Interaction of genetic predisposition and epigenetic factors in the development of anxiety

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Erster Gutachter:	Prof. Rainer Landgraf
Zweiter Gutachter:	Prof. Gisela Grupe

Angst haben wir alle. Der Unterschied liegt in der Frage wovor.

Frank Thiess († 22. Dezember 1977)

……Dedicated to the ones that I love

Abstract

It is becoming increasingly clear by current research that the continuum of physiological anxiety up to psychopathology is not merely dependent on genes, but is orchestrated by the interplay of genetic predisposition, gene x environment and epigenetic interactions. To consider this interplay, we here took advantage of the rigid genetic predisposition of a selectively bred mouse model exhibiting high anxiety-related behavior (HAB) and tested whether and how enriched environment, a manipulation of housing conditions, is capable of rescuing the genetically driven high anxiety phenotype via gene x environment and/or epigenetic interactions. Indeed, enriched environment exerts a significant anxiolytic effect on HABs of both sexes indicating for the first time that even a rigid genetic predisposition of high anxiety can be rescued by beneficial environmental stimuli. Thereby, a reduced neophobia and a bigger behavioral repertoire of HABs (e.g. social interactions) have been observed with a stronger anxiolysis in males than in females. The behavioral shift is accompanied by an attenuated release of corticosterone after application of a mild stressor. A hyperreactive hypothalamic-pituitary-adrenal (HPA) axis and amygdala constitute the most common symptoms of anxiety disorders, and decreased corticosterone release seems to entail a reduced release of noradrenaline from locus caeruleus (LC) to the medial prefrontal cortex (mPFC), thereby increasing the top-down control of mPFC on amygdala. This would entail less activation of amygdala and thus HPA axis, a consequence we indeed can observe as decreased neuronal activity flow through the amygdala of enriched housed (EE) compared to standard housed (SE) HABs. We suggest that corticotropin-releasing hormone receptor 1 (*Crhr1*) is critically involved in this phenomenon since (i) HABs compared to low anxiety-related behavior (LAB) mice exhibit higher *Crhr1* mRNA in the basolateral amygdala (BLA), (ii) this overexpression can be significantly reduced when HABs are housed in enriched environment and (iii) a bilateral application of a CRHR1 antagonist in the BLA of SE HABs induced a significant anxiolytic effect. Subsequent pyrosequencing identified that enriched environment increased methylation at a CpG site in the promoter of *Crhr1*, which is located next to a transcription factor binding site (TFB) of the epigenetic transcription factor Yin Yang 1 (*YY1*), whose mRNA levels are indeed decreased in EE HABs. *In silico* analysis identified Nr4a1 and D3Ertd300e as critical co-transcription factors, whereas Nr4a1 seems to be regulated by the quantity of available glucocorticoid receptor (GR) and D3Ertd300e positively regulates YY1. Thus, we hypothesize that reduced corticosterone release decreases the availability and thus binding of corticosterone to GR in the BLA. This, in turn decreases the binding affinity of Nr4a1 to D3Ertd300e, which then cannot positively regulate YY1 to decrease or even prevent methylation at the identified CpG site of *Crhr1*. This would finally result in a differentially methylated region (DMR) with higher methylation levels in EE HABs, which underlies the observed gene expression differences. The identified DMR might therefore be used as a biomarker for high or pathological anxiety. This hypothesized mechanism highlights the possibility that even a rigid genetic predisposition modeling pathological anxiety might be rescued by an epigenetic process that seems to be triggered by beneficial environmental stimuli, thereby raising the exciting possibility for new treatment strategies, which can be utilized complementary to already existing ones.

Table of contents

List of abbreviations

5caC	5-carboxylcytosine
5fC	5-formlycytosine
5hMC	5-hydroxymethylcytosine
5mC	5-methycytosine
ABN	arched-back nursing
ACTH	adrenocorticotropic hormone
AMP	adenosine monophosphate
ANOVA	analysis of variance
ANS	autonomous nervous system
APS	adenosine phosphosulfate
ATP	adenosine triphosphate
AVP	arginine vasopressin
B1R	β1-adrenoceptor
BCA	bicinchoninic acid
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
cDNA	complementary DNA
CA	cornu ammonis
CeA	central amygdala
CG	cingulated cortex
CGi	CpG island
CNS	central nervous system
CNV	copy number variant
CP	crossing point
CRH	corticotropin-releasing factor (CRF) or hormone
Crhr1/2	corticotropin-releasing hormone receptor 1/2
Dbh	dopamine beta hydroxylase
DC	total distance travelled in the inner zone of open field
DG	dentate gyrus
DMR	differentially methylated region
DNMT	DNA methyltransferase
DNMTi	DNA methyltransferase inhibtor
DR	dorsal raphe nuclei
DSM-IV	Diagnostic and statistical manual IV
dNTP	deoxynucleoside triphosphate
EC	total entries in the inner zone of open field
ECL	enhanced chemiluminescence
EE	enriched environment
EESE	animals tested for transgenerational inheritance
ELISA	enzyme-linked immunosorbent assay
EP	elevated platform
EPM	elevated plus maze

FST	forced swim test
GAD	generalized anxiety disorder
GxE	gene x environment
GR	glucocorticoid receptor
HAB	high anxiety-related behavior
HC	hippocampus
HCA	home cage activity
HDAC 1/2	histone deacetylase 1/2
HDACi	histone deacetylase inhibtor
HPA axis	hypothalamic-pituitary-adrenal axis
HPG axis	hypothalamic-pituitary-gonadal axis
HRP	horseradish peroxidase
HT	hypothalamus
ICD10	International classification of diseases 10
IP	intraperitoneally
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KO	knock-out
KWA	Kruskal-Wallis ANOVA
LAB	low anxiety-related behavior
LB	lysogeny broth
LC	locus caeruleus (formerly denoted as locus coeruleus)
LCe	latency to enter the inner zone of open field
LD	light dark box
LG	licking/grooming
MA	medial amygdala
MAT	methionine adenosyltransferase
MBP	methyl binding protein
MBD1/2	methyl CpG binding domain protein 1/2
MeCP2	methyl CpG binding protein 2
mPFC	medial prefrontal cortex
MR	mineralocorticoid receptor
MWU	Mann Whitney-U test
NA/NE	noradrenaline/ norepinephrine
NAB	normal anxiety-related behavior
Npsr1	neuropeptid S receptor 1
NPY	neuropeptide Y
NR	non-responder
NTS	nucleus tractus solitarii
OF	open field
PD	panic disorder
PLB	protein loading buffer
PND	postnatal day
NGFI-A	nerve growth-inducible factor A
PLB	protein loading buffer

PND	postnatal day
POMC	proopiomelanocortin
PPi	pyrophosphate
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus of the hypothalamus
OCD	obsessive compulsive disorder
OF	open field
O/N	overnight
qPCR	quantitative real-time PCR
RIA	radio immunoassay
RNAi	RNA interference
RT	room temperature
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydroxylase
SAM	sympatho-adrenergic system
SAMe	S-adenosylmethionine
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard environment
SIH	stress-induced hyperthermia
SNP	single nucleotide polymorphism
SNRI	selective noradrenaline reuptake inhibitor
ssDNA	single-stranded DNA
SSRI	selective serotonin reuptake inhibitor
SRT	stress reactivity test
T_1	body temperature measured during SIH before stress
T_2	body temperature measured during SIH after stress
TC	percent time spent in the center of open field
TF	transcription factor
TFB	transcription factor binding site
TBST	Tris-buffered saline with 0.1% Tween
TDG	thymine-DNA-glycosylase
Tet family	Ten eleven translocation family of proteins
Tmem132d	transmembrane protein 132d
TSS	transcription start site
TST	tail suspension test
UCMS	unpredictable chronic mild stress
Ucn	urocortin
USV	ultrasonic vocalization
VSDI	voltage-sensitive dye imaging
WB	western blot
YY1	Yin Yang 1

1.1 Fear, anxiety and the stress response

Evolution gave rise to at least two distinct behavioral systems, which assess emotion and originate from a common background: fear and anxiety (Flanelly et al, 2007). An increasing body of literature suggests the independence of both systems due to different neurological, hormonal, peptidergic and genetic underpinnings. Fear constitutes a reaction to an immediate threat that inherently endangers the individuals live - like a sudden sound, which might indicate the presence of a predator. These aversive stimuli are unconditioned, i.e. the animal shows an immediate innate "fight-or-flight" response to these discrete and specific stimuli. Contrary to fear, anxiety is a prolonged state of negative emotions to stimuli associated with an elevated potential risk of danger (e.g. open spaces and unfamiliarity) elicited by unavoidable stimuli characterized by the same stimulus features like stress - unpredictability and uncontrollability. Thus, fear represents a brief response to stimuli critical for survival and is mainly mediated by the amygdala, whereas anxiety is a prolonged reaction to a variety of stimuli characterized by uncertainty, which is primarily carried by the bed nucleus of the stria terminalis (BNST) and the hypothalamic-pituitary-adrenal (HPA) axis (Depue et al, 2009).

Though fear and anxiety serve different purposes, both are capable of triggering the body's stress response by sensing potentially adverse changes in the environment denoted as stress. This in turn, modifies neuronal activity and thus, behavior rapidly and enduringly in response to life-threatening stimuli, achieved through an evolutionary conserved and highly complex modulation of neuronal functioning at several levels of the central nervous system (CNS) (Joels et al, 2009). The pattern and magnitude of this complex stress response depends on "[…] the duration of stress exposure (acute versus chronic), the type of stress (physical versus psychological), the stress context (for example, time of day), the developmental stage of the animal (newborn, adult or aged), the animal's sex and genetic background" (Joels et al, 2009). The stress response *per se* comprises two waves of stress mediator actions, which are separated in most instances temporarily and spatially. The acute effects of stress take place within a time frame of seconds to minutes and are carried by monoamines like noradrenaline (NA), dopamine or serotonin as well as neuropeptides like corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). These rapid stress mediators promote appraisal of the situation, alertness, vigilance and the choice of an optimal strategy to face the situation. These actions quickly subside due to the short availability of their mediators and for this reason, the second wave of stress mediators comprises steroids like cortisol (humans) and CORT (rodents) which act within hours to days primarily upon glucocorticoid receptors (GRs) and thus facilitate the consolidation of information associated with the stressor (i.e. memory) (Joels et al, 2009). These two waves of stress mediators are carried by two different systems working complementary to guarantee an optimal function: the sympatho-adrenomedullary (SAM) system one the one and the HPA axis on the other hand.

1.2 Maintenance of homeostasis

The fast-acting SAM system is predominantly activated by fear and orchestrates the immediate "fight-or-flight" response by secreting monoamines and neuropeptides compared to the HPA axis, which finally causes the secretion of steroids to moderate the adaptive long term response that can't be controlled by the "fight-or-flight" mechanism (fig. 1). Both mechanisms aim to maintain or reinstate homeostasis during stress.

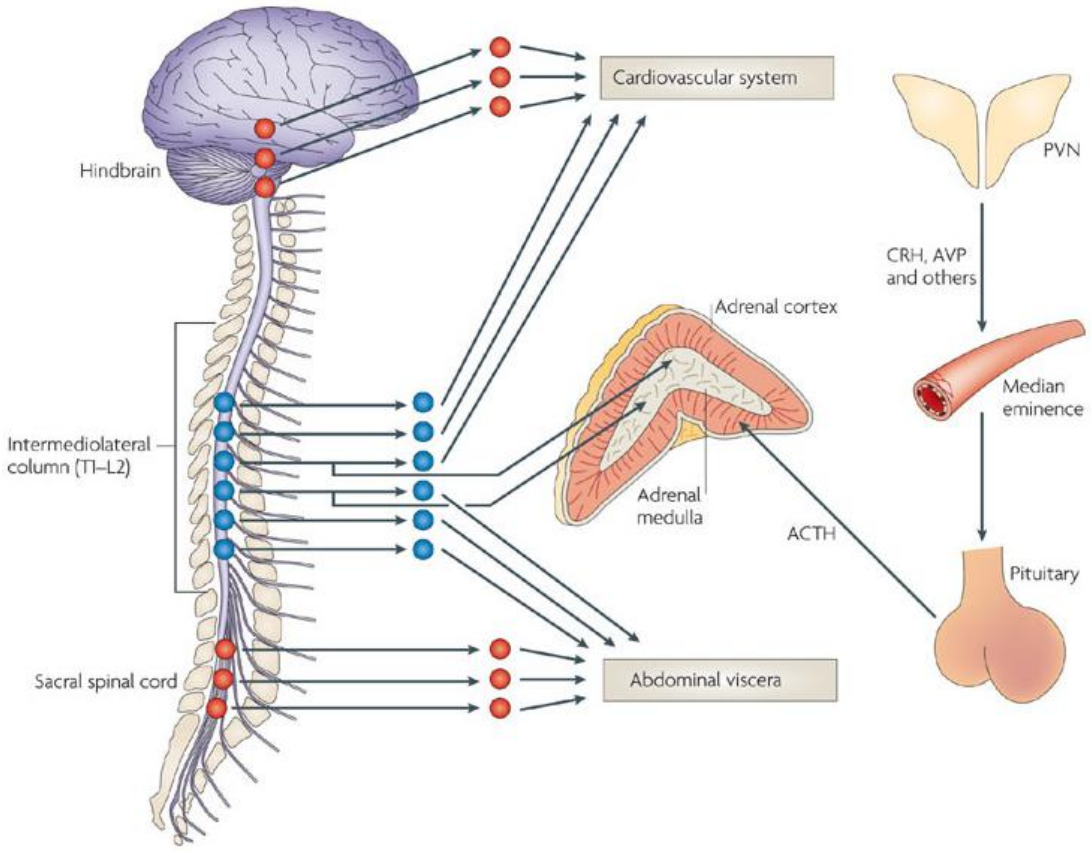


Fig. 1: Stress exposure activates the rapid SAM system (NA) and a prolonged stress response via the HPA axis, which releases a series of neuropeptides entailing the secretion of cortisol (humans) or CORT (rodents) from the adrenal cortex. Blue and red dots depict the sympathetic and parasympathetic nervous system, respectively. adrenocorticotropic hormone (ACTH), noradrenaline (NA), paraventricular nucleus of the hypothalamus (PVN). Picture adopted from Ulrich-Lai and Hermann (2009).

Every individual maintains its inner environment (e.g. temperature, pH) in an intricate equilibrium denoted as homeostasis. External threats or stimuli (i.e. stressors) aim to compromise this equilibrium every time an animal encounters a (potentially) live-threatening situation that ensues fear or anxiety, which in turn activates the SAM system and HPA-axis. Both neuroendocrine protection systems maintain or reinstate the homeostasis to a new point of equilibrium, which enables the animal to cope optimally with the encountered situation. (Engelmann et al, 2004)

Upon stress, the fast-acting SAM system activates the chromaffin cells of the adrenal medulla via a series of preganglionic sympathetic neurons and paravertebral ganglia (fig. 1,

shown in blue; Ulrich-Lai and Hermann, 2009). As a consequence, heart rate, vasoconstriction, respiratory rate and energy mobilization are increased to ensure that the animal is able to escape the situation quickly and actively (Engelmann et al, 2004; Ulrich-Lai and Hermann, 2009).

With a higher latency of ca. 2-3min., the HPA axis activates parvocellular neurons in the paraventricular nucleus of the hypothalamus (PVN), which secrete the hypophysiotrophic hormones CRH and AVP from axon terminals in the *Zona externa* of the median eminence in the hypothalamo-pituitary portal circulation and further to the adenohypophysis. These releasing hormones in turn, promote the synthesis and processing of proopiomelanocortin (POMC) to adrenocorticotropic hormone (ACTH), which travels via the blood stream to its effector organ - the adrenal cortex. Finally, glucocorticoid hormones, i.e. cortisol (humans) or CORT (rodents) are synthesized and released from the *Zona fasciculata* of the adrenal cortex into the blood stream (fig. 2) (de Kloet et al, 2005; Engelmann et al, 2004; Ulrich-Lai and Hermann, 2009).

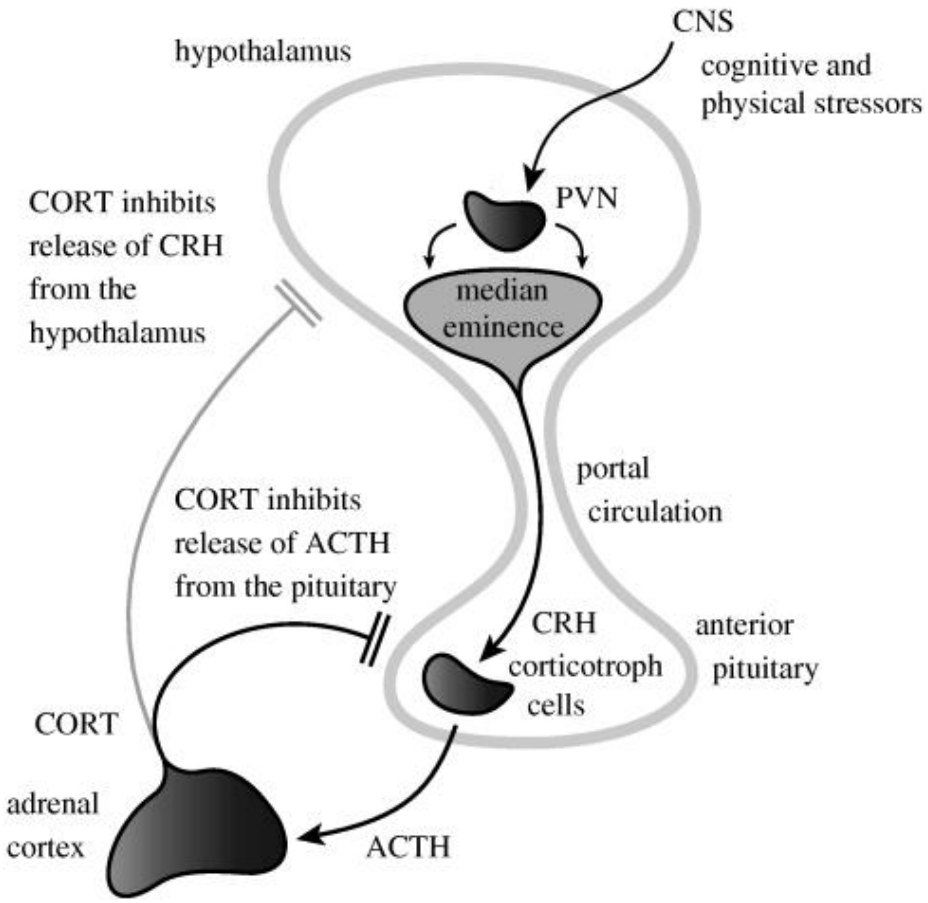


Fig. 2: Stress exposure activates the HPA axis by releasing CRH from the PVN, which in turn secretes ACTH into the blood stream to finally cause a release of cortisol (humans) or corticosterone (rodents) from the adrenal cortex. Picture adapted and modified from Walker et al (2010).

Circulating glucocorticoids then promote the mobilization of stored energy by lipolysis and gluconeogenesis, inhibition of growth and reproductive systems and induce behavioral changes like suppression of feeding, increased arousal, vigilance and cognition and

potentiate numerous sympathetically mediated effects, such as peripheral vasoconstriction (Charmandari et al, 2005; Engelmann et al, 2004; Ulrich-Lai and Herrmann, 2009). Moreover, direct innervation of the adrenal cortex by the sympathetic nervous system can regulate corticosteroid release highlighting the largely complementary actions that the SAM system and the HPA-axis have during stress (Ulrich-Lai and Herrmann, 2009).
When the animal has successfully escaped the live-threatening situation or the stressor, it is of highest importance to end the body's stress response and restore homeostasis to the pre-stress point of equilibrium. This is achieved via a negative feedback mechanism, which is tightly regulated by mineralglucocorticoid (MR) and glucocorticoid (GR) receptors. MRs are primarily localized in the hippocampus (HC), lateral septum and medial amygdala (MA), whereas GRs are mainly expressed in the PVN (Joels et al, 2004). Both receptors are expressed in the cytosol during their inactive state, each together with a complex assembly of heat shock proteins including HSP90 and members of the Fkbp5 family. The negative feedback of the HPA axis is achieved through a difference in binding affinity of glucocorticoids with MRs exhibiting a tenfold higher affinity compared to GRs. Under basal conditions, glucocorticoids diffuse passively through the phospholipid membrane to occupy MRs, whereat a stressor drastically increases glucocorticoid availability and thus, entails GR occupancy and transduction of the ligand-activated receptors from the cytosol to the nucleus to bind to glucocorticoid response elements to up- or downregulate the expression of various genes (Charmandari et al, 2005; de Kloet et al, 2005; Pariante and Miller, 2001). Thereby, glucocorticoids regulate their own production via a negative feedback loop, which inhibits the synthesis of ACTH and CRH at the level of the pituitary or HC and hypothalamus (HT), respectively (Raison and Miller, 2003).
To summarize, a stressor challenges the homeostasis of an animal facing a live-threatening situation. This encounter causes fear or anxiety, which in turn leads to the activation of two complementary systems denoted as SAM and HPA axis to adjust the equilibrium to a new set-point to maximize the chance of survival by promoting energy mobilization as well as arousal, vigilance and cognition. After successful escape, the stress response is terminated by a negative feedback mechanism at the level of PVN, HC and HT by utilizing the low affinity of GRs to glucocorticoids. Two members of the stress response, the amygdala and the release of CRH as well as the availability of the associated receptors, play an outstanding role in the regulation of anxiety and in the etiology of psychiatric disorders.

1.3 CRH system and the critical role of the amygdala

The role of CRH in the cause and consequences of anxiety, depression and chronic stress have been extensively documented and thus, may be a main pathway through which the effects of stress can shape brain development (Andrus et al., 2012; Arborelius et al, 1999, Dunn et al, 1990). Chang and Hsu (2004) propose a clear evolutionary trail for the origin of the CRH/CRH receptor system. A coevolution gave rise to diuretic hormone and its associated receptors in insects and CRH/CRH receptors in vertebrates. The latter gained the regulation of the stress response to environmental stimuli early during their evolution and exactly this regulatory mechanism of the CRH system emphasizes its importance in anxiety and may explain its homology among a huge class of species.

