Emotional and cognitive aspects in two mouse lines selectively bred for extremes in anxiety-related behavior: from trait anxiety to psychopathology

Dissertation

der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

vorgelegt von

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München, 04. August 2011

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Datum der mündlichen Prüfung: 01. Februar 2012

獻給我摯愛的家人

For my beloved family

Abstract

The studies presented in this thesis mainly address the characteristics of mice originating form a bi-directional selective breeding approach, based on behaviors on the elevated plus-maze (EPM) resulting in CD1-derived high (HAB), normal (NAB), and low (LAB) anxiety-related behavior mice. According to the binominal phenotypes and neurochemical features of HAB and LAB mice, we proposed HAB and LAB mice as useful animal models of anxiety disorder (e.g. generalized anxiety disorder) and attention-deficit hyperactivity disorder (ADHD), respectively.

Patients diagnosed for anxiety disorders often display faster acquisition and slower extinction of learned fear. HAB mice displayed pronounced cued-conditioned fear compared to NAB/CD1 and LAB mice that coincided with increased phosphorylation of the protein kinase B (AKT) in the basolateral amygdala 45 min after conditioning. This supports the notion that HAB mice formed a stronger fear memory, which might be responsible for the slower extinction and the spontaneous recovery of learned fear observed in these animals. HAB mice also displayed higher levels of contextual fear compared to NAB and LAB mice and exaggerated avoidance following step-down avoidance training.

HAB mice have been suggested to be a preclinical tool for the development of new pharmacological therapies. Here, we validated the effects of both central and intranasal administration of neuropeptide S (NPS). This newly discovered anxiolytic substance could induce anxiolytic effects in HAB mice, thus providing a promising way to deliver NPS to the brain in a therapeutic perspective. The genetic analysis revealed that the high anxiety-related behavior in HAB mice was associated with a lower NPS receptor 1 (*Npsr1*) mRNA expression in the amygdala. In addition, several genetic polymorphisms have been identified in the *Nps* and *Npsr1* gene sequences that are likely to be involved in shaping the anxiety-related phenotype.

LAB, NAB and HAB mice do not only differ in their innate anxiety but also show strong differences in locomotion in the open field (OF) test. LAB mice with elevated levels of exploratory behaviors were proposed as a useful genetic model for ADHD, which is characterized by inattention, hyperactivity, impulsivity as well as impaired cognition. In a HB

test, LAB mice displayed exceptionally increased locomotion, but showed decreased 16-hole exploration compared to HAB/NAB mice. LAB mice were heavily impaired in acquisition and relearning in the water cross-maze (WCM), indicating strong deficits in egocentric and allocentric navigation as well as behavioral flexibility. LAB mice also displayed reduced attention and, thus, reduced social memory upon exploration of conspecifics. Amphetamine administration, but not methylphenidate, exerted the same paradoxical calming effect in LAB mice as observed in human ADHD patients. Similar calming effect was observed in LAB mice after administration of AM404, an enhancer of endocannabinoid/endovanilloid action. Surprisingly, the opposite effects of amphetamine and AM404 seen in LAB *vs.* NAB/HAB mice were not mirrored by differences in dopamine release in the caudate putamen. These findings disproved the assumption that changes in dopaminergic tone are directly related to alterations in locomotor activity. However, there is still possibility that other brain circuits (e.g. medial prefrontal cortex) or mechanisms (e.g. serotonin system) may be involved in the locomotion responses to these drugs.

Taken together, our results suggest that the genetic predisposition to high anxiety-related behavior (HAB) may increase the risk of forming traumatic memories, phobic-like fear and avoidance behavior following aversive encounters, with a clear bias towards passive coping styles. In contrast, less anxious animals (LAB) that adopt active coping strategies are much bolder, showing hyperactivity, hyper-arousal and less behavioral flexibility. In combination with the behavioral and pharmacological profiles, LAB mice are highly suggested as an animal model toward ADHD. In conclusion, under selection pressure of a specific trait, the HAB/LAB animal models fit a conceptual framework of Hawk-Dove personality types, and consequently bear differential vulnerability to psychiatric diseases.

List of abbreviations

5-HT	Serotonin
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
AKT	protein kinase B
AM404	N-(4-hydroxyphenyl)arachidonoylethanolamide
Amph	Amphetamine
ANOVA	Analysis of variance
ASR	Alteration of the startle response
AVP	Vasopressin
CaMKII	Calcium/calmodulin-dependent protein kinase II
CB1	Cannabinoid receptor 1
CCW	Counter-clockwise
cDI	Central *diabetes insipidus*
CPu	Caudate putamen
CS	Conditioned stimulus
CW	Clockwise
DA	Dopamine
DAT	Dopamine transporter
DAT-KO	Dopamine transporter knockout
DER	Downstream enhancing region
DOPAC	3,-4 dihydroxyphenylacetic acid
DSM-IV	Diagnostic and statistical manual for psychiatric disorders
DT	Distance travelled
E1	Non-social empty tube 1
E2	Non-social empty tube 2
EPM	Elevated plus-maze
ERK	Extracellular-signal-regulated kinase
ESS	Evolutionary stable strategy
Ext	Extinction group
F1	First (familiar) ovariectomized female
F2	Novel ovariectomized female
FL	Free learning
FST	Forced swim test
GAD	Generalized anxiety disorder
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element
GSK-3β	Glykogen synthase kinase 3
HAB	High anxiety-related behavior
HB	Hole-board
HPA	Hypothalamo-pituitary-adrenal
HR	High resisting
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneally
IEI	Interexposure interval
IO	Input/Output
IPI	Interpulse interval

ITI	Inter-trial interval
LAB	Low anxiety-related behavior 3
LAB-I	LAB-intermediate
LAB-S	LAB-strong
LC	*Locus coeruleus*
LOD	Limit of detection
LR	Low resisting
LTD	Long-term depression
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MD	Methylphenidate
MDD	Major depressive disorder
mPFC	Medial prefrontal cortex
MPI	Max Planck Institute
NAB	Normal anxiety-related behavior
NAc	*Nucleus accumbens*
NE	Norepinephrine
NET	Norepinephrine transporter
Nomi	Nomifensine
N-P	Nose-poke
NPS/*Nps*	Neuropeptide S
NPSR1/*Npsr1*	Neuropeptide S receptor 1
NRI	Norepinephrine reuptake inhibitor
Obs/Exp	Ratio of observed and expected
OF	Open field
OXTR	Oxytocin receptor
pCREB	phosphorylated AMP response element-binding
PCR	polymerase chain reaction
PFC	Prefrontal cortex
PP	Prepulse
PPI/PPF/PP	Prepulse inhibition/Prepulse facilitation
Pre-S	Before tone-foot shock pairing
PTSD	Posttraumatic stress disorder
PVN	Paraventricular nucleus
Ret	Retention control
RLS	Restless legs syndrome
Rimo	Rimonabant (SR141716)
S	Tone-foot shock pairing
SB	SB-366791
SERT	Serotonin transporter
SHR	Spontanous hypertensive rats
SNP	Single-nucleotide polymorphism
SPECT	single-photon emission computer tomography
SPL	Sound pressure level
SR	Startle response
TESS	Transcription element search system
TH	Tomoxetine hydrochloride
TRPV1	Transient receptor potential vanilloid 1

TST	Tail suspension test
US	Unconditioned stimulus
Veh	Vehicle
WCM	Water cross-maze

Please note that the gene symbols of neuropeptide S and neuropeptide S receptor 1 are written as *Nps* and *Npsr1*.

1. Introduction

The world is just awesome. It inspires people to dedicate their lives to understanding the varieties of living beings. Personality psychology is a branch of Psychology that investigates personality (or temperament) and individual differences. The distinction between personality and temperament is hard to make. The concept of temperament dates back to philosophers in ancient Greece. It has a more restrictive meaning than personality, describing individual peculiarity or traits that are inherited, early appearing and stable over time and across situations (Budaev, 1997; Box, 1999). Personality can be defined as a dynamic and organized set of characteristics possessed by a person that uniquely influences his or her cognitions, motivations, and behaviors in various situations (Ryckman, 2004).

Besides human beings, animals also have temperament (or personality) peculiarities that indicate consistent long-term phenotypic differences among individuals (Dingemanse *et al.*, 2010). When one observes any group of animals long enough, one will start noticing individual differences among the group members, even in case of inbred (i.e. genetically identical) animals. Ecological studies on wild populations of mice, fishes and birds elucidate the individual phenotypes in their behavioral and neuroendocrine change to environmental challenge. Recently, there is growing number of literature that boots on animal models to understand personality and individual differences in a broader sense (Reale *et al.*, 2007; Koolhaas *et al.*, 2010), as well as with respect to curing psychiatric disorders (Korte *et al.*, 2005; Rettew and McKee, 2005; Whittle *et al.*, 2006). Studies investigating the link between individual difference and vulnerability to stress-related diseases started raising the following questions. How and for what reasons do individuals vary in possessing different temperament (or personality)? Why do different organisms adopt different strategies to cope with stressful events and environmental threats? And why are some individuals more vulnerable to stress-related diseases than others?

1.1. Individual difference in behavior

In a review work of Korte *et al* (2005), the authors provided evolutionary explanations why organisms adopt different behavioral strategies in order to cope with stressful events. Maynard Smith (1982) applied “Game Theory” to animal behaviors and found that natural selection

prefers to maintain a balance between different behavioral traits and strategies. To maintain the balance, the organisms ought to fit the "evolutionary stable strategies (ESS)" for being adaptive to the environment. The best example of such ESS is the "Hawk-Dove game" in which animals develop different traits preserving genes for fight-flight (Hawks) and freeze-hide (Doves) behavior strategies within a population. These two different strategies (Hawks-Doves) are widespread in the animal kingdom within the same species, e.g. great tits (Verbeek *et al.*, 1996) and rodents (van Oortmerssen and Bakker, 1981).

1.1.1. Variation in behavioral strategies in birds

Field studies suggested that the Hawk-Dove strategy could be observed in great tits. More specifically, male individuals that quickly visit all trees and explore the environment in a relatively superficial and routine way are very aggressive and bold (fast superficial explorers). In contrast, males that explore the environment more thoroughly and cautiously are non-aggressive (slow explorers) (Verbeek *et al.*, 1996; Verbeek *et al.*, 1999). Based on the discrete strategies in two populations, the fast-superficial explorers take greater risks in fighting (Hawks) and approach novel objects faster than the slow explorers (Doves) (Verbeek *et al.*, 1994; Verbeek *et al.*, 1999); however, the slow explorers (Doves) pay more attention to changes in their environment and probably get more information about it (Verbeek *et al.*, 1999).

1.1.2. Variation in behavioral strategies in rodents

Besides birds, Hawk-Dove strategies can also be found in mammals, like rodents and pigs (Hessing *et al.*, 1994; Koolhaas *et al.*, 1999). Research on aggression in rodents is probably the best-studied field with respect to Hawk-Dove strategies (Sluyter *et al.*, 1996; de Boer *et al.*, 2003). When exposed to a psychosocial stimulus (a large dominant conspecific), aggressive (Hawk-like) feral rats show fight behavior, while the non-aggressive (Dove-like) ones show freezing (de Boer *et al.*, 2003). In the other rodent species, the non-aggressive individuals tend to be cautious and flexible by displaying active burying behaviors when given sawdust from their home cages, but showing freezing response if fresh sawdust was used, i.e. reactive coping. In contrast, aggressive ones that are more likely to be bold show more active burying behaviors (i.e. proactive coping) in the defensive bury test, irrespective of bedding materials (Sluyter *et*

al., 1996). Further studies in the aggressive and non-aggressive mice support this dichotomy by the different copying strategies in the forced swim test (Veenema *et al.*, 2003). The aggressive mice displayed more escape behaviors, such as climbing and swimming, whereas the non-aggressive spent more time in floating, which can be regarded as a function of energy saving (Korte *et al.*, 1996). In consistency with showing higher immobility, the non-aggressive animals had higher anxiety levels when tested for the second time in a elevated plus-maze (EPM; Veenema *et al.*, 2003).

1.1.3. Evolutionary views of variation in behavioral strategies

In line with the results mentioned above, it has been suggested that the fundamental difference between two coping styles (proactive *vs.* reactive coping) seems to be the degree in which behavior is guided by environmental stimuli (Benus *et al.*, 1987; Benus *et al.*, 1990). Aggressive animals easily develop routines which are independent of environmental stimuli, i.e. rigid type of behavior (Bolhuis *et al.*, 2004). In contrast, non-aggressive ones are more perceptive of the environmental changes and consequently show more flexible behaviors (Benus *et al.*, 1991; Koolhaas *et al.*, 1999). Dependent on environmental conditions, Hawks and Doves show differences in emotional state, exploration rate and energy metabolism that make them more or less adaptable to the environmental changes. Early studies on a feral population of house mice suggest that a binomial distribution of behavioral phenotypes provides individuals different fitness to the environment (van Oortmerssen and Busser, 1989). The variability in the coping styles may explain why aggressive males are more successful under stable colony conditions, whereas non-aggressive ones seem to be more adapted to variable conditions, e.g. during migration.

The distinct coping styles are found not only in wild populations, but also in artificially selected animals. Interestingly, the different traits underpinning Hawk-Dove strategies (e.g. aggression *vs.* non-aggression, fight-flight *vs.* freezing, superficial *vs.* thorough exploration, rigid and routine-like *vs.* flexible behavior) may not evolve in isolation, but rather as a package caused by pleiotropy, gene-linkage or co-selection (Price and Langen, 1992). Table 1 summarizes gene-environment interactions in Hawks and Doves and the consequences for fitness.

Table 1: The Hawk-Dove personality type. The different gene-environment interactions in Hawk-Dove strategy and the consequences for fitness according to different environmental conditions (Korte *et al.*, 2005).

	Hawk	**Dove**
Behavioral strategy	Fight-flight	Freeze-hide
Coping style	Proactive	Reactive
Emotional state	Aggressive and bold	Non-aggressive and cautious
Biological role	Establish territory or defend existing territory	Adopt strategy to avoid danger within territory, e.g. immobility
Exploration	Fast and superficial	Cautious and thorough
Behavioral flexibility	Rigid and routine-like	Flexible
Energy metabolism	High energy consumption	Energy conservation
Body damage (e.g. wounds)	High risk	Low risk
Advantage according to food availability	When stable and abundant	During food scarcity
Advantage according to population cycle	When density is high	When density is low

1.1.4. Behavioral strategies and difference in disease vulnerability

Numerous studies have shown that the coping styles are not only characterized by differences in behavior, but also by differences in physiological and neuroendocrine mechanisms (De Boer *et al.*, 1990; de Ruiter *et al.*, 1992; Hessing *et al.*, 1994; Korte *et al.*, 1997). The idea of coping styles implies that animals have a differential way to adapt to various environmental conditions. If an animal fails to cope with the stressor, it would result in negative health consequences. The individual animals that adopt the proactive or reactive coping style differ in their vulnerability to stress-related disease due to the differential adaptive value of the two coping styles and the accompanying physiological/neuroendocrine differentiation (Koolhaas *et al.*, 1999). Hawk-like animals that possess proactive coping style which show relatively low hypothalamic-pituitary-adrenal (HPA) axis reactivity, low levels of serotonin (5-HT) neurotransmission and high levels of testosterone. Due to the neurochemical features mentioned above, these animals have higher tendency to express aggressive behavior and more proneness to develop impulsive disorder-related syndromes. In contrast, cautious Dove-like animals with their high HPA axis reactivity, corticosterone and tonic 5-HT neurotransmission have a higher tendency to express freezing behavior. Based on their avoidance response during attack episodes, Doves are very well to recognize contextual information and are, therefore, more aware of relevant signals in their environment compared to Hawks. These animals, due to higher susceptibility of HPA axis, have high risks to develop anxiety disorder-related syndromes (Korte *et al.*, 2005).

1.1.5. Personality trait and psychopathology

Among the various possibilities to study human psychopathologies, animal models remain one of the most popular strategies. Studies on genetic lines by artificial selection which differ in their aggression levels have provided important contributions to the relationship between aggressive behavior and physiological/neurochemical parameters (Compaan *et al.*, 1994; Sluyter *et al.*, 1994). It is a good example of employment of genetic lines of animals selected for a specific characteristic. Genetic selection for the extremes in a certain characteristic generally results in distinct phenotypes within a few generations (Koolhaas *et al.*, 1999). For the last three decades, it has been suggested that a bi-directional selection of rodents for a behavioral trait is a powerful tool to study genetic, morphological, physiological, and biochemical mechanisms underlying the particular trait.

Anxiety disorders remain one of the most common and debilitating psychiatric illnesses which induces serious socioeconomic problem in human society. It has been suggested to be genetically determined, and it is highly related to a certain personality trait (Hettema *et al.*, 2004). It has become evident that anxiety is not a unitary phenomenon but can be divided into normal/state anxiety and trait/pathological anxiety. According to current apprehension, one should not consider pathological anxiety as an extreme of normal anxiety (Belzung and Griebel, 2001); instead, it seems that the pathological fashion has a different neurobiological basis from the normal one (Engel *et al.*, 2009). Due to the lack of animal models involved in trait or pathological anxiety, it stimulates us to establish genetic animal models of anxiety sharing with endophenotypes with human psychopathology (Landgraf *et al.*, 2007).

1.2. The HAB/LAB animal model

To develop a reliable and representative animal model of anxiety disorders, Landgraf and his colleagues aimed at creating genetically selected lines for trait anxiety that seemed to be more robust and consistent. In the beginning of 1990s, they started to generated a breeding protocol with Wistar rats that were selected and mated according to the results of the EPM test, to establish two different lines termed HAB and LAB (high *vs.* low anxiety-related behavior) (Landgraf and Wigger, 2002). In addition to their robust difference in anxiety levels in various

tests, HAB and LAB rats differ in their stress coping strategies. HAB rats are more susceptible and vulnerable to stressor exposure and preferring more passive strategies (Landgraf *et al.*, 1999; Keck *et al.*, 2001; Ohl *et al.*, 2002). In contrast, LAB rats displayed increased locomotor activity and more signs of aggressive behavior (Landgraf and Wigger, 2002; Ohl *et al.*, 2002), indicative of active coping (Koolhaas *et al.*, 1999).

Since the year 2000, the focus of research has shifted from rats to mice because mice share a high level of genetic homology with humans. Starting with outbred CD1 mice, Landgraf and his colleagues used a similar approach, based on the EPM behavior, to produce HAB, NAB and LAB mice (Figure 1). In correspondence with the performance on the EPM, all three lines also revealed stable differences in a variety of anxiety paradigms including dark-light avoidance test, home cage behavior and ultrasonic vocalization while 5-day pups are separated from their mothers (Kromer *et al.*, 2005). Similar to HAB/LAB rats, HAB and LAB mice also show differences in depression-like behavior measured in the tail suspension test (TST) and forced swim test (FST). HAB mice displayed more passive, while LAB mice more active coping strategies in those tests. HAB, NAB and LAB mice do not only differ in their anxiety-related behavior as measured in the EPM test, but also display divergent levels of locomotion in home cage activity as well as total line crossings and rearing in dark-light avoidance test (Kromer *et al.*, 2005). In these tests, LAB mice displayed more spontaneous hyperactivity and excitation compared to HAB and NAB mice.

In either HAB/LAB rats or mice, when exposed to a mild stressor (5-min open arm challenge), HAB animals displayed altered expression of the immediate early gene c-Fos in prefrontal-cortical, limbic and hypothalamic areas, which are involved in regulating anxiety-related behavior (Muigg *et al.*, 2007; Muigg *et al.*, 2009). In addition to altered neuronal activation, this open arm exposure induced higher concentrations of adrenocorticotropic hormone (ACTH) and corticosterone in HAB than LAB animals (Landgraf *et al.*, 1999), indicating hyperactive HPA axis in hyperanxious animals. Not only the striking feature of hyper-reactive HPA axis, but also elevated expression of vasopressin (AVP) mRNA in the paraventricular nucleus (PVN) (Wigger

and Neumann, 2002; Bunck *et al.*, 2009) were observed in HAB animals, resembling the pathological symptoms in psychiatric patients.

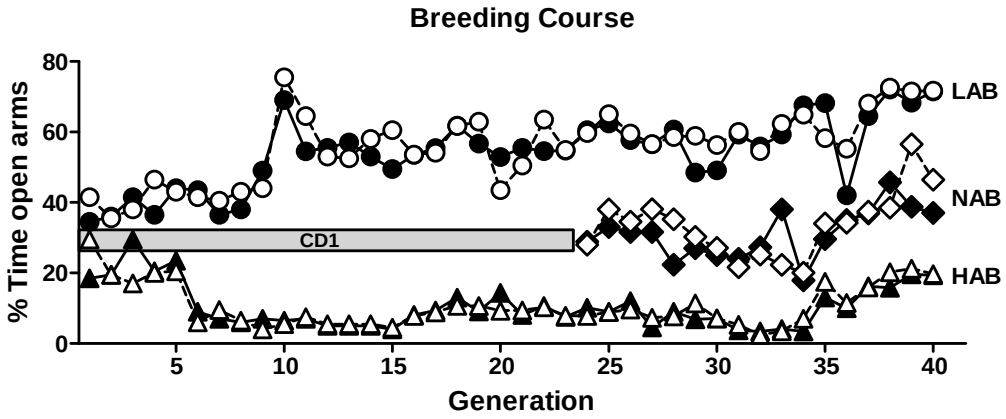


Figure 1: Breeding course of HAB, NAB and LAB mice. EPM data are presented as the percent time on the open arms in male and female HAB and LAB mice of Generation 1-40, as well as NAB mice of Generation 1-17. Male mice were indicated by solid lines and filled symbols, while female were indicated by dashed lines and open symbols. (Data are kindly provided by Markus Nußbaumer, RG Landgraf)

1.3. Extremes in trait anxiety and psychopathology

Taken together, the original data revealed that more anxious animals adopt passive coping styles when confronted with a dangerous environment and display increased immobility in FST and TST. In contrast, less anxious animals are much bolder, showing hyperactivity, hyper-arousal and less immobility. These data support the notion that extremes in trait anxiety are correlated with different coping strategies, in which HAB mice may present Dove-like personality, while LAB mice can be an example of Hawk-like personality type. According to these specific phenotypes and neurochemical features of HAB and LAB mice, ***we proposed HAB and LAB mice as useful animal models of anxiety disorder (e.g. generalized anxiety disorder) and attention-deficit hyperactivity disorder (ADHD), respectively.***

1.4. Anxiety disorder

Anxiety disorders are a well-known class of psychiatric disorder which brings a significant and serious socioeconomic problem in human society. According to the fourth edition of the

Diagnostic and Statistical Manual for Psychiatric Disorders (DSM-IV) (APA, 1995), the current classification of anxiety disorders includes generalized anxiety disorders (GAD), phobias, and posttraumatic stress disorders (PTSD), as well as panic and obsessive compulsive disorders. Patients with pathological anxiety are typically not able to react properly to environmental treats or stressful situations. Compared to normal anxiety, pathological anxiety has been defined in relation to hyperexcitability of neuronal circuits that include the amygdala and the extended amygdala (i.e. bed nucleus of the *stria terminalis*) (Rosen and Schulkin, 1998).

It is widely acknowledged that anxiety disorders are highly comorbid with depression. Among patients with major depressive disorders (MDD), 50-60 % of individuals report a lifetime history one or more episodes of certain anxiety disorders. Furthermore, clinical data also indicate that anxiety disorders rarely exist in isolation, but are accompanied by other psychiatric problems (Kaufman and Charney, 2000). In clinic, patients with depression and anxiety disorders commonly displayed high levels of personality trait of neuroticism. One subtype of anxiety disorder, GAD, has considerable overlap with neuroticism in their characteristic features. Recently, a twin study has revealed that the genetic factors underlying neuroticism are highly correlated to those that influence the liability to GAD (Hettema *et al.*, 2004).

1.4.1. Animal models of anxiety disorders

In preclinical anxiety research, animal models are used to capture various features of human conditions, from behavioral and physiological changes that are indicative of the emotional response to the etiology of the disease and therapeutic effects (Fuchs and Flügge, 2004). According to McKinney (1984), animal models are "*experimental preparations developed in one species for the purpose of studying phenomena occurring in another species. In the case of animal models in human psychopathology one seeks to develop syndromes in animals which resemble those of humans in certain ways in order to study selected aspects of human psychopathology.*"

To be suitable for research, animal models ought to fulfill three validation criteria. *Face validity* implies that the behavioral and physiological anxiety responses observed in the animal model should be identical to those observed in humans. *Predictive validity* means that the animals are

sensitive to effective drugs in clinical treatment, in this case anxiolytics. Finally, *construct validity* relates the similarity between the theoretical rationale underlying the animal model and neurobiological mechanisms in humans (Belzung and Griebel, 2001).

1.4.2. Conditioned fear in a mouse model of extremes in trait anxiety

Fear enables reflexive adaptation to threatening stimuli and situations. It is characterized by both active (e.g., startle, fight/flight) and passive (e.g., freezing, avoidance) responses. Exaggerated fear may become maladaptive and thereby contribute to the development of psychopathology (Rosen and Schulkin, 1998; Maren, 2007). Patients diagnosed with anxiety disorders show immoderate physiological reactions to aversive stimuli in comparison to healthy individuals (MacLeod *et al.*, 2002; McTeague *et al.*, 2010). In addition, they display stronger acquisition and slower extinction of learned fear behaviors (Lissek *et al.*, 2005) and an increased return of fear after treatment (Rodriguez *et al.*, 1999).

In the past three decades, the linkage between trait anxiety and learned fear has been broadly described in terms of neuroanatomical (Davis, 1992; Charney, 2003; Shin and Liberzon, 2010) and pharmacological (Santos *et al.*, 2005) parallels. It is becoming increasingly apparent that the mechanisms underlying Pavlovian fear conditioning have much in common with human anxiety disorders (Marks and Tobena, 1990; Rosen and Schulkin, 1998; Pitman *et al.*, 1999; Bouton *et al.*, 2001; Sullivan *et al.*, 2003). Due to close homologies in the anatomical and molecular signatures of the fear matrix between humans and rodents, classical fear conditioning in rats and/or mice may provide important knowledge of acquisition, expression and extinction of conditioned fear that can then be applied to humans (Walker and Davis, 2002; Ressler *et al.*, 2004; Delgado *et al.*, 2006; Monfils *et al.*, 2009; Davis *et al.*, 2010; Schiller *et al.*, 2010).

In rodents, conditioned fear is assessed by pairing of an a *priori* neutral stimulus, such as a tone or a light signal (the conditioned stimulus, or CS), with a punishment, such as an electric foot shock (the unconditioned stimulus, or US). In consequence of the CS-US association, presentation of the CS alone is capable of eliciting a conditioned fear response (e.g., freezing or fear-potentiated startle). The formation of fear memories critically depends on the amygdala (Liang *et al.*, 1994; Ledoux and Muller, 1997). Repeated presentation of the CS in absence of the

expected punishment leads to a gradual decline in fear responses. In most cases, this fear extinction process is thought to form a new memory and cannot simply be explained by forgetting or erasure of the original memory trace, since conditioned fear may reappear with the passage of time (spontaneous recovery) and/or in a different test context (renewal) (Myers and Davis, 2002; Bouton and Moody, 2004; Bouton *et al.*, 2006; Quirk and Mueller, 2008).

Fear conditioning procedures often lead to parallel formation of elemental (i.e. auditory or visually cued) and configural (i.e. contextual) fear memories. The latter process may contribute to the development of avoidance behavior (Mowrer, 1960), another core feature of anxiety disorders (Rosen and Schulkin, 1998; North *et al.*, 2009). Under experimental conditions, avoidance behavior can be studied in inhibitory (e.g. step-down or step-through) avoidance tasks.

1.4.3. Neuropeptide S (NPS)

Nowadays, the discovery of novel anxiolytic is still needed because there are high number of patients suffering from side effects of medication (Cassano and Fava, 2004) and treatment resistance (Bystritsky, 2006). Neuropeptide S (NPS) is a 20-residue peptide that was recently discovered (WO 02/31145 A1; (Sato *et al.*, 2002)) and identified as an endogenous ligand for an orphan G-protein-coupled receptor, now referred to as NPS receptor 1 (NPSR1) (Figure 2B) (Reinscheid and Xu, 2005). The name of this peptide comes from the presence of a conserved serine (S) at the N-terminal position in vertebrates (Figure 2A).

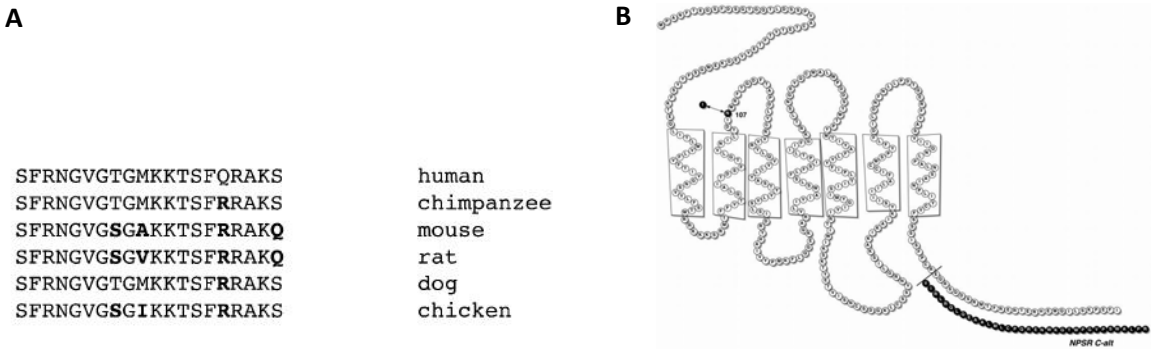


Figure 2: Neuropeptide S (NPS) and its cognate receptor (NPSR). (A) Primary structures of NPS from human, chimpanzee, mouse, rat, dog and chicken (Xu *et al.*, 2004). (B) Schematic diagram of the human NPS receptor protein (Reinscheid and Xu, 2005).

Since the NPS system was discovered, it has gained substantial interest for basic research and clinical use due to its involvement in regulation of several biological processes, particularly behavioral arousal (Xu *et al.*, 2004; Reinscheid *et al.*, 2005) and anxiety-related behavior (Xu *et al.*, 2004; Rizzi *et al.*, 2008; Vitale *et al.*, 2008). Central administration of NPS produced a unique behavioral profile of increasing arousal and of exerting anxiolytic-like behavior in rodents (Xu *et al.*, 2004; Jungling *et al.*, 2008; Leonard *et al.*, 2008), but failed to induce such effects in NPSR1 knockout animals (Duangdao *et al.*, 2009; Zhu *et al.*, 2010) or in the presence of NPSR1 antagonist (Okamura *et al.*, 2008; Camarda *et al.*, 2009; Ruzza *et al.*, 2010). Years of research from rodents have proved that the central NPS system is critically involved in the regulation of unconditioned and conditioned fear responses (Xu *et al.*, 2004; Jungling *et al.*, 2008; Meis *et al.*, 2008; Vitale *et al.*, 2008; Duangdao *et al.*, 2009). In humans, recent genetic studies also present a link between NPS system and panic disorder patients (Okamura *et al.*, 2008; Domschke *et al.*, 2010). Converging findings of NPS effects in rodents and humans, delivery of NPS into brain may have therapeutic potential in the treatment of anxiety disorders. However, the intracerebral NPS administration techniques employed in animal studies are scarcely applicable to human subjects. There are some other obstacles to the development of NPS and other peptides as CNS therapeutic agents, such as their inability to cross the blood brain barrier (BBB) and limited distribution to the blood volume following systemic administration (Pardridge, 2005). An alternative to systemic and invasive methods is the intranasal route of drug application, which is a noninvasive method of bypassing the BBB to deliver peptides and proteins to the brain (Thorne *et al.*, 2004; Domes *et al.*, 2010).

As NPS activates its cognate receptor, NPSR1, at low nanomolar concentration to stimulate mobilization of intracellular Ca^{2+} as well as activation of cAMP levels, it can be inferred that NPSR1 is coupled with Gq and Gs proteins for signal transduction mechanisms (Xu *et al.*, 2004; Reinscheid *et al.*, 2005). *In situ* hybridization studies have revealed that gene expression for both, NPS and its receptor can be detected found in the central nervous system and periphery tissues of rats (Xu *et al.*, 2004; Xu *et al.*, 2007). NPS mRNA is highly expressed in a few nuclei in the brainstem, such as the previously undefined cluster of cells located between the *locus coeruleus* (LC) and Barrington's nucleus, the principal sensory trigeminal nuclus, and the lateral

parabrachial nucleus (Xu *et al.*, 2004; Xu *et al.*, 2007). While NPS mRNA expression is limited in those few brain regions, NPSR1 mRNA is widely expressed throughout the nervous system, including the hypothalamic PVN, amygdala, *subiculum*, and various cortical regions (Xu *et al.*, 2007).

1.5. Attention-deficit hyperactivity disorder (ADHD)

According to the hyperactive phenotypes of LAB mice, the present thesis proposes this selected line as a mouse model of hyperactivity syndromes in some neuropsychiatric disorders, such as ADHD. ADHD is a heterogeneous neurobehavioral disorder affecting 2-7 % of school-age population. This disorder often occurs by the age of 7 years and is more prevalent in boys than girls. It is primarily characterized by inattention, hyperactivity, and impulsivity (Biederman, 1998). Although some argue that most cases of ADHD remit by adulthood, longitudinal studies have found that around two-thirds of ADHD children have ADHD symptoms as adults by showing a pattern of psychosocial disability, psychiatric comorbidity, neuropsychological dysfunction, familial illness and school failure (Faraone and Biederman, 2004). Recent evidences have also suggested that ADHD is associated with a range of cognitive deficits and social cognition impairments, which might be explained by fronto-striatal dysfunctions (Uekermann *et al.*, 2010).

1.5.1. Dopamine deficit hypothesis of ADHD

The catecholamine dopamine (DA) plays an important modulatory role in the central nervous system. DA in the brain is well known to influence a variety of behaviors such as locomotor activity, reward, and cognitive functions. In the clinic, the Dopaminergic changes are commonly proposed to be involved in the etiology of ADHD. It has been suggested that ADHD symptoms may be partially caused by deficits in the Dopaminergic system in cortical brain structures such as prefrontal cortex (PFC) (Sullivan and Brake, 2003) and basal ganglia such as the *nucleus accumbens* (NAc) and the *striatum* (Russell *et al.*, 1995). The three major Dopaminergic pathways, mesolimbic, mesocortical and nigrostriatal pathways are suggested to be involved in ADHD (Sullivan and Brake, 2003). There may be a dysregulation of the mesolimbic DA pathway

(from the ventral tegmental area to the nucleus accumben) in ADHD patients since they prefer to small and immediate rewards than larger and delayed ones (Sonuga-Barke *et al.*, 1992). The mesocortical DA pathway (from the ventral tegmental area to the cortical area) is also suggested to be associated with the symptoms of ADHD patients since it plays a role in selective attention and working memory. In addition to the aforementioned two pathways, the nigrostriatal pathway (from *substantia nigra* to *striatum*) is suggested to be involved in the pathogenesis of ADHD in relation to the hyperactivity and increased reaction times (Kadesjo and Gillberg, 1999).

1.5.2. Pharmacotherapy of ADHD

More than 70 years ago, Charles Bradley (Bradley, 1937) first observed that Benzedrine (a racemic mixture of d- and l-amphetamine) had a paradoxical calming effect on the behavior in hyperactive children. Since then, there are a plethora of studies testifying the paradoxical effects of the psychostimulants on the primary symptoms of ADHD. Nowadays, psychostimulants are still the most conventional treatment for ADHD (Solanto, 1998). Pharmacological studies have revealed that d-amphetamine increased general activity in adults, while exerting a paradoxical “calming effect” in ADHD patients (Greenhill, 1992). Similarly, administration of d-amphetamine at doses which increased the activity levels of normal animals induced a decrease in locomotor activity in several animal models of ADHD (e.g. DA transporter knockout (DAT-KO) mice) (Jones *et al.*, 1998a). D-amphetamine inhibits DA uptake produces facilitation of DA release into the synaptic cleft by acting on both vesicular storage of DA and directly on the DA transporter (DAT) (Floor and Meng, 1996; Sonders *et al.*, 1997).

In addition to amphetamine, both methylphenidate and atomoxetine are approved for the treatment against ADHD (Solanto, 1998; Gibson *et al.*, 2006). Methylphenidate increases the levels of DA and norepinephrine (NE) by blocking the DA and NE transporters (DAT & NET), whereas atomoxetine is a selective NE reuptake inhibitor (NRI) (Easton *et al.*, 2007). A recent single-photon emission computer tomography (SPECT) study has shown that 78 % of the striatal DAT was blocked after the administration of methylphenidate (Nikolaus *et al.*, 2005). Bymaster

et al. (Bymaster *et al.*, 2002) showed that systemic injection of methylphenidate, but not atomoxetine, increased the extracellular DA levels in the striatum and nucleus accumbens.

A call for new ADHD treatments is apparent because both amphetamine and methylphenidate are objects of drug-abuse neurotoxicity and other side unspecific effects in humans (Carter and Watson, 1994). Recently, it has been suggested that cannabinoid receptors have a role in normalizing motor activity in normal Wistar rats and DAT knockout mice (an animal model of ADHD) (Giuffrida *et al.*, 1999; Tzavara *et al.*, 2006). These investigations point to the endocannoabinoid system as a potentially important arena for drug discovery (Giuffrida *et al.*, 2001).

1.5.3. Animal models of ADHD

A variety of animal models of ADHD have been used to elucidate the molecular basis of DA disturbance in ADHD, for example, spontaneous hypertensive rats (SHR) (Sagvolden *et al.*, 1992), DA-depleted rats (Shaywitz *et al.*, 1976; Shaywitz *et al.*, 1978), and DA transporter (DAT) knockout mice (Gainetdinov *et al.*, 1999). Although animal models have numerous advantages (i.e. groups are more genetically homogeneous, the environment is easy to be controlled) compared to clinical cases, the study of human disease cannot be totally replaced by animal model exploration. Therefore, an optimal animal model is expected to be analogous to clinical cases in terms of etiology, biochemistry, symptomatology and treatment (McKinney and Bunney, 1969).

Sagvolden (Sagvolden, 2000) has proposed a list of criteria for assessing models of ADHD. First of all, an appropriate animal model of ADHD should mimic key characteristics of the disorder (face validity), such as 24-h hyperactivity in the habituated environment (Porrino *et al.*, 1983) and motor impulsiveness (Sagvolden and Sergeant, 1998). Another consideration is that this animal model should conform to a theoretical rationale for ADHD (construct validity), such as neurodevelopmental, or neurobiological background. The third criterion for good animal models is the ability to predict aspects of neuropharmacology of ADHD (predictive validity), such as similar behavioral responses to psychostimulants as observed in ADHD patients.

1.5.4. Microdialysis

Studies of the neuropharmacology, genetics and neurochemistry indicate that the neurobiological basis of ADHD is associated with alterations in the striatal Dopaminergic system (Solanto, 1998; Leo *et al.*, 2003; Russell *et al.*, 2005). The *in vivo* microdialysis is a well accepted method to measure extracellular neurotransmitter concentrations in the brain in freely moving animals. This technique is commonly used to explore neurochemical correlates to behavioral changes and to define the pharmacodynamics of drugs with relatively high spatial and temporal resolutions (Heal *et al.*, 2008). The majority of predictions about the actions of pharmacological drugs used in the treatment of ADHD have been based on results from *in vivo* microdialysis in various animal models of ADHD (Raber *et al.*, 1997; Gainetdinov *et al.*, 1999; Cheetham *et al.*, 2007). Therefore, it seems to be necessary to provide the neurochemical evidence that underlies animals' phenotypes and the pharmacological actions of ADHD in the HAB/LAB mouse model.

1.6. Scope of this thesis

Based on selective inbreeding for differences in EPM behavior, the two breeding lines can be regarded as mainly genetically distinct, providing a beneficial tool to identify genes responsible for pathologic alterations in human diseases. The two extreme HAB and LAB lines do not only show bidirectional trait anxiety, but also display tremendous difference in general locomotor activity. Therefore, a systemic survey was conducted on these anxiety-related and activity-based phenotypes in the HAB/LAB mouse model to reproduce and mimic distinct aspects of complex behaviors and neuropsychiatric diseases.

First, it was hypothesized that mice with a genetic predisposition to hyper-anxiety would have a tendency to develop phobic-like symptoms. To gain further insights into the interrelation between trait anxiety and development and maintenance of fearful memories, HAB, NAB/CD1 and LAB mice were tested for their responses in a set of fear conditioning and inhibitory avoidance paradigms. In addition, behavioral experiments were complemented by measurements of changes in protein kinase activity at level of the basolateral amygdala. In line

with the hyper-anxiety of HAB mice, a newly discovered anxiolytic peptide, NPS, was applied by using central infusion or noninvasive intranasal administration to prove its therapeutic function on pathological anxiety. In addition, the differences in brain NPS system were analyzed in HAB/LAB mice to determine whether the NPS system contributes to anxiety phenotypes.

Second, the low levels of trait anxiety seem to be associated with increased levels of exploratory behavior and arousal in LAB mice. Due to their excessive locomotion in the open field (OF), LAB mice were proposed as an animal model of ADHD. Behavioral phenotypes of LAB mice were systemically examined with respect to exploratory behavior in two OF tests, auditory startle and prepulse inhibition, spatial/habitual learning and social cognition. In addition, it was hypothesized that LAB mice may possess the same profile of behavioral responses to the level of psychostimulants as observed in ADHD patients. Therefore, the effects of various catecholaminergic and cannabinoidergic drugs were examined on locomotor activity. Also the effects of amphetamine on attention and cognitive abilities were evaluated in LAB compared to NAB mice. Finally, an in vivo microdialysis study was conducted to elucidate whether psychostimulants and endocannabinoid agents affect locomotor activity through modulation of striatal DA release.

2. Materials and Methods

2.1. Animals

Male CD1 mice used in this study were selectively inbred in the animal facilities of the Max Planck Institute (MPI) of Psychiatry as described previously (Kromer *et al.*, 2005). Briefly, > 250 animals from 25 litters of outbred Swiss CD1 mice purchased from Charles River (Sulzfeld, Germany), were used as starting point for selective and bidirectional breeding for extremes in anxiety-related behavior on the EPM. Males and females that spent either the least or most time on the open arms of the EPM were mated to establish the HAB and LAB mouse lines, respectively. The animals were routinely tested at the age of 7 weeks with HAB and LAB mice spending less than 15 % and more than 65 % of their time, respectively, on the open arms of the EPM. NAB mice were bred for intermediate anxiety-related behavior. As > 80 % of CD1 mice spent 30 % to 45 % of time on the open arms of the EPM, this range was chosen for the selection of NAB mice without any overlap with HAB or LAB animals. Data presented were obtained from animals from HAB/LAB generations 29-40 and NAB generations 6-10. All mice were single-housed in Makrolon type II cages (23 cm × 16.5 cm × 14 cm) 2 weeks prior to the experiments under standard laboratory conditions with reversed 12 h/12 h light/dark cycle (light on at 9 pm), temperature 23 ± 1 °C, and food and water *ad libitum*. Laboratory animal care and experiments were conducted in accordance with the regulations of the current version of the German Law and Animal Protection. Animal protocols were approved by the Government of upper Bavaria.

Different batches of HAB, NAB and LAB male mice bred at the MPI of Psychiatry were regularly tested in the EPM at the age of seven weeks and later subjected to various tests in the present thesis. The generation of animals used in the experiments was indicated in the legend of each figure. For example, Generation 37/7 refers to HAB and LAB mice of the 37^{th} generation and NAB mice of the 7^{th} generation. CD1 male mice purchased from Charles River were assigned into some experiments as control groups. HAB and NAB mice bred at the MPI of Biochemistry (Martinsried, Germany) were assigned to a few experiments due to the shortage of HAB and

NAB mice in MPI of Psychiatry. HAB and NAB mice from the two stocks share the same genetic background.

2.2. Elevated plus-maze (EPM) test

The test setup (Figure 3) has been described in detail previously (Kromer *et al.*, 2005; Bunck *et al.*, 2009). Briefly, the plus-shaped EPM is made of dark gray PVC and consists of two open (30 × 5 cm, 300 Lux) and two close arms (30 × 5 × 15 cm, 10 Lux) connected by a central platform (5 × 5 cm, 90 Lux). The plus-shaped platform was elevated 30 cm above the floor and surrounded by black curtain. In the beginning of each 5-min trial, the mouse was placed on the central platform facing one of the closed arms. During the 5-min test, the percentage of time spent on the open arms, the number of entries into the closed and open arms, and the latency to the first open arm entry were scored using the ANY-maze software (US Biotech, USA). Mice were considered to have entered an open or closed arm when both front paws and front shoulders were on the arm, and full entries (all four paws) also were counted. In the end of each test, the apparatus was cleaned with detergent-containing water and dried with tissue.

