorticoid receptor (GR) gene promoter in the hippocampus (Weaver *et al.*, 2004). This study provides a mechanistic link between maternal behavior and modification of the stress response system in offspring. Another study showed that methyl supplemented diets of maternal mice altered the epigenetic state of the agouti gene, leading to a change in coat color of their offspring (Wolff *et al.*, 1998). Similarly, changes in DNA methylation patterns were observed in response to pathogen attack in plants (Boyko *et al.*, 2007), and transient exposure of gestating female rats to environmental toxins, such as vinclozolin (an antiandrogenic compound) led to an increased incidence of male

infertility (Anway *et al.*, 2005). All these studies show that alterations in gene expression persist throughout lifetime and sometimes may be transmitted to the next generation (Daxinger & Whitelaw, 2010), thus altered traits and properties can be inherited (at least partially) in a Lamarckian-like way.

1.7 Allelic expression imbalance

Allelic expression imbalance (AEI) is a phenomenon where the two alleles of a given gene are expressed at different levels in a given cell, either because of epigenetic inactivation of one of the two alleles, or because of genetic variation in regulatory regions. Monoallelic expression occurs due to imprinting based on the parent-of-origin of allele. For instance, monoallelic expression has been demonstrated for a long non-coding RNA gene (H19) and insulin-like growth factor 2 (Igf2) from the maternal and paternal chromosomes, respectively, due to the presence of certain epigenetic marks, laid down during gametogenesis leading to gene silencing or genomic imprinting (Reik & Walter, 2001). Random monoallelic expression occurs due to epigenetic mechanisms like DNA methylation, where a long non-coding *Xist* mRNA has been shown to randomly envelope one copy of the X-chromosome, packaged in heterochromatin during female development, leading to inactivation of all genes on one X-chromosome in every cell of the organism. This is stably transmitted during mitosis, representing an exception to the Mendelian inhertiance (Lyon, 1961). Studies in the past decade have shown such random monoallelic expression to be common for many autosomal genes and their subsequent inheritance by daughter cells, like genes for interleukins, immunoglobulin or odorant receptor genes (Lo *et al.*, 2003; Palacios *et al.*, 2009; Zwemer *et al.*, 2012). Furthermore, other studies have shown that subtle changes in allelic expression of disease susceptibility genes can affect predisposition to a certain disease. As an example, AEI has been observed for adenomatous polyposis coli (APC) gene in colon cancer (Yan *et al.*, 2002), chitinase 3-like 1 (CHI3L1) and COMT gene in schizophrenia (Hill *et al.*, 2011). Furthermore, other illustrations of AEI include COMT (Bray *et al.*, 2003), and tryptophan hydroxylase 2 (TPH2) gene in major depression and suicide (Lim *et al.*, 2007). These AEI occur due to regulatory polymorphisms with previous studies having identified *cis*-regulatory variants by measuring total expression (both alleles) in several individuals and then using this as expression quantitative trait locus (eQTL) to identify nearby *cis*-regulatory regions (Wagner *et al.*, 2010). However, environmental influences can causes differences in interindividual differences in expression. Thus, genotyping data accompanied by measurement of relative allelic expression in the same cell, wherein alleles are exposed to the same cellular environment, allows for a more precise measurement of *cis*-regulatory sequences causing differential expression (Wagner *et al.*, 2010).

1.8 Identification of genetic variants

There are three basic approaches to identify genetic variants of mental disorders;

1. **Linkage analysis and association studies**: these methods give powerful results for single-gene disorders such as Huntington's disease, but are less appropriate for anxiety and other psychiatric disorders having multiple players of small effects. In addition, researchers found few genes with main effects that were difficult to reproduce (Caspi & Moffitt, 2006).

2. **Endophenotypes**: The term endophenotype (intermediate phenotype) is a quantitative biological trait proposed to understand the genetic contribution in the etiology of psychiatric disorders. They are associated with illness, heritable and found in affected families at higher rate than in general population (Kendler & Neale, 2010). Widely known examples of endophenotypes are the cognitive abnormalities found in schizophrenia patients which are likely modulated by genetic factors, as well as environmental factors, such as famine (Susser *et al.*, 1996) or birth complications (Jones *et al.*, 1998; Susser *et al.*, 1996). The HPA axis is also an endophenotype shown to consistently maladaptive in anxiety and depression (Kallen *et al.*, 2008; Vreeburg *et al.*, 2009).

3. **Gene-environmental (GxE) interactions** are based on the observation that mental disorders also have environmental causes and people show a wide heterogeneity in their response to these environmental stimuli (Moffitt *et al.*, 2005; Tsuang *et al.*, 2001). Gene-environmental interactions are based on the diathesis-stress hypothesis, which occur when effects of exposure to environmental stimuli is moderated based on the genotype of a particular individual. Pioneering studies by (Caspi *et al.*, 2002) showed that maltreated children carrying a functional polymorphism in the neurotransmitter-metabolizing enzyme (MAO-A) causes corresponding reduced activity and were found to have engaged in violent behavior. These studies were further met with a mixed reaction with a meta-analysis finally showing the effect to be true (Kim-Cohen *et al.*, 2006). However, another study had only confirmed in the predicted direction (Haberstick *et al.*, 2005), or the effect was observed only in a particular race (Widom & Brzustowicz, 2006), while another study failed to replicate the GxE effect (Surtees *et al.*, 2006). Another example of gene environmental interaction is the short ("s") allele of the serotonin transporter (5-HTT) gene promoter polymorphism, exhibiting higher depression and suicidal tendency following stressful life events (Caspi *et al.*, 2003). A recent study also showed how epigenetic factors play a role in mediating gene-environment interactions, here researchers showed how childhood trauma influenced DNA demethylation in regulatory regions of a stress regulatory gene, FKBP5, leading to long term dysregulation of the stress hormone system (Klengel *et al.*, 2013). Furthermore, recent studies have also started looking at neural substrates of GxE interactions *e.g.*, people

with the low MAO-A allele were found to have reduced limbic volume and a hyper-responsive amygdala during emotional arousal (Meyer-Lindenberg *et al.*, 2006) Thus, it might happen that certain other genetic variations within a cohort may dilute the GxE findings, thus imaging genomics could be used to complement the data from GxE findings, and to root out false positive data (Caspi & Moffitt, 2006). In addition, data from imaging genomics which can identify neural substrates tend to be quantitatively distributed, thus, low prevalence of environmental exposure does not constitute a problem (Caspi & Moffitt, 2006). Several studies in the past few years have utilized functional neuroimaging to compare responses of genotype groups (Egan *et al.*, 2001; Hariri *et al.*, 2006), thus, this would allow a novel population stratification, and finally, those studies with modest effects could turn out to show stronger effects (Caspi & Moffitt, 2006).

An alternative model of GXE interactions called **differential susceptibility hypothesis** (Belsky *et al.*, 2009), has been proposed to account for the deficiencies of the diathesis-stress hypothesis, which states that vulnerable individuals respond both to positive and negative stimuli for better or for worse. *i.e.*, genes are inherently neither bad nor good, but individuals vary in their susceptibility or plasticity and depending on the environment, this makes individuals succumb to psychopathology or help in coping in an enriched environment. A very interesting study showing how environment can also have positive effects was shown by (Caspi *et al.*, 2007), presenting that breastfed children had higher IQ, and this association was moderated by a genetic variant in the fatty acid desaturase 2 (FADS2) gene, which is involved in fatty acid pathways. Also coming back to the MAO-A allele, (Foley *et al.*, 2004) showed that boys with low MAO-A activity were more likely to be diagnosed with behavioral disorders if they were exposed to high levels of childhood adversity or neglect. A meta-analysis study of the MAO-A gene (Kim-Cohen *et al.*, 2006) also supported the differential susceptibility hypothesis over the diathesis-stress hypothesis (Belsky *et al.*, 2009).

Similarly, studies by (Taylor *et al.*, 2006) showed that GxE interactions were observed for the ("s") allele of 5-HTT, only when the participants experienced an early or recent adversity, but significantly less depressive symptoms if the participants experienced an early or recent positive experience compared to ("s/l") or ('ll") allele of 5 HTT, thus further supporting the differential susceptibility hypothesis. This susceptibility framework has also been documented for anxiety where individuals with ("s") allele, reported more anxiety in the evening, when daily event stress was high and *vice versa* compared to individuals with other genotype (Gunthert *et al.*, 2007).

1.9 Methods to develop animal models

To understand the neurobiological basis of affective disorders and the corresponding genetic underpinnings, we need good animal models. An animal model is supposed to recapitulate the

human physiological, pathophysiological, behavioral, anatomical and biochemical processes in a non-human organism. There are several methods utilized to develop animal models of neuropsychiatric disorders, like brain lesion, transgenic knockout and knockin, random mutagenesis, selective breeding and optogenetic manipulations of circuits (Nestler & Hyman, 2010), but a lack of proper knowledge of brain regions and candidate genes in anxiety which have variable penetrance with modest effects, does not allow the use of most of these approaches. However, selective breeding is one approach that allows the genetic variation associated with a certain trait *e.g.*, anxiety to be represented at a higher frequency after several generations, leading to homozygosity at loci conferring this trait (Falconer & Mackay, 1996). Nonetheless, an animal model should meet certain validation criteria before it could be used to study the pathophysiological mechanisms and to search for novel drug targets. Thus, McKinney and Bunney proposed four criteria to model depression-like behavior in the 1960s: first, considerable analogy to human disorder in terms of symptoms; second, reproducibility of the model; third, similar response to treatment as in human and fourth, agreement between investigators (McKinney & Bunney, 1969).These criteria were further refined by Paul Willner who proposed three criteria in the 1980s, *i.e*., construct validity, means to recreate the etiological processes that causes the disorder in animals, face validity refers to that recapitulation of behavioral, anatomical or biochemical processes and predictive validity, means the animal model should respond to pharmacological agents similar to those in humans (Willner, 1991).
These criteria reveal the difficulty of creating animal models of MDD and anxiety as the etiology, anatomical and biochemical processes of these disorders are largely unknown. However, risk factors are known and individual facets can be modeled.

1.10 Behavioral tests to measure anxiety-related and depression-like behavior

Animals cannot model every aspect of human disorders, however, the clinical heterogeneity of the anxiety disorder suggests that there are distinct neurobiological circuits for each. Thus, it is imperative to see if different tests for anxiety can reveal those differences (Bourin *et al.*, 2007). Animal models of anxiety can be classified into two groups, one involves animal's conditioned response to stressful stimuli (*e.g.*, electric foot shock), the second includes ethologically based tests involving the animal's spontaneous or natural reactions (*e.g.*, flight, avoidance and freezing) to stressful stimuli that do not explicitly involve pain or discomfort (*e.g.*, exposure to predator, novel environment or highly illuminated novel chamber). By employing ethologically valid, non-painful, aversive stimuli to induce anxiety, these tests are thought to minimize confounding factors, like state anxiety, which can vary from one moment to another and also to control for other factors, such as motivational, learning or memory (Steimer, 2011).

The open field (OF) test is a common measure of exploratory behavior and general locomotor activity in rats and mice, which generally consists of a circular enclosure with surrounding walls to prevent escape. The animal is placed in the apparatus facing the periphery and parameters, such as time spent in the center and activity within the first 5 minutes are used to measure activity and, with the correct illumination, aspects of anxiety (Gould, 2009). The light-dark (LD) box test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of animals, applying mild stressors such as novel environment and light. The test apparatus consist of one-third small dark 'secure' compartment and two-third large illuminated 'aversive' compartment (Gould, 2009). The elevated plus-maze (EPM) resembles a plus raised above the ground is one of most robust and reliable ethological test for anxiety, which consists of two open and two closed arms with enclosed walls. This test is based on the natural aversion of open spaces and uses conflict between exploration and aversion to elevated open spaces. Rodents are placed at the intersection of the four arms, the number of entries and time spent on the open arm over a 5 minute period is taken as an index of anti-anxiety-related behavior (Gould, 2009). The forced swim test (FST) is based on the fact that animals develop an immobile posture when placed in an inescapable cylinder filled with water and this immobile behavior is interpreted as a depression-like behavior or passive stress coping behavior (Petit-Demouliere *et al.*, 2005). The FST is routinely used to screen for anti-depressants and it has good predictive validity and the animals show a more active stress coping behavior such as struggling when treated with antidepressants. Tail-suspension test (TST) in which rodents are suspended by their tails with tape, such as that they cannot escape or touch any other nearby surfaces. During this test, the resulting escape oriented behavior of the rodents is taken as a measure of depression-like behavior (Castagne *et al.*, 2011).

1.11 Animal models of anxiety

There are several animal models of anxiety described previously like the Roman high- (RHA/Verh) and low-(RLA/Verh) rats (Steimer & Driscoll, 2003), the Syracuse strains (Brush, 2003), the Hatano rats (Ohta *et al.*, 1999) and HDS/LDS rats (Overstreet *et al.*, 2003) based on the avoidance behavior or receptor function. As the models based on conditioned responses, may require training and show interference with mnemonic or motivation processes, they are not ethologically relevant (Steimer, 2011). There are other animal models like the Maudsley reactive *vs*. nonreactive rat lines, bred based on the criteria of open field defecation but it shows inconsistent results on ethological tests like EPM (Blizard & Adams, 2002), but some others are more ethologically relevant, like the Tsukuba high and low emotional rat strains (Fujita *et al.*,

1994), infantile high *vs*. low ultrasonic vocalization (USV) (Brunelli, 2005) and Floripa lines (Ramos *et al.*, 2003).

1.12 Selective breeding and phenotyping of HAB *vs.* LAB rat and mice models of anxiety

Starting from an outbred Wistar rat and CD-1 mouse population, an intra-strain, selective breeding approach was utilized based on the animals performance on the EPM test over more than 20 generations to develop the HAB *vs.* LAB rat and mouse model of anxiety-related behavior (Kromer *et al.*, 2005; Landgraf & Wigger, 2002). Selective breeding leads to homozygosity at trait conferring loci, leading to two divergent populations. When this reached its maximum, strict sibling-mating was carried out to conserve the genetic polymorphisms underlying either the high or low anxiety-related traits. Overall, this approach allows us the study the risk factors at a higher penetrance compared to their outbred founder population. rHAB and rLAB (Figure 1) spend less than 5% and more than 50%, respectively, of their total test time on the open arm of the EPM (Landgraf & Wigger, 2002). Meanwhile, mHAB spend less than 10% and mLAB (Figure 2) more than 50% of total test time on open arm of the EPM (Kromer *et al.*, 2005).

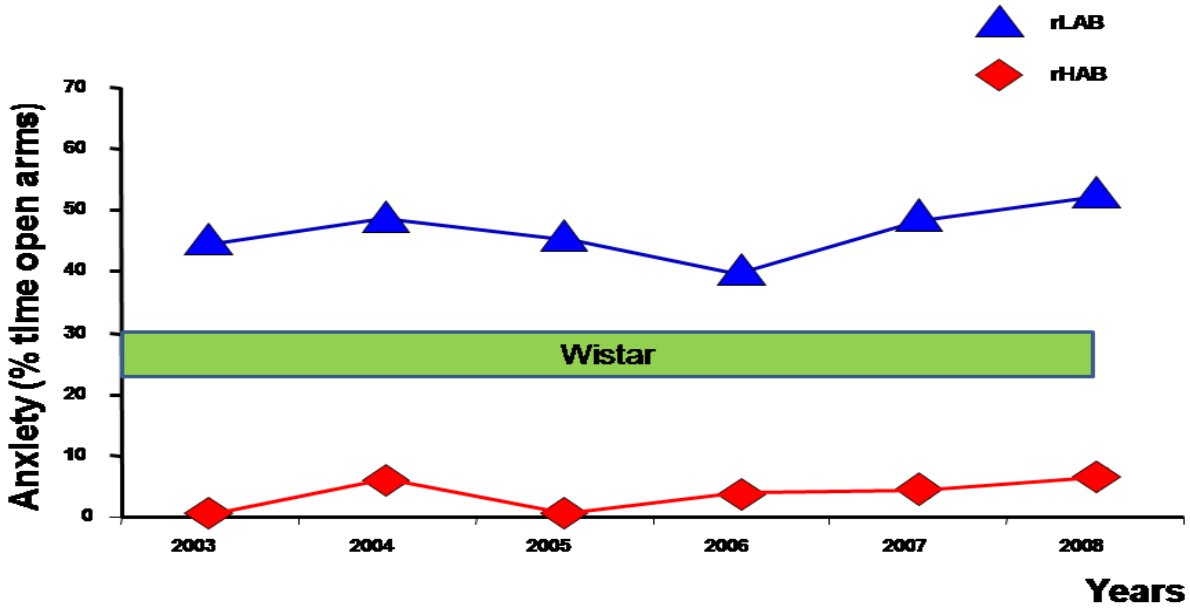


Figure 1: Selective breeding of Wistar rats based on performance in the EPM to give rHAB and rLAB. X-axis shows here years. Y-axis indicates % time spent on open arms. (adapted from (Neumann *et al.*, 2010).

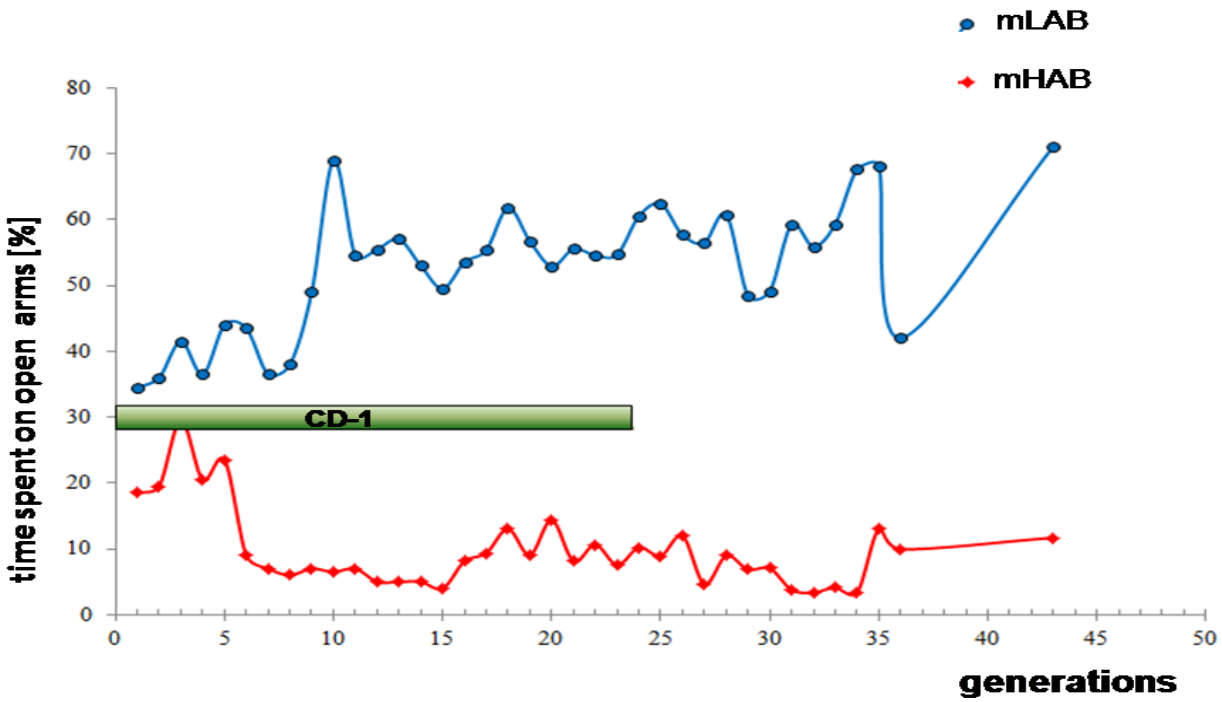


Figure 2: Selective breeding of CD-1 mice based on performance of EPM leading to divergent lines, mHAB and mLAB after several generations. X-axis indicates generations, Y-axis the % time spent on open arms.

In addition to differences in anxiety-related behavior on the EPM, these rodents also display consistent differences in trait anxiety in other behavioral tests, such as the LD box and OF (Kromer *et al.*, 2005; Landgraf & Wigger, 2002). This divergence in trait was further validated under similar conditions in four laboratories (Munich, Regensburg, Innsbruck and Lille) and found to be reliable and robust (Salome *et al.*, 2002).

In the FST, the rHAB floated more and struggled less compared to rLAB indicating passive coping strategy, nonetheless this behavior was reversed to active coping style on treatment with paroxetine (Landgraf, 2003). This indicates that the rHAB also display comorbid depression-like behavior. In addition, rHAB also exhibit an impaired extinction of fear memory in a fear conditioning task compared to rLAB (Muigg *et al.*, 2008). Treatment with diazepam, a commonly used anxiolytic drug reduced anxiety-related behavior in rHAB, thus resulting in the pharmacological validation of the rHAB *vs.* rLAB animal model (Liebsch *et al.*, 1998). Fos expression studies, which is a marker for neuronal activation, in rHAB *vs.* rLAB suggested no differences in neuronal activation under basal conditions. However, when challenged with mild stressors like open arm of EPM and OF there was higher c-fos expression in the hypothalamic nuclei including paraventricular nucleus (PVN) relative to rLABs. Meanwhile the latter showed more activation in the cingulate cortex, thalamic areas and partially the hippocampal formation (Landgraf *et al.*, 2007).

Furthermore, in a social defeat (SD) paradigm such as resident-intruder assay, rHAB displayed higher freezing behavior upon confrontation indicating passive coping style. In contrast, rLAB exhibited higher locomotion and more aggression towards resident suggesting an active stress coping behavior (Frank *et al.*, 2006). There is no difference in basal CORT between rHAB *vs.* rLAB. However, during SD rHAB secrete more CORT indicating alteration of HPA axis activity and more USV in contrast to rLAB (Frank *et al.*, 2006). The SD also enhanced c-fos expression in amygdala and hypothalamic nuclei of rHAB, while less c-fos activity in prefrontal cortex and brain stem regions in contrast to rLAB (Landgraf, 2003). Cross-mated F1 offspring of HAB *vs.* LAB rats display intermediate anxiety phenotype thus proving genetic contribution to anxiety, and even cross-fostering did not have any effect on the strong genetic predisposition, thus differential emotionality is driven autosomally (Wigger *et al.*, 2001).

On the other hand, mHAB *vs.* mLAB also displayed divergent anxiety-related behavior in the LD, OF and elevated platform (EPF) test. The behavior on the EPM was confirmed independently in three different laboratories (Munich, Regensburg and Innsbruck) under similar testing conditions (Czibere, 2008). In the USV test, which is independent of locomotion, mHAB pups emitted more calls than mLAB which was decreased by treatment with diazepam (Kromer *et al.*, 2005). mHAB also displays differences in depression-like behavior as measured by FST, TST where they exhibit more passive coping style relative to mLAB. In addition to phenotypic differences, mHAB gives a flattened CORT response relative to mLAB when exposed to strong physical stressor. Yet there is no basal difference in CORT, body weight and locomotor activity between mHAB *vs.* mLAB (Czibere, 2008). Fos expression studies revealed no difference in basal c-fos activity, however, when exposed to open arm of EPM there was altered activation patterns in prefrontal-cortical, limbic and hypothalamic areas of mHAB in contrast to mLAB (Muigg *et al.*, 2009). Interestingly, this differential pattern of c-fos activation was similar to that of rHAB *vs.* rLAB. Furthermore, cytochrome c oxidase (COX) activity suggests basal differences in long-term activity in the PVN and amygdala of mHAB *vs.* mLAB. Surprisingly, mHAB showed a decreased activity in the PVN in contrast to mLAB, whereas in the amygdala there was higher activity in comparison to mLAB (Czibere, 2008). This is interesting because previous studies have shown that amygdala is important for processing of emotional stimuli while PVN lies at the center to mediate adequate stress response (Bishop *et al.*, 2004; Herman & Cullinan, 1997). The low amygdala activity in mLAB may be crucial to maintain a low anxiety trait. Besides the high activity in PVN of mLAB might explain higher HPA axis activity and subsequent more CORT release on exposure to physical stress in contrast to mHAB (Gonik *et al.*, 2012). Cross-mated F1 offspring of HAB *vs.* LAB mice also display intermediate anxiety phenotype thus indicating genetic contribution towards anxiety (Kromer *et al.*, 2005).

1.13 Novel targets for anxiety disorders: Focus on Neuropeptides

Unlike classical neurotransmitters, neuropeptides which are small proteinaceous molecules are the most diverse class of signaling molecules that exhibit characteristic localization patterns within the central and peripheral nervous system and are thus believed to possess a wide range of physiological functions (Hokfelt *et al.*, 2000; Lin, 2012). Neuropeptide systems of brain, which are characterized by discrete synthesis and release sites, distinct receptor distribution and multiple behavioral functions, represent such potential targets (Ebner *et al.*, 2009; Landgraf *et al.*, 2007; Landgraf & Neumann, 2004; Slattery, D.A. & Neumann, I.D., 2010). Neuropeptides and their cognate receptors are encoded by DNA, thus, are vulnerable to mutations, and are believed to have evolved through either genome, gene, exon duplications or point mutations giving rise to a family of peptides performing diverse roles from invertebrates to humans (Hoyle, 2008). Most of the mammalian neuropeptide receptors are G-protein coupled with 7-transmembrane domains, an extraceullar amino (N) terminal and an intracellular carboxyl (C) terminal. Several neuropeptides, like vasopression (Landgraf *et al.*, 2007), CRH (van Gaalen *et al.*, 2002), oxytocin (Windle *et al.*, 1997), galanin (Sciolino & Holmes, 2012), substance P (Ebner & Singewald, 2006), neuropeptide Y (Primeaux *et al.*, 2005) have been shown to be important for anxiety and other mood disorders.

Previous studies have shown that rHAB have higher arginine-vasopressin (*Avp*) mRNA and corresponding neuropeptide release in the hypothalamic PVN and treatment with an AVP V1 receptor antagonist could reverse the high anxiety-related and comorbid depression-like behavior in rHAB (Wigger *et al.*, 2004). In addition, treatment with the antidepressant paroxetine and diazepam normalized *Avp* mRNA expression, HPA axis activity and consequently decreased depression-like behavior without affecting the binding properties of the AVP V1 receptor (Keck *et al.*, 2003). Furthermore, a single-nucleotide polymorphism (SNP) in the *Avp* promoter of rHAB was shown to confer lower binding of a repressor element, thus resulting in an increase in *Avp* mRNA expression and also higher AVP release in the hypothalamic PVN (Murgatroyd *et al.*, 2004).

On the other hand, in the mHAB *vs.* mLAB and corresponding F1 offspring, there was lower mLAB *Avp* mRNA allele (Bunck *et al.*, 2009) and also deficits in central release of AVP in mLAB (Kessler *et al.*, 2007) and this difference was correlated with anxiety-related behavior (Bunck *et al.*, 2009). Thus by utilizing bidirectional selective breeding approach to Wistar rats and CD-1 mice, two divergent lines, HAB *vs.* LAB rats and mice, were obtained which displayed robust phenotypic and neuroendocrinological differences in anxiety and comorbid depression like-behavior. In addition, these animal models obtained from two different species, were found to

have similar difference in at least one of the neuropeptidergic system (AVP), implicated in anxiety-related behavior.

1.14 Novel target: Neuropeptide S (*Nps*) /neuropeptide S receptor 1 (*Npsr1*)

Neuropeptide S (NPS) is a 20 amino acid mature peptide identified by a reverse pharmacological approach and named after its conserved serine residue at the N-terminal end except for the bat, which has proline at the corresponding position (Reinscheid, 2007). The primary amino acid structure of NPS is evolutionary conserved across mammals, including human and rodents, and even birds, reptiles and amphibians (Figure 3).

SFRNGVGTGMKKTSFQRAKS	human
SFRNGVGTGMKKTSF**R**RAKS	chimpanzee
SFRNGVG**S**G**A**KKTSF**R**RAK**Q**	mouse
SFRNGVG**S**G**V**KKTSF**R**RAK**Q**	rat
SFRNGVGTGMKKTSF**R**RAKS	dog
SFRNGVG**S**G**I**KKTSF**R**RAKS	chicken

Figure 3: Conservation of primary amino acid structure of 20 amino acid mature peptide across humans, chimpanzee, mouse, rat, dog and chicken. The letters in bold indicate the divergent amino acids. Figure from (Xu *et al.*, 2004)**.**

An *Nps* gene is absent from the currently available fish genomes (Reinscheid & Xu, 2005), suggesting that an *Nps* precursor gene appeared late in vertebrate evolution. The NPS precursor protein contains a hydrophobic chain following the initiator methionine residue and also a pair of basic residues (lysine (K) and arginine (R)), just before the serine residue of the mature peptide. The behavioral effects of NPS (anxiolysis coupled with arousal or wakefulness) are unique, because stimulants like cocaine, amphetamine, CRH or GABA antagonist induce arousal but are anxiogenic (Koob & Greenwell, 2004). NPS has been shown to be anxiolytic, inhibits all sleep stages (Leonard *et al.*, 2008; Xu *et al.*, 2004), activates the HPA axis (Smith *et al.*, 2006) and inhibits food intake (Cline *et al.*, 2007; Fedeli *et al.*, 2009; Niimi, 2006). NPS also works as a neuromodulator by regulating the release of several classical transmitter systems like dopamine (Si *et al.*, 2010), nor-adrenaline (Raiteri *et al.*, 2009), glycine, serotonin (Gardella *et al.*, 2013) and glutamate (Han *et al.*, 2009).

Structure-activity studies on NPS revealed that 1-10 residues from the N-terminal end mimicks the activity of the full length 20 amino acid mature peptide in *in vitro* systems. Phenylalanine (F),

arginine (R) and asparagine (N), following the serine residue, are required for the biological activity of the peptide, while residue 8 to 10 are required for activation of the corresponding receptor. However, the C-terminal (11-20) residues also are important for the *in vivo* activity of the peptide as the 1-10 length peptide had very weak effect on the locomotion in mice (Roth *et al.*, 2006).

NPS acts by activating its corresponding receptor called neuropeptide S receptor 1 (NPSR1) which is a 7- transmembrane G-protein coupled receptor sharing moderate homology with oxytocin and vasopressin receptors (Reinscheid & Xu, 2005). Phylogenetic comparative analysis of NPSR1 revealed that its sequences are orthologous to the invertebrate cardioacceleratory peptide receptor (CCAPR) and vasopressin receptor-related receptor 1 (VRR1) suggesting that NPSR1 evolved earlier in the invertebrates, then leading to functional divergence before their emergence in vertebrates (Pitti & Manoj, 2012). NPS has been shown to cause increase in intracellular calcium (Ca^{2+}) and cyclic adenosine monophosphate (cAMP) in cell lines stably expressing human NPSR1, indicating that the NPSR1 couples to the heterotrimeric Gq and Gs proteins (Reinscheid *et al.*, 2005; Xu *et al.*, 2004). Another study used chimeric receptor approach by combining NPSR1 and vasopressin V1a receptor domains and concluded that VRR1 signals through the G_q and G_s pathway (Gupte *et al.*, 2004). The expression of rat NPSR1 mRNA and protein was found to be widely abundant in central nervous tissues (Leonard & Ring, 2011; Xu *et al.*, 2007) while *Nps* mRNA is mostly localized in the peri-*locus coeruleus* (LC) area (Xu *et al.*, 2004). Previous studies could also show that intranasal application of NPS could reduce anxiety in the mHAB accompanied by explicit internalization of the NPS/NPSR1 complex in the NPSR1 expressing neurons (Ionescu *et al.*, 2012). Local injection of NPS in the amygdala of mice was shown to have anxiolytic effects and facilitates extinction of conditioned fear responses, which was reversed by application of a NPSR1 antagonist (SHA 68) (Jungling *et al.*, 2008), thus indicating the presence of an endogenous NPS system in mediating anxiety and fear response. The NPSR1 knockout mice exhibited increased anxiety-related behavior (Duangdao *et al.*, 2009), and there were no NPS-mediated effects observed (Zhu *et al.*, 2010), thus proving that NPS exerts its effects only through NPSR1.

The human NPSR1, previously called G protein-coupled receptor for asthma susceptibility (GPRA) or GPR154 has a unique set of polymorphisms found to be associated with increased risk of asthma and other allergies with high levels of serum Immunoglobulin E in Finnish and Canadian patients (Laitinen *et al.*, 2004). Furthermore, one SNP (rs324981, A/T, N107I), which causes an asparagine (N) to isoleucine (I) substitution at position 107 in the primary amino acid structure of human NPSR1 (Reinscheid *et al.*, 2005), has been associated with panic disorder, anxiety sensitivity, heightened amygdala responsiveness to aversive stimuli (Dannlowski *et al.*,

2011; Domschke *et al.*, 2011; Okamura *et al.*, 2007) and sleep and circadian phenotypes (Gottlieb *et al.*, 2007). This T allele has been also associated with increased activity in the dorsomedial prefrontal cortex, an area that supports conscious appraisal of threat stimuli (Raczka *et al.*, 2010). In *in vitro* studies, the N107I mutation was found to cause increase in cell-surface expression and subsequent increase in agonist efficacy without any effects on its binding affinity (Bernier *et al.*, 2006; Reinscheid *et al.*, 2005).