The CRH system comprises a family of peptides on the one and two types of receptors on the other hand. The CRH family of peptides includes CRH, urocortin (Ucn) as well as Ucn 2 and 3, which are distinct from the CRH and Ucn. Among these peptides, CRH is a 41 amino-acid neuropeptide, which is expressed peripherally (e.g. blood vessels, skin) and centrally (e.g. amygdala) underpinning its critical role in integrating the activity of diverse physiological systems by coordinating the behavioral, neuro-endocrine, and autonomic responses to stress. It acts in the brain through at least two different receptor subtypes, referred to as R1 (Crhr1) and R2 (Crhr2) (Binder and Nemeroff, 2010; Heinrichs et al, 1995; Rotzinger et al, 2010; Vale et al, 1981). These two receptors share approximately 70% identity on the amino acid level but exhibit significant differences at the N-terminal extracellular domain, which might account for their agonist selectivity. Fig. 3 depicts the binding affinities of the two receptor types R1 and R2 for the different peptides of the CRH family. R1 can merely bind CRH (also denoted as CRF) as well as Ucn and seems to be implicated in the stress-producing effects of CRH (Chen et al, 1993; Hsu and Hsueh, 2001; Lewis et al., 2001; Lovenberg et al, 1995; Reyes et al, 2001; Rotzinger et al, 2010; Vaughan et al, 1995). R2 binds all Ucns with significantly higher affinity in relation to CRH indicating that these peptides might be its natural ligands. Moreover, R2 is involved primarily in functions not related to anxiety or depression, e.g. suppression of feeding or decreasing blood pressure (Dautzenberg et al, 2002; Grammatopoulos et al, 2012).

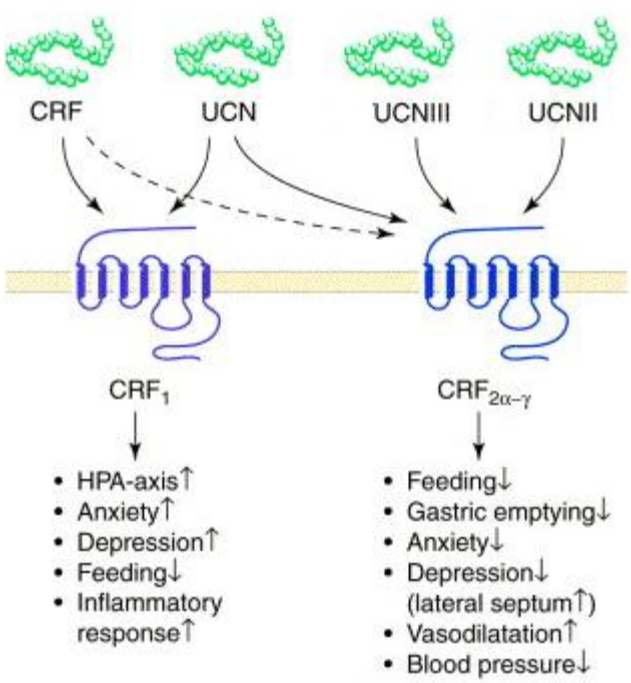


Fig. 3: The two different receptors of CRH, denoted as R1 and R2 possess different affinities for the peptides of the CRH family. Figure adapted and modified from Dautzenberg et al (2002).

The importance of the CRH system is further corroborated by studies that have shown recently that CRH is not only released after stress from axonal terminals of the median eminence but is additionally expressed in neuronal populations of the amygdala (Koob, 2008; Swanson et al, 1983), HC (Chen et al, 2001) and locus caeruleus (LC, earlier referred to as locus coeruleus) (Joels et al, 2009; Valentino and Bockstaele, 2008). CRH acts locally and exerts its neuromodulatory effects within seconds after its release to modify neuronal firing patterns (Aldenhoff et al, 1983; Baram and Hatalski, 1998; Gallagher et al, 2008), gene expression and behavior (Bale et al, 2000; Chen et al, 2006; Coste et al, 2000; Koob, 2008; Muller et al. 2003). Joels et al. (2009) propose the existence of strategic hubs denoted as “hot spots”, where different stress mediator receptors are expressed and networks, involved in different aspects of the stress response, are connected: the prefrontal cortex (PFC), amygdala, HC

and LC constitute such a network and strengthen the concept of spatial convergence of action (Bloom, 1984) (fig. 4).

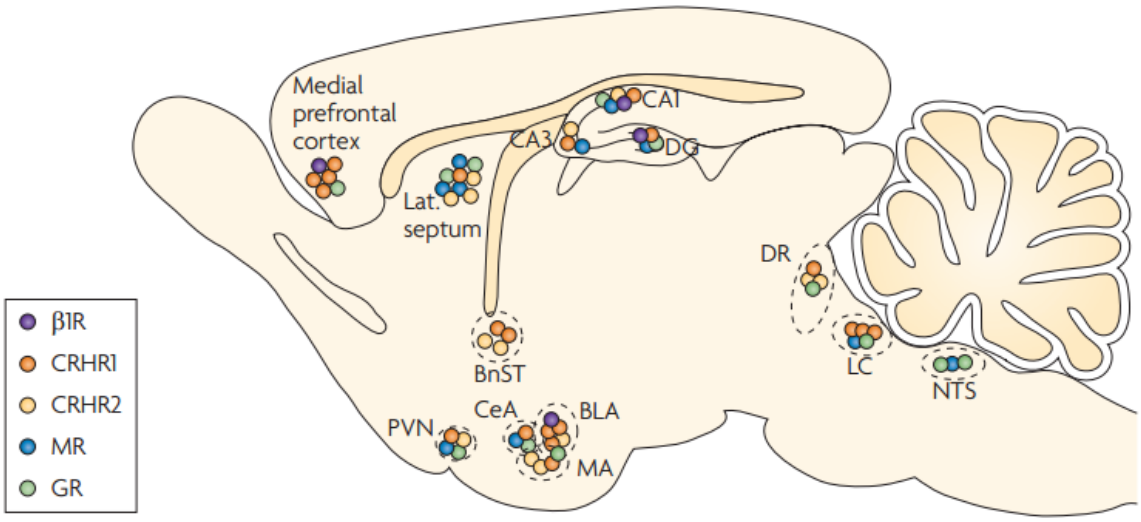


Fig. 4: The receptors MR, GR, Crhr1 and 2 as well as the β1-adrenoceptor for noradrenaline cluster in hot spots of the brain, where at least two of the former receptors are highly expressed to orchestrate different aspects of the stress response including decision making, learning and memory and autonomic and emotional responses. β1-adrenoceptor (β1R), basolateral amygdala (BLA), cornu ammonis 1/3 (CA1/3), dentate gyrus (DG), dorsal raphe nuclei (DR), medial amygdala (MA), nucleus tractus soltarii (NTS). Picture adopted from Joels et al. (2009).

The presence of monoamine (β1R), neuropeptide (Crhr1/2) and glucocorticoid receptors (MR/GR) within the three major nuclei of the amygdala (CeA, MA, BLA) emphasizes its role in anxiety and stress regulation. The amygdala seems to exhibit two main functions: first, to detect and evaluate the salience, significance, ambiguity and unpredictability of stimuli of biological relevance and to link these stimuli to current estimates of biological value (Davis and Whalen, 2001; Pessoa, 2010; Pessoa and Adolphs, 2011; Whalen, 1998). In other words, the amygdala separates the significant from the mundane by establishing affective significance by highlighting some stimuli so as to receive additional processing by other brain regions, while at the same time other stimuli are deemphasized or discarded (Pessoa, 2010). Neuroimaging studies conducted in humans have confirmed preclinical experiments performed in rodents and non-human primates: "[…] the amygdala responds to negative as well as positively valenced stimuli (Breiter et al., 1996; Somerville et al., 2004; Hennenlotter et al., 2005), suggesting it supports learning about the emotional significance of the environment in general" (Tottenham and Sheridan, 2010). Thus, the amygdala constitutes a brain region to determine the relative safety or danger of a situation, which is especially important during early life when the need to evaluate danger of novel events will be greater (Tottenham et al. 2009a). Indeed, the amygdala has a mostly modulatory role in a wide array of networks and thus, is extensively interconnected with cortical and subcortical structures like the ventral subiculum (vSub), LC, PFC and HC (Pessoa and Adolphs, 2011).

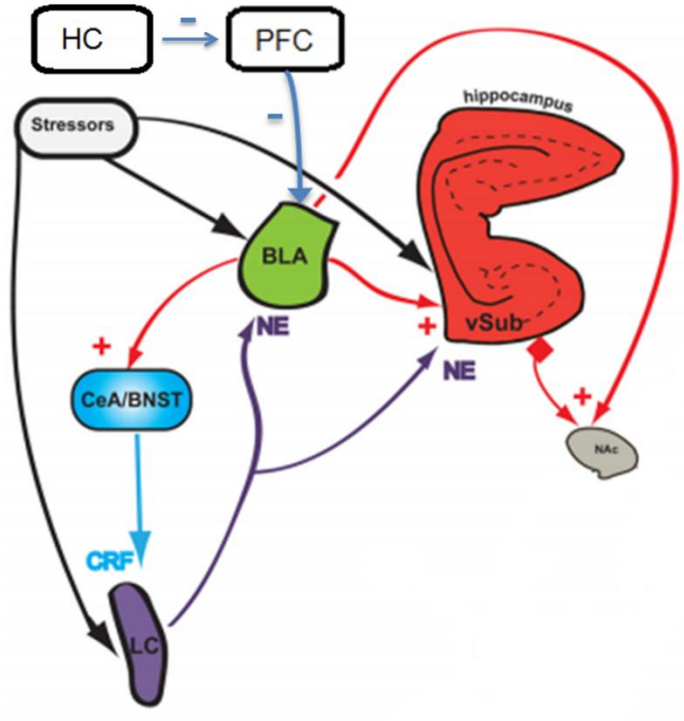


Fig. 5: The amygdala allocates processing resources to stimuli by modulating brain structures required to prioritize particular features of information processing in a given situation. Figure adapted and modified from Belujon and Grace (2011).

Fig. 5 depicts the extensive interconnectedness of the amygdala: the mPFC inhibits activation of BLA under basal conditions, whereas stressful conditions relieve BLA from cortical inhibition via dopaminergic neurotransmission (Kröner et al., 2005; Rosenkranz and Grace, 1999). This relieve initiates the HPA cascade in the amygdala, where cells within in the amygdala are quickly activated by stress and participate in the earliest reaction to environmental stressors (Honkaniemi et al., 1992). The BLA sends excitatory inputs into CeA, which in turn activates LC NA release via CRH (Belujon and Grace, 2011). Furthermore, LC and BLA have reciprocal connections by which LC can modulate BLA activity via α- and β-adrenergic receptors to allow the integration of the stress mediators NA and CRH (Belujon and Grace, 2011; Joels et al, 2009). Hippocampally-mediated memory formation can be influenced by the amygdala and the hippocampus *per se* influences the response of the amygdala when emotional stimuli are encountered (Phelps, 2004). Thereby, mPFC, amygdala and HC coordinate their actions during emotional learning: HC inhibits mPFC in new environmental contexts, followed by a release of the amygdala from mPFC inhibition (Kim and Richardson, 2010; Tottenham and Sheridan, 2010). After cessation of the stressful situation, BLA and LC modify vSub activity, which is thought to participate in the decrease of HPA axis activity (Lowry, 2002). Thus, the amygdala allocates processing resources to stimuli by modulating brain structures required to prioritize particular features of information processing in a given situation (Pessoa and Adolphs, 2011) and is not surprising that "[…] stress-induced changes in the amygdala may have downstream effects on the HPA axis that over time can change the structure and function of later stages in the axis" (Brunson et al., 2001b; Tottenham and Sheridan, 2010). It is now well established that susceptibility to psychiatric disorders is due to the combined effects of genetic, environmental and epigenetic factors. The CRH system, with the amygdala being a brain region of major importance and involvement, may serve as an epigenetic key interface between environmental stressors and the etiology of psychiatric disorders (Binder and Nemeroff, 2010). Genetic predisposition might lead to variation in an individual's response

to stressful events and as a consequence, both, the genetic load and epigenetic factors participate in the onset of anxiety disorders or depression.

1.4 Epigenetics - the missing link in psychopathology?

The last two decades of research gradually shifted the view of complex disease etiology from genocentric towards gene x environment (GxE) interactions by recognizing the importance of environmental and epigenetic mechanisms (Jaenisch and Bird, 2003; Rutter, 2006). "DNA is no more considered to be the master blueprint […] operating […] in an ecological vacuum. Rather, DNA outlines the overall adaptive potential of an organism through broadly outlined […] physical and behavior dispositions which serve as building material for the final phenotypic outcome in response to specific environmental stimuli" (Templeton 2006). Indeed, accumulating evidence suggests that epigenetics constitutes one of the main and previously missing links among genetics, environment and disease (Barros and Offenbacher, 2009). We refer to epigenetics as heritable processes that regulate the activity status of at least one gene by molecular factors and processes without altering DNA sequence (Skinner et al, 2010; Svrakic et al, 2010). These processes include DNA methylation, posttranslational modifications of histone tails and RNA interference (RNAi). Though the chronology and grade of interconnectedness of these mechanisms is still a matter of debate, it is increasingly accepted that these processes are not independent of each other (Bossdorf et al, 2008). They finally ensue inaccessibility of genes for the transcription machinery via a condensed chromatin structure (Szyf et al, 2008) enabling organisms to integrate environmental signals into their genome (Murgatroyd et al, 2009).
DNA methylation is one major epigenetic research subject (Barros and Offenbacher, 2009) and has been linked *inter alia* to:

Function	Reference(s)
alternative splicing	Shukla et al. (2011)
cellular differentiation	Illingworth and Bird (2009)
genomic imprinting	Sasaki and Matsui (2008)
genomic stability	Antequera (2003)
inactivation of alternative promoters	Illingworth and Bird (2009)
regulation of gene expression	Cedar and Bergmann (2009) Suzuki and Bird (2008)
silencing of molecular parasites	Antequera (2003)
transgenerational transmission	Jablonka et al. (2009)
X-chromosome inactivation	Sasaki and Matsui (2008)

Tab. 1: Summary of functions thought to be fulfilled by DNA methylation. Functions of particular interest for this thesis are highlighted in red.

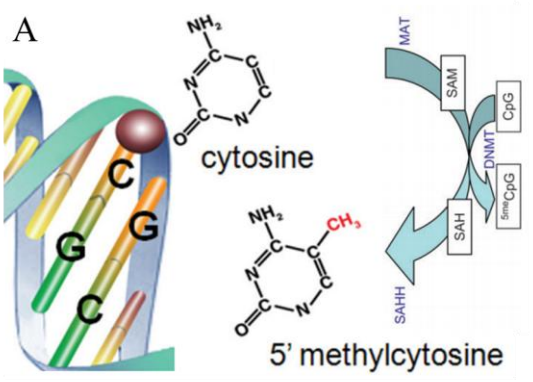
In mammals, DNA methylation predominantly occurs in CpG dinucleotides located within a region denoted as CpG island (CGi). Takai and Jones (2003) defined a CGi as a non-random distribution of methylated CpGs, which encompasses a region of ≥500bp with a

G+C content ≥55% and an observed over expected CpG ratio of ≥65% (tab. 2). This definition has been proven as the most efficient and reliable in silico analysis tool until nowadays (Zhan and Han, 2009).

Feature	CGi	Bulk DNA
G+C content	≥55%	20-25%
CpG [observed/expected]	≥65%	20-21%
methylated	no	yes
transcriptionally active	yes	no
chromatin structure	open	closed
associated with TSSs	yes	no

Tab. 2: Comparison of features of CpG islands (CGis) and bulk DNA in mammals. Transcription start site (TSS). Data adapted from Antequera, 2003; Lander et al, 2001.

Mazzio and Soliman (2012) summarize the mechanisms that are thought to cause epigenetic silencing of a gene: the enzyme DNA methyltransferase (DNMT) adds methyl groups from the methyl donor S-adenosylmethionine to the 5' carbon atom of cytosines (fig. 6A). This in turn, attracts methyl binding proteins (MBP) like methyl CpG binding domain protein (MBD) 1, MBD2 and methyl CpG binding protein 2 (MeCP2), which act as docking stations for potent repressor complexes. These complexes comprise co-repressors (e.g. Sin3, N-CoR, Mi-Nu2-NuRD) and histone deacetylases (HDACs, e.g. HDAC1/2), which alter histone stability and nucleosome positioning by controlling modifications of H3 and H4 histone tails. These histones become deacetlyated and histone cores are exchanged by more stable variants (e.g. H2A replaced by H2ABbd) to prevent nucleosomal ejection/displacement. These processes are accompanied by stabilization of the linker histone H1 via the proteins HP1α/β. These proteins tether further silencing elements to tightly crowd methylated DNA to nucleosomes. Finally, lamins position heterochromatin along the nuclear envelope, which causes permanent silencing of a gene (fig. 6B).



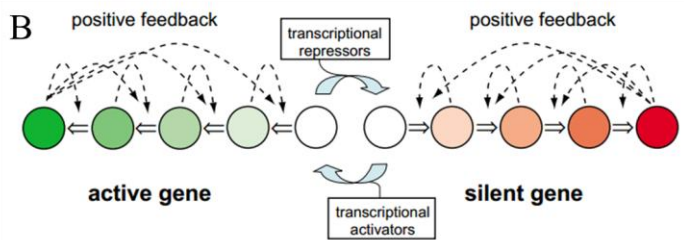


Fig. 6: The enzyme DNA methyltransferase (DNMT) transfers a CH3 group from S-adenosylmethionine (SAMe) to cytosines (A), thereby initiating a cascade, which finally silences gene expression (B). Methionine adenosyltransferase (MAT), S-adenosylhomocysteine (SAH), S-adenosylhomocysteine hydrolase (SAHH). Pictures adapted and modified from Barros et al. (2009), Foley et al. (2008), Jaenisch et al. (2003).

CGis seem to be located mainly at three different sites: most of them are associated with the promoter region of a gene, rendering them as potent targets for gene regulation. Besides the promoter region, CGis can be found in exons (often denoted as "gene body methylation"; Brenet et al, 2011) as well as in CpG shores (Doi et al, 2009). CpG shores are defined as regions within 2000bp of the TSS without being part of a CGi, explaining the term "shore".
The process of DNA methylation is highly dynamic and - of highest importance - reversible. The ten eleven translocation (Tet) family of proteins is capable to stepwise oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), further to 5-formyl-cytosine (5fC) and finally to 5-carboxylcytosine (5CaC), which is then excised by thymine-DNA-glycosylase (TDG) and replaced by cytosine (He et al, 2011; Inoue et al, 2011). DNA methylation can occur during the whole lifespan of an individual and even exerts influence prenatally and transgenerationally, thereby possibly affecting the health of future generations (Jirtle and Skinner, 2007; for a review refer to Masterpasqua, 2009). The following examples will illustrate the potential and time frame in which DNA methylation can take place: Waterland and Jirtle (2003) found evidence that maternal diet (i.e. a prenatal influence) can alter DNA methylation in the offspring: the agouti allele in mice causes a yellow coat color, overweight and diabetes. If pregnant mothers carrying the agouti allele were provided food supplemented with folic acid (a methyl donor), the agouti allele becomes silenced via DNA methylation generating offspring with brown coat color, which is not overweight and not diabetic. The research group of Meaney provided the maybe most popular evidence that the early postnatal environment and the adjacent post-weaning environment can have a dramatic influence on the phenotype of the offspring and grand-offspring (Champagne and Meaney, 2007). Rodents exhibit natural variations in maternal care (Champagne et al, 2003) seen as high vs. low levels of licking/grooming (LG) and arched-back nursing (ABN) in the first week postpartum. Offspring, which received low compared to high levels of LG and ABN had lower levels of hippocampal GR expression and an increased HPA-response to stress. This seems to be caused by high methylation levels of the transcription factor binding site (TFB) of the nerve growth-inducible factor A (NGFI-A) within the GR gene, thereby sterically hindering binding of NGFI-A and thus, expression of GR. This TFB site is highly methylated already at birth and becomes increasingly demethylated when offspring receives high levels of maternal care, whereas low levels of LG and ABN do not alter methylation causing lower expression of GR and finally an increased reactivity of the HPA-axis (Weaver et al, 2009). It was shown that the phenotype of the offspring can be reversed by manipulating housing conditions of the offspring after weaning. When offspring of high and low LG/ABN mothers was raised in an enriched environment (EE, please refer to section 1.7) or isolation respectively, their phenotype was reversed to low and high LG/ABN respectively. Importantly, the phenotype altered by housing conditions was transmitted to the next generation (Champagne and Meaney, 2007), indicating "[…] that "good" environments […] can ameliorate "bad" epigenomes and "bad" environments (i.e., those provoking fear) can pathologize "good" epigenomes" (Svrakic et al, 2011).
The discovery of epigenetic mechanisms, which allow the transmission of traits to the next generation led to the concept of modern synthesis (Bard, 2011) emphasizing that the environment acts on genes to fine-tune and adapt the organism in the best way possible to the environment it is facing (Bonduriansky et al, 2009). An increasing body of literature corroborates the existence of traits that can be inherited transgenerationally: vinclozolin-induced transgenerational adult-onset disease in rats (Anway et al, 2005), transgenerational promotion of long-term potentiation by altered environment in mice (Arai et al, 2009), transgenerational inheritance of maternal care reversible by housing conditions

(Champagne and Meaney, 2007) and gender bias in multiple sclerosis following epigenetic changes in HLA class III risk haplotypes in humans (Chao et al, 2009).
The aforementioned examples highlight the possibility that DNA methylation can act throughout the whole lifetime and beyond. Fig. 7 depicts factors thought to influence the epigenetic profile and thus, methylation of an individual:

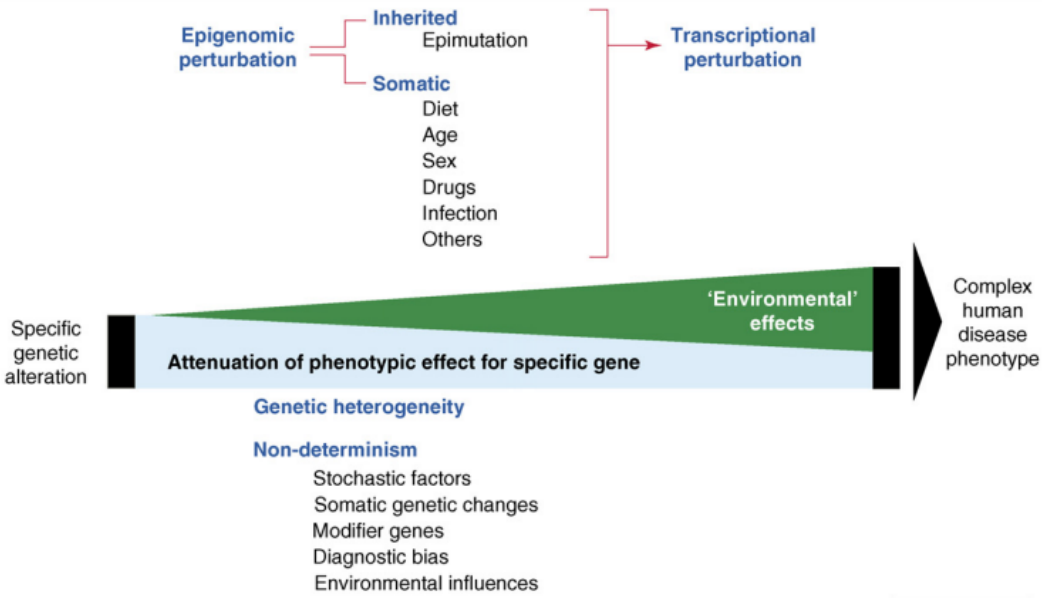


Fig. 7: At least three different categories of factors contribute to the etiology of complex human diseases: epigenetic factors, genetic heterogeneity and non-deterministic factors. Picture adapted from Hatchwell and Greally (2007).