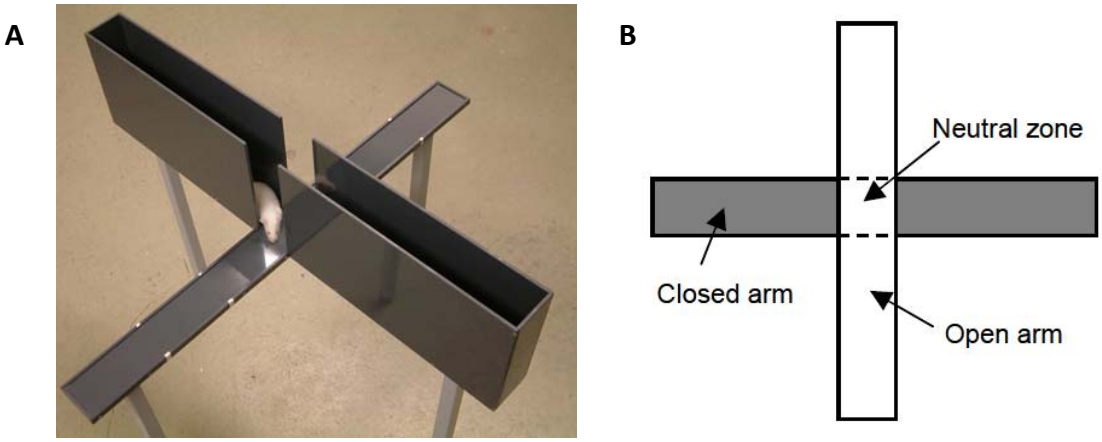


Figure 3: Elevated plus-maze (EPM) test. (A) EPM and (B) a schematic overview of the defined areas of the EPM used for the behavioral analysis.

2.3. Conditioned fear in a mouse model of extremes in trait anxiety

2.3.1. Red-light EPM test

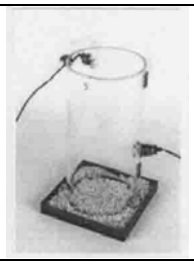
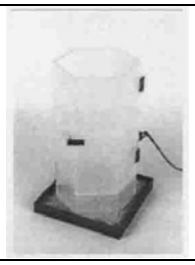
In the red-light EPM test, mice were tested in the EPM apparatus as described before (see 2.2.) with illumination of red light. The only difference between EPM and red-light EPM tests was testing light condition. The open arms were lit by red light of 20 Lux, the central platform by 10 Lux and the closed arms by 5 Lux.

2.3.2. Fear conditioning

The fear conditioning setup has been described and displayed in detail before (Kamprath and Wotjak, 2004). For fear conditioning, mice were placed into a cubic-shaped conditioning chamber with a metal grid for shock application, and the light was switched on (conditioning chamber, cf. Table 2). Three minutes later, a 20-s tone (CS: 80 dB, 9 kHz sine wave) was presented that co-terminated with a scrambled electric foot shock (US: 2 s, 0.7 mA). The conditioning procedure was repeated twice with inter-tone intervals of 30 s and 20 s, respectively. Animals were returned to their home cages 1 min after the last foot shock. To test for auditory-cued fear memory, mice were placed into a neutral test context (test context 1, cf. Table 2), which differed from the original conditioning context in shape, texture, bedding and odor. The house light was switched on and the tone presentation was started 3 min later. Mice were returned to their home cages 1 min after termination of tone presentation. To test for the intensity of contextual fear memory, mice were placed back in the conditioning context for 3 min. The specificity of contextual fear (Fanselow, 1980) was assessed by exposing the animals to a grid context, which differed from the shock context in shape, texture and odor, except for the presence of the grid floor (test context 2, cf. Table 2). The freezing response in the grid context was sought to serve as a measure of pattern completion/pattern separation. Pattern separation/completion is the ability to recall a stored representation when cued by a partial or degraded observation of the stimulus. In addition, we compared freezing responses in the conditioning context with baseline freezing during the 3 min preceding the tone presentation in the test context as a measure of context generalization.

In the fear conditioning experiments, the behavior of animals was videotaped for subsequent off-line behavioral analyses. Freezing was defined as the absence of body movements except for those related to respiration (Fanselow, 1980). Freezing responses to CSs were measured as described (Kamprath and Wotjak, 2004; Plendl and Wotjak, 2010) and expressed as percentage of the observation time (freezing [%] = freezing time/observation time × 100 %). Since thefear extinction experiment focused on between-session extinction, we analyzed the development of the freezing response to the initial CS presentation per day over the course of extinction training (Plendl and Wotjak, 2010).

Table 2: Detailed description of the three different contexts in the fear conditioning experiments. (Kamprath and Wotjak, 2004)

	Conditioning Chamber	**Test Context**	**Grid Context**
Picture			
Shape	Cubicle	Cylinder	Hexagonal Prism
Symbol			
Walls	Aluminum/Transparent Plexiglas	Transparent Plexiglas	Non-transparent Plexiglas with rough surface
Floor	Metal grids	Sawdust (same as in home cages)	Metal grids
Cleaning/order	70 % EtOH	1 % CH3COOH	1:2000 Isoamylacetate
Illumination	House light (0.6 Lux)	House light (0.3 Lux)	House light (0.3 Lux)
Dimensions	L19 × W14 × H30 cm^3	∅15 × H30 cm^3	L15 × W13 × H30 cm^3

2.3.3. Step-down avoidance

The apparatus (Figure 4) consisted of a metal grid floor (23 × 21 cm^2, 42 metal bars 3 mm in diameter, spaced apart 5 mm) inserted in a transparent Plexiglas box (L25 × W25 × H50 cm^3). The cage was illuminated with a 30 W lamp during the test resulting in ~300 Lux at floor level. A plastic platform (L10 × W10 × H2.5 cm^3) was placed on the center of the metal grid floor.

Electric shocks (0.7 mA, 2s) were delivered through the grid floor by a programmable animal shocker (San Diego Instruments, San Diego, CA, USA). The test consisted of a training session and a retention session 1 day and 7 days after training. During the training session, each mouse was placed on the platform and surrounded by a Plexiglas cylinder. After 60 s of adaptation, the cylinder was removed and an electric shock was delivered as soon as the mouse stepped down with four paws on the grid floor. The retention session was performed in a similar manner, except that no electric shock was applied. Briefly, each mouse was placed again onto the platform, and 10 s later, the cylinder was removed and the step-down latency was recorded. There was no cut-off time of step-down latency and step-down latencies were measured on-line by means of a stop watch. Animals were removed from the grid in the end of the test, and platform and grid were cleaned with detergent-containing water and dried with tissue.

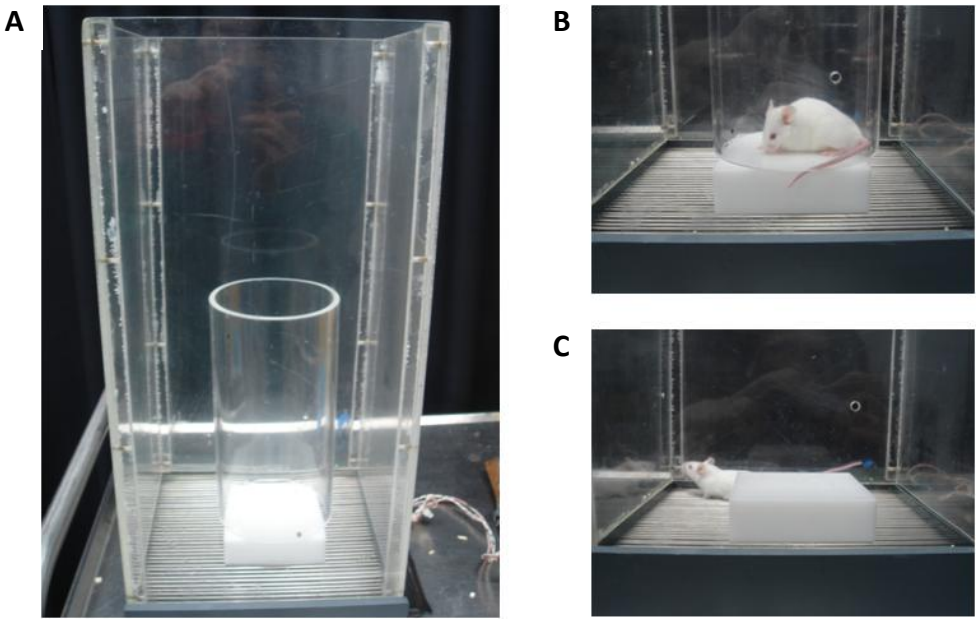


Figure 4: Step-down avoidance test. (A) Experimental setup. (B) The mouse was placed on the platform and surrounded by a Plexiglas cylinder. (C) The cylinder was removed and the step-down latency was recorded.

2.3.4. Shock sensitivity

To test for shock sensitivity, acoustic startle responses were measured essentially as described before (Golub *et al.*, 2009). In brief, mice were tested in one out of eight identical startle set-ups, consisting of a non-restrictive Plexiglas cylinder (inner diameter 4 cm, length 8 cm) mounted onto a plastic platform, each housed in a sound attenuated chamber (SR-LAB, San Diego Instruments SDI, San Diego, CA, USA; Figure 5). The cylinder movements were detected by a piezoelectric element. The voltage output of the piezo was amplified and then digitized (sampling rate 1 kHz) by a computer interface (IO-board provided by SDI). Before startle measurements, we calibrated response sensitivities for each chamber in order to assure identical output levels. Startle stimuli and background noise were delivered through a high-frequency speaker placed 20 cm above each cage. Sound pressure level (SPL) was measured using an audiometer (Radio Shack, 33-2055, RadioShack, Fort Worth, TX, USA). Plexiglas cylinders were cleaned thoroughly with soap water after each trial.

To test shock sensitivity, the response to tone-shock pairings was measured in the startle apparatus. Mice were placed into the startle apparatus with a continuous 50 dB background noise. After a 5-min adaptation to the startle chamber, 10 pairings of 20 s-tone (9 kHz, 80 dB(A)) and foot shocks (1 s, 0.7 mA) were presented with inter-stimulus intervals of 30-160 s. In the shock sensitivity test, piezoelectric accelerometers mounted under the Plexiglas platforms transduced the movements of animals, which were digitized and stored by an interface and computer assembly. Startle amplitude was taken as the highest voltage during a time window of 20 ms or the average voltage during the whole response window (tone/shock presentations). Startle amplitudes were analyzed using SR-Lab Utilities (SR-LAB, San Diego Instruments, San Diego, CA, USA).

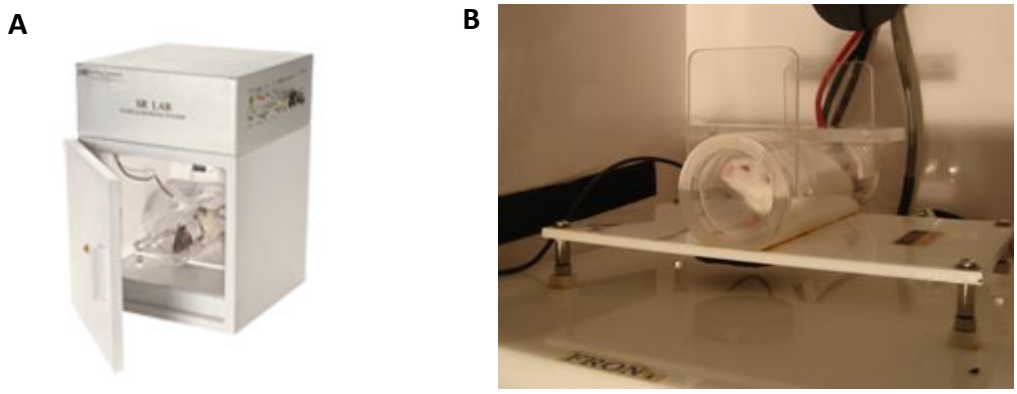


Figure 5: Startle measurements. (A) A startle apparatus and (B) the setup for measurements of startle response.

2.3.5. Western blot analysis

Mice were anesthetized with isoflurane (DeltaSelect, Germany) 45 minutes after foot shock (or the respective time in the home cage), and rapidly killed. To minimize stress levels and unspecific context reminders, mice were individually transferred to the neighboring room, anesthetized with isoflurane followed by cervical dislocation within 2-3 min after removal from the animal room. Brains were extracted on ice, snap-frozen and kept at -80°C. For brain dissection, brains were cut with a cryostat (Microm, Walldorf, Germany) up to the appearance of the amygdala. Brain specimens were isolated using cylindrical punchers (Fine Science Tools, Heidelberg, Germany). The location and diameter of the punches were chosen on basis of a stereotaxic atlas (Paxinos and Franklin, 2001) as follows. Punches started 0.8 mm posterior to bregma, with a diameter of 1.0 mm and a punch-length of 1.0 mm in order to collect the anterior part of the lateral/basolateral amygdaloid nucleus. Punches of both hemispheres were pooled per mouse and stored at -80°C until processing for Western blot analyses. The dissection site was verified by histological analyses using a stereomicroscope.

Western blot experiments have been described in detail before (Dahlhoff *et al.*, 2010). Briefly, protein was prepared from the amygdala specimens by homogenization in extraction buffer and samples with equal concentrations were electrophoresed on 12 % polyacrylamide-sodium dodecyl sulfate gels and then blotted to PVDF-membranes (GE Healthcare, Munich, Germany). Incubation with primary antibodies was carried out overnight at 4°C. Following antibodies and

dilutions (either in 5 % dry milk or in 5 % BSA) were used: rabbit anti-phospho-p44/42 mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) (Thr 202/Tyr 204; 1:2000; #9101; Cell Signaling), rabbit anti-p44/42 MAPK/ERK (1:2000; #9102; Cell Signaling), rabbit anti-phospho-calcium/calmodulin-dependent protein kinase II (CaMKII) (1:2000; #3361; Cell Signaling), rabbit anti-CaMKII (1:2000; #3357; Cell Signaling), rabbit anti-phospho-protein kinase B (AKT) (Ser 473; 1:2000; #9271; Cell Signaling), rabbit anti-AKT (1:2000; #9272; Cell Signaling), rabbit anti-phospho-GSK-3β (Ser 9; 1:2000; #9336; Cell Signaling), rabbit anti-glykogen synthase kinase 3 (GSK-3β) (1:2000; #9318; Cell Signaling), mouse anti-β-catenin (1:5000; #610154; BD Transduction Laboratories), and rabbit anti-GAPDH (1:5000; #2118; Cell Signaling). Appropriate horseradish peroxidase-conjugated secondary antibodies were used. Immunoreactive bands were visualized by chemiluminescence with ECL kit (GE Healthcare). Band intensities were quantified using the ImageQuant software package (GE Healthcare). For measurement of kinases and transcription factor activities, pERK, pAKT, pGSK-3β, pCREB and β-Catenin bands were densitometrically analysed followed by normalization to the corresponding total ERK, AKT, GSK-3β, CREB or GAPDH levels. Specimens from shocked *vs.* non-shocked LAB (blot 1) and shocked *vs.* non-shocked HAB (blot 2) were analyzed in different blots. The expression levels of each conditioned mouse were normalized to the mean expression levels obtained in non-shocked controls of the same line from the same blot.

2.4. NPS: from anxiolytic effects to molecular characterization in a mouse model of extremes in trait anxiety

2.4.1. EPM test

To investigate the effects of central and intranasal NPS, the EPM was performed as described above (see 2.2.).

2.4.2. Open field (OF) tests

A 30-min OF (OF-30) test was performed to verify the anxiolytic effects of central NPS by using the TruScan Photo Beam Activity system (Coulbourn Instruments, Whitehall, PA, USA) as described previously (Jacob *et al.*, 2009). Mice were induced into the center of a Plexiglas cage

(L26 × W26 × H38 cm^3) for 30-min testing. Each test cage, including the sensor rings, was surrounded by a box made of opaque Plexiglas side walls (L47 × W47 × H38.5 cm^3). Horizontal locomotion (i.e. distanced travelled) was automatically recorded by 2 photobeam sensor rings (2 cm and 5 cm above the floor; photobeams are spaced apart by 1.52 cm providing a 0.73 cm spatial resolution). The animals' DT (DT) (m) and center time (s), as indicators of locomotion and anxiety, respectively, were automatically recorded and analyzed by TruScan Software Version 1.1 (Coulbourn Instruments). Center time was defined as the time spent in the center zone, a region that is more than 2.5-beam (3.8 cm) space from the walls (Figure 6B).

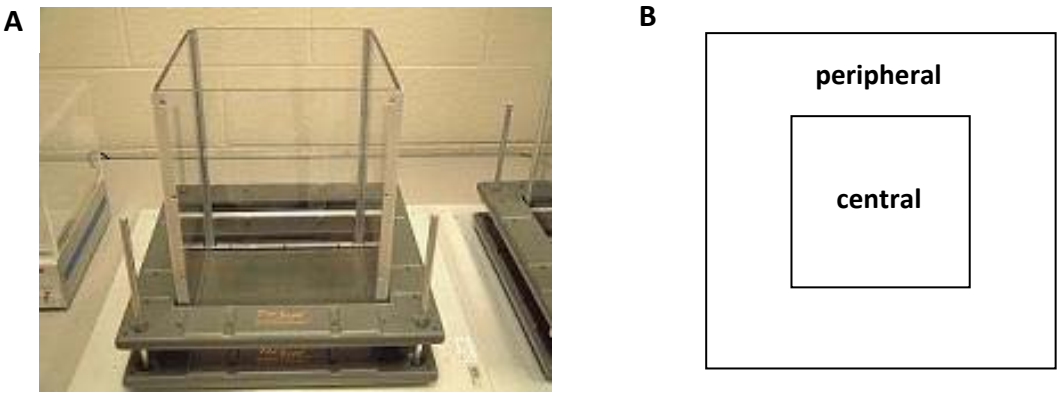


Figure 6: Open field test. (A) Experimental setup and (B) schematic overview of the central and the peripheral zones of the open field apparatus.

Another type of OF test was conducted as one test of the behavioral assay to evaluate the effects of intranasal NPS application. The OF test was described in detail previously (Bunck *et al.*, 2009). The round OF (diameter: 60 cm) was made of black gray PVC and visually divided into a central (diameter: 30 cm) and a peripheral zone to quantify the time spent in each area (Figure 7). The arena was illuminated with dim light of 60 Lux.

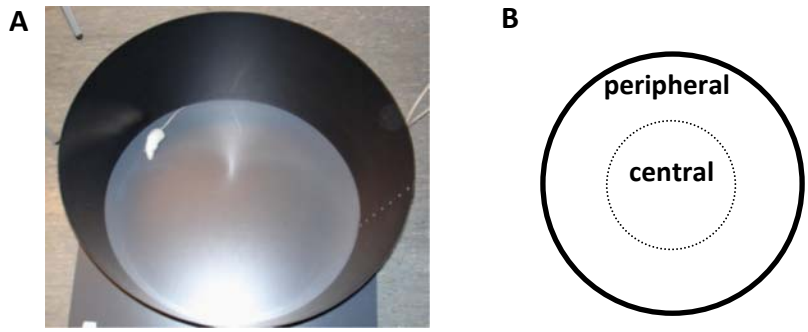


Figure 7: Open field test. (A) Experimental setup and (B) schematic overview of the central and the peripheral zones of the open field apparatus.

2.4.3. Dark-light avoidance

The dark-light avoidance test was used as one of the serial tests to evaluate the effects of intranasal NPS. The test setup has been described in detail previously (Kromer *et al.*, 2005; Bunck *et al.*, 2009). Briefly, the experimental apparatus consists of one light (29 × 20 × 26 cm^3, 650-700 Lux) and one dark (15 × 20 × 26 cm^3, 13 Lux) compartment connected via a small opening (5 × 7 cm^2) allowing transitions between the compartments (Figure 8). In each compartment, the floors were divided into 6.5 × 6.5 cm^2 squares. During the 5-min test, the percent of time an animal spent in the lit compartment is assessed as an indicator of anxiety. In addition, line crossings, transitions and vertical exploration (i.e. number of rearings) were also scored.

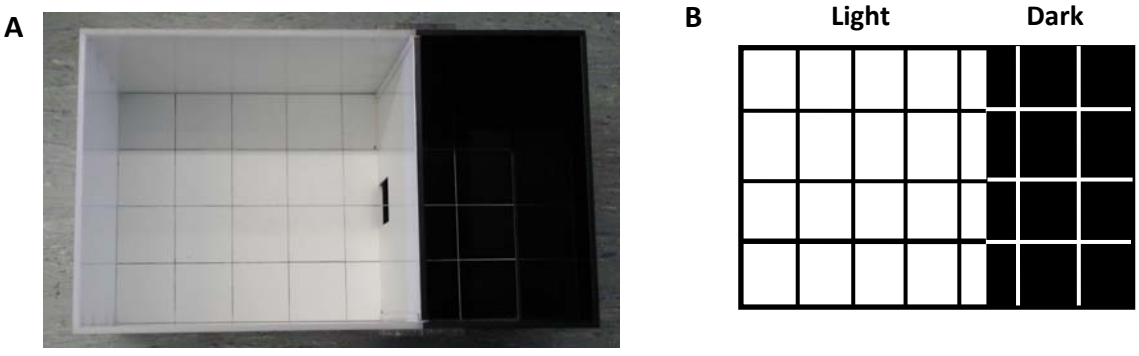


Figure 8: Dark-light avoidance test. (A) Experimental setup and (B) schematic overview of the dark and the light compartments of the behavioral test, which are connected by a small opening.

2.4.4. Behavioral assay

To test the effects of intranasal application of NPS, behavioral tests including OF, dark-light avoidance and EPM test were preformed sequentially. Each test lasted for 5 min with an inter-test interval of 5 min. Mice were tested twice in the behavioral assay 30 min and 4 h after the first intranasal application. The animal's behavior in the behaviour tests was videotaped during testing and relevant parameters were analyzed with the tracking software ANY-maze version 4.30 (Stoelting, Wood Dale, IL, USA).

2.4.5. Surgery, i.c.v. (intracerebroventricular) implantation

To investigate the effects of central NPS infusion on anxiety-related behavior, mice were implanted with an indwelling guide cannula as described previously (Kessler *et al.*, 2010). All surgical procedures were performed using isoflurane (CuraMED Pharma GmbH, Karlsruhe, Germany) anesthesia under semi-sterile conditions. Mice were fixed in a stereotaxic apparatus (TSE Systems Inc., MO, USA), anesthetized with combination of 2 % v/v isoflurane in O_2, injected intraperitoneally with 0.5 mg/kg Metacam® (Boeringer Ingelheim, Germany), and placed on a feedback controlled heating pad (37°C) (Harvard Apparatus, MA, USA). Briefly, for i.c.v. (intracerebroventricular) administration in mice, a 23-gauge guide cannula was implanted 1.5 mm above the right lateral ventricle (AP: -0.3 mm; ML: +1.1 mm from bregma; DV: -1.6 mm from the surface of the skull; Figure 9) (Paxinos and Franklin, 2001). The guide cannula was anchored to the skull by two stainless-steel skull screws (M1*3, Schrauben Preisinger, Germany) and dental cement (Kallocryl CPGM rot, Speiko - Dr. Speier GmbH, Münster, Germany).

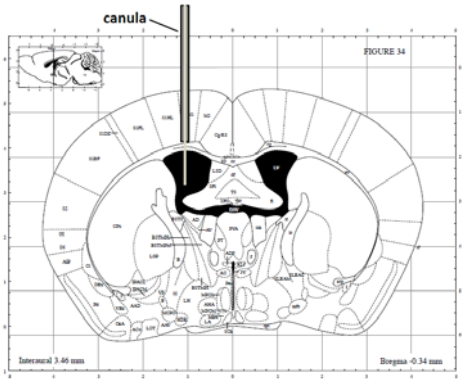


Figure 9: Schematic overview of intracerebroventricular (i.c.v.) implantation. Coronal brain section demonstrating the loci of guide canula and injection site.

2.4.6. Drug treatment

For central administration, mice were infused i.c.v. with 1.0 nmol of Neuropeptide S (rat) (Bachem GmbH, Weil am Rhein, Germany) or vehicle (Ringer solution). The injection volume was 2 µl per animal. For intranasal application, the mice were caught by the experimenter in a supine position, with the head supported at a 45 angle to the body (van den Berg *et al.*, 2002). For each intranasal application, NPS (0.5 nmol/µl) or vehicle (Ringer solution) were administered intranasally to the alert mouse with a total volume of 14 µl. 7 µl were applied to each nostril on an alternating basis, with a 5-min rest period between the administrations to the nostrils.

2.4.7. Histology/guide cannula verification

Mice were anesthetized with isoflurane (DeltaSelect, Germany), i.c.v. injected with ink and rapidly killed. Brains were removed and cut along the guide cannula to verify the guide cannula's correct anatomical position. Only if the cannula placement was found in the targeting area the respective data were included for further analysis.

2.4.8. Determination of *Nps* and *Npsr1* mRNA expression levels

Separate groups of male HAB and LAB mice were sacrificed under basal conditions and isoflurane anesthesia and the brains snap-frozen in N-methylbutane (Carl ROTH GmbH, Karlsruhe, Germany) and stored at -80°C. Total RNA was then extracted from the PVN, amygdala and peri-LC area tissue punches (2 x 500 µm punches for the amygdala complex and peri-LC area, 1 x 1000 µm punch for the PVN; Figure 10) and cDNA prepared as described previously (Bunck *et al.*, 2009). Next, based on the manufacturer's instruction for the QuantiFastSYBR Green Kit (Qiagen, Hilden, Germany) quantitative real-time polymerase chain reaction (PCR) was performed on Light Cycler 2.0 equipment (Roche Diagnostics, Mannheim, Germany).

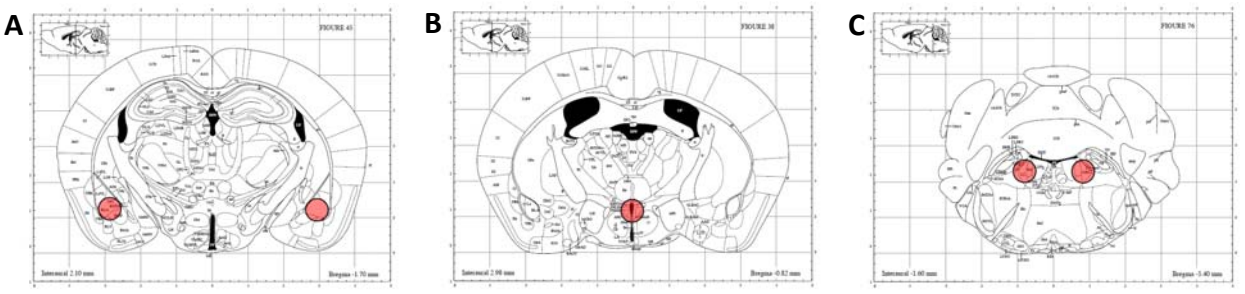


Figure 10: Overview of anatomical location of the brain regions investigated in the real-time PCRs. Red circles indicate the (A) (basolateral) amygdala (B) paraventricular nuclei (PVN) (C) peri-*locus coeruleus* (LC).

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 Table 3.

Table 3: Primer sequences used as housekeeping genes and *Nps* and *Npsr1* genes.

Gene	Primer Sequence (5'→3')	T_m	Product size(bp)
Housekeeping genes			
Rpl13a f	CACTCTGGAGGAGAAACGGAAGG	56.57	182
Rpl13a r	GCAGGCATGAGGCAAACAGTC	56.63	
B2mg f	CTATATCCTGGCTCACACTG	49.01	130
B2mg r	CATCATGATGCTTGATCACA	48.04	
Target genes			
Nps f	TGGTGTTATCCGGTCCTCTC	52.66	147
Nps r	GGACCTTTTCATCGATGTCT	49.41	
Npsr1 f	CTCTTCACTGAGGTGGGCTC	53.94	196
Npsr1 r	CCAGTGCTTCAGTGAACGTC	53.49	

2.4.9. *Nps* and *Npsr1* sequencing

Genomic DNA was extracted from cerebellar tissue or tail tips of HAB and LAB mice using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/ primer-blast) was used to design sequencing primers that were then ordered from Sigma-Aldrich (Chemie Gmbh, Munich, Germany). Primers were designed to cover about 2 kbp of the gene promoters and all exons (3 exons for Nps and 10 exons for Npsr1).

The *Npsr1* and *Nps* DNA fragments were amplified using Taq-polymerase (Fermentas, St. Leon Rot, Germany) with a cycling protocol as indicated below. 1 µl DNA and 1.5 µl of the respective primer were added in the PCR tubes (Thermofisher Scientific, Dreieich, Germany).

Supplemental table 1 and 2 provide the list of primers used for sequencing of the *Nps* and *Npsr1* genes in HAB/LAB mice. The cycling protocol was described as follows:

1. Initial denaturation for 4 min at 94°C
2. Amplification
   Denaturation for 1 min at 94°C × 35 cycles
   Annealing for 1 min at 56°C × 35 cycles
   Elongation for 1 min at 72°C × 35 cycles
3. Final elongation for 10 min at 72°C

PCR products were analyzed by agarose gel electrophoresis (1% agarose, Sigma Aldrich, Taufkirchen, Germany).

2.4.9.1. Cycle Sequencing

15-20 µl of the PCR product was transferred to a Nucleofast 96 PCR plate (Macherey-Nagel), centrifuged and then washed twice with water. Cleaned up plates were centrifuged at room temperature, 4500 g for 10 minutes. Then, samples were redissolved in 25 µl distilled water on a shaker for 10 minutes. 2.4 µl of cleaned up PCR products were used for sequencing using the Big Dye Terminator kit v3.1 (ABI Applied Biosystems, Foster City, CA, USA) and cleaned up with Millipore (Billerica, CA, USA) on a vacuum pump, and sequences were analyzed by capillary electrophoresis on a 3730 DNA analyzer (Applied Biosystems). Sequences were analyzed performed using BioEdit V 7.0.2 (Tom Hall, Ibis Therapeutics, CA, USA).

2.4.9.2. Nps sequence

For sequencing *Nps* (Figure 11), 3 exons of the unspliced transcript, ~1300 bp of the promoter and ~700 bp of DER were analyzed. The respective primers are described in Supplementary table 1. All amplified sequences were analyzed in the sequencing reaction using the indicated primers in a nested PCR reaction.

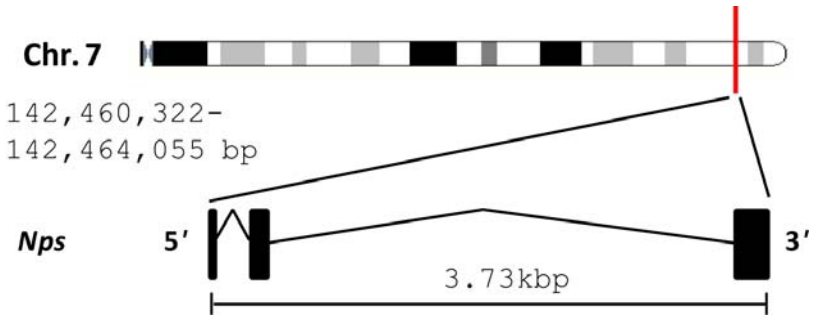


Figure 11: Neuropeptide S (*Nps*) gene. The *Nps* gene is located on mouse chromosome 7. Exons are identified by black filled boxes, and spliced introns are indicated as angled lines. Figure is based on data from Ensembl (http://www.ensembl.org, 19.11.2010).

2.4.9.3. Npsr1 sequence

For sequencing *Npsr1* (Figure 12), 10 exons of the unspliced transcript, ~2500 bp of the promoter and ~400 bp of DER were analyzed. The respective primers are described in Supplementary table 2. All amplified sequences were analyzed in the sequencing reaction using the indicated primers in a nested PCR reaction.

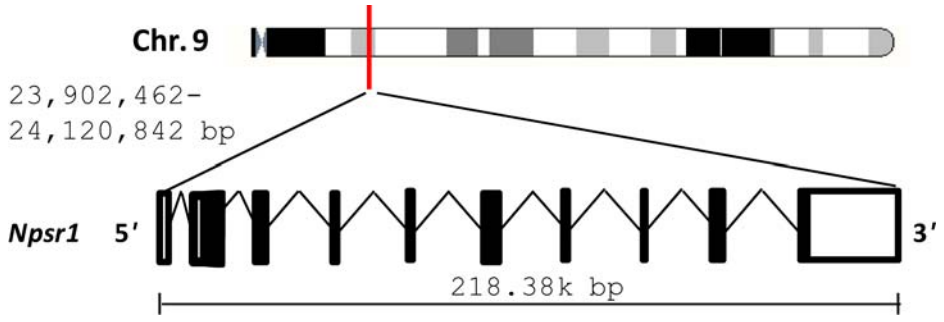


Figure 12: Neuropeptide S receptor 1 (*Npsr1*) gene. The *Npsr1* gene is located on mouse chromosome 9. Exons are indicated by black boxes, filled parts refer to translated, unfilled to untranslated regions. Spliced introns are indicated by angled lines. Figure is based on data from Ensembl (http://www.ensembl.org, 30.10.2009).

2.4.9.4. Assessing the effects of polymorphisms

All Single-nucleotide polymorphisms (SNPs) and other polymorphisms identified by sequencing were analyzed if they were located in the exons or at exon borders, if they influenced the amino acid sequence or if they were located in the promoter or the downstream enhancing region (DER). *Npsr1* was then screened for transcription factor binding motifs via the Transcription Element Search System (TESS; Schug, 2008). Putative binding sites of transcription

factors were additionally checked for their function and occurrence within the brain according to the NCBI database (http://www.ncbi.nlm.nih.gov/) (only for known murine transcription factors).

2.4.9.5. CpG island searcher

The CpG island is a short stretch of DNA in which the frequency of the CG sequence is higher than in other regions. Precisely, CpG islands are defined as a region with at least 200 bp and with a GC percentage that is higher than 50 percent and with observed/expected CpG ratio that is higher than 55 percent. The ratio observed/expected CpG (Obs/Exp) was calculated as follows: Obs/Exp CpG = (Number of CpG/number of C $\times$ number of G) $\times$ N (Gardiner-Garden and Frommer, 1987). At these locations, the CpG sequence is not methylated. By contrast, the CpG sequences in inactive genes are usually methylated to suppress their expression (Tate and Bird, 1993). The CpG island searcher is a program which screens for CpG islands which meet these criteria in the submitted DNA sequence (Takai and Jones, 2002).

2.4.9. Histology/guide cannula verification

Mice were anesthetized with isoflurane (DeltaSelect, Germany), i.c.v. injected with ink and rapidly killed. Brains were removed and cut along the guide cannula to verify the guide cannula's correct anatomical position. Only if the cannula placement was found in the targeting area the respective data were included for further analysis.

2.5. LAB mice: Towards an animal model of ADHD (behavioral phenotyping and pharmacological validation)

To test startle response in input/output (IO) curve and prepulse facilitation/prepulse inhibition (PPI/PPF) tests, mice were tested in the startle apparatus as described before (see 2.3.4.).

2.5.1. Input/Output (IO) curve test

In IO curve test, we used acoustic pulses of different intensities to induce animals' startle performance. Briefly, mice were placed into the startle apparatus with a continuous 50 dB background noise. Following an adaptation of 5 min, 136 startle trials were presented with an

inter-trial interval of 13-17 s. The intensities were 75, 90, 105, and 115 dB of white noise with 30 startle trials at each level in a pseudorandom order. Under background noise, the animals' startle responses were recorded as well for 16 times.

2.5.2. Prepulse inhibition/Prepulse facilitation (PPI/PPF) tests

PPI refers to the inhibition of a startle reflex produced by preceding the startling stimulus, or pulse, with a weak prepulse stimulus. Similarly, PPF refers to the facilitation of reflex produced by a prepulse. PPI and PPF depend on the duration of the lead interval. Plappert *et al.* (Plappert *et al.*, 2004) found facilitation of the startle response for lead intervals below 37.5 ms and inhibition for greater intervals (30-500 ms; optimal: 50-100 ms).

The session began with placing the animals into the Plexiglas enclosure. Mice were acclimated to the apparatus for 5 min before the first trial began. The first 20 trials consist of 20 startle pulses (white noise 115 dB) which served to habituate and stabilize the animals' startle response and were not included in the analysis. Each session consists of the following: 22 pulse-alone trials (115 dB), 210 prepulse (PP)-condition trials, and 18 prepulse-alone trials. The 250 discrete trials were presented in a pseudorandom order, with a variable inter-trial interval of a mean of 15 s (ranging from 13-17 s). 15 different prepulse-condition trials were presented, each for 14 times. Three different prepulse intensities were adopted (55, 65, or 75 dB white noise) with an interpulse interval (IPI, between onsets of the prepulse and pulse) of 5, 10, 25, 50 or 100 ms. The duration of the prepulse was 10 or 5 ms when the IPI was 5 ms. For prepulse-alone trials, three prepulse were presented alone, each for six times.

2.5.3. OF and hole-board (HB) tests

OF and hole-board (HB) tests were preformed using the TruScan Photo Beam Activity system (Coulbourn Instruments, Whitehall, PA, USA). For the OF test, mice were induced into Plexiglas cages as described previously (see 2.4.2.) for 80- or 90-min testing (OF-80 or OF-90). Horizontal locomotion (i.e. distanced travelled), vertical activity (i.e. the number of rearing) and immobility time were automatically recorded.

For pharmacological treatment(s) during an experiment run, the OF test was interrupted by the protocol with pause(s) and continued by pressing the “start” bar right after drug injection(s).

For HB test, additional nose poke floors were inserted into the OF Plexiglas cages (Figure 13A). Each nose poke floor contains 16 holes (4 × 4 arrays) with 16 corresponding underlying food trays (Figure 13B). At the beginning of HB test, animals were placed onto the center of the nose poke floor. Because TruScan software records coordinates by sensor rings, it is possible to analyze how many holes are accessed as well as the sequence in which the holes are accessed. The accuracy of performance is defined as the percent numbers of 16 holes which had been visited at least one time over the course of exposure, and it is calculated as [(sum of visited holes/16)*100 %].

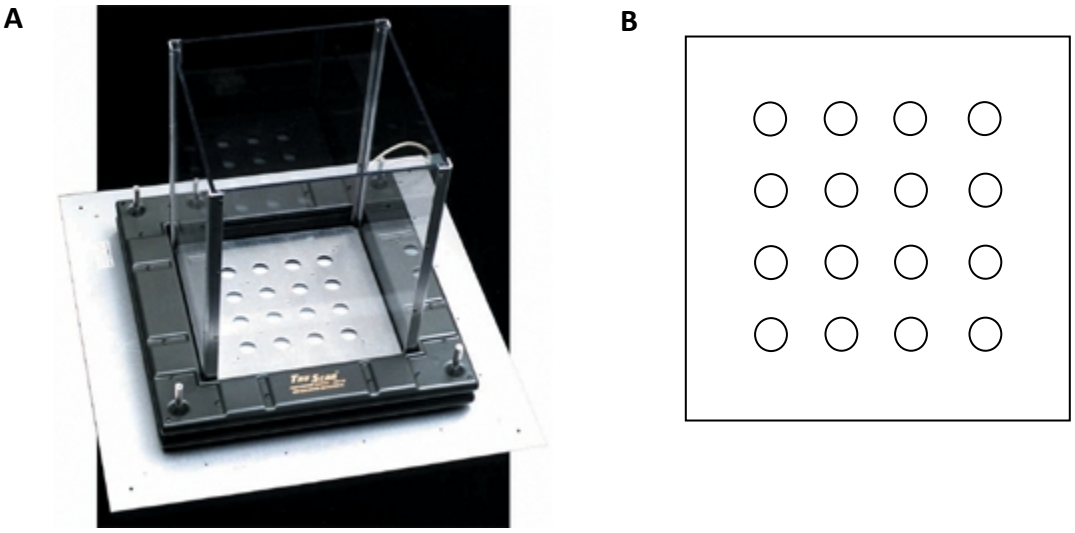


Figure 13: Hole-board (HB) test. (A) A HB apparatus and (B) schematic overview of a nose poke floor.

2.5.4. Social recognition tests

2.5.4.1. Social preference test

The social behavior test was conducted in a rectangular box made of white PVC walls and a black PVC floor. The box was separate into three compartments (L30 × W30 × H30 cm^3 each) which were connected by two opening doors (6 × 5 cm^2). The social approach behaviors were performed using essentially the same procedures methods as previously described (Moy *et al.*,

2004; Nadler *et al.*, 2004; Crawley *et al.*, 2007). In brief, the test was consisted of four 10-min trial sessions (Figure 14). During the first 10 min, mice were placed into the center compartment with both doors closed, in order to familiarize the subject mouse with the testing environment and the center compartment. During the next 10 min, the doors were open and the subject mouse could habituate to three compartments and two empty perforated 50 ml plastic tube (SARSTEDT AG & Co., Nümbrecht, Germany) located in the center of side compartments. The next 10 min served as a sampling session, furing which one empty tube was removed and replaced by an ovariectomized female (F1) mouse in an identical tube. This 10 min session was designed to see difference between the sniffing time spent in social stimulus and non-social stimulus. The last 10 min period was the testing session, during which the other empty tube was removed and replaced by a novel ovariectomized female (F2). This session was designed to test the ability of the subject mouse to distinguish between two female mice.

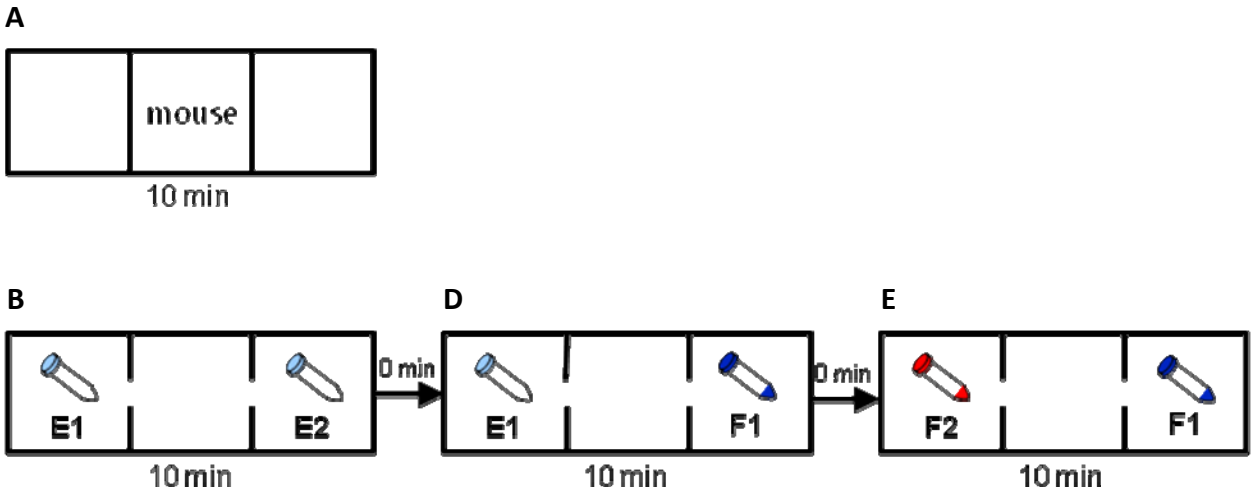


Figure 14: Social preference test. The test was conducted in a 3-chamber apparatus. (A) Adaptation: the subject mouse was kept in the center chamber with both doors closed. (B) Habituation phase: two empty tubes were placed in the left and the right chamber (E1, E2). (C) Sampling phase: one empty tube was replaced by a tube containing a female mouse (F1). (D) Testing phase: the second empty tube was replaced by a tube containing a second female (F2).

2.5.4.2. Social discrimination test

The social discrimination test was established in rat studies (Engelmann *et al.*, 1995) and was also applied in mice (Richter *et al.*, 2005). Here we modified this procedure by using

ovariectomized females in empty tubes which were immobile over the cross of testing. Figure 14B depicts the experimental procedures. After being transferred to the experimental cage (L25 × W22 × H38 cm^3) with an empty perforated 50 ml plastic tube (SARSTEDT AG & Co., Nümbrecht, Germany) for 60 min of habituation, the subject animal was introduced to the first stimulus animal, protected in a perforated plastic tube for five min. After IEI (interexposure interval) of 15 min, 30 min, 2h or 4 h respectively, the first (F1) ovariectomized female was reintroduced for five min to the test mouse together with a novel (F2) stimulus animal (also in a plastic tube). According to Engelmann *et al.* (1995), a significantly increased olfactory investigation of the novel stimulus female during the second exposure was taken as a parameter of the animals' social discrimination ability.