1.15 Novel target: Transmembrane protein 132D (Tmem132D)

TMEM132D, also called mature oligodendrocyte transmembrane (MOLT) or KIAA1944 (Nagase *et al.*, 2001) encodes a single-pass type 1 integral membrane protein belonging to the TMEM132 protein family. The human *Tmem132d* gene is located on chromosome 12, has two transcripts and the protein product is approximately 130 kDa in size. TMEM132D is predicted to contain a N-terminal hydrophobic signal peptide, a single pass transmembrane region and several posttranslational modifications like N- and O-glycosylation and phosphorylation. Primary cultures of rat oligodendrocyte precursor cells show no expression of either *Tmem132d* mRNA or protein; however, on differentiation they start to express the TMEM132D protein (Nomoto *et al.*, 2003). Notably, there was prominent expression of TMEM132D protein in the cytoplasmic processes of mature oligodendrocytes in both gray and white matter, and thus they may serve as a cell surface marker for mature oligodendrocytes (Nomoto *et al.*, 2003). In addition, *Tmem132d* mRNA was observed in the human pancreas, testis and lungs (Nomoto *et al.*, 2003). However, another preliminary study shows that TMEM132D is a putative cell adhesion molecule and its colocalization with actin and neuronal markers may imply their role in neuronal sprouting (Walser *et al.*, 2011). In mice, *Tmem132d* is located on chromosome 5 and the primary amino acid sequence of TMEM132D is highly conserved with more than 80% sequence identity across humans, rats and mice.

In a genome-wide association study, a haplotype containing two intronic SNPs rs7309727 and rs11060369 in the human *Tmem132d* were associated with panic disorder, and three other SNPs were associated with severity of anxiety symptoms in German patients (Erhardt *et al.*, 2011). The two intronic SNPs were further replicated in additional samples of panic disorder patients and subsequent meta-analysis (Erhardt *et al.*, 2012). These risk genotypes were associated with higher *Tmem132d* mRNA in the frontal cortex of panic disorder and unipolar depressed patients (Erhardt *et al.*, 2011). In addition, next generation sequencing of *Tmem132d* revealed an overrepresentation of rare variants in healthy controls as compared to panic disorder patients, thus suggesting that rare variants decreasing the functionality of the corresponding gene might have a protective effect (Quast *et al.*, 2012). Furthermore, a recent

study by (Sämann *et al.*, 2012) who used functional magnetic resonance imaging (fMRI) to study the genotype-dependent connectivity in healthy individuals found that rs7309727 risk allele carriers have a lower whole brain connectivity in the ventromedial prefrontal cortex. In addition, *Tmem132d* has been identified as a candidate gene in children with attention-deficit hyperactivity disorder (ADHD) (Mick *et al.*, 2011). On the other hand, in the mHAB *vs.* mLAB and the corresponding F2 panel obtained by cross-mating F1 offspring among each other, *Tmem132d* mRNA expression and a corresponding SNP were associated with anxiety-related behavior in the anterior cingulate cortex, central to the processing of anxiety/fear-related stimuli (Erhardt *et al.*, 2011). Likewise a *Tmem132d* SNP in outbred CD-1 mice has been associated with exploratory behavior in the LD box test (Czibere, personal communication).

1.16 Aims of the present thesis

1. To study the behavioral and molecular architecture of a novel candidate gene, NPS/NPSR1 in anxiety-related behavior.

The anxiolytic and fear attenuating effects of NPS was first confirmed in HAB rodents by central administration. Similarly, central administration of NPSR1-A (antagonist) was found to cause higher anxiety in LAB rodents. This encouraged me to study the molecular basis of NPS mediated anxiety-related behavior.

Thus I measured basal *Nps* and *Npsr1* mRNA expression by qPCR in selective brain regions of HAB *vs.* LAB, implicated in anxiety-related behavior and NPS synthesis. This was followed by sequencing of *Npsr1* and *Nps* gene to determine polymorphisms that might underlie differential expression of the gene. The expression of any gene can be modulated by different *trans*-acting factors like synaptic input or chromatin modifications impinging on every cell of HAB or LAB animal. Thus, I took advantage of HAB *vs.* LAB crosses to analyze the differential pattern of *Npsr1* mRNA alleles in the same cell, wherein each allele acts as an internal standard for the other. This method allows differential expression to be pinpointed on the *cis*-acting variations as the expression of alleles is studied in the same pool of *trans*-acting factors. Then to assess the molecular and functional differences, techniques such as *in vitro* promoter assays, protein qualitative assays and Western blots were utilized. *Npsr1* mRNA expression was also studied in mHAB, mLAB subjected to EE and UCMS, respectively to determine its plasticity.

2. To study the behavioral and molecular underpinnings of a second novel candidate gene, *Tmem132d* implicated in anxiety-related behavior.

Earlier studies from our group have shown higher expression of *Tmem132d* gene in the cingulate cortex of mHAB in contrast to mLAB. Also, two polymorphisms were found in the corresponding promoter region. Here, I again utilized *in vitro* promoter assays to explain the observed differential *in vivo Tmem132d* expression in mHABs *vs.* mLABs. The presence of a CpG island in the *Tmem132d* promoter region prompted me to do bisulfite sequencing in search of additional factors underlying differential expression. The plastic nature of the gene was also studied in mHAB, mLAB subjected to EE and UCMS, respectively. To study GXE interactions and negate the confounding *trans*-acting factors, mHAB *vs.* mLAB crosses were subjugated to EE and UCMS and their corresponding effects on anxiety-related and comorbid depression-like behavior analyzed. Subsequently, *Tmem132d* mRNA alleles were studied *via* qPCR in these animals. Methylation specific PCR was utilized to determine the epigenetic factors mediating the plastic nature of the *Tmem132d* gene.

2.0 Materials and methods

2.1Animals

Adult HAB, LAB rats and mice selectively bred based on performance in EPM over more than 25 generations were utilized for these studies. Rats and mice were housed in groups of four, until undergoing surgical procedures or one week before tissue harvesting, when they were single-housed. Similarly, F1 offspring of both rat and mice were obtained by cross-mating of HAB mothers with LAB fathers and *vice versa* to rule out any imprinting effects. Animals were maintained on a 12-h light/dark cycle (lights on at 06:00 a.m. (rats) and 08:00 a.m. (mice)) in a temperature controlled colony (21-23°C, 55 % humidity). The animals had free access to food and water. All experimental procedures were performed in the morning (08:30 – 11:30) and conducted in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and the approval of the local governments of the Oberpfalz and Oberbayern.

2.2 Behavioral testing of HAB, LAB and F1 offspring of rats and mice

The primary testing for the selection of experimental HAB, LAB and F1 offspring of rats and mice was performed on the EPM at the age of 9 and 7 weeks, respectively, as follows.

2.2.1 Elevated plus-maze

The EPM was utilized to test anxiety-related behavior in both rats and mice. The 5 minute test was carried out on a plus-shaped maze, which was elevated (rats: 70 cm; mice: 37 cm) from the ground consisting of two closed arms (rats: 50 x 10 x 40 cm; 25-30 Lux; mice: 30 x 5 x 15 cm; 10 Lux) and two open arms (rats: 50 x 10 cm; 90-100 Lux; mice: 30 x 5 cm; 300 Lux) joined by a central neutral zone (rats. 10 x 10 cm; mice: 5 x 5 cm). A camera above the maze allowed assessment of behavior. The test commenced by placing the animal in the neutral zone facing a closed arm, and the percentage time spent on the open arms, as an anxiety index, was determined by an observer blind to treatment. The number of closed arm entries (rats) or total distance travelled (mice; Any-maze 4.82; Stoelting co., Illinois, USA) was determined as an indicator of locomotor activity. Only rats spending less than 10 % and more than 35 % of their time on the open arms, and only mice spending less than 15% and more than 55% of their time on the open arms of the EPM were considered for experimental use as HAB or LAB animals, respectively.

2.2.2 Surgical and drug infusion procedures

To assess the effects of intracerebroventricular (*icv*) NPS and D-Cys(tBu)^5-NPS (an NPSR1 antagonist [NPSR1-A] (Camarda *et al.*, 2009)) infusion on anxiety-related behavior of HAB and LAB, respectively, rats and mice were fixed in a stereotaxic apparatus and implanted with an indwelling guide cannula using isoflurane anaesthesia under semi-sterile conditions as previously described (Kessler *et al.*, 2010; Slattery & Neumann, 2010). Briefly, a guide cannula (rat: 21-gauge, 12 mm long; mice 23-gauge, 8 mm long) was implanted 2 or 1.5 mm above the right lateral ventricle (rat: AP: -1.0 mm from bregma; ML: +1.6 mm; DV: +1.8 mm (Paxinos & Watson, 1998); mice. AP: -0.3 mm; ML: +1.1 mm; DV: +1.6 mm) (Franklin & Paxinos, 2007), and anchored to two stainless-steel skull screws using dental acrylic.

Animals were allowed to recover for at least 7 days before undergoing behavioral testing. In rat studies, vehicle (5 µl; Ringer's solution, pH 7.4, B. Braun Melsungen AG, Melsungen, Germany), NPS (0.1 – 1 nmol/5 µl, Bachem GmbH, Weil am Rhein, Germany) or NPSR1-A; 10 nmol/5 µl,) were infused *icv* acutely prior to the behavioral testing (45 min prior to the EPM) via an 28 g-infusion cannula, which extended 2 mm beyond the tip of the guide cannula, attached via polyethylene tubing to a 5 µl Hamilton syringe. In mice, vehicle (2 µl; Ringer's solution, pH 7.4, B. Braun Melsungen AG, Melsungen, Germany) or NPS (1 nmol/2 µl, Bachem GmbH, Weil am Rhein, Germany) were infused *icv* 25 min before behavioral testing via a 30 g-cannula.

2.2.3 mHAB *vs.* mLAB crosses for GXE interaction studies

Another cohort of F1 offspring from mHAB *vs.* mLAB crosses and *vice versa* were left with mother in the cage from postnatal day (PND) 1 to PND 15. Subsequently, the F1 offspring were divided into three groups, each for UCMS, EE and a control group. For EE, the size and complexity of cage was enhanced by providing more nesting material such as wood chips, insets for climbing (ladder) or hiding (tube), raised platform and group housing with three animals per cage over a total period of 27 days (Markt, 2012) as these improved conditions have been shown to have dramatic effects on physiology and behavior of animal as shown by increased exploration, locomotion, foraging and more positive social interactions and less agonistic behavior (Baumans, 2005; Olsson & Dahlborn, 2002). On the other hand, for UCMS, F1 offspring at PND 15 were subjected to different types of stressors like wet bedding, cage tilting, stroboscopic light illumination, inversion of the light/dark cycle, etc., over the same time period as EE to elicit anxiety and depression-like behavior (Markt *et al.,* (submitted); Mineur *et al.*, 2006). After this, behavioral testing was carried out using EPM, LD box, TST and FST. The EPM was carried out as described above.

2.2.4 Light-dark box test

The LD box test was carried out for 5 minute to measure percent time, distance travelled in the light compartment and entries, latency to enter the light compartment (Hascoët & Bourin 2009). The LD box was composed of a dark (16 x 27 x 27 cm) and light compartment (32 x 27 x 27 cm) illuminated with 400 Lux and <20 Lux, respectively.

2.2.5 Tail suspension test

The mouse was suspended by its tail to a bar that was 35 cm above the ground. The mouse behavior was videotaped during the 6 minute trial and the duration of total immobility was scored.

2.2.6 Forced swim test

The FST was performed for 6 minutes by placing the mouse in a glass beaker filled with 2 liters of water at room temperature (22.5±1 °C). The dimensions of the beaker were 13.5 cm (diameter) and 28 cm (height). During the trial the mouse tail did not touch the floor of the cylinder and sight barriers prevented its distraction. Parameters such as total amount and percent time spent immobile were measured. All behavioral tests were videotaped and analyzed by Any-maze 4.82 (Stoelting co., Illinois, USA) or Eventlog 1.0 (EMCO software, Devon, UK). Further details on rat EPM test and all the other mouse behavioral tests are available here (Hascoët & Bourin 2009; Kromer *et al.*, 2005; Neumann *et al.*, 2010)

2.3 Determination of *Npsr1, Nps* and *Tmem132d* mRNA expression levels

Male and female rats and mice were sacrificed under basal conditions and brief 20-sec isoflurane (Curamed Pharma GmbH, Karlsruhe, Germany) anaesthesia, the brains snap-frozen in 2-methylbutane (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), stored at -80°C, and 3 x 200 µm PVN-targeted, 3 x 200 µm basolateral amygdala-targeted, 5 x 200 µm LC-targeted and 6 x 200 µm cingulate cortex (Cg)-targeted sections were mounted on slides with the aid of histological staining and atlases (Franklin & Paxinos, 2007; Paxinos & Watson, 1998). Frozen tissue punches of 1.8 mm inner diameter (rats) and 0.8 mm (mice; Fine Science Tools, Heidelberg, Germany) from the PVN, basolateral amygdala, LC area and Cg were harvested.

2.3.1 Quantitative PCR (qPCR) to measure mRNA expression

Total RNA was separately extracted from PVN, amygdala, LC area or Cg punches in rats and mice, kept frozen at -80°C as described here. To homogenize the tissue punches, 300 µl of

precooled TRI® (Sigma-Aldrich GmbH, Munich, Germany) and 30 µl nuclease-free water was added to each tube and mixed up and down at least 40X with a 200 µl pipette tip. Subsequently, 1 µl linear acrylamide as a coprecipitant (Life technologies, Darmstadt, Germany) and 60 µl chloroform which helps to remove phenol (Sigma-Aldrich GmbH, Munich, Germany) were added and vortexed for 30 sec. Then centrifuged for 5 minutes at 18^0C at 13000 rpm and the upper aqueous phase containing total RNA was transferred to a clean Eppendorf tube. Equal volume of isopropanol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added and the tube was left to precipitate overnight at -20^0C.

Next day, the tubes were centrifuged at 13 000 rpm at 4^0C for 30 minutes to pellet the total RNA., the supernatant was removed and the RNA pellet was washed twice with ice cold 70 % ethanol. Then the supernatant was removed and pellet dried in a heated block at 50^0C for 5 minutes with open lids. Finally, the pellet was redissolved in the required volume of nuclease-free water and heated at 95^0C for 5 minutes before proceeding for cDNA conversion. For cDNA conversion, we utilized the High Capacity cDNA Reverse Transcription Kit (Life technologies, Darmstadt, Germany) and followed the manufacturer's instructions. For qPCR, QuantiFastSYBR Green Kit (Qiagen, Hilden, Germany) was utilized and based on the manufacturer's instructions, a master mix was prepared with each sample carrying 1 µl of nuclease-free water, 1 µl of the 10 µM respective forward and reverse primers and 5 µl QuantiFastSYBR Green reagent and then 2 µl of cDNA was utilized.

The qPCR was performed on Light Cycler 2.0 equipment (Roche Diagnostics, Mannheim, Germany) using the following PCR conditions: Hot start to activate polymerase at 95^0C for 5 min, amplification with 40 cycles (X) (Denaturation at 95^0C for 10 sec, Combined annealing and extension at 60^0C for 30 sec) and melting curve 1X (95^0C, 50^0C for 10 sec and 95^0C) and then cooling (42^0C for 30 sec). Experiments were performed in duplicates and every run included a 1:5 and 1:25 diluted sample to generate a standard curve as well as a negative control. The primers used are indicated in Tables 1 and 2 for rats and mice, respectively. Relative transcript concentrations were calculated using the $2^{(-\Delta\Delta Ct)}$ method (Livak & Schmittgen, 2001).

Table 1: List of primers used for mRNA expression studies in the mHAB and mLAB.

Gene	Orientation	Primer sequence (5'→3')
Housekeeping genes		
Rpl13a	(+)	CACTCTGGAGGAGAAACGGAAGG
Rpl13a	(-)	GCAGGCATGAGGCAAACAGTC
B2mg	(+)	CTATATCCTGGCTCACACTG
B2mg	(-)	CATCATGATGCTTGATCACA
Target genes		
Nps	(+)	TGGTGTTATCCGGTCCTCTC
Nps	(-)	GGACCTTTTCATCGATGTCT
Npsr1	(+)	CTCTTCACTGAGGTGGGCTC
Npsr1	(-)	CCAGTGCTTCAGTGAACGTC
Tmem132d	(+)	CATCCCTTCTTCAGCCAGAG
Tmem132d	(-)	AGTGAGAACCGCTGAATGCT

Table 2: List of primers used for mRNA expression studies in the rHAB and rLAB.

Gene	Orientation	Primer sequence (5'→3')
Target and housekeeping genes for rat *Nps* measurements		
Polr2b	(+)	GAAGCCAGGTTAAGAAATCTC
Polr2b	(-)	GACACTCATTCAGCTCACAC
Gapdh	(+)	TGGAGTCTACTGGCGTCTT
Gapdh	(-)	TGTCATATTTCTCGTGGTTCA
Actb	(+)	GGCACCACCATGTACCCAGGC
Actb	(-)	CGATGGAGGGGCCGGACTCA
Nps	(+)	ATCTTAGCTCTGTCGCTGTC
Nps	(-)	CGACGTCTTCTCCAAAATTG
Target and housekeeping genes for rat *Npsr1* measurements		
18srRNA	(+)	ACGGACCAGAGCGAAAGCAT
18srRNA	(-)	TGTCAATCCTGTCCGTGTCC
Actb	(+)	TGTCACCAACTGGGACGATA
Actb	(-)	GGGGTGTTGAAGGTCTCAAA
CycA	(+)	AGCACTGGGGAGAAAGGATT
CycA	(-)	AGCCACTCAGTCTTGGCAGT
Gapdh	(+)	TCACCACCATGGAGAAGGC
Gapdh	(-)	GCTAAGCAGTTGGTGGTGCA
Hmbs	(+)	TCCTGGCTTTACCATTGGAG
Hmbs	(-)	TGAATTCCAGGTGAGGGAAC
Hprt1	(+)	GCAGACTTTGCTTTCCTTGG
Hprt1	(-)	CGAGAGGTCCTTTTCACCAG
Rpl13a	(+)	ACAAGAAAAAGCGGATGGTG
Rpl13a	(-)	TTCCGGTAATGGATCTTTGC
Ywhaz	(+)	TTGAGCAGAAGACGGAAGGT
Ywhaz	(-)	GAAGCATTGGGGATCAAGAA
Npsr1	(+)	CTGTTCTCCATCCCCACACT
Npsr1	(-)	GCAGTTGGAAATCACCGTCT

2.4 PCR amplification of the *Nps* and *Npsr1* gene products

Genomic DNA was extracted from cerebellar tissue or tail tips of HAB and LAB rodents using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co., Düren, Germany) as per manufacturer's instructions. Then, NCBI/Primer-BLAST was used to design sequencing primers for the ten exons and approximately 2,000 bp promoter region of the rat and mouse *Nps* and *Npsr1* gene (Sigma-Aldrich GmbH, Munich, Germany). The *Npsr1* and *Nps* DNA fragments were amplified using Taq-polymerase (see Tables 3 – 6 for a list of the primers used; Fermentas, St. LeonRot, Germany) as follows:2.5 µl of 10X PCR buffer containing $(NH_4)_2SO_4$, 1.5 µl of 10 mM dNTP mix, 1.5 µl of 2 µM respective forward and reverse primers, 3 µl of 25 mM $MgCl_2$, 1 µl of Taq polymerase (1 U/µl), 1 µl DNA and nuclease-free water up to 25 µl. The PCR conditions were as follows: Initial denaturation at 95°C for 5 min, 35X (denaturation at 95°C for 1 min, annealing at (52-60)°C for 1 min, extension at 72°C for 1 min), Final extension at 72°C for 10 min and 4°C overnight.

2.4.1 Cycle sequencing of the *Npsr1* and *Nps* gene products

15 µl of PCR product was loaded onto Nucleofast 96 PCR clean-up plate (Macherey-Nagel GmbH & Co., Düren, Germany). Then 100 µl nuclease-free water was added to each well and the plate centrifuged at 4500 g for 10 minutes at room temperature. This was repeated two times. Then the samples were resolved in 25 µl nuclease-free water on a shaker for 10 minutes. Subsequently, BigDye Terminator kit v3.1 (Applied Biosystems, California, USA) was utilized for sequencing reaction as follows per sample:

1.2 µl sequencing buffer (5X), 0.4 µl BigDye reagent, 1 µl corresponding forward or reverse primer (2 µM) and 2.4 µl of cleaned PCR product were subjected to the following PCR conditions: Initial denaturation at 96°C for 1 min, 35X (denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 4 min). Then the reaction mixture was loaded onto Montage Seq 96 plate (Millipore GmbH, Schwalbach, Germany) and washed twice with 20 µl Montage injection solution (Millipore GmbH, Schwalbach, Germany). Finally the PCR product was dissolved in 20 µl injection solution and then resolved by capillary electrophoresis on a ABI 3730 DNA analyzer (Life technologies, Darmstadt, Germany) at the Helmholtz Zentrum's Institute of Human Genetics (Neuherberg, Germany).

Table 3: List of primers used for sequencing of the rat *Npsr1* gene with location and exons/promoter/downstream enhancing region (DER) the primers hybridized to.

Sequential order	Location	Orientation	Primer sequence (5'→3')
1	Intron1	(+)	GGTGAGCAATAGCCAGAAGC
1	Intron2	(-)	CAGAATTTAAAGCCAGGGCA
2	Intron2	(+)	GCGCAAGTGACTGTGTCATC
2	Intron3	(-)	CTTCTCTCCCGCTGGTACTG
3	Intron3	(+)	TCCATGCCTCACTTTTCCTC
3	Intron4	(-)	AGCTAGGGAGAAAGGCGTGT
4	Intron4	(+)	AGCCCAGATCTGCTTCCAGT
4	Intron5	(-)	ATGGCGTGAGGATCAGGTAG
5	Intron5	(+)	GTCCTAGTGACTCCCAGCCA
5	Intron6	(+)	GTTCCCACAAGGAGTTTGGA
6	Intron6	(-)	TGGCACCTTCAGTATGAGCA
6	Intron7	(+)	CCAGATACCCCTATTTCCAGC
7	Intron7	(-)	AGCCGCCACTAATCCATCTT
7	Intron8	(+)	ACACTCCTTCCCTGCATGTC
8	Intron8	(+)	AAGAGGGATGCTTCTGGTGA
8	Intron9	(-)	GAGCATTGGGAGCACAACTT
9	Intron9	(+)	TGGAGGAAGAGGTCCAGTTG
9	Exon10	(-)	ATGGTGAAGGTCTGGGTGAG
10	Exon10	(+)	CTCTCCAAGCCTGAATTCATC
10	Exon10	(-)	CTAACATCTTCTCCTCCACATG
11	Exon10	(+)	GGAGGACAACAAAGGTTAGAC
11	Exon10	(-)	ATAAGACCAGCACTTCCTTG
12	Exon10	(+)	AAATAGTGATAGACCCTGGC
12	Exon10	(-)	ACATGTTAACGACTGAACGA
13	Exon10	(+)	CCCACAGCCCTATGACGCACG
13	Exon10	(-)	TGCTAGCTAGGACACCCGCCA
14	Exon10	(+)	GCTGACGGCTCGTTCAGTCG
14	Exon10	(-)	AGGGGATGGTGTCGGCATGTG
15	Exon10	(+)	ACAGGACTGGTGCTGAAATCGC
15	Exon10	(-)	ACTTCAACATCCTCTGCTACACTGC
16	Exon10	(+)	GTCCTATGATGCTGGATGAATCATGC
16	Exon10	(-)	CCTGAAAGGAGAGGATCTTTCGCCA
17	Exon10	(+)	GGTGCCCACCTTCCACACCAAGATG
17	DER	(-)	GGCCATCAGACGTGTGGCTTCC
18	DER	(+)	CCAGCTTCATAGAGACAGCTCTGC
18	DER	(-)	ACCCCCATTCTCCCACCCCAC
19	DER	(+)	TCATTATCCACAACAGGGCTGGACC
19	DER	(-)	ATGGCCTGCAAGGCTAAGGCG
20	DER	(+)	CAGCACTTGGGAGACAGAGA
20	DER	(-)	TGCTGAGCTAAATGTCAAAG
-1	Promoter	(+)	AGACAAACACAGACCCCTGC
-1	Intron1	(-)	GAGTTCAGTTAGCCAGGGCA
-2	Promoter	(+)	TGTCATGTCGAAACCCTTCA
-2	Promoter	(-)	CAGCTGAGATCGCTTTTGTCT
-3	Promoter	(+)	GTCAGCAGCTTCTGTGCATC
-3	Promoter	(-)	AAGGGGTATGTCCCAGGAAG
-4	Promoter	(+)	TGCACCCATTTTTAGTTCCC
-4	Promoter	(-)	AGGGGTATGTCCCAGGAAGT

Table 3 continued: List of primers used for sequencing of the rat *Npsr1* gene with location and exons/promoter/downstream enhancing region (DER) the primers hybridized to.

Sequential order	Location	Orientation	Primer sequence (5'→3')
-5	Promoter	(+)	GATCCTACTTTGGGCCTGTCG
-5	Promoter	(-)	GAAGATGCTCAACCACATTATTAGC
-6	Promoter	(+)	GTCCACCCCTGAGAGTTCCAG
-6	Promoter	(-)	CAGGGCATCAAGTGAGGGCATC
-7	Promoter	(+)	GGTGTGGATTTGTGAGGGAGGT
-7	Promoter	(-)	GTTCAGTCAGGGAAGATGCATC

Table 4: List of primers used for sequencing of the mouse *Nps* gene with location and exons/promoter/downstream enhancing region (DER).

Sequential order	Location	Orientation	Primer sequence (5'→3')
-4	Promoter	(+)	CCAGGCTTCCAGCTTGGCAC
-4	Promoter	(-)	GCTGCTATTGCTGCTGTTTCTGAAG
-3	Promoter	(+)	GGGTATCTTTGCCCTCCAAAAGGTG
-3	Promoter	(-)	GGCAATCTGTTGTCACTGGTCCCTG
-2	Promoter	(+)	TCCCTGCTCAACACCCCAAACC
-2	Promoter	(-)	ACTGGTTGGCCTGGCTGTGG
-1	Promoter	(+)	GAGGCTCCTGGCCACCCATG
-1	Intron2	(-)	GGGCCCTCCACCATCCTGATCA
1	Promoter	(+)	TGGCAAGCTCTGAGTGAAGTCAACC
1	Intron2	(-)	TTTGGGCCCTCCACCATCCTGA
2	Promoter,Exon1	(+)	CCCATCTGCGCAGGTCTCGG
2	Intron2	(-)	TCCACTGTGCGGGTTTTTGGT
3	Promoter,Exon1	(+)	CATCTGCGCAGGTCTCGG
3	Intron2	(-)	CCAGAGTTACCTACTGTCACATAC
4	Intron2	(+)	AGCCGGTGGTAGCCCTACACT
4	Exon3	(-)	ACTCTGAGCCCGTTAGGAGAAGGG
5	Exon3	(+)	CCTTTCGCAACGGAGTCGGCT
5	Exon3	(-)	CGAGCCCTTGCTGCAGGTACC
6	Exon3	(+)	GTGCCACCAAGTGCAGTGGC
6	DER	(-)	GCTGGTGACCAAGGACAGGGT

Table 5: List of primers used for sequencing of the mouse *Npsr1* gene with location and exons/promoter/downstream enhancing region (DER).

Sequential order	Location	Orientation	Primer sequence (5'→3')
-7	Promoter	(+)	GCAGAGGAGACCACACTGGCG
-7	Promoter	(-)	GCCTGACGACAAGGAAGATCCACG
-6	Promoter	(+)	TTGTCATCTCCTGTCTGTGCCCCT
-6	Promoter	(-)	CGCCAGTGTGGTCTCCTCTGC
-5	Promoter	(+)	TGCAGCGTAATGAACACCCCCA
-5	Promoter	(-)	GTAGGCCAACCTTTGCTTTACTGCC
-4	Promoter	(+)	CTGTATGTGCAAATGTGTGTC
-4	Promoter	(-)	GGAGAGCAGAATGTCATGAG
-3	Promoter	(+)	AAGCCCTCATCTCTAACCTG
-3	Promoter	(-)	TCATGGTTTCCCCTCCTCCA
-2	Promoter	(+)	GGGCAAACAAACACTATTGATC
-2	Promoter	(-)	ACATCCCCTAAATACCACTGAGT
-1	Promoter	(+)	CACCTACAAACTTTTCCATC
-1	Promoter	(-)	AATCTCCACATTTCCCTGAG
1	Promoter	(+)	GGGCAGGTCTGTGGGATGGTG
1	Intron2	(-)	GCCTCCCTAGCAGCAGCTAAGACT
2	Intron2	(+)	CCTGGGCATTTGCTGGGCGG
2	Intron3	(-)	TGTGAGGACACTGAAGGTGGCA
3	Intron3	(+)	AGCAAGCCCTCTCCTGGGACC
3	Intron4	(-)	AAGGAGTGTCTGATTGTGCAGGAGC
4	Intron4	(+)	CTGCTTCCAGCAGGGAGGGC
4	Intron5	(-)	TGGGGTGAGGATCAGGCAGCA
5	Intron5	(+)	AGGTAGGTGGGCCTGCACCC
5	Intron6	(-)	AAGCAGGGTCCAGCCCGTGG
6	Intron6	(+)	CAAGCAGAGCTGTCAAGGATGGT
6	Intron7	(-)	GCTTTCAGGGAGGCCGAGTGG
7	Intron7	(+)	TGGGCATTTGCATTGGGTTGC
7	Intron8	(-)	TGGCTCTTGCAGCAGTCAAACAC
8	Intron8	(+)	TGTTAGCACACCCAAGGCCAC
8	Intron9	(-)	GGAAGTGTACGGAGGTTCGCAGC
9	Intron9	(+)	ACTGTCCACTAGGCTGTGATGGC
9	Exon10	(-)	TGCAGGTGCTGGGCTAACGG
10	Exon10	(+)	TGCCACCTGCAATTCACGCAC
10	Exon10	(-)	TGTGCCTGCATGGTGTCCTTGT
11	Exon10	(+)	AGCAAGAGCAAACTCCCAAGCA
11	Exon10	(-)	GCATCATAGGGCTGTGGGTGG
12	Exon10	(+)	GGCACCTCTGGCACCTCTGC
12	Exon10	(-)	CCACCATGACCTTAAGCAGGCAGTC
13	Exon10	(+)	TGGCTGACTGCTGGTTGAGTCG
13	Exon10	(-)	CAAGGGCCTGGGCCTCCTGT
14	Exon10	(+)	AGCAAGCAGAAGCATTGAGTGGC
14	Exon10	(-)	GTGGTGCCCAGAGACACAGCA
15	Exon10	(+)	GCCATCTATGCAGAACTTGCTCTACG
15	Exon10	(-)	AACACATTTGCCCGATCAGCCT
16	Exon10	(+)	AGGTGCCTACCTTCCACACCAAG
16	DER	(-)	GGCTGTCAAATGTGCAGCTTCCCT

Table 6: List of primers used for sequencing of the rat *Nps* gene with location and exons/introns/promoter/downstream enhancing region (DER) the primers hybridized to.