Hatchwell and Greally (2007) suggest at least three categories of factors, which influence the phenotype of an individual: i) epigenetic factors like age, sex or drug use, ii) genetic heterogeneity comprising single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) and iii) non-deterministic factors including stochastic factors (e.g. developmental noise) and environmental influences.
Taken together, DNA methylation can activate or silence genes throughout the whole lifetime and beyond and is able to translate environmental influences into the genome. These features enable DNA methylation to crucially influence the phenotype of an individual to the better or the worse (Belsky et al, 2009), depending on the genetic predisposition and the environment the individual is living in and thus, makes it a promising candidate to identify new mechanisms contributing to the onset of anxiety disorders.

1.5 From normal to pathological anxiety

Mental illnesses, with anxiety disorders being the most common in Europe and the USA, constitute the leading cause of disability worldwide (Andlin-Sobocki et al, 2005; Kessler et al, 2005). A lifetime prevalence of 28.8%, the high comorbidity of anxiety with, amongst others, depression (Bateson et al, 2011), substance abuse and tobacco dependence (Thayer and Kuzawa, 2011; Tottenham and Sheridan, 2010) as well as a high rate of individuals responding only partially or not at all to the prescribed drug treatment might indicate why affected individuals suffer extremely (Bateson et al, 2011; Kessler et al, 2005). Thus, it is not surprising that both disorders rank among the most common and proliferating health problems in the world and cause direct (treatment) and indirect (e.g. absence to work) costs

in the range of hundreds of millions dollar worldwide (Johnston et al, 2009; Wong et al, 2001, 2004). Mental disorders are classified either via the "international classification of diseases" (ICD-10-GM2012) of the WHO or the "diagnostic and statistical manual" (DSM IV) of the American Psychiatric Association. Despite intense characterization, correct classification and subsequent treatment remains challenging due to overlapping symptoms (Svrakic et al., 2011) and patients not responding to treatment - either due to a wrong diagnosis or since they do not respond to the prescribed drug(s). Nowadays, six forms of pathological anxiety are described: social and simple phobia, panic disorder (PD), generalized anxiety disorder (GAD), obsessive compulsive disorders (OCD) and posttraumatic stress disorder (PTSD; ICD-10). The diagnostic and treatment difficulties highlight the multigenic and complex nature of anxiety, which is likely to be shaped by environmentally-driven plasticity at the genomic level. In other words, "a single gene may contribute additively and interchangeably to vulnerability […], but its contribution is neither necessary nor sufficient for manifesting the expression of the phenotype of […]" (Lee et al, 2005) an anxiety disorder.

Affected individuals show one to several behavioral, physiological and (epi-) genetic alterations absent in healthy persons with "normal" or average anxiety: persons suffering from anxiety disorders exhibit a bias to interpret harmless or neutral stimuli as rather dangerous (Kim and Gorman, 2005), a bias to favor a negative association when assessing the emotional quality of a situation (Landgraf, 2001) as well as a hyperreactive HPA-axis (Reul and Holsboer, 2002) and amygdala (Shin and Liberzon, 2010). A hyperreactive HPA-axis causes a prolonged release of glucocorticoids, which might damage brain regions important for the negative feedback mechanism (e.g. HIP) leading to a feed-forward mechanism that drives glucocorticoid synthesis indefinitely under the presence of ongoing stressors (Raison and Miller, 2003). Anxiety *per se* is protective in many settings, whereas an excessive form promotes disability. Pathological anxiety ensues a series of behavioral manifestations comprising phases of "excessive anxiety and worries" that are consistent over at least 6 months (DSM-IV). Disruption of sleep (hyper- or hyposomnia), weight gain or loss, withdrawal from usual activities, concentration problems, fatigue, feeling tensed or restless represent some, but by far not all, criteria (DSM-IV; Leonardo and Hen, 2006).

Despite intensive research over the last decades, a lot of questions remain merely partially or not answered at all: What are the factors contributing to the onset of pathological anxiety? Is pathological anxiety merely an extreme form of normal anxiety? And what does "normal" mean? Svrakic et al. (2011) propose the term adaption disorders instead of personality disorders since maladaptation might reflect the core deficit. A poor or deviant adaptation to the environment and not extreme behavioral traits (though extreme traits may have interfered with successful adaptation) seem to constitute the gist of the matter. Many researchers agree that extreme behavioral traits *per se* do not necessarily reflect a maladaption or an evolved dysfunction. These traits are operating functionally if they maximize survival and reproduction within a given environment, even if a mechanism is producing distress or impairs quality of life (Bateson et al, 2011; Sachser et al, 2011). Moreover, the term adaption disorders explicitly include the environment as an important source in the etiology of psychiatric disorders.

The crucial question is which mechanisms might be amenable and/or show a maladaption in pathological anxiety? In particular, evidence is provided that stress facilitates or causes a dysregulation of the endocrine system (Elizalde et. al, 2010; Toth et al, 2008) rendering regulation of the HPA-axis as a promising candidate. Myriads of papers indicate that psychiatric disorders indeed cause a severe dysregulation of the HPA-axis, which seems to be mediated, at least partially, by the CRH/CRH receptor system. CRH is the major

regulator of HPA-axis activity and alterations in CNS CRH-containing neuronal circuits are implicated in the pathopsychology of anxiety and depression (Binder and Nemeroff, 2010; Mathew et al, 2008; Reul and Holsboer, 2002, Risbrough and Stein, 2006). Patients suffering from psychiatric disorders show - in most cases - a hyperreactive HPA-axis likely due to a hyperreactive central CRH system (Reul and Holsboer, 2002).
In addition, patients suffering from anxiety disorders (PTSD, PD, GAD, specific and social phobia) often show a hyperreactive amygdala likely due to a decreased inhibition of mPFC (Shin and Liberzon, 2010) as well as a dysregulation of monoamine systems in limbic structures (Elizalde et. al, 2010; Toth et al, 2008) highlighting amygdala as another potential candidate brain region important for pathological anxiety (Tottenham and Sheridan, 2010). LC and amygdala are closely connected (Itoi and Sugimoto, 2010) and the CRH system innervates both brain regions, which in turn activate the SAM system and CNS noradrenergic production (Valentino et al, 1983). Increased fear and alertness, responses relevant for the fight-or-flight reaction are associated with the release of CRH in limbic brain regions, which is mainly mediated by CRHR1 and is dysregulated in depression and anxiety disorders (Arborelious et al, 1999; Heinrichs et al, 1997; Nemeroff, 2009; Reul and Holsboer, 2002).
Less synapses formed in the hippocampus (Bessa et al, 2009), a smaller hippocampal volume (Gilbertson et al., 2002) and a dysregulated negative feedback of the HPA-axis in patients suffering from anxiety disorders (Reul and Holsboer, 2002) qualify HIP as an additional candidate important for adaption processes and thus, regulation of anxiety. The negative feedback of the HPA-axis (i.e. termination of the stress response) is controlled by MRs and GRs in HIP and indeed, affected persons seem to have lower levels of GRs and a reduced neurogenesis in HIP (Elizalde et. al, 2010; Reul and Holsboer, 2002; Toth et al, 2008). The dysregulation of amygdala, HIP and the CRH system in affected individuals offers the possibility that HPA-axis dysregulation can occur at several stages or levels.
Research has begun to identify environmental conditions, which are likely to be associated and/or contribute to the onset of psychiatric disorders. An increasing number of researchers emphasize the existence of genetically influenced individual variations in exposure to risky or protective environments (Jaffe and Price, 2007; Kendler and Baker, 2007). "With respect to depression and anxiety, the key focus is on the fact that environments are not randomly distributed. Social selection means that there needs to be a concern regarding the origins of risk environments as well as focus on their effects" (Rutter, 2010). This hypothesis is strengthened by the fact that an individual's social position is closely related to the amount of perceived psychosocial stress with a low social position linked to an increased amount of psychosocial stress (Thayer and Kuzawa, 2011). This increase in perceived stress further impacts on blood pressure, stress hormone metabolism and immune function (Bindon et al, 1997; Flinn and England, 1997). Recent animal studies have begun to unravel first mechanisms. Chronic stress exposure in adult mice demethylates the *Crh* gene within the hypothalamus (Elliott et al, 2010) and highlights the potential of psychosocial stress to induce epigenetic changes linked to pathological physiology and behavior (Thayer and Kuzawa, 2011). Moreover, adverse early life experiences (i.e. traumas) like physical assault or sexual abuse, especially in combination with negative immediate onset-provoking adolescent or adult life experiences (e.g. death of a loved one, job loss) are thought to contribute to the onset of psychiatric disorders (Rutter, 2010). These studies highlight that the life history, i.e. the interaction of environmental and epigenetic factors with the genetic predisposition, is crucial for the onset of a psychiatric disorder. Further evidence comes from twin studies consistently showing that environmental influences account for a substantial proportion of the population variance including anxiety and depression (Plomin et al. 2008).

The interplay of brain regions involved in emotional regulation (e.g. amygdala, HIP, LC, mPFC), neuropeptides (CRH, UCN) and receptors (CRHR1) seems to indicate that chronic stress - in combination with and/or because of the exposure to adverse (i.e. stressful) environments - might cause a dysregulation of the HPA-axis, which causes a gradual shift from normal to pathological anxiety and comorbid depression. Therefore, it is not surprising that the majority of treatment strategies tries to reverse the aforementioned pathological alterations.

1.6 Limitation of actual treatment strategies for pathological anxiety

In general, actual treatment strategies can be divided in two main fields: the prescription of one or a combination of drugs aiming to restore neurotransmitter levels in a specific brain region and non-pharmacological therapies. The latter comprise electroconvulsive therapy, transcranial magnetic stimulation, deep brain stimulation, sleep deprivation and different forms of therapies offered by psychotherapists trying to reverse the maladaptive behavioral repertoire acquired by affected individuals (Fava and Kendler, 2000). Since anxiety and depression show a high level of comorbidity, it is assumed that they share some neurobiological features and thus, many drugs prescribed for depression are also used for the treatment of anxiety disorders (Moehler, 2011).
This highlights the first problem in actual treatment for pathological anxiety. Current research still has not identified exact mechanisms, environmental stimuli or a combination thereof to develop a more effective treatment strategy for pathological anxiety. This hypothesis is corroborated by the estimation that up to 60% of patients suffering from anxiety disorders are resistant or refractory to first-line treatment (Lanouette and Stein, 2010). Nonetheless, affected patients must be taken care of in order to attenuate their suffering. Thereby, benzodiazepines (e.g. Alprazolam, Diazepam, Flurazepam) and selective serotonin reuptake inhibitors (SSRIs, e.g. citalopram, fluoxetine, paroxetine) are the current first-line treatment for most anxiety-disorders (Cryan and Sweeney, 2011; Ravindram and Stein, 2010; Sartori et al, 2011) though tricyclic antidepressants, monoamine oxidase inhibitors and serotonin norepinephrine reuptake inhibitors are also used as treatment options (Black, 2006). All of these drugs have in common that they aim to restore or maintain one or more neurotransmitters in a specific brain region. The second and major problem of these drugs and therefore actual treatment is that they act throughout the whole brain at their target receptors (e.g. $GABA_A$ receptors for benzodiazepines) and not solely in the brain region where it is required. These drugs induce - partially very heavy - side effects, which sometimes cause patients to drop out from treatment or severely impairs their quality of life (though their actual symptoms might be reduced) (personal communication with Dr. Rohrbacher). Drug treatment tries to reestablish brain physiology from pathological back to pre-pathological (i.e. normal or physiological) conditions, whereas psychotherapy follows a different strategy.
Up to now, there are a plethora of different psychotherapies comprising *inter alia* mindfulness-based therapy (Segal, Williams, & Teasdale, 2002), cognitive behavioral therapy (Black, 2006), psychoeducation (Rummel-Kluge et al, 2009), cognitive therapy, exposure therapy, ritual prevention therapies and psychoanalysis (Black, 2006). Most of these psychotherapeutic methodologies try to identify crucial situations in the life history of an individual, which likely contributed to the onset of pathological anxiety and finally led to a behavioral repertoire, which enables the affected person to circumvent aversive conditions. This is exemplified by the following example: affected persons retreat to their homes instead of pursuing their work - by doing so, they try to circumvent even small problems, which regularly occur during every day work, since even the smallest problem

can cause feelings of helplessness and/or panic due to their bias to interpret neutral stimuli as dangerous (personal communication with Dr. Rohrbacher). Psychotherapies enable the individual to relearn or change behavioral strategies and to adopt techniques to circumvent or attenuate feelings of anxiety and panic (e.g. mindfulness, i.e. focusing on a specific task so extensively and exclusively that the patient has no resources to feel anxious or think about adverse situations).
The third problem complicating the treatment of anxiety disorders is the high comorbidity with other disorders like depression or substance abuse (particularly alcoholism) (Tottenham and Sheridan, 2010). Interestingly, alcoholism, binge drinking and anxiety disorders seem to share molecular underpinnings, namely CRHR1 (Treutlein et al, 2006). Animal studies in alcohol preferring msP rats indicate that *ad libitum* access to alcohol downregulates *Crhr1* in the amygdala and *nucleus accumbens* (Hansson et al, 2007), whereas a history of alcohol dependence in male Wistar rats causes an upregulation of *Crhr1* within the amygdala (Sommer et al, 2008). Both brains regions are involved in the control of emotionality. This highlights the possibility that improved treatment not only improves the quality of life from people suffering from pathological anxiety but that individuals suffering from other disorders may be treated too.
Though it is not clear whether a combinatory treatment of drugs and psychotherapy is superior to monotherapy (Black, 2006), many experts recommend it likely since both treatments pursue a different treatment strategy. Drug treatment tries to establish a pre-pathological brain physiology, whereas psychotherapy changes the way an individual behaves when facing a potential anxiety or panic-provoking situation. Both treatment options aim to improve or reverse the consequences of pathological anxiety and to identify the adverse environmental situations contributing or causing anxiety disorders. Importantly, there is a third possible treatment strategy - to identify beneficial environments, which might prevent or ameliorate pathological anxiety. This strategy is of preventive nature and might be well combined with already existing treatments. In the recent years, positive psychology was paid increasing attention likely belonging to the aforementioned third category since it exactly focuses on the effects of positive environments, situations or behaviors (Proyer et al, 2012). In animal research, EE seems to fulfill the same criteria like positive psychology in human research. Thayer and Kuzuwa (2011) state that "[…] we need to know more about whether EE might have positive effects on biology and health via epigenetic modification […] as an addition to the current focus on strategies to avoid the negative health effects of environmental stress. […] Based upon past work on the epigenetic effects of environmental stressors, one exciting possibility is that the health benefits of EE might also carry across multiple generations through epigenetic inheritance". This statement clearly emphasizes the potential of EE to identify protective environments and new targets for drug development.

1.7 The beneficial effects of enriched environment

Rosenzweig et al. (1978) were the first researchers who examined the consequences of beneficial housing conditions combining "complex inanimate and social stimulation" on rodent behavior and physiology. They therewith created a paradigm nowadays denoted as EE. It offers laboratory mice an enlarged home environment and provides biological relevant stimuli like group housing, shelter, additional nesting material, climbing structures and deep bedding to facilitate manipulation of their microenvironment. These arrangements create a semi-natural environment with a higher complexity, predictability and controllability. It allows physical exercise to improve motor performance and enhances sensory (e.g. visual; Sale et al, 2004) and cognitive capacities (Olsson and Dahlborn, 2002;

Würbel and Garner, 2007). It is of utmost importance to recognize that the design of EE must be according to the needs of the underlying research subject. There are no universal but just rough guidelines how to design an appropriate EE since the designing process is as unique as the research subject and keeps some fallacies (Würbel and Garner, 2007). Many researchers agree that the plurality of different EEs and utilized mouse strains has led to partially controversial results (Chapillon et al, 1999; Nevison et al, 1999; Van de Weerd et al, 1994), which can be circumvented by a well-chosen and self-designed paradigm (fig. 8; it is hard to imagine that every researcher will use the same paradigm or mouse strain).
A well designed EE induces a variety of beneficial effects, which can be attributed to either the "arousal" (Walsh and Cummins, 1975) or "learning and memory" hypothesis (tab. 3; Rosenzweig and Bennett, 1996):

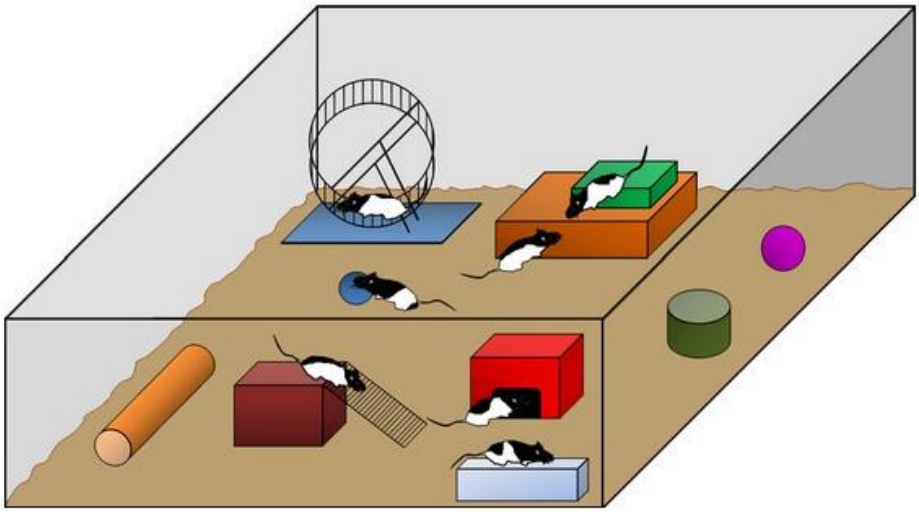


Fig. 8: Stylized picture depicting toys with different color and shape, which may be used to design an EE. Picture adapted and modified from Bengoetxea et al, 2012.

Hypothesis	Effect	Reference(s)
learning & memory	learning and memory	Nithianantharajah and Hannan (2006)
	neural connectivity	Nakamura et al. (1999)
	increased neurogenesis	Bruel-Jungerman et al. (2005)
	altered brain region activity	van Praag et al. (1999)
arousal	more naturalistic behavioral pattern	Kempermann et al. (2010)
	object exploration	Renner and Rosenzweig (1986)
	increase in locomotor and exploratory activity	Prior and Sachser (1995)
	anxiolytic and anti-depressive effects	Benaroya-Milshtein et al. (2004); Meshi et al, 2006; Olsson and Dahlborn, 2002

Tab. 3: Beneficial effects elicited after housing rodents in EE. Topics of major interest for this thesis are highlighted in red.

The arousal hypothesis favors the possibility that animals housed in an EE are confronted with higher environmental complexity and often novelty (e.g. changed toys), whereas the learning and memory hypothesis tries to attribute observed changes in brain structures and cellular mechanisms to underlying learning processes. Both hypotheses do not encompass

all possible factors nor do they rule out each other and should be viewed complementary to our view. Independent of the hypothesis, EE does not solely induce a variety of beneficial effects, it can also offset many of the negative neurobehavioral and physiological consequences of early life adversity (Bredy et al, 2003, 2004; Francis et al, 2002; Laviola et al., 2004; Morley-Fletcher et al, 2003).

Though EE can induce effects during the whole lifespan of an individual, there seems to be a sensitive period in mammals where animals are more susceptible to environmental influences. Champagne and Curley (2005) suggest the postnatal phase as this period since brain circuits are highly plastic as synaptic connections are elaborated and refined. This phase includes the formation of neuronal circuits mediating anxiety and depression-like behavior in mice and raises the possibility that this period may be critical for setting HPA-axis reactivity (Leonardo and Hen, 2006). In spite of intense studies, researchers were not able to delineate variables (i.e. no specific toy, cage size etc.) contributing to or eliciting beneficial effects, pointing towards EE as an emergent phenomenon strengthening the importance of a well-suited design.

Despite difficulties to identify variables important for the design, researchers have begun to unravel mechanisms likely to contribute to one of the most robust findings of EE - anxiolysis (Sztainberg et al, 2010a). The following examples highlight some of the different mechanisms thought to cause or contribute to the observed anxiolytic effect elicited EE. A study performed by Sztainberg et al. (2010b) indicated that EE decreased *Crhr1* mRNA levels in the BLA of C57BL/6 mice compared to controls and might be accounted for the anxiolytic effect. Findings from Okuda et al. (2009) support the potential of EE to influence the amygdala, a brain region of major importance for the regulation of anxiety. They were able to show that progenitor cell proliferation and differentiation are increased in enriched housed C57BL/6 mice compared to controls and suggest that these effects contribute to the anxiolytic effects of their EE procedure.