All social experiments were performed between 8 am and 12 pm and videotaped for later analysis. The duration of olfactory investigation towards the respective stimulus animal in both sessions was quantified by an observer blind to the genotype using the computer software Eventlog 1.0 (EMCO Software, Eden Prairie, MN, USA). The total investigation time during the first exposure was quantified to exclude nonspecific effects on learning due to group differences.

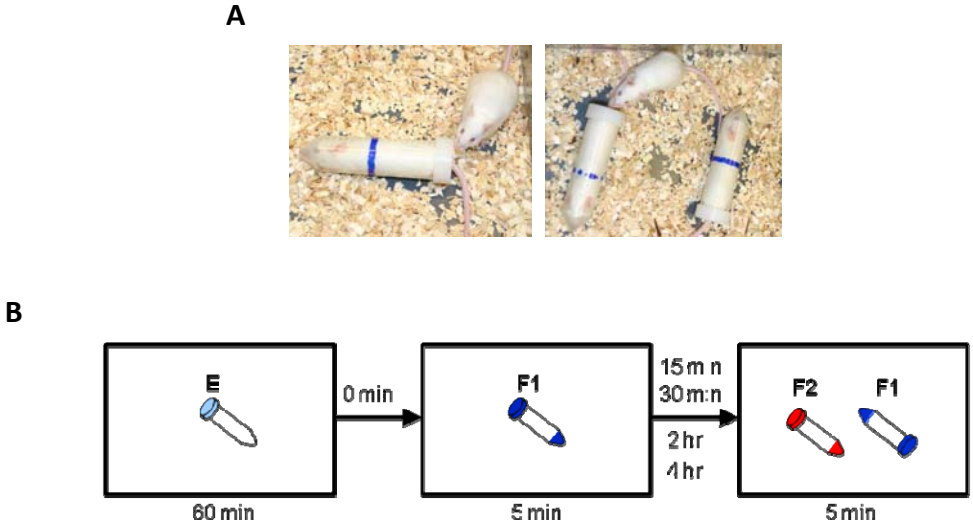


Figure 15: Social discrimination test. (A) Real experimental setup and (B) diagram of experimental procedures: after a 60-min habituation to the experimental cage with an empty tube (E), a female mouse (F1) was introduced to the subject mouse for 5 min. After different inter-exposure intervals (IEIs), the first mouse (F1) and a novel female mouse (F2) were introduced to the subject animal for another 5-min exposure.

2.5.5. Water cross-maze (WCM)

The water cross-maze (WCM, custom made, MPI of Psychiatry, Germany; Figure 16A) is made of 1-cm thick transparent Plexiglas and consists of four identical arms (50 × 10 cm^2, 30 cm height; corresponding to North, East, South, and West arms in clockwise order). The maze was filled with fresh tap water at 23 °C before testing and a Plexiglas platform (9 × 9 cm^2, 10 cm height) was located at the end of West or East arms depending on the testing phases. The top of the platform was submerged 1.5 cm below the water surface. The testing room was illuminated by indirect spectrum light of four lamps, resulting in light intensity of 20 Lux at the upper edge of the maze and 14 Lux at the level of water surface. The room contained sufficient distal visual cues (i.e. sink, small grey cabinet etc.) for animals to orient during the test. In each testing trial, after placing animals into the maze, the experimenter consistently stood at the same position (the end of South arm) to avoid altering the testing environment.

The animals were trained in groups of six for six trials per day with equal inter-trial intervals (ITIs) of 10 min in free learning (FL) protocol (Figure 17). The FL protocol is a dual-choice protocol, which allows animals to solve WCM by using either spatial-allocentric or response-egocentric strategies. In the first week of 4- or 5-day training, the North arm was always blocked by a partition during testing and animals were trained to swim from the end of South arm and navigate the platform at the end of West arm. In the second week of reverse training, the platform was removed to the East arm, and the animals started from the same position (South arm) but need to shift their navigation from the West to the East arm. In each trial, the mice were allowed to find the submerged platform within 30 s. The time from placing the animal into the water until it has reached on the platform was measured as escape latency. If the mouse failed to reach the platform by 30 s, it was guided onto the platform by a metal stick, and a score of 31 s was assigned for that trial. After mounting on the platform, the animals were allowed to remain there for 5 s, and were then taken by the metal stick back to their home cages until the start of next trial.

In addition to the escape latency, accuracy and wrong platform were taken as measures of animals' learning performance in WCM. As soon as the animals mounted on the platform in the

goal arm without visiting the arm opposite to the goal arm or reentering the start arm within 30 s, the trial was recorded as accurate. Accuracy defines as the percent accurate trials of 6 testing trials on each day. The accurate rate is calculated as [(sum of correct trials/6) × 100 %]. Animals were assigned as accurate learners if they performed accurately in 5 or 6 out of 6 trials per day (≥ 83.3 %). Wrong platform visits were counted when the animal entered the outer third of the arm opposite to the goal arm (Figure 16B).

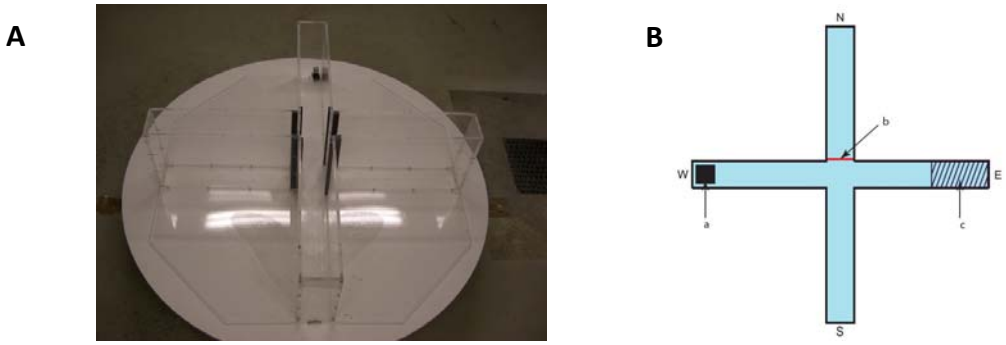


Figure 16: Water cross-maze (WCM) test. (A) WCM apparatus and (B) diagram of water cross-maze (a) submerged platform (b) partition (c) wrong platform area.

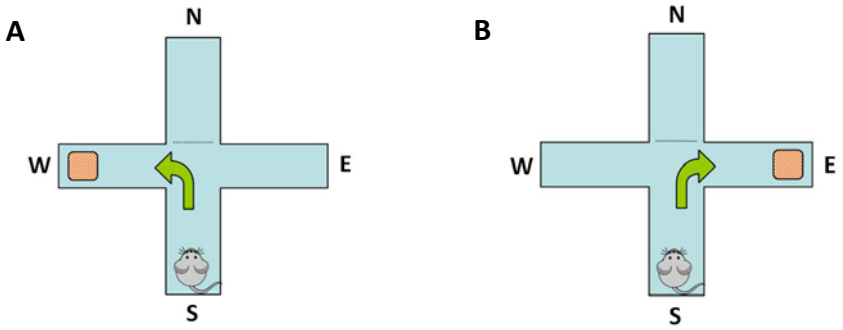


Figure 17: Free learning (FL) protocol. (A) Acquisition phase: mice started from the southern arm (S) and trained to navigate a hidden platform (red square) located in the end of the western arm (W) with 6 trials per day during the first week. (B) Relearning phase: the platform was moved to the opposite eastern arm (E) and the mice were trained to relearn the platform position during the second week. The north arm (N) is always blocked by a partition (dashed line).

2.5.6. Drugs

For systemic drug administration mice were treated intraperitoneally (i.p.) with 0.5, 1.0 or 2.0 mg/kg d-amphetamine sulfate (Sigma-Aldrich, USA), 3.0 or 10.0 mg/kg Tomoxetine

hydrochloride (BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany), 10.0 mg/kg d-threo-methylphenidate (Sigma-Aldrich, USA), 1.0 mg/kg haloperidol (Haldol, Janssen-Cilag GmbH, Neuss, Germany) dissolved in sterile saline, 3.0 mg/kg SR141716 (Rimonabant) (supplied by National Institute of Mental Health Chemical Synthesis and Drug Supply Program, USA) dissolved in a mixed solution of 2.5% DMSO, Tween 80 and saline, N-(4-hydroxyphenyl)-arachidonylamide (AM404) (Sigma-Aldrich, USA) dissolved in the mixture of absolute ethanol, Cremophore® (Sigma-Aldrich, USA), and saline to the final concentration of 0.1 or 0.3 mg/mL, and SB 366791 (Sigma-Aldrich, USA) dissolved in the mixture of DMSO (Sigma-Aldrich, USA) and saline to the final concentration of 0.5mg/mL.

2.6. LAB mice: towards an animal model of ADHD (characterization of basal and stimulated DA release in the dorsal striatum)

2.6.1. Behavioral validation

Before surgery, all animals were tested in the OF test as described before (see 2.4.2.) for 30 min (OF-30).

2.6.2. Drugs

D-amphetamine sulfate and d-threo-methylphenidate hydrochloride (Sigma-Aldrich, USA) were dissolved in sterile saline. Mice were treated i.p. with 1.0 mg/kg D-amphetamine in two separate trials (day 1 and day 3) and 10.0 mg/kg methylphenidate. The selective DAT Nomefensine (Sigma-Aldrich, USA) was also dissolved in sterile saline and administered at a dose of 10.0 mg/kg (i.p.). AM404 (Sigma-Aldrich, USA) was dissolved in the mixture of absolute ethanol and Cremophore® (Sigma-Aldrich, USA) and saline to the final concentration of 0.3 mg/mL and administered at a dose of 3.0 mg/kg (i.p.).

2.6.3. Surgery, probe implantation and microdialysis

One week before the probe implantation, the surgical procedures are conducted as i.c.v. implantation (see 2.4.5.). Each mouse was fixed in a stereotaxic apparatus and a guide cannula (MAB 4.15.IC, Microbiotech/se AB, Sweden) for a dialysis probe was implanted into right caudate putamen (CPu, AP – 0.5 mm; ML + 2.0 mm; DV – 2.25 mm; Figure 18) (Paxinos and

Franklin, 2001). Animals were left for recovery in the square Plexiglas home cages (L16 × W16 × H32 cm^3) for 1 week under reversed light-dark cycle (light "on" at 7 pm). Metacam® (0.25 mg/100mL) had been added to the drinking water for three consecutive days after surgery for anti-inflammatory and analgetic purposes. One day before the experiment, probes of 3-mm length (MAB 4.15.3.Cu, Microbiotech/se AB, Sweden) were inserted under slight isofluran anesthesia. Since the moment of implantation, probes were continually perfused with sterile aCSF (containing 145 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM Na_2HPO_4, set at pH = 7.4) at a flow rate of 0.3 μL/min. The high content of potassium aCSF (100 mM KCl) used at day 3 was compensated with reduced content of NaCl to keep normal osmolarity. Before used, the aCSF and aCSF-high potassium solutions were filtered through a 0.22 μm filter.

Microdialysis experiments were conducted on three consecutive days. On each day, one hour before the beginning of the sample collection, flow rate was increased up to 1.0 μL/min and probes were left for equilibration period. Microdialysis samples (20 μL) were collected every 20 min and three basal samples were collected within 1 hour. 20-min fractions were collected into the microdialysis tubes and kept in a refrigerated autosampler (Univentor 820 Microsampler, Univentor, Malta). At the end of the experimental procedure, perfusion flow rate was reduced to 0.3 μL/min for overnight continuous dialysis.

The dead volume of the outlet line (8.5 μL) was compensated with a delay in fraction harvesting (7 min). Therefore, all microdialysis fractions carefully corresponded to actual time of the experimental schedule.

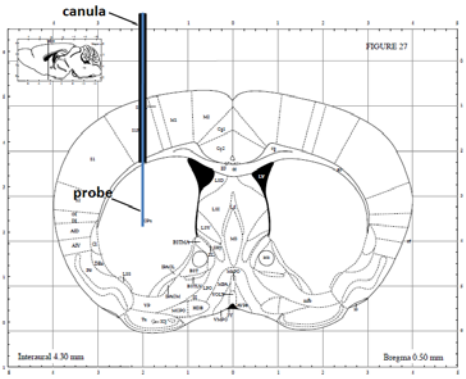


Figure 18: Schematic overview of probe implantation. Coronal brain section demonstrating the loci of guide canula and dialysis probe.

2.6.4. Pharmacological treatment procedures

On the first day of MD experiment after the basal sample collection, animals were transferred into square Plexiglas chambers (L25 × W25 × H32 cm^3) which mimic the condition of OF test. 20 min later, animals were treated with saline (i.p.). One hour later, they were administered with amphetamine (1.0 mg/kg, i.p.) and samples were continuously collected for the next 2 hours. Then mice were moved to their home cages. After a 60-min break and collection of 2 basal samples, animals were transferred again into the OF chambers. 20 min later, animals were treated with methylphenidate (10.0 mg/kg, i.p.) and samples were collected for another 2 hours (Figure 19A).

On the second day, 60 min after the start of collection and 20 min after being transferred into the OF, animals were treated with AM404 (3.0 mg/kg, i.p.) and then samples were continuously collected for 2 hours (Figure 19B).

On the third day, the normal aCSF was replaced by switching to a stream of modified aCSF solution using a low dead-volume liquid switch (Univentor Ltd, Malta). Animals were locally perfused for 10 min with aCSF containing high concentration of K^+. After perfusion with normal aCSF for 80 min, mice were transferred into the OF chambers. Twenty min later, animals were injected first with nomifensine (10.0 mg/kg, i.p.) and then d-amphetamine (1.0 mg/kg, i.p.) with 20 min interval (Figure 19C).

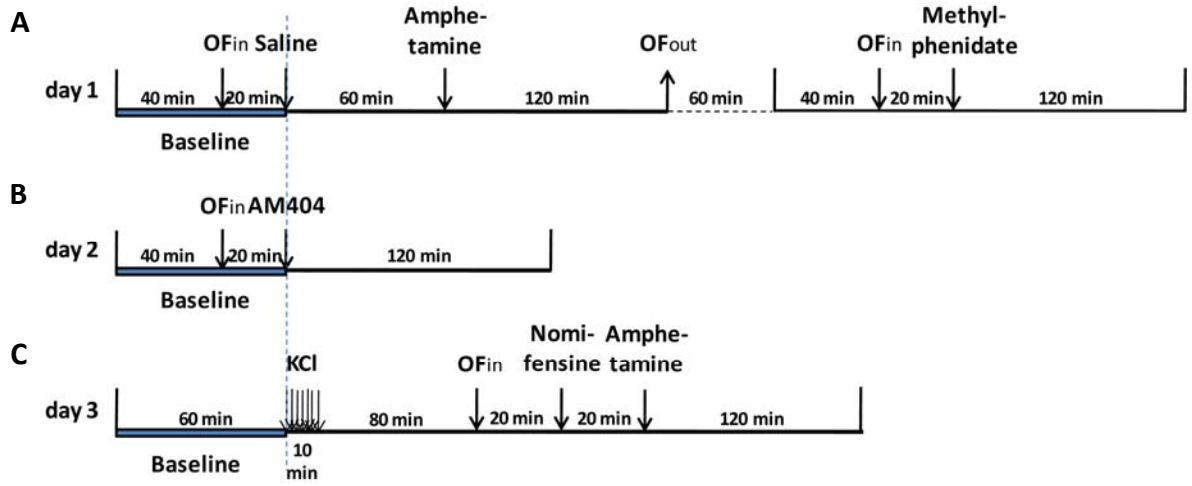


Figure 19: Schedule of microdialysis experiment. The experimental procedures on (A) day 1, (B) day 2 and (C) day 3. Note that OFin represents a moment of transferring animals into the open field chambers and OFout indicates a moment of transferring animals out of the open field chambers.

2.6.5. Monoamine assays

Once collected, all microdialysis samples were stored at -80°C and analyzed within 1-2 weeks following the sampling. DA and 3,-4 dihydroxyphenylacetic acid (DOPAC) were determined by reverse-phase HPLC coupled with amperometric detection. A solvent delivery isocratic system was SunFlow100 (SunChrom, Germany) and an amperometric detector was Decade (ANTEC Leyden, the Netherlands). The mobile phase contained 0.09 M sodium phosphate, 0.05 M sodium citrate, 1.7 mM sodium octane sulfate, 0.05 mM Na_2-EDTA and 15% acetonitrile (v/v) and adjusted to pH = 3.0 with 10M NaOH. All reagents used for the mobile phase were of analytical grade. Mobile phase was filtered through a 0.22 μm nylon filter and pumped through the system at a flow rate of 0.45 ml/min. Monoamines were separated on an analytical column (C18, 150mm x 3.2 mm, 3 μm, YMC-PackProC18, YMC Europe, Germany). Detection was performed at glassy carbon electrode at oxidation potential sat at +650 mV, against Ag/AgCl electrode. Injection volume was 20 μL and limit of detection (LOD) for DA was 0.018 nM (3.6 fM on column). LOD for DOPAC was not examined since the microdialysate concentration for the metabolite is usually in the ten-hundred nM range and peak identification offer no difficulty. The monoamine levels were quantified by external standard curve calibration using peak area for quantification.

2.6.6. Histology/probe placement verification

After completing the experiment, mice were anesthetized with isoflurane, and after the decapitation brains were removed. Forty μm sections were verified under microscope for probe placement using Paxinos and Franklin (Paxinos and Franklin, 2001) mouse atlas. All probes implanted were found in the caudate putamen and used in the analysis.

2.7. Data analysis and statistics

Data are shown as mean ± S.E.M. Statistical analyses were performed using Statistica (StatSoft, Inc., Tulsa, Oklahoma, USA).

Statistical evaluation of fear acquisition and extinction was performed by either one-way or two-way analysis of variance (ANOVA) with repeated measures. A two-way ANOVA was applied to reveal differences of the avoidance learning, mobility changes to tone-foot shock pairings. The Newman-Keuls test was used for post-hoc comparisons, if appropriate. Western blots were analyzed by t-test separately per kinase.

The effects of central and intranasal NPS were analyzed using either an unpaired t-test or a two-way ANOVA followed by a post-hoc Newman-Keuls test. Molecular characterizations of *Nps* and *Npsr1* were analyzed using an unpaired t-test.

Statistical evaluation of OF, HB, IO curve and WCM tests was performed by either one-way or two-way ANOVA with repeated measures followed by a post-hoc Newman-Keuls test. In PPI/PPF tests, we calculated alteration of the startle response (SR) with preceding pulse (PP) as percental change (% ASR) according to the following formula % ASR = (SR(PP+P)-SR(P))/SR(P) × 100 %. At different prepulse intensities, two-way ANOVA with repeated measures were conducted for statistical evaluation. Social cognitive functions were analyzed by dependent t-test separately per line. For the matter of clarity, in a few experiments, we refrained from showing the results of post-hoc analyses in the figures. Instead, we mentioned them in the text.

Original HPLC data were analyzed by means of Clarity software, version 2.8.2.648 (DataApex Ltd, Czech Republic). The absolute extracellular levels of DA and DOPA in dialysates were expressed in nanomoles. In the pharmacological tests, HPLC data were standardized and presented as percent from basal concentrations (defined as the average of three fractions before saline/drug administration). In the experiments with an acute treatment, data were analyzed using a two-way ANOVA, with subsequent post-hoc analysis (Newman-Keuls test), when appropriate.

A $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Conditioned fear in a mouse model of extremes in trait anxiety

3.1.1. Trait anxiety in HAB, NAB and LAB mice under different lighting conditions

Before elucidating the linkage between trait anxiety and fear learning, we first examine whether HAB, NAB and LAB mice perform robust anxiety-related behavior. Lighting has been reported to have a significant impact on general locomotor activity and therefore, alter the anxiety-related behavior on the EPM (Bertoglio and Carobrez, 2002; Strekalova *et al.*, 2005). To exclude the confounding factor of light-induced hyperactivity on anxiety-related behaviors, mice were tested on the EPM under normal and dim lighting conditions. We regularly tested 11 HAB, 12 NAB and 10 LAB on standard EPM at the age of 7 weeks. Two weeks later, these animals were tested again on EPM under red lighting. Figures 20A and 20B show the anxiety levels of HAB, NAB and LAB mice in the EPM test under normal and red light. The two-way ANOVA revealed significant different levels of anxiety between the three lines ($F_{2,30} = 78.73$, $p < 0.001$). Post-hoc comparisons showed that percent time on the open arms under both normal and red lighting was higher in LAB than in HAB and NAB mice ($p < 0.001$), as well as higher in NAB than in HAB mice (normal lighting: $p < 0.001$; red lighting: $p < 0.05$). No difference was found in HAB and LAB mice between two lighting conditions, however, a sight decrease in NAB mice was detected under red lighting versus normal lighting ($p < 0.05$).

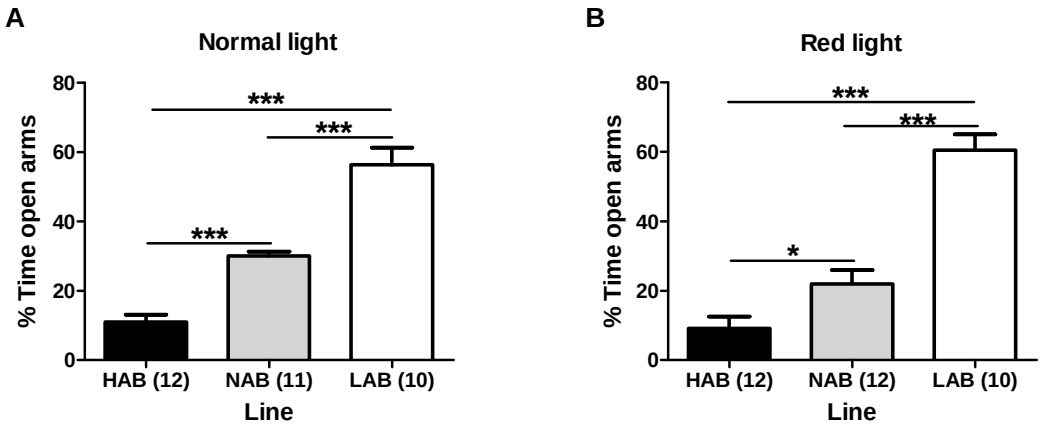


Figure 20: Anxiety-related behaviors under different light conditions. Behavior on the elevated plus-maze of HAB (n = 12), NAB (n = 11) and LAB (n = 10) mice under (A) normal lighting (open arms: 300 Lux) and (B) red lighting (open arms: 20 Lux). Data were obtained from mice of Generation 30/7. * $p < 0.05$; *** $p < 0.001$ (ANOVA followed by post-hoc Newman-Keuls test).

3.1.2. Acquisition/expression of conditioned fear

First experiment was aimed at testing HAB, NAB, and LAB mice for general processes related to fear conditioning including habituation and contextual memory. The experimental schedule is summarized in Figure 21A. On day 1 after conditioning, HAB mice showed higher freezing responses to the shock context than NAB and LAB mice ($F_{2,27} = 5.318$ $p < 0.05$), but not to the grid context ($F_{2,27} = 1.890$, $p > 0.05$) and the test context ($F_{2,27} = 1.976$, $p > 0.05$; Figure 21B). This indicates differences in contextual fear, but not in pattern separation/completion or fear generalization, even though we cannot entirely rule out that the order of testing might have contributed to the differences in freezing levels due to extinction of contextual fear. Freezing to the 3-min tone was more pronounced in HAB compared to NAB and LAB mice both at day 1 ($F_{2,27} = 24.36$, $p < 0.001$) and day 7 ($F_{2,27} = 11.57$, $p < 0.001$; Figure 21B) with HAB mice showing the highest levels, NAB mice intermediate levels and LAB mice virtually no freezing at all. If analyzed in 20-s intervals (Figure 3.2C), both HAB and NAB, but not LAB mice showed a comparable decline in freezing over the course of acute tone presentation ($p < 0.01$) on day 1, thus pointing to intact short-term habituation of the fear response in the two lines (Kamprath and Wotjak, 2004; Kamprath *et al.*, 2006; Plendl and Wotjak, 2010).

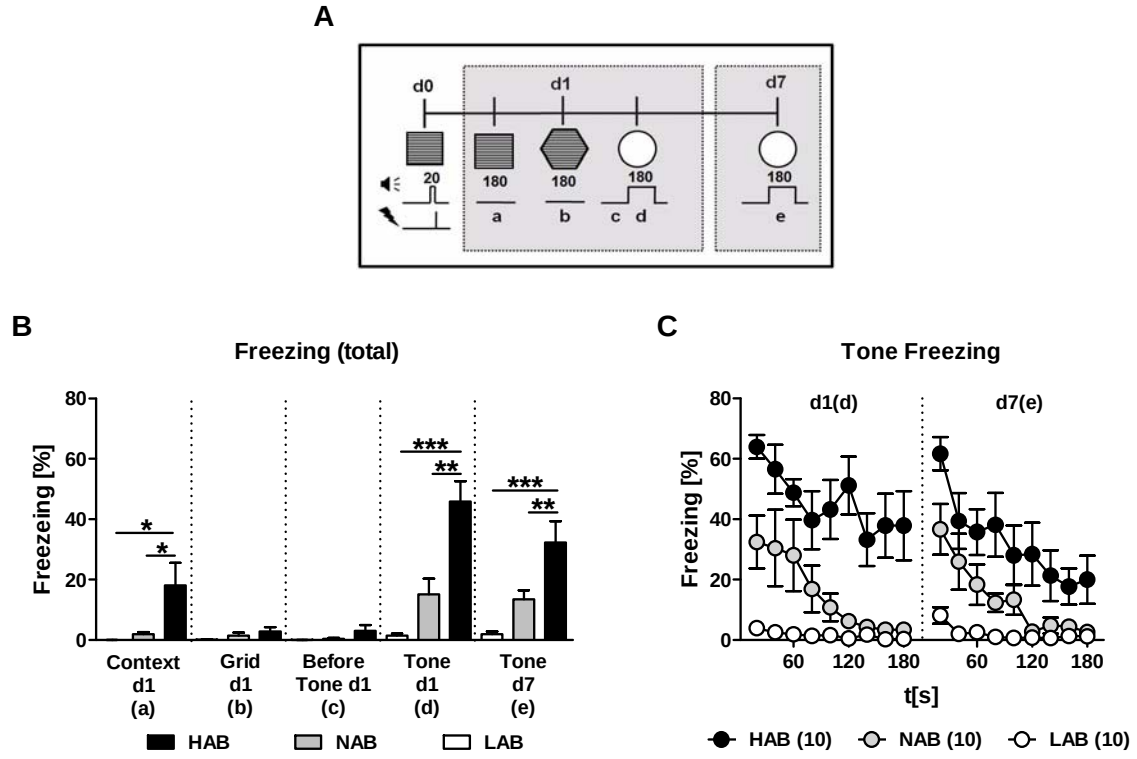


Figure 21: Acquisition and expression of conditioned fear. (A) Experimental schedule of fear conditioning with all mice receiving three tone-shock pairings. One day later, all animals were exposed to the shock context, a hexagonal chamber containing the grid floor, and to a cylinder. Freezing responses to (a) shock context, (b) grid context, (c) before and (d) during tone presentation in the cylinder on day 1 were scored. On day 7, freezing responses to (e) 180-s tone in the cylinder were measured. The freezing responses have been analyzed (B) as the sum of freezing to context and different conditioned cues, respectively, and (C) in 20-s intervals during tone presentation on day 1 and day 7. HAB differed from both NAB and LAB through out the 180-s period. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (ANOVA followed by post-hoc Newman-Keuls test).

3.1.3. Inhibitory avoidance learning

Naïve HAB (n = 16) and NAB (n = 7) mice were tested in step-down avoidance test. Two-way ANOVA revealed main effects of line ($F_{1,21} = 28.42$, $p < 0.001$) and time ($F_{1,21} = 26.07$, $p < 0.001$) as well as a significant interaction ($F_{1,21} = 18.30$, $p < 0.001$). As shown in Figure 22, HAB and NAB mice did not differ significantly in their step-down latencies during training ($p > 0.05$). During training, all mice received an electric foot shock once they stepped down with four paws on the grid floor. One day later, however, HAB mice displayed pronounced avoidance as reflected by extraordinarily high step-down latencies compared to NAB mice ($p < 0.001$).

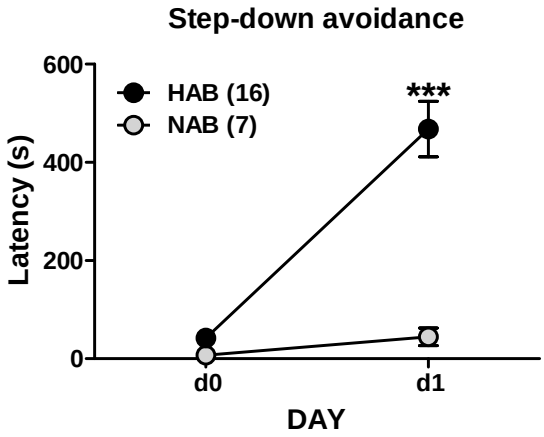


Figure 22: Inhibitory avoidance learning in HAB and NAB mice. At day 0, HAB (n = 16) and NAB (n = 7) mice received an electric foot shock as soon as they stepped down from a platform onto a metal grid floor. The next day, mice were again placed onto the platform and step-down latencies served as measures of inhibitory avoidance memory. *** $p < 0.001$ *vs.* NAB (d1) (ANOVA followed by post-hoc Newman-Keuls test). Data were obtained from mice of MPI of Biochemistry (Martinsried).

3.1.4. Kinase activity in the basolateral amygdala following conditioning

Western blot experiment investigated the molecular signature of differences in acquisition/consolidation of conditioned fear. Naïve HAB and NAB mice were randomly assigned to two groups (n = 6 per group). One group of each line was conditioned as in the previous experiment (see 3.1.2.), the other group served as home cage control. All animals were killed 45 min after conditioning (or the respective time in the home cages). Conditioned NAB mice showed decreased levels of pCaMKII ($p < 0.01$) and increased levels of β-catenin ($p < 0.01$) 45 min after conditioning, compared to non-shocked NAB controls with no changes in pERK, pAKT and pGSK-3β (Figure 23). Conditioned HAB mice showed essentially the same changes except for significantly elevated levels of pAKT ($p < 0.05$; Figure 24). Direct comparison of the changes in kinase activity between conditioned NAB and conditioned HAB confirmed significantly increased levels of pAKT in HAB as compared to NAB ($p < 0.01$; Figure 25).

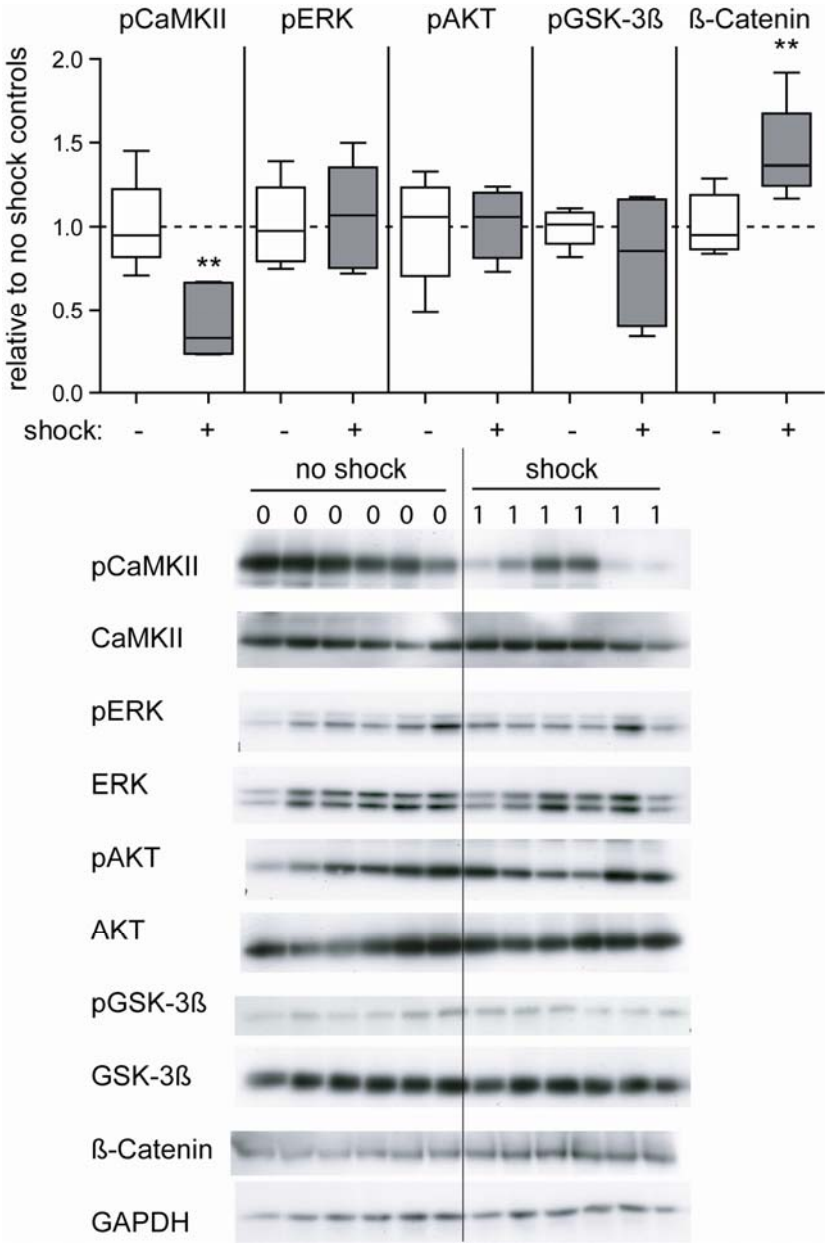


Figure 23: Western blot analyses of kinase activity in NAB mice. Western blot analyses of kinase activity and transcription factor levels measured in specimens of the lateral/basolateral amygdala of shocked *vs.* non-shocked NAB mice 45 min after conditioning. Bands of the Western blots were densitometrically analyzed, normalized to corresponding total kinase [pCaMKII/CaMKII, pERK/ERK (=pMAPK/MAPK), pAKT/AKT or pGSK-3β/GSK-3β] or GAPDH (β-catenin) and expressed relative to the mean expression levels of non-shocked controls. Data are shown as box-and-whisker blots. Data were obtained from mice mice of MPI of Biochemistry (Martinsried). ** $p < 0.01$ *vs.* non-shocked controls (unpaired t-test).

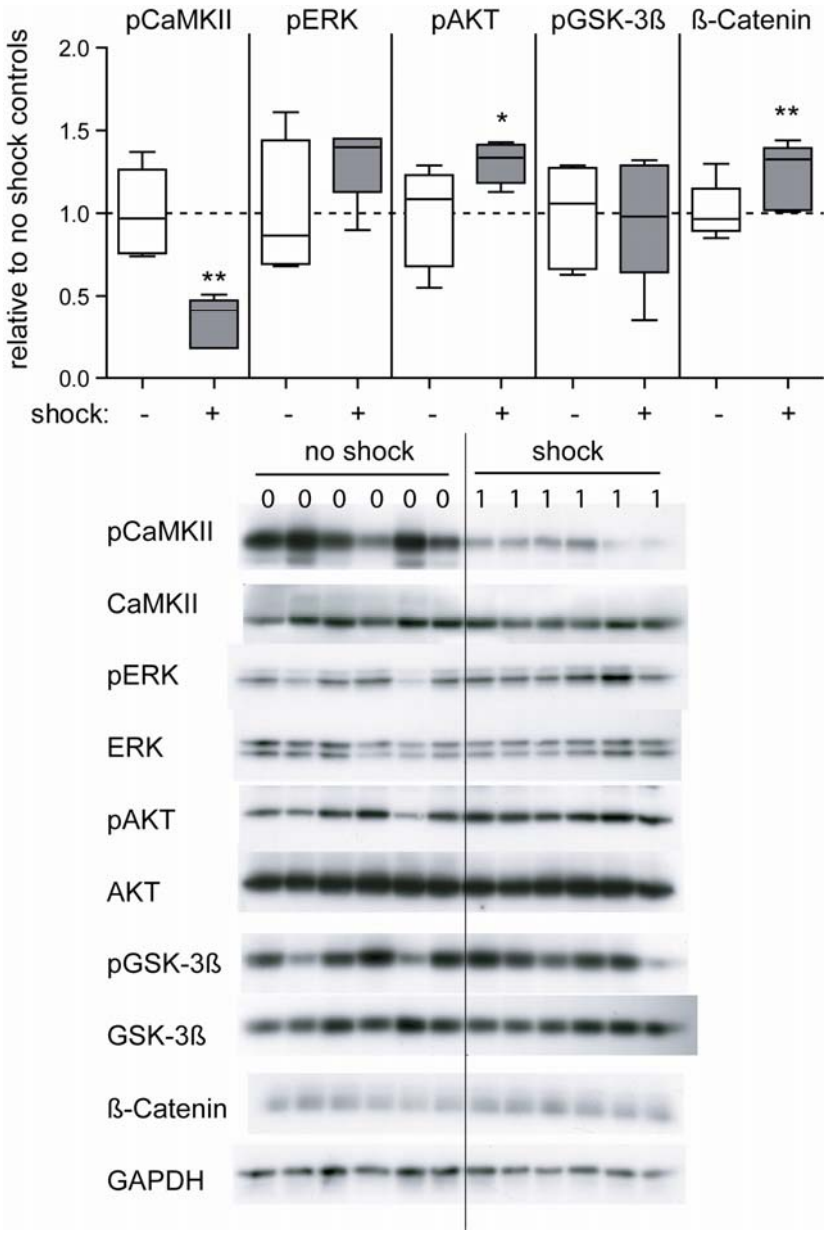


Figure 24: Western blot analyses of kinase activity in HAB mice. Western blot analyses of kinase activity and transcription factor levels measured in specimens of the lateral/basolateral amygdala of shocked *vs.* non-shocked HAB mice 45 min after conditioning. Data were obtained from mice mice of MPI of Biochemistry (Martinsried). * $p < 0.05$, ** $p < 0.01$ *vs.* non-shocked controls (unpaired t-test). For further details see Figure 23.

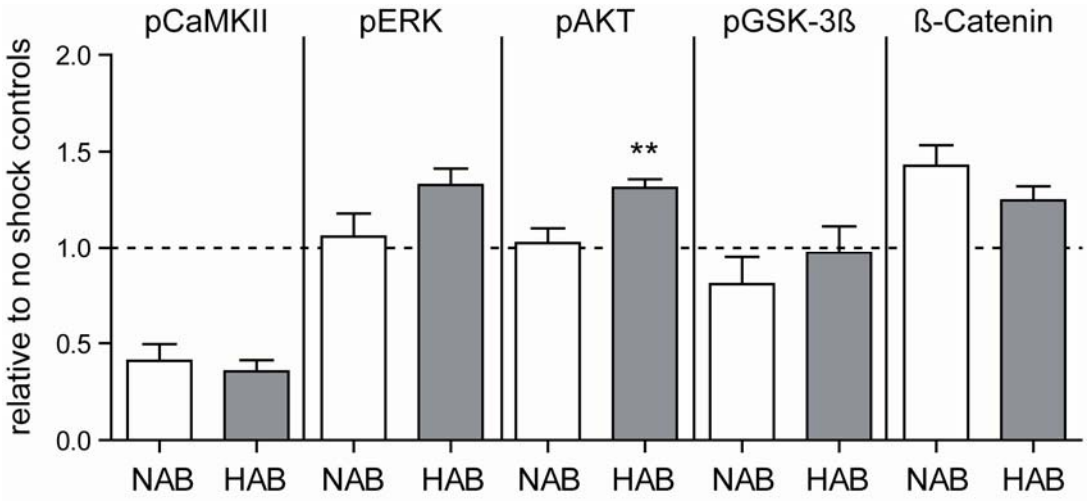


Figure 25: Expression levels of kinases/transcription factors in HAB *vs.* NAB mice. Expression levels of kinases/transcription factors measured in specimens of the lateral/basolateral amygdala of shocked *vs.* no-shocked HAB mice. Data were obtained from Figure 23 (NAB) and Figure 24 (HAB). Data were obtained from mice mice of MPI of Biochemistry (Martinsried). Mean ± SEM; ** $p < 0.01$ *vs.* NAB (unpaired t-test).

3.1.5. Shock sensitivity

The aim of present experiment was to measure the tone-foot shock pairings. We tested the sensitivity to tone-foot shock pairings in the three lines. Briefly, mice were placed into the startle apparatus with a continuous 50 dB background noise. Following an adaptation of 5 min, 10 pairings of 20 s-tone (9k Hz, 80 dB) and foot shocks (1 s, 0.7 mA) were presented with inter-stimulus intervals of 30-160 s.

To rule out that the differences between HAB, NAB and LAB in conditioned fear are simply related to differences in tone and/or foot shock perception, we measured mobility changes to the tone-foot shock pairings in new groups of naïve animals (HAB, n = 20; NAB, n = 8; LAB, n = 20). Figure 26A illustrates animals' mobility changes during the tone-foot shock pairings as assessed in the startle apparatus. Figure 26B summarizes the mobility 10 s before tone-foot shock pairings (Pre-S) and during tone-foot shock pairings (S). The two-way ANOVA showed significant differences between the three lines ($F_{2,45} = 21.78$, $p < 0.001$). Post-hoc comparisons revealed that baseline mobility was higher in LAB than in HAB and NAB mice during the 10 s before tone-foot shock pairings ($p < 0.001$), whereas the startle responses to the conditioning procedures were comparable between the three lines. The latter suggests a similar perception of the foot shocks among HAB, NAB and LAB mice. The relative changes in startle responses

triggered by the conditioning procedure, however, were less pronounced in LAB (80 ± 115%) as compared to HAB (649 ± 123%) and NAB (801 ± 163%) mice ($F_{2, 47}$ = 13.93, p < 0.001), pointing to altered perception of the foot shock in LAB mice. The similarities in the responses of HAB and NAB mice, however, render it highly unlikely that the differences in conditioned fear (Figure 21) and kinase activity (Figure 25) between those two lines simply relate to altered pain perception.

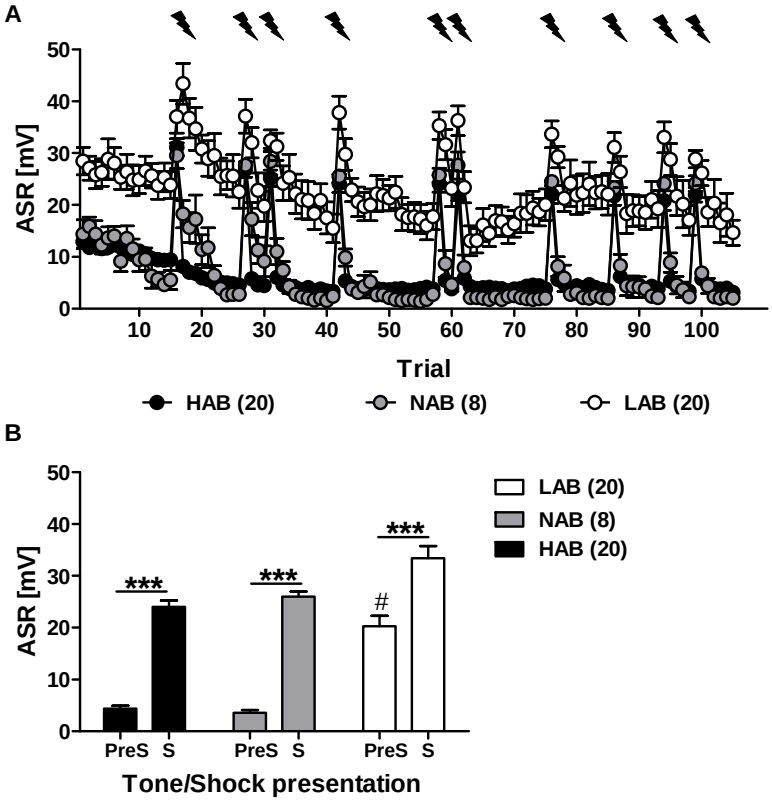


Figure 26: Shock sensitivity in HAB, NAB and LAB mice. (A) Time course of mobility changes during tone-foot shock pairings in HAB (n = 20), LAB (n = 20), and NAB (n = 8) mice. Mice were placed into the startle apparatus with a continuous 50 dB background noise. Following an adaptation of 5 min, 10 pairings of 20 s-tone (9k Hz, 80 dB) and foot shocks (1 s, 0.7 mA) were presented with inter-stimulus intervals of 30-160 s. (B) Mobility 10 s before tone-foot shock pairings (Pre-S) and during tone-foot shock pairings (S) in HAB, LAB, and NAB mice. Data were obtained from mice of Generation 32/9. LAB *vs.* HAB/NAB: # p < 0.001; Pre-S *vs.* S: *** p < 0.001 (ANOVA followed by post-hoc Newman-Keuls test).