Sequential order	Location	Orientation	Primer sequence (5'→3')
1	Promoter	(+)	CCCCTGGCCACCCATGTCAC
1	Exon2, Intron2	(-)	AGCCGTGAAGCCCTTACCTTGGA
2	Promoter	(+)	GCAGGCTCAGACAGCGAGCG
2	Intron2	(-)	GAAACAGCCATTTCCATGTGCAGG
3	Intron2	(+)	TCAGGATGGTGGAGTGCCCAA
3	Intron2	(-)	GCTCATGGCATAGGAGCAAGGACA
4	Intron2	(+)	AAATGATTGCCTTTCTTCGGGGGT
4	Intron2	(-)	ACACCACCTTGTGGCCCAGGA
5	Intron2	(+)	TCCAAGTGGCAACTCCTGCAAGC
5	Intron2	(-)	AGGCAGCACCATCGCTCACC
6	Intron2	(+)	TGTCCCTAAAGGTTTGCTCACCGC
6	Intron2	(-)	ACTGCCCATTTTAAGTCTTGAGCCACC
7	Intron2	(+)	AGTGGCCTCTGGGAAGAGTGG
7	Intron2	(-)	GCCCTGGCTGAGTGAATGACTGG
8	Intron2	(+)	TGCACATCTTCTTTCCTCCAGAGCCA
8	Intron2	(-)	GCCTCCGATGGGAGCTGCTG
9	Intron2	(+)	TCCCAACCCCCAAACAGAGCG
9	Intron2	(-)	ACCGGGCCAAAGGAACCTGC
10	Intron2	(+)	TGGCTCTGGCGCTTGGCTTC
10	Intron2	(-)	AGCCCTAGGTTTAGCCCCCAGC
11	Intron2	(+)	CGGCCTGCCCATGCACACTTA
11	Exon3	(-)	GCCTGGCTGGGCAGGTACTC
12	Intron2	(+)	GCTGTGTTTCAGTGATGTTTCTCCCCA
12	DER	(-)	GGCGGAAGTTTGAGACAGGTTTGC
13	Exon3	(+)	ACGGAGTCGGCTCAGGGGTG
13	DER	(-)	CGCTGGCGATCCCTTGCTGC
14	DER	(+)	ACGACGCGTGGGCGTTTCTAC
14	DER	(-)	TGACCTGGCAGGGACAGCGA
15	DER	(+)	CCTGGGTCTGTTTCTCCCCCTC
15	DER	(-)	CTGGAAGCTGGTGCCAAGGATAC
-6	Promoter	(+)	GGAGCTGCAGGCAAAGCCTCA
-6	Promoter	(-)	ACCCAAACCAAGGTTCCTCACCA
-5	Promoter	(+)	GATACAGCAAACAGGAGGGA
-5	Promoter	(-)	TCTCCAAAGAACAGAGCTCC
-4	Promoter	(+)	CAAGAAGAAGGGAAGTGATGTGGCA
-4	Promoter	(-)	AGGACAAGGAGGTGACCCAGCT
-3	Promoter	(+)	CCCAGGCTTCCAGCTTGGCA
-3	Promoter	(-)	CGGCAGAGGAAAACGTCAGAGGG
-2	Promoter	(+)	CGGATCCTTGTGCTTGCATGGC
-2	Promoter	(-)	GGCCAGGGGCCTCCAAAGGA
-1	Promoter	(+)	CAGCCCTGTCAGCCTGCATCA
-1	Intron1	(-)	AGGACCTTGGGTGGGATCTCACAC

2.5 Copy number variation (CNV) measurement of *Nps* and *Npsr1*

To determine if CNV plays any role in differential regulation, genomic DNA was also used to measure corresponding gene copies using qPCR. The corresponding primers are listed in Table 7.

Table 7: List of primers used for the measurement of CNV of *Nps* and *Npsr1.*

Species, gene	Orientation	Primersequence(5'→3')
Rat, *Npsr1*	(+)	GCTGCTGCTGCCCTGGCTAA
Rat, *Npsr1*	(-)	GCCCTCTGTGAGGTTGGCCG
Rat, *Nps*	(+)	AGCTCTGTCGCTGTCCGTGGT
Rat, *Nps*	(-)	AGCCGTGAAGCCCTTACCTTGG
Rat, *Gapdh*	(+)	CGTGTGTAGCGGGCTGCTGT
Rat, *Gapdh*	(-)	CCAGGCGTCCGATACGGCCA
Mouse, *Npsr1*	(+)	CAGCTGCTGCCCCGGCTAAC
Mouse, *Npsr1*	(-)	GGTTGGCTGGCATGGCTCAGG
Mouse, *Nps*	(+)	ACGTGCTTTGGTGTTATCCGGTCC
Mouse, *Nps*	(-)	TTGGGCCCTCCACCATCCTGA
Mouse, *Gapdh*	(+)	TCCCCCTATCAGTTCGGAGC
Mouse, *Gapdh*	(-)	AGTAGCTGGGCCTCTCTCAT

2.6 Bioinformatic analysis of DNA sequences

Rat and mouse *Nps* and *Npsr1* DNA sequences from at least three each HAB *vs.* LAB were analyzed using BioEdit V 7.0.2 (Hall, 1999) for polymorphisms. CpG island searcher (Takai & Jones, 2002, 2003) was utilized to search for CpG islands using default settings (http://cpgislands.usc.edu/): GC%: 55%, ObsCpG/ExpCpG: 0.65, length: 500 bp and gap between adjacent islands: 100 bp. CpG islands are usually prone to DNA methylation and they may play a role in differential regulation of the gene. Potential transcription factor binding sites on *Npsr1* and *Tmem132d* promoter were predicted using the Transcription Element Search System (TESS) (http://www.cbil.upenn.edu/cgi-bin/tess/tess) (Schug, 2008) and PROMO database (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) (Farre *et al.*, 2003; Messeguer *et al.*, 2002). Genomic coordinates are based on genome builds RGSC3.4 (EnsEMBL release 68) for rats and Mm10 (GRCm38, EnsEMBL release 68) for mice. VISTA genome browser was used for *Npsr1* comparative genome analysis (Dubchak *et al.*, 2000; Frazer *et al.*, 2004).

2.7 Measurement of allele expression imbalance (AEI)

In order to assess interactions between *cis*- and *trans*-acting factors, PVN and amygdala tissues were punched, as described above, from F1 offspring of crossed HAB *vs.*LAB rats or mice, respectively. Both crosses (i.e. HAB father or LAB father) were performed to rule out any imprinting-based effects. Study of HAB and LAB alleles in the F1 offspring is a unique approach

where each allele acts as an internal standard for the other allele. Cross-mating of HAB and LAB would result in presence of both alleles in the same pool of *trans*-acting factors such as synaptic input, chromatin remodeling, factors encoded by other chromosomes, hormonal influences, etc. Thus any difference in allelic expression in such a system can be solely attributed to *cis*-acting variations present in the regulatory regions of the gene. Total RNA was isolated and the converted cDNA was used to quantify HAB and LAB alleles by qPCR as described above. The primers used to differentiate between the *Npsr1* HAB and LAB alleles were obtained by incorporating the observed transcribed SNPs between the rat (A(227,016)G) and mouse (A(156,453)G; rs37572071) lines (Table 8) at the 3' end of the respective primers and a mismatch nucleotide at penultimate position to favor allelic discrimination and a common primer (Figure 4).

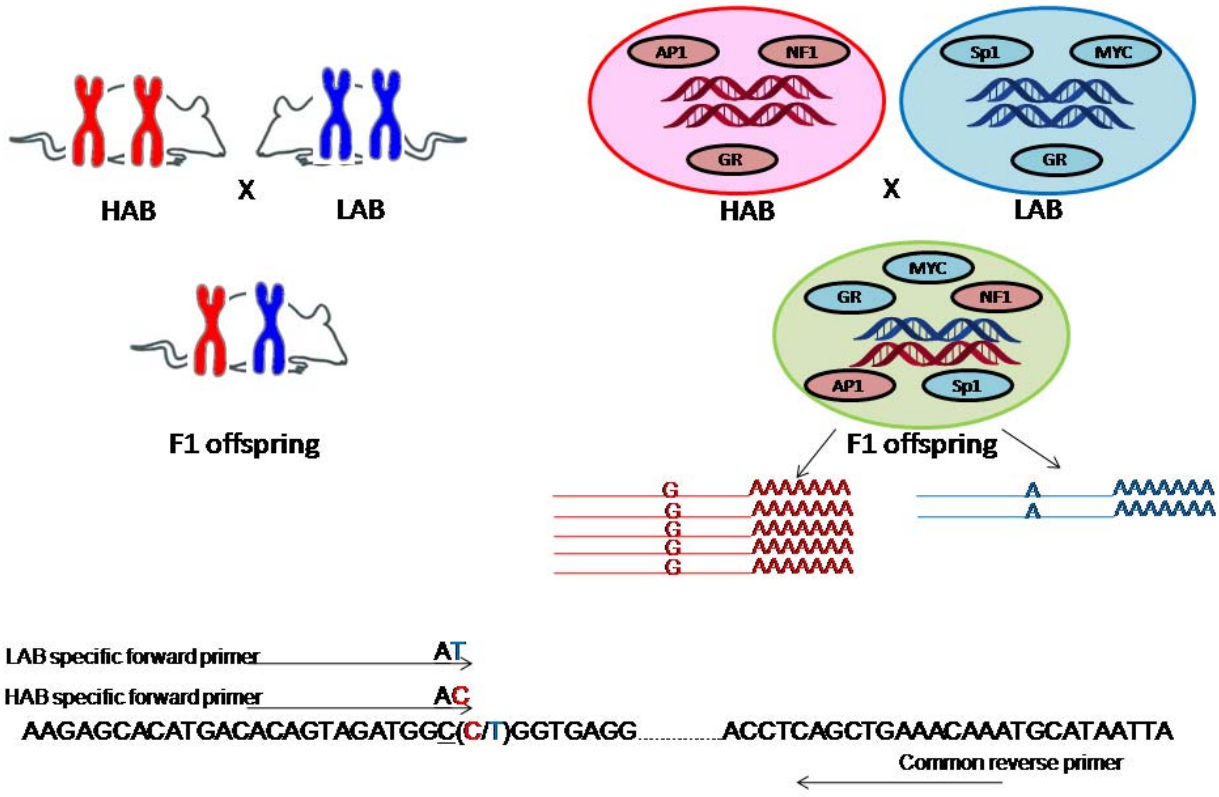


Figure 4: Schematic diagram showing cross-mated F1 offspring carrying half each of HAB, LAB chromosomal DNA and representative transcription factors. However, sometimes due to regulatory factors, there could be an allele expression imbalance, which can be detected using allele specific primer by incorporating respective SNP and a mismatch nucleotide at penultimate position.

A standard curve (linear regression line) was generated by making predefined mixtures of homozygous HAB vs. LAB cDNA in the ratio of 1:9, 5:5, 3:7 and 9:1. Then the crossing points (Cps) obtained from the test samples were plotted on the standard curves to obtain percentage of respective alleles.

Similarly, for *Tmem132d*, Cg was punched from UCMS, EE and control group of F1 offspring and these were homogenized in 400 µl Chaotropic buffer (4.5 M Guanidinium thiocynate, 2% N-laurylsarcosine, 50 mM EDTA, pH 8.0, 25 mM Tris-HCl, 0.1 M ß mercaptoethanol, 0.2% antifoam A) (Triant & Whitehead, 2009) and lysate was distributed equally for total RNA and DNA extraction using Qiagen's DNeasy Blood & Tissue Kit and RNeasy Plus Micro Kit (Qiagen GmbH, Hilden, Germany). For RNA extraction, 20 µl of 2 M sodium acetate (pH 4.0) was added to the homogenized 200 µl chaos buffer lysate, vortexed, 200 µl acidic phenol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) prewarmed to room temperature, 100 µl of chloroform/isoamyl alcohol (23:1) (both from Sigma-Aldrich GmbH, Munich, Germany) were added to each sample. Then samples were incubated on ice for 10 min and centrifuged at 13200 rpm for 15 min at 4^0C. Subsequently, the upper aqueous phase was drawn out, mixed with 1 µl carrier RNA (20 ng/µl) and up to 350 µl of RLT plus buffer (Qiagen GmbH, Hilden, Germany). Later, the lysate was processed as given by manufacturer's instructions. For the measurement of AEI, primers were designed using the following transcribed SNP, (C(593)T) in the mouse *Tmem132d* gene (Table 8).

Table 8: List of primers used for measurement of AEI of HAB, LAB alleles in F1 offspring of rats and mice.

Species, gene, specificity	Orientation	Primer sequence (5'→3')
Rat, *Npsr1*, common	(+)	ACTGTGGCCAGACGACTCCT
Rat, *Npsr1*, rHAB-specific	(-)	CCTCGGTTGTAGCTGCAGCATATC
Rat, *Npsr1*, rLAB-specific	(-)	CCTCGGTTGTAGCTGCAGCATACT
Mouse, *Npsr1*, common	(+)	GTGCTGTTCTCCACGTGCAG
Mouse, *Npsr1*, mLAB-specific	(-)	TCAGGGGCCATGAAGTCTCGT
Mouse, *Npsr1*, mHAB-specific	(-)	TCAGGGGCCATGAAGTCTCGC
Mouse,*Tmem132d*, common	(-)	CTGATGAGCACGGGAGACCAGAG
Mouse,*Tmem132d*, mHAB-specific	(+)	GCCCTGCTGCTCGGGTAT
Mouse,*Tmem132d*, mLAB-specific	(+)	GCCCTGCTGCTCGGGTAC

2.8 Bisulfite sequencing of mHAB *vs.* mLAB *Tmem132d* promoter region

After having discovered a CpG island in *Tmem132d* promoter, genomic DNA was isolated from Cg of mHAB *vs.* mLAB to assess the corresponding DNA methylation using the Qiagen's DNeasy protocol (Qiagen GmbH, Hilden, Germany). 400 ng of genomic DNA was sheared 5 times with a 26 G needle (Josef Peske GmbH & Co. KG, Aindling-Arnhofen, Germany) and subjected to bisulfite conversion using the Qiagen's EpiTect Bisulfite kit (Qiagen GmbH, Hilden, Germany) for 3 each HAB, LAB DNA sample from Cg as per manufacturer's instructions.

Bisulfite sequencing primers were designed using Methyl Primer Express v 1.0 Software (Life technologies, Darmstadt, Germany) to cover the whole approximately 600 bp CpG island upstream from the transcription start site. The primers were designed (Table 9) using the default parameters and finally tailed with M13 forward and reverse primers to provide a universal primer binding site. These universal primers contains all the four nucleotides and after first round of PCR amplification the longer primer binding site provides higher melting temperature and specificity of the product (Life technologies, Darmstadt, Germany, catalogue: cms_039258). In addition, corresponding primers for genomic DNA tailed with M13 were also designed to check for any false positives due to incomplete bisulfite conversion. PCR composition and conditions were adapted from Invitrogen handbook (cms_039258:

http://tools.invitrogen.com/content/sfs/manuals/cms_039258.pdf) and are as follows: 5 µl of Dream Taq buffer (10X), 1 µl of 10 mM dNTP mix, 4 µl of 25 mM $MgCl_2$, all from Thermoscientific GmbH, Bonn, Germany and 2.5 µl of each 5 µM primers, BSA-glycerol mix (5 mg/ml BSA+5% glycerol), 5µl of bisulfite products and nuclease-free water up to 50µl. The following are PCR condition: 5X (denaturation at 95°C for 30 sec, annealing at 60°C for 2 min, extension at 72°C for 3 min), 35X (denaturation at 95°C for 30 sec, annealing at 65°C for 1 min, extension at 72°C for 3 min), 60°C for 60 min for poly A tail addition, 4°C overnight. Subsequently, the bisulfite products were purified using $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2), 1 µl glycogen (10 mg/ml) and 2.5 volumes of 95% ethanol. Then the mixture was centrifuged at 13000 rpm for 30 min at 4°C, washed with 300 µl 70% ethanol, centrifuged for 5 min again at 13000 rpm, 4°C. Then the supernatant was decanted, the dried pellet resuspended in nuclease-free water. Then products were ligated into a pGEM®-T vector (Promega GmbH, Mannheim, Germany) system for blue/white screening as per manufacturer's instructions with insert:vector ratio (3:1) and mixture incubated overnight at 4°C. Next day, transformation of ligation product into chemically competent DH5alpha cells was carried out as follows: 10 min incubation of ligation mixture along with 50 µl DH5alpha cells on ice, then mixture was placed on a heating block at 42°C for 45 sec and immediately cooled down on ice for 2 min. After addition of 500 µl LB medium (20 g/l) (Serva electrophoresis GmbH, Heidelberg, Germany), the cells were incubated at 37°C for 90 min. Then the tubes were centrifuged at 1000 g for 10 min at room temperature, pellet was resuspended in minimum volume and whole cell suspension was plated to LB agar (1%) plates containing X-gal (20 mg/ml) and IPTG (100 mM) (Thermoscientific GmbH, Bonn, Germany), and ampicillin (50µg/ml, Sigma-Aldrich GmbH, Munich, Germany). Then white clones were picked and colony PCR carried out as follows: initial denaturation at 94°C for 5 min, 5X (denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 45 sec), 35X (denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and extension at 72°C for 45 sec) and a final extension at 72°C for 10 min and then 4°C overnight. Then all the positive clonal mixtures were loaded onto Nucleofast 96 plate (Macherey-Nagel GmbH & Co., Düren, Germany), washed twice with nuclease-free water and centrifuged at 4000 rpm for 10 min, resuspended in 25 µl nuclease-free water and then T7 universal primer was used for cycle sequencing as described above in section 2.4.1. Subsequently, the cleaned PCR products were resolved by capillary electrophoresis on a ABI 3730 DNA analyzer (Life technologies, Darmstadt, Germany) at the Max Planck institute of Biochemistry (Martinsried, Munich, Germany). Furthermore, the bisulfite sequence reads were analyzed using BiQ Analyzer (Max-Planck-Institut Informatik, Saarbrücken, Germany).

2.9 Quantitative Methylation specific PCR (qMSP) for mHAB, mLAB *Tmem132d* alleles of F1 offspring

Primers were designed to detect differential methylation of mHAB, mLAB *Tmem132d* alleles using selective SNPs, A(519)G and A(310)G at the 3' and 5' ends for forward and reverse primers, respectively (Table 9). In addition, there was a penultimate mismatch base and one CpG dinucleotide in the primer sequence. Genomic DNA fractions isolated from remaining chaos buffer homogenates of the Cg of F1 offspring were subjected to bisulfite conversion and purification as per manufacturer's instructions. Then, QuantiFastSYBR Green Kit (Qiagen, Hilden, Germany) was utilized for qPCR on a Light Cycler 2.0 equipment (Roche Diagnostics, Mannheim, Germany) using a protocol adapted from the following (Chan *et al.*, 2004): Hot start to activate polymerase at 94°C for 6 min, 38X (denaturation at 94°C for 30 sec, annealing at 64°C for 25 sec and extension at 72°C for 25 sec). The melting curve analysis and relative transcript concentration measurements were as described above using 2(-ΔΔCt) method (Livak & Schmittgen, 2001).

Table 9: List of primers used for bisulfite sequencing and qMSP of *Tmem132d* gene in mHAB *vs.* mLAB.

Species, gene, designation		Orientation	Primer sequence (5'→3')
Mouse,*Tmem132d*, tailed bisulfite	M13	(+)	TGTAAAACGACGGCCAGTGGAGTGATGTTGGGTTTTTTTT
Mouse,*Tmem132d*, tailed bisulfite	M13	(-)	CAGGAAACAGCTATGACCTTTTAAACCCCACCCTTCTAAA
Mouse,*Tmem132d*, tailed genomic DNA	M13	(+)	TGTAAAACGACGGCCAGTGGAGTGATGCTGGGTTTCCTCT
Mouse,*Tmem132d*, tailed genomic DNA	M13	(-)	CAGGAAACAGCTATGACCTTTTAAGCCCCACCCTTCTGGA
Mouse,*Tmem132d*, MSP-519	HAB-	(+)	ATGGGTATTATGTATTTTGGTGTGAGTTCGTTTCG
Mouse,*Tmem132d*, MSP-310	HAB-	(-)	CGCAAAAACCCCTAACATAAACTAAAATATTTCCG
Mouse,*Tmem132d*, MSP-519	LAB-	(+)	ATGGGTATTATGTATTTTGGTGTGAGTTCGTTTCA
Mouse,*Tmem132d*, MSP-310	LAB-	(-)	TGCAAAAACCCCTAACATAAACTAAAATATTTCCG

2.10 Promoter constructs, cell culture, transfection and reporter gene assay

2.10.1 Amplification of promoter constructs and cloning them into pGL3 basic vector

To assess the role of polymorphisms found in the putative *Npsr1* promoter, homologous sequences up to 2 kbp upstream of *Npsr1* sequence of HAB and LAB animals were amplified by PCR using Phusion DNA polymerase (New England Biolabs), which included exon 1, intron 1 and a part of exon 2 until the ATG start codon as follows: 10 µl of 5X Phusion HF buffer, 1 µl of

10 mM dNTP mix, 1 µl of 5% DMSO, 1 µl each respective 2 µM forward and reverse primers, 1 µl DNA and 0.5 µl of Phusion DNA polymerase (2 U/µl) and nuclease-free water up to 50 µl. The purified PCR products and pGL3 basic luciferase vectors were digested with *KpnI*^{HF} and *NheI*^{HF} restriction enzymes (New England Biolabs GmbH, Frankfurt, Germany) and then ligated together as per manufacturer's instructions (see Table 10 for a list of all primers used). The ligated mixture was then transformed into DH5alpha chemically competent cells as described above. Similarly, mHAB and mLAB *Tmem132d* promoter constructs were amplified and cloned into pGL3 luciferase vectors.

Table 10: list of primers used for cloning rat and mice *Npsr1* and *Tmem132d* promoter fragments into luciferase vector.

Species, gene, designation	**Orientation**	**Primer sequence (5'→3')**
Rat,*Npsr1,*Frag.E	(+)	ATCGGTACCGATGGTGAGGGCTGTGCTGG
Rat,*Npsr1*,Frag.D	(+)	ATCGGTACCCGATCTCAGCTGAAACAAACTCATAACTC
Rat,*Npsr1*,Frag.C	(+)	ATCGGTACCCTCTAGGAATGCACACTTACTCAGCTCTG
Rat,*Npsr1*,Frag.B	(+)	ATCGGTACCCCCACTCTAGGGCCTTTCATCTAGG
Rat,*Npsr1*,Frag.A	(+)	ATCGGTACCCTGGGTCCTCCAGTCTCTTGAGG
Rat,*Npsr1*,common	(-)	CAGGCTAGCGGCTCAGGCAGGGTCAAGTCTTA
Mouse,*Npsr1*,Frag.S	(+)	ATCGGTACCGTGATACCAGCTGAAACAAACACATAACTGAC
Mouse,*Npsr1*,Frag.R	(+)	ATCGGTACCGAGACAAACACAGACTCCTACCTC
Mouse,*Npsr1*,Frag.Q	(+)	ATCGGTACCGCAAAGGTTGGCCTACATGGCTC
Mouse,*Npsr1*,Frag.P	(+)	ATCGGTACCGGATTGTCATCTCCTGTCTGTGCC
Mouse,*Npsr1*,common	(-)	CAGGCTAGCGGCTCAGGCAGGGTCAGGTC
--,--,RVprimer3	(+)	CTAGCAAAATAGGCTGTCCC
--,--,GLprimer2	(-)	CTTTATGTTTTTGGCGTCTTCCA
Mouse, *Tmem132d*	(+)	GCAGGTACCCAAGGCTCTGCGGAGCAGTG
Mouse, *Tmem132d*	(-)	GCTAGCAATTTCTCTCTCTTCCTCTCTCCC
Mouse, *Tmem132d,* 310 A sub	(+)	GTTAGGGGTTCCTGAACTGTCCTTGCCTGAAG
Mouse, *Tmem132d,* 310 A sub	(-)	CTTCAGGCAAGGACAGTTCAGGAACCCCTAAC
Mouse, *Tmem132d,* 310 del	(+)	GACGGTACCTCAGGGACAGGAATTTGAGG
Mouse, *Tmem132d,* 519 A sub	(+)	GTGTGAGTTCGCCTTAGATACCCTGGAAGG
Mouse, *Tmem132d,* 519 A sub	(-)	CCTTCCAGGGTATCTAAGGCGAACTCACAC

Next day, clones were analyzed for positive insert, and then a single colony was inoculated in 10 ml LB broth overnight.

2.10.2 Plasmid isolation using alkaline lysis with SDS: midipreparation

Plasmid isolation was carried out using alkaline lysis with SDS using the following reagents and protocol by (Sambrook & Russell, 2001).

1. **Alkaline lysis solution I**

50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0).

2. **Alkaline lysis solution II**

0.2 N NaOH, 1% (w/v) SDS

3. **Alkaline lysis solution III**

5 M potassium acetate, glacial acetic acid and distilled water up to 100 ml.

Protocol: The 10 ml LB broth containing bacterial suspension was centrifuged to recover bacteria at 2000 g for 10 min at 4°C. Then the pellet was resuspended in 200 µl ice-cold alkaline lysis solution I by vigorous vortexing and the pellet transferred to an Eppendorf tube. Next, 400 µl of alkaline lysis solution II was added to each bacterial suspension for lysis and the contents were mixed by inverting the tube five times. Then to this mixture, 300 µl of alkaline lysis solution III was added to renature the plasmid DNA and the mixture incubated on ice for5 minutes. Subsequently, the lysate was centrifuged at 13000 rpm for 5 minutes at 4°C. Then 600 µl of supernatant containing the plasmid DNA was mixed with an equal volume of phenol:chloroform (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The organic and aqueous phases were mixed by vortexing and then the emulsion was centrifuged at 13000 rpm for 2 minutes at 4°C. Next, the aqueous phase was drawn out and equal volume of isopropanol (Sigma-aldrich GmbH, Munich, Germany) was added to precipitate the plasmid DNA. After 2 minutes, the mixture was centrifuged at 13000 rpm for 5 minutes at room temperature to collect the plasmid DNA pellet. Then the pellet was washed with 1 ml of 70% ethanol, air dried and resuspended in nuclease-free water. All plasmids were sequence-verified before further use.

2.10.3 Transfection of promoter-pGL3 constructs

The mouse Neuro-2a cells were seeded in 96 well plates at a density of 8000 cells per well in DMEM containing 10% FBS, 1% sodium pyruvate and 1% antibiotic-antimycotic, all purchased from Life technologies, Darmstadt, Germany. Next day, when the cells reached a density of 50-70%, for each well 300 ng *Npsr1*/*Tmem132d*-pGL3 vector constructs and 10 ng pRK5-Gaussia-KDEL expression vector (internal control vector) were diluted with 150 mM NaCl and then mixed

with 0.99 µl Exgen 500 *in vitro* reagent (Thermoscientific, Braunschweig, Germany). The pRK5-Gaussia-KDEL expression vector was used to normalize transfection efficiency and a SV40-pGL3 vector was also used as a positive control. The reaction mixture was vortexed for 15 sec and incubated at room temperature for 20 minutes to allow formation of complex. The mixture was then added to each well, some wells were left untransfected to measure background signal during luciferase assay. Subsequently, the plate was centrifuged for 5 minutes at 280 g at room temperature to improve transfection efficiency. Then the plate was incubated at 37°C, 5% CO_2 for 48 hr. As sequencing data suggested the introduction of a GR transcription binding site in the mHAB and rHAB *Npsr1* promoters, shorter constructs of approximately 500 bp were stimulated with 1, 10 µM dexamethasone (DEX; Ratiopharm, Ulm, Germany) and water as control for 24 h before carrying out the dual reporter assay. Firefly and Gaussia luciferase activites were measured after 48 h as described in the following section.

2.10.4 Dual luciferase assay

Following are the composition of reagents required for dual luciferase assays

0.5 M phosphate buffer (KPO_4) pH 7.8

0.5 M KH_2PO_4 (pH 4.1) and

0.5 M K_2HPO_4 (pH 9.5), pH adjusted to 7.8

Passive lysis buffer

100 mM KPO_4 buffer pH 7.8

0.2 % Triton X-100 and then volume made up to 200 ml with distilled water.

Firefly substrate solution

2.5 mM $MgCl_2$

2 mM ATP (Sigma-aldrich GmbH, Munich, Germany)

100 µM D-Luciferin (P.J.K. GmbH, Kleinblittersdorf, Germany)

dissolved in distilled water.

0.5 M Phosphate (KPO_4) buffer pH 5.1

0.5 M KH_2PO_4 (pH 4.1) and

0.5 M K_2HPO_4 (pH 9.5), pH adjusted to 5.1

Gaussia substrate solution (2X)

2.2 M NaCl

4.4 mM Na_2EDTA
0.22 M KPO_4 buffer pH 5.1
0.88 mg/ml BSA
6 *µ*g/ml Coelenterazin (P.J.K. GmbH, Kleinblittersdorf, Germany)

At the end of 48 h, the supernatant was gently aspirated and 50 µl passive lysis buffer was added to each well. Then the plate was incubated at 37°C for 30 minutes at 800 rpm. Subsequently, 20 µl lysate was transferred into white Nunc96 plate (Nunc GmbH & Co. KG, Langenselbold, Germany). The luciferase signal from Firefly and Gaussia were measured on a TriStar LB 941 multimode microplate reader (Berthold technologies, Bad Wildbad, Germany) as follows: The luminometer first injected 50 µl of firefly substrate solution and after shaking for 1 sec, measured the Firefly luciferase signal over the period of 10 sec. Then, 50 µl Gaussia substrate solution was injected into the same well, causing a decrease in pH and subsequent quenching of the Firefly signal. Next, the plate was shaken for 1 sec and after a 2 sec delay, the Gaussia luciferase signal was measured over a period of 5 sec. The data analysis commenced after subtracting the background signal intensity of lysates from untransfected cells from each data point. The promoter activity represents Firefly data normalized to Gaussia activities + SEM of at least three independent experiments, each performed in triplicate.

2.10.5 Rat and mouse *Npsr1* cDNA amplification and site-directed mutagenesis

To determine the functional role of a single synonymous SNP found in the coding region of rHAB and mHAB *Npsr1* gene.

Both rat and mice HAB,LAB *Npsr1* cDNA were amplified using Phusion DNA polymerase (New England Biolabs, Frankfurt, Germany), and the sequences were verified. Additional hemagglutinin signal peptide and FLAG tag were added at the N-terminal of the *Npsr1* cDNA sequences as described (Bernier *et al.*, 2006). Finally, the LAB *Npsr1* cDNAs were cloned into the KpnI/NotI sites of pcDNA™3.1/Zeo $^{(+)}$ (Life Technologies, Darmstadt, Germany).Then LAB *Npsr1* cDNA plasmid was used to selectively mutate an adenine (A) residue in the LAB sequences at 227,016 and 156,453 bp relative to the ATG start codon to guanine (G) in HAB rats and mice, respectively, to nullify any other PCR-based errors. Then Phusion DNA polymerase (New England Biolabs, Frankfurt, Germany) was used to introduce these site-specific mutations according to the instructions of the QuikChange site-directed mutagenesis kit (Agilent technologies, Oberhaching, Germany). The primers are listed in listed in Table 11.

Table 11: List of primers used for cDNA amplification, site-directed mutagenesis and addition of hemagglutinin signalpeptide (HA-SP) flag tag to rat and mouse *Npsr1* cDNA sequences.

Species, gene, designation	Orientation	Primer sequence (5'→3')
Rat, *Npsr1*,complete cDNA	(+)	CTGCAGGTGCAGAGACATAAGACTTGA
Rat, *Npsr1*,complete cDNA	(-)	CTGAGCTCAGCCTAGCACTGTTGC
Rat, *Npsr1*, nested Tag	(+)	GTCAGGTACCGCCATGCCGGCCAACC
Rat, *Npsr1*, nested Tag	(-)	GCAGCGGCCGCTTAGATGAATTCAGGCTTGGAGAGAATC
Rat,*Npsr1*, A(227,016)G mutated to G in rHAB	(+)	CCAACTGCTCAGATGGAGAGCTATGCTGCAGCTACAACC
Rat, *Npsr1*, A(227,016)G mutated to G in rHAB	(-)	GGTTGTAGCTGCAGCATAGCTCTCCATCTGAGCAGTTGG
--,HA-SP,flag tag, containing rat *Npsr1* sequence	(+)	CTGCCTGGTATTCGCCGACTACAAGGACGATGATGACGCCCCGG CCAACCTCACAGAGGG
--,HA-SP,flag tag, common	(+)	GTAGGTACCACCATGAAGACGATCATCGCCCTGAGCTACATCTT CTGCCTGGTATTCGCCGACTAC
Mouse, *Npsr1*, complete cDNA	(+)	CAGGGAGGGCTCTGTGC
Mouse, *Npsr1*, complete cDNA	(-)	GGAGAGCTGACTAAGTTCAGC
Mouse,*Npsr1*, rs37572071 mutated to G in mHAB	(+)	CATTATTTGGCGATTCACGGGAGACTTCATGGC
Mouse,*Npsr1*, rs37572071 mutated to G in mHAB	(-)	GCCATGAAGTCTCCCGTGAATCGCCAAATAATG
HA-SP,flag tag, containing mouse *Npsr1* sequence	(+)	CTGCCTGGTATTCGCCGACTACAAGGACGATGATGACGCCCCAG CCAACCTCACAGAGGG
Mouse, *Npsr1*, cDNA, common	(-)	GCAGCGGCCGCTTAGATGAATTCCGGCTTGGAGAGAATCTGCAT CTCGTGTCTCTCGCTTCTCTCTCGG
--,--,T7	(+)	TAATACGACTCACTATAGGG
--,--,Bgh	(-)	TAGAAGGCACAGTCGAGG

2.10.6 Functional reporter assay

Principle of the assay: NPS on binding NPSR1 is known to activate the downstream cAMP pathway (Reinscheid *et al.*, 2005). This property is harnessed here to determine if the SNP has any functional impact on the HAB NPSR1 protein. Thus HAB and LAB NPSR1 cDNA was cotransfected with a plasmid that has cAMP response element (CRE) upstream of the luciferase gene. When the agonist (NPS) is added to the cells expressing HAB and LAB NPSR1, there would be activation of the downstream cAMP pathway. This cAMP would in turn bind at the CRE and cause an increase or decrease of downstream luciferase signal to indicate the functional impact of SNP on the NPSR1.