Besides the amygdala, HIP seems to be another brain region amenable for the effects of EE. Increased hippocampal neurogenesis is of the most robust findings elicited by EE and there is increasing evidence that it may contribute to the observed anxiolytic effects (Revest et al, 2009). The beneficial effects of EE on neurogenesis are thought to be multifactorial comprising *inter alia* brain-derived neurotropic factor (BDNF; Rossi et al, 2006), which is known to be regulated epigenetically. Indeed, EE increases hippocampal BDNF mRNA expression compared to controls by increasing the trimethylation at lysine 4 of histone 3 (H3K4me3, marker for actively transcribed genes) and reduces lysine 9 and lysine 27 histone 3 trimethylation (H3k9me3 and H3k27me3, respectively; markers for silenced genes) (Kuzumaki et al, 2011). EE is capable of modulating HIP by altering epigenetic modifications highlighting the possibility of EE as a tool to study epigenetic and GxE interactions. Importantly, HIP - like amygdala - is another brain region important for the regulation of emotion

A plethora of studies shows that EE reverses adverse early life or prenatal stress effects *inter alia* by normalizing a dysregulated HPA-axis (Francis et al, 2002) indicating that EE has the potential to attenuate detrimental effects thought to contribute to pathological anxiety. An even more fascinating and intriguing possibility has been revealed by recent literature. It suggests that EE has the potential to pass its beneficial effects from one generation to the next. Arai et al. (2009) were able to show that two weeks of EE not merely increased the long-term potential (LTP) in the hippocampus of ras-grf 1/2 knock-out (KO) mice via the activation of an alternative cAMP/p38 MAP kinase signaling pathway, in addition increased LTP was passed from EE-housed ras-grf 1/2 KO mothers to their offspring, which has never been housed in an EE. An experiment by Leshem and Schulkin (2011) strengthens the possibility of transgenerational inheritance. To evaluate

whether EE can reverse the effects of prenatal stress, female rats were exposed to stress from PND 27-29 and subsequently split in two groups: one was housed in EE and the other in a standard environment SE until mating. The offspring of prenatally stressed rats housed in SE until mating was further distributed to EE or SE. By doing so, the authors were able to evaluate whether the prenatal effect of EE on mothers on the one and the postnatal effect of EE on the offspring of prenatally stressed mothers on the other hand can reverse the effects of prenatal stress. Indeed, prenatal stress caused a transgenerational effect of adversity in SE offspring of prenatally stressed mothers, which was ameliorated by both, parental and offspring EE. This highlights the exciting possibility of EE to influence the health of future generations by attenuating or rescuing pathological phenotypes.
Taken together, EE raises the possibility to be a valuable tool to study the impact of epigenetic and GxE interactions on pathological anxiety since it exerts significant impact on nearly all brain regions or metabolic processes important in the regulation of emotion - amygdala, HIP and regulation of the HPA-axis - not only in the present, but likely in future generations too. Thus, it is surprising that almost all EE studies performed so far rely on "normal" rodents to identify mechanism contributing to pathological anxiety. This raises several problems: first, normal laboratory animals do not exhibit the pathological genetic predisposition of affected individuals and second, these studies miss the possibility to explore the interaction how environmental stimuli may act on and shape a rigid genetic predisposition, thereby creating a whole new scenario almost completely neglected so far.

1.8 High anxiety-related behavior mice - a mouse model of pathological anxiety.

Before considering any model organism, one should pay attention to the criteria a good model organism has to meet in order to produce valid and valuable results. A model organism suited for the study of pathological anxiety must fulfill validity criteria and should meet an acceptable cost-benefit ratio. Mice are easy to breed, have a short-reproductive cycle, are amenable for genetic and environmental manipulations and exhibit low maintenance costs, i.e. exhibit an excellent cost-benefit ratio (Cryan and Holmes, 2005). They possess all prerequisites to detect threatening stimuli - perception of a threat *per se*, association with a specific context and recalling of the respective memory - and thereby, to experience anxiety (Belzung and Philippot, 2007). Moreover, mice offer remarkable similarities with humans at the molecular, anatomical and physiological level (Leonardo and Hen, 2006) empowering them as good animal models for psychiatric disorders. Clément et al. (2002) propose that an appropriate animal model should meet three validity criteria: the same underlying rationale (construct validity), a close approximation of symptoms including psychopathology (e.g. genetic, neuroendocrine and behavioral concomitants of trait anxiety; face validity) and a reverse of symptoms after pharmacological intervention, i.e. an anxiolytic response after receiving anxiolytics (face validity) (Finn et al, 2003; Gordon and Hen, 2004). It is generally accepted that a complex disorder like depression or certain characteristics thereof can't be adequately mimicked or modeled in mice (e.g. a mouse is not "depressed" or has suicidal thoughts), which led to the concept of "endophenotypes". Endophenotypes represent a characteristic or symptom of a disorder that can indeed be modeled in the mouse (e.g. hyposomnia, HPA-axis dysregulation etc.) or reflects a behavioral dimension that is necessary to study psychiatric disorders in a preclinical environment (Cryan et al, 2002). Therefore, a good animal exhibits multiple phenotypes on the one and meets as many validity criteria as possible on the other hand.
Almost all prerequisites are met by a mouse model of pathological anxiety, which has been bred for >45 generations at the Max Planck Institute of Psychiatry in Munich. These mice

were bidirectionally and selectively bred from a genetically heterogeneous CD-1 population for high (HAB), "normal" (NAB) or low (LAB) anxiety-related behavior with percent time spent on open arms as key criterion (fig. 9).

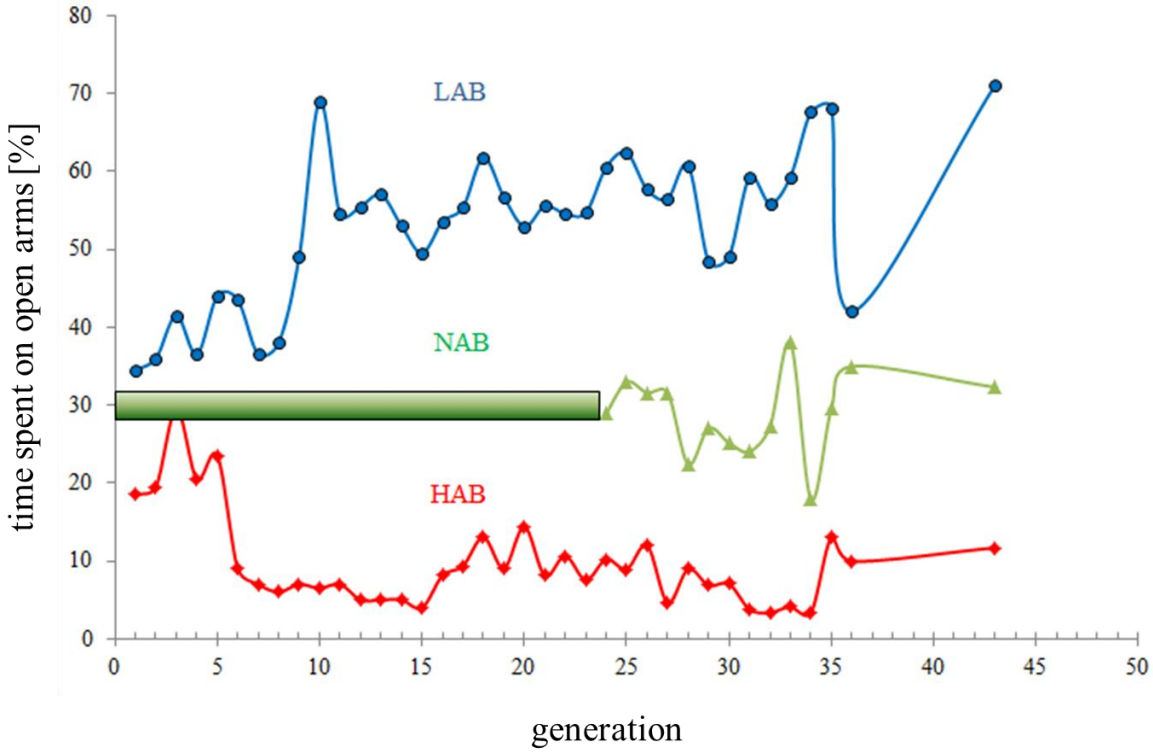


Fig. 9: Breeding course of high (HAB), "normal" (NAB) and low (LAB) anxiety-related behavior mice with percent time spent on open arms a key breeding criterion. All sublines originate from an outbred CD1 population.

Intrastrain comparisons by selective bidirectional breeding approaches hold the potential to investigate the genetic variability of complex, polygenic traits like anxiety (Sartori et al, 2011; Swallow and Garland, 2005) and thus, to unravel mechanisms contributing to pathological anxiety by focusing on particular traits associated with anxiety disorders (Landgraf and Wigger, 2003), differences in receptor functions likely to be associated with differences in anxiety (Overstreet et al, 2003) or avoidance behavior (Brush, 2003). Selective bidirectional breeding increases the frequency of genes associated with a particular trait by shifting the animals' phenotype bidirectionally from the strain mean (Falconer and Mackay, 1996; Krömer et al, 2005) and clusters it around the extremes of the total spectrum typically observed in an outbred strain (Sartori et al, 2011). HAB mice (HABs) were generated by using exactly this procedure and indeed exhibit a series of endophenotypes closely mimicking pathological characteristics of patients suffering from anxiety disorders and - at the same time - meet a variety of criteria of construct, face and predictive validity.

Converging evidence from more than one behavioral test confirms the face validity of a modeled endophenotype (Cryan et al, 2002). Indeed, the high anxiety trait of HABs compared to NABs and LABs has been confirmed by a reduced time spent in the aversive zone in a multitude of tests including *inter alia* open field (OF), elevated plus maze (EPM), light dark box (LD) (Kromer et al, 2005; Markt unpublished data, 2009; Markt and Sotnikov, in preparation; Muigg et al, 2009) as well as by an increased aversion to fox odor (Sotnikov, Markt et al, 2011) and an increased number of ultrasonic vocalizations (Kessler,

2006). These findings are largely independent of sex, age, maternal care and intrauterine environment (Landgraf et al, 2007). Recent studies (Gaburro et al, 2011; Sartori et al, 2011b) succeeded to show that HABs display enhanced fear learning in contextual and learned conditioning paradigms, which is associated with a lower heart rate variability in comparison to NABs and might indicate a weaker ability to inhibit fear responses or a stronger fear memory. This positive association has been confirmed in patients with anxiety disorders (Lissek et al, 2005). Importantly, the high anxiety phenotype has been confirmed independently in different laboratories corroborating the robustness of the trait (Landgraf et al, 2007; Muigg et al, 2009).

Further evidence comes from genetic studies showing that a two-SNP haplotype of the recently identified candidate gene *Tmem123d* is associated with PD in three independent human samples. In addition, risk genotypes of PD were associated with higher mRNA levels of *Tmem123d* in the frontal cortex. Likewise, HABs exhibit high mRNA expression levels of *Tmem123d* in the mPFC with the SNP rs13478518 being positively correlated with anxiety-related behavior on the EPM (Erhardt et al, 2011). Neuropeptide S receptor 1 (*Npsr1*) is associated with PD too (Okamura et al, 2007) and similar to *Tmem132d*, Slattery, Naik et al. (in preparation) were able to show that *Npsr1* plays an important role for the high anxiety phenotype of HABs.

Neuronal activation (i.e. brain metabolism) within the amygdala and neuronal propagation are suggested as biomarkers of endophenotypes associated with anxiety disorders (Norrholm and Ressler, 2009). Indeed, voltage-sensitive dye imaging (VSDI) studies revealed that HABs compared to LABs show an increased neuronal propagation within the amygdala indicating an inherent stronger activation (i.e. more fear and/or anxiety) independent of the stimulus (Avrabos, Markt et al, in preparation). Exposure of HABs to a mild stressor (open arm exposure or novel environment) ensued an increased c-Fos expression (i.e. a hyperactivation) *inter alia* of the amygdala, several hypothalamic nuclei and LC (Muigg et al, 2007; Sotnikov, unpublished data). All of these brain regions are thought to be involved in the etiology of anxiety disorders. Remember, for example, the hyperreactive amygdala in patients suffering from PD, GAD, OCD, social or specific phobia (Shin and Liberzon, 2010).

As expected, predictive validity is met by application of a benzodiazepine or the selective neurokinin-1 receptor antagonist L-822,429, which reversed the enhanced fear- and anxiety-related behavior (Sartori et al, 2001b). It has to be mentioned that some drugs (e.g. Paroxetine, Fluoxetine) seems to exert no anxiolytic effect on male adult HABs (Landgraf, unpublished data) indicating that several pathways - likely involving GxE or epigenetic interactions - contribute to the overall high anxiety trait phenotype.

These examples highlight the excellent validity of the HAB mouse model and the high degree of similarity with characteristics of pathological anxiety indicated by shared genetic and neuronal underpinnings. We would like to take advantage of the rigid and pathological genetic predisposition towards high anxiety to study the potential of environmental influences to mitigate even seemingly rigid genetic drives, thus revealing genetic X epigenetic interactions at multiple levels.

2 Aims of the thesis

This thesis focuses on the interaction of gene x environment and/or epigenetic interactions. There is increasing evidence that the interplay of these factors possesses an important role in the etiology of psychiatric disorders. EE might be one way to model exactly these GxE interactions and thereby expand our knowledge about mechanisms likely to be important for mental disorders. Thus, we would like to combine both aspects, a rigid genetic predisposition and an environmental manipulation like EE to study the interplay of all factors contributing to pathological anxiety. Therefore, the following aims were specifically addressed:

a) We would like to verify whether even the high anxiety and comorbid depression phenotype of our selectively bred HAB mice can be rescued by a beneficial environmental manipulation like EE. Male adult HABs do not respond to a variety of drugs like Diazepam or Paroxetine though they respond as juveniles and thus, we are interested whether it is possible to rescue a rigid genetic predisposition with EE though it is difficult to do so by pharmacological intervention.
   Thus, we subjected male and female HABs to EE lasting 4 weeks and utilized OF, EPM, LD and SIH to assess the anxiety-related behavior as well as FST and TST to evaluate coping style. Moreover, we monitored home cage locomotion and exploration to identify a possibly masking effect thereof.

b) Since we conducted EE several times, we are interested to evaluate effect sizes as well as reproducibility and reliability of our paradigm. Therefore, we performed a meta-analysis to identify the overall impact of EE on HABs.
   We used z-scores to compare effect sizes between different experiments and to assess the overall effect on anxiety-related behavior and coping style.

c) We would like to compare the impact of EE on genetically heterogeneous CD1 mice to that of HAB and NAB mice exhibiting a strong genetic predisposition with only the former showing high anxiety-related behavior.
   CD1 and NAB mice were housed for 4 weeks in EE and behaviorally phenotyped similar to HABs.

d) In addition to the possibility to identify possible new targets or mechanisms likely to be involved in anxiety, we would like to verify whether it is possible to increase the anxiolytic effect size elicited by EE.
   To test our hypothesis, we conducted EE for 10 instead of 4 weeks.

e) We would like to identify factors contributing to the beneficial effects of EE since the environmental stimuli eliciting a certain effect are as important as the effect *per se*.
   Thus, we intensively monitored maternal, pup and early adolescent behavior and controlled whether mice indeed use the provided toys and possibility to manipulate their environment.

f) After the intense behavioral phenotyping, we are interested to unravel which genes and proteins might be involved in the behavioral shift observed after EE.
We used qPCR and if necessary, adjacent WB to verify the involvement of several candidate genes and their associated protein products.

g) One of our major aims is to identify which epigenetic mechanism(s) may contribute to the transcriptional regulation of identified candidate genes.
We used *in silico* analyses to detect genes amenable for epigenetic regulation via 5-mC and subsequently used a pyrosequencing service to verify promoter methylation. Finally, we were interested to identify transcription factors possibly regulating gene transcription together with 5-mC.

h) We are interested in shifting the rigid genetic phenotype of HABs from SE to EE or vice versa by applying epigenetic drugs. Is it possible to pharmacologically mimic the phenotype created by EE? Beyond that, we would like to identify whether there might be an additive effect of environmental manipulation and drug treatment. Do pharmacological and environmental manipulations act on the same or different molecular underpinnings?
Thus, we applied either the DNMTi valproic acid or the HDACi 5-Aza-2'-deoxyuridine IP three times weekly in the 2^{nd} and 3^{rd} week of EE to SE and EE HABs.

i) We would like to know whether the anxiolytic effect of EE can be passed on to the next generation. Does a transgenerational effect exist for such a complex disease like anxiety and comorbid depression?
Therefore, we mated male and female HABs housed for 4 weeks in EE to generate F1, which has never been in contact with EE and was raised in SE directly from birth. We continued mating to F3 and assessed anxiety-related behavior with OF, EPM and LD in all generations compared to respective SE controls. Moreover, we monitored coping style via FST in F3 to detect a possible additive effect of EE by housing up to 3 generations successively in it.

3 Material and Methods
3.1 Animals and housing conditions

Normal and high anxiety-related animals used for experiments were either bred in the facility of the Max Planck Institute of Psychiatry or Biochemistry, Munich and originated generations 33-43. CD1 mice were purchased from Charles River, Sulzfeld, Germany. Prior to experiments, all mice were transferred from their respective breeding facility to a room adjacent to the testing room and kept under standard housing conditions (room temperature 22.5±1°C, relative air humidity 45±5%, 12h/12h light/dark cycle with lights on at 8 a.m.) and were provided with food (Altromin 1314 TPF; protein 22.5%, fat 5%, fibre 4.5%, ash 6%) and tap water *ad libitum*. Housing conditions in the respective breeding facilities and the testing room were kept as similar as possible and all animals were granted a habituation of at least 5 days after transfer and before the beginning of any behavioral testing. Mice were housed in same sex groups of three animals, denoted as trios, either under standard or enriched housing conditions. Both included the use of wood chips and litter (Lie E – 001 and NBG E – 012, ABEDD-LAB and VET Service GmbH, Vienna, Austria) and macrolone cages with their respective lids (Ehret Labor- und Pharmatechnik GmbH und Co.KG, Emmendingen, Germany)

Standard housing: Type two cages (207 x 140 x 265mm) providing a floor space of 363cm² were covered with an iron chopper bar breech lid (article no. 15050). The cage bottom was covered with wood chips and a handful of wood wool was provided to ensure proper nest building and to meet animal welfare guidelines.

Enriched housing: Type IV cages (380 x 200 x 590mm) offering a floor space of 1815cm² were covered with an iron lid with heightened design (article no. 40051) to further improve cage size since movement in all three spatial directions is important for mice (e.g. climbing). To increase predictability, controllability and complexity, biologically relevant stimuli were provided: i) a 5cm layer of wood chips to facilitate manipulation of the microenvironment, ii) a plastic inset (22 x 16 x 8cm) and tunnel (19.5 x 6 x 6cm) as retreat possibilities as well as iii) a wooden latter and scaffold to offer climbing structures. These manipulanda, in combination with a bigger home cage, allowed mice to accomplish highly motivated natural behaviors, while creating as few stressors as possible. To circumvent an increase in CORT and testosterone levels, accompanied by increased aggression, areas and toys that might lead to monopolization of cage structures, toys or nutrition were omitted (Olsson & Dahlborn, 2002; Nevison et al., 1999). Therefore, we did not supply the animals with a running wheel to bypass locomotor-induced anxiolysis (Henderson et al., 2004). Furthermore, transfer of half of the nesting material during the weekly cage change allowed preservation of olfactory designation for the mice, which additionally ensured less aggression (Olsson & Dahlborn, 2002). Design of EE was adapted from Touma (2008) and modified to further increase the observed anxiolytic effect. The EE paradigm was adapted from Arai et al. (2009) and expanded by a self-designed enrichment paradigm lasting for 14 more days. Thus, EE comprised two 14-day periods, called partial and full enrichment: from PND 15-28, pups and their respective damn were transferred for 6h/day to EE (partial enrichment, adapted from Arai et al., 2009), whereas from PND28 pups were weaned, grouped in trios and subjected to EE permanently until PND 42 (full enrichment, self-designed paradigm).

All experiments were conducted with the approval of the local authorities (Regierung von Oberbayern, §9 Abs. 1 Satz 4 referring to BGBI.IS.1105 as amended on May 25th, 1998).

3.2 Behavioral Testing

All behavioral tests were conducted between 09:00 a.m. and 01:00 p.m. to minimize differences in basal CORT levels induced by the daily rhythm - variations in these levels offer profound effects on the behavior of the tested animals and should be circumvented (Romero, 2004). To exclude any confounding effects, all three animals of a particular cage were tested simultaneously in the respective test and the experimental design was counterbalanced for all known variables (e.g. sex, housing condition, litter size, weight etc.). To minimize olfactory effects between tested mice, the open field (OF), elevated plus maze (EPM) and light-dark box (LD) apparatuses were cleaned with water containing detergent after every tested animal and additionally with 10% ethanol before the sex of the tested mice changed from male to female or vice versa. To ensure complete volatilization, a pause of 5min. was maintained. To guarantee identical test conditions during the tail suspension (TST) and elevated platform (EP) test, secreted fecal boli and urine were removed from the test chamber after every tested cage. Likewise, water used during the forced swim test was renewed after every tested mouse.

Tests of OF, EPM and LD were videotaped and analyzed using Anymaze (version 4.84, Stoelting Co., Wood Dale, USA); TST, FST and EP were analyzed via Event Log event recorder (version 1.0, EMCO, Robert Henderson). The criteria for entering a compartment during a behavioral test was met when a mouse entered it with ≥40% of its body (related to its barycenter), while an exit was scored when ≤25% of its body remained in a compartment or area, respectively. These criteria approximated a two-paw entry.

To obtain a broad overview of the behavioral changes elicited by EE, a wide series of behavioral tests, assessing the following parameters, were conducted: anxiety-related behavior, exploratory behavior and locomotion, anhedonia, coping style and stress reactivity.

Assessment of anxiety-related behavior: Marker of enhanced anxiety like avoidance, escape and freezing behavior are thought to reflect the emotional component of anxiety in mice and are easily accessible. Therefore, these behaviors are denoted as anxiety-related behavior rather than anxiety *per se* and are categorized in ethological based unconditioned and conditioned, i.e. learned paradigms (Hadley, 1995; Gray, 1982; Sartori et al., 2011). The first utilize a spontaneous, natural-like innate approach-avoidance conflict by providing areas of relative safety contrasting open, brightly lit spaces. Conditioned paradigms pair a neutral with a stress- and painful stimulus (e.g. electric foot shock) and require several training sessions (Hascoët and Bourin, 2009). We omitted conditioned tests to minimize possible confounding effects of motivational or perceptual states arising from interference with learning/memory, hunger/thirst or nociceptive mechanisms (Rodgers et al., 1997), but performed a variety of unconditioned paradigms since different tests of anxiety-related behavior may represent different forms of murine anxiety. For this reason, we conducted the most established tests exploiting an approach-avoidance conflict: OF, EPM and LD (Bouwknecht and Paylor, 2008; Sartori et al., 2011). In addition, we performed one non-exploratory driven test referred to as stress-induced hyperthermia (SIH), which used a physiological instead of a behavioral readout.

Elevated plus maze: Percent time spent on and entries made into open arms (File, 1992; Rodgers and Cole 1994) were recorded as anxiety-related indices. Entries into closed arms were used as a locomotor index during the 5min. test interval. The EPM was made of gray polyvinyl chloride and consisted of two open (30 x 5cm) and closed arms (30 x 5 x 15cm), connected via a central platform (5 x 5cm) 40cm above the floor. Light intensity attenuated gradually from 300lux on the most outer part of the open arms to 50lux on the central

platform, whereas illumination was <10lux in the closed arms. Animals with high levels of anxiety will quickly cross from one closed arm to the other and avoid the brightly lit open arms, whereas less anxious animals will spent more time on the open arms (Bouwknecht and Paylor, 2008).

Light dark box: The test was conducted for 5min., measuring percent time and distance travelled in the light compartment as well as entries and latency to enter the light compartment. The LD box comprised a dark (16 x 27 x 27cm) and light compartment (32 x 27 x 27cm) illuminated with 400lux and <20lux, respectively. Compartments were separated by a wall offering a small opening (5 x 5cm) to enable travelling between both compartments. Mice exhibiting lower levels of anxiety will enter the light compartment faster, more often, spend more time and travel more distance in it (Hascoët and Bourin, 2009).