3.1.6. Extinction of learned fear

The schedule of experiment, in which extinction of cued-conditioned fear of HAB and CD1 mice was tested, is depicted in Figure 27A. LAB mice were not included because these animals had shown no freezing at all after fear conditioning (Figure 21B, C), and CD1 mice were used as control representing the normal population due to breeding limitations of NAB mice.

The decrease in freezing over the course of the 3-min tone presentation in the previous experiment (see 3.1.2.; Figure 21C) can be largely ascribed to habituation-like processes (Kamprath and Wotjak, 2004; Kamprath *et al.*, 2006; Plendl and Wotjak, 2010). To assess 'true' between-session extinction of conditioned fear, the last experiment investigated in new batches of HAB mice and CD1 controls whether high levels of innate anxiety coincided with attenuation of the decline in the initial freezing responses (i.e. freezing shown during the first 20-s tone presentation per day; (Plendl and Wotjak, 2010)) over the course of repeated extinction training. During fear conditioning of repeated CS/US pairings on day 0, the percentage of freezing behavior remained unaltered with no differences between HAB and CD1 mice. Both lines showed less than 7% freezing within the three tone presentations (data not shown). During the extinction training (day 1), no freezing behavior was displayed 20 s before the first CS presentation (Figure 27B); nevertheless, HAB as compared to CD1 mice displayed a pronounced fear expression during the first CS presentation (2 (group) $\times$ 2 (trial) ANOVA: $F_{1,14\ group} = 30.56$ $p < 0.001$; $F_{1,14\ trial} = 209.33$, $p < 0.001$, $F_{1,14\ group \times trial} = 31.92$, $p < 0.001$; Figure 27B). Extinction training significantly decreased the levels of freezing in both HAB ($F_{2,24} = 56.56$, $p < 0.001$) and CD1 mice ($F_{2,21} = 24.19$, $p < 0.001$); however, HAB mice failed to reach the freezing levels of non-shock controls on day 4 (post-hoc comparison: $p < 0.05$, Figure 27C). This deficit could be overcome by more intensified extinction training (i.e. exposure to 20 instead of 10 CS at day 2; $p < 0.001$; Figure 27C). Interestingly, the freezing response to the tone spontaneously recovered in HAB Ext mice within 6 weeks (d45: $F_{3,25} = 10.32$, $p < 0.001$, Figure 27B), reaching approximately 35 % freezing levels, which were significantly lower than those displayed by non-extinguished HAB mice ($p < 0.01$), but significantly more pronounced compared to the respective CD1 Ext cohort ($p < 0.001$; Figure 27B). HAB mice, which underwent more intensified extinction training, showed the same spontaneous recovery of conditioned fear ($p < 0.05$;

Figure 27C). After repeated CS representations on days 4 and 9, a rapid decrease in freezing behavior was seen in CD1 Ret mice down to very low levels (5 %), whereas HAB Ret mice continued to display higher levels of freezing (~60 %) even on day 45 (Figure 27B), indicating that older fear memories in HAB mice showed more resistance to extinction than in CD1 mice (2 (group) × 3 (day) ANOVA: $F_{1,19\ group} = 58.43$ $p < 0.001$; $F_{2,38\ day} = 23.12$, $p < 0.001$, $F_{2,38\ group \times day} = 7.26$, $p < 0.01$).

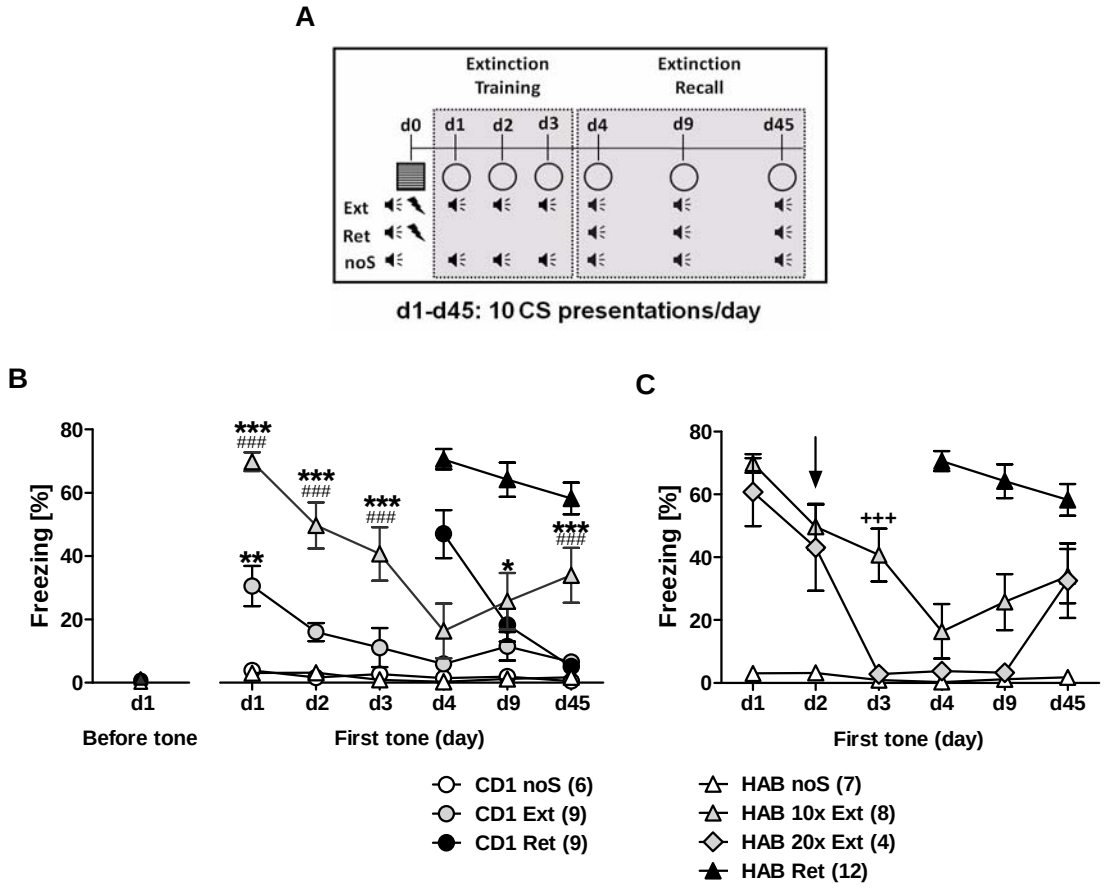


Figure 27: Extinction of learned fear. (A) Experimental schedule of acquisition and extinction of learned fear: HAB (n = 31) and CD1 (n = 24) mice were randomly assigned to one out of three groups. Two groups received three tone-shock pairings (Ext, Ret), the third group remained un-shocked (noS). For extinction training (Ext), animals were placed into a cylinder on three consecutive days and received 10 CS presentations (20s each) per day. A subset of HAB mice received 20 instead of 10 CS presentations at day 2 for studying consequences of intensified extinction training. As retention controls (Ret), mice of the other shocked group remained in their home cages. Extinction retention was performed on days 4, 9 and 45, again with exposure to 10 CS. (B) Freezing to the first 20-s tone during extinction training and extinction recall in HAB and CD1 mice as a measure of between-session extinction (Plendl and Wotjak, 2010). (C) Freezing to the first 20-s tone in HAB mice during extinction training with an intensified protocol at day 2 (arrow: 20 CS presentations; n = 4) compared to the standard protocol (10 CS presentations; n = 8; from panel B). Data were obtained from mice of Generation 33 and 34. Ext: extinction group; Ret: retention control; noS: control group; Ext/Ret *vs.* noS: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; HAB *vs.* CD1: # $p < 0.05$, ### $p < 0.001$; 10 CS *vs.* 20 CS: +++ $p < 0.001$.

3.2. NPS: from anxiolytic effects to molecular characterization in a mouse model of extremes in trait anxiety

3.2.1. Behavioral effects of central administration of NPS

The anxiolytic effect of NPS could be demonstrated in male CD1 mice, replicating the findings in C57BL/6N and BALB/c mice (Guerrini *et al.*, 2010), and we also could extend this finding to HAB mice. Central administration of NPS (1.0 nmol) significantly increased the percent time spent in the open arms of the EPM in both HAB (t_{29} = -3.48; $p < 0.01$; Figure 28A) and CD1 (t_{15} = -2.87; $p < 0.05$; Figure 29A) mice. Also, in the OF, NPS treatment increased the time spent in the central zone in CD1 mice during the 30-min test session ($F_{3,21}$ = 4.31; $p < 0.05$; Figure 30A), consistent with an anxiolytic-like effect found in the EPM test. Post-hoc analysis revealed that central administration of NPS at three different doses (0.1, 1 and 2nmol) significantly increased the time spent in the central zone ($p < 0.05$). For both behavioral tests, the anxiolytic effects of NPS on HAB and CD1 mice were not accompanied with changes in locomotor activity, i.e. DT (Figures 28B, 29B and 30B).

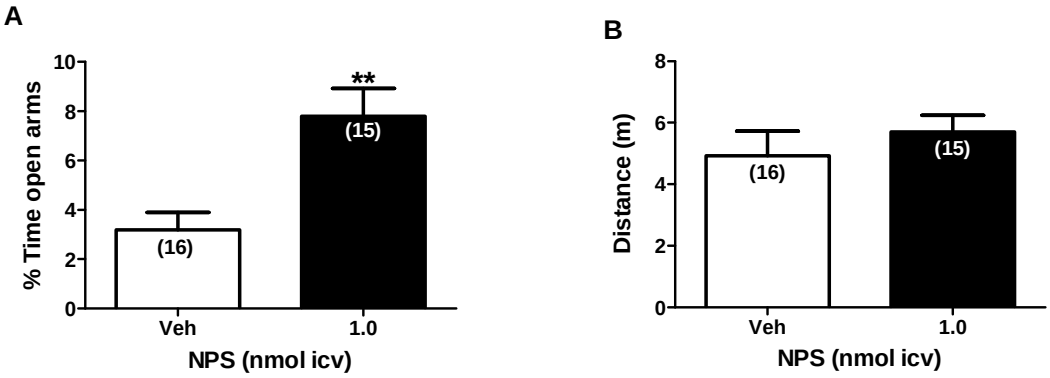


Figure 28: Effects of central NPS on the EPM test in HAB mice. I.c.v. administration of NPS (A) significantly increased percent time on the open arm, but (B) did not alter the distance traveled. Data were obtained from mice of Generation 32 and 33.** $p < 0.01$ (unpaired t-test).

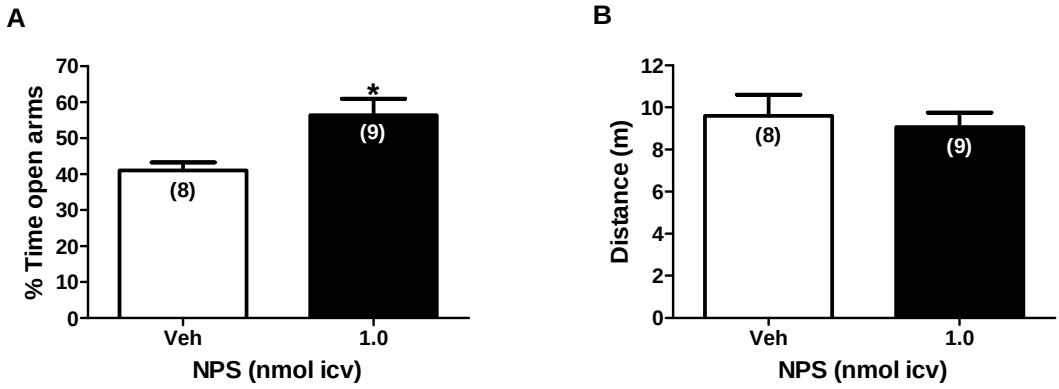


Figure 29: Effects of central neuropeptide S (NPS) on the EPM test in CD1 mice. I.c.v. administration of NPS (A) significantly increased percent time on the open arm, but (B) did not alter the distance traveled. * $p < 0.05$ (unpaired t-test).

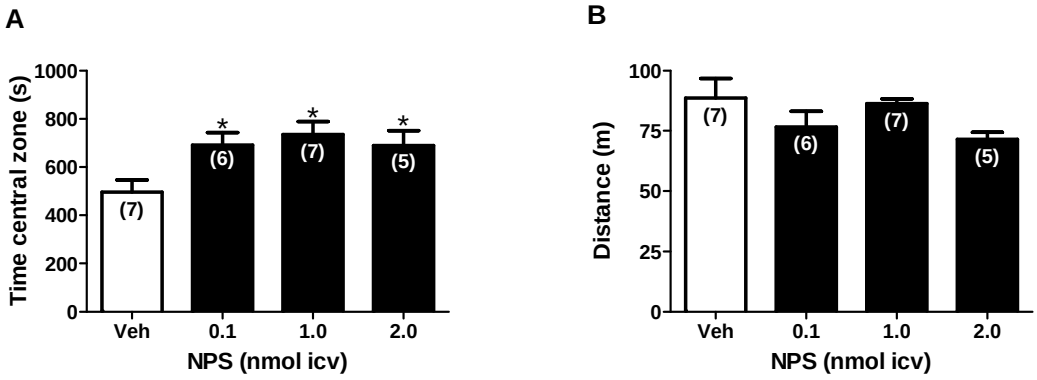


Figure 30: Effects of centrally administered neuropeptide S (NPS) on the open field in CD1 mice. Central administration of NPS (A) significantly increased time spent in the central zone at different doses of NPS, but (B) did not affect travelled distance. * $p < 0.05$ (one-way ANOVA followed by post-hoc Newman-Keuls test).

3.2.2. Behavioral effects of intranasal administration of NPS

HAB mice were treated with NPS by intranasal application, a noninvasive method. 30 min and 4 hr after the first application, animals were tested twice in a behavioral assay, including OF, dark-light avoidance and EPM tests. In the first assay, none of the three behavioral tests were influenced by NPS treatment. In the second assay, intranasal NPS significantly increased the time spent in the light chamber during the dark-light test ($p < 0.05$; Figure 31A), but had no effect on the other two behavioral tests (data not shown). The DT in the dark-light test was not

influenced by treatment with NPS, indicating that the anxiolytic effect of intranasal NPS is independent of changes in locomotor activity (Figure 31B).

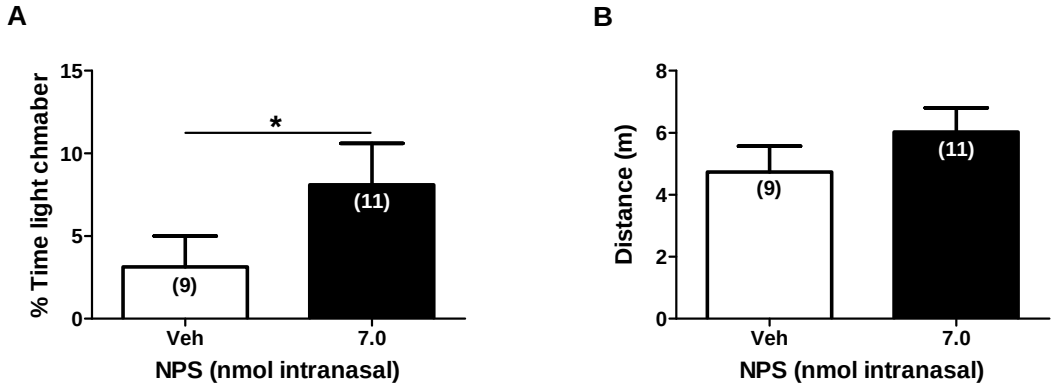


Figure 31: Effects of intranasal neuropeptide S (NPS) on the dark-light avoidance test in HAB mice. Intranasal administration of NPS (A) significantly increased time spent in the light chamber in the dark-light avoidance test, but (B) did not affect travelled distance. Data were obtained from mice mice of MPI of Biochemistry (Martinsried). * $p < 0.05$ (unpaired t-test).

3.2.3. Expression profile of *Nps/Npsr1* in the brain

The behavioral evidence suggests a role of the NPS/NPSR1 system in the profound differences in innate anxiety between HAB and LAB mice. Therefore, we next compared the expression of *Nps* and *Npsr1* mRNA in HAB and LAB mice.

3.2.3.1. Nps expression in the peri-LC (locus ceruleus) area

In order to characterize the NPS system in the brain, *Nps* expression within the peri-LC area was compared between HAB and LAB mice. *Nps* mRNA expression in the peri-LC area did not differ between HAB and LAB mice (Figure 32A).

3.2.3.2. Npsr1 expression in the paraventricular nucleus (PVN) and amygala

However, line differences were found in LAB mice, with an almost 5-fold higher *Npsr1* mRNA in the amygdala ($t_9 = -2.83$; $p < 0.05$; Figure 32B), but not in the PVN ($t_8 = -0.63$; $p > 0.05$; Figure 32C), compared to HAB mice.

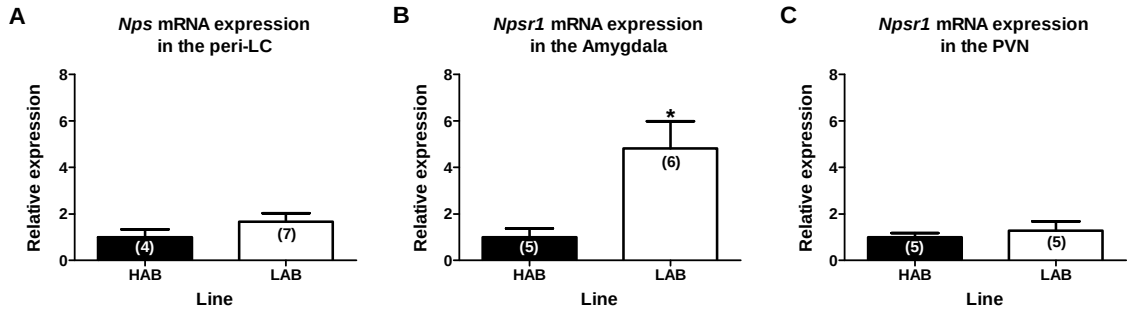


Figure 32: Gene expression levels of neuropeptide S (*Nps*) and neuropeptide S receptor 1(*Npsr1*). Gene expression levels of (A) neuropeptide S (*Nps*) as confirmed in tissue punches from the peri-LC (*locus ceruleus*), (B) neuropeptide S receptor 1 (*Npsr1*) from the amygdala complex and (C) from the paraventricular nucleus (PVN) of male HAB and LAB mice under basal conditions. Data were obtained from mice of Generation 34. Data

3.2.4. *Nps* and *Npsr1* DNA sequence analysis

In total, 35 SNPs and 4 insertions were found in the Nps gene in HAB *vs.* LAB mice. In the promoter region, 14 SNPs and one insertion were found. The gene-coding locus contained one synonymous polymorphism and three SNPs leading to amino acid changes: position T(125)A with HABs carrying leucine and LABs isoleucine, G(140)A with HABs carrying valine and LABs isoleucine, A(3624)G with HABs carrying arginine and LABs glycine, and A(3659)G with both HABs and LABs carrying threonine (Figure 33). The DERs also contained several SNPs (Table 4).

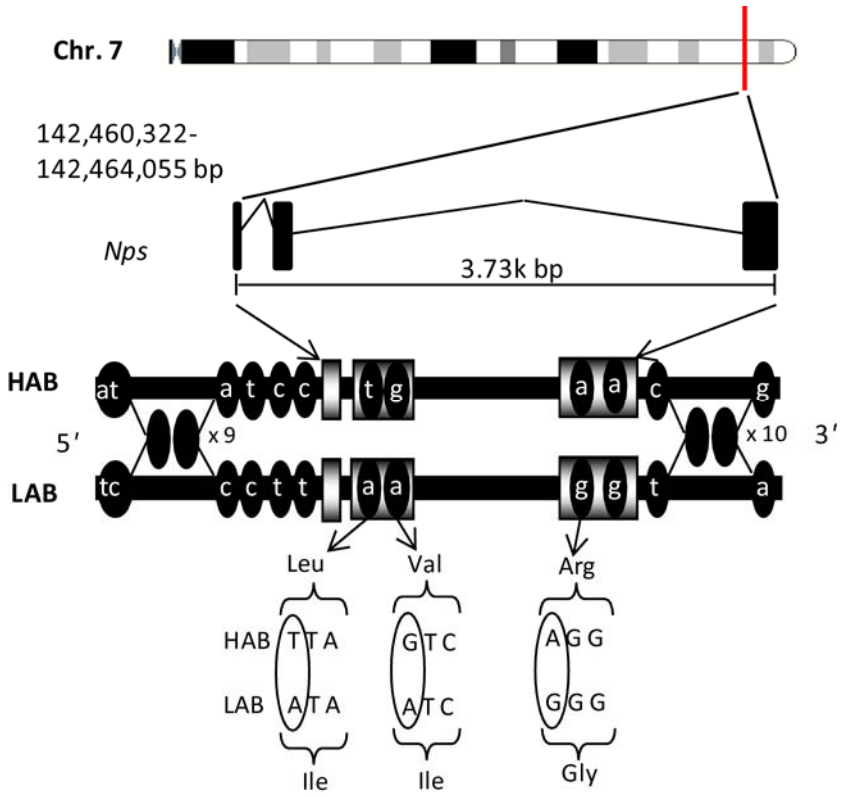


Figure 33 Neuropeptide S (*Nps*) gene sequence of HAB *vs.* LAB mice. Exons and untranslated regions (UTRs) are indicated by boxes (exons shaded). Polymorphic sites and three amino acid changes are indicated. Figure is based on the data from Ensembl (www.ensembl.org, 19.11.2010).

Sequencing of the mouse *Npsr1* gene resulted in the identification of 47 polymorphic sites. All identified polymorphisms are described in Table 5. Altogether, 41 SNPs, 4 deletions and 2 insertions were found. The definition of insertion and deletion was made in reference to the mouse strain C57BL/6J. The upstream promoter region contained 31 SNPs, one insertion and several deletions. In the ten exons, 3 polymorphic sites were identified. Interestingly, HAB mice missed a 38 bp segment between -1402 and -1365. Three further SNPs were identified in exons, with G(156657)A in exon 4 (rs37572071), G(216712)A and G(217986)A in the untranslated region. The other variations in the introns and the DER are also listed in Table 5.

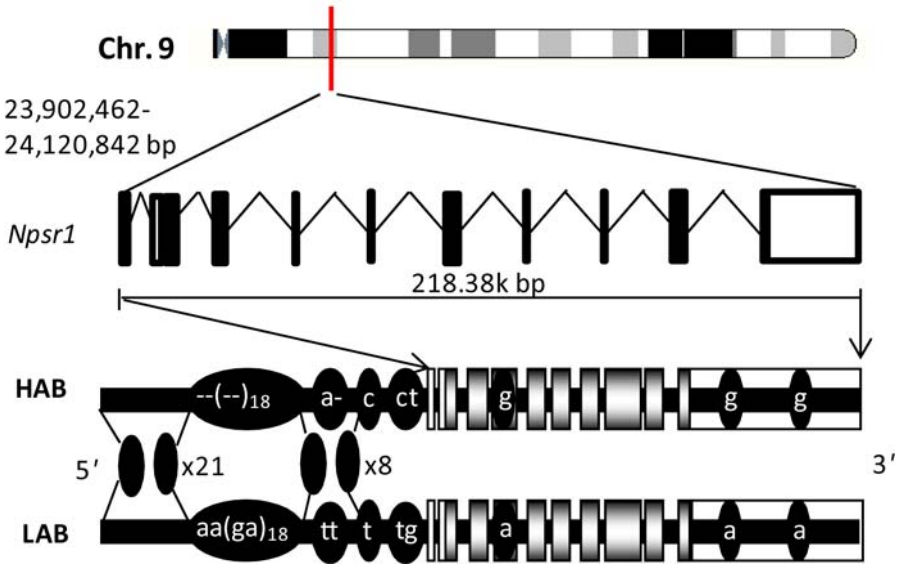


Figure 34 Neuropeptide S receptor 1 (*Npsr1*) gene sequence of HAB *vs.* LAB mice. Exons and untranslated regions (UTRs) are indicated by boxes (exons shaded, UTRs completely white). Selected polymorphic sites are indicated. Figure is based on the data from Ensembl (www.ensembl.org, 30.10.2009).

3.2.5. Screening for transcription factor binding sites

A detailed *in silico* search for potential transcription factor binding sites at the polymorphic loci at the *Nps* locus led to the identification of a number of candidates that are summarized in Supplementary table 3.

For the polymorphisms in *Npsr1*, TESS analysis of HAB/LAB mice revealed the presence of transcription factor binding sites at particular SNP positions. *In silico* analysis resulted in numerous transcription factors (Supplementary table 4). In particular a deletion of $(GA)_{18}$ repeat was found in HAB mice.

Table 4: Variations identified in the neuropeptide S (*Nps*) gene. Variation type refers to single nucleotide polymorphisms (SNPs), deletions or insertions, the HAB and LAB to their line specific allele, location in the gene to the functional structure of the variation locus (DER: downstream enhancer region), relative position to the *Nps* locus and SNP identifier to already described polymorphisms. Functional structures are indicated by horizontal disjunctions (www.ensembl.org, 21.01.2011).

Variation type	HAB	LAB	Location in the gene	Relative position	SNP identifier
SNP	A	T	Promoter	-1031	
SNP	T	C	Promoter	-1030	
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	3624	rs33470378
SNP	A	G	Exon3	3659	rs33470381
SNP	C	T	DER	3937	rs33471194
SNP	A	G	DER	4022	rs33471197
SNP	T	C	DER	4127	rs33471203
SNP	A	G	DER	4155	rs33471946
SNP	C	A	DER	4195	rs50157889
SNP	C	G	DER	4196	rs33466004
SNP	C	T	DER	4215	rs47207120
SNP	A	G	DER	4217	rs46716508
SNP	G	A	DER	4240	rs49462104
SNP	T	C	DER	4264	rs51623072
SNP	T	A	DER	4321	rs33466010
SNP	G	A	DER	4369	

Table 5: Variations identified in the neuropeptide S receptor 1 (*Npsr1*) gene. Variation type refers to single nucleotide polymorphisms (SNPs), deletions or insertions, the HAB and LAB to their line specific allele, location in the gene to the functional structure of the variation locus (DER: downstream enhancer region), relative position to the *Npsr1* locus and SNP identifier to already described polymorphisms (www.ensembl.org, 21.01.2011).

Variation type	HAB	LAB	Location in *Npsr1* gene	Relative position	SNP identifier
SNP	G	A	Promoter	-2045	rs50949943
SNP	C	T	Promoter	-2042	rs48292984
SNP	C	G	Promoter	-1917	rs47083749
SNP	T	C	Promoter	-1906	rs49887483
SNP	T	A	Promoter	-1898	rs47000117
SNP	T	C	Promoter	-1840	rs48022291
SNP	G	A	Promoter	-1736	rs46860992
SNP	T	C	Promoter	-1657	rs51840884
SNP	T	A	Promoter	-1636	rs45839541
SNP	C	T	Promoter	-1569	rs52096988
SNP	G	A	Promoter	-1461	
Deletion		AA $(GA)_{18}$	Promoter	-1402~-1365	
SNP	G	A	Promoter	-1310	
SNP	T	C	Promoter	-1263	
SNP	G	A	Promoter	-1212	
SNP	A	G	Promoter	-1170	
SNP	A	T	Promoter	-1109	
SNP	C	T	Promoter	-1030	rs48864073
Deletion		T	Promoter	-1021	
SNP	A	T	Promoter	-1020	rs36643873
SNP	A	T	Promoter	-926	
SNP	T	G	Promoter	-866	rs51941766
SNP	G	A	Promoter	-826	
SNP	C	T	Promoter	-654	rs45719875
SNP	G	A	Promoter	-610	rs37067240
SNP	C	T	Promoter	-525	rs50871983
SNP	C	T	Promoter	-508	rs48580633
SNP	T	G	Promoter	-507	rs47842102
SNP	C	G	Promoter	-468	rs46047101
Deletion		T	Promoter	-442	
SNP	T	A	Promoter	-431	rs45879530
SNP	T	C	Promoter	-406	rs51858460
SNP	T	C	Promoter	-385	rs46930781
SNP	C	A	Promoter	-351	rs50633535
Insertion	TC		Promoter	-274	
SNP	A	T	Intron1	123	rs48722200
Insertion	A		Intron3	156437	
SNP	G	A	Exon4	156657	rs37572071
SNP	C	T	Intron7	202552	
Deletion		C	Intron7	202622	
SNP	T	C	Intron8	212144	
SNP	G	C	Intron9	215447	
SNP	G	A	Intron9	215448	
SNP	A	G	Intron9	215449	

SNP	G	A	Exon10	216712	rs49543460
SNP	G	A	Exon10	217986	rs49030747
SNP	A	G	DER	218747	

3.2.6. In search for CpG islands in the *Nps* and *Npsr1* sequence

There were only a few CG bases in the promoter and exon sequences of both HAB and LAB *Nps* and *Npsr1* DNA sequence. This CG bases were much less than the required 55 percent GC and thus there was no CpG island.

3.3. LAB mice: Towards an animal model of ADHD (behavioral phenotyping and pharmacological validation)

3.3.1. EPM test

HAB (n = 13), NAB (n = 21) and LAB (n = 31) mice were tested in the EPM test at the age of seven weeks. Figures 35A and 35B respectively depict percentage of time spent on the open arms and DT in the EPM test. Statistical analysis revealed a significant main effect of line in the percent time on the open arms (one-way ANOVA: $F_{2,62}$ = 127.04, p < 0.001). Post-hoc comparisons revealed significant differences between the three lines (all ps < 0.001). Accordingly, HAB mice spent lower and LAB mice higher percent time on the open arms than did NAB animals. We also measured DT as an index of EPM-related locomotion, which indicated significant differences between lines (one-way ANOVA: $F_{2,62}$ = 5.26, p < 0.01). Post-hoc analyses revealed more DT in LAB mice than in HAB mice during the EPM testing (p < 0.05).

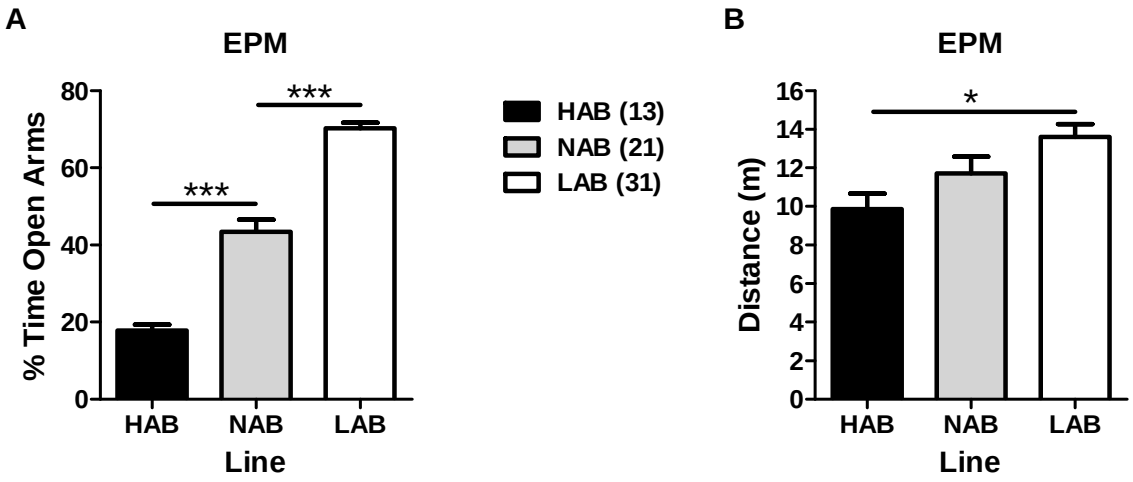


Figure 35: Anxiety-related behaviors in the elevated plus-maze (EPM) test. (A) Mice originating from selectively bred lines showed low (LAB), intermediate (NAB) or high (HAB) levels of innate anxiety. (B) LAB mice displayed more distance travelled than HAB mice in the EPM test. Data were obtained from mice of Generation 40/10. *** p < 0.001; * p < 0.05 (one-way ANOVA followed by post-hoc Newman-Keuls test).

3.3.2. Startle measurements

3.3.2.1. IO curve

We tested IO curves of the startle responses in HAB, NAB and LAB mice from two different batches of animals to evaluate their sensation upon the acoustic stimuli. Figure 36A and 36B show the mean amplitude of startle responses elicited by acoustic stimuli of Generation 37/7 and Generation 40/10, respectively. In Generation 37/7, the two-way ANOVA revealed significant differences between the three lines ($F_{2,27} = 16.44$, $p < 0.001$) as well as a significant line $\times$ intensity interaction ($F_{8,108} = 13.77$, $p < 0.001$). Post-hoc analyses showed that the acoustic startle responses were higher in LAB mice than in HAB and NAB at 105 and 115 dB (all $p < 0.001$), as well as in NAB than HAB at 105 and 115 dB (all $p < 0.01$). In Generation 40/10, there were also a main effect of line ($F_{2,63} = 28.86$, $p < 0.001$) and a significant line $\times$ intensity interaction ($F_{8,252} = 18.25$, $p < 0.001$). Further analyses revealed that HAB mice showed lower startle responses than both NAB and LAB mice at 105 and 115 dB (all $p < 0.001$). Moreover, the acoustic startle responses were higher in LAB mice than in HAB and NAB at all stimulus intensities.

3.3.2.2. PPI/PPF tests

With a validated protocol of PPI and PPF measurement, 13 HAB, 23 NAB and 32 LAB mice of Generation 40/10 were tested in PPI/PPF tests to evaluate animals' sensorimotor gating. Figures 36C-E illustrate the percental change of startle response at different prepulse (PP) intensities. While significant differences were not found between the lines at 55 dB ($F < 1$; Figure 36C) and 65 dB ($F2, 63 = 1.03$, $p > 0.05$; Figure 36D) PP intensities, the two-way ANOVA revealed significant difference between the three lines at 75 dB ($F_{2,63} = 7.87$, $p < 0.001$; Figure 36E) PP intensity. There was also a significant line $\times$ IPI interaction ($F_{8, 252} = 4.81$, $p < 0.001$) at 75 dB PP intensity. In comparison with NAB mice, LAB mice displayed pronounced PPI with IPI of 10 ms ($p < 0.001$) but HAB mice showed less PPI with IPIs of 25 and 50 ms (all $p < 0.05$).

The aforementioned startle measurements demonstrate robust differences in acoustic startle responses between the three lines at higher stimulus intensities (105 and 115 dB), as well as

pronounced PPI in LAB mice for which one would expect an impairment in PPI, implying the unlikeliness of LAB as an animal model of schizophrenia.

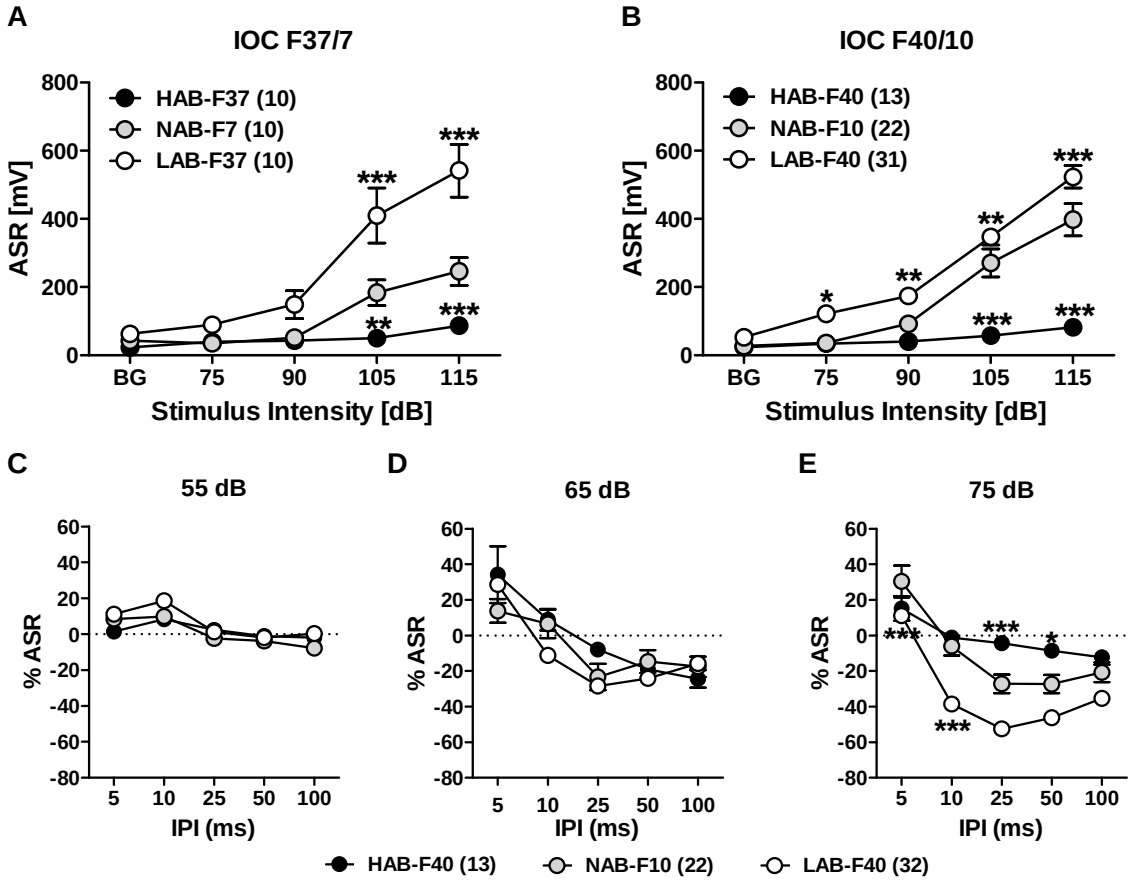


Figure 36: Startle measurements. Acoustic startle responses were elicited by acoustic stimuli in HAB, NAB, and LAB mice of (A) Generation 37/7 and (B) Generation 40/10. LAB mice showed higher, but HAB mice displayed lower startle responses than NAB mice at 105 and 115 dB. Percental change of startle response (% ASR) in HAB, NAB and LAB mice of Generation 40/10 at prepulse intensities of (C) 55, (E) 65 and (F) 75 dB across five interpulse intervals (IPIs). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared with NAB (ANOVA followed by post-hoc Newman-Keuls test).

3.3.3. OF test

We used an OF-80 test to measure the baseline of exploratory behavior in HAB, NAB and LAB mice. Exploratory behavior was analyzed in terms of horizontal locomotion (i.e. DT), immobility time and vertical exploration (i.e. number of rearing). Rotational behavior was also measured

and analyzed as the difference between the total number of clockwise and counter-clockwise turns. Results are shown in Figure 4.3.

3.3.3.1. Locomotion

DT in the OF test was defined as a general measure of horizontal activity. During the entire 80-min exposure, the three lines displayed difference in the overall DT ($F_{2,62}$ = 17.32, $p < 0.001$; Figure 37A). Post-hoc comparisons revealed that no significant difference was found between HAB and NAB mice, whereas LAB mice displayed significantly more locomotion compared to HAB and NAB mice (all $p < 0.001$). The difference in locomotion between lines in the first 5-min ($F_{2,62}$ = 9.97, $p < 0.001$) could reflect their difference in anxiety levels. HAB and NAB mice exhibited habituation of locomotion towards the end of the exposure (one-way ANOVA, HAB: $F_{15,180}$ = 46.90, $p < 0.001$; NAB: $F_{15,300}$ = 107.87, $p < 0.001$), whereas LAB mice showed exceptional increased locomotion without habituation over the course of the exposure ($F < 1$).

3.3.3.2. Rearing

In addition to horizontal activity, we also estimated animals' vertical exploratory behavior in the OF test. Rearing is defined as animals' "standing up" with their hind legs and is regarded as an index of vertical exploration. Analysis of number of rearing revealed significant difference between the three lines ($F_{2,62}$ = 14.11, $p < 0.001$) and significant line $\times$ time interaction ($F_{30,930}$ = 3.67, $p < 0.001$; Figure 37B). Further comparisons showed that HAB exhibited less rearing compared to NAB and LAB mice (all $p < 0.001$), with no difference between NAB and LAB mice ($p > 0.05$). HAB mice showed the lowest level of rearing over the entire course of the exposure. Significant difference was found between LAB and NAB mice in the first 30-min exposure, whereas no difference was shown between two lines in the latter 50 min.

3.3.3.3. Immobility

Immobility time mainly mirrored the data obtained concerning locomotion. Results showed that total time of immobility significantly differed between all three lines ($F_{2,62}$ = 86.64, $p < 0.001$) as well as significant line $\times$ time interaction ($F_{30,930}$ = 7.72, $p < 0.001$; Figure 37C). Post-hoc comparisons revealed that LAB mice showed less immobility than HAB and NAB mice, with

significant difference between HAB and NAB mice (all ps < 0.001). The difference between three lines was more pronounced towards the end of exposure (Figure 37C).

3.3.3.4. Rotations

Rotational behavior was measured as the difference between the number of clockwise and counter-clockwise circling turns. One-way ANOVA revealed significant difference between the three lines ($F_{2,62}$ = 8.52, p < 0.001; Figure 37D). Post-hoc comparisons showed that LAB mice exhibited significantly more rotational behavior than HAB and NAB mice (all p < 0.01). Similar behavioral differences between the three lines have been shown by the total numbers of turns (mean ± standard deviation; HAB: 32.46 ± 12.46; NAB: 32.48 ± 8.35; LAB: 138.5 ± 124.3).

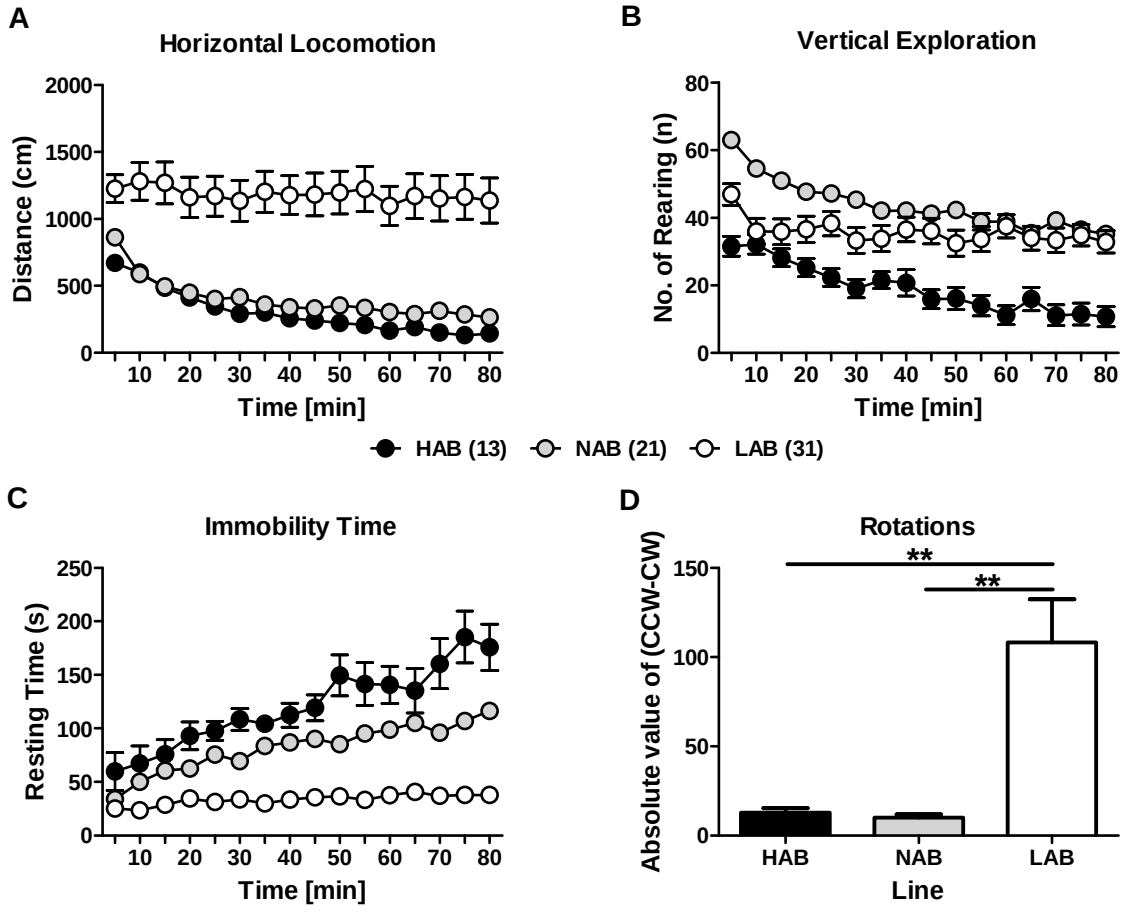


Figure 37: Exploratory behaviors of HAB, NAB and LAB mice in the open field test. HAB, NAB and LAB mice were tested in the 80-min open field test. Animals' exploration was tested in terms of (A) distance travelled, (B) number of rearing, and (C) immobility in 5-min bins for the 80-min exposure. (D) Rotation was measured as the difference between the total number of clockwise and counter-clockwise circling turns during the entire investigation. Data were obtained from mice of Generation 40/10. ** p < 0.01 (ANOVA followed by post-hoc Newman-Keuls test).