HAB and LAB *Npsr1* cDNA constructs carrying the exonic SNPs [A(227,016)G and [A(156,453)G; rs37572071] were cotransfected with either a mixture of CRE-luciferase and Gaussia vector (20:1) in HEK 293 cells or cDNA constructs along with Gaussia in HEK 293 CRE-luciferase cells using lipofectamine 2000 (Life technologies, Darmstadt, Germany) using the following protocol:

60,000 HEK 293 cells or HEK 293 CRE-luciferase cells were seeded into 24 well plates using antibiotic free DMEM+10% FBS medium (Life technologies, Darmstadt, Germany). Next day, when the cells reached approximately 95% density, 700 ng *Npsr1* cDNA vector and 100 ng either Gaussia or a mixture of CRE-luciferase + Gaussia vector (20:1) per well were diluted with opti-MEM medium (Life technologies, Darmstadt, Germany). Similarly, for each well, 2 µl lipofectamine 2000 (Life technologies, Darmstadt, Germany) was diluted in 50 µl opti-MEM medium (Life technologies, Darmstadt, Germany) and incubated at room temperature for 5 minutes. Then plasmid DNA and lipofectamine 2000 were mixed together and incubated at room temperature for 20 minutes to form the DNA-liposome complex. Then the mixture was added to each well and plate incubated at 37°C, 5% CO_2. After 5 hr media was replaced and incubation continued until 40 hr when the cells were stimulated with 1 nmol NPS (1mg/ml stock solution; Bachem, Weil am Rhein, Germany). Finally the dual luciferase assay was carried out at 48 h as described in section 2.10.4.

2.11 Western Blotting for NPSR1 protein

For assessment of NPSR1, protein was extracted using RIPA buffer supplemented with protease inhibitors (Sigma-aldrich GmbH, Munich, Germany) from PVN of rats and amygdala of mice. Then 60 and 15 µg of total proteins from rats and mice, respectively, were electrophoresed on a 8% separating gel. The proteins were transferred on a nitrocellulose membrane (Millipore GmbH, Schwalbach, Germany) and blocking for non-specific antibody binding carried out with 5% milk in tris-buffered saline (TBS) for 1 hr at room temperature. Then the membrane was incubated overnight with primary NPSR1 antibody (Ab1) (Leonard & Ring, 2011): 1:500 for rats and 1:200 for mice in 2.5% milk in TBS-T, overnight at 4°C. Next day, 3X washes with TBS containing tween 20 (TBS-T) (Sigma-aldrich GmbH, Munich, Germany). Then the membranes were incubated in a 1:5000 dilution of HRP-conjugated secondary antibody (New England Biolabs, Frankfurt, Germany) for 2 hr at room temperature, washed 3X with TBS-T, and then bands were visualized by chemiluminescence (PerkinElmer, Massachusetts, USA). β-tubulin (1:1000) was used as a loading control (New England Biolabs, Frankfurt, Germany).

2.12 Statistical analyses

The molecular characterization of *Nps* and *Npsr1* were analyzed using a one-way (factor line), two-way analysis of variance (ANOVA; factors line x DEX) or unpaired t-test. EPM data were analyzed using either a two-way ANOVA (factors line x treatment) followed by a Fisher's LSD *post-hoc* test or an unpaired t-test.

For molecular characterization of *Tmem132d* one-way (factor line) ANOVA test was utilized. Analysis of behavior of mHAB, mLAB and F1 offspring subjected to EE, UCMS and control group was done by one-way ANOVA (factor treatment). Subsequently, Bonferroni *post-hoc* test was used to verify group effects. Spearman's rank correlation coefficient (rho) was used to measure the correlation between mRNA expression and promoter methylation. All results are presented as mean+SEM and data was considered statistically significant at $p< 0.05$. Statistical analyses were performed using SPSS v19.0 (IBM GmbH, Ehningen, Germany) and graphs were prepared in graphpad prism v5 (GraphPad software, Inc., California, USA).

3. Results

3.1.1 NPS-NPSR1 system

3.1.2 Behavioral studies

The behavioral effects of NPS and NPSR1-A administration was studied in HAB and LAB rodents with genetic predisposition to extremes in anxiety. The anxiolytic effect of *icv* NPS (1 nmol) administration in both outbred Wistar rats and CD-1 mice (data not shown) was confirmed. In rHAB central NPS administration dose-dependently altered the percentage time spent in the open arms of the EPM ($F_{2,26}$ = 17.7; P < 0.001) with 1 nmol (p < 0.001), but not 0.1 nmol leading to an anxiolytic effect (Figure 5A). Whereas both doses ($F_{2,26}$= 12.0; p < 0.001) increased the number of closed arm entries (0.1 nmol; p < 0.01; 1 nmol; p < 0.001; Figure 5B).

Central NPS administration also significantly increased the percent time spent in the open arms of the EPM in mHAB (p < 0.001; Figure 5C) without altering locomotor activity (Figure 5D).

Both of these studies demonstrate that NPS is an efficient anxiolytic agent even in genetically predisposed HAB rodents. The anxiolytic effect was readily distinguishable from any locomotor induced effects except in rHAB where dose response distinguished anxiety from locomotion.

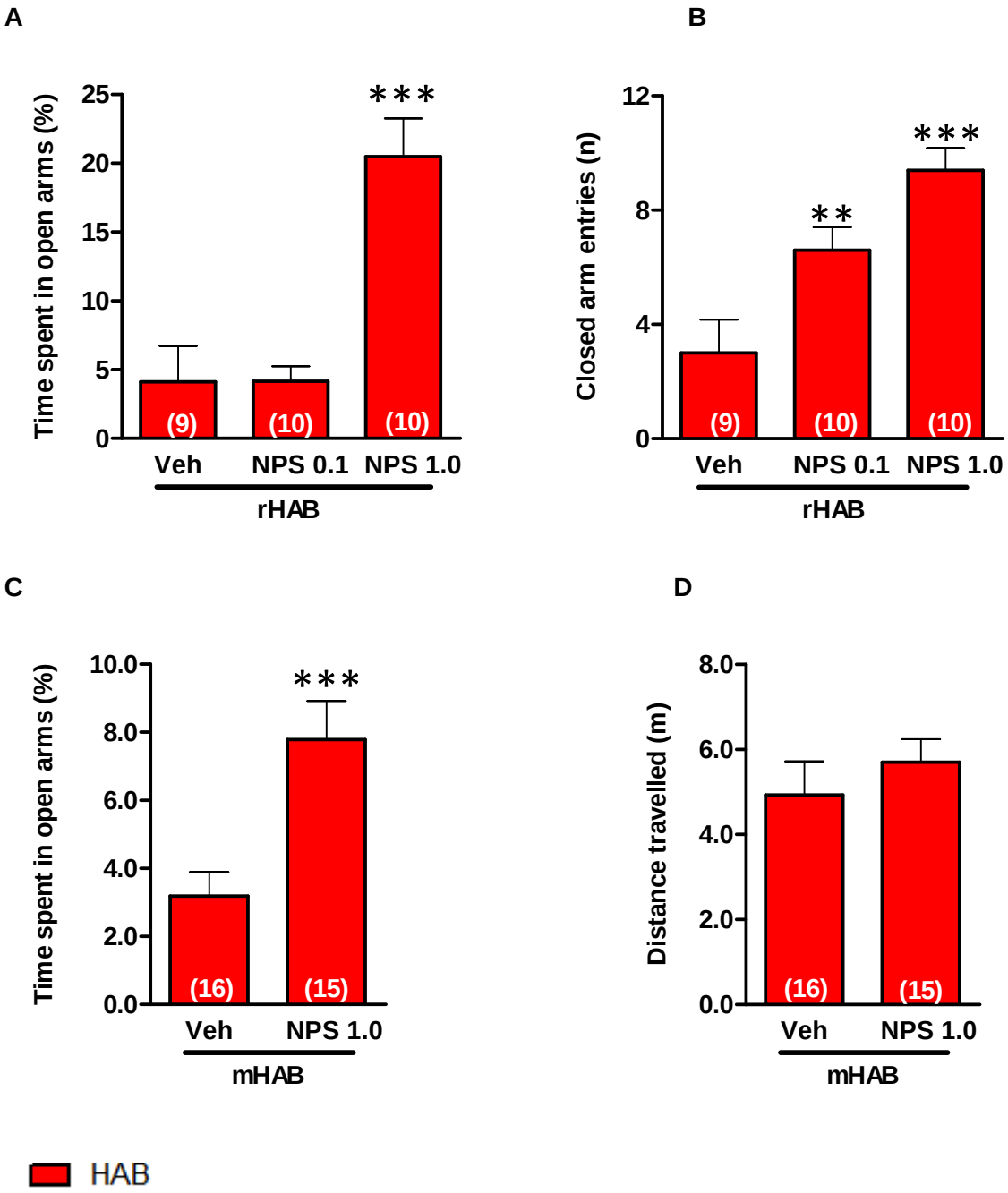


Figure 5: Effect of *icv* NPS administration on anxiety-related behavior in **(A)** rHAB-time spent in open arm **(B)** rHAB-closed arm entries, **(C) mHAB-** time spent in open arm and **(D) mHAB-** distance travelled in the EPM. Data represents mean+SEM. Numbers in parentheses indicate group size. ** $p < 0.01$, *** $p < 0.001$ compared with vehicle group.

On the other hand, *icv* administration of D-Cys(tBu)^5-NPS, NPSR1-A (10 nmol) decreased the percent time rLABs ($p < 0.05$; Figure 6A) and mLABs ($p < 0.05$; Figure 6C) spent on the open arms of the EPM while not affecting locomotion (Figures 6B and 6D).

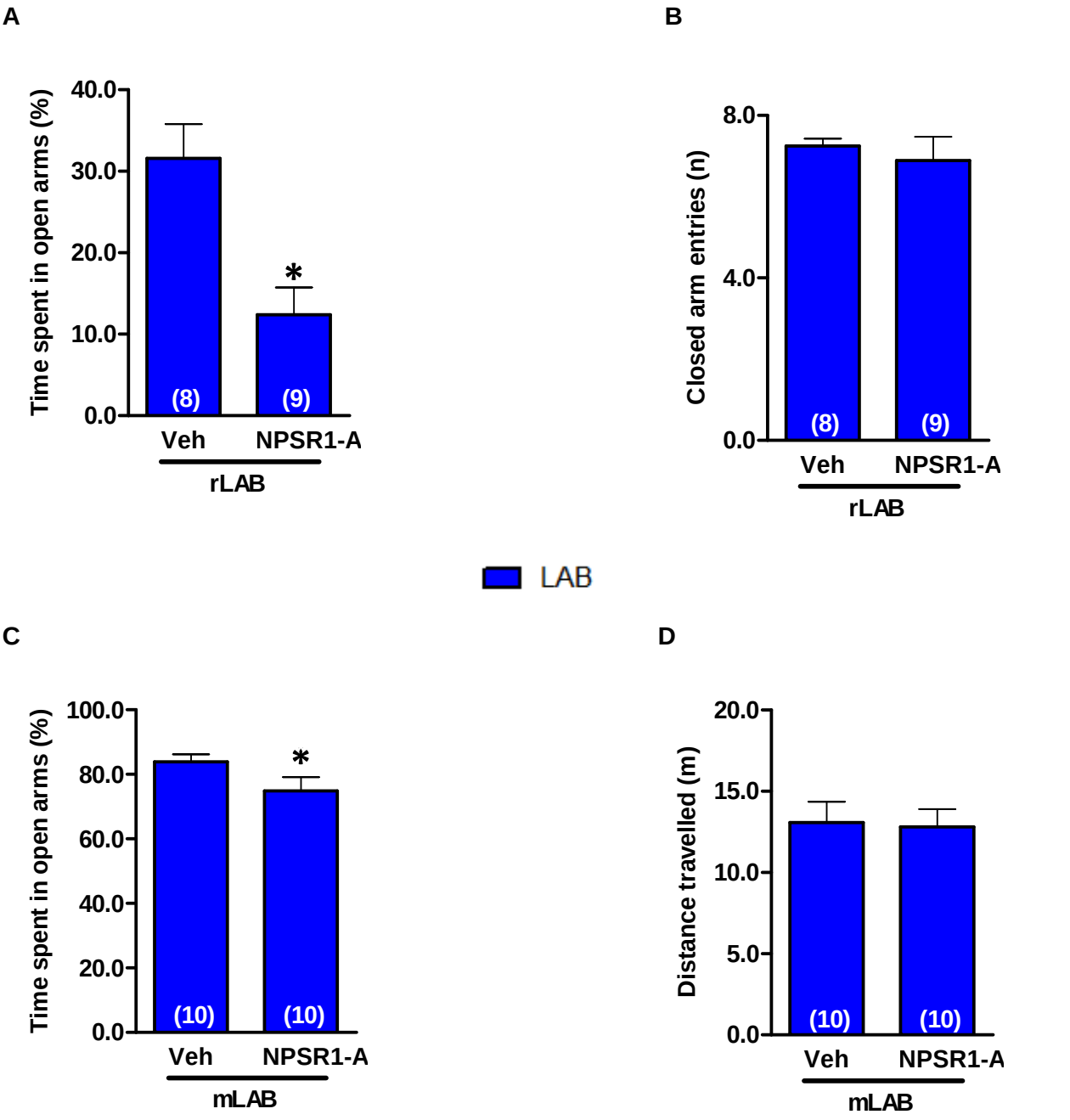


Figure 6: Effect of *icv* NPSR1-A administration on anxiety-related behavior **(A)** rLAB- time spent in open arm **(B)** rLAB- closed arm entries, **(C)** mLAB-time spent in open arm, **(D)** mLAB-distance travelled in EPM. Data represent mean+SEM. Numbers in parentheses indicate group size. * $p < 0.05$ compared with vehicle group.

The anxiogenic effect observed in LAB rodents suggests involvement of an endogenous NPS system in mediating anxiety-related behavior without any effects on locomotion. This encouraged me to study the molecular underpinnings of NPS mediated anxiety-related behavior.

3.1.3 Measurement of basal *Nps* mRNA expression

A nearly three-fold higher expression of *Nps* was detected in the LC area of rHAB compared with rLAB ($p < 0.05$; Figure 7A), while levels did not differ between mHAB *vs.* mLAB (Figure 7B).

3.1.4 Measurement of basal *Npsr1* mRNA expression

Within the PVN, rHAB displayed lower *Npsr1* mRNA expression than rLAB in both males ($p < 0.001$; Figure 7C) and females ($p < 0.01$; Figure 7D).

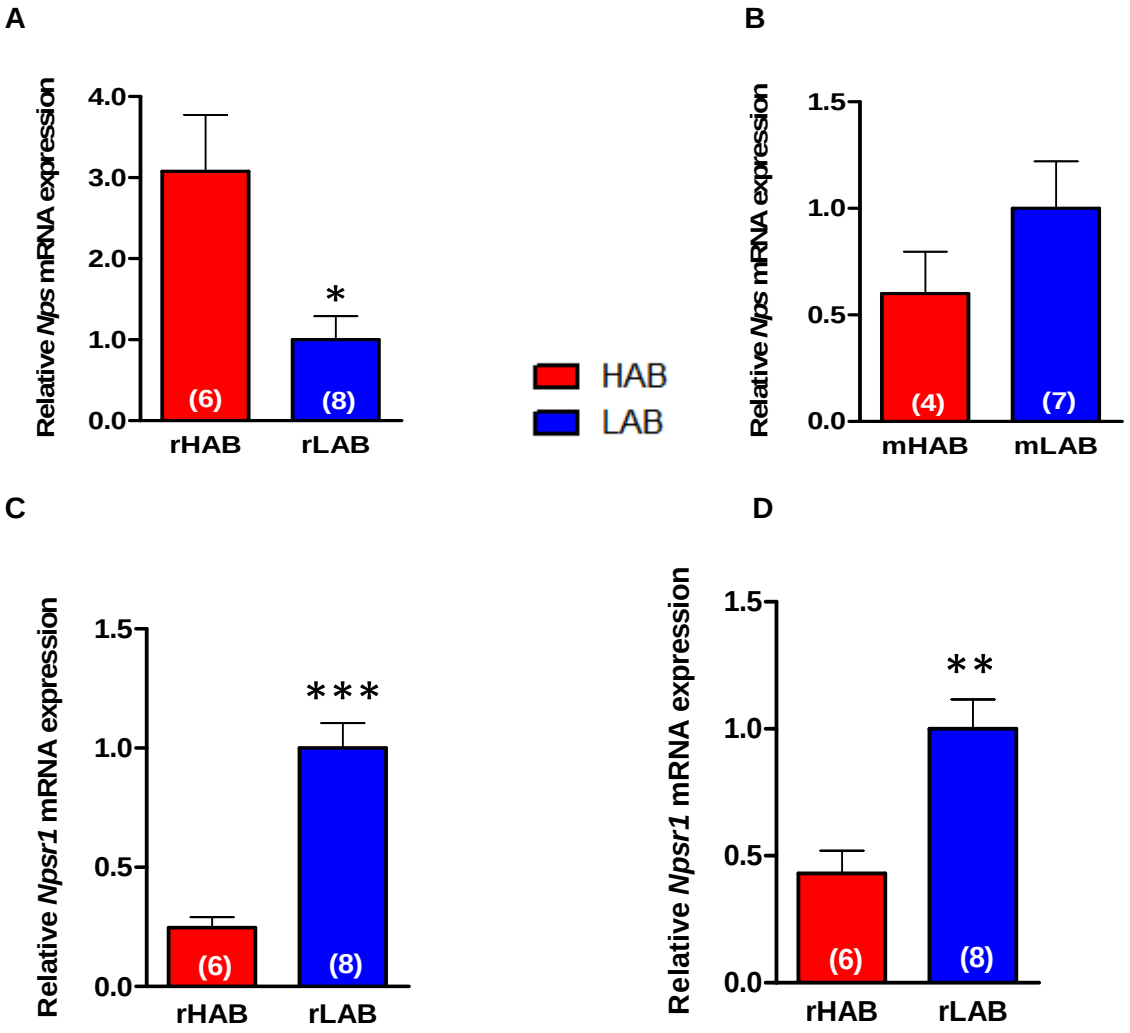


Figure 7: Basal *Nps* mRNA expression in the LC area of **(A)** rHAB *vs.* rLAB and **(B)** mHAB *vs.* mLAB. Basal *Npsr1* mRNA expression in the PVN of **(C)** male and **(D)** female rHAB *vs.* rLAB, respectively. Number in parentheses indicates group numbers. Data are represented as mean+SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with respective HABs.

In contrast, *Npsr1* mRNA expression within the amygdala did not differ between rat lines (Figure 8A). Differences in *Npsr1* mRNA levels were also found in mice, with mHAB displaying lower expression in the amygdala ($p < 0.05$; Figure 8B), but not within the PVN (Figure 8C) compared with mLAB.

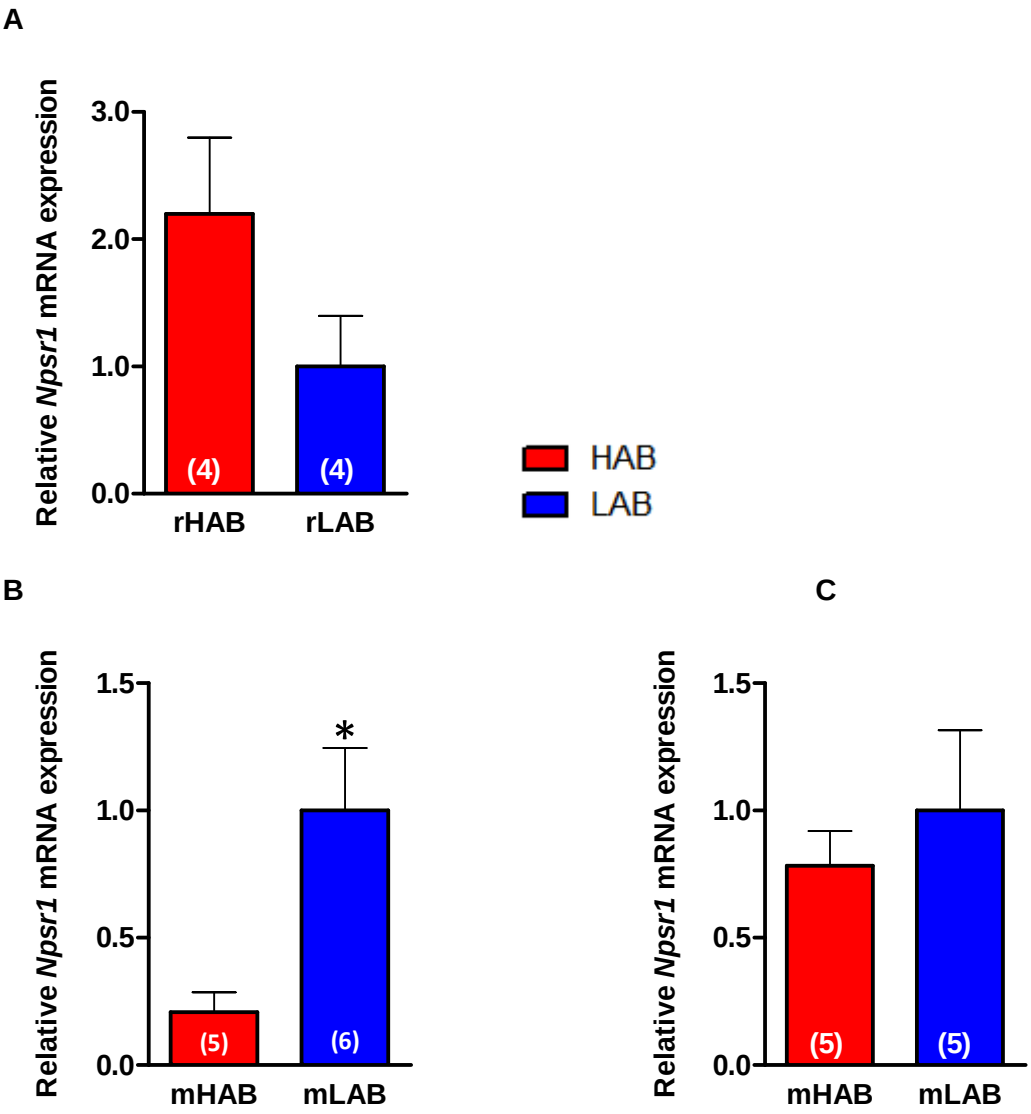


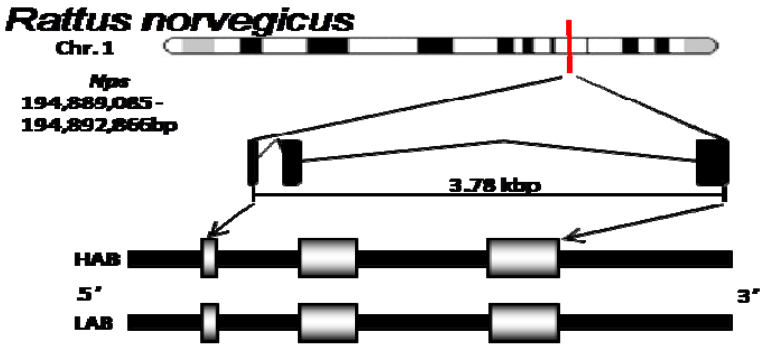
Figure 8: Basal *Npsr1* mRNA expression in the **(A)** amygdala of rHAB *vs.* rLAB. Basal *Npsr1* mRNA expression in the **(B)** amygdala and **(C)** PVN of mHAB *vs.* mLAB. Number in parentheses indicates group numbers. Data are represented as mean+SEM. *$p < 0.05$ compared with respective HABs.

Overall, there was a lower basal *Npsr1* mRNA expression in the PVN of rHAB and amygdala of mHAB in contrast to their corresponding LABs. Thus, the next step was DNA sequencing of *Nps* and *Npsr1* gene to determine the genetic factors that might underlie their differential expression.

3.1.5 *Nps* DNA sequence analysis

The *Nps* DNA sequence of rHAB *vs.* rLAB did not differ (Figure 9A), and thus, both were identical to the reference strain (BN/NHsdMcwi) in the Ensembl database (release 70). While, the mouse *Nps* mRNA did not differ but the corresponding DNA sequence differed significantly between the mHAB *vs.* mLAB lines (Figure 9B). In total, 35 SNPs and one insertion were found within the mLAB *Nps* sequence, whereas the mHAB *Nps* sequence was identical to the reference mouse strain (C57BL/6J). The gene-coding locus of mLAB carried four SNPs leading to amino acid changes at position leucine(5)isoleucine, valine(10)isoleucine, arginine(54)glycine and a synonymous mutation coding for threonine at position 65 in the amino acid sequence – all prior to the mature 20 amino acid peptide (Table 12).

A



B

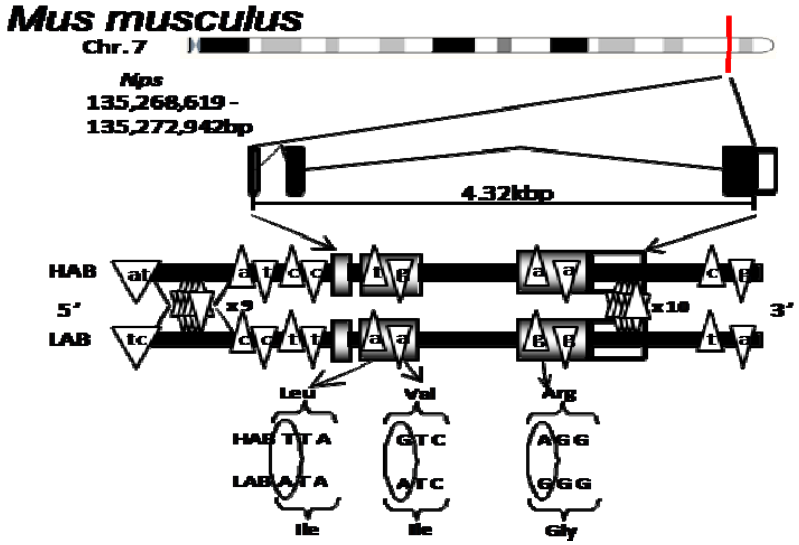


Figure 9: Schematic representation of **(A)** rHAB *vs.* rLAB and **(B)** mHAB *vs.* mLAB *Nps* gene (adapted from Ensembl database and (Yen, 2011)). Polymorphic sites are indicated as unfilled triangles, exons including untranslated regions (UTRs) are indicated by boxes (exons shaded, UTRs are white), intronic variations are not shown.

Table 12: *Nps* polymorphisms in mHAB *vs.* mLAB : SNPs, Deletions and Insertions. Positions relative to ATG start codon.

Variation type	HAB	LAB	Location in *Nps* gene	Relative position	SNP identifier
SNP	A	T	Promoter	-1,031	
SNP	T	C	Promoter	-1,030	
Insertion		GTGT	Promoter	-995	
SNP	T	C	Promoter	-924	rs49048062
SNP	A	G	Promoter	-920	rs42460586
SNP	T	C	Promoter	-871	
SNP	C	T	Promoter	-868	
SNP	T	G	Promoter	-867	
SNP	A	G	Promoter	-819	rs50307957
SNP	C	T	Promoter	-788	rs50890340
SNP	G	A	Promoter	-712	rs49326925
SNP	A	C	Promoter	-621	rs52014995
SNP	T	C	Promoter	-316	
SNP	C	T	Promoter	-163	
SNP	C	T	Promoter	-13	rs33467230
SNP	T	A	Exon2	125	
SNP	G	A	Exon2	140	
SNP	G	A	Intron2	250	
SNP	G	A	Intron2	273	
SNP	A	T	Intron2	380	
SNP	A	C	Intron2	460	
SNP	C	T	Intron2	502	
SNP	A	G	Exon3	3,624	rs33470378
SNP	A	G	Exon3	3,659	rs33470381
SNP	C	T	Exon3	3,937	rs33471194
SNP	A	G	Exon3	4,022	rs33471197
SNP	T	C	Exon3	4,127	rs33471203
SNP	A	G	Exon3	4,155	rs33471946
SNP	C	A	Exon3	4,195	rs50157889
SNP	C	G	Exon3	4,196	rs33466004
SNP	C	T	Exon3	4,215	rs47207120
SNP	A	G	Exon3	4,217	rs46716508
SNP	G	A	Exon3	4,240	rs49462104
SNP	T	C	Exon3	4,264	rs51623072
SNP	T	A	DER	4,321	rs33466010
SNP	G	A	DER	4,369	

3.1.6 *Npsr1* DNA sequence analyses

rHAB *vs.* rLAB showed several polymorphisms across the *Npsr1* DNA sequence (Figure 10; Table 13) with rLAB *Npsr1* sequence identical to the reference rat strain (BN/NHsdMcwi).

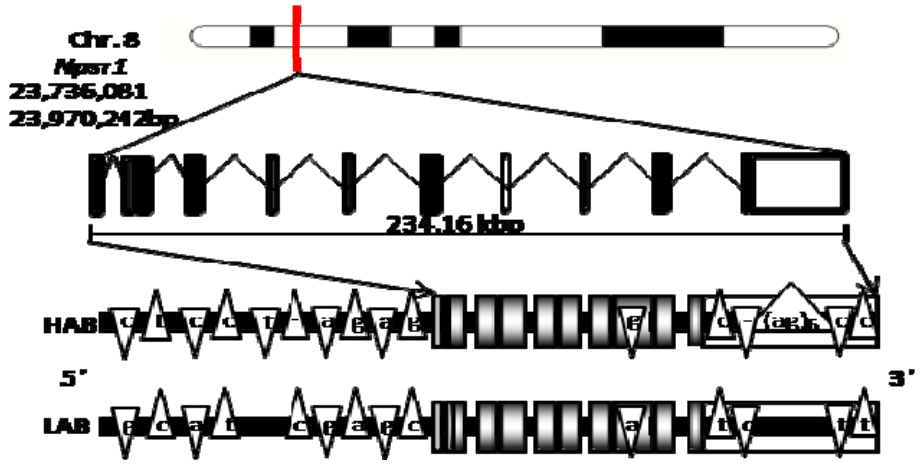


Figure 10: Polymorphisms and SNPs in *Npsr1* of rHAB *vs.* rLAB. Polymorphic sites are indicated as unfilled triangles, exons including UTRs are indicated by boxes (exons shaded, UTRs are white), intronic variations are not shown.

Table 13: *Npsr1* polymorphisms in rHAB *vs.* rLAB: SNPs, Deletions and Insertions. Positions relative to ATG start codon.

Variation type	HAB	LAB	Location in *Npsr1* gene	Relative position
SNP	C	G	Promoter	-1,926
SNP	T	C	Promoter	-1,856
SNP	C	A	Promoter	-1,813
SNP	C	T	Promoter	-1,593
Insertion	T		Promoter	-1,366
Deletion	-	C	Promoter	-1,300
SNP	A	G	Promoter	-908
SNP	G	A	Promoter	-867
SNP	A	G	Promoter	-388
SNP	G	C	Promoter	-221
Deletion	-	A	Intron4	168,623
SNP	T	A	Intron4	198,121
SNP	C	T	Intron5	198,323
Deletion	-	CTT	Intron5	198,338~198,340
SNP	G	A	Exon8	227,016
SNP	C	T	Intron8	227,154
SNP	C	T	Exon10	231,601
Deletion	-	C	Exon10	231,993
Insertion	AGAGAGAGAGAG		Exon10	232,152
SNP	C	T	Exon10	232,218
SNP	C	T	Exon10	232,505
Insertion	TGTCTCTCTCT		DER	234,193
SNP	A	G	DER	234,331
SNP	A	G	DER	234,985
SNP	C	T	DER	235,041
SNP	G	A	DER	235,223
SNP	T	C	DER	235.279

Similarly, the *Npsr1* DNA sequence of mHAB *vs.* mLAB also showed several polymorphisms in the promoter and downstream regions (Table 14; Figure 11) with mLAB *Npsr1* sequence identical to reference mouse strain (C57BL/6J).