Stress induced hyperthermia: To prepare for a fight-or-flight reaction, body temperature is increased as a consequence of a physiological or psychological stressor. SIH takes advantage of the stress-induced activation of the autonomic nervous system by measuring the body temperature rectally before (T_1) and after stress exposure (T_2). The increase in body temperature, denoted as psychogenic fever, rises body temperature within 15min. up to 1.5°C and usually returns to basal levels within 2h. The SIH response (ΔT) is calculated by subtracting the stress from basal temperature ($\Delta T = T_2 - T_1$) (Vinkers et al., 2009; Zethof et al., 1995). It seems to be relatively independent of locomotor activity, its curve parallels HPA-axis activity (Groenink et al., 1994; Spooren et al., 2002; Veening et al., 2004) and anxiolytic drugs like CRF receptor antagonists have been shown to selectively and dose-dependently reduce the SIH response. Any treatment reducing the SIH response is indicative of an anxiolytic effect (Bouwknecht el., 2007).

Evaluation of exploratory behavior and locomotion: Animals with high locomotor activity usually display lower levels of anxiety-related and depression-like behavior (Ferguson et al., 2004), determining suppression of locomotion as one of the cross-test dimensions of anxiety (Henderson et al., 2004). We analyzed locomotor activity during the OF as well as during a more ethological situation like in the home cage since both analyses contribute with different aspects to facilitate interpretation of differences related to locomotion.

Open field: We used this test to measure the quality and quantity of the general activity in rodents. Quality comprised exploratory and stress-related behavior, quantity comprehended the total distance travelled (Miyata et al., 2007). At the beginning of the test, every animal was placed into the OF with its head facing the south wall and was allowed to freely explore the arena for 5min. This short length of time emphasizes exploratory behavior and response to novelty, rather than baseline activity (Gould et al., 2009) and is an ideal extension to home cage activity. Total distance travelled, percent time and distance travelled in the inner zone, latency to enter and total entries to the inner zone were scored (Belzung and Prut, 2002). EE seems to increase exploratory behavior and thus, we counted rearings performed during the test (Gould et al., 2009). Rearing was defined as a behavior where the mouse was standing solely on its hind limbs in an upright posture to actively explore its environment, either at the wall or in the arena itself. The OF consisted of a grey, circular PVC arena with a diameter of 60cm for the outer and 30cm for the virtual inner zone. Illumination decreased successively from 50lux in the center to ca. 15lux near the wall by using a spot-like white light source. Highly anxious animals show higher levels of thigmotaxis (i.e. walking close to the wall using their whiskers to detect it), enter the inner zone with a higher latency and spent less time within (Bouwknecht and Paylor, 2008).

Elevated platform: At the beginning of the test, each mouse was placed on the platform with its head facing towards the camera. The apparatus consisted of a wooden cylinder 40cm in height, with a circular platform (Ø 10cm) fixed on top. Light intensity during the trials was 300lux. The height and small size of the platform were used as a psychological stressor to monitor duration and frequency of freezing behavior as well as the total amount of rearings and head-dippings during the 5min. test interval. Freezing was defined as absence of movement excluding respiration (Miyata et al. 2007); head dipping was counted when a mouse lowered its head completely below the circular platform, i.e. snout, scull and ears were unambiguously lower than the bottom edge of the circular platform. The primary use of EP is to detect differences related to exploration, but freezing behavior can be used as an index of anxiety, especially because this readout seems to be sensitive to serotonergic anxiolytics (Miyata et al. 2007), in contrast to EPM, where drugs that affect serotonergic neurotransmission vary greatly depending on the report (Dunn et al, 1989; Hascoët et al., 2000; Koks et al., 2001; Kostowski et al., 1992; Moser, 1989; Pinheiro et al., 2007; Treit et al., 1993). Thus, it is an excellent supplement to control whether the serotonergic system is involved in observed behavioral changes caused by EE.

Behavioral tests of anhedonia, coping style and stress reactivity: Individual variation in stress reactivity is an important factor, which determines vulnerability for stress-related diseases and is influenced by environmental demands and the capacity to cope with them. "Understanding the origin and underlying mechanisms of this individual coping capacity and hence individual disease vulnerability is one of the major challenges of modern biomedical research" (Koolhaas, 2007). Some, but not all, important characteristics of psychiatric disorders include anhedonia (disability to feel joy or delight), psychomotor retardation (significant decrease of activity) and an in- or decreased HPA-axis reactivity (Diagnostic and Statistical Manual of Mental Disorders IV). Because anxiety often entails depression as a comorbid disorder (Johnston et al., 2009; O' Leary and Cryan, 2009) and the onset of both diseases seem to depend to a big extend on the experienced life-stress, it is essential to perform behavioral tests assessing i) coping style (formerly denoted as depression-like behavior), ii) anhedonia and iii) to determine reactivity of the HPA-axis. Therefore, we conducted TST and FST to assess coping style, the sucrose consumption test to evaluate anhedonic behavior and the stress reactivity test (SRT) to determine HPA-axis reactivity.

Tail suspension test: Mice were suspended to an iron bar located 75cm above the floor for 6min. by fixing the last 3-4cm of their tail via adhesive tape to one of four 15cm long appendages protruding from the iron bar. The suspension of the mouse represented an inescapable stressor that caused escape-orientated behavior like running movements, body jerks and torsions (summarized as struggling) attempting to catch the suspending apparatus, followed by increasing bouts of immobility (O' Leary and Cryan, 2009). Duration of immobility was originally described as an index of "behavioral despair'' (Porsolt et al., 1977; 1978) at which the animal stopped its effort to escape the situation. More recent literature suggests immobility as a measure of coping or adaptation, whereby immobility disengages the animal from active forms of coping with a stressful situation (Lucki, 2001; O' Leary and Cryan, 2009; Thierry et al., 1984). An animal was considered immobile when it stopped all limb and body movements except breathing and minor, slow head movements. In general, two different coping styles are described in the literature: the active response, represented by high levels of escape-orientated behavior, aggression, sympathetic nervous system and often HPA-axis activity (Henry and Stephens, 1977; Koolhaas, 1997) and the passive response characterized by low levels of aggression, escape-orientated behavior and high levels of immobility (Engel and Schmale, 1972;

Koolhaas, 1997). TST exhibits a high predictive value since the following antidepressants significantly increased the time animals engaged in escape-orientated behavior (O'Leary and Cryan, 2009): selective norepinephrine reuptake inhibitors (SNRIs) (Cryan et al., 2004), tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs) (Cryan et al., 2005; O'Leary et al., 2007), atypical antidepressants such as bupropion (Steru et al., 1987), monoamine oxidase inhibitors, as well as electroconvulsive shock (Teste et al., 1990). Importantly, aforementioned antidepressants belong to different classes and act on different neurotransmitter systems. Animals exhibiting an active coping style (or reduced depression-like behavior) engage more often (frequency) and for a longer total time in struggling behavior.

Forced swim test: FST was performed for 6min., scoring the total amount and percent time spent immobile. Immobility was considered when the animal was floating or made minor limb movements to avoid drowning. Two liters of water with room temperature (22.5±1°C), filled in a cylinder with a diameter of 135mm and height of 280mm were used. Importantly, the mouse was not able to touch the ground of the cylinder by its tail and sight barriers circumvented distraction of animals during testing. Though both, TST and FST assess coping style and their predictive validity overlaps to a certain extent, research suggests that they obviously measure different aspects of coping behavior (Chatterjee et al., 2012; Cryan et al., 2005). Thus, we additionally used this test to guarantee a better interpretation of coping style.

Stress reactivity test: To evaluate HPA-axis reactivity to a stressor, two different versions of a SRT were employed: a moderate (Touma et al., 2008) and more severe (Sotnikov, in preparation) version. We used for the moderate version a 15min. restraint period in a 50ml plastic tube, with a hole for ventilation and an aperture in the cap for the tail. Basal samples were collected prior to and reactive samples immediately after restraint stress. Instead of restraint stress, we used a 6min. FST for the more severe version. Reactive samples were collected 30min. after the end of the FST, while basal samples were taken two days later, prior to killing animals. Blood samples were collected from the ventral tail vessel in less than two minutes to ensure basal CORT levels according to Dürschlag et al. (1996, with slight modifications) using Microvette® CB300 coated with potassium-EDTA (code: 16444, Sarstedt, Nümbrecht, Germany). Cellular constituents were removed by centrifugation (10min., 4000rpm, 4°C) and plasma was frozen at -20 °C until further analysis.

Home cage activity: Existing behavioral assays mostly examine a single behavioral domain over a short period of time (de Visser et al., 2006; Tecott and Nestler, 2004) and completely neglect the rich behavioral repertoire of mice that can merely be analyzed in their home cage. These behaviors are highly motivated, possess exceptionally ethological validity and reflect the function and interaction of numerous behavioral and physiological systems. "Detailed assessment of these patterns thus has the potential to provide a powerful tool for understanding basic aspects of behavioral regulation and their perturbation by disease processes" (Goulding et al., 2008). Because litter size and sex composition influences maternal behavior in rodents (Lonstein and Flemming, 2001), we culled litters on PND 1 to a size of eight, comprising in each case 4 male and female pups. To complement the read out of classical behavioral tests, we analyzed four different aspects of home cage activity (HCA): maternal, juvenile and early adolescent behavior as well as circadian rhythm:

Type of HCA	PND	Time of observation	Housing condition
maternal behavior	15 and 17	09:00 a.m. - 03:00 p.m.	EE or SE
	16	08:00 p.m. - 02:00 a.m.	home cage
juvenile behavior	22 and 24	09:00 a.m. - 03:00 p.m.	EE or SE
	23	08:00 p.m. - 02:00 a.m.	
early adolescence behavior	35	09:00 a.m. - 03:00 p.m.	EE or SE
circadian rhythm	35-42	72h per animal	type 3 cage

Maternal behavior: A vast body of literature indicates that maternal behavior can influence behavior of the offspring. A prominent example was given by Champagne et al. (2003): it was shown that LG and ABN in rats have profound effects on anxiety-related behavior and HPA-axis reactivity of the offspring. In adulthood, pups of high licking/grooming and ABN mothers showed increased expression of GR in the hippocampus and decreased expression of CRH in the hypothalamus (Weaver, 2007). These expression changes caused by maternal behavior are of epigenetic nature and ensued an enhanced glucocorticoid feedback sensitivity, which resulted in a less anxious phenotype of the offspring (for a detailed review see Weaver, 2007). In our paradigm, different treatment of SE and EE offspring started on PND 15. SE mice were transferred for 6h per day to a new cage identical with their home cage (to exclude handling effects), whereas EE mice were allowed to explore an EE cage during that time. Thereby, dams together with their pups were transferred since offspring wasn't capable to take care for itself. The different environments might have caused changes in maternal behavior, which contributed to effects elicited by EE. Thus, we analyzed the two general categories of maternal responses: active and quiescent behaviors (Lonstein and Flemming, 2001).

Active behaviors	Quiescent behaviors
licking/grooming	sitting together with body contact
time spent with pups (licking/grooming/body contact/nursing)	nursing
locomotion	
digging	

Licking/grooming was defined as an active caretaking behavior of the damn by using her tongue and/or paws to groom her pups. We counted "time spent with pups" when the damn performed licking/grooming or was sitting together with body contact with her pups. If the mother is in body contact with pups, she lies either on top of the pups (sitting together with body contact) or nurses them. We omitted counting of different nursing styles because Kessler (2006) showed that from PND15 onwards HAB dams were merely engaged in the nursing style "lying on the side". Litters were videotaped each for 6h during the light and 6h during the dark phase to investigate whether maternal behaviors differed when dams were housed either in their respective environment (SE or EE, light phase) or their home cage (dark phase). We analyzed the videotaped behavior every 10min. for 60s via 1/0 sampling in 15s intervals (Martin and Bateson, 2007) giving rise to 144 monitored intervals in total. 1 and 0 were noted when a certain behavior was conducted during a 15s interval or not, respectively. This procedure ignored multiple occurrences of a certain behavior per 15s interval, but this drawback might be neglected almost completely since parameters we monitored were performed for a considerable amount of time (e.g. sitting together with body contact). All occurrences of a respective behavior were added from the 144 intervals and expressed as arbitrary units. A similar procedure was performed earlier

by Kessler (2006) and yielded reliable results. Thus, we used this method too, to analyze juvenile and early adolescence behavior.

Juvenile behavior: Pups open their eyes around PND 15 and actively start to explore their environment and to engage in social interactions. Therefore, the time period from PND 15-25 is denoted as socialization period and the perceived stimuli during this time are of utmost importance for the development and refinement of a species-specific behavioral repertoire including motor, cognitive and social skills. This seems to be necessary to foster a general disposition for neurobehavioral plasticity (Martin and Caro, 1985; Terranova and Laviola, 2005). A key component of the natural behavioral repertoire of developing animals is amicable and playful behavior like chasing and horizontal and vertical jumping. Interestingly, EE facilitates cognitive, sensory and motor stimulation and leads to the expression of a much wider range of social interactions. Moreover, the enhanced spatial complexity leads to increased exploratory behavior and reduced neophobia since animals are confronted regularly with novel objects. The defense of territories, marking and sheltering indicate that these animals indeed demonstrate a more naturalistic behavioral pattern (Kempermann et al. 2010). Thus, we monitored juvenile behavior on PND 22 and 24 during the light phase and on PND 23 during the dark phase by observing the following behaviors:

Monitored behavior	**Category**	**Definition**
sitting together with body contact	sociopositive	direct body contact of animals without movement
play/amicable behavior	sociopositive	chasing, horizontal and vertical jumping, grooming
toy use/environmental manipulation	exploration	digging and burying, usage of provided toys

Sitting together with body contact implied direct body contact of animals without movement excepted very short movements like head raising (e.g. due to noise), moaning or changing position. Play/amicable behavior included chasing, horizontal and vertical jumping as well as grooming of a companion (allogrooming). Toy use/environmental manipulation comprehended manipulation of the microenvironment via digging and burying and usage of provided toys for a variety of possible behaviors like retreat, exploration etc.

Early adolescent behavior: Juvenile animals were weaned on PND 28 to transfer them in their respective environment in same sex groups of three animals (beginning of permanent enrichment). Thus, mice were confronted with two important changes: reduction of group size from eight (different sex) to three (same sex) and the onset of early adolescence. To investigate the effect of EE on these changes, we videotaped the animals on PND 35 and scored the following additional behaviors compared to juvenile behavior (tab. 4):

Monitored behavior	**Category**	**Definition**
activity	locomotion	movement for 3s consecutively
eating/drinking	energy balance	food intake and/or water consumption

Tab. 4: Summary and definitions of behaviors monitored during early adolescent behavior.

Animals were housed in trios from PND 28 – 42 and a certain behavior was counted when 2 out of 3 animals were engaged in it. Activity was defined as a behavior which comprised movement for at least 3s consecutively (e.g. patrolling). This precaution minimized

confounding movements like head lifting or changing position when sitting together with body contact to be counted as activity.

Circadian rhythm: Activity, i.e. locomotion and exploration significantly varies depending on the scene of measurement. Activity in the home cage is predominantly influenced by the activity rhythm, in contrast to behavioral tests, where mice are placed in an unfamiliar environment, which affects fear and anxiety due to neophobia. We quantified home cage activity via an automated system (Inframot; TSE, Bad Homburg, Germany) over a period of 72h. Eight animals were tracked simultaneously, each in a type 3 cage offering 825cm² (265 x 150 x420mm). The cage was covered with an iron lid harboring a photo beam sensor on top. Every time an animal passed the sensor, an activity was counted. Activity was analyzed by the hour for 72h in total. The first 12h of observation were granted to permit habituation of animals to the new cage: thus, three dark and two light cycles were completely analyzed.

Assessment of transgenerational inheritance: Epigenetic or transgenerational inheritance is defined as transmission of phenotypic variations to subsequent generations of cells (mitotic) or organisms (meiotic) and does not stem from variations in DNA sequence (Jablonka et al., 2009). The idea of passing on acquired traits was originally coined by Lamarck (1809) and stands vis-à-vis the evolutionary synthesis proposed by Darwin (1859) suggesting that mutations within individuals lead to genetic variation within a population (Bard, 2011). For this reason, Lamarck's theory is denoted as "soft inheritance" (non-genomic inheritance) in contrast to Darwin's "hard inheritance" (genomic inheritance like SNPs etc.). It is becoming increasingly clear that epigenetic inheritance seems indeed to exist and moreover, might be ubiquitously present as examples in bacteria, protists, fungi, plants and animals corroborate (for a review see Jablonka et al., 2009). As a consequence, current biology tries to unite both theories to a new concept referred to as "modern synthesis", allowing fast (epigenetic) and slow (genomic) adaption to environmental demands by inherited phenotypic variation. This implies that the health of future generations might be compromised and highlights the importance to provide novel preventative and therapeutic approaches before the disease systems develop (Jirtle and Skinner, 2007). Thus, we tried to determine whether beneficial effects of EE might be transmitted to the offspring (fig. 10):

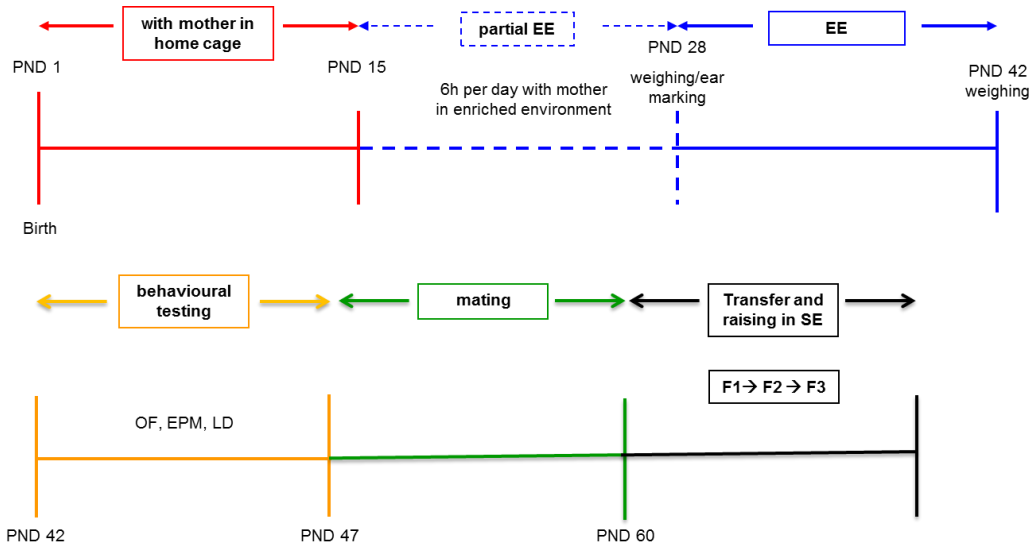


Fig. 10: Experimental schedule depicting the set-up to evaluate possible transgenerational inheritance of anxiety-related behavior in HAB mice.

Animals were housed and tested as described until PND 47. Subsequently, animals of the respective environment were mated according to their performance in the behavioral tests. We used the parameters "% time spent in inner zone" (OF), "% time spent on open arms" (EPM) and "% time spent in light compartment" (LD) to rank mice. Therefore, we calculated the average time for every of the three parameters for SE housed mice and attributed points according to the following rules:

Ranking rule	**Points**
EE mouse spent more time in a respective compartment compared to the SE average	2
EE mouse spent the same amount of time (±1%) in a respective compartment compared to the SE average	1
EE mouse spent less time in a respective compartment compared to the SE average	0

Whenever applicable, EE animals that scored ≥4 and SE mice that scored ≤1 point(s) (ranking was conducted vice versa to EE) were mated to generate offspring. Mating was allowed for 14 days, whereat SE mice were mated in type 3 and EE animals in EE cages, respectively. Males were removed from their respective females when pregnancy was observed during the daily examination. Pregnant EE females were transferred by an experienced person 2-3 days before parturition to SE to exclude any contact of neonates with EE. From that day on, offspring of EE and SE was raised and tested as described in SE housing. Breeding was continued until F3 and anxiety-related behavior of every generation was assessed in a behavioral test battery comprising OF, EPM and LD.

Killing of animals, tissue harvesting and blood collection: Animals were deeply anesthetized with Forene (ABBOTT GmbH, Wiesbaden, Germany) before decapitation. We harvested brains from experimental animals for further molecular analyses. Blood was collected either 48h prior to killing from the tail vessel (Dürschlag et al., 1996; with slight modifications) or after decapitation (trunk blood) to determine CORT concentration.

3.3 *in silico* and molecular analyses

***In silico* analysis to identify candidate genes:** Czibere (2008) performed gene expression profiling (MPI24K-platform) covering the whole genome to identify expression differences between HAB and LAB mice. A second genome-wide screening approach identified 267 SNPs differing between the two sublines, to finally achieve a list of candidate genes relevant for anxiety-related and depression-like behavior in our mouse model of extreme trait anxiety. To expand our list of candidate genes by another layer of transcriptional regulation in addition to SNPs, we performed *in silico* analyses to identify genes amenable for epigenetic regulation. Therefore, we used CpG Island Searcher (default settings, Release 29.10.04) to identify CpGIs within promoter regions of earlier identified candidate genes. These epigenetic candidate genes were further analyzed for expression differences via qPCR and, if differences were detected, subsequently analyzed for differences in promoter methylation (fig. 11).

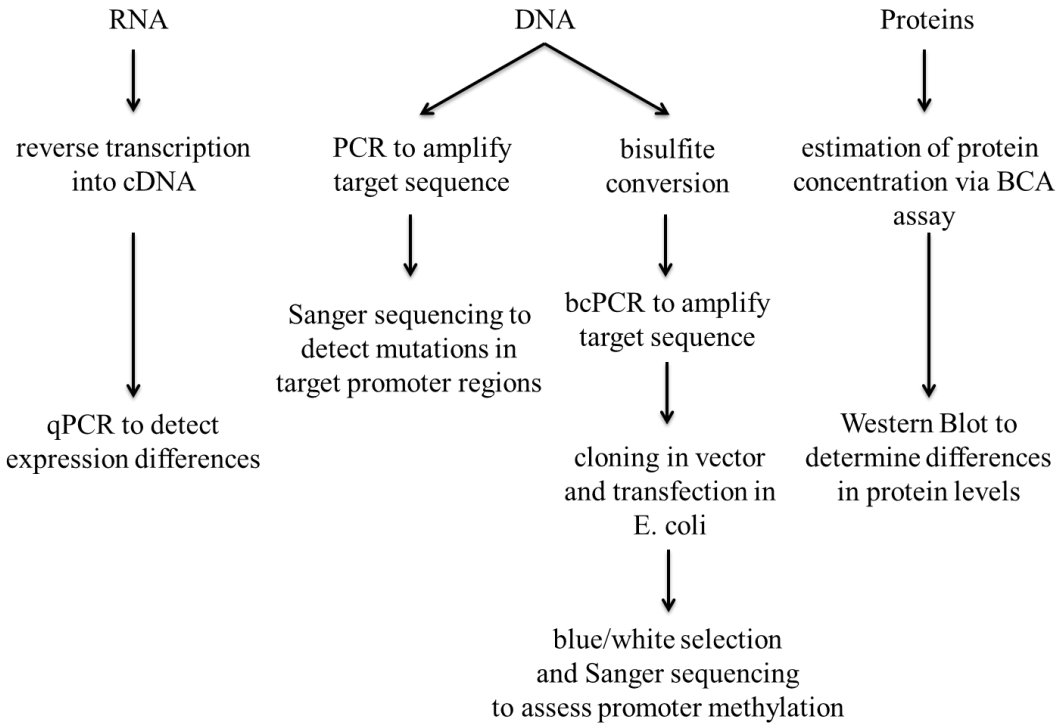


Fig. 11: Workflow depicting the conducted procedures after extraction of RNA, DNA and proteins to infer from methylation status to gene transcription.