3.3.4. OF test (distinction of LAB-S and LAB-I)

Figure 38A depicts the individual data plot of total DT of HAB, NAB and LAB mice in the OF during the entire 80-min observation period. None of the HAB and NAB mice displayed more than 15000 cm of DT, however, two clusters of data points were observed in the LAB population, which could be distinguished by a threshold of 15000 cm of DT. Figure 38B shows the locomotion of LAB mice by different litters. In each litter, the percentage of LAB offspring which displayed notably an extraordinary level of locomotion (> 15000 cm) ranged form 0 to 67.7 %. Based on the above selection criterion, 13 out of 31 LAB mice (38.7 %) were assigned as LAB-S (LAB-strong) group which showed higher level of DT, whereas 18 out of 31 LAB animals (61.3 %) were assigned as LAB-I (LAB-intermediate) group because of their intermediate locomotion.

Figures 38C-F depict the exploratory behavior of HAB, NAB, and LAB mice in Figure 37 in terms of different indices after the assignment of LAB-S and LAB-I mice.

3.3.4.1. Locomotion

For total DT, the results revealed a significant difference between lines ($F_{3,61}$ = 116.44, $p < 0.001$; Figure 38C). Further analysis indicated that LAB-S mice differed significantly from the other three lines (all $p < 0.001$). The other LAB line, LAB-I mice displayed significantly more DT compared to HAB mice ($p < 0.05$), but only a tendency for increased locomotion compared to NAB mice ($p = 0.058$).

3.3.4.2. Rearing

The number of rearing in the OF was lower for HAB mice than for NAB, LAB-I and LAB-S animals ($F_{3,61}$ = 9.86, $p < 0.001$; Figure 38D). No difference was found between NAB, LAB-I and LAB-S mice, however, the number of rearing was slightly lower for LAB-S than for NAB mice ($p = 0.055$) that might be due to their extremely high horizontal locomotion.

3.3.4.3. Immobility

Total time of immobility that was depicted in Figure 38E significantly differed between all four lines ($F_{3,61}$ = 89.14, $p < 0.001$). HAB mice spent significantly more time immobile compared to the other three lines (all $p < 0.001$), as well as NAB mice exhibited more immobility time than both LAB-I and LAB-S mice (all $p < 0.001$). Moreover, significant difference was also found between LAB-I and LAB-S animals ($p < 0.001$). Therefore, immobility may not only be the index to mirror the data of locomotion, but also the best measure to distinguish the different selected lines. However, because of the convention in the literature, we decided to focus on locomotion in the subsequent pharmacological studies to ensure the comparability with other published data.

3.3.4.4. Rotations

Analysis of rotational behavior resulted in significant difference between the four lines ($F_{3,61}$ = 13.33, $p < 0.001$). The number of rotations was significantly higher for LAB-S mice than for HAB, NAB and LAB-I animals (LAB-S *vs.* HAB & NAB: $p < 0.001$; LAB-S *vs.* LAB-I: $p < 0.01$; Figure 38F). However, no difference was found between HAB, NAB and LAB-I mice.

To sum up, according to the bimodal phenotypes in LAB population in the OF test, separation of LAB-I and LAB-S groups is needed to testify their respective behavioral phenotypes and pharmacological responses.

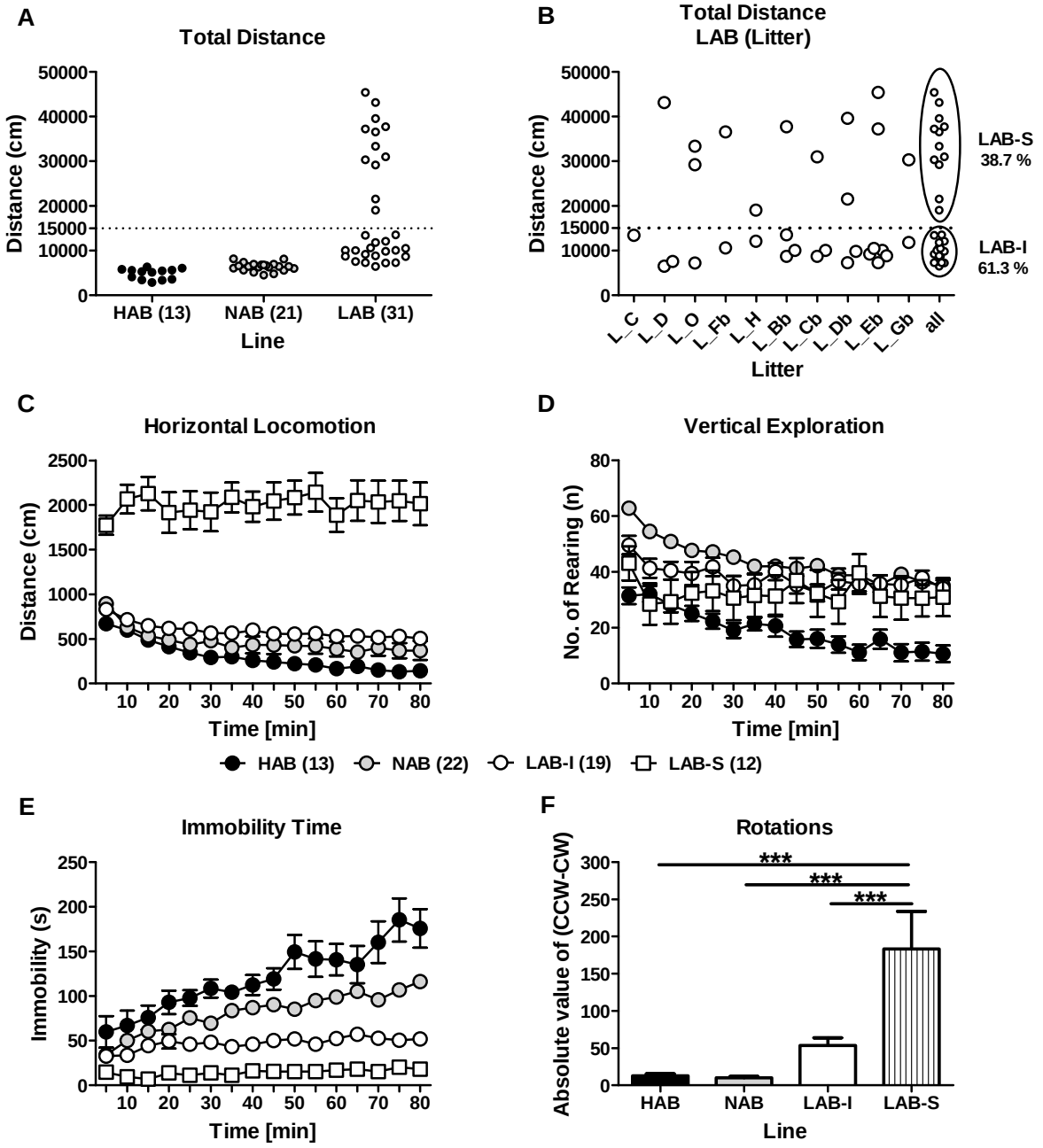


Figure 38: Selection of LAB-I and LAB-S mice and exploratory behaviors of HAB, NAB LAB-I and LAB-S mice in the open field test. Distribution of distance travelled in (A) HAB, NAB and LAB mice and (B) LAB mice by different litters. Distance travelled of 15000 cm was set as the criterion (dashed lines) for the selection of LAB-I and LAB-S mice. After selection, animals' exploration was measured in terms of (C) distance travelled, (D) number of rearing, and (E) immobility in 5-min bins for the 80-min exposure. (F) Rotational behavior of LAB-S mice was observed higher than that of HAB, NAB and LAB-I animals (reanalysis of the data shown in Figure 4.3). Data were obtained from mice of Generation 40/10. *** $p < 0.001$ (ANOVA followed by post-hoc Newman-Keuls test).

3.3.5. Persistence of locomotion

Given the fact that mice were repeatedly tested in the OF tests over the course of pharmacological treatments, we used the availability of no-treatment trials to examine the persistence. The experimental schedule is summarized in the upper panel of Figure 39. On day 0, animals were tested in the OF test for the first time followed by several times of OF testing with pharmacological treatments and WCM test (c.f. Figure 38C). 3 LAB-S and 1 LAB-S mice were not included in the analysis due to their death during the WCM testing. 62 days later, the same batches of HAB and NAB mice as well the rest of LAB-I and LAB-S animals were again tested in the OF-80 test without treatments. Analysis of DT revealed a significant difference between lines ($F_{3,57} = 43.64$, $p < 0.001$; Figure 39A). Further analysis indicated that LAB-S mice differed significantly from the other three lines (all $p < 0.001$) and LAB-I mice displayed significantly more DT in comparison with HAB and NAB mice (all $p < 0.001$). Figure 39B describes the change of DT between the two OF-80 exposures without treatment. There was a significant effect of line on DT ($F_{3,57} = 43.64$, $p < 0.001$), and an approaching significance of line by day interaction ($F_{3,56} = 2.56$, $p = 0.064$) as LAB-I mice travelled slightly farther across the days ($p = 0.058$) while HAB, NAB and LAB-S mice showed no change over days. The data suggest that HAB, NAB and LAB-S mice showed inter-individual persistence over time, whereas LAB-I displayed increased locomotion over time resulting in more distinction between LAB-I and HAB/NAB mice.

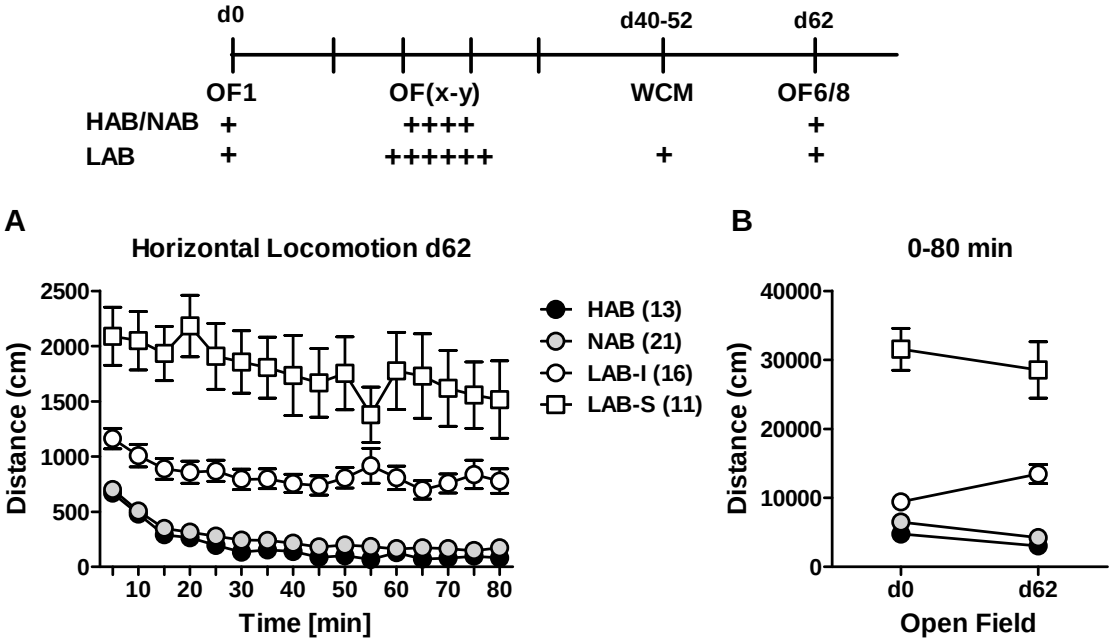


Figure 39: Persistence of phenotypes over the time. Animals were repeatedly tested in the OF-80 test without (d0, d62) or with pharmacological treatments (OF(x-y)) and in the water cross-maze (WCM) test in between. (A) Distance travelled in HAB, NAB, LAB-I and LAB-S mice on day 62 (corresponding to the 6th OF exposure in HAB and NAB mice and the 8th OF exposure in LAB mice). (B) Total distance travelled on day 0 and day 62 over the entire observation period. Data were obtained from mice of Generation 40/10.

3.3.6. HB Test

OF test is a common behavioral tool used to measure the general activity such as locomotor activity, hyperactivity, and exploratory behaviors. In addition to general measures of activity, we were also curious about how the animals respond to a more complex environment. Therefore, we tested HAB (n = 10), NAB (n = 10) and LAB (n = 10) mice of Generation 37/7 in a HB test which contains nose poke floors.

3.3.6.1. Locomotion

For total DT, one-way ANOVA showed significant differences between lines ($F_{2,27}$ = 12.49, $p < 0.001$; Figure 40B) in which LAB mice displayed significantly more locomotion compared to HAB and NAB mice during the entire 30-min exposure (all $p < 0.001$) with no significant difference between HAB and NAB mice ($p > 0.05$). Analyses of DT at 5-min bins revealed significant main

effect of line ($F_{2,27}$ = 12.49, p < 0.001) and line × time interaction ($F_{10,135}$ = 7.79, p < 0.001). Post-hoc comparisons revealed no difference in locomotion between any of the three lines during the first 5-min exposure; however, in the following 5-min bins towards the end of the exposure, LAB mice showed more DT than HAB and LAB mice (all ps < 0.001; Figure 40A).

3.3.6.2. Rearing

Investigating vertical exploration by analysis of the number of rearings showed significant line difference during the entire exposure ($F_{2,27}$ = 12.13, p < 0.001; Figure 40D). Having a close look at the number of rearing over the course of exposure, we observed significant differences between LAB/NAB mice and HAB mice, but no difference between NAB and LAB mice in the beginning of exposure (both ps < 0.01; Figure 40C). There was a significant line × time interaction ($F_{10,135}$ = 2.05, p < 0.05). In the last two 5-min bins of exposure, LAB showed more rearing compared to HAB and NAB mice (all p < 0.05), with no significant difference between HAB and NAB mice (Figure 40C).

3.3.6.3. Nose-poke (N-P) behavior

The number of nose pokes into holes (N-P entries) is shown in Figures 41A and 41B. For the total number of N-P entries, significant differences were found between lines ($F_{2,27}$ = 31.66, p < 0.001; Figure 41B), and further analyses showed that LAB mice showed less exploration of the 16 holes compared to HAB and NAB mice in terms of N-P entries (all p < 0.001) and the time spent in nose poking (data not shown). Two-way ANOVA revealed a significant line × time interaction ($F_{10,135}$ = 2.29, p < 0.05). Post-hoc comparisons revealed that HAB and NAB mice showed significantly more N-P entries than LAB mice over the course of exposure (all p < 0.05). In the first two 5-min bins of exposure, NAB mice displayed a higher number of N-P entries than HAB mice (all p < 0.05; Figure 41A).

Not only number and duration of nose pokes was reduced in LAB mice, but also the accuracy of performance, which describes how many of the total 16 holes had been visited at least once over the course of the exposure ($F_{2,27}$ = 13.48, p < 0.001, Figure 41C). It is of note that there was

an inverse relationship between locomotor activity (cf. Figure 40B) and accuracy of hole exploration in LAB mice ($r^2 = 0.852$, $p < 0.001$), but not in HAB and NAB mice.

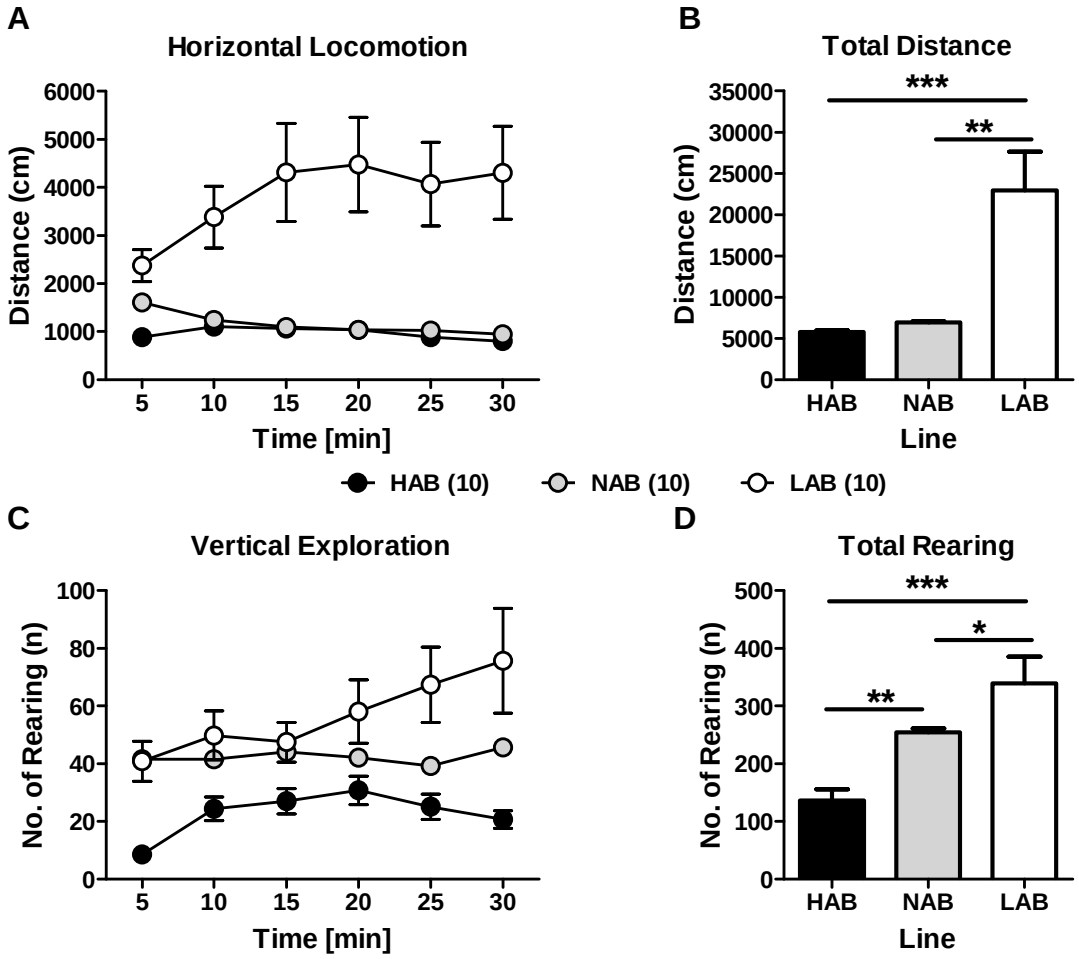


Figure 40: Horizontal and vertical exploration of HAB, NAB and LAB mice in the hole-board test (HBT). Horizontal locomotor activity was measured as (A) distance travelled in 5-min bins for the 30-min exposure and (B) total distance travelled during the entire investigation. Vertical exploratory behaviors in HAB, NAB and LAB mice were indicated by the number of rearing (C) in 5-min bins for the 30-min exposure and (D) during the entire exposure. Note that this experiment was performed with different batches of mice other than those used in the previous open field exposures. Data were obtained from mice of Generation 37/7. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ (ANOVA followed by post-hoc Newman-Keuls test).

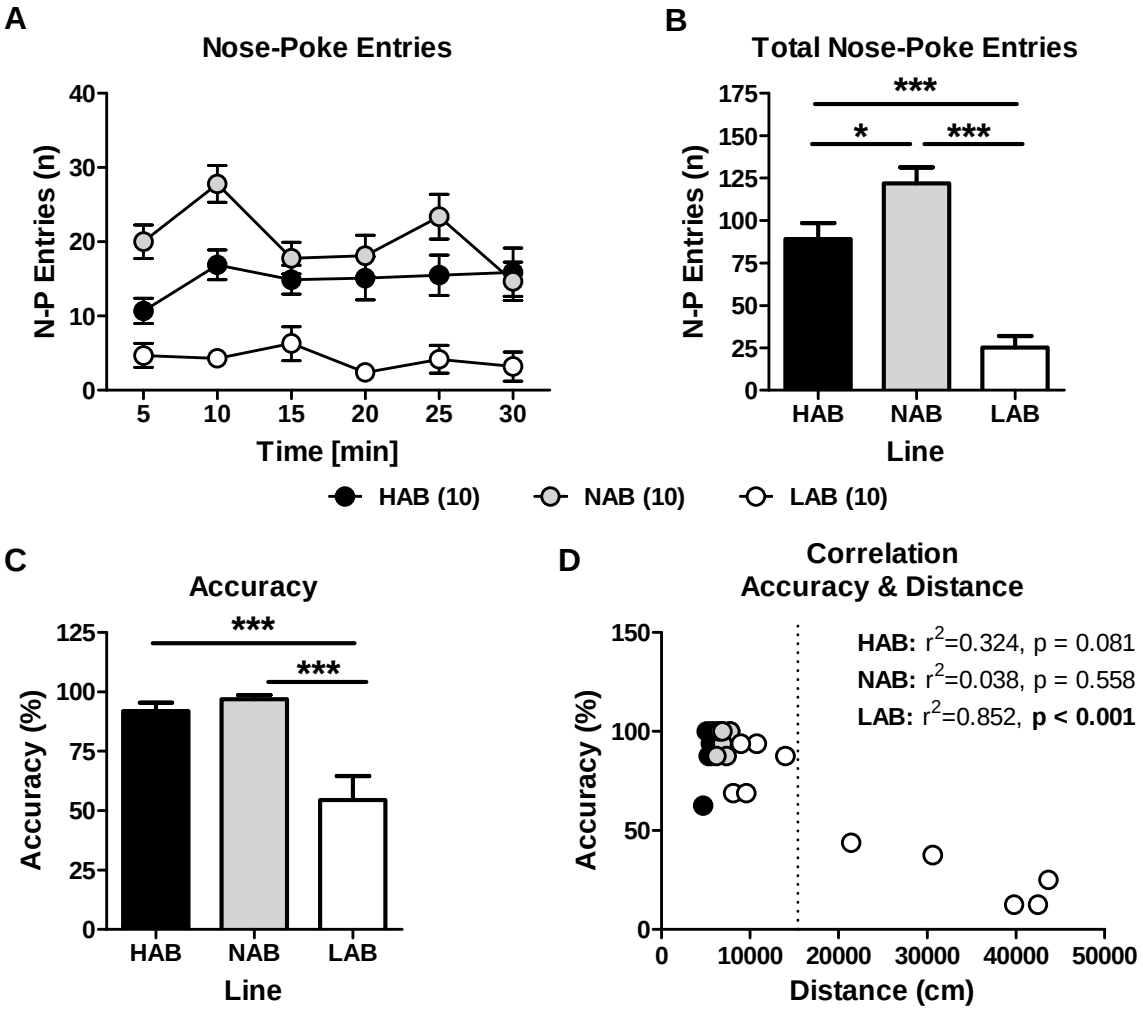


Figure 41: Nose-poke behaviors of HAB, NAB and LAB mice in the hole-board test. Downward exploration was estimated as the number of nose-poke entries (A) in 5-min bins for the 30-min exposure and (B) during the entire exposure. (C) Nose-poke accuracy was calculated as percentage of holes visited by the animals during the entire exposure. (D) Correlation between accuracy and distance travelled. Note that LAB-I and LAB-S could be also differentiated among LAB mice by a threshold of 15000 cm (dashed line). Data were obtained from mice of Generation 37/7. *** $p < 0.001$; * $p < 0.05$ (ANOVA followed by post-hoc Newman-Keuls test).

3.3.7. Pharmacological treatment and locomotion

3.3.7.1. Intraperitoneal (i.p.) injection of amphetamine

Before amphetamine administration, no difference was observed between the two identical groups in all four lines during 20-min baseline OF exposure. Two-way repeated measure ANOVA revealed that administration of amphetamine (2.0 mg/kg, i.p.) resulted in increased locomotor activity in HAB ($F_{1,11}$ = 44.83, $p < 0.001$; Figure 42A) and NAB ($F_{1,20}$ = 6.75, $p < 0.05$; Figure 42B) mice. In contrast, amphetamine treatment significantly decreased hyperlocomotor activity in both LAB-I ($F_{1,17}$ = 14.34, $p < 0.01$; Figure 42C) and LAB-S ($F_{1,10}$ = 8.39, $p < 0.05$; Figure 42D) mice. Figure 4.8E shows the percent change of DT after amphetamine administration in comparison with Veh treatment. The percent change of DT (DT) after vehicle or drug treatment was calculated as [(DT-drug – DT-Veh $_{mean}$)/DT-Veh $_{mean}$] $\times$ 100 %. Analyses of the percent change of DT during 60 min showed that amphetamine at doses of 0.5 (not shown as line graphs) and 2.0 mg/kg caused significant increases in locomotion in HAB ($F_{2,16}$ = 18.73, $p < 0.001$) and NAB ($F_{2,30}$ = 4.41, $p < 0.05$) mice. In contrast, decreased locomotor activity was observed in LAB-I after acute administration of amphetamine at doses of 0.5 and 2.0 mg/kg ($F_{2,24}$ = 4.20, $p < 0.05$) and in LAB-S mice at a dose of 2.0 mg/kg ($F_{2,16}$ = 4.62, $p < 0.05$).

3.3.7.2. i.p. injection of methylphenidate

Mice of each line were reassigned to control (Veh) and methylphenidate (MD) groups based on the locomotion data during drug-free period (-20~0 min) of the previous test. During the 20-min OF exposure before MD administration, no difference was found between MD-treated and Veh-control animals in lines HAB, NAB and LAB-S. By chance, the two LAB-S groups displayed difference in DT before drug administration, which however, did not hamper the interpretation of the data. Statistical analyses revealed that methylphenidate (10.0 mg/kg) induced increased DT in HAB ($F_{1,11}$ = 47.55, $p < 0.001$; Figure 43A), NAB ($F_{1,20}$ = 135.68, $p < 0.001$; Figure 43B) and LAB-I ($F_{1,16}$ = 17.28, $p < 0.001$; Figure 43C) mice, but no change in LAB-S mice ($F_{1,10}$ = 1.01, $p > 0.05$; Figure 43D). Change of DT was summarized in Figure 43E which validates the effects of methylphenidate on locomotion in HAB, NAB and LAB-I mice.

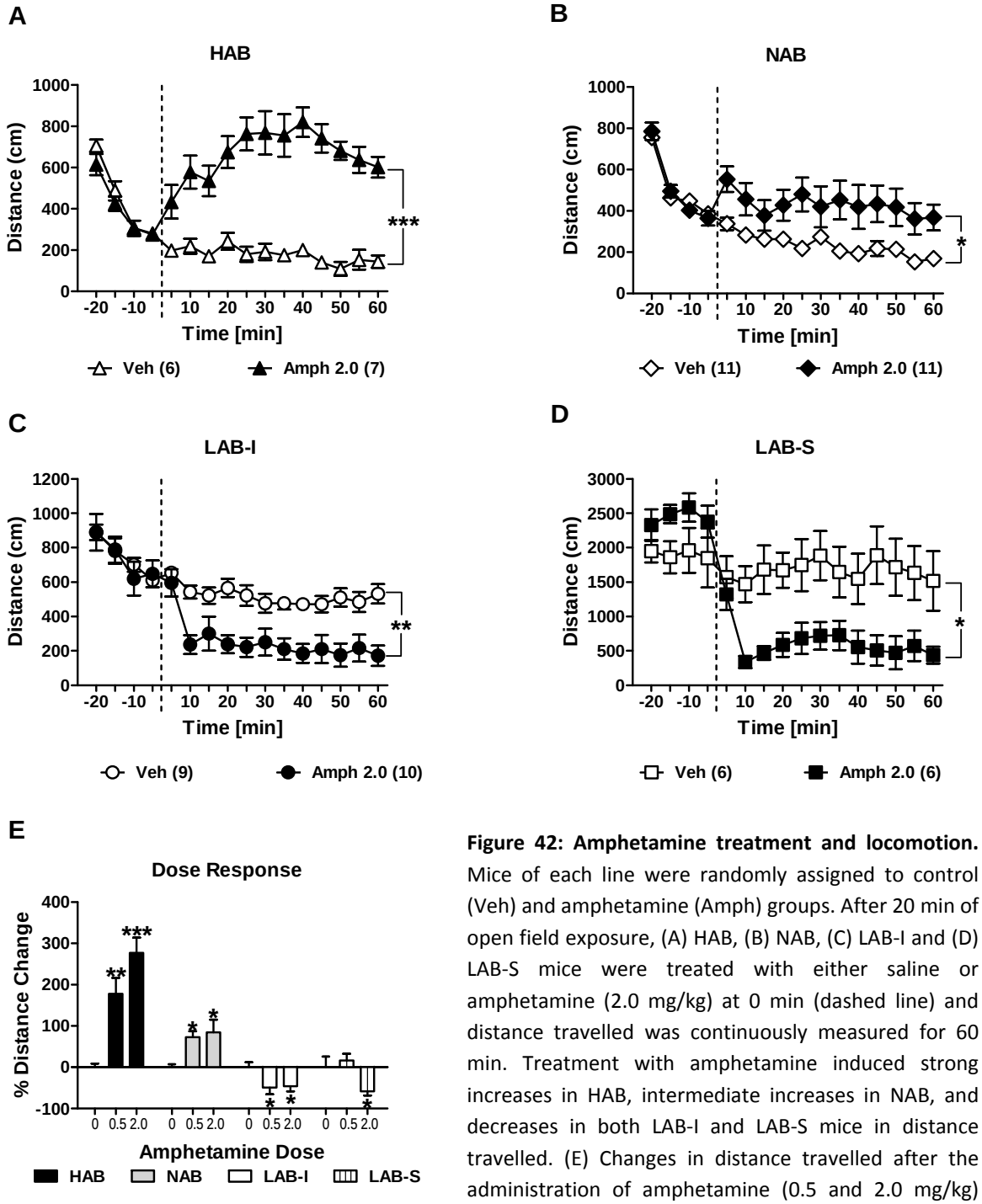


Figure 42: Amphetamine treatment and locomotion. Mice of each line were randomly assigned to control (Veh) and amphetamine (Amph) groups. After 20 min of open field exposure, (A) HAB, (B) NAB, (C) LAB-I and (D) LAB-S mice were treated with either saline or amphetamine (2.0 mg/kg) at 0 min (dashed line) and distance travelled was continuously measured for 60 min. Treatment with amphetamine induced strong increases in HAB, intermediate increases in NAB, and decreases in both LAB-I and LAB-S mice in distance travelled. (E) Changes in distance travelled after the administration of amphetamine (0.5 and 2.0 mg/kg) were calculated as percentage of changes relative to the mean of Veh-treated controls. Data are shown either in 5-min bins from (A) to (D), or the mean of distance changes in (E). Data were obtained from mice of Generation 40/10. *** $p < .001$; ** $p < .01$; * $p < .05$ compared with Veh (ANOVA followed by post-hoc Newman-Keuls test).

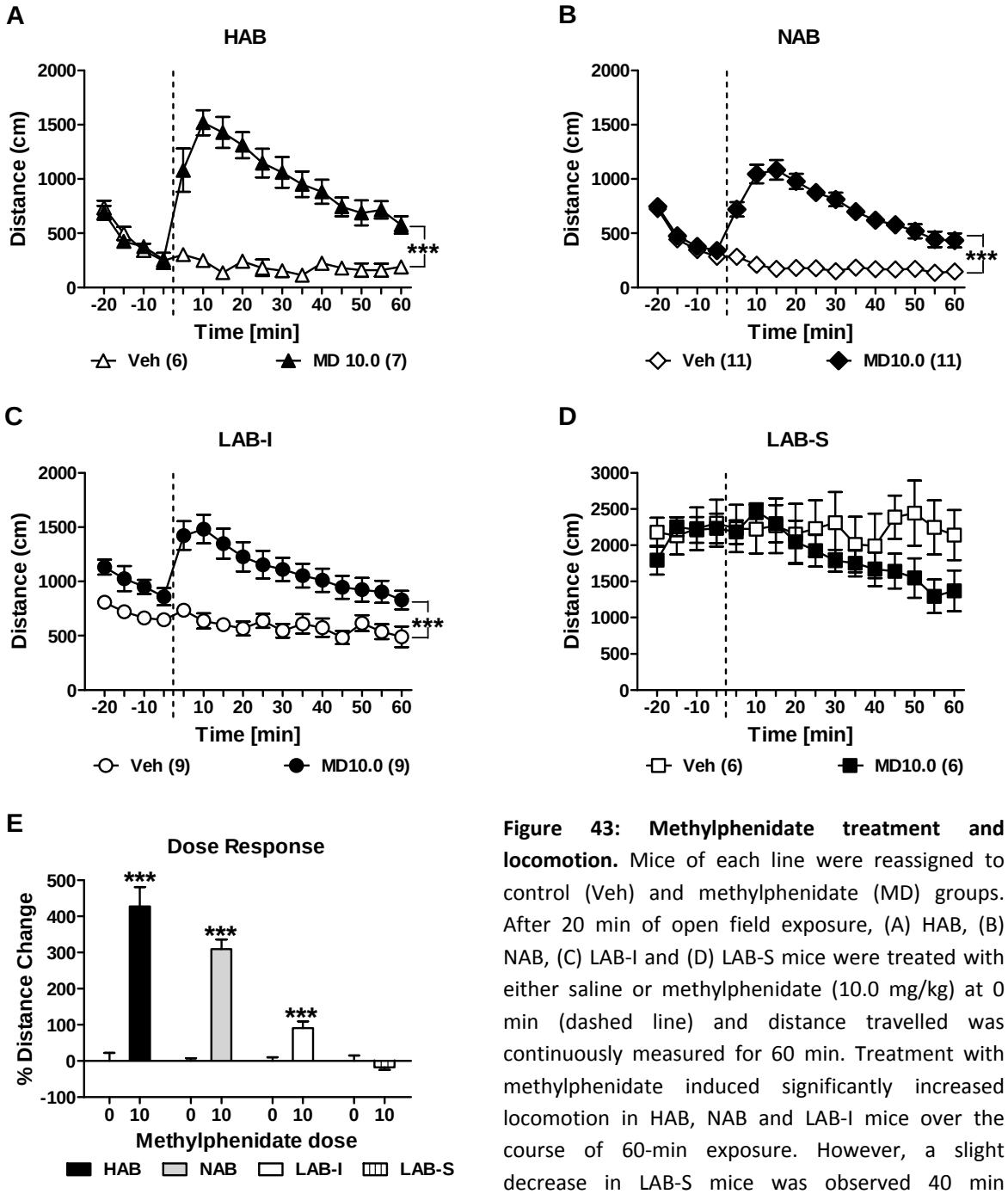


Figure 43: Methylphenidate treatment and locomotion. Mice of each line were reassigned to control (Veh) and methylphenidate (MD) groups. After 20 min of open field exposure, (A) HAB, (B) NAB, (C) LAB-I and (D) LAB-S mice were treated with either saline or methylphenidate (10.0 mg/kg) at 0 min (dashed line) and distance travelled was continuously measured for 60 min. Treatment with methylphenidate induced significantly increased locomotion in HAB, NAB and LAB-I mice over the course of 60-min exposure. However, a slight decrease in LAB-S mice was observed 40 min following the methylphenidate administration. (E) Summary of distance change (c.f. Figure 4.7E) after methylphenidate treatment for 60 min. Data are shown either in 5-min bins from (A) to (D), or the mean of distance changes in (E). Data were obtained from mice of Generation 40/10. *** $p < 0.001$ compared with Veh (ANOVA followed by post-hoc Newman-Keuls test).

3.3.7.3. i.p. injection of tomoxetine hydrochloride (TH)

To investigate the effects of noradrenaline system on locomotion, NAB and LAB mice of Generation 39/9 were tested in the OF test. In this batch of LAB mice, there were only three LAB-S mice which were excluded from testing because of the insufficient sample size. Administration of the selective NRI, tomoxetine hydrochloride, at doses of either 3.0 (Figure 44A) or 10.0 mg/kg (Figure 4.10B) had no effect on locomotor activity of NAB and LAB-I mice (statistics not shown).

3.3.7.4. i.p. injection of haloperidol

To elucidate whether Dopaminergic transmission is required for the presence of locomotor activity, mice of NAB and LAB-I were subsequently treated with a DA receptor 2 antagonist, haloperidol (first run), and saline (second run) in separate OF-80 tests. Two tests were performed with one-week break to avoid confounding influences of lasting drug effects. Before drug administration, no difference was observed between the two runs in LAB-I mice, but a significant difference was found between the two runs in NAB mice ($F_{1,14}$ = 28.34, $p < 0.001$). Fortunately, this difference did not impede the explanation of drug effects. Statistical analyses revealed that treatment with haloperidol significantly suppressed locomotor activity in both NAB ($F_{1,14}$ = 33.77, $p < 0.001$; Figure 44C) and LAB-I ($F_{1,12}$ = 23.63, $p < 0.001$; Figure 44D) mice.

3.3.7.5. i.p. injection of AM404

To examine the effects of endocannabinoid system on locomotion, we administered an endocannabinoid uptake inhibitor AM404 in HAB, NAB and LAB mice of Generation 40/10. Mice of each line were reassigned to Veh or AM404 groups. During the 20-min OF exposure before AM404 administration, no difference was found between AM404-treated and Veh-control animals in all four lines (Figure 45A-D). Statistical analyses revealed that AM404 (3.0 mg/kg) had no effect on DT of HAB and NAB mice (Fs < 1). However, AM404 treatment induced a significant reduction in locomotor activity in LAB-I mice ($F_{1,16}$ = 9.95, $p < 0.01$; Figure 45C), and a tendency of decrease in hyperlocomotor activity in LAB-S mice ($F_{1,11}$ = 4.59, $p = 0.055$; Figure 4.11D) as well as a significant treatment $\times$ line interaction ($F_{3,33}$ = 5.54, $p < 0.01$). Change of DT was

summarized in Figure 45E which double proves the effects of AM404 on locomotion in HAB, NAB and LAB-I mice.

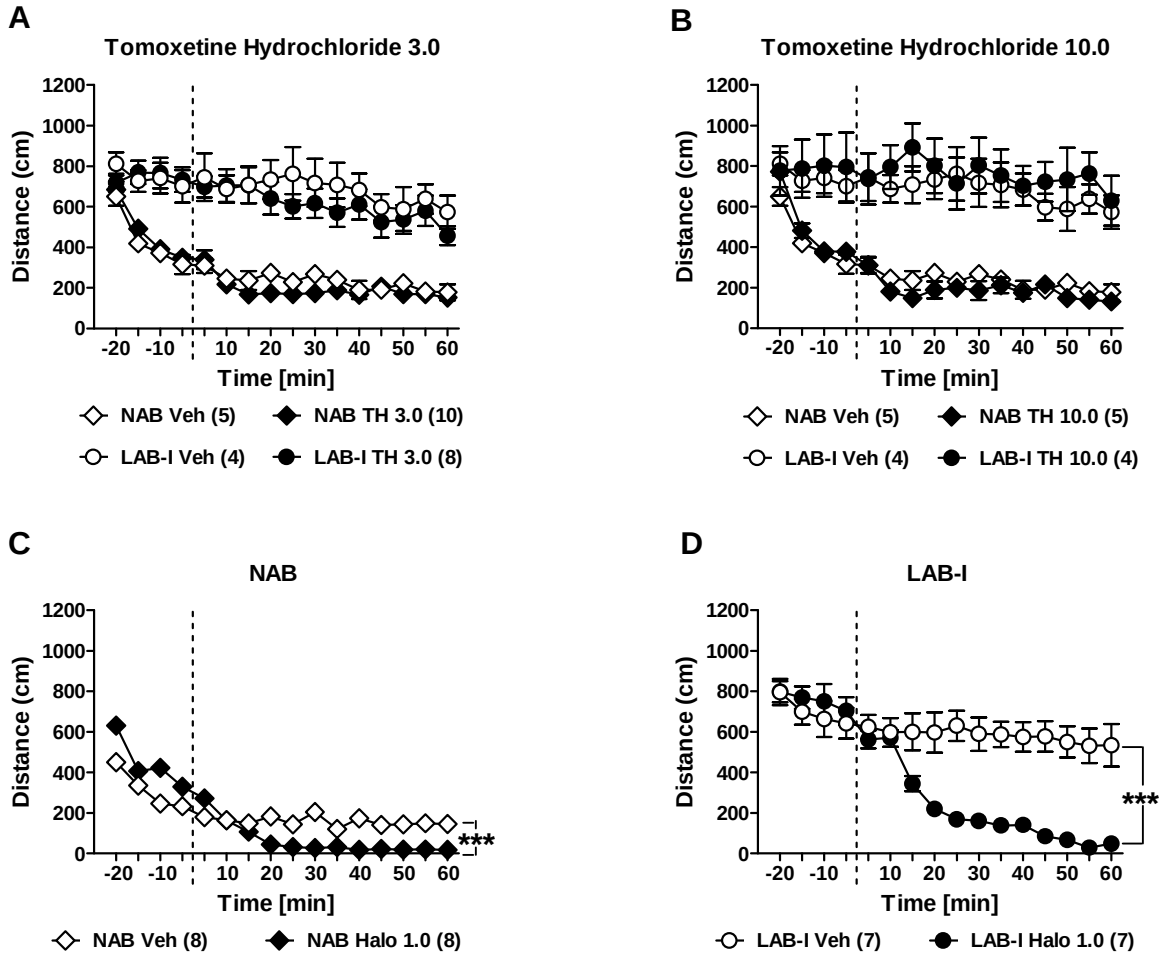


Figure 44: Tomoxetine hydrochloride/haloperidol treatments and locomotion. NAB and LAB-I mice were used for testifying the effects of tomoxetine hydrochloride and haloperidol. Mice of each line were randomly assigned to control (Veh) and tomoxetine hydrochloride (TH) groups. After 20 min of open field exposure, both NAB and LAB mice were treated with either saline or tomoxetine hydrochloride of (A) 3.0 mg/kg and (B) 10.0 mg/kg at 0 min (dashed line) and distance travelled was continuously measured for 60 min. Treatment with tomoxetine hydrochloride didn't induce any change of locomotion in NAB and LAB-I mice. Moreover, mice from the same batches of (C) NAB and (D) LAB-I mice were treated with saline and haloperidol (1.0 mg/kg). After 20 min of open field exposure, haloperidol treatment significantly induced a reduction in locomotion of HAB and LAB-I mice over the course of 60-min exposure. Data were obtained from mice of Generation 39/9. *** $p < 0.001$ compared with Veh (ANOVA followed by post-hoc Newman-Keuls test).