Table 14: *Npsr1* polymorphisms in mHAB *vs.* mLAB : SNPs, Deletions and Insertions. Positions relative to ATG start codon.

Variation type	HAB	LAB	Location in *Npsr1* gene	Relative position	SNP identifier
SNP	G	A	Promoter	-2,251	rs50949943
SNP	C	T	Promoter	-2,248	rs48292984
SNP	C	G	Promoter	-2,123	rs47083749
SNP	T	C	Promoter	-2,112	rs49887483
SNP	T	A	Promoter	-2,104	rs47000117
SNP	T	C	Promoter	-2,046	rs48022291
SNP	G	A	Promoter	-1,942	rs46860992
SNP	T	C	Promoter	-1,863	rs51840884
SNP	T	A	Promoter	-1,842	rs45839541
SNP	C	T	Promoter	-1,775	rs52096988
SNP	G	A	Promoter	-1,667	
Deletion		AA(GA)x18	Promoter	-1,608~-1,571	
SNP	G	A	Promoter	-1,516	
SNP	T	C	Promoter	-1,469	
SNP	G	A	Promoter	-1,418	
SNP	A	G	Promoter	-1,376	
SNP	A	T	Promoter	-1,315	
SNP	C	T	Promoter	-1,236	rs48864073
Deletion		T	Promoter	-1,227	
SNP	A	T	Promoter	-1,226	rs36643873
SNP	A	T	Promoter	-1,132	
SNP	T	G	Promoter	-1,072	rs51941766
SNP	G	A	Promoter	-1,032	
SNP	C	T	Promoter	-860	rs45719875
SNP	G	A	Promoter	-816	rs37067240
SNP	C	T	Promoter	-731	rs50871983
SNP	C	T	Promoter	-714	rs48580633
SNP	T	G	Promoter	-713	rs47842102
SNP	C	G	Promoter	-674	rs46047101
Deletion		T	Promoter	-648	
SNP	T	A	Promoter	-637	rs45879530
SNP	T	C	Promoter	-612	rs51858460
SNP	T	C	Promoter	-591	rs46930781
SNP	C	A	Promoter	-557	rs50633535
Insertion	C		Promoter	-479	
Insertion	T		Promoter	-478	
SNP	A	T	Intron1	-82	rs48722200
Insertion	A		Intron3	156,233	
SNP	G	A	Exon4	156,453	rs37572071
SNP	C	T	Intron7	205,648	
Deletion		C	Intron7	205,718	
SNP	T	C	Intron8	215,240	
SNP	G	C	Intron9	215,243	
SNP	G	A	Intron9	215,244	
SNP	A	G	Intron9	215,245	
SNP	G	A	Exon10	216,508	rs49543460
SNP	G	A	Exon10	217,782	rs49030747
SNP	A	G	DER	218,543	

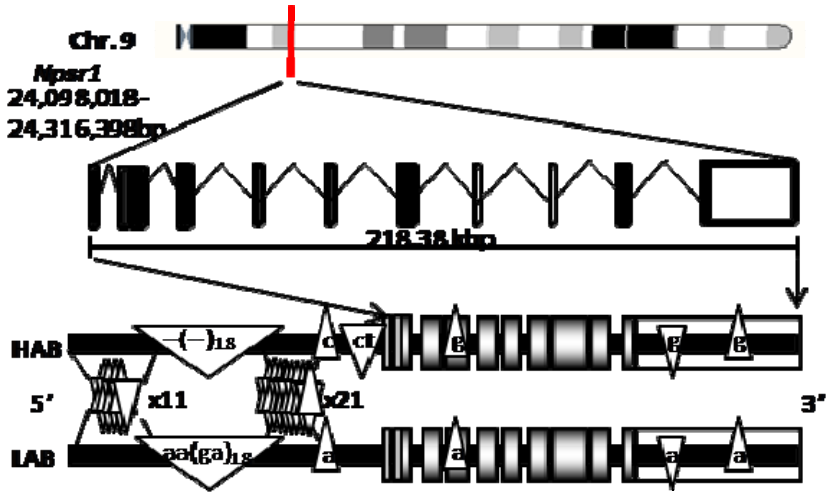


Figure 11: Polymorphisms and SNPs in *Npsr1* of mHAB *vs.* mLAB. Polymorphic sites are indicated as unfilled triangles, exons including UTRs are indicated by boxes (exons shaded, UTRs are white), intronic variations are not shown. (Adapted from (Yen, 2011)).

Overall, DNA sequencing of both rat and mice *Npsr1* revealed several polymorphisms like SNPs, insertion, deletion in the upstream promoter and downstream exonic and intronic regions. TESS analysis accompanied by literature survey to study the role of putative transcription factors revealed two interesting candidates: At G(-388)A, there was nuclear factor-1 (NF-1) binding at G residue in rLAB, while in rHAB A residue favors binding of GR transcription factor. In addition, at A(-1813)C there was TATA binding protein in rLAB which on mutation allows GR binding at corresponding position in rHAB.

Similarly, TESS analysis of mouse *Npsr1* DNA followed by literature search also found two transcription factors. Surprisingly, there was an insertion of CT residue (-479,-478) in mHAB which also favor GR binding while in mLAB at the corresponding position there was adjacent binding of activator protein 1 (AP-1). A SNP (G(-1082)T) in the distal region of mHAB *Npsr1*, allowed binding of another nuclear factor NF 3-β while there was no putative transcription factor found for corresponding LAB position. In the downstream coding region there was a synonymous SNP [A(227016)G and A(156453)G; rs37572071] in exon 8 and 4 of rHAB and mHAB, respectively at the 3rd wobble position.

3.1.7 Measurement of allelic expression imbalance

As the expression of any gene can be due to an interaction between *cis*- and *trans*-acting factors, HAB and LAB allelic expression was studied in cross mated F1 offspring where each allele acts as an internal standard for the other allele. In contrast to the lower HAB *Npsr1* mRNA expression, both F1 rats ($p < 0.001$; Figure 12A) and F1 mice ($p < 0.05$; Figure 12B) displayed higher HAB than LAB allelic expression, irrespective of the maternal line.

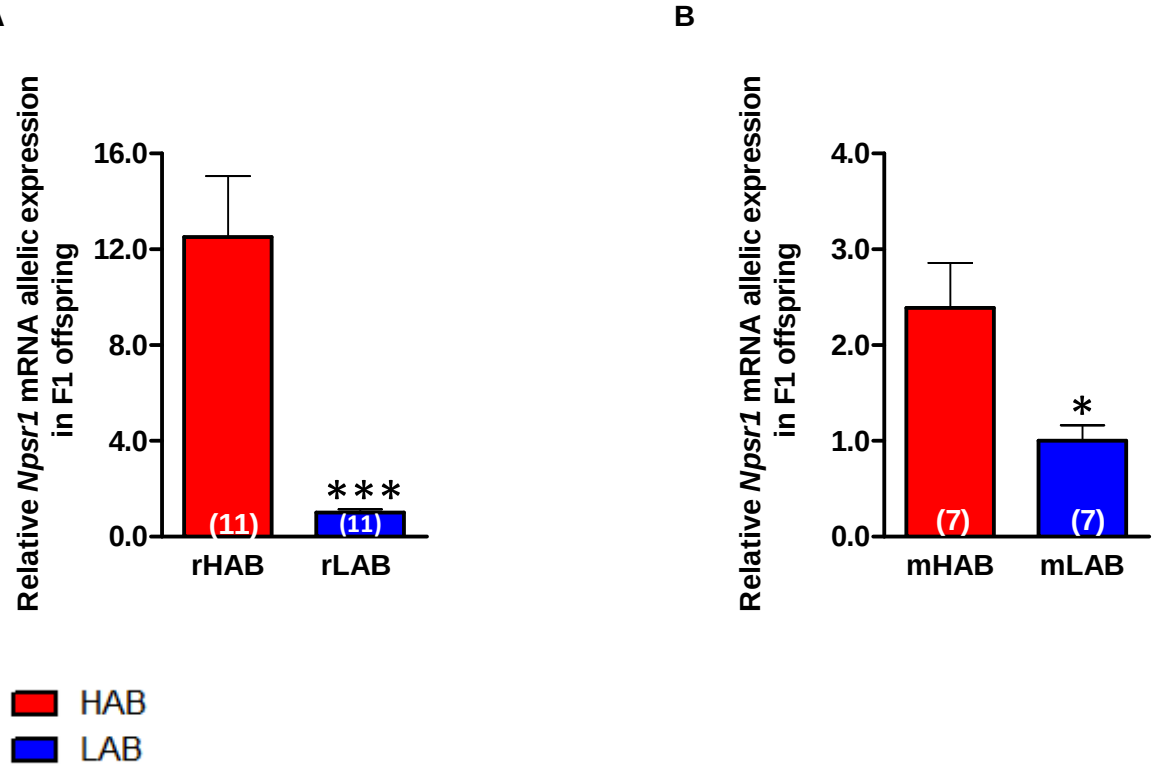


Figure 12: Relative *Npsr1* mRNA allelic expression in F1 offspring of **(A)** rHAB *vs.* rLAB and **(B)** mHAB *vs.* mLAB. Number in parentheses indicates group numbers. Data are represented as mean+SEM. *** $p < 0.001$, * $p < 0.05$ compared with HABs.

The AEI data was in contrary to the observed lower basal *Npsr1* mRNA expression in HAB rodents relative to LABs. Thus this suggests that in HABs there is an interaction between *cis*-variations and *trans*-acting factors. Next, *in vitro* promoter assays were utilized to study the promoter activity.

3.1.8 Measurement of *Npsr1* promoter activity to assess the role of polymorphisms

When rat promoter constructs of approximately 2000 bp upstream of the ATG translation start site were assessed, rHAB promoter activity was half that of the corresponding rLAB promoter ($p < 0.001$; Figure 13). The activity of promoter-deletion constructs was also assessed to deduce

the contribution of individual SNPs. No difference was observed between rHAB *vs.* rLAB Fragment B while a higher activity in rLAB than rHAB was seen in Fragment C ($p < 0.01$). However, Fragment D had nearly 2-fold higher rHAB promoter activity in comparison to rLAB ($p < 0.001$); analogous to the higher HAB-specific allele expression in F1 offspring. Subsequent deletion of the G(-388)A SNP led to Fragment E with the C(-221)G SNP, where promoter activity did not differ between rHAB and rLAB (Figure 13).

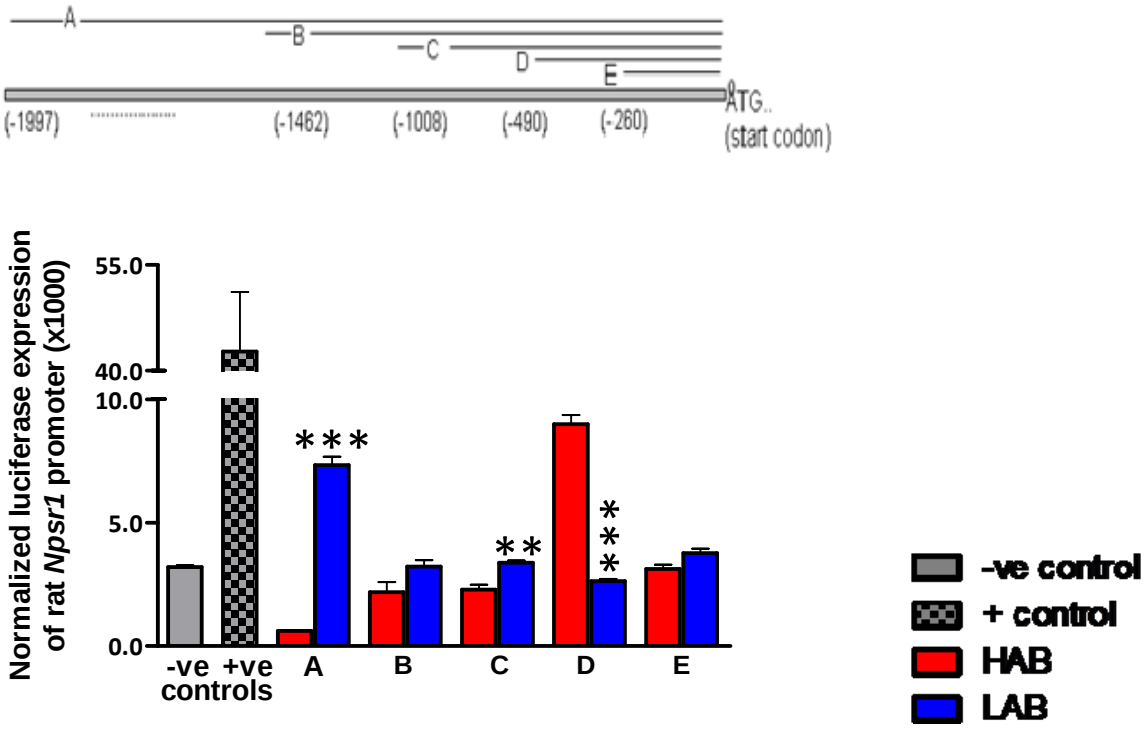


Figure 13: *in vitro* analyses of *Npsr1* promoter fragments. Promoter constructs A to E depict the longest to the shortest rat *Npsr1* fragments. Dual luciferase assays with rHAB *vs.* rLAB *Npsr1* promoter constructs (A-E) in pGL3 basic vector. Data are shown as means+SEM; ** $p < 0.01$; *** $p < 0.001$ compared with corresponding rHAB. Firefly luciferase data were normalized to Gaussia activities and are presented as relative expression + SEM of three independent experiments performed in triplicate.

Similarly, using homologous mouse *Npsr1* promoter constructs, the activity of the full length mHAB construct was found to be lower than the corresponding mLAB (Fragment P: $p < 0.01$; Figure 14). As the putative promoter length decreased, no difference in the corresponding promoter activity between mHAB *vs.* mLAB was observed (Fragments Q and R). However, Fragment S, which harbors the putative GR binding site, displayed higher mHAB promoter activity than the corresponding mLAB construct ($p < 0.01$; Figure 14). The higher activity of mHAB fragment S was analogous to the higher HAB specific allelic expression in the F1 offspring.

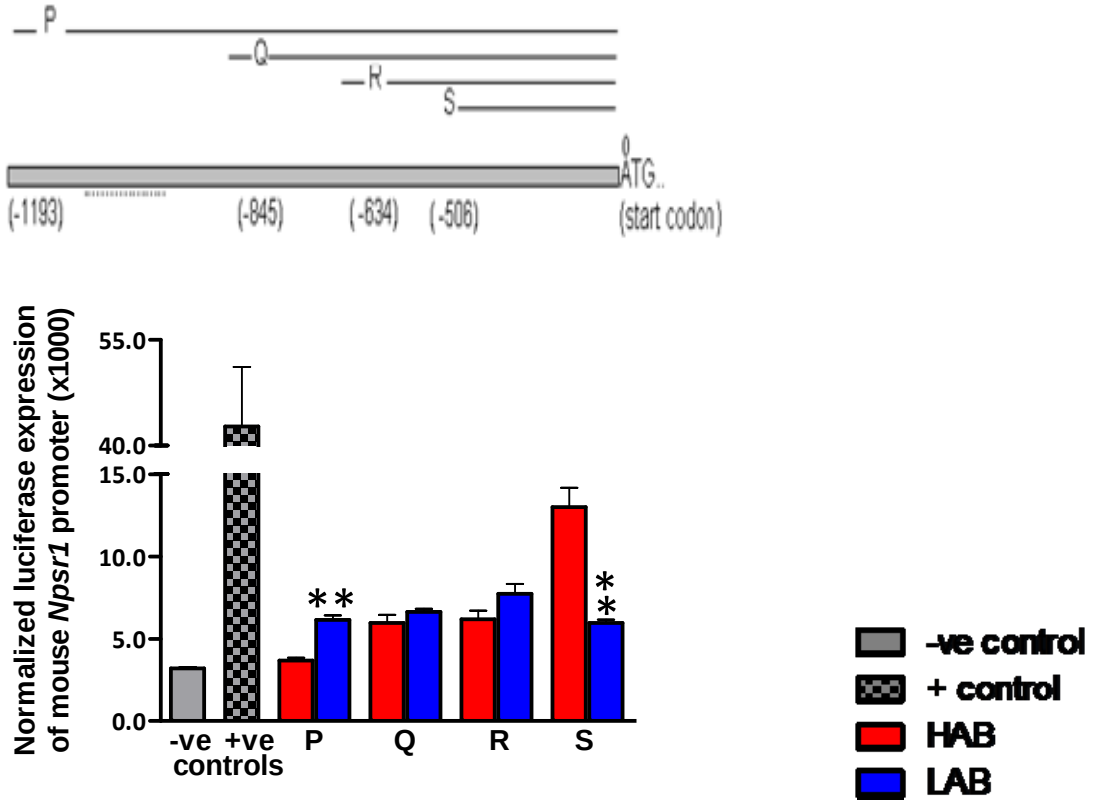
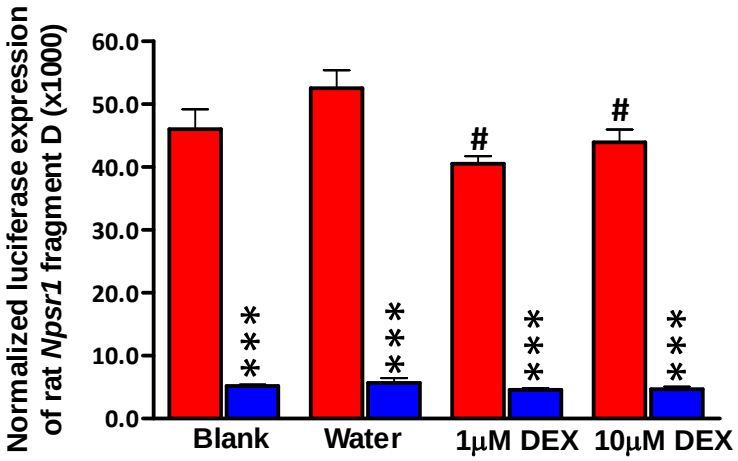


Figure 14: *in vitro* analyses of mouse *Npsr1* promoter fragments. Promoter constructs P to S depict the longest to the shortest *Npsr1* fragments .Dual luciferase assays with mHAB *vs.* mLAB *Npsr1* promoter constructs (P-S) in pGL3 basic vector. Data are shown as mean+SEM; ** $p < 0.01$ compared with corresponding mHAB. Firefly luciferase data were normalized to Gaussia activities and are presented as relative expression + SEM of three independent experiments performed in triplicate.

Thus, *in vitro* promoter assay could recapitulate the *in vivo* lower basal *Npsr1* mRNA expression observed in HAB rodents in contrast to LABs. This data indicates that there are distal repressor elements that down regulates HAB *Npsr1* mRNA expression. However, the shortest constructs (Fragment D and S of rats and mice, respectively) had higher HAB specific allelic expression similar to that observed in F1 offspring. Next step was to verify the presence of GR binding in HABs as suggested by TESS analysis.

The *in silico* data of GR binding site was further validated by showing that the high activity of HAB Fragments D ($F_{3,16}$ = 3.37; p = 0.045) and S ($F_{3,16}$ = 4.12; p = 0.023), which was recapitulated here (p < 0.001 for all HAB *vs.* LAB comparisons) could be reduced by both 1 and 10 µM DEX administration (p < 0.05 *vs.* water; Figure 15A and 15B).

A



B

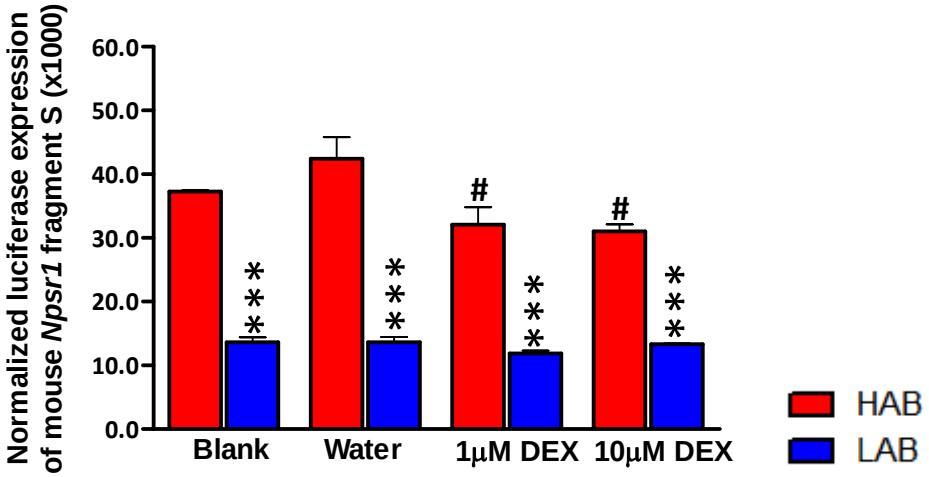


Figure 15: (A) rHAB *vs.* rLAB fragment D treated with 1, 10 µM DEX and water as control for 24 h and **(B)** mHAB *vs.* mLAB fragment S carrying the putative GR binding site treated with 1, 10 µM DEX and water as control for 24 h before the luciferase assay. Data are shown as mean+SEM; *** p < 0.001 compared with HAB; # p < 0.05 when compared to water. Firefly luciferase data were normalized to Gaussia activities and are presented as relative expression + SEM of three independent experiments performed in triplicate.

This study could confirm GR binding. However, the lower activity of DEX administration suggests that GR interferes with activity of basal transcription factors like NF-1 or AP-1.

3.1.9 Comparative genome analysis of *Npsr1* DNA sequences using VISTA browser

The similar promoter activity observed for rats and mice *Npsr1* prompted me to do a comparative genome analysis to search for possible homologous regions.

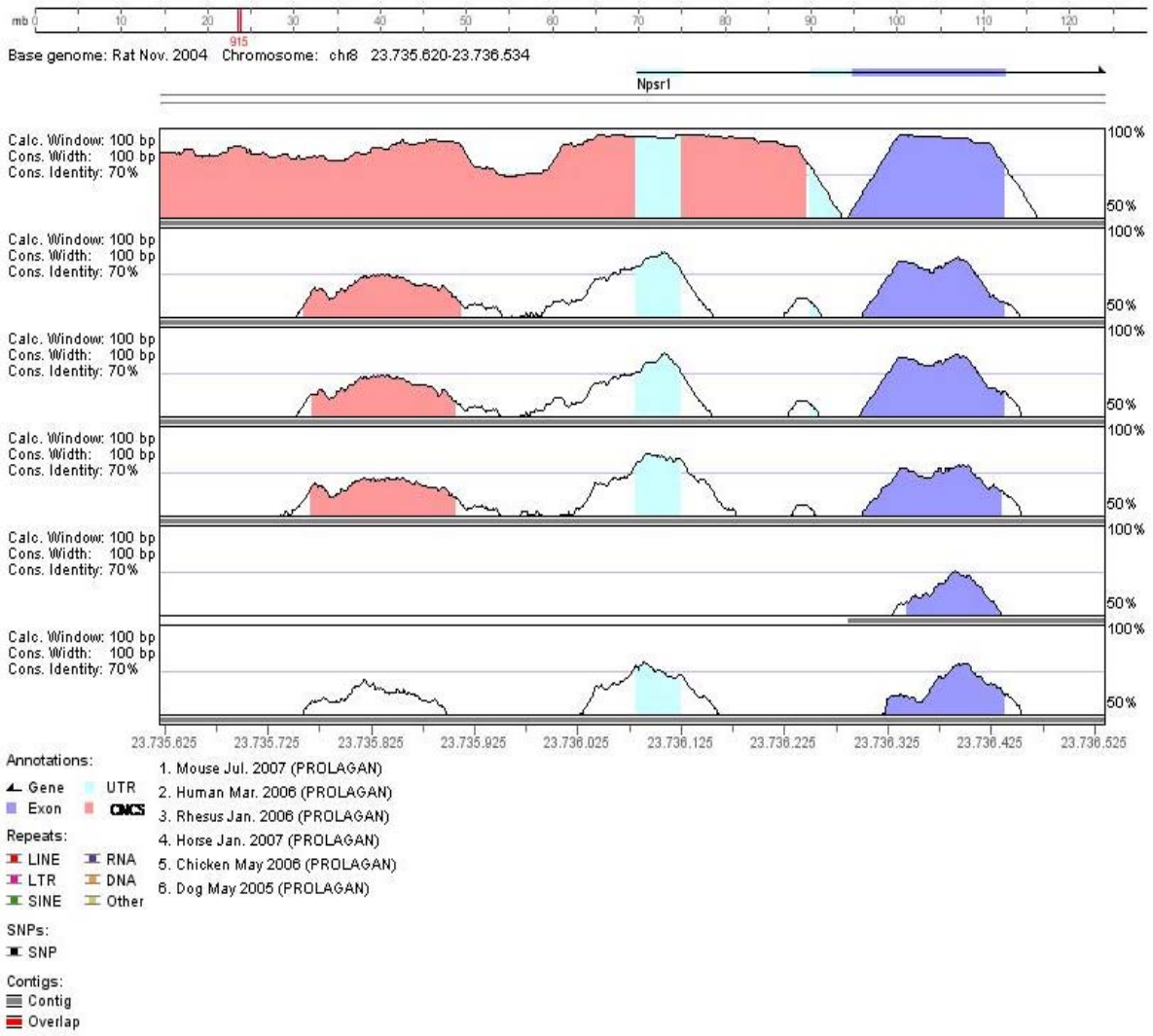


Figure 16: Comparative genome analysis between mouse, human, rhesus monkey, horse, chicken, rats and dog *Npsr1* DNA sequence. Dark blue, pink and light blue colored regions are exons, conserved non-coding sequences (CNCS) and UTR, respectively.

There is high degree of homology in the UTR, CNCS and the exonic region of mouse, human, rhesus monkey, horse, chicken, rats and dog *Npsr1* gene (Figure 16). However, in dogs there was no homologous CNCS region found and in chicken no similar domains were found both in CNCS and UTR.

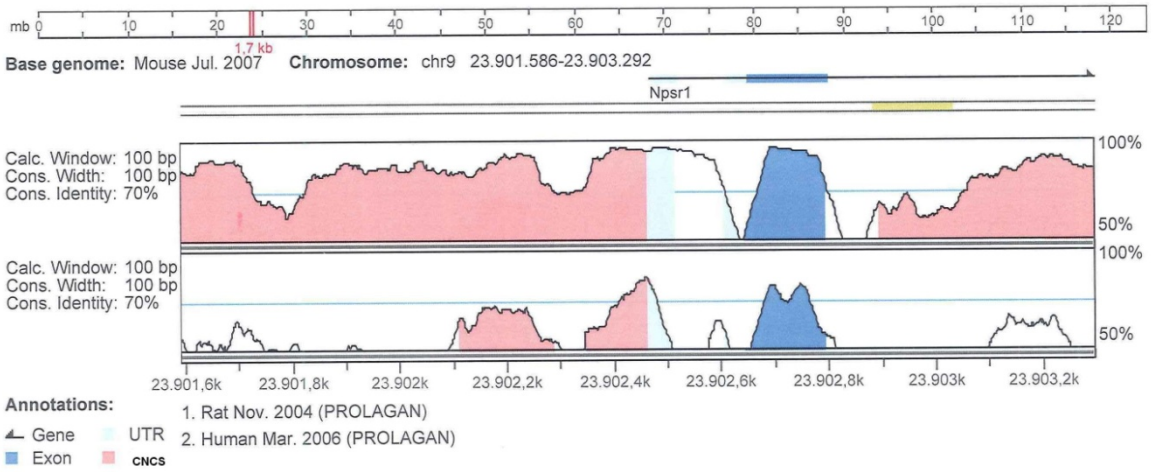


Figure 17: Comparative genome analysis between mouse, human and rats *Npsr1* DNA sequence. Dark blue, pink and light blue colored regions are exons, conserved non-coding sequences (CNCS) and UTR, respectively.

A detailed look at the VISTA browser genome analysis data among rat, mice and human also revealed presence of homologous sequences in upstream 5' UTR region.

3.1.10 Copy number variation and search for CpG islands

To search for additional factors that might mediate differential gene regulation.

There was no difference in CNV in both rats and mouse *Nps* and *Npsr1* gene (data not shown). There were also no CpG islands in both rat and mouse *Npsr1* DNA sequence. The CpG islands are usually prone to DNA methylation based epigenetic processes. Thus CNV or DNA methylation based epigenetic processes as a factor causing differential regulation of these genes is ruled out.

3.1.11 Functional characterization of HAB *vs.* LAB NPSR1 and assessment of protein expression

There was higher NPS-dependent luciferase expression (*i.e.* downstream cAMP response) in cells expressing the HAB NPSR1 protein in both rats and mice (both $p < 0.01$; Figure 18A and 18B) akin to the human Ile[107] risk isoform.

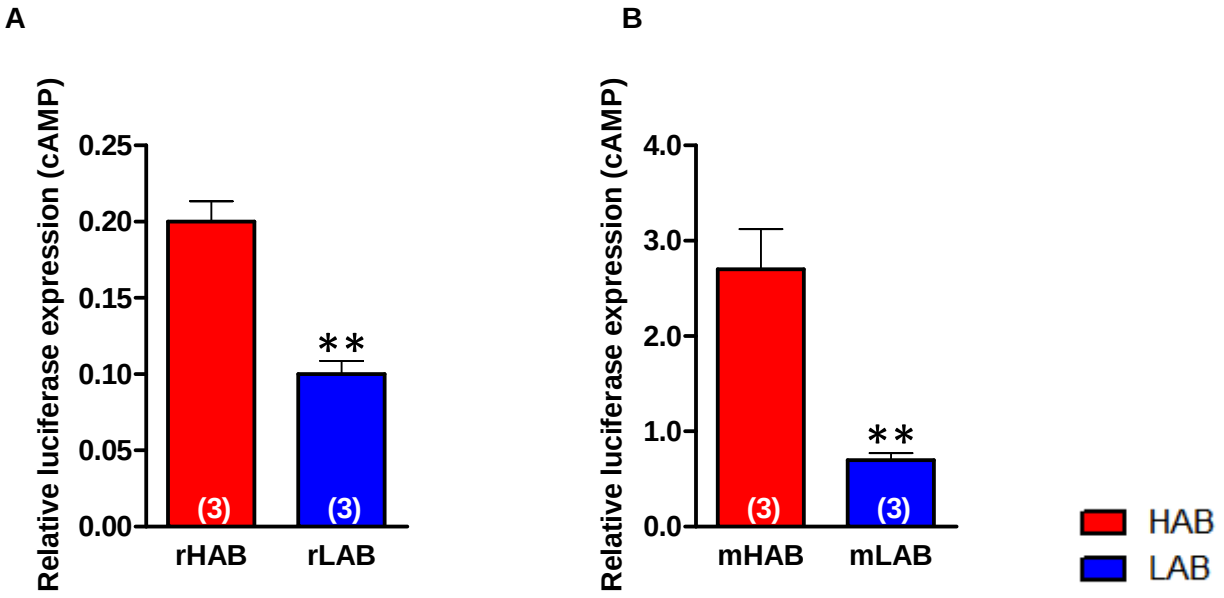


Figure 18: *in vitro* analyses of *Npsr1* coding SNPs.
(A) HEK 293 cells were co-transfected with **(A)** rHAB and rLAB *Npsr1* cDNA constructs carrying A(227,016)G along with CRE-luciferase: Gaussia vector (20:1) and
HEK 293 CRE-luciferase cells were cotransfected with **(B)** mHAB and mLAB *Npsr1* cDNA constructs carrying A(156,453)G; rs37572071 along with Gaussia vector. Then these cells were stimulated with 1 nmol NPS at 40h post-transfection until assay at 48 h. Number in parentheses indicates group numbers. Data are represented as mean+SEM. **$p < 0.01$ compared with respective HABs.

3.1.12 Semi-quantitative Western blot analysis for NPSR1

However, there was no difference in total NPSR1 protein expression in the PVN of rats (Figure 19A) or amygdala of mice (Figure 19B).