Tissue collection and micropunching: Harvested brains were cut from rostral to caudal into 200µm slices using a cryostat (MH50, Microm, Walldorf, Germany) and mounted to Superfrost microscope slides (Menzel, Braunschweig, Germany). Desired brain regions (tab. 5) were acquired from the slices by micropuncture through a method described by Palkovites (1973) utilizing autoclaved punchers with a diameter of either 0.5mm or 1.0mm (Fine Science Tools, Heidelberg, Germany).

Brain region	Interaural (mm)	Bregma (mm)	Ø of puncher (mm)
cingulate cortex	4.90 - 3.58	1.10 to -0.22	1.0
basolateral amygdala	3.22 - 1.98	-0.58 to -1.82	0.5
paraventricular nucleus of the hypothalamus	3.22 - 2.86	-0.58 to -0.94	0.5
locus caeruleus	-1.54 to -1.88	-5.34 to -5.68	0.5

Tab. 5: Stereotaxic coordinates of brain regions extracted via micropunching. Coordinates originate from Allen Brain Atlas 2nd edition.

These tissue punches were used either for the extraction of proteins or simultaneous extraction of RNA and DNA.

Simultaneous extraction of DNA and RNA: To give a reliable statement about the relation of promoter methylation and gene expression, it is of highest importance to extract DNA and RNA simultaneously from the desired tissue. Therefore, we employed a protocol established by Bettscheider et al. (2011): tissue punches were homogenized in 400µl of guanidinium thiocyanate buffer (4.5M guanidium thiocyanate, 2% N-lauroylsarcosine, 50mM EDTA pH 8, 25mM Tris-HCl pH 7.5, 0.1M beta-mercaptoethanol, 0.2% antifoam A) by passing five times through a hypodermic syringe (30G) and split in equal parts for RNA and DNA isolation.

RNA extraction: 20µl of 3M sodium acetate pH 5.2, 200µl of acidic phenol (product number A980.3, Carl Roth) and 100µl of chloroform:isoamyl alcohol (24:1) were added to every sample and mixed vigorously. Samples were incubated on ice for 10min. and subsequently centrifuged for 20min. at 4°C with 13000rpm. Aqueous phase was transferred into a new sterile 1.5ml Eppendorf tube and 350µl of 70% ethanol were added. Samples were transferred into spin columns (RNeasy mini Kit, Qiagen GmbH) and further steps were performed according to the manufacturer's protocol. Samples were stored at -80°C until further used.

DNA extraction: 200µl of Buffer AL and 200µl of 100% ethanol were added to every sample and mixed vigorously. Samples were transferred into spin columns and further steps were performed according to manufacturer's protocol (Qiagen, DNeasy Blood and Tissue Kit). Samples were eluted with 200µl of pre-warmed (70°C) Buffer AE to maximize yield. To increase DNA concentration, all samples were vacuum-centrifuged (Speed Vac Plus SC210A, Savant, Bachofer Laboratoriumsgeräte, Germany) until the remaining liquid evaporated and finally resuspended in 20µl of water. Samples were stored at -20°C until further used.

Reverse transcription: 100ng of extracted total RNA was reverse transcribed to complementary DNA (cDNA) using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Darmstadt, Germany) according to manufacturer's instructions. Reverse transcription uses a retroviral enzyme called "reverse transcriptase" to generate cDNA from a RNA template by using random primers to amplify all existing RNA.

Quantitative real-time PCR: The following genes (tab. 6) were analyzed by utilizing the QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions:

Gene symbol	Region	Orien-tation	Primer sequence 5'- 3'	Product size
Avp	PVN	forward reverse	TCG CCA GGA TGC TCA ACA C TTG GTC CGA AGC AGC GTC	164
Crhr1	BLA	forward reverse	GCC CCA TGA TCC TGG TCC TGC CCA TCG CCG CCA CCT CTT CC	187
Crh	BLA	forward reverse	GCA GTG CGG GCT CAC CTA CC GGC AGG CAG GAC GAC AGA GC	109
Dbh	LC	forward reverse	AGA GAG CCC CTT CCC CTA CCA CAT C TTT CCG GTC ACT CCA GGC ATC	232
Npsr1	BLA	forward reverse	CTC TTC ACT GAG GTG GGC TC CCA GTC CTT CAG TGA ACG TC	196
Tmem132d	CG	forward reverse	CAT CCC TTC TTC AGC CAG AG AGT GAG AAC CGC TGA ATG CT	187
Ucn	BLA	forward reverse	CAC TGG GCA GAC ACT CCG GCA GCC AGT GGA GCC C	121

Tab. 6: List of genes analyzed via quantitative real-time PCR.

Primer pairs were designed using "Primer 3" (Rozen and Skaletzky, 2000) with default settings except "primers must span an exon-exon junction" and "Mus musculus (taxid: 10090)" was selected as organism. Spanning of an exon-exon junction is important to avoid co-amplification of genomic DNA. Experiments were performed in duplicates on the Lightcycler®2.0 instrument (Roche Diagnostics, Mannheim, Germany) under the following

PCR conditions: 10min at 95°C for initial denaturation, followed by 40 cycles of denaturation (95°C for 10s) and a combined annealing and extension phase (60°C for 30s). To ensure the quality of the PCR product, a melting curve (50-95°C with 0.1°C/s) was generated at the end of every run. LightCycler® Software 4.05 (Roche Diagnostics) calculated crossing points (CP) by utilizing the absolute quantification fit points method. For this, noise band and threshold were set to the same level in all compared runs (Czibere, 2008). Relative gene expression was determined by the comparative Ct method (Livak and Schmittgen, 2001) and CPs were normalized to the housekeeping genes *Pol13*, *B2mg* and *Rflp13a* or any combination thereof. If expression differences were confirmed for a gene, proteins were extracted from tissue punches to perform WB to evaluate whether expression differences ensued differences in protein quantity.

Protein extraction: Proteins were extracted from tissue punches either as total fraction using an in-house protocol or separated by nuclear, mitochondrial and cytosolic fraction with a method described earlier by Djordjevic et al. (2009).

Total protein extraction: Punches were homogenized in 200µl of ice-cold RIPA buffer (R0278, Sigma-Aldrich, Hamburg, Germany) comprising 2µl of both, protease and phosphatase inhibitor cocktail (P8340 and P0044, Sigma Aldrich, Hamburg, Germany). Homogenization was achieved by passing five times through a hypodermic syringe (30G). Afterwards, samples were put on ice for 5 minutes before centrifugation was performed with 8000g for 10min at 4°C to pellet the cell debris. Supernatant (proteins) was transferred to an ice-cold new 1.5ml Eppendorf tube and stored at -20°C until further used.

Protein extraction by fractions: Frozen tissues punches from every sample were homogenized in 200µl of ice-cold homogenization buffer comprising 2µl of each, protease and phosphatase inhibitor cocktail (P8340 and P0044, Sigma-Aldrich, Hamburg, Germany). Inhibitors were added just before use and homogenization was acquired by passing five times through a hypodermic syringe (30G). Subsequently, samples were centrifuged at 2000rpm for 10min. at 4°C to collect the nuclear pellet. Supernatant was further centrifuged at 20000g for 30min. to provide a crude mitochondrial pellet. The resulting supernatant of this centrifugation was filled with homogenization buffer up to 500µl and ultracentrifuged at 105000g for 1h to separate the membrane from the cytoplasmic fraction (Optima XL 90, Beckman Coulter, Rotor SW55, Tubes 344090 and adaptor 356860). Nuclear pellets were washed three times in 0.5ml of homogenization buffer and resuspended in homogenization buffer with 0.5M KCl. Samples were incubated for 1h on ice (with frequent vortexing) and centrifuged for 10min with 8000rpm at 4°C. The resulting supernatant was used as nuclear extract. The crude mitochondrial pellets were washed three times in 0.5ml of homogenization buffer and centrifuged at 20000g for 30min., lysed in lysis buffer comprising 2µl of each, protease and phosphatase inhibitor cocktail (P8340 and P0044, Sigma-Aldrich, Hamburg, Germany) and incubated on ice for 1.5h with frequent vortexing. The resulting fraction was used as a final mitochondrial extract. Respective fractions were stored at -20°C until further used.

Homogenization buffer		**Lysis buffer**	
Substance	**Conc.**	**Substance**	**Conc.**
Tris-HCl, pH 7.2	20mM	Tris-HCl pH 7.4	50mM
Glycerol	10%	glycerol	5%
NaCl	50mM	EDTA	1mM
EDTA	1mM	DTT	5mM
EGTA	1mM	Triton X-100	0.05%
DTT	2mM		

Estimation of protein concentration: Protein concentration was estimated by utilizing the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Illinois, USA). All samples were measured in triplicates to increase accuracy and reliability of the obtained protein concentrations. Per sample, 200µl of reagent A and 4µl of reagent B were mixed to obtain a working solution. 10µl of both, BSA standards and samples (protein solution) were pipetted in a 96-well tissue culture test plate (Product number 92696, Techno Plastic Products, Trasadingen, Switzerland). 200µl of working solution was added to all samples and the 96-well plate was incubated for 30min. at 37°C. Samples were measured at 562nm in an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR7000).

Western Blot: Western blots were performed in protein blotting cells from Bio-Rad (Mini Protean Tetra Cell, Munich, Germany). Prior to use, samples were diluted to the same protein concentration and 20% of final volume of 5x protein loading buffer (PLB) was added. Samples were heated for 5min. at 95°C and 20µg of protein per sample was loaded to perform sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) page at 4°C (electrophoresis buffer: Tris 25mM, glycine 192mM) for 15min. with 70V followed by 90min. with 120V. Subsequently, samples were blotted (blotting buffer: Tris 25mM, glycine 190mM, 20% methanol) at 4°C for 60min. with 400mA on a nitrocellulose membrane (Protran, Whatman, Dassel, Germany). Transfer of proteins from the SDS gel to the nitrocellulose membrane was verified by incubation with Ponceau S solution (Applichem, Darmstadt, Germany) for 3min on a shaker (Mini Rocker MR-1, Peqlab, Erlangen, Germany). Next, membrane was incubated for 60min. in 50ml of 5% milk solution in Tris-buffered saline with 0.1% Tween (TBST) to dissociate Poinceau S solution from the positive charged amino-groups and to block unsaturated binding sites for proteins. The protein ladder (Page Ruler prestained protein ladder, Fermentas, Leon-Rot, Germany) was used to determine the exact position for cutting the membranes in two parts to separate protein of interest and housekeeping or marker protein, respectively. Membranes were each incubated overnight (O/N) with 10ml of 2.5% milk solution in TBST comprising the respective primary antibody:

Primary antibody	Application	Dilution	Molecular weight (kD)	Host	Type
GAPDH	housekeeper	1:4000	35	goat	polyclonal IgG
H3(Lys9)	nuclear marker	1:2500	17	rabbit	polyclonal IgG
α-Tubulin	cytosolic marker	1:1000	55	mouse	monoclonal IgG
CRHR1	protein of interest	1:2500	48	goat	polyclonal IgG
GR	protein of interest	1:500	95	rabbit	polyclonal IgG

Next, membranes were washed three times by shaking in TBST to remove unbound primary antibody and incubated each for 2h at RT with 10ml of 2.5% milk solution in TBST comprising the respective secondary antibody:

Secondary antibody	Dilution	Nature
GAPDH	1:10000	donkey anti-goat IgG-HRP
H3(Lys9)		goat anti-rabbit IgG-HRP
α-Tubulin		goat anti-mouse IgG-HRP (F_{ab} specific)
CRHR1		donkey anti-goat IgG-HRP
GR		goat anti-rabbit IgG-HRP

Again, membranes were washed three times by shaking in TBST to remove unbound secondary antibody. Membranes were incubated with 33ml of enhanced

chemiluminescence (ECL) solution for 2min on a shaker. ECL solution was prepared just before use by mixing 30ml of solution A (0.1M Tris, 50mg luminol per liter), 3ml of solution B (DMSO, 0.11% para-hydroxycoumarin acid) and 11µl of hydrogen peroxide (H_2O_2). All secondary antibodies were conjugated to horseradish peroxidase (HRP), which catalyzed the oxidation of luminol to 3-aminophthalate under the presence of H_2O_2. This reaction caused chemoluminescent emission of light at 428nm, which was further enhanced by the presence of para-hydroxycoumarin acid up to several 100-fold (Carlsson et al., 2005). The intensity of emitted light correlates with the amount of "protein-primary-secondary-antibody-HRP-complex". Proteins were visualized using the ChemiDoc MP system and finally quantified via ImageLab 4.0 software (Bio-Rad, Munich, Germany).

Analysis of mutations in the promoter of *Crhr1*: To unambiguously attribute changes in gene expression to differences in promoter methylation, we performed Sanger sequencing (tab. 7) for the proximate promoter region (-2069bp to +93bp relative to TSS, NCBIM 37) to detect SNPs and indels (insertions and deletions):

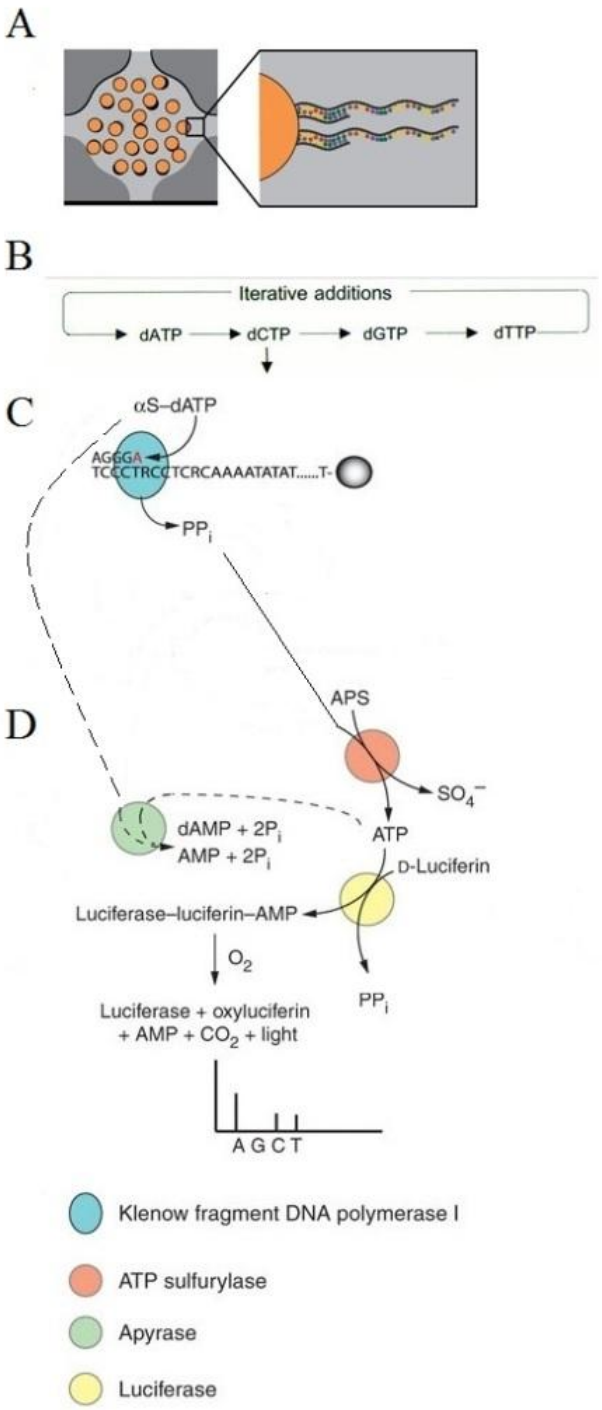
Region amplified	Orientation	Primer sequence 5'- 3'	Product size
A (-2069 to -1584)	forward	GCCCACTCTATCTTGATGAT	485
	reverse	CCTCCTTCCTAATTCCCAAC	
B (-1732 to -1136)	forward	CTTCAGGACTTTGCTTCACTG	596
	reverse	TTCTAATTCCACTTCCAGCC	
C (-1296 to -738)	forward	CCTGAGAGGTGAAGATGTTTC	558
	reverse	CAATTTAGTGGGGAGGGGAG	
D (-954 to -263)	forward	CCGCTGTCACCACTTATCTT	691
	reverse	TCGTGTCCCCTCCTCTTTCT	
E (-479 to 93)	forward	TTTTCCCTAGCTGCGGTGGC	572
	reverse	GTCCTCTCTTACCTTCACGA	

Tab. 7: Primers used to monitor the existence of mutations, which might influence gene transcription of *Crhr1*. (corticotropin-releasing hormone receptor 1).

These mutations occur regularly within the genome and are well known to alter gene expression and this fact corroborates the necessity to determine their possible regulatory effects.

Analysis of promoter methylation: We employed two different techniques to analyze promoter methylation of *Crhr1*: pyrosequencing and bisulfite sequencing of a bacterial vector carrying the region of interest.

Pyrosequencing: This technique is based on the "sequencing by synthesis" principle, i.e. the desired sequence can be analyzed by light emission due to the iterative incorporation of complementary nucleotides into the template: single-stranded DNA (ssDNA) of the region of interest, generated by a first round of PCR. The intensity of emitted light represents the number of identical nucleotides in a row within the analyzed sequence (e.g. light emission of a GG peak would be ca. double the height compared to a GC, GA or GT peak). Therefore, 12-15µl of the respective template was immobilized to 2µl Streptavidin Sepharose (GE Healthcare, Munich, Germany) followed by annealing to 0.8 - 1.0µl of the respective biotinylated sequencing primer (5µM) for 2min. at 80°C (fig. 12a). To allow light emission by incorporation of a complementary nucleotide, the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase were added concomitantly with the substrates adenosine phosphosulfate (APS) and luciferin. The sequencing reaction *per se* was started by addition of one of the four deoxynucleoside triphosphates (dNTPs) (fig. 12b), which was incorporated by the Klenow fragment of DNA polymerase I at the 3'-end



of the pyrosequencing primer and led to the release of PPi (fig. 12c). This, in turn, converted APS into ATP, which provided the energy to form an unstable luciferase–luciferin–AMP complex. In the presence of oxygen, light was released proportional to the available amount of ATP and thus PPi (fig. 12d). Apyrase degraded unincorporated α-S- dATP and the sequencing reaction was continued by adding a different dNTP. A methylated CpG site is represented as "R" in the template sequence. The ratio of incorporated C (methylated cytosine before bisulfite treatment) or T (unmethylated cytosine before bisulfite treatment) yields the methylation degree at this position (fig. 12 C and D) (Gharizadeh et al., 2001; Tost and Gut 2007). The following primers and PCR routines were designed by Varionostic GmbH. The CpGi ranging from -1336bp to +1323bp comprised 186 CpG sites in total and was sequenced with eight biotinylated primers (P1-8). Every region amplified by a biotinylated primer was sequenced finally with 1-4 sequencing primers (S1-4), generating a nomenclature like P1S1, P1S2, P1S3, P2S1 etc. Due to the short read length (ca. 100bp) of pyrosequencing, several sequencing primers are needed per analyzed region, i.e. per biotinylated primer (average length of ca. 350bp). The nucleotide adenine of the start codon ATG was counted as +1bp, the first nucleotide before adenine was counted as -1bp (tab. 8).

Fig. 12: Pyrosequencing is based on the iterative addition of dNTPs causing a release of PPi, which in turn converted APS into ATP to provide the energy to form an unstable luciferase-luciferin-AMP complex. In the presence of oxygen, light is released proportional to the amount of ATP. Abbreviation: AMP (adenosine monophosphate), APS (adenosine phosphosulfate), (dNTP (deoxynucleoside), PPi (pyrophosphate). Adapted and modified from Tost and Gut, 2007.

Region sequenced	Assay	Orientation	Sequencing primer (Biotin = B)	Product length	CpGs covered
-1412 to -998	P1	forward reverse	gttggttttttattagga B-aaccaactaaacacctaatcta	414	1-18
-1027 to -724	P2	forward reverse	B-ttttttatagattaggtgtttag actaaaaactacatttaaataattc	303	19-35
-757 to -560	P3	forward reverse	B-tagggagggaattattt aaacaacctttcttctctaa	197	36-43
-588 to -216	P4	forward reverse	ataggaggttagagaagaaa B-cccacaactacctctctc	372	44-75
-247 to +70	P5	forward reverse	gggatttaggtaggagaga B-aacccctctaattaccc	317	76-104
+24 to +362	P6	forward reverse	gttagtgaaggtaagagagga B-atcccatccaaaacct	339	105-130
+348 to +735	P7	forward reverse	ggttttggatgggattt B-aactaccaaacacctaactctt	388	131-160
+700 to +1164	P8	forward reverse	ggatagagttagggaagagtta B-tccaacccttaaattcacta	465	161-186

Tab. 8: List of sequencing primers used for pyrosequencing of *Crhr1*. (corticotropin-releasing hormone receptor 1)

Varionostic GmbH (Ulm, Germany) used the Q24 system (Qiagen, Hilden, Germany) to perform pyrosequencing and the PyroMark Q24 software to conduct analyses of CpG sites.

Bisulfite sequencing of clones: This technique is considered as the gold standard to analyze bisulfite modified DNA (Mikeska et al., 2010). It utilizes the different conversion of unmethylated and methylated cytosine residues when DNA is treated with sodium bisulfite: the CH_3-group attached to the 5th carbon atom protects the NH_2-group from deamination, whereas unmethylated cytosine residues are converted to uracil (fig. 13a). During PCR, uracil is replaced by the nucleotide thymine and this evolved difference in sequence can be subsequently determined by Sanger sequencing (fig. 13b).