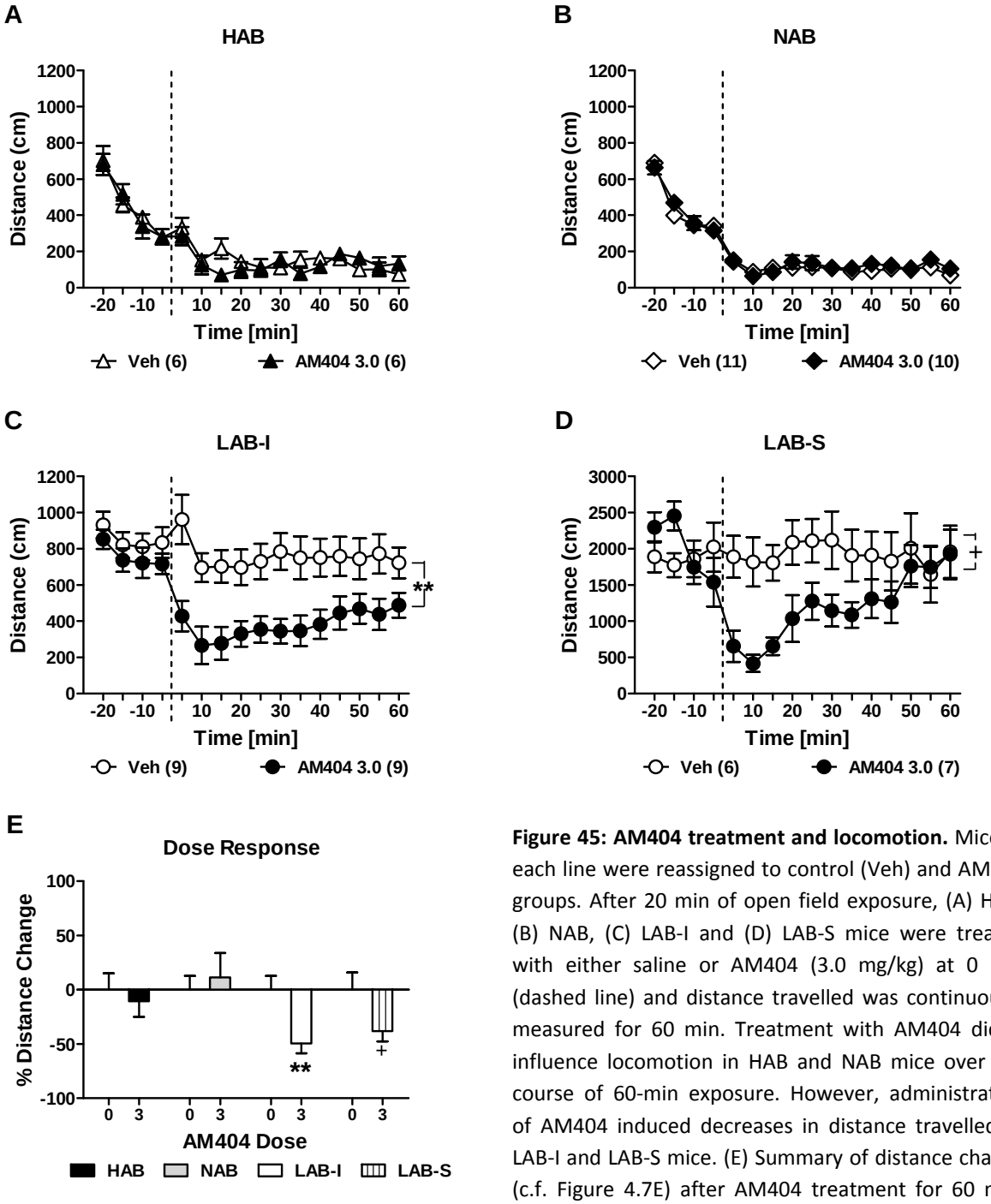


Figure 45: AM404 treatment and locomotion. Mice of each line were reassigned to control (Veh) and AM404 groups. After 20 min of open field exposure, (A) HAB, (B) NAB, (C) LAB-I and (D) LAB-S mice were treated with either saline or AM404 (3.0 mg/kg) at 0 min (dashed line) and distance travelled was continuously measured for 60 min. Treatment with AM404 didn't influence locomotion in HAB and NAB mice over the course of 60-min exposure. However, administration of AM404 induced decreases in distance travelled of LAB-I and LAB-S mice. (E) Summary of distance change (c.f. Figure 4.7E) after AM404 treatment for 60 min. Data are shown either in 5-min bins from (A) to (D), or the mean of distance changes in (E). Data were obtained from mice of Generation 40/10. ** $p < 0.01$; + $p < 0.1$ compared with Veh (ANOVA followed by post-hoc Newman-Keuls test).

3.3.7.6. The involvement of cannabinoid receptor 1 (CB1) receptors in the hypolocomotor effects of AM404

To evaluate the modulating role of CB1 receptors on the locomotor-attenuating effects of AM404 in LAB mice, we injected an inverse agonist for the cannabinoid receptor, Rimonabant (Rimo, SR141716), or Veh 30 min before administration of AM404. During the 20-min OF exposure before both administrations, no difference was found between SR-treated and Veh-control LAB-I mice ($F < 1$; Figure 46A). Administration of Rimo (3.0 mg/kg) induced a tendency of decreased locomotor activity ($F1, 6 = 5.06$, $p = 0.066$), but did not exert any effect on AM404-induced hypolocomotion ($F < 1$).

3.3.7.7. The involvement of transient receptor potential vanilloid 1 (TRPV1) in the hypolocomotor effects of AM404

In order to delineate the functional involvement of TRPV1 receptors in mediating the hypolocomotor effects of AM404 in LAB mice, we first administered SB-366791 (SB) at dose of 1.0 mg/kg 30 min before the administration of AM404 at 3.0 mg/kg, a dose efficient in attenuating hyperlocomotion in LAB-I and LAB-S mice. Treatment with SB at 1.0 mg/kg per se had no effect on locomotor activity ($F < 1$), but also failed to block the AM404-induced effects in LAB-I mice ($F < 1$; Figure 46B). We increased SB dose and modified the experimental paradigm by administering SB before the OF test and injecting AM404 (3.0 mg/kg) 30 min later. Treatment with SB at dose of 5.0 mg/kg significantly attenuated locomotor activity in both LAB-I ($F_{1,14} = 20.62$, $p < 0.001$) and LAB-S ($F_{1,11} = 34.87$, $p < 0.001$) mice, and further potentiated the hypolocomotor effects of AM404 (Figure 46C & D). Based on the improper SB doses used in the previous experiments, we applied SB at 3.0 mg/kg, a dose per se that had no effect on locomotor activity in LAB-I and LAB-S mice ($Fs < 1$), but that induced different consequences for the hypolocomotor effects of AM404 in LAB-I and LAB-S mice (Figure 46E & F). Taken together, blockade of TRPV1 induced hypolocomotion at high doses but failed to attenuate the effects of AM404. In fact, there was even evidence of additive effects for the two drugs.

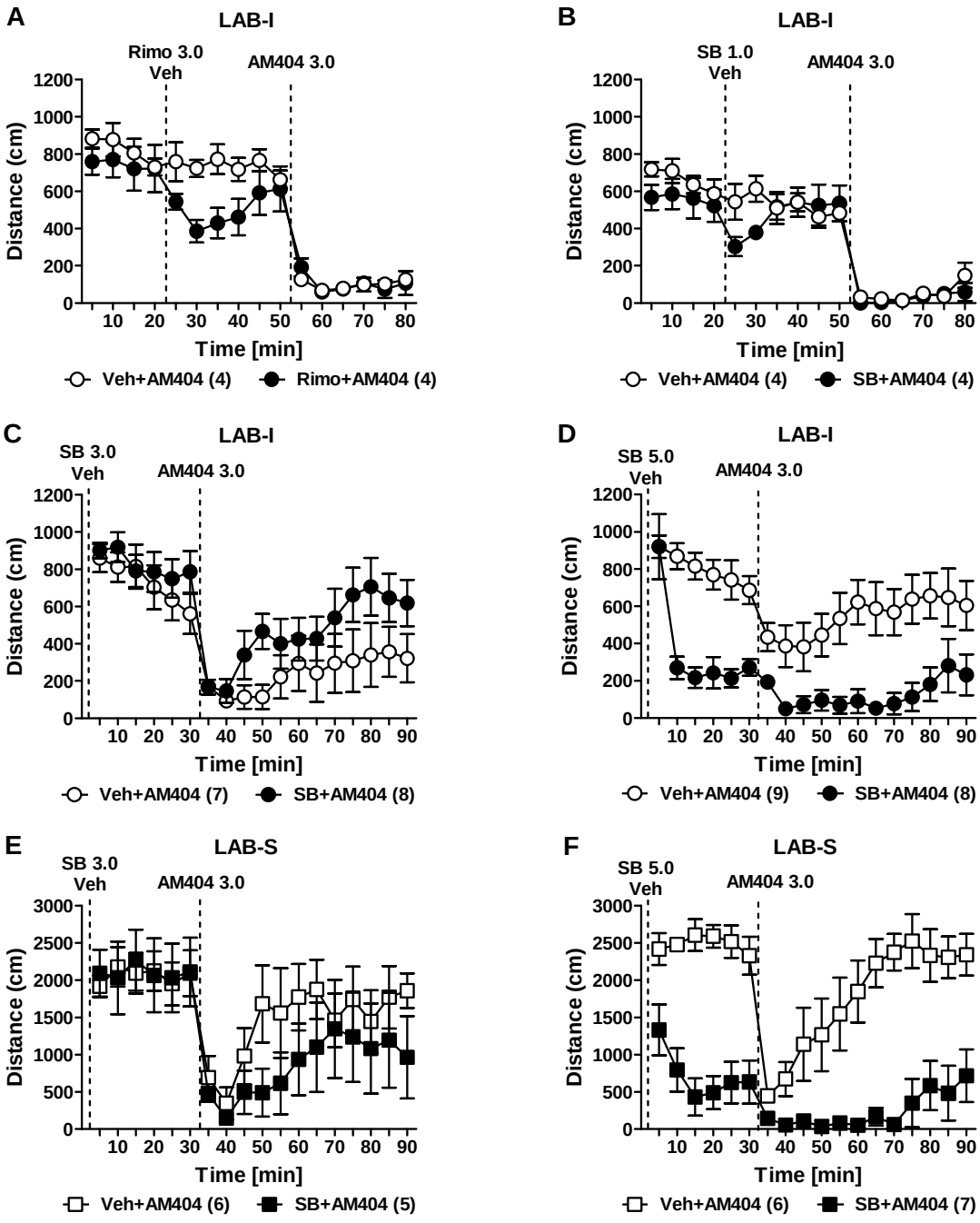


Figure 46: The involvement of CB1 & TRPV1 receptors in AM404 treatment and locomotion. Mice of each line were reassigned to control (Veh) or drug-treated groups. After 20 min of open field exposure, LAB-I mice were treated with either saline or (A) Rimonbant (Rimo) (3.0 mg/kg) (B) SB-366791 (SB) (1.0 mg/kg) at 20 min (dashed line). 30 min later, both groups were treated with AM404 (3.0 mg/kg) and distance travelled was continuously measured for 30 min. Neither SR nor SB mediated the AM404-induced hypolocomotion in LAB-I mice. In LAB-I mice, administration of SB at (C) 3.0 mg/kg and (D) 5.0 mg/kg potentiated the hypolocomotor effects of AM404. In LAB-S mice, treatment of SB at (E) 3.0 mg/kg slightly blocked the hypolocomotor effects of AM404; however, administration of SB at (F) 5.0 mg/kg potentiated AM404-induced effects. Data are shown either in 5-min bins from (A) to (F). Data of (A) and (B) were obtained from mice of Generation 39/9, and the rest from Generation 40/10.

3.3.8. WCM test

10 HAB, 10 NAB and 10 LAB mice were trained in a WCM test with FL protocol. Only animals that successfully learned during the first week of training were used for reversal training in the second week. During the first week of training, all three lines showed a decrease in escape latencies ($F_{3,81} = 45.42$, $p < 0.001$), implying learning of the task. However, the escape latencies of HAB and NAB mice were significantly lower than those of LAB mice (all $p < 0.01$). Statistical analysis revealed a significant main effect of line in the levels of accuracy ($F_{2,27} = 5.41$, $p < 0.001$). Concerning the levels of accuracy on day 4, 90 percent of HAB and 70 percent of NAB mice reached the accuracy criterion of ≥ 5 accurate out of 6 trials, whereas only 40 percent of LAB mice reached the accuracy criterion of 83.3 % (Figure 47D). The number of wrong platform visits that is depicted in Figure 47C didn't differ between the three lines ($F < 1$).

During the second week, the accurate learners of week 1 underwent reversal training with relocation of the platform to the opposite arm. On the first day of reversal learning, all animals showed memory perseveration, as reflected by the high number of visits into the original platform position and the resultant increase in escape latencies and low levels of accuracy. Ongoing training processes led to progressing relearning in HAB and NAB mice; however, there is virtually no reversal learning in LAB mice (Figure 48, relearning). This phenomenon was reflected by significant main effect of line for all three learning parameters (escape latency: $F_{2,17} = 20.28$, $p < 0.001$; accuracy: $F_{2,17} = 11.24$, $p < 0.001$; wrong platform visit: $F_{2,17} = 24.94$, $p < 0.001$; Figure 48A-C). Further analyses revealed that LAB mice displayed impairment in relearning, as reflected by the higher escape latency, the poor accuracy and the high number of wrong platform visit. Notably, on day 4 of reversal learning, all LAB mice still showed perseveration of the original platform position, as indicated by ≤ 1 accurate out of 6 trials and 5 to 6 wrong platform visits (Figure 48B & C).

To sum up, LAB mice are heavily impaired in learning acquisition and, in particular, relearning in the WCM by the evidence that 4 out of 10 LAB mice acquired the test at all, as well as none of these four learners showed relearning. The most parsimonious explanation would be that those

4 mice only acquired habitual memory on the basis of egocentric response learning strategies during the acquisition phase and were strongly impaired in place learning, which is supposed to be a prerequisite for relearning (Kleinknecht & Wotjak, in preparation).

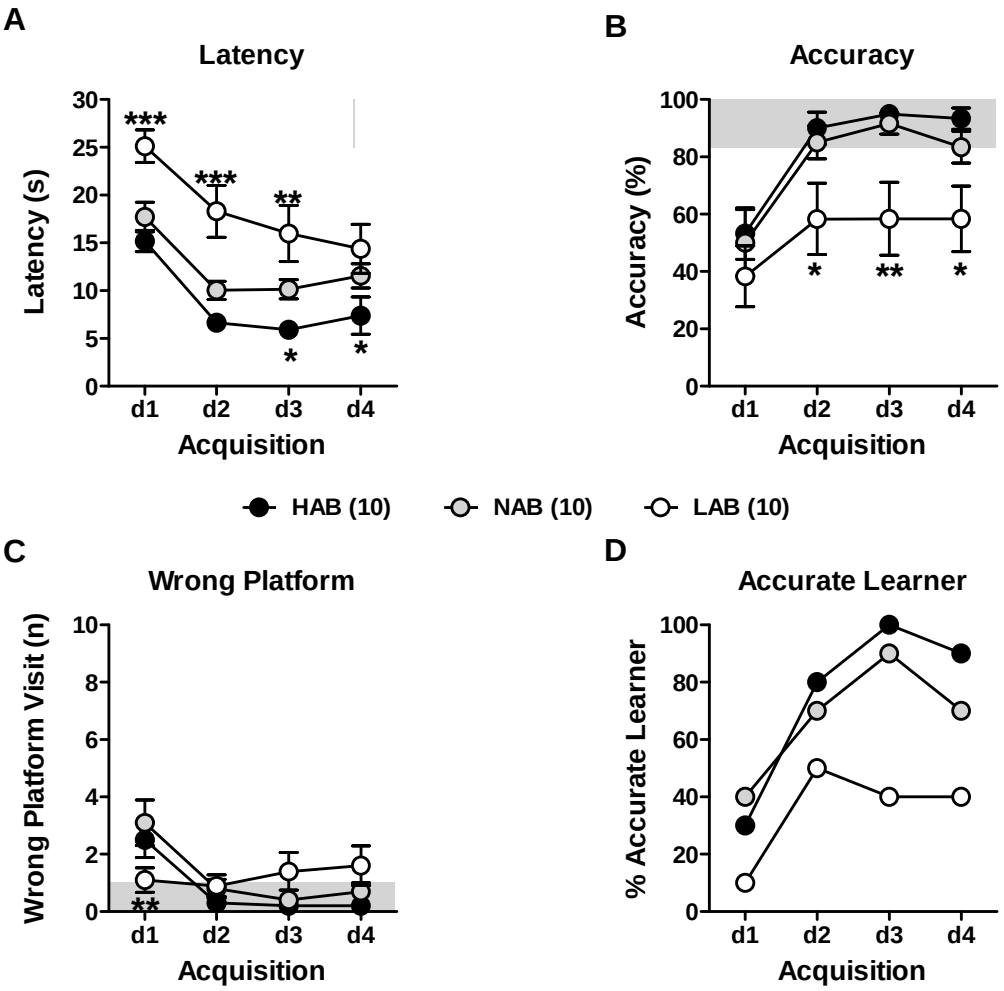


Figure 47: Water cross-maze in all animals (acquisition phase). HAB, NAB and LAB mice were started from the South arm of the maze and trained to navigate to a hidden platform localized in the end of the West arm with 6 trials per day over the course of 4 consecutive days (d1-d4; free learning protocol). The performance of learning acquisition was described in terms of (A) escape latency, (B) % accuracy, (C) number of wrong platform visits and (D) accurate learners. HAB and NAB mice acquired that test, while LAB mice displayed impairment in acquisition by the higher escape latency and lower levels of accuracy. Data were obtained from mice of Generation 37/7. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared with NAB mice (ANOVA followed by post-hoc Newman-Keuls test).

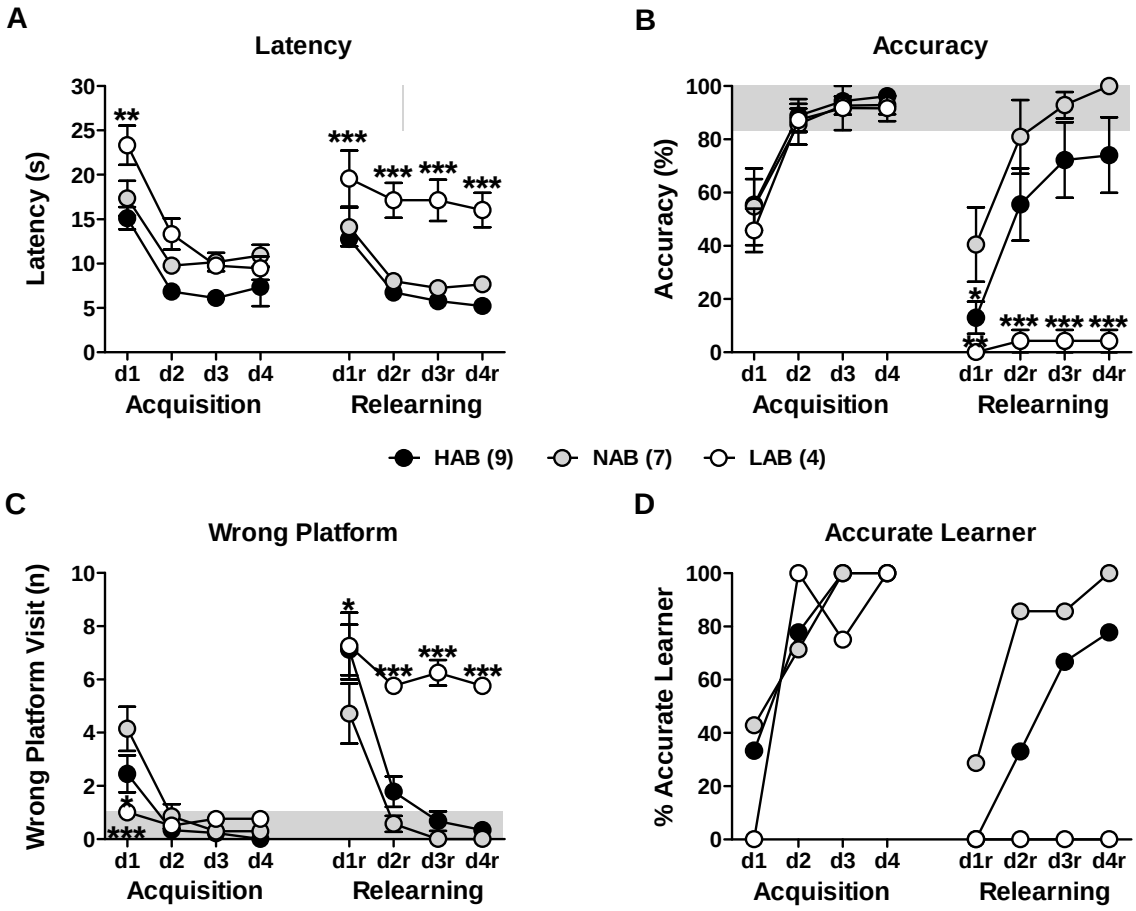


Figure 48: Water cross-maze in accurate learners (acquisition and relearning phases). 9 HAB, 7 NAB and 4 LAB accurate learners during the acquisition were tested for the reversal training, and only the data of those animals were analyzed in terms of (A) escape latency, (B) % accuracy, (C) wrong platform visit and (D) accurate learners. During the learning acquisition phase (left panels), even though three lines showed significant difference on the first day, no difference was found in any of the four parameters between three lines at the end of the acquisition phase (i.e. d4). Thereafter, the platform was moved to the opposite East arm and mice were trained for relearning of the platform position for another 4 days (d1r-d4r). During the second week of relearning phase (right panels), 7 out of 9 HAB mice (77.77 %) and 7 out of 7 NAB mice (100 %) showed relearning during reversal training. In contrast, none of LAB mice (0 %) displayed relearning, as reflected by the poor performance in all parameters. Data were obtained from mice of Generation 37/7. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared with NAB mice (ANOVA followed by post-hoc Newman-Keuls test).

3.3.9. Social recognition tests

3.3.9.1. Social preference test

10 HAB, 10 NAB and 10 LAB were subjected to the social preference test by using a 3-chamber apparatus. During the 10 min of habituation phase, none of the three lines showed side preference in the 3-chamber apparatus as reflected by no differences between time spent in the left versus the right chamber (all $p > 0.05$; inserted in Figure 49A left panel), confirming the absence of a prior side preference in the testing environment in all three lines. Moreover, no difference was found between time spent in sniffing two empty tubes (E1, E2) in the left and the right chambers (all $p > 0.05$; Figure 49A left panel). In the next 10 min sampling phase, all three lines spent significantly more time in sniffing the tube containing an ovariectomized female mouse (social stimulus, F1) than an empty tube (non-social stimulus, E1) ($t_9 = -5.41$, $p < 0.001$ for HAB; $t_9 = -4.83$, $p < 0.001$ for NAB; $t_9 = -10.42$, $p < 0.001$ for LAB; Figure 49A middle panel). During the last 10 min testing phase, HAB and NAB mice spent significantly more time in sniffing the tube containing a novel ovariectomized female mouse (F2) than that containing the familiar female mouse (F1) ($t_9 = -6.60$, $p < 0.001$ for HAB; $t_9 = -4.10$, $p < 0.01$ for NAB; Figure 49A right panel), confirming a preference for social novelty in HAB and NAB mice. However, LAB mice failed to show preference for social novelty as mirrored by no difference between time spent in sniffing the novel and the familiar female mouse ($t_9 = -1.28$, $p = 0.23$; Figure 49A right panel).

3.3.9.2. Social discrimination test

In the social discrimination task, all three lines were introduced to an ovariectomised female (F1) for 5 min, and after different IEIs, they were exposed to the familiar female (F1) and a novel ovariectomised female (F2) for another 5 min. During the sampling phase, results showed that sniffing time did not differ between all three lines ($F < 1$). With IEIs of 15 min (Figure 49B) and 30 min (data not shown), only HAB and NAB mice were able to discriminate between the familiar (F1) and the novel (F2) female ($t11 = 3.93$, $p < 0.01$ for HAB; $t11 = 3.06$, $p < 0.05$ for NAB), whereas LAB mice failed to discriminate two different social stimuli. Interestingly, with an IEI of 2 h, only HAB mice could distinguish the novel female (F2) from the familiar one (F1) (F2:

94.25 ± 25.99, F1: 56.38 ± 26.20; t = -3.02, p < 0.05). This ability was vanished after an IEI of 4h in HAB mice (F2: 82.63 ± 25.56, F1: 62.12 ± 30.28; t = 1.75, p > 0.05).

Together these data suggest that LAB mice show impairments in social recognition and seem to lose interest in social exploration with ongoing exposure or display no attention to the social change of the environment.

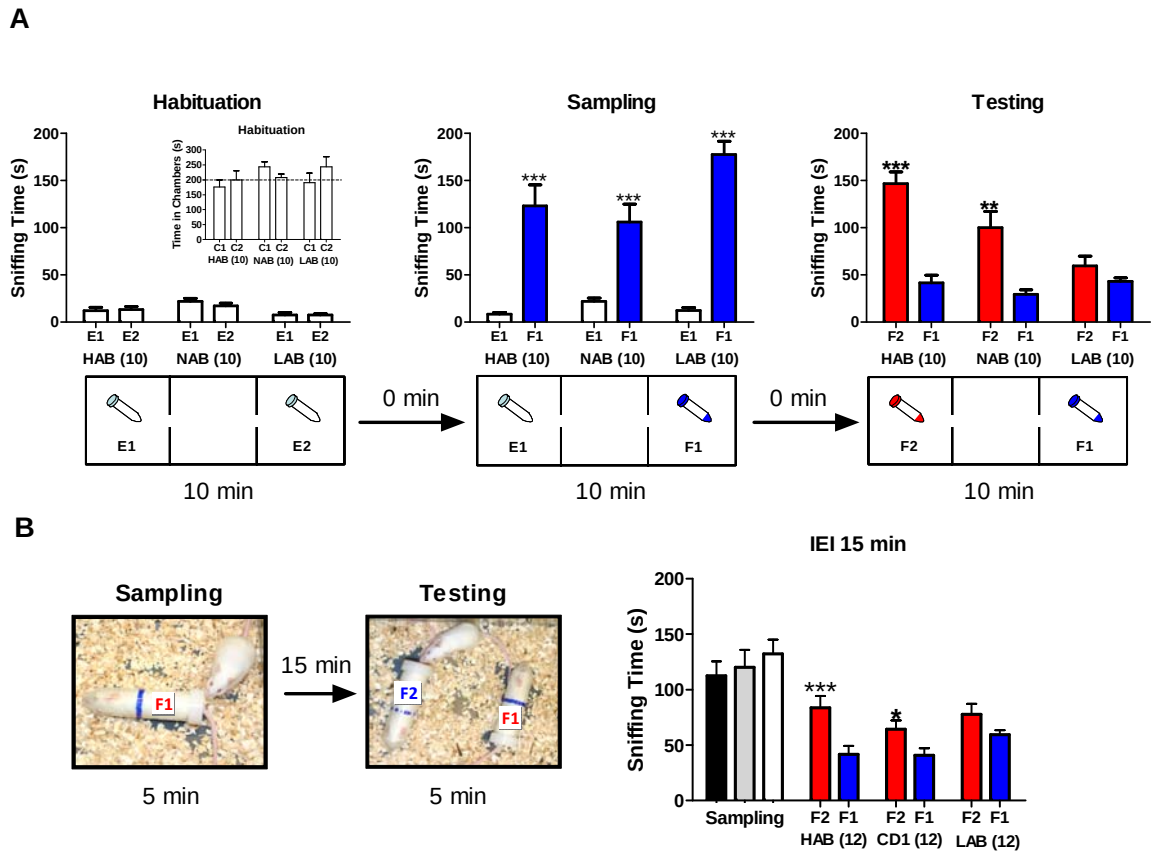


Figure 49: Social recognition tests. (A) In a 3-chamber apparatus, mice were tested in 3 consecutive phases: a habituation phase with empty tubes in the left and the right chamber (E1, E2), a sampling phase, in which one empty tube was replaced by an identical tube containing a female (F1), and a testing phase, during which the second empty tube was replaced by a tube containing another female (F2). During habituation phase, all three lines displayed no difference in investigation time of side chambers (C1, C2, inset) as well as in sniffing time towards two empty tubes, indicating no side preference of these animals. All three lines spent more time in sniffing the female (F1) exposed during the sampling phase compared to the empty tube (E1) and the habituation phase. LAB mice apparently lost interest in social investigation during the testing phase (as mirrored by the reduced total sniffing time) and failed to show a preference for the novel female (F2). (B) In a social discrimination task, all three lines were introduced to an ovariectomised female (F1) for 5 min, and after an interexposure interval (IEI) of 15 min, they were exposed to the familiar female (F1) and a novel ovariectomised female (F2) for another 5 min. Sniffing time did not differ between all three lines during the sampling phase. After 15 min, both HAB and NAB could discriminate between the F1 and F2, whereas LAB mice failed to do so. *** p < 0.001; ** p < 0.01; * p < 0.05 (paired t-test).

3.3.10. Pharmacological treatment and WCM test

To elucidate whether amphetamine could be a potential cognitive enhancer, we only focus on testing LAB mice that were heavily impaired in the WCM test. Lower lose of amphetamine (1.0 mg/kg) was chosen because of the repeated treatments over the course of the WCM test. 30 LAB mice were assigned to control (Veh) and amphetamine (Amph) groups based on their locomotor activity in the OF. One animal was excluded from the analysis since it died during the acquisition phase. 5 min before the first trial, animals were treated with either saline or amphetamine (1.0 mg/kg) and tested with 6 trials per day over the course of 5 consecutive days (d1-d5). There were significant overall "day" effects on escape latency ($F_{4,108} = 25.08$, $p < 0.001$), accuracy ($F_{4,108} = 5.46$, $p < 0.001$) and wrong platform ($F_{4,108} = 2.74$, $p < 0.05$), indicating an acquisition curve during training phase. Treatment with amphetamine had no effects on the escape latency ($F_{1,27} = 1.35$, $p = 0.25$; Figure 50A) and the number of wrong platform visits ($F_{1,27} = 1.23$, $p = 0.28$; Figure 50C). However, animals treated with amphetamine showed impairment in acquisition, evidenced by a tendency of decrease in the accuracy level ($F_{1,27} = 3.57$, $p = 0.070$; Figure 50B).

During the second week, only animals that successfully learned during the first week of training were tested in the reversal training. One Veh-control animal and one Amph-treated animal were excluded because of their death during the reversal training. Similar to acquisition phase, animals were treated with either saline or amphetamine (1.0 mg/kg) 5 min before the first trial and tested with 6 trials per day over the course of 5 consecutive days (d1r-d5r). Neither Veh-control nor Amph-treated groups showed appropriate relearning till the end of training (i.e. d5r). The escape latency was slightly shorter in animals treated with amphetamine than in those treated with saline ($F_{1,10} = 3.29$, $p = 0.099$; Figure 51A). However, treatment with amphetamine failed to induce an improvement of relearning performance in terms of accuracy levels and wrong platform visits ($Fs < 1$).

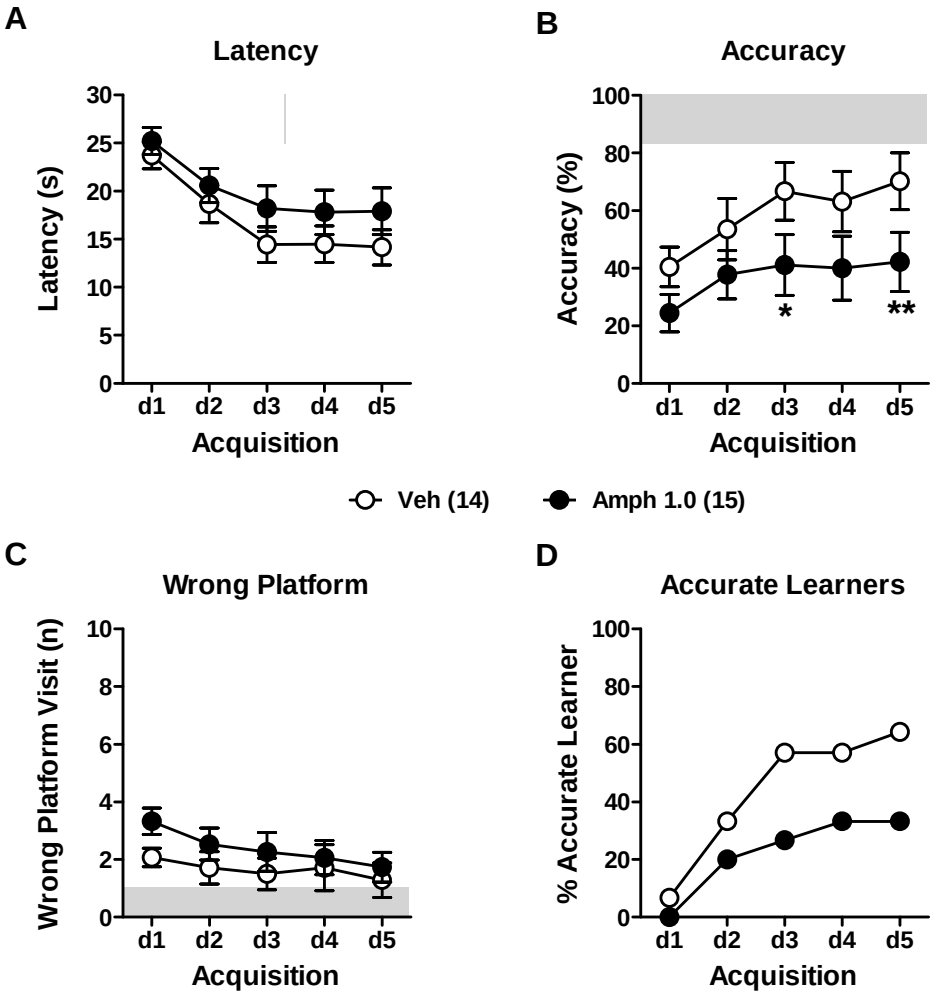


Figure 50: Amphetamine treatment and water cross-maze (water cross-maze) in all animals (acquisition phase). LAB mice were randomly assigned to control (Veh) and amphetamine (Amph) groups and were trained in the WCM with free learning protocol (c.f. Figure 4.12). On each testing day 5 min before the first trial, animals were treated with either saline or amphetamine (1.0 mg/kg) and tested with 6 trials per day over the course of 5 consecutive days (d1-d5). The performance of learning acquisition was described in terms of (A) escape latency, (B) % accuracy, (C) number of wrong platform visits and (D) accurate learners. No significant difference was found in escape latency and wrong platform visit between Veh-control and Amph-treated groups; however, treatment with amphetamine resulted in decreased levels of accuracy. Data were obtained from mice of Generation 40/10. ** $p < 0.01$; * $p < 0.05$ compared with Veh-control group (ANOVA followed by post-hoc Newman-Keuls test).

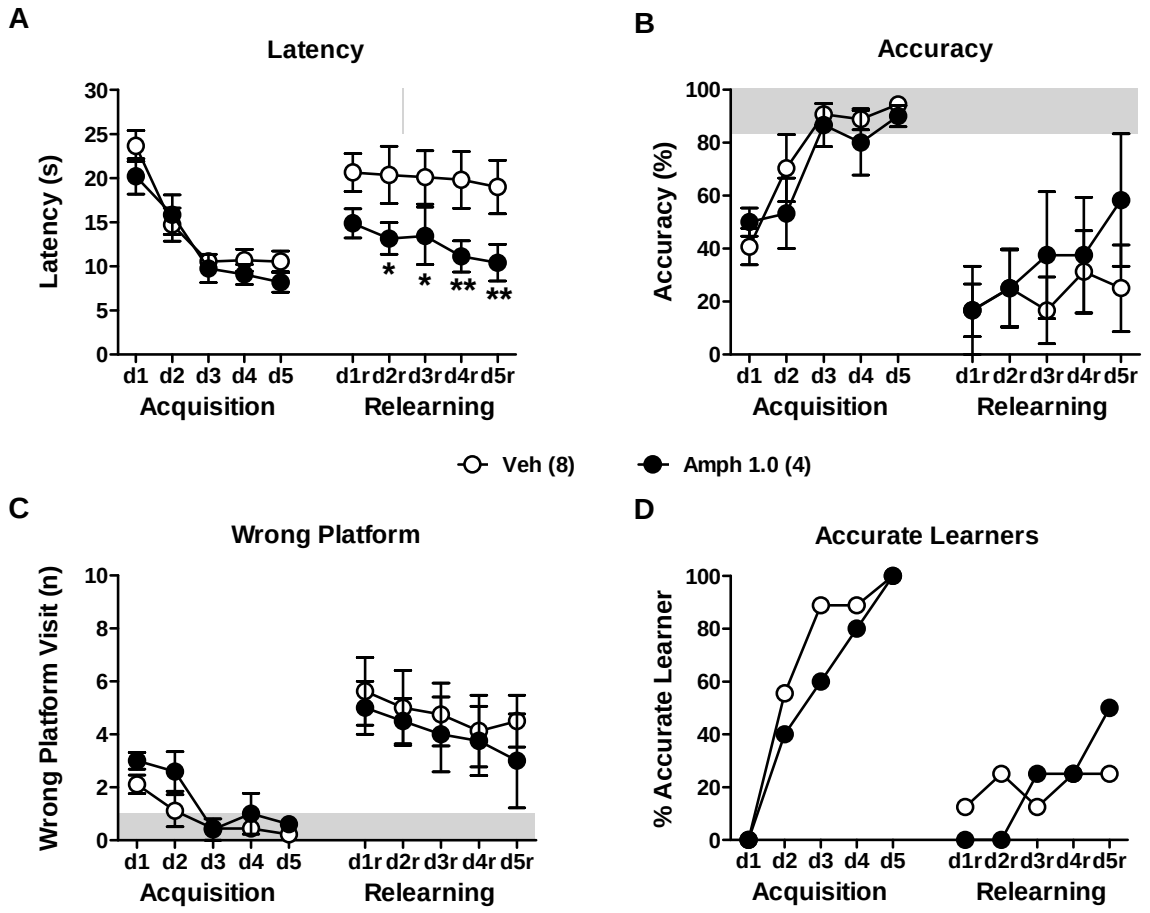


Figure 51: Amphetamine treatment and water cross maze in accurate learners (acquisition and relearning phases). 8 Veh-control and 4 Amph-treated LAB accurate learners during the acquisition were tested for the reversal training, and only the data of those animals were analyzed in terms of (A) escape latency, (B) % accuracy, (C) wrong platform visit and (D) accurate learners. During the learning acquisition phase (d1-d5, left panels), no difference was found between Veh-control and Amph-treated groups in any parameter. During the second week of relearning phase (d1r-d5r, right panels), both LAB groups showed no relearning. Animals treated with amphetamine showed shorter escape latencies for finding the platform; however, treatment with amphetamine didn't facilitate relearning by improving the other learning parameters (i.e. accuracy level). Data were obtained from mice of Generation 40/10. ** $p < 0.01$; * $p < 0.05$ compared with Veh (ANOVA followed by post-hoc Newman-Keuls test).

3.3.11. Pharmacological treatment and social recognition tests

LAB mice that displayed impairments in the social recognition tests were used to examine the effects of amphetamine on social cognition with NAB mice as control group. NAB and LAB mice were randomly assigned to vehicle control (Veh) and amphetamine (Amph) groups. Mice were treated with saline or amphetamine (1.0 mg/kg) immediately before being introduced into the

3-chamber apparatus. During the habituation phase, all groups showed no difference in sniffing two non-social empty tubes (E1, E2) in the left and the right chambers (all $p > 0.05$; Figure 52 left panel). In sampling phase, NAB and LAB mice of Veh-control spent significantly more time in sniffing a female mouse (F1) than an empty tube (E1) ($t_4 = -11.23$, $p < 0.001$ for NAB-Veh; $t_4 = -5.23$, $p < 0.01$ for LAB-Veh); however, preference of social animals could not be confirmed in Amph-treated NAB and LAB mice ($t_3 = -11.23$, $p = 0.13$ for NAB-Amph; $t_3 = -2.75$, $p = 0.07$ for LAB-Amph; Figure 52 middle panel). During the testing phase, NAB-Veh mice spent significantly more time sniffing a novel female mouse (F2) than the familiar one (F1) ($t_4 = 4.04$, $p < 0.05$), but either LAB-Veh or Amph-treated animals failed to display preference for social novelty ($t_4 = 1.32$ for LAB-Veh; $t_3 = -0.12$ for NAB-Amph; $t_3 = 0.63$ for LAB-Amph, $ps > 0.05$; Figure 52 right panel).

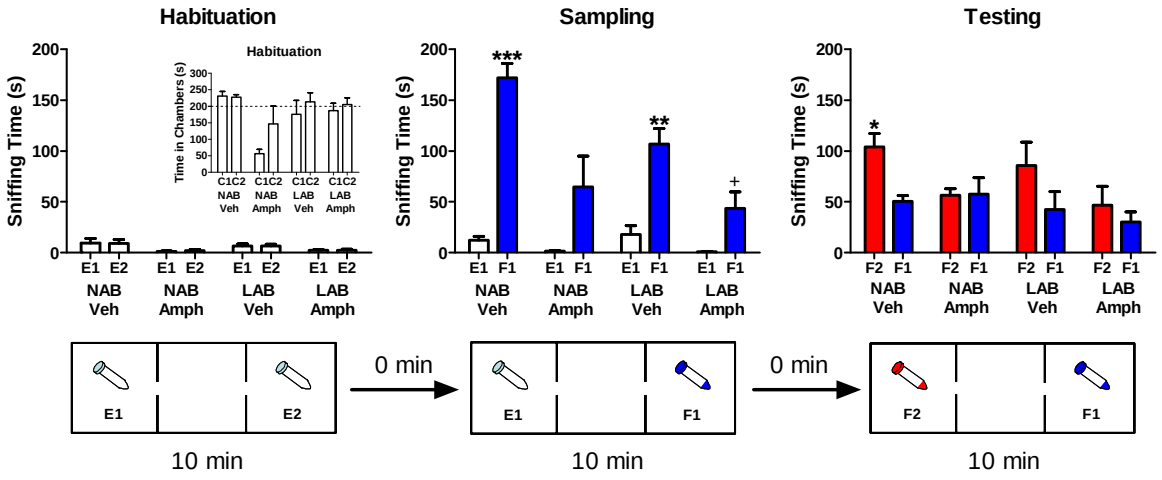


Figure 52: Amphetamine treatment and social behavior test. 9 NAB and 11 LAB mice were randomly assigned to control (Veh) and amphetamine (Amph) groups and were trained in the social behavioral test by using a 3-chamber apparatus (c.f. Figure 4.14A). Animals of each line were treated with either saline or amphetamine (1.0 mg/kg) immediately before introduction into the 3-chamber apparatus. During the habituation phase, all animals displayed no difference in sniffing two empty tubes, indicating no side preference of these animals. NAB and LAB mice treated with saline spent more time in sniffing the female (F1) exposed compared to the empty tube (E1) during the sampling phase, while those treated with amphetamine failed to show preference to the social stimulus. During the testing phase, NAB-Veh mice showed a preference for the novel female (F2), whereas either LAB-Veh or Amph-treated animals failed to prefer exploration of the novel social stimulus. Data were obtained from mice of Generation 40/10. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; + $p < 0.1$ (paired t-test).

3.4. LAB mice: Towards an animal model of ADHD (characterization of basal and stimulated DA release in the dorsal striatum)

3.4.1. OF test

Before microdialysis experiment, HAB and LAB of Generation 41 were tested in the OF test. During the entire 30-min exposure, the two lines, HAB and LAB, displayed a line effect in the overall DT ($F_{1,16}$ = 9.00, $p < 0.01$; Figure 53A). Post-hoc comparisons revealed that LAB mice displayed significantly higher locomotion than HAB mice ($p < 0.001$). Unpaired t-test revealed significant difference in the rotational behavior between two lines (t_{16} = -2.67, $p < 0.05$; Figure 53B).

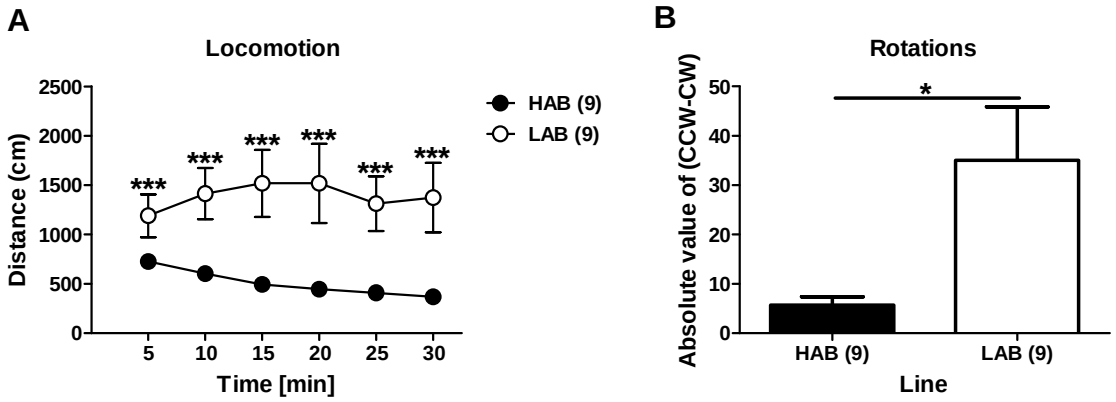


Figure 53: Exploratory and rotational behaviors of HAB, CD1 and LAB mice in the open field . HAB and LAB mice were tested in the 30-min open field test. (A) Distance travelled was measured in 5-min bins for the 30-min exposure. (B) Rotation was measured as the difference between the total number of clockwise (CW) and counter-clockwise (CCW) turns during the entire session. Data were obtained from mice of Generation 41. *** $p < 0.001$ compared with HAB mice (ANOVA followed by post-hoc Newman-Keuls test); * $p < 0.05$ (unpaired t-test).