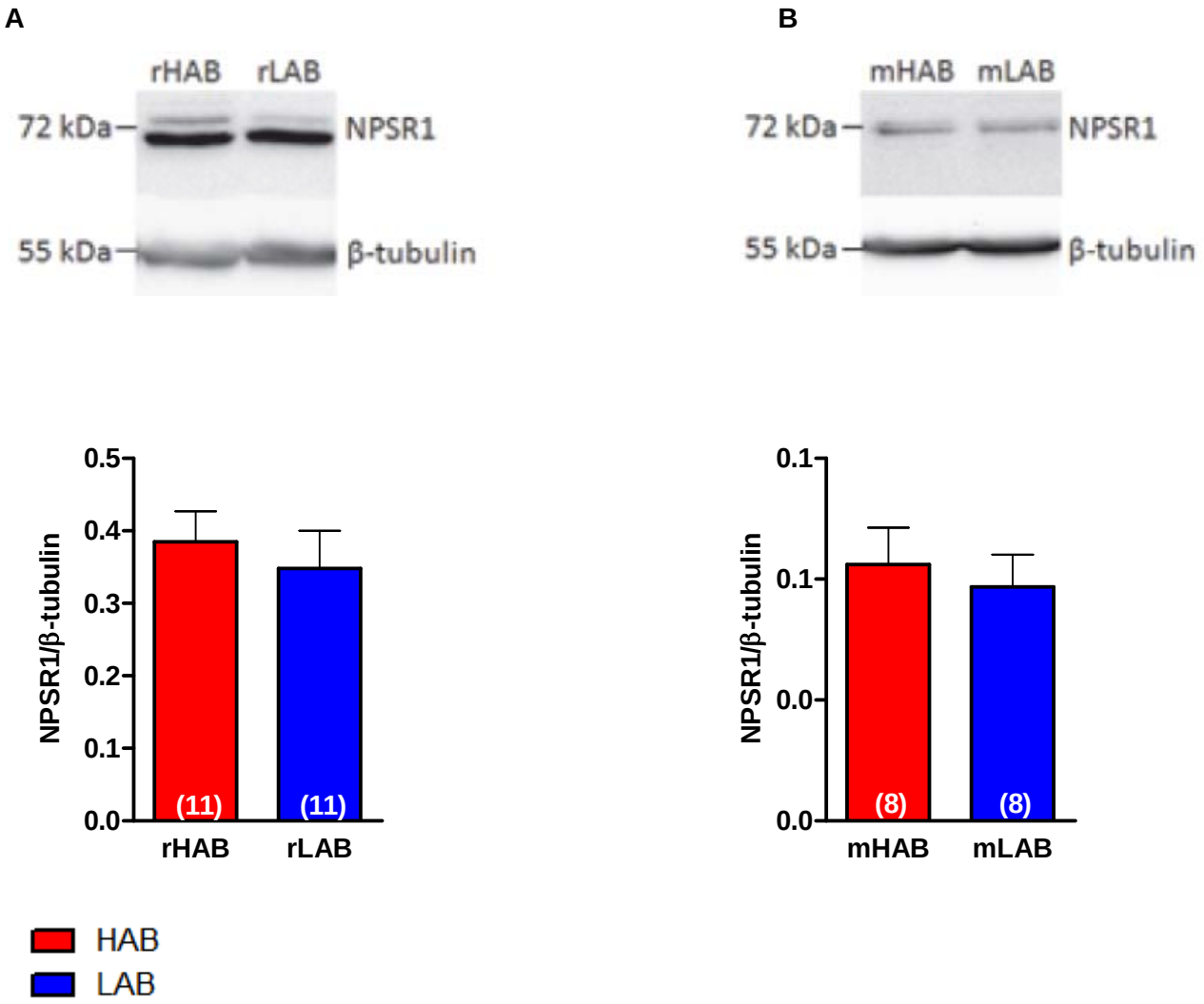
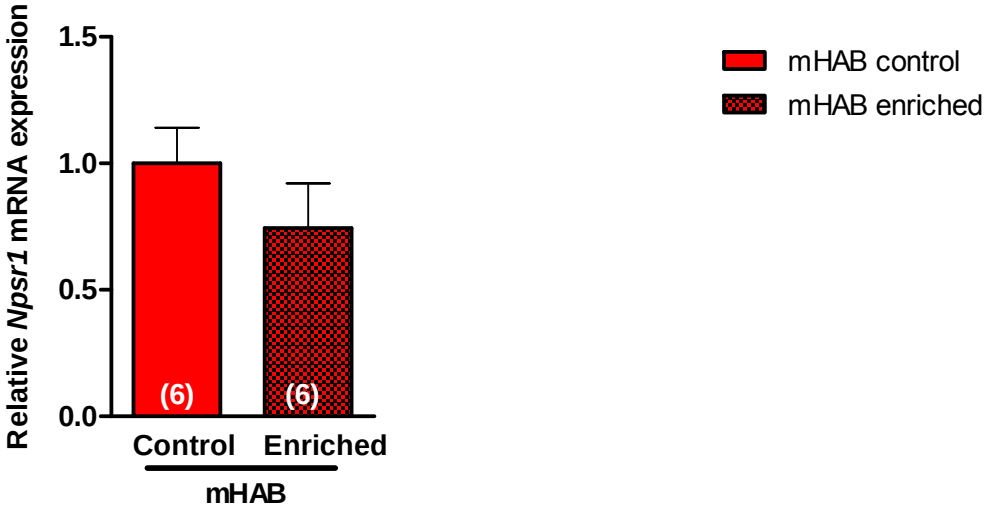


Figure 19: Semi-quantitative Western blots for total NPSR1 protein **(A)** of rHAB, rLAB in the hypothalamic PVN and **(B)** of mHAB, mLAB in the amygdala with ß-tubulin as loading control. Data are shown as mean+SEM, numbers in parentheses indicate the group numbers.

3.1.13 To determine the plasticity of *Npsr1* gene

To determine if *Npsr1* gene responds to the environmental challenges faced by animal we measured its mRNA expression in the corresponding animals. There was no difference in *Npsr1* mRNA expression in mHAB or mLAB subjected to EE or UCMS (Figure 20A and 20B), respectively. This suggests that *Npsr1* is a non-plastic gene that is not responsive to the environmental manipulations applied to the animals.

A



B

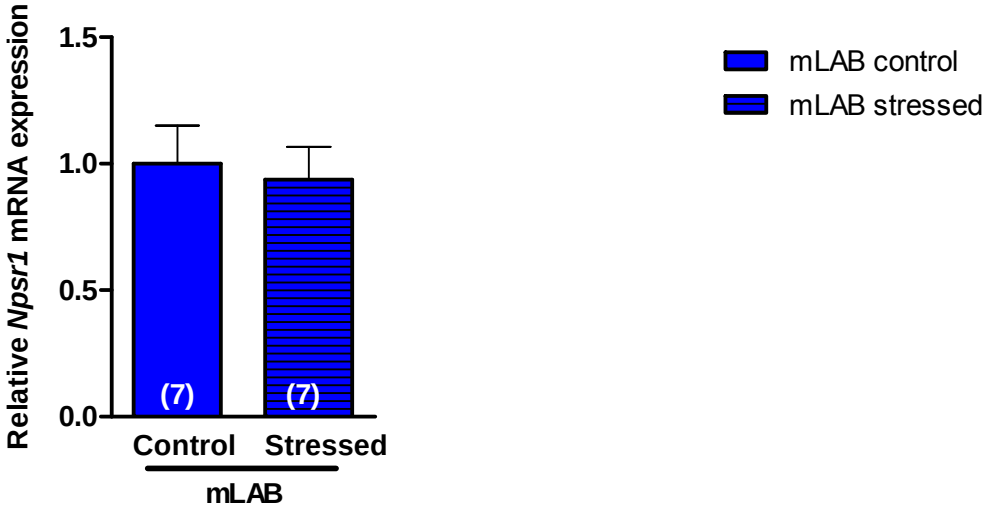


Figure 20: qPCR measurement of *Npsr1* mRNA in **(A)** mHAB control *vs.* mHAB enriched and **(B)** mLAB control *vs.* mLAB stressed. Numbers in parentheses indicates group size. Data are represented as mean+SEM.

3.2. *Tmem132d* system

3.2.1 Measurement of promoter activity of *Tmem132d* in mHAB *vs.* mLAB

Earlier studies by (Erhardt *et al.*, 2011) have shown that there is higher expression of *Tmem132d* mRNA in the anterior cingulate cotex of mHAB in comparison to mLAB. Thus to dissect the genetic underpinnings of this differential expression, mHAB *vs.* mLAB *Tmem132d* promoter carrying the A(-519)G and A(-310)G SNPs (Figure 21) were cloned into pGL3 basic vector and promoter activity was measured using dual luciferase assay.

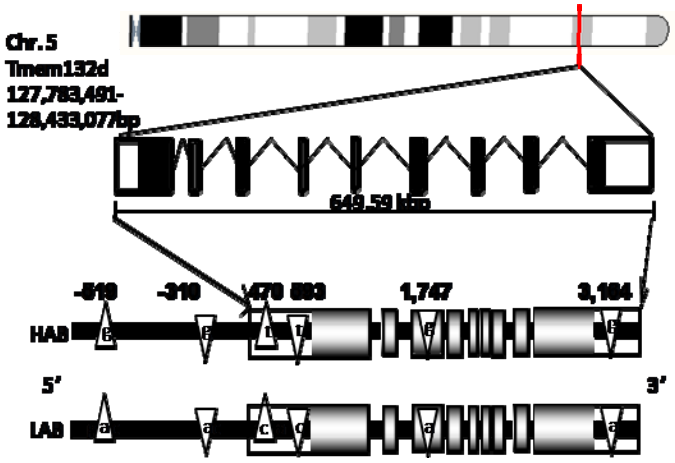


Figure 21: Polymorphisms in *Tmem132d* of mHAB *vs.* mLAB. Polymorphic sites are indicated as unfilled triangles, exons and UTRs are indicated by boxes (exons shaded, UTRs are white), intronic variations are not shown. The SNP position is relative to transcription start site (Figure adapted from (Czibere, 2008)).

The whole mHAB *Tmem132d* promoter constructs containing a G residue at -519 and -310 position had higher promoter activity (** $p < 0.01$; Figure 22) compared to corresponding mLAB constructs. This explains the *in vivo* findings of higher *Tmem132d* mRNA expression in mHAB compared to mLAB. To analyze the individual contribution of SNPs, -519 or -310 G residue when mutated to A caused a significant decrease in promoter activity (*** $p < 0.001$) or (** $p < 0.01$), respectively in comparison to whole mHAB constructs. This suggests that both G residues are required for the higher mHAB promoter activity. On the other hand, deletion of -519 A residue in the mLAB construct did not have any effect on its promoter activity. While, deletion of -519 G residue in the mHAB construct again led to significant decrease in its promoter activity (** $p < 0.01$; Figure 22), thus proving the importance of the -519 G residue. Thus, it would be interesting to know the putative transcription factor binding sites at these two positions.

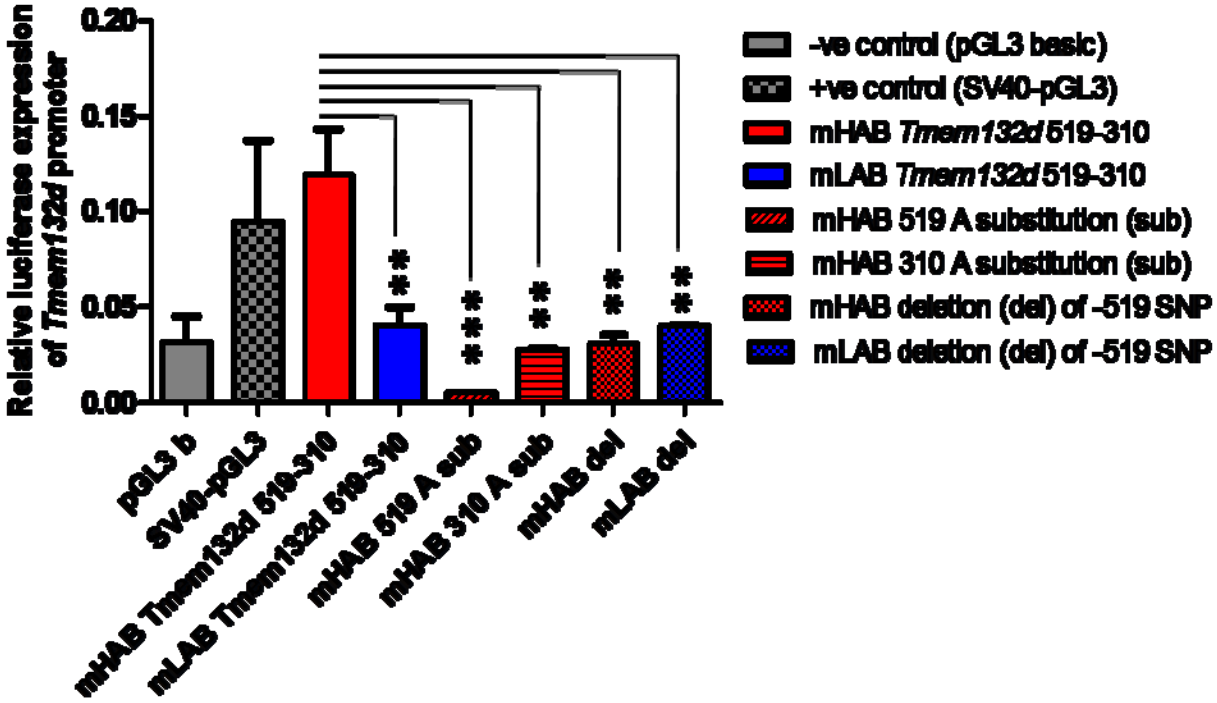


Figure 22: Dual luciferase assay with mHAB *vs.* mLAB *Tmem132d* promoter constructs. Data are shown as mean+SEM; ** $p < 0.01$; *** $p < 0.001$ in comparison with mHAB *Tmem132d* 519-310. Firefly luciferase data were normalized to Gaussia activities and are presented as relative expression + SEM of three independent experiments performed in triplicate.

3.2.2 *in silico* analysis of *Tmem132d* promoter region

Assessment of putative transcription factor binding sites revealed binding of nuclear factor I/C (CCAAT-box binding transcription factor), also known as NF-I or CTF at -519 position. This nuclear factor has been shown to be involved in vertebrate brain development (Singh *et al.*, 2011), eukaryotic transcription (Santoro *et al.*, 1988) and was found to colocalize with RNA polymerase II (Zhao *et al.*, 2005). Meanwhile, at position -310 there was binding of TFIIB which is one of the general transcription factor that makes the RNA polymerase II preinitiation complex (Kostrewa *et al.*, 2009). Moreover, the *Tmem132d* gene was scanned using *in silico* tools for the presence of CpG islands and interestingly there was a 600 bp CpG island in the promoter region which encompasses the above described two SNPs.

3.2.3 Bisulfite sequencing of mHAB *vs.* mLAB *Tmem132d* promoter region

To determine if DNA methylation also plays any role in the differential gene regulation, genomic DNA from Cg was subjected to bisulfite conversion. Two types of primers were utilized, bisulfite specific primers that only bind bisulfite DNA and wild type primers, binding to genomic DNA to check for incomplete bisulfite conversion. Aliquots of bisulfite DNA were subjected to PCR with bisulfite specific or wild type primers. Bisulfite DNA covering 600 bp of *Tmem132d* gene was amplified only with bisulfite primers (Figure 23). There was no product observed with wild type primers suggesting complete bisulfite conversion.

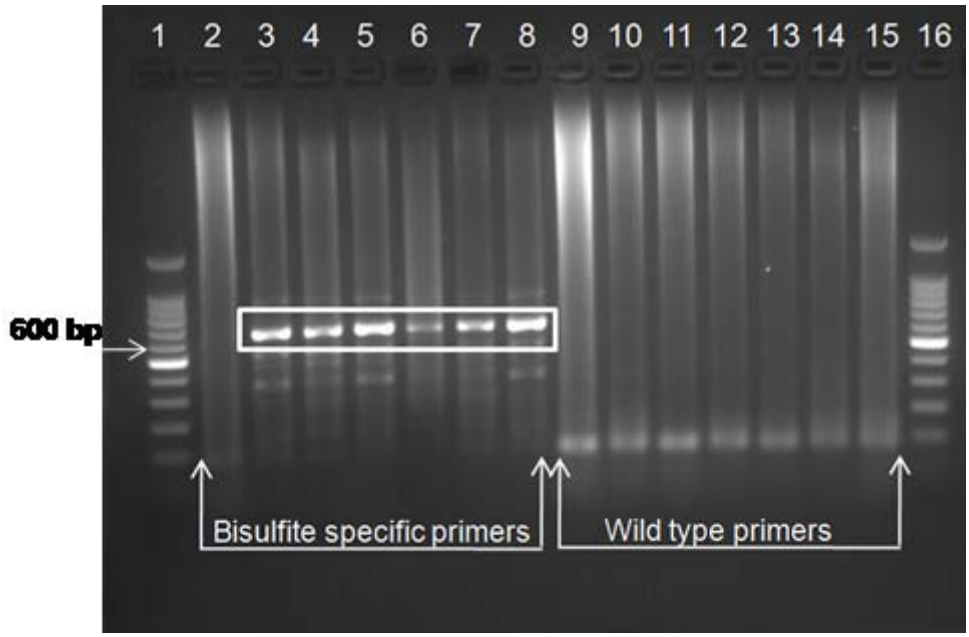
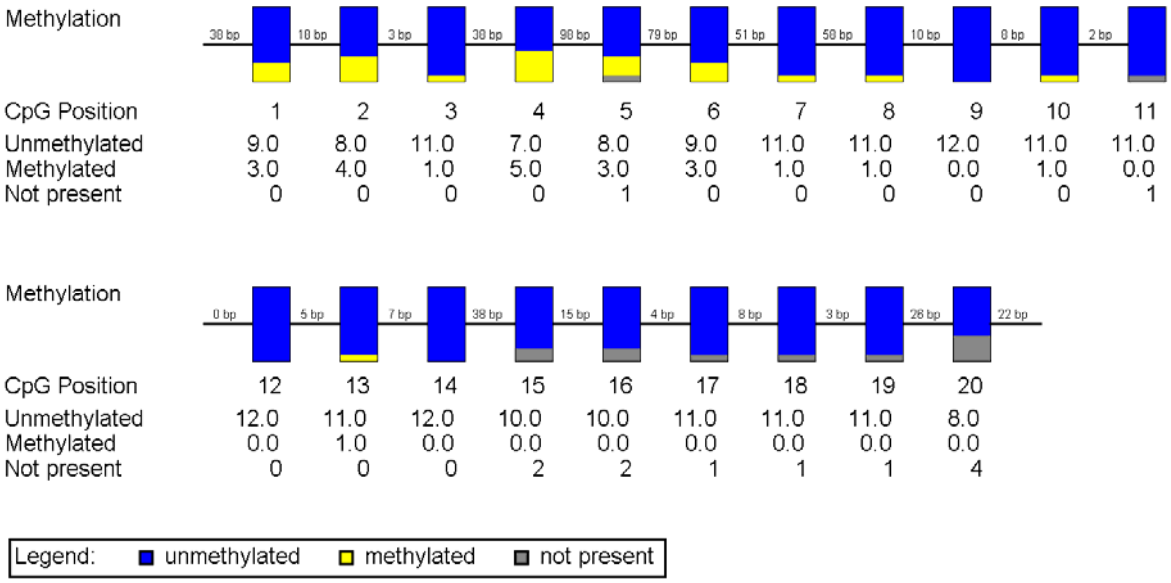


Figure 23: Quality control for bisulifte conversion of mHAB *vs.* mLAB *Tmem132d* promoter DNA. Lane 1 and 16 are DNA ladder, lane 2 and 9 are no template control (NTC) for bisulfite specific and wild type primers. Lane 3-5 are mHAB and 6-8 are mLAB bisulfite PCR products at approximately 600 bp obtained with bisulfite specific primers. Lane 10-15 are corresponding mHAB, mLAB bisulfite products subjected to PCR with wild type primers. Here, no products, at least at the expected 600 bp suggest complete conversion of genomic DNA to bisulfite DNA. Thus, this acts as quality control for bisulfite conversion.

3.2.4 Analysis of mHAB *vs.* mLAB *Tmem132d* promoter DNA methylation with BiQ analyzer

Overall, there was DNA methylation observed in the distal regions especially around the -519 and -310 SNP, which decreased upon nearing the transcription start site (Figure 24A, 24B). However, there was no difference in total percentage of methylation between basal mHAB *vs.* mLAB *Tmem132d* promoter (Figure 25), and not even any difference at individual CpG positions between mHAB *vs.* mLAB (data not shown).

A



B

mLAB *Tmem132d* promoter region (22B)

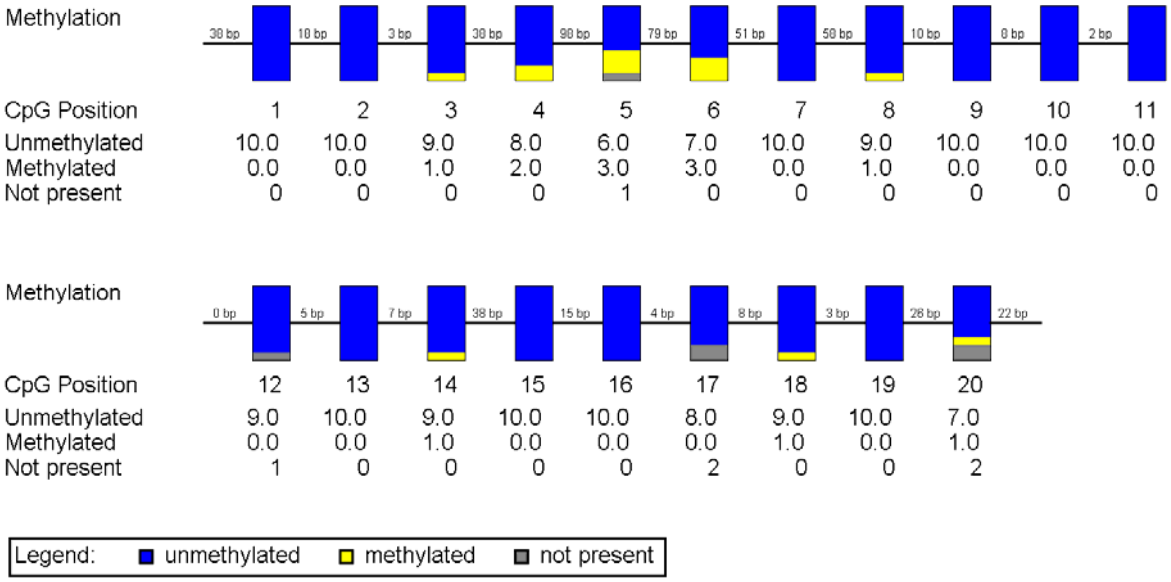


Figure 24: Aggregated representation of methylation data of a single **(A)** mHAB and **(B)** mLAB *Tmem132d* promoter region containing 20 CpG positions. Each box corresponds to one CpG position in the genomic sequence. The colored bars summarize the methylation states of all sequences at that position.

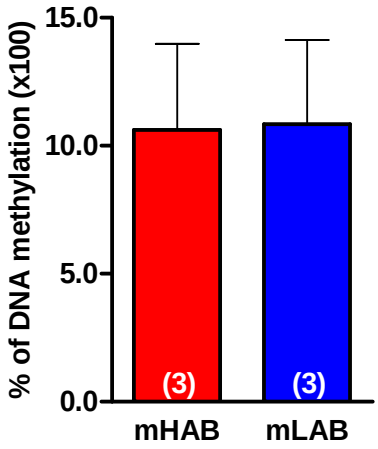


Figure 25: Percentage of DNA methylation of mHAB *vs.* mLAB *Tmem132d* promoter region encompassing a CpG island of approximately 600 bp. There were at least 10 clones per animal except one. Data are shown as mean+SEM.

3.2.5 To determine the plasticity of the *Tmem132d* gene

To determine if *Tmem132d* gene responds to the environmental challenges faced by the animals corresponding mRNA in the Cg was measured. EE mHABs spent a higher percentage of time in the light compartment ($p < 0.001$; Figure 26A), and there was higher corresponding *Tmem132d* mRNA expression ($p < 0.05$; Figure 26B) in comparison to control mHABs. Conversely, stressed mLABs spent less percentage of time in the light compartment ($p < 0.001$; Figure 26C), and there was lower corresponding *Tmem132d* mRNA expression ($p < 0.05$; Figure 26D) in comparison to control mLABs. It is worth noting that the time spent by EE HABs in light zone is similar to that of outbred CD-1 mice. Thus EE can exert anxiolytic effects even in a genetically predisposed animal model (Figure 26A).

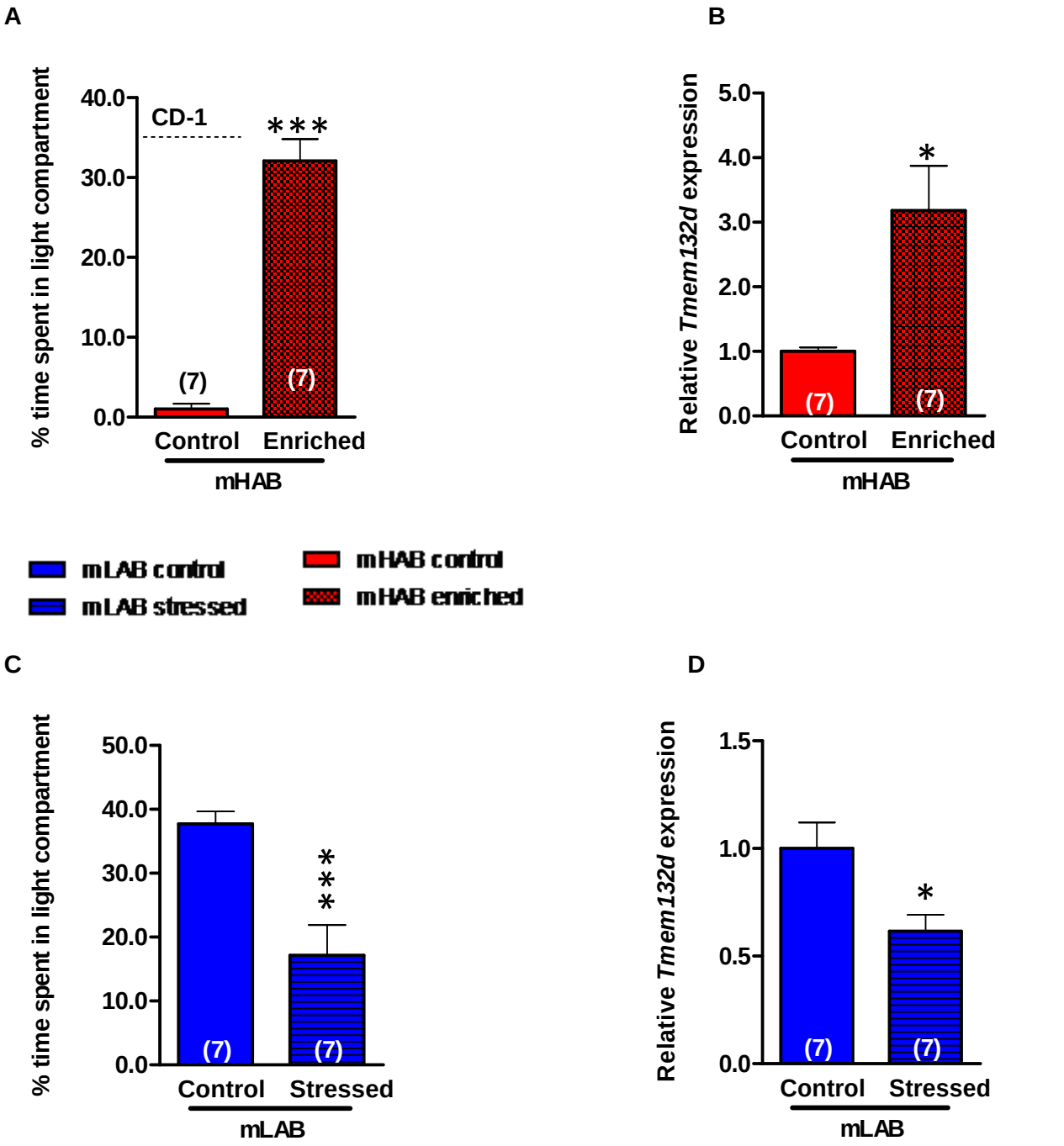


Figure 26: Percent time spent in the light compartment of LD box test and corresponding *Tmem132d* expression changes: (**A** & **B**) enriched mHAB *vs.* control mHABs. (**C** & **D**) stressed mLABs *vs.* control mLABs. Numbers in parentheses indicates group size. Data are indicated as mean+SEM. * $p < 0.05$; *** $p < 0.001$ compared with respective control.

This suggests possible environmentally modulated epigenetic mechanisms underlying differential expression. Thus, to rule out any environmentally modulated factors, we cross-mated mHAB with mLAB and *vice versa* to study the relative allelic expression in the same cell.

3.2.6 Measurement of behavior of F1 offspring

F1 offspring of HAB mother genotype were analyzed on a battery of behavioral tests including neuroendocrinological parameters such as corticosterone measurement.

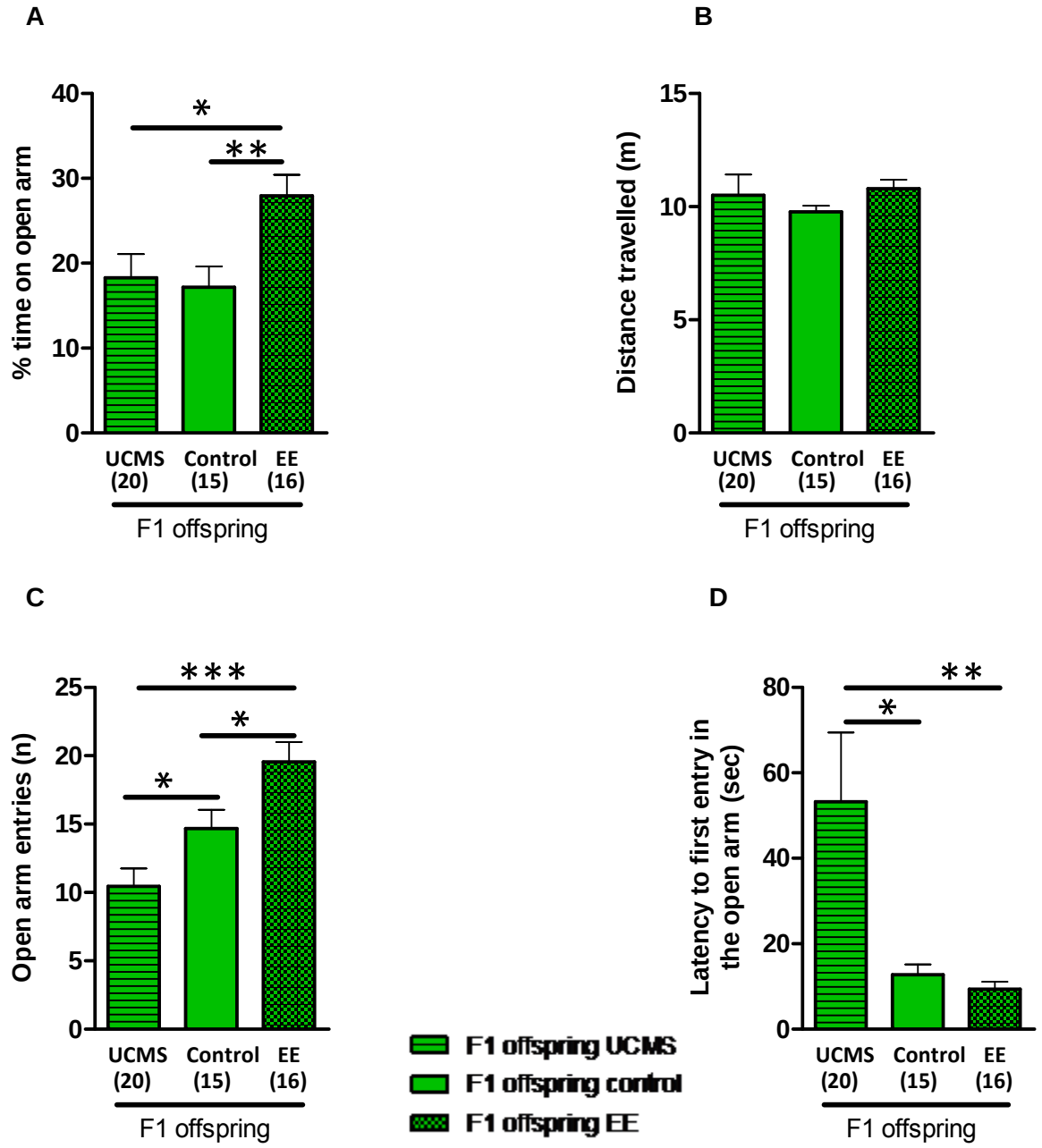


Figure 27: Effect of UCMS and EE on F1 offspring in **(A)** percent time on open arm **(B)** distance travelled **(C)** open arm entries and **(D)** latency to first entry in the open arm of EPM. Data represented as mean+SEM. Numbers in parentheses indicate group size. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

F1 offspring subjected to EE or UCMS had significant percent time spent in the open arm ($F_{2,48}$=4.86; $p < 0.01$; Figure 27A), open arm entries ($F_{2,48}$= 11.30; $p < 0.001$; Figure 27C) and

latency to enter open arm ($F_{2,48}$=5.40; $p < 0.01$; Figure 27D). While there was no difference in the distance travelled between all three groups ($F_{2,48}$=0.555; p=0.578; Figure 27B). In addition, EE group spend higher percent time in open arm ($p < 0.01$; Figure 27A) and open arm entries ($p < 0.05$; Figure 27C) in comparison to control group. Likewise the UCMS group had lower open arm entries ($p < 0.05$; Figure 27C) and higher latency to entry in open arm ($p < 0.05$; Figure 27D) in comparison to control F1 offspring.