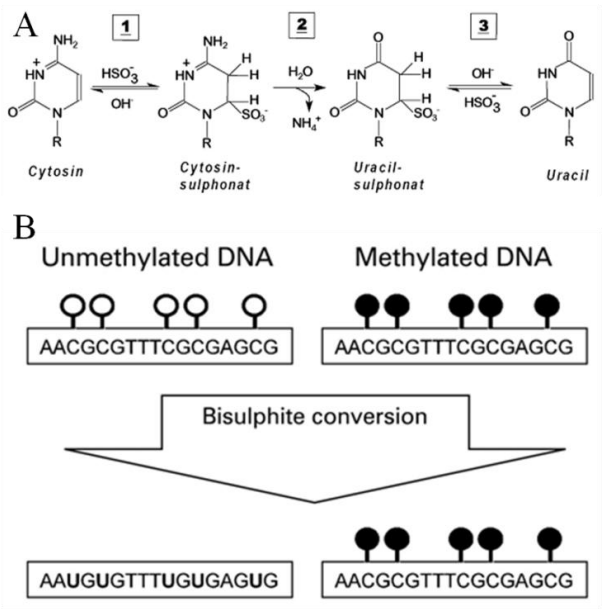


Fig. 13: Treatment with sodium bisulfite deaminates cytosine to Uracil (a), which can be sequenced subsequently to determine methylation status due to different nucleotide sequences (b). A adapted and modified from Schumacher, 2007. B adapted and modified from Hung et al., 2009.

Bisulfite conversion and bcPCR: We used the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol to convert extracted DNA from tissue punches. To amplify the complete CpGi, we designed primers using BiSearch (Tusnády et al., 2005) with default settings except:

primer length	**min. 20**	**opt. 25**	**max. 30**
melting temp.	min. 55	opt. 60	max. 65
Max. Tm diff.		3°C	
Database		Mus musculus	

The following list depicts all primers used to amplify the complete CpGi. Drawbacks of bisulfite conversion to differentiate between methylated and unmethylated cytosine residues are: i) the harsh conditions during bisulfite conversion were DNA was randomly fragmented to an average length of 300-400bp, giving rise to the necessity to utilize several overlapping primer pairs for Sanger sequencing of clones, ii) the reduced complexity of the genome due to the presence of merely three instead of four different nucleotides (cytosines are converted to uracil and finally to thymine after PCR) and iii) the necessity to create primers with so called wobble positions, i.e. a primer containing a CpG site mustn't favor methylated or unmethylated sites to avoid an amplification bias. For this reason, a mismatch denoted as "Y" was created using cytosine or thymine at this position. (tab. 9) To verify correct amplifications of the respective amplicons, we performed agarose gel electrophoresis.

Region sequenced	**Orientation**	**Primer sequence 5'- 3'**	**Product length**	**CpGs covered**
-1505bp to -1247bp	forward reverse	ATTTTGTTTAGTGTGTTGAG ATTCTTTTAATTTCCTTCCC	258	1-3
-1471bp to -1180bp	forward reverse	AAGGGGAGTTGTATAAAGTA TAACTTTCTAATTCCACTTC	291	1-4
-1345bp to -965bp	forward reverse	TTTGAGAGGTGAAGATGTTTT TCTTTTTAATCCAAACCCCA	380	2-19
-1198bp to -856bp	forward reverse	AAGTGGAATTAGAAAGTTAA CCTAAAATATACTAAAACACT	342	5-26
-981bp to -732bp	forward reverse	GGTTTGGATTAAAAAGATAG ACTACATTTAAATAATTCCC	249	20-34
-875bp to -557bp	forward reverse	GTGTTTTAGTATATTTTAGGTG AAACAAACAACCTTTCTTCT	318	27-42
-576bp to -225bp	forward reverse	AGAAGAAAGGTTGTTTGTTT TACCTCTCTCCTACCTAAATC	352	43-74
-398bp to -8bp	forward reverse	AGGAGATTGGAGTTTGTAG TCACTCTATCAACATCCTAA	390	61-96
-171bp to 215bp	forward reverse	GAGTAAGAGTTTGTTGGTGG AATTATCCCTCTATCTCCAA	387	79-118
105bp to 522bp	forward reverse	GGGTGTTGGAGGAGAGGATT GCACACCATCACCTCTCAAAA	418	111-144
347bp to 675bp	forward reverse	AGGTTTTGGATGGGATTTTG CCCAACTTTAACCAATAAACACTA	329	130-155
502bp to 836bp	forward reverse	AGGTTTTGGATGGGATTTTG CCCAACTTTAACCAATAAACACTA	335	143-171

622bp to 957bp	forward reverse	GAGTTTTAGAAAGTTTTTAG CTTTCCTAACCACAATTAAC	335	155-178
798bp to 1150bp	forward reverse	GGGTATTAGTATTTTAGTTTTGG TCACTAAAACTCCCTTAAAT	352	172-186

Tab. 9: Primers used to identify the methylation of *Crhr1* via bisulfite sequencing of clones. (corticotropin-releasing hormone receptor 1).

Clean-up and subsequent ligation: Amplicon was run on a 1.5% agarose gel to verify correct product size and subsequently cleaned up using the Nucleo Spin Extract II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Removal of all fragments shorter than the desired product was controlled on a 1.5% agarose gel. Next, the amplicon was cloned into the pGEM® T-vector system I (Promega, Mannheim, Germany) according to the manufacturer's instructions (samples were incubated overnight together with amplicon in a 1.5ml Eppendorf tube to increase maximum number of transformants). 10µl of overnight incubated pGEM® T-vectors were purified by an in-house protocol: 20µl of 100% ethanol, 1µl glycogen (10mg/ml) and 1µl of sodium acetate 3M pH 5.2 were added before samples were shock-frozen for 30s on dry ice and centrifuged for 15min. at 4°C with maximum speed. Supernatant was removed carefully with a pipette, 200µl of 100% ethanol were added and samples were centrifuged for 15min. at 4°C with maximum speed. This washing step was repeated with 70% ethanol and supernatant was removed carefully with a pipette. 1.5ml Eppendorf tubes containing the pGEM® T-vectors were dried at RT until remaining liquid was evaporated. Cleaned-up vectors were resuspended in 10µl of water and stored on ice to prepare lysogeny broth (LB) agar plates for following transfection and blue/white selection.

Transfection and blue/white selection: Petri dishes (100 x 15mm) were prepared the day before by filling ca. 25ml of LB-Lennox agar pH 7 comprising 100mg of ampicillin sodium salt (Sigma-Aldrich, Hamburg, Germany) into them. This LB-Lennox agar was autoclaved for 2h (Vakulab S3000, Münchner Medizin Mechanik GmbH, Germany) and 2ml of an ampicillin stock solution (50mg/ml) was added when LB-Lennox agar cooled down to ca. 50-60°C to prevent degradation of ampicillin. These prepared LG agar plates were covered with 100µl of a blue/white selection mix using a Drygalski spatle and petri dish rotary plate (petri turn-M, Schuett-biotec.de, Germany):

blue/white selection mix

Substance	Volume (µl)
RNAse free H_2O	52,5
DMSO	20
IPTG, 100mM	15
x-Gal, ready to use solution	12,5

Petri dishes prepared for blue/white selection were stored upside down for 45min. at 37°C. We used highly competent JM109 cells from Promega for transfection (genotype: recA1, endA1, gyrA96, thi, hsdR17 (rK–,mK+), relA1, supE44, Δ(lac-proAB), [F´, traD36, proAB, lacI qZΔM15]). Competent cells were thawed on ice and 100µl of cells were added to 5µl of every sample (pGEM® T-vectors carrying the amplicon) and gently mixed by tapping. Samples were incubated on ice for 30min. to increase transfection efficiency. Samples, i.e. competent cells and pGEM® T-vectors carrying the amplicon, received a heat shock at 42°C for 45s and were allowed to recover 2min. on ice to reduce damage to the competent cells. 250µl of pre-warmed (37°C) SOB medium without antibiotic were added to every sample and incubated on a thermomixer (model 5436, Eppendorf, Hamburg,

Deutschland) for 1h at 37°C with 250rpm. Transfected cells were gently resuspended by tapping and 120µl were spread on LB-Lennox plates prepared with blue/white selection mix as described before. JM109 cells were grown overnight at 37°C and positive colonies were picked about 12-16 hours later. pGEM® T-vector confers resistance to ampicillin to the competent cells to enable only growth of those cells, which were successfully transfected. The vector also contains a *lacZ* gene, which enables the JM109 cells to use the x-Gal substrate and confers them a blue appearance. If the amplicon was ligated successfully into the pGEM® T-vector, the *lacZ* gene was disrupted and the colonies appeared white instead of blue. Since white colonies still might carry no or a wrong insert (due to inserted primer dimers, linearized vectors etc.) we performed colony PCR to unambiguously identify vectors harboring the desired amplicon.

Colony PCR and bisulfite sequencing: Thus, white colonies were picked and transferred to a non-skirted 96-well plate (Thermofast 96, Thermo Scientific, Illinois, USA) containing the following PCR mix per sample:

Substance	Primer sequence 5'- 3'	Volume (µl)
$MgCl_2$ (25mM)		3
Taq Buffer with $(NH_4)_2SO_4$		2,5
dNTPs (10mM each)		1,5
T7 primer (4µM)	TAA TAC GAC TCA CTA TAG GG	1,5
SP6 primer (4µM)	ATT TAG GTG ACA CTA TAG	1,5
Taq polymerase 1U/µl		1
RNAse free H_2O		14

PCR was performed under the following conditions: initial denaturation for 5min at 94°C, followed by 5 cycles of denaturation (94°C for 30s), annealing (56°C for 30s) and an extension phase (72°C for 45s). Next, we performed 35 cycles of denaturation (94°C for 30s), annealing (48°C for 30s) and an extension phase (72°C for 45s). Final elongation was performed for 10min. at 70°C. For clean-up, samples were transferred to a NucleoFast 96 PCR plate (Macherey-Nagel, Düren, Germany) and 100µl of RNase-free water was added to every sample. Centrifugation was performed at 9°C with 4.500g for 10min. (Heraeus Multifuge 4KR, Thermo Fisher Scientific, Waltham, MA). Flow-through was discarded and washing step was repeated. Samples were resolved by shaking 10min. on a thermomixer with 25µl of RNAse-free H2O. Sequencing reaction was performed by adding the following substances to a ThermoFast 96 PCR plate (ABgene, Hamburg, Germany):

Substance	**Composition**	**Volume (µl)**
sequencing buffer	Tris 350mM, pH 8.8 $MgCl_2$ 2.5mM	1
Big Dye Terminator v3.1		0.5
T7 primer (forward) or SP6 primer (reverse)		1
sample		2.5

Big Dye Terminator v3.1 cycle sequencing kit (Life Technologies, Darmstadt, Germany) was used to perform PCR under the following conditions: initial denaturation for 1min at 96°C, followed by 35 cycles of denaturation (96°C for 10s), annealing (50°C for 5s) and an extension phase (60°C for 240s). Samples were transferred to a Montage SEQ96 plate (Millipore, Billerica, MA) and cleaned-up by washing twice with 20µl of injection solution (Millipore, California, USA) via a vacuum pump (Biomek 2000 Laboratory Automation

Workstation, Beckman Coulter, Fullerton, CA) for 5 min. Samples were resuspended in 20µl of RNAse free water by shaking for 10 minutes (Biomek 2000) and sequenced in the genome analysis center located within the Helmholtz Center (Neuherberg, Germany).

Radio immunoassay (RIA): 10µl of blood plasma were used to determine the concentration of CORT via a RIA kit from MP Biomedicals (article number 07120103, Solon, Ohio, USA) according to the manufacturer's instructions, except by diluting all samples 1:200, basal and reactive samples were diluted 1:13.5 and 1:100, respectively. All samples were measured in duplicates. Intra- and inter-assay coefficients were below 10%. RIA is based on the competition between ^{125}I-labeled and non-radioactive-labeled CORT within the samples for a limited amount of binding sites – the higher the CORT concentration in the samples, the fewer binding sites can be bound by the radioactive-labeled CORT. Secondary antibody was added in excess and unbound antigen was decanted after centrifugation. Radioactivity within the precipitate was measured via a gamma-counter.

3.4 Pharmacological manipulation: We used pharmacological treatment to elucidate whether the anxiolytic phenotype elicited by EE can be mimicked pharmacologically. To achieve this, we used two different approaches: firstly, we used a CRHR1 antagonist known as a classical target from psychiatric research and, secondly by injecting drugs known to exert epigenetic alterations to reduce anxiety-related and depression like behavior.

CRHR1 antagonist: 24 Male mice were housed under standard conditions in groups of three until week 10. Subsequently, animals were transferred next to the testing room and habituated for 3d before they were single-housed and randomly assigned either to the treatment or vehicle group. Six animals per day underwent surgery by two trained and skilled persons, implicating four days of surgery in total (PND 73-76). For this purpose, mice were anesthetized for 10s with Forene and quickly fixed into a stereotaxic apparatus (Technical & Scientific Equipment GmbH, Bad Homburg, Germany). Anesthesia was continued via a self-made face mask supplying the animals with an oxygen/forene mixture. In addition, each animal received 0.1ml Metacam (Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany) subcutaneously and additionally 100µl/200ml in drinking water to minimize post-surgical pain. Prior to surgery, eyes were covered with a thin layer of panthenol eye ointment (Jenapharm, Jena, Germany) to avert searing. Fur on the head was removed via an electrical shaver (Wahl GmbH, Unterkirnach, Germany), followed by disinfection of the exposed skin (Kodan, Schülke & Mayr GmbH, Norderstedt, Germany). Afterwards, fur was transected longitudinally with a scalpel starting medial behind the eyes to the beginning of the neck. Incision was kept as short as possible, approximately 2-3cm. Bregma and Lambda were visualized via a cotton bud soaked with 3% H2O2. Exact coordinates of Bregma were determined using a magnifying glass and a guide cannula (Microlance canula 21G, BD Bioscience, Heidelberg, Germany) was implanted bilaterally in the BLA (-1.00mm rostral, ±3mm lateral and -4.00mm dorsoventral; mouse brain in stereotaxic coordinates 2nd ed., 2001). Holes for implanting the two guide cannulas and one screw for stabilizing purposes were drilled via a drephine (EWL type 970 K9, Kavo Dental GmbH, Biberach/Riß, Germany). The guide cannulas and screw were connected and fixed with multiple layers of dental cement (Kallokryl, Dr. Speier GmbH, Münster, Germany). After 5-10min. of hardening, animals were removed from the stereotaxic apparatus, transferred back to their home cage and were allowed to recover for 4 days. Starting on PND 77, animals were injected three times within a time interval of 36h either with 2µl of CRHR1 antagonist (α-helical CRF 9-41, Sigma-Aldrich, Hamburg, Germany) or vehicle (ringer solution) bilaterally in the BLA. Therefore, a glass

fiber (Polymicro Technologies, Arizona, USA, ID 74µm and OD 154µm), a priori adjusted to the length necessary to penetrate the BLA, was inserted into the guide cannula. The glass fiber was connected to a tubing (Smiths Medical International Ltd, Haye, USA, ID 0.28mm and OD 0.61mm) filled with an air bubble at the end to determine the amount of delivered liquid.

PND	Time	behavioral testing
77	05:00 p.m.	yes
78	05:00 a.m.	no
	05:00 p.m.	yes

We performed a behavioral battery consisting of OF, EPM and LD to assess anxiety-related behavior either 40min. after a single or after three injections because total amount of antagonist necessary to trigger an effect was unknown. Since both groups, i.e. treatment and vehicle were retested, test-retest reliability should not be compromised. After behavioral testing, animals were killed as described and correct injection into BLA was verified by injecting 1µl of 1% methylene blue B solution (Merck, Darmstadt, Germany) into the guide cannula. Mice were excluded from statistical analysis when the distance of one or both guide cannulas from BLA deviated ≥0.5mm.

Application of epigenetic drugs: Accumulating evidence suggests that epigenetics might constitute the previously missing link among genetics, environment and disease (Barros and Offenbacher, 2009). *Per definitionem*, it comprises heritable changes of molecular factors and processes that regulate genome activity without altering the DNA sequence (Skinner et al, 2010) like methylation of CpG dinucleotides and/or covalent modifications of histones. As a consequence, genes are silenced via a condensed chromatin structure inaccessible for the transcription machinery (Szyf et al, 2008) and these mechanisms allow regulation of gene expression by integrating environmental signals in the genome (Murgatroyd et al, 2009) - Interindividual differences in epigenetic marks would thus result in interindividual phenotypic differences (Szyf et al, 2008). Indeed, distinct profiles of histone acetylation and DNA methylation patterns that arise during the lifetime of monozygotic twins corroborate that environmental influences account for a substantial proportion of the population variance for depression and anxiety (Davis et al, 2009). These differences might contribute to some of their phenotypic discordances and the differential frequency/onset of common diseases (Barros and Offenbacher, 2009). Moreover, the epigenome seems to react to a variety of environmental influences including maternal care and responses to environmental stimuli (Szyf et al., 2008) throughout the whole life span of an individual: fetal development, the plastic phase of early childhood and with increasing evidence in adulthood. Thus, it is of outstanding interest to identify new drugs, which alter the pathological epigenetic state of risk genes for psychiatric disorders. Several new and already known drugs like valproic acid exhibit their effects via epigenetic mechanisms and are tested in clinical trials at the moment. This is true for both, inhibitors of histone deacetylase (HDACi) and DNA methyltransferase (DNTMi) (fig. 14).

Table 2 **Epigenetic drugs**

Target	Drug	Clinical trials
DNA methylation	5-Azacytidine	Phase I/II/III
	5-Aza-2′-deoxycytidine	Phase I/II/III
	FCDR	
	Zebularine	
	Procainamide	
	EGCG	Phase I
	Psammaplin A	
	Antisense oligomers	Phase I
Histone deacetylase	Many[55], including:	
	Phenylbutyric acid	Phase I/II
	SAHA	Phase I/II
	Depsipeptide	Phase I/II
	Valproic acid	Phase I/II

EGCG, epigallocatechin-3-gallate; FCDR, 5-fluoro-2′-deoxycytidine; SAHA, suberoylanilide hydroxamic acid.

Fig. 14: Depicted are epigenetic drugs, which are tested currently in clinical trials.

Interestingly, trichostatin A was shown to exert an anxiolytic (Weaver et al., 2006) and 5-Aza-2'-deoxycytidine an antidepressive (Sales et al., 2011) effect in animal studies. These are striking examples of an epigenetic reprogramming to rescue the behavioral phenotype of mice. Therefore, we injected our animals intraperitoneally (IP) from PND 28-42 three times a week either with valproic acid or 5-Aza-2'-deoxyuridine (both from Sigma-Aldrich, Hamburg, Germany) to investigate whether i) the behavioral phenotype can be shifted from EE to SE or vice versa and ii) whether there is an additive anxiolytic and/or antidepressive effect as a combination of concomitant environmental and pharmacological manipulation (fig. 15).

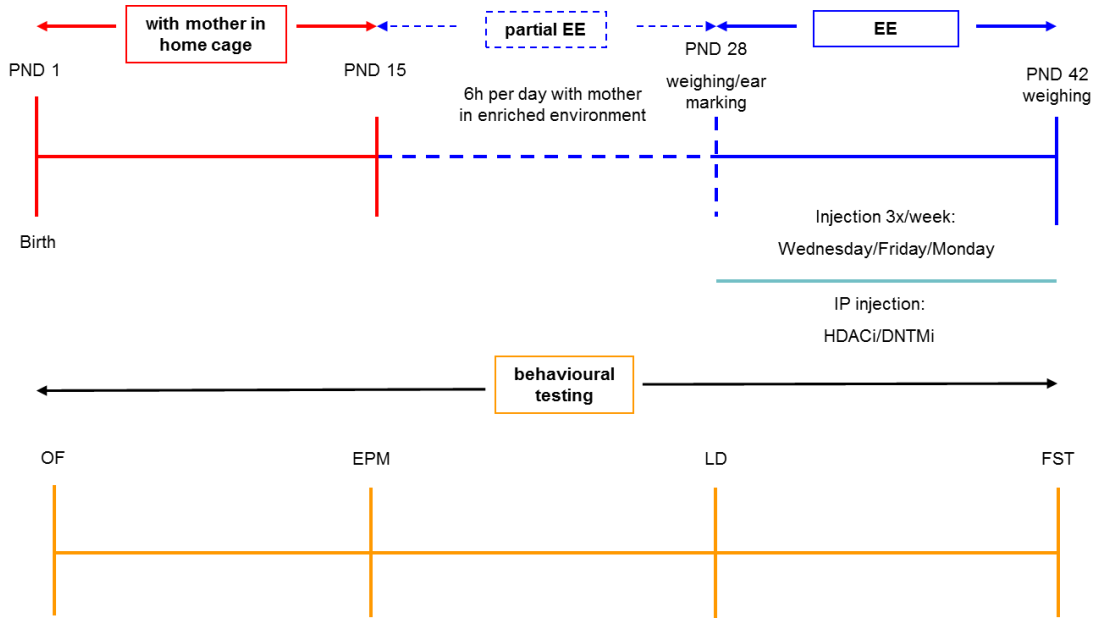


Fig. 15: Time schedule depicting the experimental procedure to assess the effects of treatment with epigenetic drugs.

3.5 Statistical analyses

All data are presented as mean ± SEM and one-, two- or three-way analyses of variance (ANOVAs) were used for analysis, followed by either Tukey or Fisher as *post-hoc* test. Repeated measures ANOVA was used to analyze independent groups for which at least one parameter was measured repeatedly (e.g. home cage activity). Three independent groups were analyzed via Kruskal-Wallis ANOVA (KWA) with Mann-Whitney-U (MWU) as *post*-hoc test followed by Dunn-Šidák correction for multiple testing. When testing multiple times, statistical significance will occur by chance in every 20th test. Thus, one has to correct for multiple testing by adjusting the significance level to counteract this effect. The Dunn-Šidák method uses the following formula to adjust the significance level:

$$1 - (1 - \alpha)^{\frac{1}{n}}$$

Thus, to still reach statistical significance when multiple parameters are tested simultaneously, their p-value must be below the new significance level α of:

number of parameters tested	p-value	Dunn-Šidák significance level
1	0.05	0.05
2		0.025
3		0.017
4		0.013

The Dunn-Šidák method should be preferred over Bonferroni correction for multiple testing due to the high chance of false negatives for the latter (Abdi, 2007). The following table summarizes the used statistical tests:

Statistical test	*Post-hoc* test	Multiple correction	Type of test
MWU	x	Dunn-Šidák	non-parametric
KWA	MWU	Dunn-Šidák	non-parametric
repeated measures ANOVA	Tukey or Fisher	x	parametric
2/3-way ANOVA	Tukey or Fisher	x	parametric
χ^2-test	x	x	sample distribution
Z-score	2/3-way ANOVA	x	parametric

Tab. 10: Summary of statistical tests performed to assess statistical significance.