3.4.2. Absolute basal levels of DA in dialysates

On the day 1 of microdialysis experiment, before any injection, three samples were collected as baseline. The basal extracellular levels of DA in the caudate putamen significantly differed between both lines ($F_{1,16}$ = 2.77, $p < 0.01$). For 120-min testing before drug treatment (e.g. amphetamine), repeated measures two-way ANOVA showed significant line effect ($F_{1,16}$ = 9.00, $p < 0.01$) and interaction between the line and time ($F_{5,80}$ = 3.02, $p < 0.05$). Either a transfer of

mouse into the OF or administration of saline did not affect DA levels in both HAB and LAB mice ($F_{5,80}$ = 1.05, p > 0.05). Post-hoc comparisons revealed that basal levels of DA in LAB mice were significantly lower than that in HAB mice either before or after saline administration (p < 0.001; Figure 54).

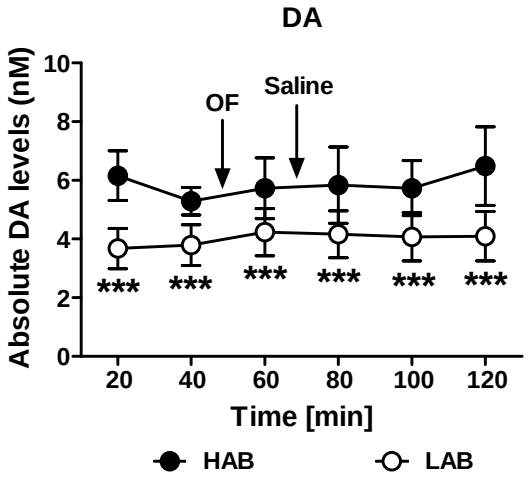


Figure 54: The extracellular DA (home cage-OF-saline administration) in the caudate putamen. The first two samples were collected from the freely moving animals in home cage environment, and then mice were transferred into the OF (the first arrow). Twenty min later, mice were treated with saline (the second arrow). The basal extracellular levels of DA in the caudate putamen of both lines were not affected by the transfer into OF or treatment with saline. *** p < 0.001 compared with HAB mice (ANOVA followed by post-hoc Newman-Keuls test).

3.4.3. Effects of OF exposure and saline treatment on the relative DA and DOPAC contents in the caudate putamen (CPu)

On day 1, the relative levels of DA and DOPAC remained stable within the first 6 samples in mice of all three lines. Neither transfer into the OF (sample 3) nor saline administration (sample 4) coincided with changes in levels of DA and DOPAC (Figure 55A, B).

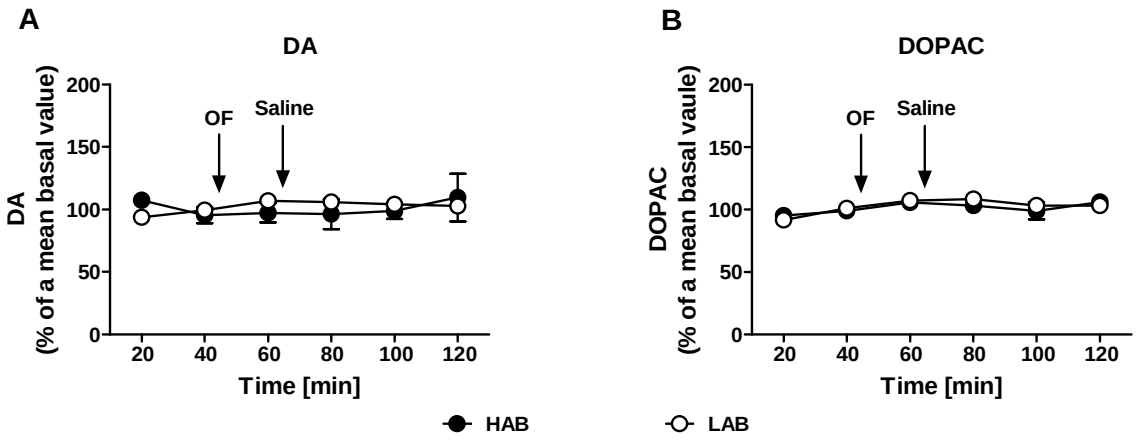


Figure 55: Effect of the OF exposure and saline administration on DA and DOPAC contents. The first two samples were collected from the freely moving animals in home cage environment, and then mice were transferred into the OF (the first arrow). 20 min later, mice were treated with saline (second arrow) and samples were taken every 20 min for a period of 60 min. The relative changes of extracellular (A) DA and (B) DOPAC in the caudate putamen of both lines were not affected by OF exposure or saline treatment. (Data were presented as a percentage of the averaged two baseline samples.)

3.4.4. Effect of systemic treatment with amphetamine on the relative DA and DOPAC contents in the CPu

In the caudate putamen, amphetamine equipotently elicited significant increases in the DA levels in HAB and LAB mice (Figure 56A). Repeated measures two-way ANOVA revealed a significant time effect ($F_{11,99}$ = 68.08, $p < 0.001$) but no significant line effect and interaction between time and line (line: $F < 1$; line $\times$ time: F11,99 = 1.005, $p > 0.05$). Subsequent post-hoc analysis proved that amphetamine induced significant increases in DA contents in both lines ($p < 0.001$) without line difference.

Treatment with amphetamine induced significant decreases in the DOPAC levels in HAB and LAB mice. The analysis of changes in the DOPAC levels in the caudate putamen revealed a significant difference between two lines ($F_{1,9}$ = 10.98, $p < 0.01$; Figure 56B). Repeated measures two-way ANVOA showed a significant interaction of line and time for the amphetamine effect in the caudate putamen ($F_{11,99}$ = 10.84, $p < 0.001$). Post-hoc comparisons indicated that

amphetamine elicited a more pronounced decrease in DOPAC levels in LAB mice than HAB/CD1 mice ($p < 0.05$; 40-120 min).

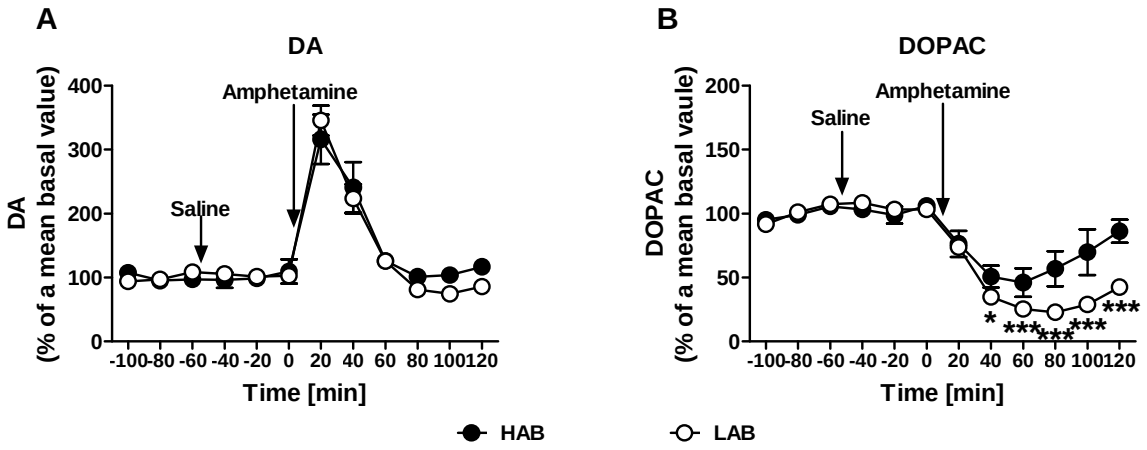


Figure 56: Effect of amphetamine treatment on DA and DOPAC levels. The effect of amphetamine on relative changes of extracellular (A) DA and (B) DOPAC in the caudate putamen was measured in freely moving animals. Sixty min after the administration of saline, both lines were treated with amphetamine (1.0 mg/kg, i.p.). After amphetamine administration, samples were taken every 20 min for a period of 120 min. *** $p < 0.001$, * $p < 0.05$ compared with HAB mice (ANOVA followed by post-hoc Newman-Keuls test).

3.4.5. Effect of systemic methylphenidate treatment on the relative levels of DA and DOPAC in the CPu.

In the caudate putamen, treatment with methylphenidate equally increased the extracellular levels of DA in HAB and LAB mice (two-way ANOVA, line: $F < 1$; time: $F_{8,80} = 43.21$, $p < 0.001$; Figure 57A). Repeated measures two-way ANOVA revealed no significant interaction between line and time ($F_{8,80} = 1.11$, $p > 0.05$). Post-hoc comparisons revealed that methylphenidate caused significant increases in the extracellular levels of DA in both lines ($p < 0.001$).

Two-way ANOVA showed significant main effects of line and time for methylphenidate effects on DOPAC level (line: $F_{1,8} = 9.17$, $p < 0.05$; time: $F_{8,64} = 6.05$, $p < 0.001$), but no significant interaction between line and time ($F < 1$). Post-hoc analysis proved that extracellular levels of DOPAC dropped in LAB ($p < 0.05$), but not in HAB mice ($p > 0.05$) after methylphenidate administration (Figure 57B).

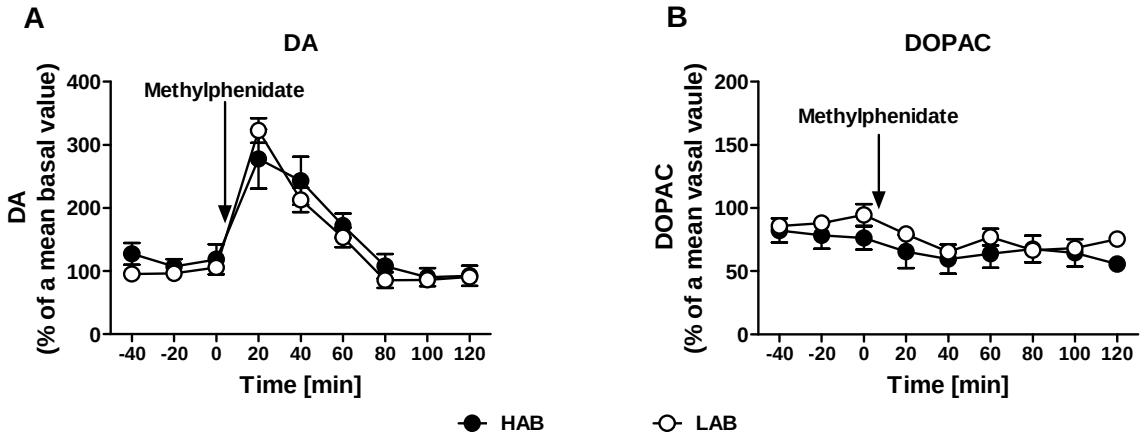


Figure 57: Effect of treatment with methylphenidate on DA and DOPAC levels. The relative changes of extracellular concentrations of (A) DA and (B) DOPAC in the caudate putamen were measured in freely moving animals. Twenty min after being transferred into the open field, all animals were treated with methylphenidate (10.0 mg/kg, i.p.). Samples were taken every 20 min for a period of 120 min after methylphenidate treatment.

3.4.6. Effect of combined treatment with nomifensine and amphetamine on the relative levels of DA and DOPAC in the CPu.

To examine the mechanism of amphetamine action, we performed additional experiments with amphetamine administration when DAT was blocked by nomifensine (Nomi). Repeated measures two-way ANOVA showed a main effect of time ($F_{9,45}$ = 12.47, $p < 0.001$), but no significant line effect and interaction between line and time (line: $F < 1$; line × time: $F < 1$). Post-hoc comparison revealed an elevation in the extracellular levels of DA (~250 % of the basal level) (HAB: $p < 0.001$; LAB: $p < 0.05$) after administration of nomifensine, comparable to that seen after methylphenidate treatment (~300 % of the basal level; Figure 57A). The subsequent administration of amphetamine did not evoke a further increase in the DA levels in LAB mice ($p > 0.05$), but significantly decreased DA levels in HAB mice. For extracellular levels of DOPAC, repeated measures two-way ANOVA showed significant time effect ($F_{9,54}$ = 23.22, $p < 0.001$) and interaction ($F_{9,45}$ = 2.68, $p < 0.05$), but no significant line effect ($F_{1,6}$ = 2.19, $p > 0.05$). Treatment with Nomi only induced a decrease in the extracellular levels of DOPAC only in LAB mice ($p < 0.05$); however, subsequent amphetamine administration induced a strong decline in the DOPAC levels in both lines (all $p < 0.05$).

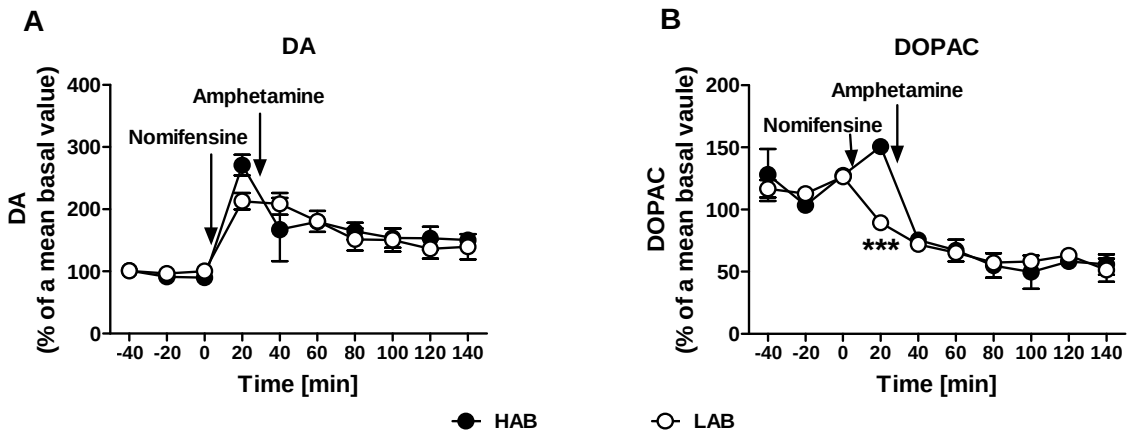


Figure 58: Effect of combined treatment with nomifensine and amphetamine on DA and DOPAC levels. The relative changes of extracellular contents of (A) DA and (B) DOPAC in the caudate putamen were estimated in freely moving animals. Twenty min after being transferred into the OF, all animals were treated with nomifensine (10.0 mg/kg, i.p.). Twenty min later, all three lines were treated with amphetamine (1.0 mg/kg, i.p.) and samples were taken every 20 min for a period of 120 min after amphetmiane treatment. *** $p < 0.001$ compared with HAB mice (ANOVA followed by post-hoc Newman-Keuls test).

3.4.7. Effect of systemic administration of AM404 on the relative levels of DA and DOPAC in the CPu.

To determine whether endocannabinoid agents affect locomotor activity through modulation of striatal DA release, we measured the extracellular contents of DA in the caudate putament after AM404 treatment. Treatment with AM404 did not affect either the extracellular DA (line: $F < 1$; time: $F < 1$; line $\times$ time: $F < 1$) or DOPAC levels (line: $F < 1$; time: $F_{8,56} = 2.08$, $p > 0.05$; time $\times$ line: $F < 1$; Figure 59B). The slight potency of AM404 to decrease the DA levels in the LAB mice was not confirmed with respective post-hoc comparisons.

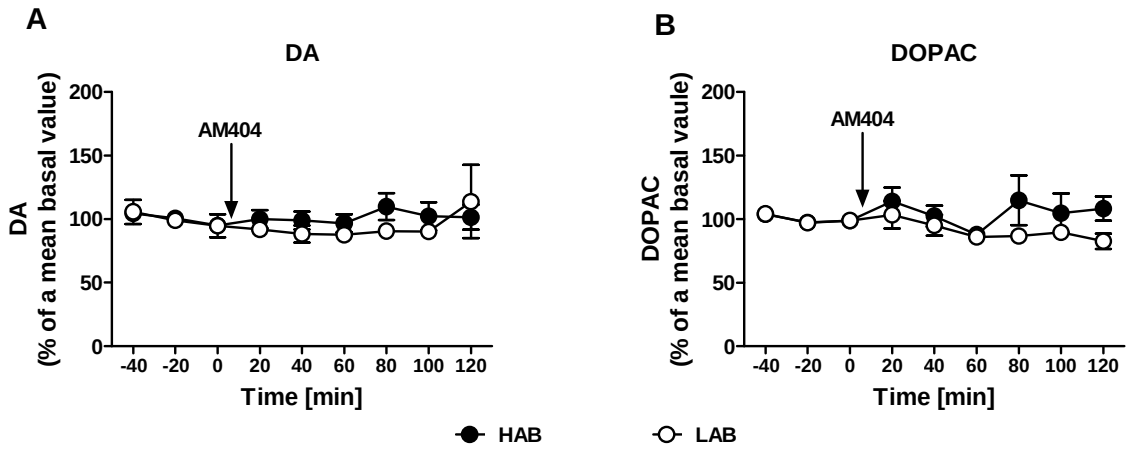


Figure 59: Effect of treatment with AM404 on DA and the DOPAC levels. The relative changes of extracellular contents of (A) DA and (B) DOPAC in the caudate putamen were estimated in freely moving animals. Twenty min after being transferred into the open field, all animals were treated with AM404 (3.0 mg/kg, i.p.). Samples were taken every 20 min for a period of 120 min after AM404 treatment.

3.4.8. Effect of KCl-induced depolarization on the relative levels of DA and DOPAC in the CPu

Ten-minute perfusion with aCSF containing 40-times higher content of K^+ (100 mM) through the dialysis probes elicited an immediate increase in the levels of extracellular DA in the caudate putamen of HAB and LAB mice (two-way ANOVA, time: $F_{7,56} = 30.20$, $p < 0.001$; Figure 60A). The DA levels returned to the baseline once the perfusion was stopped. For effects of K^+ on DOPAC levels, repeated measures two-way ANOVA revealed a significant time effect ($F_{7,35} = 3.96$, $p < 0.01$) but no significant line effect and interaction between time and line (line: $F_{1,5} = 4.12$, $p > 0.05$; time × line: $F_{7,35} = 1.70$, $p > 0.05$). Since depolarization exhausts the exocytotic vesicular pool of DA, this physiologically relevant increase in neurotransmitter concentration in the extracellular space coincided with a slight elevation in DOPAC levels in HAB ($p < 0.5$) but not in LAB mice ($p > 0.05$) (Figure 60B).

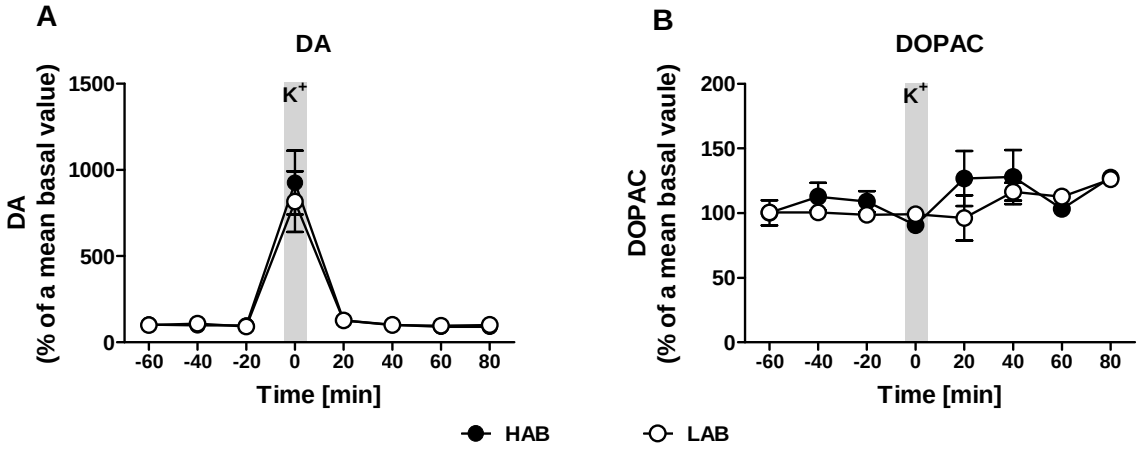


Figure 60: Effect of KCl-induced depolarization on DA and DOPAC levels. aCSF with high concentration of K^+ (100 mM) was perfused through the dialysis probes for 10 min (gray bar). (A) The relative changes of extracellular DA contents in the caudate putament strikingly increased during the K^+ stimulation, and (B) the extracellular levels of DOPAC in HAB mice slightly increased 20-40 min after KCl perfusion.

3.4.9. The involvement of dopamine transporter (DAT) in the hypolocomotor effects of amphetamine

To examine the effects of interaction of DAT blocker (Nomi) and the non-selective releaser of DA (amphetamine), an additional behavioral OF test was performed. During the first 20 min of drug-free period, no difference was found between Nomi-treated and Veh-control animals (NAB: $F_{1,9} = 2.91$, $p > 0.05$; LAB-I: $F < 1$; Figure 61). Administration of Nomi (10.0 mg/kg) induced significant increases in locomotor activity in both NAB ($F_{1,9} = 44.01$, $p < 0.001$) and LAB-I mice ($F_{1,12} = 10.59$, $p < 0.01$). All animals were treated with amphetamine 30 min after the administration of Nomi. In NAB mice, treatment with amphetamine did not increase locomotor activity in Veh-control group ($p > 0.05$), but significantly decreased the Nomi-induced hyperactivity in Nomi-treated group ($p < 0.001$). However, in LAB-I mice, amphetamine induced significant decreases in both Veh-control and Nomi-treated groups ($p < 0.001$), with significant difference between two groups ($F_{1, 12} = 6.26$, $p < 0.05$).

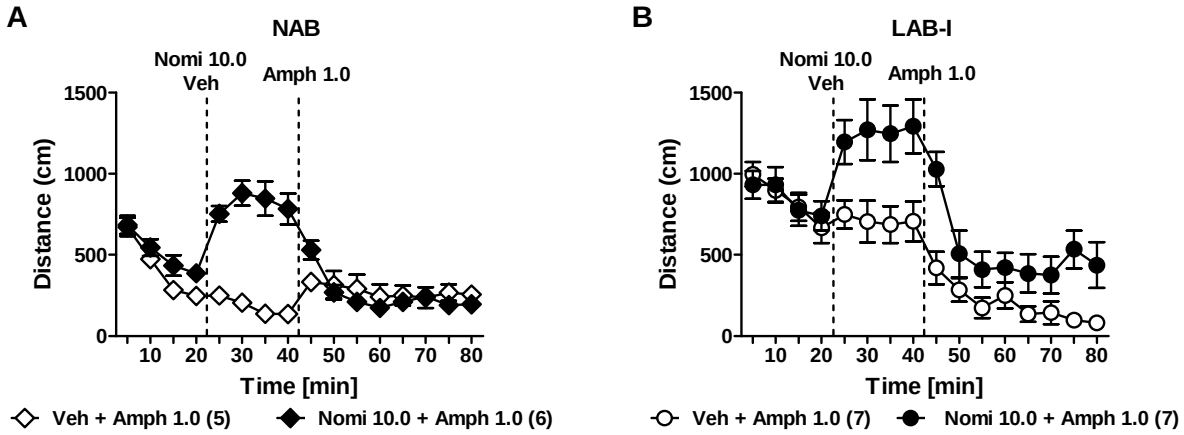


Figure 61: The involvement of dopamine transporter (DAT) blocker in amphetamine treatment and locomotion. Mice of each line were reassigned to control (Veh) or drug-treated groups. After 20 min of open field exposure, (A) NAB and (B) LAB-I mice were treated with either saline or nomifensine (Nomi; 10.0 mg/kg) (dashed line). 30 min later, both groups were treated with amphetamine (1.0 mg/kg) and distance travelled was continuously measured for 30 min. Treatment with Nomi significantly increased locomotion in both NAB and LAB-I mice. In NAB mice, amphetamine induced a slight increase in locomotion in Veh-control group, whereas attenuated the Nomi-induced hyperactivity in Nomi-treated group. However, amphetamine induced decreases in both Veh-control and Nomi-treated LAB-I mice. Data were obtained from mice of generation 40/10.

4. Discussion

The present thesis demonstrated the characteristics of HAB/LAB mice, a selectively bred animal model of extremes in trait anxiety based on their performance on the EPM. The EPM test has been widely used as screening test for putative anxiolytic/anxiogenic compounds in genetically modified mice and rats (Bourin, 1997; Belzung and Griebel, 2001; Holmes, 2001). Although the test offers a number of advantages over the other behavioral paradigms in neurobiological research, the animals' performance on the EPM may be influenced by various test conditions, especially illumination (Jones and King, 2001). Here, we proved that HAB, NAB and LAB mice exhibit robust phenotypes, independent of the lighting condition. This stable diversity of HAB/LAB mice in trait anxiety can actually be harnessed as a reliable tool to further investigate phenotypic and genotypic characteristics related to psychiatric diseases.

4.1. Conditioned fear in HAB mice

By using a classical cued fear conditioning paradigm and a step-down avoidance task, we observed that HAB mice with a genetic predisposition to hyper-anxiety showed pronounced acquisition of conditioned fear in terms of auditory-cued fear, contextual fear and inhibitory avoidance memory, compared to both NAB and LAB mice, which was not due to different levels of sensitivity to tone and/or foot shocks. After conditioning, increased phosphorylation of AKT in HAB but not NAB mice, provided molecular evidence for the stronger fear memory in HAB mice, which also required more extinction training trials than CD1 controls to reach the freezing levels of non-shocked animals. Furthermore, HAB mice showed spontaneous recovery after successful extinction training and were impaired in extinction of older fear memories. Taken together, the data of the present study demonstrates that mice with high trait anxiety display stronger fear acquisition, which, in turn, results in exaggerated avoidance behavior, slower fear extinction and proneness to relapse of conditioned fear.

Data of both fear conditioning and step-down avoidance experiments indicate that the acquisition/consolidation of fear memories was stronger in HAB mice than in the other two lines. Both NAB and HAB mice showed decreased levels of pCaMKII and increased levels of β-catenin within the basolateral amygdala after conditioning. Whereas the latter is in agreement

with recent observations about the role of β-catenin in consolidation of auditory-cued fear memories (Maguschak and Ressler, 2008), the strong decrease in pCamKII is striking and deserves further investigations. Compared to NAB mice, HAB mice showed increased phosphorylation of AKT following conditioning. AKT is known of being involved not only in a variety of signaling cascades, which lead to cell proliferation, survival and growth (Manning and Cantley, 2007), but also in fear conditioning processes in various brain structures including the *nucleus accumbens* (Krishnan *et al.*, 2007), dorsal hippocampus and basolateral amygdala (Dahlhoff *et al.*, 2010). In this study AKT is the sole investigated kinase which is specifically altered in conditioned HAB mice, suggesting AKT phosphorylation as a molecular marker of exaggerated fear memories at early stages following fear conditioning.

In a series of control experiments we could rule out that the line differences in acquisition of fear memories cannot merely be explained by higher sensitivity to the unconditioned stimulus (foot shock), as all three lines showed increased startle responses to the foot shock with no differences between HAB and NAB mice (Figure 26C). It is also highly unlikely that the pronounced freezing response shown by HAB mice simply relates to a *priori* higher sensitivity to the conditioned stimulus, since HAB mice showed the lowest acoustic startle responses as compared to NAB and LAB mice (Figure 36A, B).

The observed differences in learned fear strongly support the proposed genetic relationship between fear learning and anxiety-related behavior (Lissek *et al.*, 2005). In line with this idea, animals displaying stronger fear responses also show higher emotionality on the EPM (Ponder *et al.*, 2007). Studies investigating the neural mechanisms of fear conditioning across species indicate that the amygdala has a critical function in the acquisition, storage and expression of conditioned fear (Pare *et al.*, 2004), additionally making fear memories “erase”-resistant (Pizzorusso, 2009). Interestingly, the recent data showed that acute open arm challenge induced a pronounced c-fos expression in the medial and lateral amygdala of HAB but not LAB animals (Muigg *et al.*, 2009), which may be associated with increased fear expression and the inborn anxiety-related phenotype of this line. This activation of the amygdala has been associated with a hypo-activation of the medial prefrontal cortex (Muigg *et al.*, 2008).

In recent years, fear extinction has been acknowledged as a preclinical model for behavioral therapy of human anxiety (Barad, 2005; Herry *et al.*, 2010). Thus, we also examined the extinction of learned fear in HAB and CD1 mice, the latter of which show the same anxiety-related phenotype as NAB mice (Landgraf *et al.*, 2007). We found no major differences in extinction processes, suggesting that fear memory in both HAB and CD1 mice can be efficiently extinguished (Figure 27B), particularly, if extinction training is intensified (Figure 27C). However, HAB mice did not reach the same freezing levels as non-shocked animals, indicating that it takes longer for a complete extinction of freezing behavior relative to CD1 controls. Furthermore, our extinction experiment points to resistance to extinction in HAB mice, as older fear memory formed in HAB Ret mice failed to be dampened by repeated CS presentations, whereas the rate in which the fear response decayed was much faster in CD1 Ret mice. These findings support the notion that the strength of fear memory may be correlated with resistance to extinction (Bieszczad and Weinberger, 2010). Consistent with impaired extinction of learned fear in HAB rats (Muigg *et al.*, 2008), the current data suggest that HAB mice show clear signs of exaggerated fear learning. These two independent models confirm the importance of innate anxiety for the development of phobic-like fear.

Interestingly, in contrast to CD1 controls, HAB mice showed reemergence of extinguished fear, i.e. spontaneous recovery, within 6 weeks (Figure 27B). While fear extinction leads to progressive reduction in the expression of fear, it is not permanent because extinction does not modify the existing memory, but instead leads to the formation of a new memory that depends on the context (Bouton and Moody, 2004; Bouton *et al.*, 2006) and suppresses the activation of the initial trace (Rescorla and Heth, 1975; Robbins, 1990; Westbrook *et al.*, 2002; Effting and Kindt, 2007). In clinical settings, extinction-based exposure therapy used as treatment for a number of anxiety-related disorders is effective in some but not all cases, and those who do benefit, may show a return of fear due to spontaneous recovery (Effting and Kindt, 2007; Schiller *et al.*, 2008). This raises the possibility that our HAB mice may serve as a psychopathological animal model for traumatic fear memories.

It has been shown that LTP in the medial prefrontal cortex (mPFC) is associated with long-term maintenance of extinction, whereas prefrontal long-term depression (LTD) is predictive of spontaneous recovery of conditioned fear (Herry and Garcia, 2002; Milad and Quirk, 2002; Herry and Garcia, 2003). These data suggest that activation of mPFC may be a critical factor for recall of extinction memory. Inversely, the lack of prefrontal activation after extinction may be critically involved in spontaneous recovery of conditioned fear. The observation of diminished challenge-induced c-Fos expression in prefrontal-cortical and enhanced expression in limbic areas of HAB mice (Muigg *et al.*, 2009) supports the phenomena described here.

In conclusion, HAB mice display a phenotypic combination of stronger acquisition, slower extinction and spontaneous recovery of learned fear, resembling the symptoms of anxiety disorders (face validity). Construct validity is based on the involvement of amygdala-prefrontal cortex circuitry in regulating the confrontation of stressful events (Muigg *et al.*, 2009). The present results also have significant applications for the mechanisms underlying the critical symptoms of anxiety disorders. First, Western blot analyses suggest phosphorylation of AKT as a therapeutic target for the prevention of exaggerated fear memories. Second, it will be important to pursue further how the return of fear might be prevented by appropriate behavioral manipulations. For example, recent studies have shown promising non-invasive techniques to persistently attenuate fear memories of rats (Monfils *et al.*, 2009), which also work out in anxiety patients after treatment (Schiller *et al.*, 2010). The clinical efficacy of these approaches remains to be determined, especially for those anxiety patients, who fail to respond to the extinction-based exposure therapy. The HAB animals, therefore, could represent a preclinical tool to further elucidate the neural mechanism underlying the phenomena of exaggerated fear memories and resistance or phobic-like states (Sartori *et al.*, 2011) to fear extinction.

In addition to face and construct validities, predictive validity is also given, as anxiolytic drugs could normalize the hyper-anxiety of HAB line. Previous evidence has shown that HAB mice were sensitive to central administration of corticotrophin-releasing hormone receptor 1 antagonist (Bunck, unpublished), a recommended anxiolytic substance in both rodents and

humans. These findings provide solid evidence for the pharmacological usage of HAB mice in preclinical research, such as screening of putative anxiolytic compounds.

4.2. NPS

To further investigate a candidate substance to rescue anxiety-related phenotype, we validated the anxiolytic action of central NPS in unselected CD1 mice and animals with a genetic predisposition to hyper-anxiety (HAB), like the previous findings in Bl6 mice (Leonard *et al.*, 2008). In addition, we developed a non-invasive NPS administration method to investigate the effects of NPS on hyperanxious HAB mice. For the first time we testified that intranasal NPS could induce anxiolytic effects in the pathological animal model, providing a promising way to deliver NPS to the brain in a therapeutic perspective. To further analyze the involvement of inherited mechanisms, we compared the *Nps* mRNA expression within the peri-LC area as well as *Npsr1* expression in the PVN and amygdala between HAB and LAB mice, and sequenced both genes to search for genetic polymorphisms in HAB *vs.* LAB mice. Finally, we determined the genetic differences in the brain NPS system in HAB/LAB mouse model which may contribute to their trait anxiety phenotypes.

Recently, NPS has gained much interest due to its promising anxiolytic and arousing effects (Xu *et al.*, 2004). As human studies suggested a detrimental role of enhanced brain NPS signaling in the context of anxiety-related diseases (Domschke *et al.*, 2010; Donner *et al.*, 2010; Raczka *et al.*, 2010), we determined the effect of NPS in a mouse model of extremes in anxiety. Initially, we replicated the anxiolytic effects of i.c.v. administered NPS (Xu *et al.*, 2004; Leonard *et al.*, 2008; Rizzi *et al.*, 2008) in normal CD1 mice, and extended the effect to HAB mice with a genetic predisposition of hyper-anxiety. As central administration of drugs is not feasible in human patients, developing a non-invasive delivery method is essential for translational research. Here, for the first time we successfully demonstrated that intranasal administration of NPS induced anxiolytic effect in conscious HAB mice, suggesting a potential basis for intranasal NPS administration to humans. A variety of substances among the neuropeptides, have been shown to reach the brain to a certain extent by nasal delivery (Ozsoy *et al.*, 2009; Dhuria *et al.*, 2010). Although the nasal bioavailability of hydrophilic peptides and proteins is usually less than 1%

(Ozsoy *et al.*, 2009), a significant amount of hydrophilic NPS obviously manages to bypass the neuropeptide-degrading enzymes of the murine nasal mucosa to enter the mouse brain (Ohkubo *et al.*, 1994). Other proofs for the effectiveness of intranasal NPS treatment in HAB mice were made by the regulation of proteins in the prefrontal cortex (Ionescu *et al.*, submitted) and the modulation of synaptic transmission in the ventral hippocampus (Dine, unpublished) induced by intranasal NPS delivery. The anxiolytic effects of centrally and intranasally administered NPS in HAB mice provide support for a role of NPS in regulating innate anxiety levels.

Further molecular analysis revealed no differences in the *Nps* mRNA expression in the peri-LC region (the only brain region where *Nps* is expressed at well-detected levels) between HAB and LAB mice, suggesting that the line-dependent divergences in behavior and expression may not be associated with *Nps* mRNA levels. However, the present data show that *Npsr1* mRNA expression in the amygdala but not in the PVN was increased in LAB mice compared to HAB mice. This observation was consistent with the microarray experiment showing that higher amount of RNA was observed in the basolateral amygdala in LAB mice compared to HAB mice (GEO Dataset: GSE29015; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29015). In addition, lower *Npsr1* mRNA expression within the PVN, but not within the amygdala was found in HAB rats compared to LAB rats (Slattery, unpublished). In two different species, high trait anxiety is associated with lower *Npsr1* expression levels within brain regions essential for the modulation of anxiety. These findings provide evidence that the NPS-NPSR1 system plays an important role in determining innate anxiety levels.

Results from genetic analyses in HAB and LAB mice underline our hypothesis that *Nps* and *Npsr1* may modulate the predisposition to high trait anxiety. With respect to line-dependent genetic difference in the *Nps* sequence, 35 SNPs and 4 insertions were found within the *Nps* gene sequence in HAB *vs.* LAB mice. Among these polymorphic sites, four exonic *Nps* SNPs were shown to lead to 3 amino acid substitutions in the HAB sequence compared to the LAB sequence. An analysis concerning the SNPs in the peptides of the NPS precursor gene, HAB mice were identified as homozygous for the mutant allele, and LAB mice as homozygous for the wild-

type allele (wild-type refers to the C57/BL6 sequence). The three identified SNPs in the NPS precursor gene, exchanging isoleucine to leucine, isoleucine to valine in the signal peptide and glycine to arginine in a yet undefined region in HAB mice, are likely to be involved in alteration of the secondary structure of the precursor protein (Figure 62). These structural alterations may lead to an incorrect folding of the precursor and inadequate binding of neuropeptide S to the carrier protein, whereby the misfolded propeptide may accumulate in the endoplasmic reticulum (Ito and Jameson, 1997; Siggaard *et al.*, 1999). The incorrect folding might also lead to faster degradation of the precursor (Nijenhuis *et al.*, 1999) before the mature NPS could be acting on receptors.

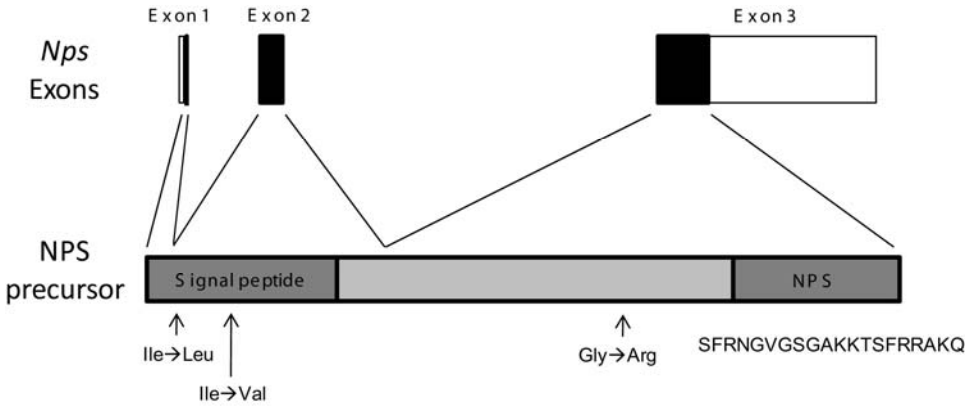


Figure 62: Two SNPs in the second exon and one SNP in the third exon of the NPS precursor gene cause two amino acid exchanges in the signal peptide and one in a yet undefined peptide region (light gray bar). Arrows indicate the three amino acid exchanges from isoleucine (Ile) to leucine (Leu), isoleucine (Ile) to valine (Val) at the signal peptide and glycine (Gly) to arginine (Arg) in the undefined region in HAB mice.

Furthermore, a recent human genetic association study has implicated that single nucleotide polymorphisms within the *Nps* sequence were found to be associated with panic disorders in humans (Donner *et al.*, 2010). Intriguingly, there are some similarities between the finding in humans and our observation in HAB/LAB animal model, e.g. SNPs were found in the second exon and the second intron of both human and mouse *Nps* sequences (Table 6).

Table 6: Comparison of SNPs in the *Nps* sequence between mouse and human. The alleles in bold indicate the polymorphic sites in HAB mice (left panel) and in people with panic disorder (right panel) (Donner *et al.*, 2010). Exon 2 and intron 2 are separated by horizontal disjunctions.

Mouse				Human			
Location in the gene	**Relative position**	**Allele**	**SNP identifier**	**Location in the gene**	**Relative position**	**Allele**	**SNP identifier**
Exon 2	125	T/**A**	N/A	Exon 2	33	C/**T**	rs990310
Exon 2	140	G/**A**	N/A				
Intron 2	250	G/**A**	N/A	Intron 2	495	G/**A**	rs1999635
Intron 2	273	G/**A**	N/A	Intron 2	2112	A/**G**	rs11018195
Intron 2	380	A/**T**	N/A				
Intron 2	460	A/**C**	N/A				
Intron 2	502	C/**T**	N/A				

Even more sequential variations were observed in *Npsr1* gene sequence between HAB and LAB mice. In detail, 47 polymorphic sites were identified including a 38 bp deletion in the promoter region in HAB mice. *In silico* analysis of potential transcription factor binding sites using the TESS (http://www.cbil.upenn.edu/cgi-bin/tess/) revealed a glucocorticoid responsive element (GRE), which is inserted by the HAB-specific alleles (TC) of the first polymorphic site in the promoter region (InsTC(-274)-). Glucocorticoid receptors (GRs) have been suggested to be the target site to modulate gene expression. GRs are known to mediate both transcriptional activation (Chen *et al.*, 2003) and inhibition (Drouin *et al.*, 1989) in a cell-type specific manner. The latter is best described for the feedback regulation of the HPA axis but also accountable for repression of genes not involved in stress response (Guertin *et al.*, 1988; De Kloet *et al.*, 1998). In case of inhibitory properties, such factors provide histone deacetylase activity, protein methyltransferase activity or ATP-dependent chromatin-remodeling abilities. It is conceivable that the additional GR binding factor due to the inserted SNPs in the HAB sequence results in stronger inhibition of *Nps* in this line, thus leading to a lower expression in HAB compared to LAB mice. Moreover, we also demonstrated that HAB mice displayed a long deletion of a GA repeat. Analysis with TESS-database revealed a GAGA factor binding site, a factor known to facilitate DNA loop formation and to regulate gene expression at multiple levels, is disrupted by the long deletion of HAB promoter region (Δ(-1400~-1365)$(GA)_{18}$). The GAGA factor plays a role for both activator/antirepressor and repressor activity, depending on its target genomic

location (Adkins *et al.*, 2006). Given the importance of the GAGA factor for gene expression (Volpi *et al.*, 2002), we hypothesized that the GAGA factor can induce gene activation of *Nps* mouse sequence so that the deletion sites of $(GA)_{18}$ may reduce transcription and, consequently, contribute to the lower expression of *Npsr1* mRNA in the amygdala of HAB mice. However, since the GAGA factor is located over a distance of 1000 bp (-1400~-1365) from the transcription start, the possibility of GAGA factor-mediated modulation of gene expression might be reduced. Therefore, further experiment of GAGA factor expression in the amygdala is needed to testify this hypothesis. Similar to the *Nps* sequence, some SNPs were also found in the promoter region and Exon 1 in both mouse and human *Npsr1* sequences (Donner *et al.*, 2010; Table 7), making the polymorphic profile of HAB mice a useful model to investigate similar effects in panic disorder patients.

Table 7: Comparison of SNPs in the *Npsr1* sequence between mouse and human. The alleles in bold indicate the polymorphic sites in HAB mice (left panel) and in people with panic disorder (right panel) (Donner *et al.*, 2010). The promoter and exon 1 are separated by horizontal disjunctions.

Mouse				Human			
Location in the gene	**Relative position**	**Allele**	**SNP identifier**	**Location in the gene**	**Relative position**	**Allele**	**SNP identifier**
Promoter	-468	G/**C**	rs46047101	Promoter	-463	A/**C**	rs2125404
Promoter	-442	T/**-**		Promoter	-423	C/**A**	rs2168891
Promoter	-431	A/**T**	rs45879530	Promoter	-391	G/**A**	rs1963499
Promoter	-406	C/**T**	rs51858460				
Promoter	-385	C/**T**	rs46930781				
Promoter	-351	A/**C**	rs50633535				
Promoter	-274	-/**TC**					
Exon 1	123	T/**A**	rs48722200	Exon 1	72	C/**T**	rs2530547
				Exon 1	132	G/**A**	rs887020

In conclusion, these data suggest that both central and intranasal administrations of NPS induced promising anxiolytic effects in HAB mice, a robust animal model for anxiety disorder. The analyses in HAB and LAB mice suggest the involvement of specific genomic loci and polymorphisms in shaping the anxiety-related behavior, thus providing a mechanistic explanation.

4.3. LAB mice: an animal model of ADHD?

Selective breeding with outbred CD1 mice for low anxiety-related behaviors (Kromer *et al.*, 2005) resulted in LAB mice that were hyperactive which is a key feature of models for ADHD. In the OF test, LAB mice displayed hyperactivity without habituation by reflecting significant increases in horizontal locomotion, mobility and rotational behavior compared to HAB and NAB mice. Interestingly, LAB mice showed higher levels of acoustic startle responses compared to NAB and HAB mice, but no PPI deficit was observed in LAB mice. In a HB task, in addition to their exceptional increased locomotion, LAB mice showed decreased 16-hole exploration compared to HAB/NAB mice. LAB mice were heavily impaired in acquisition and relearning in the water cross maze, indicating strong deficits in egocentric and allocentric navigation as well as behavioral flexibility. LAB mice also displayed reduced attention and, thus, reduced social memory upon exploration of conspecifics. Amphetamine administration increased locomotion in HAB/NAB mice but decreased locomotion in LAB mice; however, it failed to reverse the LAB's cognitive deficits in the water cross maze and social memory tests. Moreover, hyperactivity of LAB mice was also alleviated by AM404, which is proposed to be a potential drug for the treatment of ADHD.