In the LD box test, there was significant effect of environmental challenges in the percent time in the light compartment ($F_{2,48}$=10.10; $p < 0.001$; Figure 28A). F1 offspring confronted with UCMS had higher latency to first entry in the light compartment ($p < 0.01$; Figure 28B), lower entries in light zone ($p < 0.05$; Figure 28C) and less rearing ($p < 0.05$; Figure 28D) in comparison to control.

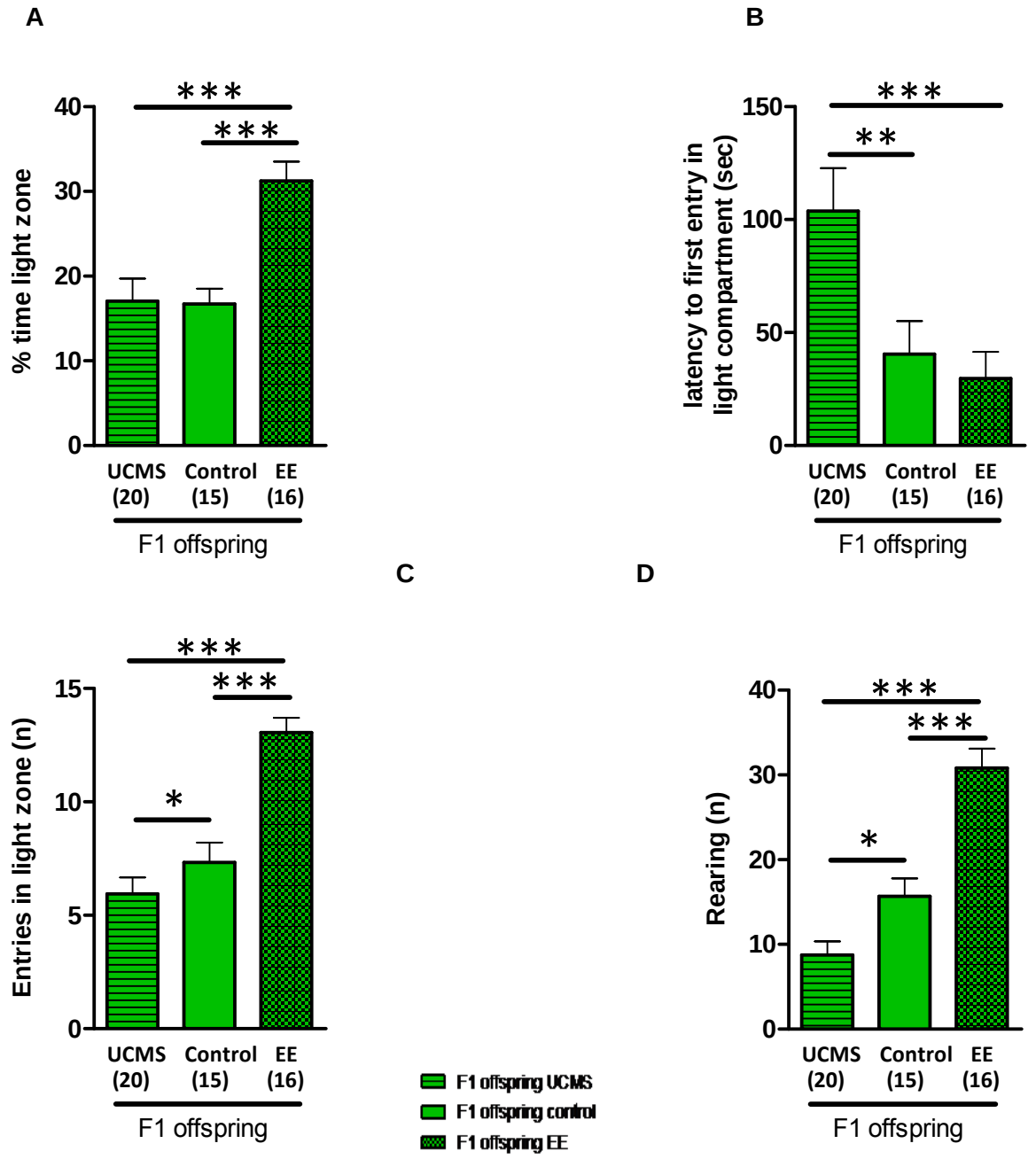


Figure 28: Effect of UCMS, EE on F1 offspring in **(A)** percent time light zone **(B)** latency to first entry in light compartment **(C)** entries in light zone and **(D)** rearing in LD box test. Data represented as mean+SEM. Numbers in parentheses indicate group size. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

On the contrary, F1 offspring subjugated to EE spend more time in light zone ($p < 0.001$; Figure 28A), greater entries in light zone ($p < 0.001$; Figure 28C) and more rearing ($p < 0.001$; Figure 28D) in comparison to control.

To determine depression-like or stress coping behavior in different F1 groups TST and FST parameters were measured. Both tests show significant difference in passive coping mechanism

in terms of time spent immobile ($F_{2,48}$=4.93; $p < 0.05$; Figure 29A) and $F_{2,48}$=3.37, $p < 0.05$, Figure 29C), respectively between EE and UCMS groups. Especially, the UCMS group had significantly higher time spent immobile ($p < 0.01$; Figure 29A) and ($p < 0.05$; Figure 29C) in comparison to control. Besides, only UCMS group differed in latency to first immobility ($p < 0.05$; Figure 29B and Figure 29D), respectively in comparison to controls.

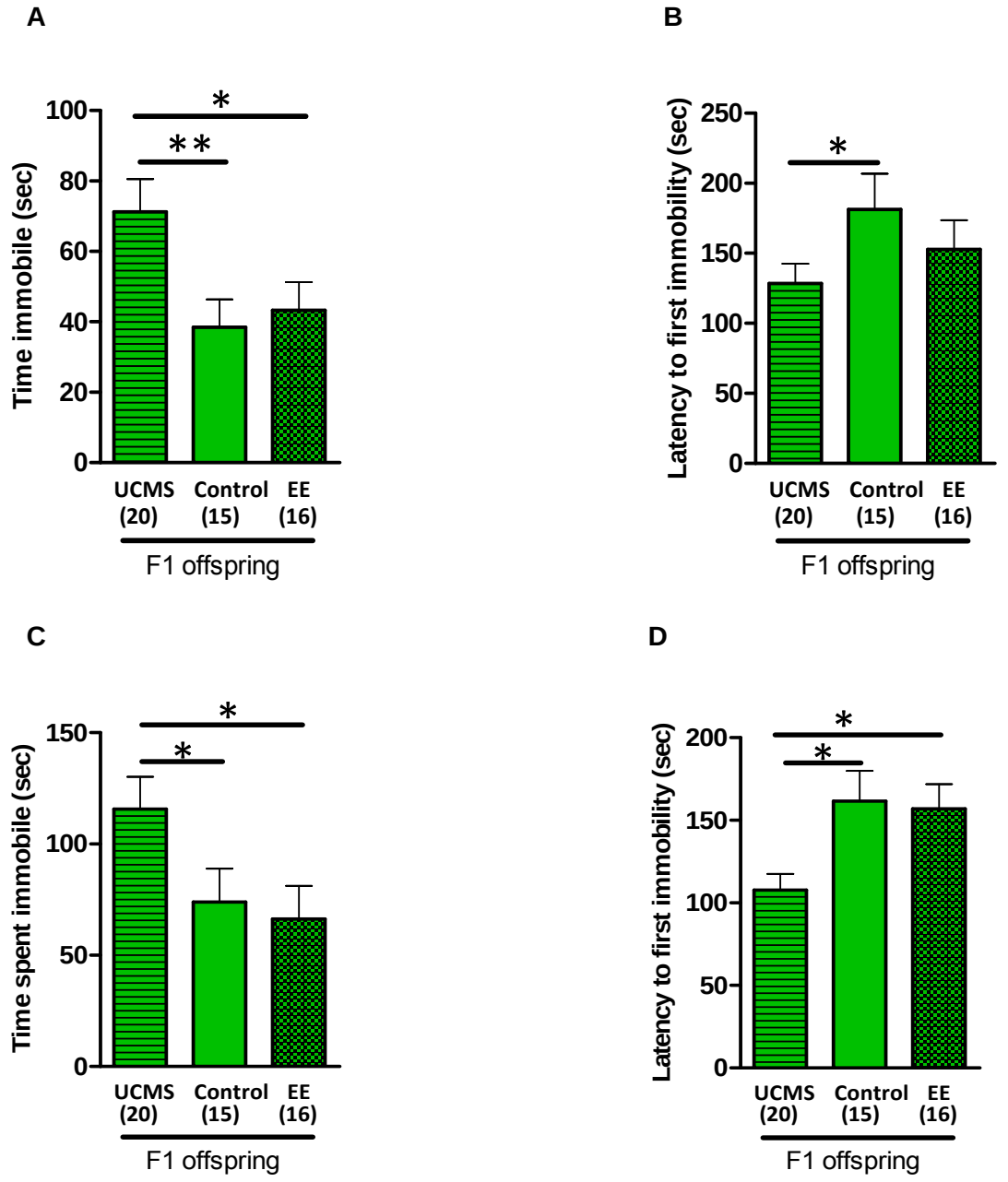


Figure 29: Effect of UCMS, EE on F1 offspring in (**A** & **C**) time immobile (**B** & **D**) latency to first immobility in TST and FST, respectively. Data represented as mean+SEM. Numbers in parentheses indicate group size. (* $p < 0.05$; ** $p < 0.01$).

Similarly, there was a significant difference in blood CORT measurement between the three groups ($F_{2,27}$=3.86; $p < 0.05$; Figure 30A). This was coupled with significant changes in body weight both between UCMS and EE, UCMS and control (both $p < 0.001$, Figure 30B).

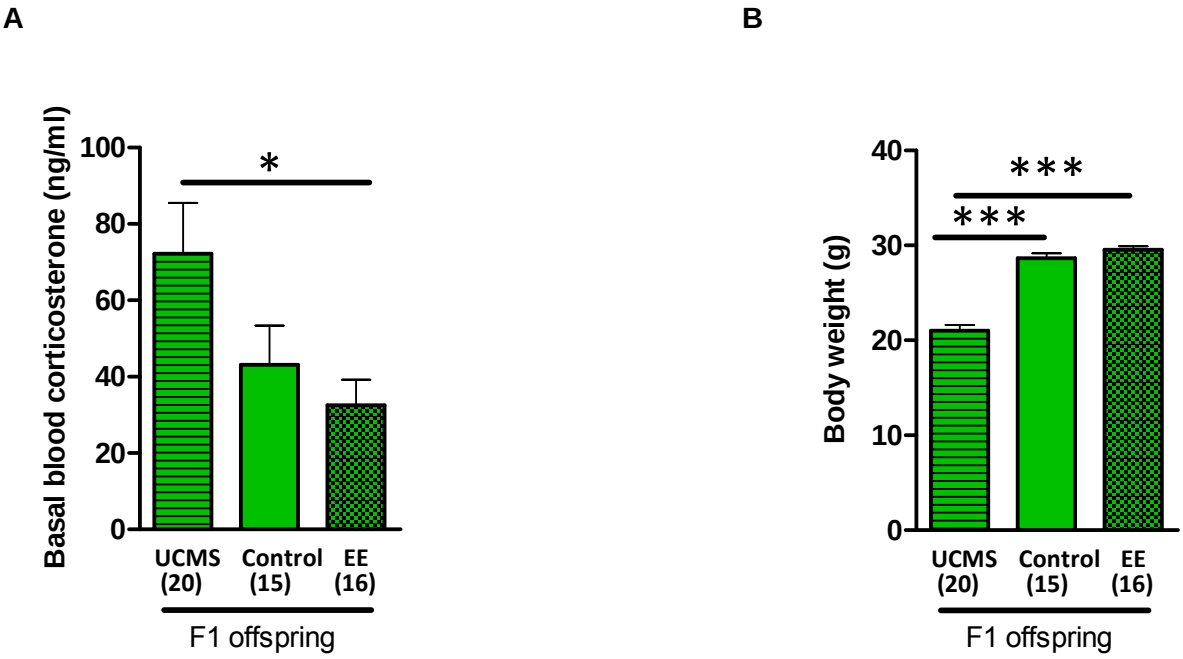


Figure 30: Effect of UCMS, EE on **(A)** blood CORT and **(B)** body weight. Data represented as mean+SEM. Numbers in parentheses indicate group size. (* $p < 0.05$; *** $p < 0.001$).

Comparatively, F1 offspring derived from LAB mother and HAB father were also subjected to EE and UCMS and their behavioral parameters measured on EPM, LD box, TST and FST.

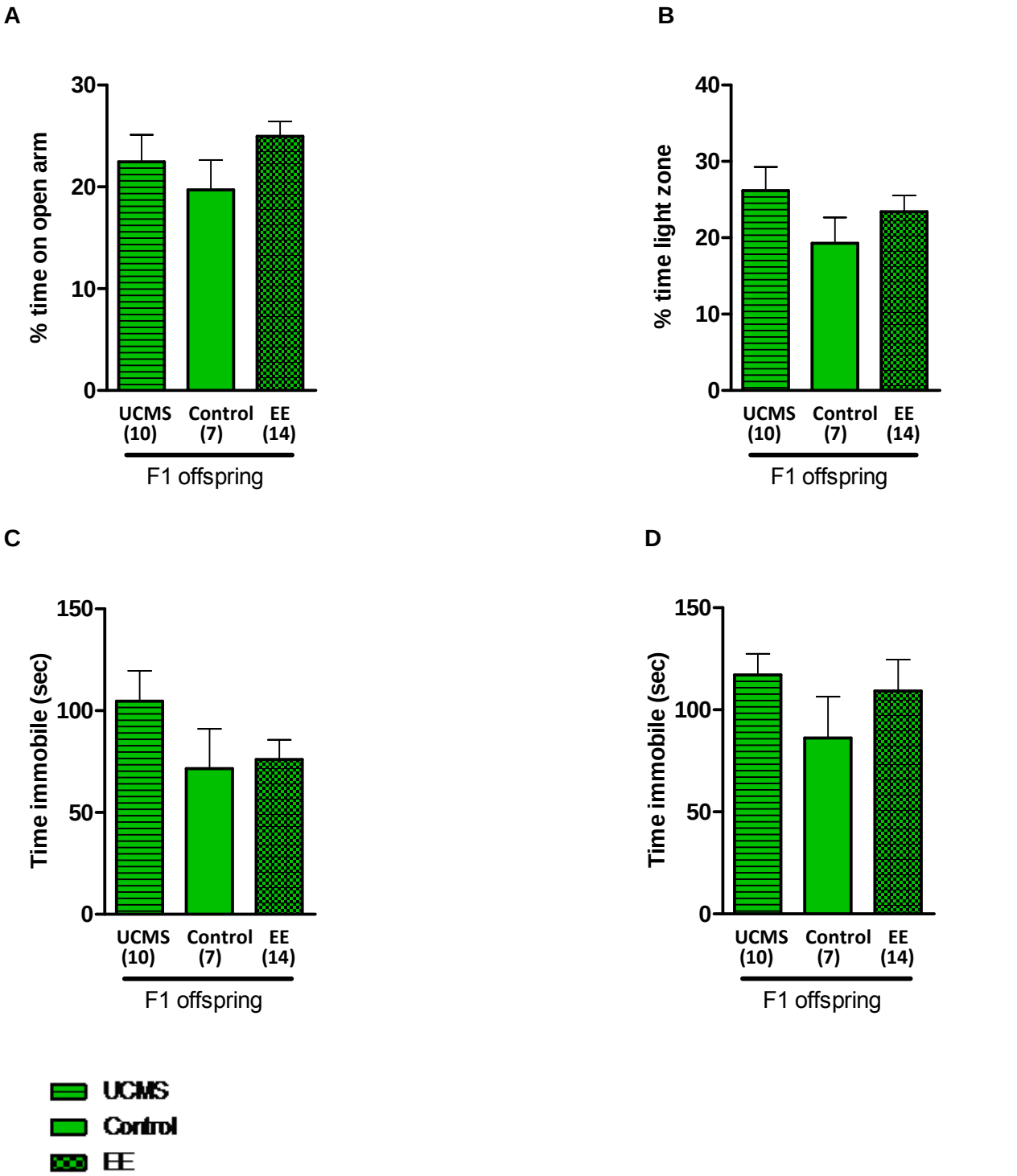


Figure 31: F1 offspring of LAB mother subjected to EE, UCMS on **(A)** percent time open arm of EPM **(B)** percent time light zone in LD box **(C)** time immobile in TST and **(D)** time immobile in FST. Data represented as mean+SEM. Numbers in parentheses indicate group size.

F1 offspring of LAB mother exposed to EE and UCMS had no difference in percent time in open arm ($F_{2,29}$=1.202; p=0.315; Figure 31A), percent time in light zone ($F_{2,27}$=1.197; p=0.318; Figure

31B), and time immobile in TST($F_{2,28}$=0.334; p=0.719; Figure 31C) and time immobile in FST ($F_{2,28}$=0.833; p=0.445; Figure 31D) between the three groups.

Overall, cross-mated F1 offspring, in particular with HAB mother genotype displayed a robust behavioral and neuroendocrinological phenotype with the stressed group showing higher anxiety and more passive stress coping behavior, which was accompanied by higher blood CORT levels and lower body weight. On the other hand, the EE F1 offspring displayed lower anxiety, more active stress coping behavior and corresponding lower blood CORT and higher body weight. However, F1 offspring with LAB mother genotype were resilient to both UCMS or EE.

3.2.7 Measurement of *Tmem132d* AEI in F1 offspring subjected to EE or UCMS

After having observed differential *Tmem132d* mRNA expression in the Cg of mHAB and. mLAB subjected to EE and UCMS, respectively its corresponding allelic expression was measured in F1 offspring subjugated to similar environmental challenges. F1 offspring allows us to have the two alleles in the same pool of *trans*-acting factors, thus any difference in allelic expression can be attributed to *cis*-variations.

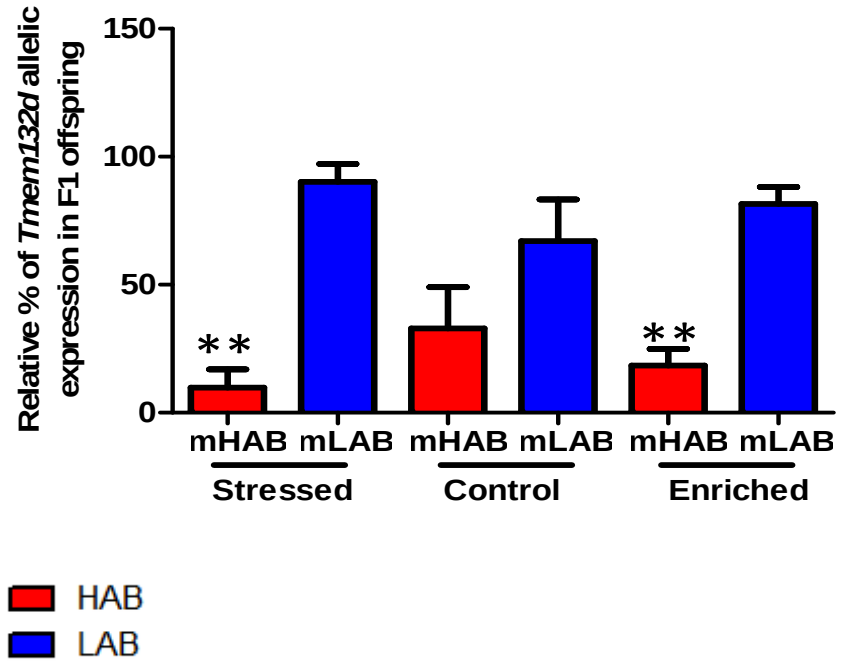


Figure 32: Relative percentage of *Tmem132d* allelic expression in F1 offspring subjected to EE or UCMS. Six animals per group were utilized for measurement of AEI. Data represents mean+SEM. (** p < 0.01 in comparison to corresponding LAB alleles).

There was no statistical difference between mHAB *vs.* mLAB *Tmem132d* mRNA alleles in the control F1 offspring (p=0.515). Nonetheless, both the F1 offspring subjected to EE and UCMS

show a significant higher mLAB specific allele expression compared to mHABs (Figure 32; $p < 0.01$). This is in contrary to the observed higher *Tmem132d* mRNA expression in the Cg of mHAB. This may either suggest a non-specific effect of environmental manipulations or a crosstalk of genetic and environmentally mediated epigenetic factors leading to an overall higher LAB specific allele expression. Thus, qMSP was utilized to measure mHAB, mLAB DNA methylation changes in the *Tmem132d* promoter of these F1 offspring.

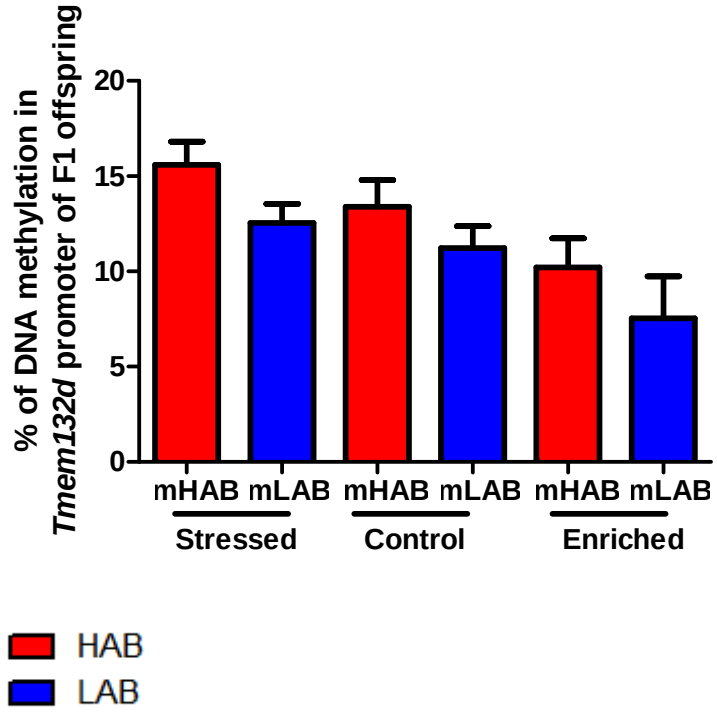


Figure 33: Percentage of DNA methylation in the *Tmem132d* promoter region in F1 offspring subjected to EE, UCMS and control. There were 6 animals used for measurement of AEI. Data represents mean+SEM.

The total percentage of DNA methylation of each mLAB allele is lower than corresponding mHAB allelic expression, albeit not statistically significant ($F_{5,29}$=3.644; p=0.011; Figure 33). Rho was calculated for percentage of *Tmem132d* mRNA and corresponding methylated promoter alleles. Comparison of mHAB *Tmem132d* (mRNA *vs.* methylated promoter alleles) gave (Rho = -0.690; ** $p < 0.01$). Thus, there is a negative correlation between mHAB *Tmem132d* mRNA allelic expression and corresponding promoter methylation. However, comparison of corresponding mLAB *Tmem132d* (mRNA *vs.* methylated promoter alleles) gave Rho=0.584; p=0.014).

Please note that the following data were obtained in collaboration with other people:

- Figure 7C and 8A - Dr. Gregers Wegener, Aarhus University, Denmark.
- Figure 5C, 5D, 6C, 6D, 7B, 8B, 8C, 9B(Table 12), 11(Table 14)- Dr. Yi-Chun Yen, MPI psychiatry. These data were also part of her Ph.D. thesis.
- Figure 5A, 5B, 6A, 6B, 19A, 19B - Dr. David Slattery, University of Regensburg.
- Figure 26A, 26C, 27-31- Sergey Sotnikov and Natalia Chekmareva, MPI psychiatry.

4.0 Discussion

Earlier studies with rHAB *vs.* rLAB have discovered *Avp* (Murgatroyd *et al.*, 2004) and with mHAB *vs.* mLAB also *Avp* (Bunck *et al.*, 2009), glyoxalase-I (Kromer *et al.*, 2005), enolase phosphatase (Ditzen *et al.*, 2010) and cathepsin B (Czibere *et al.*, 2011) as candidate genes for anxiety-related behavior. In the current study, these selectively bred rats and mice called HAB *vs.* LAB showing stark differences in anxiety-related and comorbid depression-like behavior were utilized to unearth their molecular differences in the Nps/Npsr1 and Tmem132d system. These studies revealed robust differences in both systems, which may contribute to their behavioral phenotypes and mark these genes as candidates for novel treatment options or aetiological factors in depression and anxiety disorders.

4.1 Nps/Npsr1 system

The anxiolytic and fear attenuating role of NPS in rodents (Jungling *et al.*, 2008; Xu *et al.*, 2004) prompted us to test their effects in HAB rodents. Central administration of NPS was found to cause decrease in anxiety as indicated by higher percent time on the open arm of EPM (Figure 5A and 5C). Acting in the opposite direction, central administration of NPSR1-A in LABs caused a significant decrease in percent time on open arm of EPM, thus leading to higher anxiety (Figure 6A and 6C). Importantly in both cases, NPS and NPSR1-A application, locomotion was generally not affected, except for rHAB (Figure 5B, 5D, 6B, 6D), which is often a confounding factor in distinguishing trait anxiety (Escorihuela *et al.*, 1999; Fraser *et al.*, 2010; Rodgers *et al.*, 1997). However, dose-response separated anxiety and locomotor effects of NPS in rHAB. The HAB *vs.* LAB lines were found to have several differences in the NPS system. The NPS mRNA, which is moderately expressed in lateral parabrachial nucleus, principal sensory 5 nucleus and strongest expression in the LC area (Xu *et al.*, 2004) was three fold higher in rHAB *vs.* rLAB (Figure 7A). However, there was no difference in the corresponding DNA, which was identical to the reference rat stain (BN/NHsdMcwi). Thus, the differential expression cannot currently be explained. Comparative studies revealed no difference in mHAB *vs.* mLAB *Nps* mRNA expression (Figure 7B). Nevertheless, there were several polymorphisms like SNPs and a insertion found in the promoter and coding region of the cognate DNA sequence of mLAB (Table 12). The mHAB *Nps* sequence was identical to the reference mouse strain (C57BL/6J). The SNPs in the mLAB promoter do not seem to affect *Nps* mRNA expression, at least in the LC area. The four SNPs in the coding region included three missense mutations (leucine(5)isoleucine, valine(10)isoleucine, arginine(54)glycine) and a synonymous mutation coding for threonine at position 65, were all prior to the mature 20 amino acid peptide. The

leucine(5)isoleucine and valine(10)isoleucine substitutions occur in the presumed hydrophobic signal peptide sequence of the mouse NPS precursor (Reinscheid & Xu, 2005). A mutation in the signal peptide for example, in the dentin sialophosphoprotein (*Dspp*) gene has been shown to affect protein translocation to the endoplasmic reticulum and subsequent defective dentine biomineralization (Rajpar *et al.*, 2002). However, the functional implications of the SNPs in the mouse NPS precursor are currently unknown. There were also no CNV or CpG islands, in the rat and mice *Nps* gene that could probably explain its differential expression or copy numbers. A recent study utilized transgenic enhanced green fluorescent protein (EGFP) expressing mouse line under the control of *Nps* promoter and found that stress, and in particular CRH causes release of the NPS (Jungling *et al.*, 2012). A previous study has also found higher CRH mRNA expression in the LC area of rHAB compared to rLAB (Plotsky *et al.*, 2000). Thus, the high NPS mRNA expression observed in the LC area of rHAB in contrast to rLAB could be attributed to higher CRH expression in the same region. However, whether this translates to protein level and/or NPS release remains unknown currently. The LC area housed in the brain stem has projections going into amygdala and PVN, and this has been linked with autonomic sympathetic response to stress (Damasio, 1998; Sands & Morilak, 1999). Activation of LC area can lead to activation of amygdala and higher anxiety, and conversely, anxious stimuli that enhance amygdalar activity can also increase LC activity (Samuels & Szabadi, 2008).

As NPS acts by binding to its cognate transmembrane protein called NPSR1, its subsequent mRNA expression was measured in the limbic brain regions, particularly in the hypothalamic PVN and the amygdalar region. These two brain regions were earlier implicated in local NPS-mediated anxiety, fear response and activation of HPA axis (Jungling *et al.*, 2008; Meis *et al.*, 2008; Smith *et al.*, 2006). In rHAB there was lower expression of *Npsr1* mRNA only in the PVN of both, males and females (Figure 7C and 7D) but not in amygdala (Figure 8A) in comparison to rLAB. On the other hand, there was lower *Npsr1* mRNA expression only in the amygdala of mHAB (Figure 8B), while not in the PVN (Figure 8C) in contrast to mLAB.

To dissect the genetic underpinnings of this differential expression *Npsr1* gene was sequenced. The *Npsr1* gene is located on chromosome 8 and 9 of rat and mouse, respectively and contains 10 exons. Sequencing of approximately 2000 bp promoter and downstream *Npsr1* DNA revealed several polymorphisms such as SNPs, insertions, deletions in the promoter, exon, intron and DER (Table 13 and 14) with the LAB sequence similar to the reference strain.

The differential expression of any gene can be due to interaction between *cis*- and *trans*-acting factors in the cellular milieu of HAB or LAB animals. Thus, simultaneously HAB *vs.* LAB were cross-mated to obtain F1 offspring which allows us to study the expression of each allele in the same cellular environment, where each allele acts as an internal standard for the other. Both

crosses *i.e.*, HAB mother with LAB father and *vice versa* were utilized to rule out any imprinting based mechanism underlying differential expression of the *Npsr1* gene. Interestingly, in the F1 offspring of both, rats and mice, there was a higher HAB-specific allele expression (Figure 12A and 12B) in comparison to the LAB-specific one.

The *Npsr1* promoter and deletion fragments of rat and homologous mouse were cloned into luciferase vector to measure their promoter activity. The dual luciferase assays recapitulated the *in vivo* findings as whole HAB rat and mouse constructs had lower promoter activity than their respective LAB construct (Figure 13 and 14). *In silico* TESS analyses suggest that the introduction of a GR binding site A(-1813)C in rHAB at a putative TATA binding protein (TBP) site may be responsible for the lower activity, as TBP is an essential componentto make pre-initiation complex which is required for downstream gene transcription (Meyer *et al.*, 1997). In mHAB, the introduction of a hepatocyte NF 3-β may also lead to repression of transcription, as described previously for glucagon (Philippe *et al.*, 1994). However, both these hypotheses are yet to be tested. In contrast, as the putative promoter length was decreased, there was a 2-fold higher promoter activity in rHAB and mHAB (Figure 13 and 14), analogous to the higher HAB-specific allelic expression in F1 offspring. This indicates the importance of the whole promoter and a cross-talk between *cis*-acting polymorphisms and *trans*-acting factors. Intriguingly, TESS analyses revealed the introduction of plausible GR binding sites in the HAB sequence in place of a NF-1 or AP-1 site in the corresponding LAB promoters. The *cis-trans* interaction was confirmed by *in vitro* stimulation of promoter constructs with DEX, which only decreased HAB promoter activity (Figure 15A and 15B). A previous study by (Mori *et al.*, 1997) demonstrated that GR causes suppression of downstream interleukin-5 gene expression by interfering with activities of AP-1 or NF-kB. Moreover, it has also been shown that the presence of AP-1 and NF-1 can enhance chromatin accessibility and resultant GR binding, which in turn helps to recruit other co-activators in absence of glucocorticoids (Belikov *et al.*, 2004; Biddie *et al.*, 2011). Thus, in HAB rodents, it is possible that the adjacent NF-1 or AP-1 site enhances GR binding and that this would, in turn, probably recruit other co-activators causing an enhanced HAB-specific expression of *Npsr1*. In contrast, DEX stimulation leads to GR activation, which may interfere with activity of basal transcription factors like AP-1, NF-kB leading to trans-repression (Newton & Holden, 2007).