4.3.1. Startle measurements

Noteworthy, LAB mice showed the highest level of acoustic startle responses, which strikingly resembles the phenotype observed in LAB rats (Yilmazer-Hanke *et al.*, 2004). These data support the notion that extremes in trait anxiety are correlated with different coping strategies, with higher anxious animals adopting a more passive coping style when confronted with a dangerous environment (Korte *et al.*, 2005), and this is associated with less hyper-arousal, increased immobility in TST and FST (Landgraf and Wigger, 2002; Kromer *et al.*, 2005) and higher freezing levels. Accordingly, lower anxious animals that adopt active coping style could be accompanied by hyper-arousal and less freezing and immobility when compared with NAB and HAB animals.

Here, we reported that the PPI in LAB mice was similar to that in HAB and NAB mice when the prepulse level was 5 or 15 dB over the 50-dB background (Figure 36A). LAB mice even showed more pronounced PPI compared to NAB controls at a higher prepulse level (25 dB over the 50-dB background; Figure 36C), but this effect may be confounded by the elevated startle response elicited by 75-dB startle stimulus (Figure 36A, B). The PPI in the most widely studied animal for ADHD (e.g. SHR) is not conclusive. Van den Buuse (2004) argued that SHR showed similar levels of PPI to their respective controls, whereas Li *et al.* (2007) reported that SHR showed pronounce PPI deficits compared to control animals at higher prepulse levels. In humans, the PPI of ADHD adults was not significantly different from that of healthy control subjects in any of the PPI conditions, suggesting that PPI is not a general feature of ADHD which possess attention abnormalities (Feifel *et al.*, 2009). Based on the aforementioned data from animals and humans, ADHD may have a neurobiological substrate somewhat distinct from schizophrenia and other neuropsychiatric disorders that are associated with PPI deficits (Hawk *et al.*, 2003).

4.3.2. Hyperactivity

In the OF test, LAB, NAB and HAB mice revealed line differences in horizontal locomotion, immobility time and rotational behavior. Overall, LAB mice are more active than NAB and HAB mice, as can be seen by higher levels of locomotion, mobility time and rotations. Similar difference in exploratory behaviors has been found in HAB *vs.* LAB rats (Ohl *et al.*, 2002). During the 80-min OF testing course, HAB and NAB mice showed habituation within daily test course, whereas LAB mice failed to habituate to the testing environment as indicated a consistent level of locomotion. The lack of habituation makes LAB mice fulfill the key criterion of ADHD disorder that is hyperactivity in the habituated environment (Porrino *et al.*, 1983). A number of LAB mice also displayed striking rotational behavior, which was positively correlated with horizontal locomotion. Abnormal rotational behavior in rodents has been reported to be indicative of an imbalance of the nigrostriatal DA system (Pycock, 1980). Furthermore, disturbances in caudate asymmetry have been suggested to be involved in the etiology of several psychiatric disorders, including ADHD (Schrimsher *et al.*, 2002). Therefore, this extreme rotational behavior in LAB mice may be due to a loss of balance in the striatal DA system.

In the LAB population, two sub-lines (LAB-S and LAB-I) could be distinguished. LAB-S mice displayed much higher levels of horizontal locomotion compared to HAB and NAB as well as LAB-I lines. On the other hand, the DT of LAB-I, even though lower than that of LAB-S mice, was by far more pronounced than HAB and NAB mice, in particular upon revealed OF exposure. Noteworthy, about one-third of LAB mice developed as LAB-S line in each generation. Analysis of LAB's individual locomotion by each breeding pair revealed that LAB-S mice are not bred from specific maternal/paternal combination. Instead, the probability of LAB-S mice almost equally distributes in these batches, which suggests that the bimodal phenotypes in LAB population are not likely the readout of genetic mutations. The development of the two sub-lines might result from postnatal rearing environment and epigenetic regulation.

In addition, HAB, NAB and LAB-S mice displayed no change in DT over long-term test course (62 days), implicating their inter-individual persistence in activity-related phenotypes. Although LAB-I mice slightly increased their DT over time, the elevated locomotion results in more distinction in LAB-I *vs.* HAB/NAB lines and more proneness to LAB-S line. The increase in locomotor activity may compare well to the gradual development of hyperactivity in children with ADHD over testing time (Sagvolden and Sergeant, 1998). However, we can not rule out that the altered locomotion in LAB-I mice may be influenced by the various tests and multiple treatments.

LAB mice also displayed higher levels of general exploration than HAB and NAB mice in the HB test, a more complicated OF testing environment. In contrast to their elevated locomotion and rearing, LAB displayed significantly less 16-hole exploration in terms of N-P entries (Figure 41A & B) and N-P time (data not shown), suggesting their inattention to the major feature of the test environment. Interestingly, there was an inverse relationship between horizontal locomotion and the accuracy of hole exploration in LAB mice. These data suggest that hyperactivity is correlated with attention deficits, which is also observed in ADHD patients.

4.3.3. Cognitive deficits

In the WCM experiment, general processes of spatial learning and memory were impaired in LAB mice. During the first week of training, 60 % of LAB mice failed to acquire the task. It may be due to their inability in motor control. Only accurate learners in the last day of training were used for relearning to avoid the influence of different performance in acquisition. Relearning only differed from acquisition in the additional requirement to inhibit an established response pattern. Analysis of perseveration errors (wrong platform visits) of each line during the relearning phase indicated that the great majority of LAB mice with poor performance in relearning made the same perseverative set of errors: they swam into the previously rewarded arm and then turned quickly into the other correct arm. These findings are in accordance with results of a previous study demonstrating that LAB rats are impaired in declarative memory performance by showing more wrong choices than HAB rats (Ohl *et al.*, 2002). The evidence of perseveration in LAB animals suggested their poor behavioral flexibility that they may be not able to inhibit inappropriate responses. Interestingly, a certain amount of ADHD patients suffer from a deficit in behavioral inhibition such that they respond to various stimuli in an improper manner (Barkley, 1997). Impairments of behavioral flexibility, such as in set-shifting and reversal learning, are associated with ADHD and have been attributed to a disruption in frontal lobe functioning (Cubillo *et al.*, 2010).

Intriguingly, similar perseveration problem was reported in a study in pigs diverging for their Backtest response, high-resisting (HR) and low-resisting (LR) responses (Bolhuis *et al.*, 2004). In a spatial discrimination (T-maze) task, HR and LR pigs displayed different behavioral patterns. Noteworthy, HR pigs are less successful in reversal learning then LR pigs, which is consistent with poor behavioral flexibility in LAB animals. Converging above evidence in two species, the fact that the dichotomy of cognitive processing may also be interpreted by their differential coping strategies (Hawk *vs.* Dove). In line with this framework, the different behavioral strategies shown in Hawks and Doves, especially behavioral flexibility, may be related to changes in their hippocampal morphology (Schwegler *et al.*, 1981).

Besides the impaired spatial learning and memory, LAB mice also displayed social cognition deficits by reflecting reduced social discrimination ability and social memory. LAB mice, like NAB and HAB mice, displayed an intact social preference to the social stimulus *vs.* non-social stimulus. However, compared to HAB and NAB mice, LAB mice failed to show social discrimination ability and displayed inferior social memory. In social preference test, LAB mice may lose interest in social investigation during the testing phase (as mirrored by the reduced total sniffing time) and failed to show a preference for the novel female. In the social discrimination test, they may be unable to maintain the olfactory information and then to discriminate two females properly. Based on LAB's poor performance in both tests, one would speculate that LAB mice might be inattentive to the cues in their environment. These findings are consistent with recent evidence suggesting that ADHD is associated with social cognition impairments, such as social interaction and communication (Uekermann *et al.*, 2010). Data from behavioral and brain imaging studies have implicated fronto-stiatal structures in cognitive impairments in ADHD (Hermens *et al.*, 2006), including social cognition. Furthermore, evidence from a genetic study on humans suggests that the oxytocine receptor (*OXTR*) gene is somehow involved in the social cognitive deficits seen in some ADHD children (Park *et al.*, 2010).

4.3.4. Other phenotypes

A high incidence of voiding dysfunction (e.g. nocturnal polyuria) in children with ADHD was reported in some urological studies (Baeyens *et al.*, 2004; Chertin *et al.*, 2007). Coincidently, previous data indicated that LAB mice with a deficit in vasopressin showed signs of central diabetes insipidus (cDI) (Kessler *et al.*, 2007). Although the comorbidity between symptoms of cDI and signs of ADHD is not described in literature, nocturnal polyuria can be caused by numerous diseases, such as cDI (Asplund, 2002). Furthermore, the two clusters of symptoms are commonly associated with obesity (Campbell and Eisenberg, 2007).

ADHD is known to be highly comorbid with a broad range of child neuropsychiatric disorders (Gillberg *et al.*, 2004), including sleep disorders. Among these sleep disorders, restless legs syndrome (RLS) appears to be more frequent in children with ADHD than in controls (Silvestri *et al.*, 2009). Interestingly, increased muscle tone was also observed in LAB mice during sleeping

(Jakubcakova & Kimura, unpublished). This promising co-occurrence of RLS in ADHD and increased muscle tone in LAB mice further highlights the usage of LAB mice as an animal of ADHD.

Overall, the face validity of LAB mice is excellent as hyperactivity, cognitive deficits, social cognition impairments as well as signs of comorbid disorders were all found in this line. Even though attention-related behavior was not measured, several signs reflecting inattention could be observed in LAB mice, such as their poor performance in N-P task, WCM and social cognitive tests. The measure of impulsivity is missing in that serial of behavioral tests in LAB mice. However, LAB mice showed perseveration and poor response inhibition possibly reflecting increased impulsivity (Yan *et al.*, 2011).

4.3.5. Pharmacological validation

4.3.5.1. Amphetamine and tomoxetine/methylphenidate

Amphetamine is commonly used in the treatment of ADHD symptoms. As predicted, treatment with amphetamine increased locomotor activity in HAB/NAB mice, but exerted a calming effect on locomotion in both LAB-I and LAB-S mice. Interestingly, response to amphetamine seemed to depend on level of trait anxiety (respectively basal locomotor activity in the OF), with HAB mice being highly stimulated, NAB mice being intermediately stimulated, and LAB mice being depressed. With respect to other predictive validity markers, tomoxetine failed to affect hyperactivity in LAB mice, and methylphenidate even increased locomotor activity in both LAB-I and HAB/NAB mice. The reason why there is a difference in response to various ADHD treatments in LAB-I mice is unclear. It may involve some differential effects on other monoamines that are seen after amphetamine, but not tomoxetine or methylphenidate administration. The effects of amphetamine have been suggested to be modulated by noradrenergic or serotonergic transmission as DA is hyperactive with respect to noradrenalin metabolism, but hypoactive to 5-HT metabolism (Oades, 2002).

In the current studies, amphetamine was not able to compensate LAB animals' deficits in spatial learning and social memory. This is in line with increasing studies in humans in which stimulant

drugs fail to reverse cognitive dysfunctions in ADHD (Advokat, 2010). In rats, administration of amphetamine at low doses is able to restore attention in PFC-lesioned rats. However, the same or escalating dosage of amphetamine causes impairments in memory (Chudasama *et al.*, 2005). The trade-off of amphetamine effect on attention and memory may explain why the cognitive deficits in LAB mice cannot be rescued by amphetamine treatment. In addition, clinical evidence supported that the most effective duration of the pharmacological treatment for ADHD can begin in early childhood (Zito *et al.*, 2000). Several studies have proposed that both the timing of treatment onset and treatment duration have been proven to influence the therapeutic effects of ADHD drug on cognitive processes during development (Andersen *et al.*, 2002; Thanos *et al.*, 2007). A review work has suggested that the cognitive deficits could be efficiently improved by repeated treatment during early development in rodents (Britton, 2011). Therefore, the timing of treatment may be too late to modify the cognitive deficits of adult subjects in the present study. Furthermore, since only few studies in rodents have demonstrated the facilitating effects of psychostimulants on cognitive ability (Shaywitz *et al.*, 1978), robust approaches have to be developed to assess the effects of psychostimulants and other clinically effective compounds in the cognitive tasks relevant for ADHD in mice.

4.3.5.2. Haloperidol

The locomotor activity of both LAB and NAB mice was attenuated by haloperidol, a DA D_2 receptor antagonist. As haloperidol produces a state of catalepsy by reducing Dopaminergic transmission in the basal ganglion (Klemm, 1985), it is not recommended be feasible for treatment of ADHD.

4.3.5.3. AM404

Psychostimulants are the most conventional treatment for ADHD patients; however, they have a bad reputation because these drugs have a potential for abuse. Therefore, there is a strong urge for the search of alternative therapeutic strategies for ADHD. A previous study on DAT KO mice has pinpointed that a dysregulated striatal endocannabinoid transmission is associated with hyperdopaminergic state (Tzavara *et al.*, 2006). Restoring endocannabinoid transmission by AM404 was found to rescue the hyperactivity induced by hyperdopaminergia, in which the

transient receptor potential vanilloid 1 (TRPV1) receptors seem to play a critical role. Here, administration of AM404 exerted a calming effect in the LAB mice, indicating the endocannabinoid system on locomotion in LAB mouse line. However, neither CB1 nor TRPV1 receptor was involved in the AM404-induced hypolocomotion. Surprisingly, TRPV1 receptor antagonist even potentiated the hypolocomotion effects of AM404. This discrepancy between DAT KO mice and LAB mice might be due to their different levels of dopaminergic function, with DAT KO mice showing a hyperactive, but LAB mice a hypoactive DA system.

4.3.6. Microdialysis

In our microdialysis experiments, we compared the drug-induced neurochemical changes in the extracellular DA in LAB mice with that in HAB mice. The present data revealed that the basal level of extracellular DA in LAB mice was significantly lower than that in HAB mice (Figure 54). Therefore, the neurochemical basis of the hyperactivity in LAB mice may be related to decreased levels of DA in the CPu of LAB line. Several animal models of ADHD suggested that the dopaminergic system is functionally impaired (Luthman *et al.*, 1989; Russell *et al.*, 2005). Some animal models for ADHD have decreased levels of extracellular DA concentrations (Sagvolden *et al.*, 2005) while others show increased levels of extracellular DA concentrations (Gainetdinov *et al.*, 1999). Consistent with the aforementioned animal models, LAB mouse model is suggested to be a model with hypoactive DA system.

We investigated the effects of acute treatment with the psychostimulants (amphetamine and methylphenidate) on the extracellular levels of DA in the CPu of HAB and LAB mice. Stimulation with amphetamine (Figure 56A) and methylphenidate (Figure 57A) increased DA accumulation in the CPu eliciting an equipotent increase in both HAB and LAB mice. It is commonly believed that changes in dopaminergic tone are highly related to alterations in locomotion. Surprisingly, the equal increase in the DA levels was not corresponding to the distinctive amphetamine effects on locomotion in both lines. Interestingly, administration of amphetamine but not methylphenidate induced a larger and longer DOPAC decrease in LAB than in HAB mice (Figure 56B). The decrease in the DOPAC content indicated that amphetamine inhibited monoamine oxidase (MAO) in the CPu in both mouse lines with different overall effect. As the DA-DOPAC

interaction has a reciprocal feature, amphetamine may induce different intracellular metabolisms or transmission of DA. It could be attributed to a prior difference in MAO levels (HAB > LAB mice) or more efficient effects of amphetamine on LAB MAO then HAB MAO.

Since AM404 was discovered to attenuate the hyperactivity in LAB mice, the extracellular levels of DA were also evaluated after administration of AM404, consistent with the hypothesis of a direct involvement of DA in regulation of locomotion. However, treatment with AM404 did not induce significant changes in either DA or DOPAC levels in both HAB and LAB mice. The data demonstrated that AM404 can modulate the hyperactivity without producing concurrent changes in the extracellular DA concentrations in the CPu. Similarity in the increase of DA extracellular level was revealed by perfusion with KCl (100 mM) in HAB and LAB lines, indicating no difference in vesicular pool capacity between the two lines.

To investigate the role of DAT and NET on amphetamine effect, a DAT and NET blocker, nomifensine, was injected 20 min before amphetamine administration. The data revealed that blockade of DAT and NET with nomifensine attenuated the increase of the extracellular DA concentrations in the CPu in HAB and LAB mice. However, nomifensine did not abolish the calming effects of amphetamine. Based on the above mentioned findings, one could conclude that the calming effects of amphetamine are not mediated by dopaminergic or noradrenergic transmission.

Psychostimulants have been reported to interact with the 5-HT transporters (SERTs) (Kuczenski and Segal, 1997). There is increasing evidence on animal models suggesting that psychostimulants reduce hyperactivity not by inhibiting DAT but by inhibiting NET and SERT (Davids *et al.*, 2002; Russell *et al.*, 2005). The studies with tomoxetine and methylphenidate revealed it unlikely that the noradrenergic system plays an important role in the calming effects amphetamine. In line with this notion, pharmacological dissection of molecular targets points to a possible involvement of disturbed 5-HT neurotransmission (Gainetdinov *et al.*, 1999) in the genesis of LAB phenotype.

Table 8 summarizes the effects of various drugs on locomotor activity in the OF and on the DA levels in the CPu. These findings indicate the hyperlocomotion and calming effects of

amphetamine and AM404 in LAB mice do not correspond to the extracellular DA levels. There may be other important neurochemical changes produced by amphetamine- and AM404-attenuated hyperlocomotor activity, or perhaps a different brain region or multiple brain regional effects are involved in the behavioral effects of amphetamine and AM404. So far, there is no evidence for a regulating effect on the side of the neurotransmitter, but this does not preclude the existence of differences of the receptive side (e.g. DA receptors).

Table 8: Summary of behavioral (black symbols) and neurochemical (open symbols) effects of selected drugs. Note that N/A indicates not applicable.

Line	Amphetamine		Methylphenidate		Nomifensine		Nomifensine + Amphetamine		AM404	
HAB	⬆⬆	⇧	⬆⬆⬆	⇧	N/A	⇧	N/A	⇩	⬌	⇔
NAB	⬆	N/A	⬆⬆	N/A	⬆⬆	N/A	⬇	N/A	⬌	N/A
LAB	⬇	⇧	⬆	⇧	⬆	⇧	⬇	⇔	⬇	⇔

4.3.7. Validation of animal models of ADHD

Table 9 summarizes the comparable characteristics of the LAB mouse model and ADHD. It can be concluded that LAB line is a potential animal model of ADHD demonstrating excellent face validity as proved by hyperactivity, cognitive deficits (including preservation and poor social cognitive abilities) as well as signs of comorbid disorders. The predictive validity of LAB mice is intermediate as only amphetamine, but not methylphenidate and tomoxetine, was found to attenuate hyperactivity. It is well-accepted to evaluate the predictive validity of a certain animal model on the basis of their respondence to psychostimulants. However, it should be noted that in clinic, approximately 30 % of children with ADHD do not respond to psychostimulant treatment (Klein *et al.*, 1988).

Current validation of animal models relies heavily on behavioral phenotypes due to a poor understanding of construct validity for ADHD itself. For example, it is still unknown how genetic alterations in ADHD influence the neural mechanisms and result in behavioral changes. Although the entire genetic framework of ADHD has not been established, possible involvement of the DAT and D4 receptor or other markers (e.g. 5-TH transporter) which seem

to be important and valuable targets to confirm the construct validity of proposed animal models.

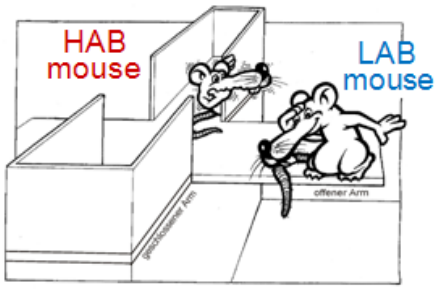
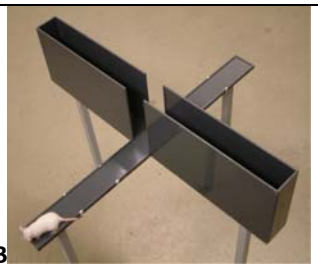
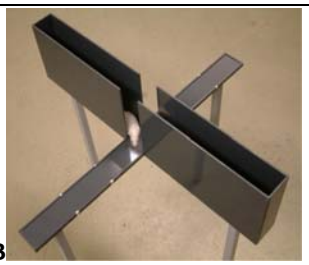
Table 9: Summary of the characteristics of ADHD and LAB mice. Note that N/A indicates not applicable.

ADHD	LAB mice
Inattention	Attention deficits
Hyperactivity	Hyperlocomotion
Impulsivity	N/A
Cognitive deficits	Impaired habitual and/or spatial learning
Social cognition impairments	Reduced social discrimination abilities
Paradoxical calming effects of psychostimulants	Calming response to amphetamine treatment
Restless leg syndrome	Increased muscle tone during sleeping
Increased incidence of nocturnal polyuria	cDI symptoms

4.3.8. Other animal model of ADHD

Several other animal models of ADHD have been proposed, and they were developed by genetic manipulation, neurotoxic lesion or selective breeding. In a review study by van Kooij and Glennon (2007), the neonatal 6-OHDA lesioned rat and DAT-KO mice have the highest degree of validity for ADHD as they preferably respond to both amphetamine and methylphenidate. However, it is clear that no rodent model would be able to recapitulate fully the complex nature of ADHD and each model has its strengths and limitations. In DAT-KO mice, the level of DAT dysregulation and DA dysfunction is too extreme so that DAT-KO mice have some other phenotypes, for example, dwarfism (Bosse *et al.*, 1997), hormonal dysregulation and remarkable neurochemical alterations (Jones *et al.*, 1998b) that may not be relevant for classical ADHD. Moreover, since the pathogenesis is likely to be polygenic in nature, single receptor knockout would not represent a proper animal model of ADHD. In 6-OHDA lesioned mice, DAT and D1 receptor sites were reduced after intrastriatal 6-OHDA, but only very minor alterations have been found in ADHD patients compared to the massive loss in this animal model (Frohna *et al.*, 1997; Thomas *et al.*, 1998). While there may be no single prefect animal model of ADHD, further research on animal models will undoubtedly promote a better understanding of underlying mechanism for ADHD. Thus, it is absolutely necessary to identify the strengths and limitations of each model (Gainetdinov, 2010) in order to use the model efficiently in investigating the pathogenesis of the disorder and development of new therapies.

Table 10: HAB/LAB mouse model and Hawk-Dove personality framework. Summary of characteristics of HAB/LB animal model corresponding to the framework of Hawk-Dove personalities. The star symbol indicates the evidence provided in the present study.

Breeding by EPM performance		
Extremes in anxiety-related behavior	**LAB** 	**HAB** 
Personality type	**Hawk** 	**Dove** 
Behavioral strategy	Fight-flight ✡	Freeze-hide✡
Coping style	Proactive ✡	Reactive✡
Emotional state	Aggressive	Non-aggressive
	Bold ✡	Cautious ✡
Biological role	Establish territory or defend existing territory	Adopt strategy to avoid danger within territory, e.g. immobility ✡
Exploration	Fast and superficial ✡	Cautious and thorough ✡
Behavioral flexibility	Rigid and routine-like ✡	Flexible ✡
Energy metabolism	High energy consumption	Energy conservation
Exploratory behavior	Hyperlocomotion ✡	Hypolocomotion ✡
Psychopathology	Impulse control disorder ✡ e.g. Attention deficit/hyperactivity disorder (ADHD) 	Anxiety disorders ✡ Generalized anxiety disorder (GAD) (post stress) Panic-like symptoms 

5. Perspectives

Selective breeding for anxiety-related behavior has resulted in two extreme lines with different clusters of characteristics and neurochemical features and these two breeding lines represent an animal model to decode and analyze different psychiatric disorders (see Table 10).

The present data demonstrated a direct relationship between trait anxiety and stronger fear acquisition, slower fear extinction and susceptibility to relapse of conditioned fear. HAB mice displayed pronounced conditioned fear compared to NAB/CD1 and LAB mice that coincided with increased phosphorylation of AKT in the basolateral amygdala. Having indentified AKT phosphorylation as a molecular marker of exaggerated fear memories in a psychopathological animal model of trait anxiety, one major focus in further studies should be the testing of an AKT phosphorylation inhibitor that is capable to downregulate the described protein, thus possibly attenuating pronounced conditioned fear and avoidance behavior in HAB mice.

With respect to NPS, the molecular and cellular effects of the SNPs on peptide processing and cell viability have to be analyzed to prove the functional impact of the mutations on NPS release. In addition, measuring the levels of NPS in certain brain regions in HAB *vs.* LAB by mass spectrometry or HPLC would be necessary for better understanding of the direct role of NPS in determining the levels of trait anxiety. Furthermore, the current data has addressed that NPS in the ventral hippocampus also played a critical role on anxiolytic effects (Dine, unpublished). This finding emphasizes the need to study the NPS receptors in brain regions other than the basolateral amygdala, such as the ventral hippocampus.

Since LAB mice fulfill face validity (hyperlocomotion and cognitive impairments) and predictive validity (response to amphetamine), they have been suggested to represent a novel animal model of ADHD. Besides the pronounced phenotypes mentioned above, additional behavioral tests have to be developed to directly measure attention and impulsivity of LAB mice. With respect to predictive validity of LAB animal model, further research should focus on determining the neuropharmacological functions of psychostimulant drugs and AM404 as these drugs reverted hyperactivity in LAB mice by altering off-target effects. Besides hyperactivity, cognitive impairment is also a key feature of ADHD. However, there are only few studies

addressing the cognitive improvement for ADHD animal models, suggesting the lack of evidence for effective cognitive enhancers. Therefore, the evaluation of neural mechanisms underlying cognitive deficits and the development of proper application methods (e.g. repeated treatment during early development) in LAB mice would help to identify optimal cognitive enhancers for ADHD.

Taken together, the HAB/LAB mouse is a valuable and promising tool for understanding the association between personality trait and psychopathology. With respect to the concept of endophenotypes, the HAB line provides a unique opportunity to identify the underlying mechmisms of developing traumatic memories in psychiatric patients with trait anxiety. On the other hand, the LAB line highlights a potential tool for the preclinical researches in the field of ADHD.

6. Supplementary Tables

Supplementary table 1: Primer sequences used for sequencing of the neuropeptide S (*Nps*) gene. The primer sequences are listed below with location in the *Nps* gene (including exons/promoter/downstream enhancing region (DER), melting temperature (T_m), PCR fragment length and orientation).

Primer	Location	Type	Primer Sequence (5'→3')	T_m	Product size (bp)
Nps af	Promoter	Fwd	CCAGGCTTCCAGCTTGGCAC	58.16	542
Nps ar	Promoter	Rev	GCTGCTATTGCTGCTGTTTCTGAAG	57.24	
Nps bf	Promoter	Fwd	GGGTATCTTTGCCCTCCAAAAGGTG	57.78	588
Nps br	Promoter	Rev	GGCAATCTGTTGTCACTGGTCCCTG	59.72	
Nps cf	Promoter	Fwd	TCCCTGCTCAACACCCCAAACC	58.94	523
Nps cr	Promoter	Rev	ACTGGTTGGCCTGGCTGTGG	59.19	
Nps 1f	Exon 1,2	Fwd	GAGGCTCCTGGCCACCCATG	59.12	572
Nps 1r	Exon 1,2	Rev	GGGCCCTCCACCATCCTGATCA	59.72	
Nps 2f	Exon 1,2	Fwd	TGGCAAGCTCTGAGTGAAGTCAACC	59.16	507
Nps 2r	Exon 1,2	Rev	TTTGGGCCCTCCACCATCCTGA	59.51	
Nps 3f	Exon 1,2	Fwd	CCCATCTGCGCAGGTCTCGG	59.56	594
Nps 3r	Exon 1,2	Rev	TCCACTGTGCGGGTTTTTGGT	57.07	
Nps 4f	Exon 1,2	Fwd	CATCTGCGCAGGTCTCGG	55.00	419
Nps 4r	Exon 1,2	Rev	CCAGAGTTACCTACTGTCACATAC	52.24	
Nps 5f	Exon 3	Fwd	AGCCGGTGGTAGCCCTACACT	59.02	500
Nps 5r	Exon 3	Rev	ACTCTGAGCCCGTTAGGAGAAGGG	59.22	
Nps 6f	DER	Fwd	CCTTTCGCAACGGAGTCGGCT	59.72	568
Nps 6r	DER	Rev	CGAGCCCTTGCTGCAGGTACC	59.78	
Nps 7f	DER	Fwd	GTGCCACCAAGTGCAGTGGC	59.01	525
Nps 7r	DER	Rev	GCTGGTGACCAAGGACAGGGT	58.34	

Supplementary table 2: Primer sequences used for sequencing of the neuropeptide S receptor 1 (*Npsr1*) gene. The primer sequences are listed below with location in the *Nps1r* gene (including exons/promoter/downstream enhancing region (DER), melting temperature (T_m), PCR fragment length and orientation).

Primer	Location	Type	Primer Sequence (5'→3')	T_m	Product size (bp)
Npsr1 af	Promoter	Fwd	GCAGAGGAGACCACACTGGCG	59.46	509
Npsr1 ar	Promoter	Rev	GCCTGACGACAAGGAAGATCCACG	59.60	
Npsr1 bf	Promoter	Fwd	TTGTCATCTCCTGTCTGTGCCCCT	59.46	519
Npsr1 br	Promoter	Rev	CGCCAGTGTGGTCTCCTCTGC	59.33	
Npsr1 cf	Promoter	Fwd	TGCAGCGTAATGAACACCCCCA	58.76	551
Npsr1 cr	Promoter	Rev	GTAGGCCAACCTTTGCTTTACTGCC	58.55	
Npsr1 df	Promoter	Fwd	CTGTATGTGCAAATGTGTGTC	58.6	495
Npsr1 dr	Promoter	Rev	GGAGAGCAGAATGTCATGAG	58.6	
Npsr1 ef	Promoter	Fwd	AAGCCCTCATCTCTAACCTG	60.8	571
Npsr1 er	Promoter	Rev	TCATGGTTTCCCCTCCTCCA	62.8	
Npsr1 ff	Promoter	Fwd	GGGCAAACAAACACTATTGATC	61.1	574
Npsr1 fr	Promoter	Rev	ACATCCCCTAAATACCACTGAGT	62.1	
Npsr1 gf	Promoter	Fwd	CACCTACAAACTTTTCCATC	57.7	433
Npsr1 gr	Promoter	Rev	AATCTCCACATTTCCCTGAG	58.6	
Npsr1 1f	Exon 1,2	Fwd	GGGCAGGTCTGTGGGATGGTG	59.10	500
Npsr1 1r	Exon 1,2	Rev	GCCTCCCTAGCAGCAGCTAAGACT	59.82	
Npsr1 2f	Exon 3	Fwd	CCTGGGCATTTGCTGGGCGG	60.94	485
Npsr1 2r	Exon 3	Rev	TGTGAGGACACTGAAGGTGGCA	57.77	
Npsr1 3f	Exon 4	Fwd	AGCAAGCCCTCTCCTGGGACC	59.90	468
Npsr1 3r	Exon 4	Rev	AAGGAGTGTCTGATTGTGCAGGAGC	58.99	

Npsr1 4f	Exon 5	Fwd	CTGCTTCCAGCAGGGAGGGC	59.41	477
Npsr1 4r	Exon 5	Rev	TGGGGTGAGGATCAGGCAGCA	59.56	
Npsr1 5f	Exon 6	Fwd	AGGTAGGTGGGCCTGCACCC	60.18	427
Npsr1 5r	Exon 6	Fwd	AAGCAGGGTCCAGCCCGTGG	61.10	
Npsr1 6f	Exon 7	Rev	CAAGCAGAGCTGTCAAGGATGGT	57.04	404
Npsr1 6r	Exon 7	Fwd	GCTTTCAGGGAGGCCGAGTGG	59.45	
Npsr1 7f	Exon 8	Rev	TGGGCATTTGCATTGGGTTGC	57.03	426
Npsr1 7r	Exon 8	Fwd	TGGCTCTTGCAGCAGTCAAACAC	58.30	
Npsr1 8f	Exon 9	Fwd	TGTTAGCACACCCAAGGCCAC	57.22	452
Npsr1 8r	Exon 9	Rev	GGAAGTGTACGGAGGTTCGCAGC	59.82	
Npsr1 9f	Exon 10	Fwd	ACTGTCCACTAGGCTGTGATGGC	58.39	536
Npsr1 9r	Exon 10	Rev	TGCAGGTGCTGGGCTAACGG	59.34	
Npsr1 10f	Exon 10	Fwd	TGCCACCTGCAATTCACGCAC	58.80	489
Npsr1 10r	Exon 10	Rev	TGTGCCTGCATGGTGTCCTTGT	58.95	
Npsr1 11f	Exon 10	Fwd	AGCAAGAGCAAACTCCCAAGCA	57.01	610
Npsr1 11r	Exon 10	Rev	GCATCATAGGGCTGTGGGTGG	57.23	
Npsr1 12f	Exon 10	Fwd	GGCACCTCTGGCACCTCTGC	59.69	400
Npsr1 12r	Exon 10	Rev	CCACCATGACCTTAAGCAGGCAGTC	69.55	
Npsr1 13f	Exon 10	Fwd	TGGCTGACTGCTGGTTGAGTCG	59.10	467
Npsr1 13r	Exon 10	Rev	CAAGGGCCTGGGCCTCCTGT	60.47	
Npsr1 14f	Exon 10	Fwd	AGCAAGCAGAAGCATTGAGTGGC	58.42	544
Npsr1 14r	Exon 10	Rev	GTGGTGCCCAGAGACACAGCA	58.98	
Npsr1 15f	Exon 10	Fwd	GCCATCTATGCAGAACTTGCTCTACG	57.93	571
Npsr1 15r	Exon 10	Rev	AACACATTTGCCCGATCAGCCT	57.14	
Npsr1 16f	DER	Fwd	AGGTGCCTACCTTCCACACCAAG	58.25	569
Npsr1 16r	DER	Rev	GGCTGTCAAATGTGCAGCTTCCCT	59.94	

Supplementary table 3: Polymorphisms in the promoter of *Nps* between the HAB and LAB specific sequence, with probably binding factors. Location refers to position from transcription start in the promoter region.

Location	HAB SNP	LAB SNP	HAB binding factor	LAB binding factor
-13	C	T	RAF	RC2, NF-1
-163	C	T	GR alpha, GR beta, PR A, PR, GR/PR, HSF1, AR, GR, PR B, TCF-4E	-
-316	T	C	GR, HSF1, Dof2, Dof3, MNB1a, PBF, LEF-1, TCF-1(P), TCF-1, TCF-1A, TCF-1B, TCF-1C, TCF-1E, TCF-1F, TCF-1G, TCF-2alpha, Hb	c-Ets-2, LEF-1, TCF-1 (P), TCF-1, TCF-1A, TCF-1B, TCF-1C, TCF-1E, TCF-1F, TCF-1G, TCF-2alpha, Dof2, Dof3, MNB1a, PBF
-621	A	C	NF-1/L, GR, Sp1, LEF-1, TCF-1(P), TCF-1, TCF-1A, TCF-1B, TCF-1C, TCF-1E, TCF-1F, TCF-1G, TCF-2alpha	T-Ag
-712	G	A	-	c-Myb, LEF-1, TCF-1(P), TCF-1, TCF-1A, TCF-1B, TCF-1C, TCF-1E, TCF-1F, TCF-1G,

				TCF-2alpha, GT-IIBa
-788	C	T	NF-1, NF-1/L, EcR, c-Myc, c-Myb	EcR, MYB1, c-Myb
-819	A	G	Zeste	H4TF2, H4TF-2
-867	T	G	NF-E2, AP-1, c-Jun, v-Jun, Zeste, Zta, c-Fos, YAP1, Fra-1	TTF-1, NF-1, Ap-1
-868	C	T	NF-E2	TTF-1, NF-1
-871	T	C	-	TTF-1
-920	A	G	CCAAT-binding factor, CP1, CTF, CP2, alpha-CBF, alpha-CP1, alpha-CP2a, alpha-CP2b, alpha-IRP, CDP2, Clox, CUTL1, Cut11, H1TF2, NF-1, NF-E, SRF, TGGCA-binding protein, CBP/CRF, C/EBPalpha, CBF (1), CBF (2), CBF-A, CBF-B, CDF, CRF, NF-Y', NF-Y, NF-1	-
-924	T	C	-	GCN4, AP-1
-995	-	GTGT		
-1030	T	C	SGF-1, ETF, TFIID, POU3F2	HSTF
-1031	A	T	SGF-1, ETF, TFIID, POU1F1a, POU3F2	HSTF

Supplementary table 4: Polymorphisms in the promoter of *Npsr1* between the HAB and LAB specific sequence, with probably binding factors. Location refers to position from transcription start in the promoter region.

Location	**HAB SNP**	**LAB SNP**	**HAB binding factor**	**LAB binding factor**
-274	TC	-	GR, AP-1	-
-351	C	A	DTF-1	POU3F2, YY1
-385	T	C	-	GCN4
-406	T	C	-	NF-1, twi
-431	T	A	-	c-Myc
-442	-	T	-	AP-1, YAP1, v-Jun, c-Jun, JunD, GCN4
-507	T	G	-	-
-508	C	T	-	-
-525	C	T	GAL4	-
-610	G	A	CCAAT binding factor, CP1, CTF, alpha-CBF, alpha-CP1, alpha-CP2a, alpha-CP2b, alpha-IRP, CDP2, C/EBPalpha, CBF(1), CBF(2), CBF-A, CBF-	GCN4, AP-1

			B, CP2, NF-1, CBP/CRF, GT-IIBa	
-654	C	T	ABF2, GAL4, TBP, BEAF-32A, BEAF-32B, RAF	CBP/CRF, alpha-CBF, alpha-CP1, alpha-CP2a, alpha-CP2b, alpha-IRP, CDP2, Clox, C/EBPalpha, CBF(1), CBF(2), CBF-A, CBF-B, CCAAT binding factor, CD, CP1, CP2
-826	G	A	GR, C/EBPbeta, H-APF-1, IL-6.RE-BP	MCBF
-866	T	G	HNF-3alpha, HNF-3B, SRY	-
-926	A	T	NF-1/L, CTF, C/EBPalpha, CBF(1), CBF(2), CBF-A, CBF-B, CCAAT binding factor, CDF, CRF, NF-Y', NF-Y, CP2, alpha-CBF, alpha-CP1, CP2, alpha-CP2a, alpha-CP2b, alpha-IRP, CDP2, Clox, CUTL1, Cut1, CBP/CRF	
-1020	A	T	delta factor, YY1, TBP, F2F, POU1F1a, NPTC-II	TBP, Hb, POU1F1a
-1021	-	T		TBP, Hb, POU1F1a
-1030	C	T	CCAAT binding factor, gammaCAC1, gammaCAC2, YY1 , delta factor	GCN4, Zesta
-1109	A	T	-	YY1
-1170	A	G	YY1	IPF1
-1212	G	A	HOXD10, Cad	IPF1
-1263	T	C	GATA-1, GATA-1A, GATA-1B, GATA-2, GATA-3, NF-E1b	NIT2, BEAF-32A, BEAF-32B
-1310	G	A	-	delta factor, YY1
-1400~-1365	-	(GA)x18	-	TFII-I, GAGA factor
-1401	-	A	-	HSTF, Dof2, Dof3, MNB1a, PBF, TFII-I
-1402	-	A	-	HSTF, Dof2, Dof3, MNB1a, PBF
-1461	G	A	NF-1	-
-1569	C	T	-	HSTF, IHF
-1636	T	A	LEF-1, TCF-1(P), TCF-1, TCF-1A, TCF-	-

			1B, TCF-1C, TCF-1E, TCF-1F, TCF-1G, TCF-2alpha, Sp1	
-1657	T	C	-	-
-1736	G	A	RC2, IHF	MYB1, c-Myb
-1840	T	C	HOXA5	v-Jun, Zta, AP-1, c-Jun, YAP1, GR, HES-1
-1898	T	A	HSF1	-
-1906	T	C	EFII	-
-1917	C	G	-	GR
-2042	C	T	GR, Sp1, ER-alpha, LF-A1, T-Ag	NF-1
-2045	G	A	GCR1, GR, Sp1	-

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8. Acknowledgment

First of all, I thank Prof. Dr. Landgraf for giving me the opportunity to pursue my PhD degree in his research group at the Max Planck Institute of Psychiatry. More importantly, I'd like to thank him for his supervision and support through all of different problems I had while working on the projects and on my dissertation. I would also like to thank Prof. Dr. Conradt for her kind willingness to take over the second evaluation of my dissertation.

Furthermore, I express my sincere gratitude to Dr. Carsten T. Wotjak for his supervision and support. He always brings me new insights in doing scientific researches and motivates me with his unconditional encouragement and brilliant enthusiasm. Without his advice, I would not have improved myself that much.

I would like to show my gratitude to Dr. Mirjam Bunck and Dr. Rudolph Marsch, for their kind supervision during the tuff year, the beginning of my study. Despite their absence in the institute, they kept on supporting me psychologically during my PhD study.

It is a pleasure to thank those co-wokers who participated in the described experiments. I owe my deepest gratitude to Chritoph P. Mauch for his great contribution in fear conditioned experiments. I thank Dr. Elmira Anderzhanova for her professional advice in establishment of microdialysis experiments, and Dr. Vincenzo Micale for his help with setting up the step-down avidance. Also thank Dr. Maik Dahlhoff for the analysis of western blotting, Roshan Naik for great assistance in gene sequence and rtPCR, as well as Irina Ionescu for partly conducting the intranasal application of NPS.

I am indebted to all of my colleagues to assist me in any respect of the lab work. Especially thank Dr. Ludwig Czibere for his unconditional support and professional advice for my work. I would like to point out the helping hands of our technicians Marina Zimbelmann and Mukus Nuβbaumer. Thank Marina for her help with HPLC injection and Mukus for breeding and providing HAB/LAB mice.

Special thanks go to Carola Hetzel, a secretary of Max Planck Institute of Psychiatry. She is a superwoman in my mind, who always saves my life from any trouble to have a happy life in Gemany. Therefore, I would like to express my gratitude to her.

No man is isolated. I'd like to thank Christoph P. Mauch, Regina Widner and Kathrin Henes for their friendship and warm regards. Many thanks to my friends from the Taiwanese Student Association in Munich and the Chinese Church in Munich for their psychological support and greetings.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of my dissertation.

特別感謝我的家人，若不是有你們一路上對我的支持，並包容我不能常陪伴在你們左右，我不可能走到這麼遠完成我的夢想與學位，在異**鄉負笈求學的日子並非盡皆一帆風順，每逢挫折**，總會想起你們給我的愛與**溫暖，讓我又有勇氣的繼續接受挑戰**。謹將本論文獻予你們，我愛你們！

最後，感謝神的帶領與保守，一路上的經**歷，都是祢賜下的恩典**！

9. Curriculum vitae

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- Yen YC, Mauch CP, Dahlhoff M, Micale V, Bunck M, Sartori SB, Singewald N, Landgraf R, Wotjak CT. Increased levels of conditioned fear and avoidance behavior coincide with elevated phosphorylation of the protein kinase B (AKT) within the amygdala in a mouse model of extremes in trait anxiety (in revision).

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- Gonik M, Frank E, Keβler MS, Czamara D, Touma C, Bunck M, Yen YC, Pütz B, Holsboer F, Bettecken T, Landgraf R, Müller-Myhsok B, Czibere L. Endocrine stress response is linked to one specific locus on chromosome 3 in a mouse model of extremes in trait anxiety (2011, submitted).

- Slattery DA, Yen YC, Naik R, Finger BC, Czibere L, Fuechsl A, Reber SO, Elfving B, Maloumby R, Mathé AA, Calo G, Landgraf R, Wegener G, Neumann ID. The Neuropeptide S system contributes to the in-born anxiety phenotype of two animal models of high-anxiety (in preparation).

- Micale V, Marsch R, Pamplona FA, Yen YC, Drago F, Landgraf R, Holsboer F, Wotjak CT. Extinction of avoidance behavior by safety learning depends on endocannabinoid signaling (in preparation).

- Kleinknecht K, Bedenk B, Kaltwasser SF, Yen YC, Czisch M, Wotjak CT. Flexibility of spatial learning requires hippocampus-dependent allocentric navigation (in preparation).

Declaration / Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet. Auch habe ich nicht anderweitig versucht eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

München, 04 August 2011

Yi-Chun Yen