The similar *in vitro* and *in vivo Npsr1* data in rat and mouse prompted me to do comparative genome analysis along with human *Npsr1* DNA. Indeed, there was high degree of sequence conservation in the 5' UTR, promoter, CNCS and downstream exonic region (Figure 17). This is an interesting finding, because of a previous study also suggesting binding of AP-1 transcription factor in the 500 bp upstream region of human *Npsr1* gene (Anedda *et al.*, 2011). Besides *Npsr1*

polymorphisms, other factors such as possible CNV and CpG islands were estimated but they were not differing between HAB *vs.* LAB lines. Thus, CNV or CpG islands as additional causal elements in the differential expression of the gene could be ruled out. The role of SNPs in the downstream coding region of *Npsr1* was further pursued due to their putative functional role on the final protein product. There was a deletion of exon 4 observed in the rLAB *Npsr1* cDNA sequence. The deletion of a single exon in the rat *Npsr1* can give rise to different corresponding mRNA isoforms as described for the human *Npsr1* gene (Laitinen *et al.*, 2004). There are two human *Npsr1* isoforms described, which differ in their intracellular carboxyl terminus and show distinct expression patterns and differ in their downstream signaling properties (Pietras *et al.*, 2011). As an illustration, deletion of a particular exon in human epidermal growth factor receptor (EGFR) gene has been reported to improve treatment outcome in lung cancer patients (Jackman *et al.*, 2006). Likewise, a selective deletion of exon in mouse ferrochelatase gene causes mild protoporphyria (Magness *et al.*, 2002). Similarly, the deletion of exon 4 in rHAB *vs.* rLAB may give rise to a different *Npsr1* isoform, which exhibits differential expression only in the hypothalamic PVN. Meanwhile, the corresponding mHAB *vs.* mLAB without any deletion of exonic region in *Npsr1* may exhibit distinct properties only in the amygdala.

Furthermore, there was a synonymous SNP [A(227016)G and A(156453)G; rs37572071] in exon 8 and 4 of rHAB and mHAB, respectively at the third base wobble position. As per Francis Crick's 'Wobble hyothesis' only the first two bases of codon have precise pairing with corresponding anticodon bases in the mRNA. While the pairing between the 3rd base of codon and anticodon may wobble *i.e.* nonspecific binding. This may lead to different translation efficiency, if HAB (G) or LAB (A) alleles are recognized at different frequency or they might influence stability of codon-anticodon bonds (Angov, 2011). Specifically the guanine (G) nucleotides are more frequently observed than adenine (A) nucleotides in the third position of synonymous substitutions (Hunt *et al.*, 2009) and that this may impact both, the incorporation rate of amino acids into newly synthesized proteins as well as its subsequent translocation. Although synonymous mutations do not lead to altered amino acid sequences, they are not always silent and have been shown to affect mRNA splicing, stability, protein structure, synthesis and folding (Cartegni *et al.*, 2002; Cartegni & Krainer, 2002). For instance, a synonymous mutation due to codon usage bias in the human dopamine receptor D2 (DRD2) altered mRNA folding, leading to decrease in mRNA stability and translation (Duan *et al.*, 2003). This, in turn, affected the dopamine induced upregulation of the DRD2 expression in *in vitro* studies (Duan *et al.*, 2003). Another synonymous SNP in the same gene annulled the effects of above SNP, which was consequently found in linkage disequilibrium (LD) with other polymorphisms, previously associated with schizophrenia and alcoholism (Lafuente *et al.*, 2008;

Rodriguez-Jimenez *et al.*, 2006). Similarly, other studies have shown how a synonymous SNP changes substrate specificity in the multidrug resistance 1 (MDR1) gene (Kimchi-Sarfaty *et al.*, 2007), and another study showed a synonymous codon in the Lamin A/C (LMNA) gene, which altered mRNA splicing leading to limb girdle muscular dystrophy type 1B (Todorova *et al.*, 2003). This encouraged testing of both rat and mice HAB *vs.* LAB NPSR1 protein carrying the synonymous SNP, and in an *in vitro* system, the HAB NPSR1 protein carrying the G residue was found to produce higher luciferase expression *i.e.*, cAMP response on stimulation with NPS (Figure 18A and 18B). This may be due to higher surface receptor expression in HABs but there was no difference in total receptor expression in HAB *vs.* LAB; at least *in vivo* (Figure 19A and 19B). These studies are analogous to the human NPSR1 Ile^{107} isoform which causes higher surface receptor expression and NPS efficacy without any difference in the total receptor expression (Bernier *et al.*, 2006; Reinscheid *et al.*, 2005). Thus, the *in vitro* promoter and protein assay indicates that the lower HAB *Npsr1* mRNA expression due to SNPs in the promoter region is overridden by a functional SNP in the coding region. These results along with those of others, unlike in the past, would motivate the researchers to include synonymous SNPs in genome-wide association studies (Duan *et al.*, 2003).

Moreover, there were also few polymorphisms in the 3' UTR or DER region of the *Npsr1* gene which could be of functional significance. For example, a SNP in the 3' UTR region of thrombin-activable fibrinolysis inhibitor (TAFI) has been shown to affect mRNA stability and consequently its concentration in the human plasma (Boffa *et al.*, 2008). Similarly, microRNAs are known to bind at 3' UTR position and regulate mRNA degradation and translation control (Valencia-Sanchez *et al.*, 2006). Likewise, Encyclopedia Of DNA Elements (ENCODE) project has suggested enrichment of functional disease causing polymorphisms in the non-coding parts of genes ("An integrated encyclopedia of DNA elements in the human genome," 2012). Thus the polymorphisms in the 3' UTR or DER region of *Npsr1* might as well affect mRNA degradation or protein translation leading finally to the observed similar protein expression between HAB *vs.* LAB lines.

The anxiolytic effect of NPS is intriguing because adult mHAB have been shown to be resistant to benzodiazepine (Sartori *et al.*, 2011). On the other hand, *icv* administration of NPSR1-A increased anxiety in rLAB and mLAB, at a dose that effectively antagonized the anxiolytic effects of NPS in rNAB (Slattery *et al.*, (submitted)).

This provides evidence for an involvement of the endogenous NPS system in hypoanxiety particularly as no *icv* effects of an NPSR1-A alone have been reported to date and that NPSR1 knockout mice do not display an overtly anxious phenotype (Fendt *et al.*, 2011; Pulga *et al.*, 2012; Ruzza *et al.*, 2012).

The fact that NPS administration can rescue the phenotypes of HAB rodents, coupled with the above molecular findings, strongly suggests that a lack of endogenous NPSR1 activity, probably *via* a lack of NPS release, may underlie the high anxiety- phenotype. A similar situation may exist in higher risk Ile^{107} NPSR1 carriers, therefore, future studies should assess both basal and stress-induced NPS release in HAB rodents and human Ile^{107} NPSR1 allele carriers (centrally in rodents and peripherally in humans to give an indication of central release). Interestingly, a study from our institute showed the effects of intranasal NPS application after 4 h in mHAB, and decrease in anxiety was accompanied by explicit internalization of the NPS-NPSR1 complex (Ionescu *et al.*, 2012). In search of novel drug targets owing to drug resistance, NPS might work as an easy, cost effective target to treat patients carrying the Ile^{107} risk form. Finally, these results support the notion that HAB rodents are a useful tool to resolve discrepancies between preclinical studies showing pronounced anxiolytic effects mediated by NPS and clinical association studies, emphasizing the association of the human NPSR1 Ile^{107} polymorphism with anxiety or panic disorder (Domschke *et al.*, 2011; Okamura *et al.*, 2007).

These findings suggest that differences in the brain NPS system underlie, at least partly, the HAB *vs*. LAB behavioral phenotypes, which seems all the more probable given that genetic and expression differences have evolved in both rat and mouse lines underlining their evolutionary impact. After having identified and characterized an interesting candidate gene for anxiety-related behavior, the plasticity nature of the gene was studied in animals facing different environmental challenges. mHAB and mLAB subjected to EE or UCMS did not differ in *Npsr1* mRNA in relative to their corresponding controls (Figure 20A, 20B). Thus, *Npsr1* is a non-plastic gene in line with the differential susceptibility hypothesis proposed by (Belsky *et al.*, 2009).

4.2 TMEM132D system

The second candidate gene in the focus of this thesis was *Tmem132d*, as a recent study emphasized its importance in the frontal cortex of panic disorder and unipolar depressed patients. The higher *Tmem132d* mRNA expression in the frontal cortex of panic disorder patients was associated with risk alleles in the corresponding gene (Erhardt *et al.*, 2011). Genome-wide association studies revealed a haplotype containing two intronic SNPs namely, rs7309727 and rs11060369 associated with panic disorder across three independent German samples (Erhardt *et al.*, 2011). Furthermore, three independent SNPs, also in the intronic region, chiefly, rs900256, rs879560 and rs10847832 were associated with severity of anticipatory anxiety (Erhardt *et al.*, 2011). The three SNPs are in high LD and their structure suggests that functional variants earmarked by these associations are unlikely to lie in exonic or upstream regulatory

regions of the gene (Erhardt *et al.*, 2011). Regulatory regions in introns have been found for instance, to affect transcription of human dopamine transporter gene (Greenwood & Kelsoe, 2003), and an intronic SNP has been linked with overexpression of thyroid hormone receptor β2 in thyroid hormone resistance syndrome (Alberobello *et al.*, 2011). The rs7309727 and rs11060369 intronic SNPs were further replicated in additional panic disorder patients of European ancestory (EA), and a meta-analysis finally confirmed that *Tmem132d* gene contributes to genetic susceptibility to panic disorder patients of EA (Erhardt *et al.*, 2012).

TMEM132D also called mature oligodendrocyte transmembrane (MOLT) or KIAA1944 encodes a single-pass type 1 integral membrane protein. The human *Tmem132d* contains 9 exons and encodes a protein of 130 kDa in size. Previous studies by (Nagase *et al.*, 2001) reported substantial expression of *Tmem132d* mRNA in the caudate nucleus and lower expression in the amygdala, corpus callosum, hippocampus, substantia nigra, subthalamic nucleus, and thalamus of fetal and adult brain. This diverse expression suggests that *Tmem132d* probably plays a crucial role in the brain.

The corresponding protein is predicted to contain an N-terminal hydrophobic signal peptide, seven N-glycosylation sites, two O-glycosylation sites, a number of phosphorylation sites, and a C-terminal transmembrane domain (Nomoto *et al.*, 2003). The rat and mouse *Tmem132d* gene is located on chromosome 12 and 5 and its protein product shares 83.4% and 83.2% amino acid identities, respectively, with the corresponding human protein (Nomoto *et al.*, 2003). The high degree of protein homology among rat, mouse and human indicates that TMEM132D function is likely evolutionary conserved. Rat oligodendrocytic precursor cells show no expression of either *Tmem132d* mRNA or protein, however on differentiation they start to express the corresponding protein (Nomoto *et al.*, 2003). Thus, TMEM132D has been proposed as a cell surface marker for mature oligodendrocytes. However, another study also found *Tmem132d* expression in neurons and their colocalization with actin filaments suggests their role in cell-cell adhesion (Walser *et al.*, 2011). Interestingly, TMEM132D shares moderate homology with neural cell adhesion molecule (NCAM) (NCBI database). Thus the involvement of *Tmem132d* in oligodendrocyte maturation and/or cell adhesion may be important for efficient connection of Cg with other brain regions implicated in anxiety-related behavior.

Characterization of the human *Tmem132d* promoter in an oligodendrocytic cell line has identified several inhibitory transcription factors such as MYT1, SP1, HES1, ZNF219 whose expression is reduced in differentiating oligodendrocytic cells (Herrmann, 2012). Except ZNF 219, the other

transcription factors have been earlier shown to be involved in oligodendrocytic differentiation (Armstrong *et al.*, 1995; Guo *et al.*, 2010; Wu *et al.*, 2003).

In mHAB *vs.* mLAB, *Tmem132d* mRNA expression was studied in several brain regions like PVN, basolateral amygdala, central amygdala, dentate gyrus, but difference was found only in the Cg (Czibere, 2008). In more detail, there was higher expression of *Tmem132d* mRNA in mHABs compared to mLAB with intermediate levels in the CD-1 mice. This expression difference was consistent throughout in microarray, qPCR (Czibere, 2008) and also in *in situ* hybridization experiments (Steiner, 2009) irrespective of gender. The Cg has intricate connections with amygdala, where heightened responses to anticipatory signals are associated with the Cg and treatment response in anxiety disorders (Nitschke *et al.*, 2009). At the synaptic level, fear conditioning has been found to exhibit synaptic plasticity changes in the amygdala and Cg (Toyoda *et al.*, 2011). Several fMRI studies have also suggested connections between Cg and amygdala during emotional processing events in anxiety disorder patients (Kim & Whalen, 2009; Stein, M. B. *et al.*, 2007). As anxiety-related brain circuits are conserved across species (Cryan & Sweeney, 2011), the altered expression profile of *Tmem132d* in the Cg may contribute to differential modulation of anxious stimuli in this brain region and subsequent predisposition to higher anxiety in HABs. This is the first oligodendrocytic gene, to the best of my knowledge, to be involved in anxiety-related phenotype.

Apart from expression differences, two SNPs A(-519)G and A(-310)G from the transcription start site were described in the *Tmem132d* promoter up to 1000 bp (Czibere, 2008). To understand the genetic foundation of this differential expression, mHAB or mLAB promoters containing two G or A residues at positions -519 and -310 each, respectively, were tested in a dual luciferase assay. The mHAB fragment containing both G residues had significantly higher promoter activity than corresponding mLAB (Figure 22). When either -519 or -310 G residue was mutated to A, there was complete loss of promoter activity suggesting the importance of these two loci. However, deletion of the -519 A residue in mLAB did not have any effect on its promoter activity. *In silico* analysis of *Tmem132d* promoter predicted binding of nuclear factor I/C at -519 position. This transcription factor has been shown to be involved in eukaryotic transcription (Santoro *et al.*, 1988), co-localization with RNA polymerase II (Zhao *et al.*, 2005) and participation in vertebrate brain development (Singh *et al.*, 2011). A previous study showed that binding of nuclear factor I/C is blocked by CpG methylation causing an increase in promoter activity of Igf2 (Jaenisch & Bird, 2003). Whereas the -310 SNP was also found to bind TFIIB, which is a general transcription factor involved in formation of RNA polymerase II preinitiation complex (Kostrewa *et al.*, 2009; Sainsbury *et al.*, 2013)**.** Thus, both the G residue in mHAB seem to interact with RNA

polymerase II and interestingly, a recent study found that presence of RNA polymerase II, stalled or active, predicts the epigenetic fate of promoter CpG islands (Takeshima *et al.*, 2009).

Other genetic factors were studied that might act either synergistically or alone in *Tmem132d* gene regulation. Among them CNV of *Tmem132d* was measured, which was not different between mHAB *vs.* mLAB (Steiner, 2009). In addition, there was a 600 bp CpG island in the promoter region encompassing the above described two SNPs. These CpG islands are usually prone to DNA methylation based epigenetic mechanism, but bisulfite sequencing of *Tmem13d* promoter did not reveal any difference in total percentage of DNA methylation between basal mHAB *vs.* mLAB lines (Figure 25). Thus, this suggests that SNPs in the *Tmem132d* promoter are sufficient to cause differential gene regulation in accordance with the observed *in vivo* expression data.

Moreover, in the coding region two SNPs in the 5' UTR (C(470)T, C(593)T), one SNP each in exon 3 (A/G, rs36596918) and exon 9 (A/G, rs13478518) of *Tmem132d* have been described (Steiner, 2009). The rs36596918 causes substitution from arginine to lysine (Czibere, 2008). However, the functional significance of these SNPs is currently unknown. The rs13478518 of *Tmem132d* was found to co-segregate with anxiety-related behavior in an F2 panel, independent of depression-like behavior and locomotor activity (Czibere, 2008), advocating causal role of the *Tmem132d* gene in anxiety-related behavior. The F2 panel is obtained by cross mating F1 offspring among each other and the subsequent alleles and traits should segregate freely to resolve genetic contribution of the involved loci. However, the small number of meiosis and resultant recombination events from the parental mHAB *vs.* mLAB to the F2 generation may lead to false positive association of alleles with anxiety-related behavior (Steiner, 2009). Subsequently, in a group of 80 outbred CD-1 mice, this SNP was also associated with anxiety-related behavior (Czibere, 2008). As the alleles in outbred CD-1 mice got separated by recombination over several generations, a positive association in this group of mice validates the F2 panel studies (Steiner, 2009).

Subsequently, in search for a plasticity gene that is responsive to environmental challenges, *Tmem132d* mRNA expression was measured in mHAB and mLAB subjected to EE or UCMS, respectively. Interestingly, *Tmem132d mRNA* was found to be differentially regulated with EE mHAB showing higher expression while, in contrast, UCMS mLAB had lower expression compared to their respective controls (Figure 26B and 26D). Thus, we have here a good example of plasticity gene in accordance with the Belsky's differential susceptibility hypothesis (Belsky et al 2009). At the behavioral level, there were corresponding increases or decreases of percent time spent in light compartment in mHAB or mLAB, respectively (Figure 26A and 26C) *i.e.*, decreased and increased anxiety, respectively. However, the expression data was in

contrary, with EE mHAB expected to have lower *Tmem132d* expression and *vice versa* for stressed LABs, as higher *Tmem132d* expression previously associated with higher anxiety-related behavior.

Earlier studies in our group have shown that homozygous mHAB and mLAB subjected to EE and UCMS, respectively causes a bidirectional shift in their anxiety-related behavior towards normality (Markt *et al* (submitted)). Thus, in the next step, cross -mated F1 offspring were similarly subjugated to groups of EE, UCMS and control. The idea here was to simulate an outbred situation where the risk (mHAB) and protective (mLAB) alleles interact with other factors, to help the animal cope with different environmental challenges. As mouse behavior is multimodal, a battery of behavioral tests was utilized to measure anxiety and comorbid depression-like behavior (Crawley *et al.*, 1997). F1 offspring of HAB mother genotype experiencing EE or UCMS had significant percent time in the open arm, open arm entries and latency to enter open arm without affecting locomotion on the EPM. In particular, the EE group spent more time in open arm (Figure 27A) and had more open arm entries (Figure 27C) in contrast to control F1 offspring, indicating decreased anxiety. Likewise, the UCMS group had lower open arm entries (Figure 27C) and higher latency to entry in open arm (Figure 27D) in comparison to control F1 offspring; suggesting an anxiogenic effect. Besides in the LD box test, significant effects of environmental challenges were observed in percent time in light compartment.

In the LD box test, F1 offspring from EE group unlike their control batch spend more time (Figure 28A) and exhibited more entries (Figure 28C) into light zone, including more rearing (Figure 28D), which is an index of exploratory behavior in mice. The F1 offspring confronted with UCMS had higher latency to enter (Figure 28B) and fewer entries (Figure 28C) into light zone, in contrast to control F1. There was also less corresponding rearing (Figure 28D) compared to control F1 offspring. This again shows the contrasting effects of EE and UCMS on the same F1 offspring.

Regarding depression-like or stress-coping behavior, F1 offspring of EE and UCMS differed in their passive stress-coping as reflected by higher time spent immobile in both, TST and FST (Figure 29A and 29C). Consequently, blood CORT and body weight also differed significantly between the three groups (Figure 30A, 30B), In particular, UCMS batch exhibited higher CORT and lower body weight in contrast to their control F1 offspring. Decrease in body weight has been found earlier in UCMS exposed mice which was reversed on SSRI treatment and has been used as a marker for UCMS evoked conditions (Surget *et al.*, 2011). Increased CORT suggests hyperactivity of HPA axis which is an endophenotype and impairment of this has been found in anxiety disorders (Kallen *et al.*, 2008). Interestingly, CORT usually goes hand in hand with

depression in human patients (Holsboer & Barden, 1996; Seckl *et al.*, 1990). The higher depression-like behavior has also been shown in animals repeatedly administered with CORT (Kalynchuk *et al.*, 2004), and in an animal model selectively bred for differences in CORT response to a stressor, the mice

with higher CORT in blood plasma also displays higher depression-like behavior in FST (Touma *et al.*, 2008). However, the lower body weight observed in UCMS F1 group is in contrast to studies in depressed human patients where higher body weight has been reported (Berlin & Lavergne, 2003; Shioiri *et al.*, 1993). Nonetheless, studies have also shown that the body weight differences are due to different subtypes of depression in human patients (Saarni *et al.*, 2011). Conversely, F1 offspring of LAB mothers subjected to EE or UCMS were also analyzed on several behavioral tests. Here, these F1 offspring did not differ in any parameter such as EPM, LD box test, FST and TST (Figure 31). Thus F1 offspring of LAB mothers were mostly resilient to different environmental challenges suggesting that LAB mothers probably confer a protective effect. F1 offspring of LAB mothers when cross-fostered with HAB mother, it did not have any effect on its anxiety-related behavior (Sotnikov, personal communication). However, F1 offspring of HAB mothers when cross-fostered with LAB mothers there was a significant decrease in anxiety-related behavior (Sotnikov, personal communication). Thus this suggests that F1 offspring of LAB mother are somehow resilient owing to their genetic make up or they learn to cope with different environmental challenges.

As *Tmem132d* was found to be a plastic gene in mHAB *vs.* mLAB, its corresponding allelic expression was measured in these F1 offspring, too. F1 offspring from both mHAB and mLAB mother subjected to EE or UCMS had significantly higher mLAB allelic expression in contrast to mHAB (Figure 32). Meanwhile, there was no statistical difference between mHAB and mLAB alleles in the control F1 offspring in contrast to studies by (Czibere *et al.*, 2011), which showed higher expression of mHAB allele overriding the corresponding mLAB.

This was an unexpected finding owing to *in vitro* data showing that SNPs in mHAB cause higher *Tmem132d* mRNA expression, thus this suggests probably non-specific effect or a probable GXE interaction. Since there was a CpG island in the promoter region of this gene, qMSP was utilized to measure DNA methylation differences in the *Tmem132d* mHAB and mLAB alleles. Surprisingly, the total percentage of DNA methylation of *Tmem132d* promoter of each mLAB allele is lower than corresponding mHAB allelic expression, although not statistically significant (Figure 33). Nevertheless, this is in line with the above higher mLAB *Tmem132d* mRNA allelic expression as there was a significant negative correlation with each other at least in mHAB alleles. Thus, these studies indicate an interaction between genetic and environmentally mediated epigenetic factors like DNA methylation. Also, a recent study presented at a

conference reported higher symptom severity and lower methylation in intronic region of human *Tmem132d* suggesting overexpression of corresponding mRNA in PTSD patients (Erhardt, 2012).

It is worth mentioning that studies by (Quast *et al.*, 2012) in the human *Tmem132d* gene of healthy controls found an overrepresentation of rare variants with protective effects. Thus in a similar way, in the heterozygous F1 offspring, the mLAB *Tmem132d* allele might have a protective role to buffer the animals against any kind of environmental challenges. Thus, the subsequent observed higher mLAB *Tmem132d* allelic expression. Studies by (Taylor *et al.*, 2006) showed that the combination and proportion of 5-HTT polymorphic alleles confer either susceptibility or protective effects based on the prevailing situation. Thus, although there was higher mLAB allelic expression in both EE and UCMS group of F1 offspring compared to mHAB, the proportion of each allele may determine at least partially the animal's response to the environmental challenges faced by it. Although I would like to add that *Tmem132d* is not the first plasticity gene discovered for anxiety-related behavior. Studies by (Sztainberg *et al.*, 2010) showed that the high *Crhr1* mRNA expression which is associated with higher anxiety-related behavior, is reduced in the amygdala when the mice are exposed to EE with a concomitant reduction in anxiety-related behavior. Subsequently, in our group *Crhr1* mRNA was found to be differentially expressed with basal mHAB having higher *Crhr1* mRNA expression relative to mLAB in the amygdala (Markt *et al.,* (submitted)). Interestingly, when the mHAB was subjected to EE there was a corresponding decrease in *Crhr1* mRNA expression relative to its control in the amygdala. In contrary, mLAB experiencing UCMS had higher *Crhr1* mRNA expression compared to their respective controls in the amygdala. The *Crhr1* promoter was further characterized for differential methylation in EE mHAB and UCMS mLAB and the dissimilar expression was suggested to be a result of an interaction between DNA methylation and Yin-Yang (YY1) transcription factor binding in the upstream *Crhr1* promoter region (Markt *et al* (submitted)). These findings support the notion of differential susceptibility hypothesis (Belsky *et al.*, 2009) that there is neither good nor bad genes. They simply respond to environmental challenges thereby increasing phenotypic plasticity for adaptation and aid in the survival of the species.

Thus the above behavioral and molecular findings further emphasize the use of selective breeding to study anxiety-related and depression-like behavior. The study of these two candidate genes *i.e.*, Nps/Npsr1 and Tmem132d would pave the way for further studies in humans to determine their translational potential.

5.0 Perspectives

The high degree of similarity among the mature 20 amino acid NPS peptide suggests conservation of its function across different phyla. This is also indicated by the fact that the commercial NPS peptide, which is based on the corresponding human sequence, nonetheless works well in rats and mice. The mutations observed in the mLAB NPS precursor could be artificially engineered and its post-translational processing studied in an *in vitro* set up. The effects of NPSR1-A showing higher anxiety in LAB rodents suggested an involvement of an endogenous NPS system. On the contrary, in HAB rodents, these data hint towards a dysfunctional endogenous NPS. This could be verified by measuring basal NPS release in target brain regions of HAB *vs.* LAB rodents and also it would be interesting to look at corresponding protein levels, localized within CRH positive cells. This idea is based on the fact that *Crh* mRNA was higher in rHAB and thus this maybe a reason for increased *Nps* mRNA. The interesting study by (Jungling *et al.*, 2012) demonstrating release of NPS by CRH, further warrants its investigation in the HAB *vs.* LAB rodents, because of CRH involvement in HPA axis activity, which is also altered in these animal models. This would enhance understanding of HPA axis activity and its corresponding interactions with other peptide molecules.

On the other hand, in *Npsr1* gene the conservation of exonic and 5' regulatory region along with our promoter characterization data would help other researchers working in diverse fields such as sleep medicine, respiratory disease and inflammatory bowel disease, where the role of *Npsr1* is currently being investigated. In addition, the fact the a single exonic SNP in NPSR1 has similar behavioral and physiological correlates with its human NPSR1 Ile^{107} isoform, advocates use of NPS in anxiety or panic disorder patients carrying the risk allele. Nevertheless, it is worth noting that in humans NPSR1 the exchange of hydrophilic asparagine to hydrophobic isoleucine residue may additionally affect protein folding, conformation, etc. Thus, normal rats and mice which carry non-polar hydrophobic tryptophan and isoleucine, respectively, in NPSR1 at the corresponding position could be mutated to asparagine and its corresponding behavioral and physiological outcomes studied. Furthermore, the *in vitro* demonstration of increase in cAMP in HAB NPSR1 relative to LAB in rats and mice could be utilized to find other downstream coplayers that might modulate the activity of this GPCR. Also, it would be interesting to do an enzyme-linked immuno sorbent assay (ELISA) in cells overexpressing HAB, LAB NPSR1 to verify if difference in cAMP response is due to differences in total and surface receptor expression. Finally, the fact that *Npsr1* mRNA expression did not differ in mHAB *vs.* mLAB subjugated to EE or UCMS, suggests that not all candidate genes are plastic but they have other roles as discussed above.

The second candidate gene, TMEM132D which is expressed on mature oligodendrocytes and neurons further emphasizes the role of neuron-glia interactions and cell-cell adhesion in governing the physiology of brain. There is a need to do bisulfite sequencing of *Tmem132d* promoter in mHAB, mLAB and the corresponding F1 offspring subjected to EE or UCMS, owing to its observed differential mRNA expression. The identification of TFIIB and nuclear factor I/C in the *Tmem132d* promoter region warrants use of electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipiation (ChIP) techniques to verify the binding of these factors and its interaction with adjacent methylation groups as suggested by MSP in mHAB *vs.* mLAB alleles of F1 offspring. In addition, the Cre-Lox site-specific recombinase technology could be utilized to achieve deletion of *Tmem132d* and subsequent evaluation of behavioral consequences in animals. Additionally reverse pharmacology or chimeric receptor (combining TMEM132D and NCAM domains) approaches could be used to discover ligands for TMEM132D. There is a need to verify the protein expression differences using Western blot once a suitable antibody is available.

Furthermore, to elucidate functional aspects of TMEM13D, the yeast two hybrid system (Y2H) could be utilized with *Tmem132d* cDNA as a bait and a random library as the prey. The protein-protein interactions could be further validated in a mass spectrometry set up once a good antibody is available. The differential expression of *Tmem132d* in light of the environmental challenges faced by animal and subsequent detection of epigenetic factors like DNA methylation further highlights the role of GxE interactions in mediating complex phenotypes.

To conclude, identification and functional characterization of risk factors at a higher penetrance in these animal models would help in finding novel biomarkers for anxiety and comorbid depression-like behavior. The hope is that, once these risk factors are also discovered in corresponding patient group as for the above two candidates, the preclinical studies would contribute to the diagnosis and treatment of these disorders. Also, HAB NPSR1 or TMEM132D could be overexpressed into wild-type mice to see the corresponding phenotype. Similarly, small interfering RNA (siRNA) could be used to see if alterations in Nps, Npsr1 or Tmem132d observed in HAB, LAB rodents leads to similar phenotype when only that one change introduced.

6. References: American Psychological Association (APA) style, 6th edition used here)

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8.0 *Curriculum vitae*

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Objective

I plan to develop and utilize tools & techniques to understand the physiology and chemistry of the brain.

Education

Since Apr 10 PhD student at the Max Planck Institute of Psychiatry, enrolled at the Ludwig Maximilians University, Faculty of Biology, Munich

Sep 08-Mar 10 University of Regensburg, Germany
M.Sc. in Experimental and Clinical Neuroscience
Thesis: Genetic analysis of the brain Neuropeptide S system in selectively bred rats.

Jun 04-May 06 Goa University, Panaji-Goa, India
M.Sc. in Marine Biotechnology
Thesis: Strain improvement and optimization of antibiotic production of two halotolerant micro organisms.

Work experience

Jan 07-Aug 08 United Breweries Limited, Ponda-Goa, India
Process Chemist: All of the physical and chemical analyses of raw materials and beer in process performed.

Jul 06-Dec 06 Gujarati Samaj Educational Trust School for the handicapped, Margao-Goa, India
Trainee teacher for mentally challenged children. Provided vocational training, periodic assessment & teaching.

Honors & Activities

1. Received travel award to present poster at the International Behavioral and Neural Genetics Society (IBANGS) 2013 meeting to be held at Leuven, Belgium.
2. Max Planck Society PhD fellowship since 2010.
3. Qualified Graduate Aptitude Test in Engineering (GATE) life science exam with 90.83 percentile in 2011.
4. Awarded scholarship by the Department of Sainik Welfare, Government of Goa, India during B.Sc. 1st, 2nd & M.Sc. 1st, 2nd year.

Conferences

1. Presented a poster titled, ‘‘The voice of silent mutations: regulatory SNPs in the HAB *Npsr1* have implications for panic disorder patients’’ at the Interact munich 2013 conference.

2. Presented a talk titled ’Phenomena involved in the regulation of Neuropeptide S in rats and mice selectively bred for extremes in trait anxiety’ at the ‘‘Biomarkers, Functional Genomics & Novel Antidepressants’’ symposium at the Ringberg castle, Germany in september 2011.

3. Attended 8th Göttingen meeting of the German Neuroscience Society in March 2009.

Publications or other contributions

Slattery D.A.*, **Naik R.R.***, Yen Y-C., Sartori S.B., Fuechsl A., Finger B.C., Elfving B., Nordemann U., Guerrini R., Girolamo Calo G., Wegener G., Mathé A.M., Singewald N., Czibere L., Landgraf R. & Neumann I.D. Functional alterations in the neuropeptide S receptor are conserved across two animal models of high anxiety: implications for human anxiety disorders. (in preparation). #

Naik R.R., Steiner A., Brehm N., Stündl A., Landgraf R., Czibere L. Tmem132d: Functionality of structural gene variations in a candidate gene of anxiety phenotypes. (in preparation). #

Sotnikov S.V.*, Markt P.O.*, Malik V., Chekmareva N.U., **Naik R.R.**, Sah A., Singewald N., Holsboer F., Landgraf R. Bidirectional epigenetic rescue of extreme genetic predispositions to anxiety: impact of CRH receptor 1 as plasticity gene in the amygdala. Molecular Psychiatry (submitted).

Sotnikov S.V.*,Chekmareva N.U.*, **Naik R.R.**, Diepold R., Czibere L., Landgraf R. Allelic expression imbalance and parent of origin effect in anxiety-related behavior. (in preparation). #

* : These authors contributed equally

: Parts of this thesis were published or will be published in these articles.

9.0 Declaration/ Erklärung

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

München, den 09.04.2013

(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 09.04.2013

(Unterschrift)