A trend or significance were accepted when $p \leq 0.1$ or $p \leq 0.05$, respectively. When the Dunn-Šidák correction for multiple testing was used, the new significance level is explicitly mentioned in the test. For the sake of clarity, significance levels mentioned in the text or depicted in graphics are categorized in $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ and are shown as *, ** or *** respectively.

EE was performed several times to assess different behavioral aspects and to evaluate reproducibility and reliability of the obtained data. Meta-analysis was performed by using "z-score", […] "which standardizes observations obtained across experiments and from different cohorts, thereby allowing their compilation and/or comparison. Z-scores are standardized scores, which indicate how many standard deviations (σ, SD) an observation (X) is above or below the mean of a control group (μ)" (Guilloux et al., 2011).

$$Z = \frac{X - \mu}{\sigma}$$

Mouse behavior is multimodal, changes rapidly between emotional states (Ramos et al., 2008) and can only be fully quantified by utilizing multiple behavioral tests covering a wide range of behaviors on several days (Crawley et al., 1997; Crawley and Paylor, 1997). Z-scores allow the integration of several parameters per test, like percent time spent in center (TC), distance travelled in the center (DC), latency to enter center (LCe), total entries light center (EC) and total distance travelled (TD):

$$Zopen\ field = \frac{\left(X - \frac{\mu}{\sigma}\right)TC + \left(X - \frac{\mu}{\sigma}\right)DC + \left(X - \frac{\mu}{\sigma}\right)LCe + \left(X - \frac{\mu}{\sigma}\right)EC + \left(X - \frac{\mu}{\sigma}\right)TD}{number\ of\ parameters}$$

Moreover, it is possible to generate a final score averaging the observed effect sizes of multiple tests (e.g.: OF, EPM, LD, SIH etc.):

$$Zemotionality = \frac{Z(OF) + Z(EPM) + Z(LD) + Z(SIH)}{number\ of\ tests}$$

The directionality of z-scores was adjusted so that a decrease reflects an anxiolytic and an increase an anxiogenic effect, respectively. Importantly, psychiatric disorders like depression are diagnosed by a set of variable symptoms (4-5 out of 10) over an extended time period since changes of emotionality can manifest via different aspects over time (Guilloux et al., 2011). Thus, a method like z-score, taking advantage of several parameters per test and multiple tests reflects the human situation indeed in a more realistic fashion. Z-scores are more resistant to fluctuating behavior ("behavioral noise") by testing whether an experimental group deviates from mean behaviors in converging directions across tests and time. Z-scores were calculated for parameters assessing emotionality and locomotor activity, thereby eliminating the latter as confounding factor (for a review see Guilloux et al., 2011).

We used a 2x3 contingency table to perform a χ^2 goodness of fit test to evaluate whether the sample distribution of effect sizes was shifted after EE. This test is used when data are present as mutually exclusive categorical variables and utilizes observed versus expected frequencies. Thereby, Cramers V is used as a measure of effect size:

Effect size	**Cramers V**
small	V<0.1
medium	0.1≤V≤0.5
high	V≥1.0

Murine gene symbols and the respective mRNA are written in italicized letters with the first letter in capital. Murine peptides and proteins are held in non-italicized capital letters. As human and murine, genes, proteins and peptides apply to the same script conventions, the associated organism is explicitly mentioned in the respective text. The symbols and gene definitions are based on the information provided by the Mouse Genome Database (MGD, Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, ME. World Wide Web URL: http://www.informatics.jax.org; September, 2012) and are subject to change.

All data are presented as bar plots with male HAB mice being displayed as red and female HAB as pink bars. Male and female NAB and CD1 are depicted as dark and light green and grey bars, respectively. Male and female LABs are shown in dark and light blue. In addition, bars of EE animals are hatched, bricks were used for non-responders (NRs) and bars of animals tested for transgenerational inheritance (EESE) are dotted. P-values in tables highlighted in red or blue indicate significance or a trend, respectively. All data were analyzed via Statistica 8 (Statsoft, Hamburg, Germany).

4. Results

4.1.1. Effect of EE on anxiety-related behavior

EE exhibits significant anxiolytic effects in a mouse model with a rigid genetic predisposition resembling pathological anxiety and comorbid depression.

We performed OF, EPM, LD and SIH to assess as many aspects of anxiety-related behavior as possible. All results and effects presented in this section are representative for the beneficial effects of EE and originate from multiple experiments since the variety of tests was never performed in a single experiment.

We observed significant anxiolytic effects of housing on percent time spent in the center ($F_{(1,79)}$ = 17.43; p≤0.05), percent distance travelled in the center ($F_{(1,79)}$ = 17.48; p≤0.001) and total entries into center ($F_{(1,79)}$ = 15.36; p≤0.001) without effects of sex or interaction of housing x sex. No effects were seen for latency to enter the inner zone and importantly, total distance travelled was not different between EE and SE mice, thus excluding locomotion as a masking factor for the observed anxiolytic effects:

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
total distance travelled	0.321	0.802	0.233
latency to enter inner zone	0.844	0.250	0.729
percent time spent in inner zone	0.025	0.350	0.385
percent distance travelled in inner zone	≤0.001	0.741	0.743
total entries inner zone	≤0.001	0.409	0.495

Tukey *post-hoc* tests revealed that EE significantly increased percent distance travelled in the inner zone for both, males (p≤0.01) and females (p≤0.01) and total entries inner zone for males (p≤0.01), whereas females were close to a trend (p = 0.011). Significance of percent time spent in inner zone did not survive *post-hoc* testing with Tukey (fig. 16)

To assess the overall anxiolytic effect of EE on anxiety-related behavior in the OF, we calculated "Z-open field" (fig. 17) by normalizing all measured parameters to z-scores and dividing them by the number of parameters. A 2-way ANOVA ($F_{(1,79)}$ = 15.13; P≤0.001) followed by a Fisher *post-hoc* test revealed that EE significantly reduced anxiety in males (p≤0.01) and females (p≤0.05) by 0.78 SDs and 0.56 SDs below the mean of standard housed mice, respectively.

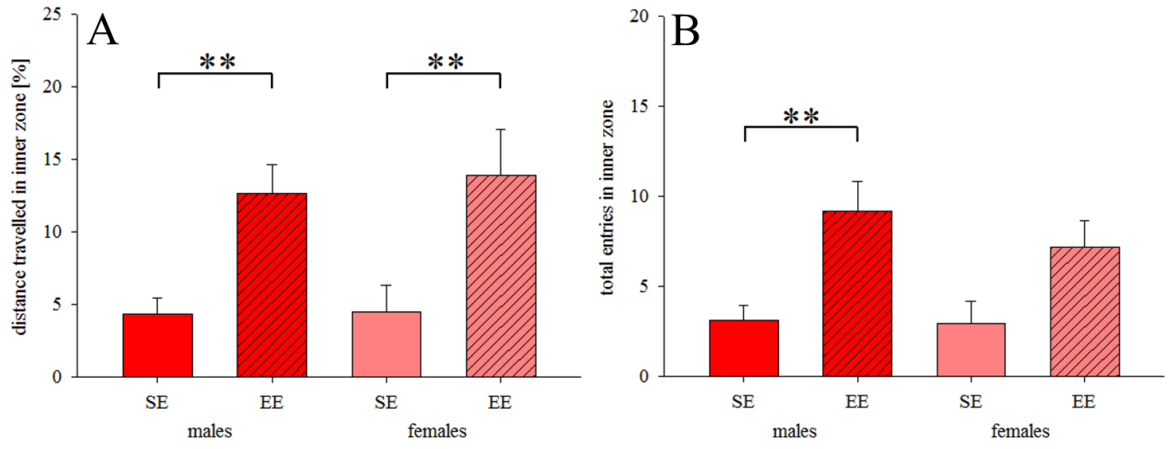


Fig. 16: EE exhibits an anxiolytic effect indicated by an increased distance travelled in the inner zone for both sexes (A), whereas only males entered more often into it. N (males) = 21 (SE, EE); N (females) = 21 SE and 20 EE

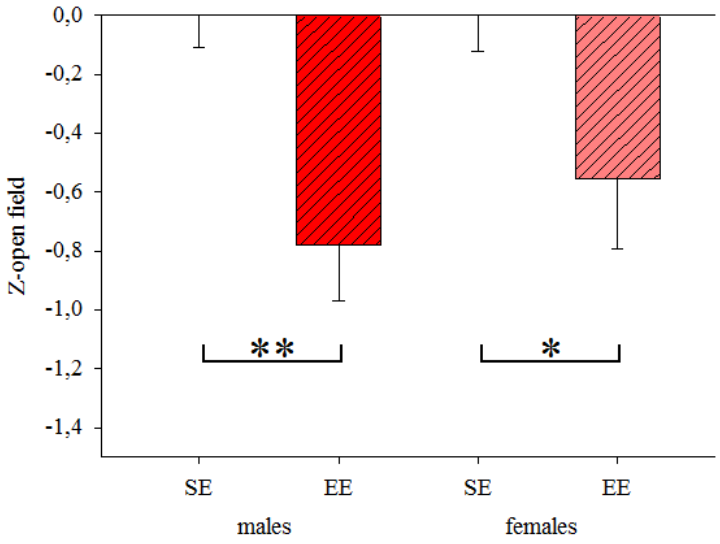


Fig. 17: Z-open field reveals a strong anxiolytic effect of EE for males and females suggested by a decrease of 0.78 SDs and 0.56 SDS below the mean of respective controls. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

The beneficial effect of EE observed in the OF is corroborated by an anxiolytic effect in the EPM. There is a significant effect of housing on percent time spent on the open arms ($F_{(1,66)}$ = 19.17; p≤0.001) and significant effects of sex and housing x sex for percent entries into open arms ($F_{(1,66)}$ = 15.36; p≤0.001) without a difference for total distance travelled ($F_{(1,66)}$ = 0.758; p=0.387).

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
total distance travelled	0.387	0.720	0.685
percent time spent on open arms	≤0.001	0.450	0.830
percent entries into open arms	0.612	≤0.001	0.001

Tukey *post-hoc* tests offered that EE increases the percent time spent on open arms for both sexes (p≤0.05), in contrast to percent entries into open arms whereat only EE females showed a strong trend to enter the open arms more often (p=0.058) but not males (p=0.158) (fig. 18).

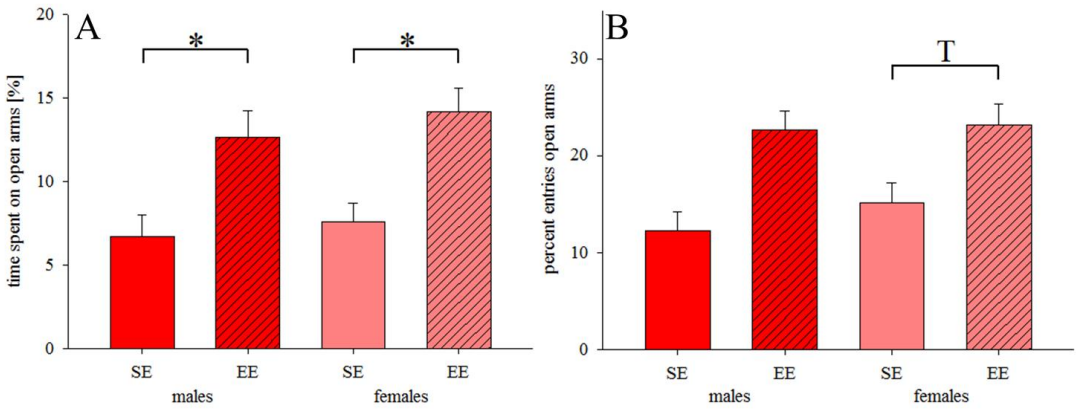


Fig. 18: EE significantly increases "percent time spent on open arms" for both sexes (A), while only EE females offer a trend towards entering the open arms more often (B). N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

2-way ANOVA for Z-elevated plus maze reveals a strong overall anxiolytic effect of EE ($F_{(1,66)}$ = 19.17; p≤0.001), confirmed by Tukey *post-hoc* test for males (p≤0.001) and females (p≤0.001). Males and females exhibit an overall reduction of 0.87 and 0.85 standard deviations from the mean compared to their standard housed controls (fig. 19).

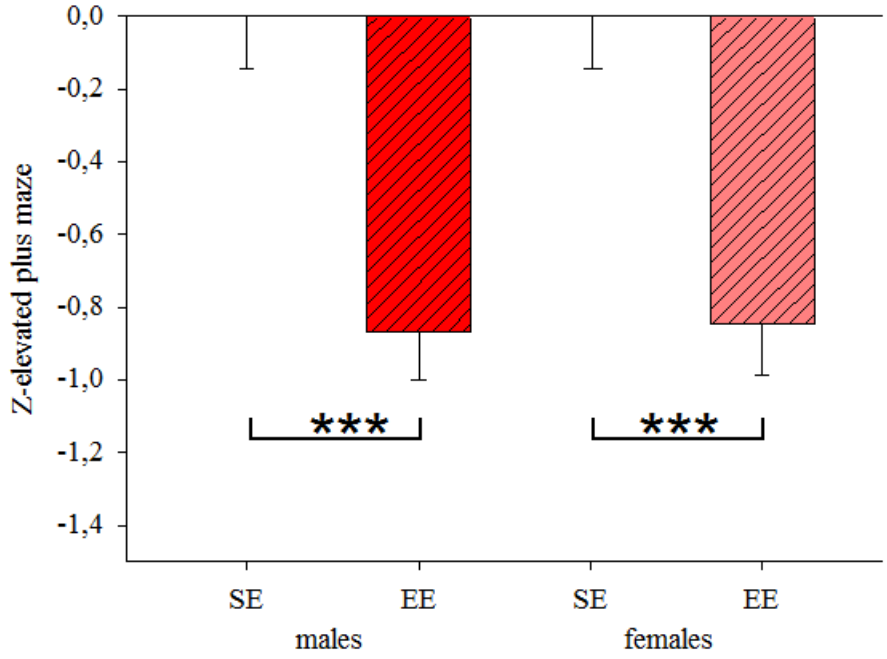


Fig. 19: Z-elevated plus maze reveals an anxiolytic effect of EE for both sexes by a reduction of 0.87SDs and 08.85SDs for males and females, respectively below the mean of SE controls. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

Like OF and EPM, LD uncovers an anxiolytic effect of EE indicated by an effect of housing and a trend of sex on percent time spent in the light compartment ($F_{(1,79)}$ = 12.04; p≤0.001 and $F_{(1,79)}$ = 19.17; p=0.097, respectively) and effects of housing on percent distance travelled in the light compartment ($F_{(1,79)}$ = 27.93; p≤0.001), latency to enter the light compartment ($F_{(1,79)}$ = 7.494; p≤0.01) and total entries into light compartment ($F_{(1,79)}$ = 7.764; p≤0.01). Again, total distance travelled was not significantly different between EE and SE housed animals ($F_{(1,79)}$ = 0.223; p=0.638).

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
total distance travelled	0.638	0.157	0.315
latency to enter light compartment	0.008	0.604	0.826
percent time spent in light compartment	0.001	0.097	0.294
percent distance travelled in light compartment	≤0.001	0.222	0.927
total entries in light compartment	0.007	0.165	0.343

As revealed by *Tukey post-hoc* tests, EE significantly increases percent time spent in light compartment for females (p≤0.05) but not males (p=0.322), percent distance travelled in light compartment for both sexes (p≤0.01) and total entries in light compartment for females (p≤0.05) without affecting males (p=0.563). Significance of latency to enter the light compartment did not survive *post-hoc* testing. Z-score for LD ($F_{(1,79)}$ = 10.17; p≤0.01) confirms the anxiolytic effect by Fisher *post-hoc* test for females (p≤0.01) and with a trend for males (p=0.084) with 0.76 and 0.48 standard deviations below the mean of SE mice, respectively (fig. 20).

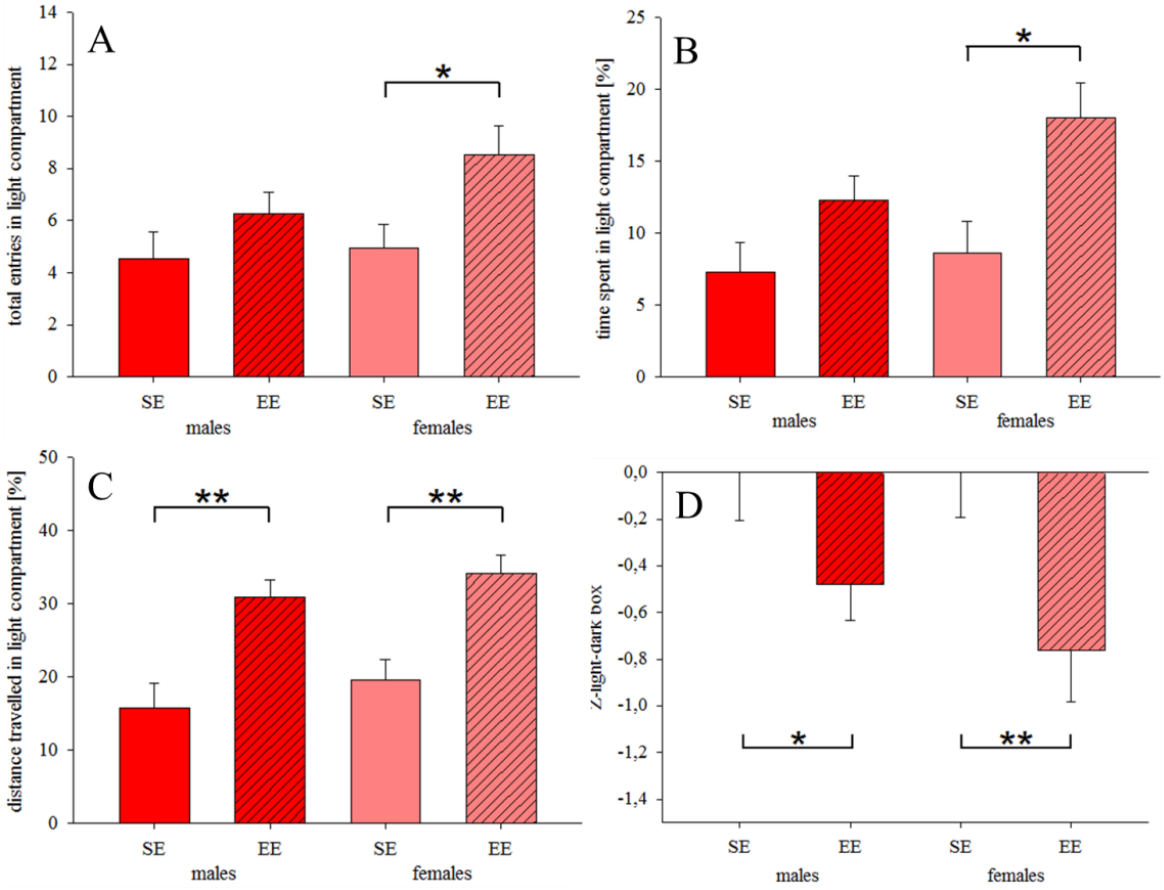


Fig. 20: EE increases entries (A) and percent time in light compartment (B) for females, while increasing percent distance light compartment (C) and decreasing z-score (D) for both sexes, clearly indicating an anxiolytic effect. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

We performed SIH to assess whether the SAM-system is involved in the beneficial effects elicited by EE. A significant reduction of temperature increase, tantamount to an anxiolytic effect, was revealed for EE mice by a 2-way ANOVA ($F_{(1,68)} = 11.51$; $p \leq 0.001$). A 3-way ANOVA identified the following factors and the combination thereof as related to differences in basal and stress body temperature ($F_{(1,136)} = 14.05$; $p \leq 0.001$).

Factors	**P-value for main effect:**
housing	≤0.001
time (basal or stress)	≤0.001
sex	≤0.001
housing x time	0.011
housing x sex	0.149
time x sex	0.028
housing x time x sex	0.356

Tukey *post-hoc* tests show a significant lower increase of body temperature after stress for males ($p \leq 0.01$) and a trend for females ($p = 0.10$) (fig. 21a). We observe no significant differences of body temperature after stress for both sexes ($p = 0.993$ for males; $p = 0.999$ for females) and no difference concerning basal temperature between EE and SE females ($p = 0.401$). Only EE males exhibit an increased basal body temperature compared to SE mice ($p \leq 0.01$) (fig. 21b). Stress significantly increased body temperature for both housing conditions and sexes ($p \leq 0.001$ for all).

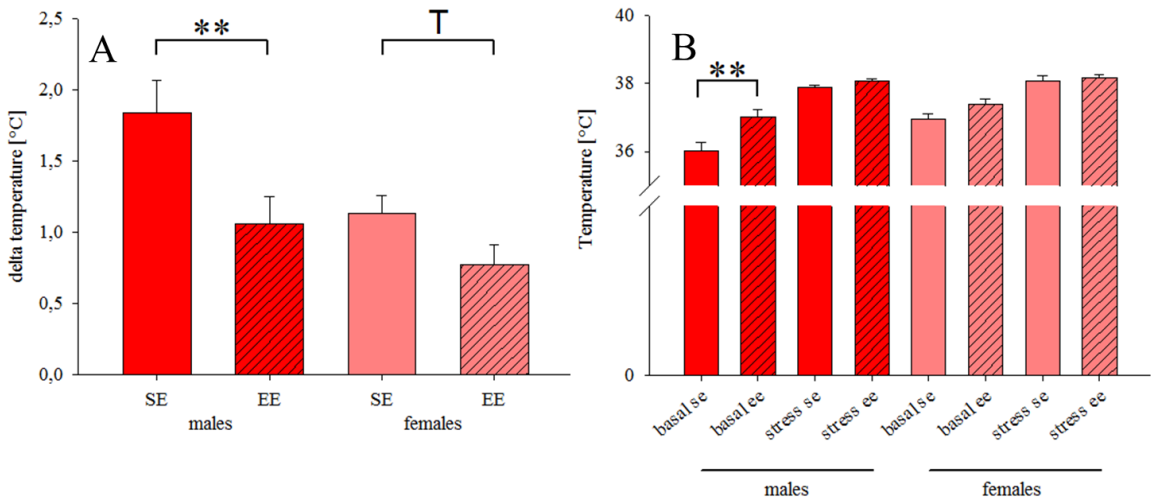


Fig. 21: EE causes a significantly smaller temperature increase after a stressor for males and shows a trend for females (A) - This indicates an anxiolytic effect. Merely EE males show a higher basal body temperature compared to their SE controls. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

To evaluate the overall anxiolytic effect of EE, we averaged the z-scores from OF, EPM. LD and SIH. In more detail, we calculated the z-scores for the parameters measured in a respective test (e.g. EPM: percent time and entries open into arms, total distance travelled), and averaged these z-scores (fig. 22) to obtain a parameter denoted as "emotionality score".

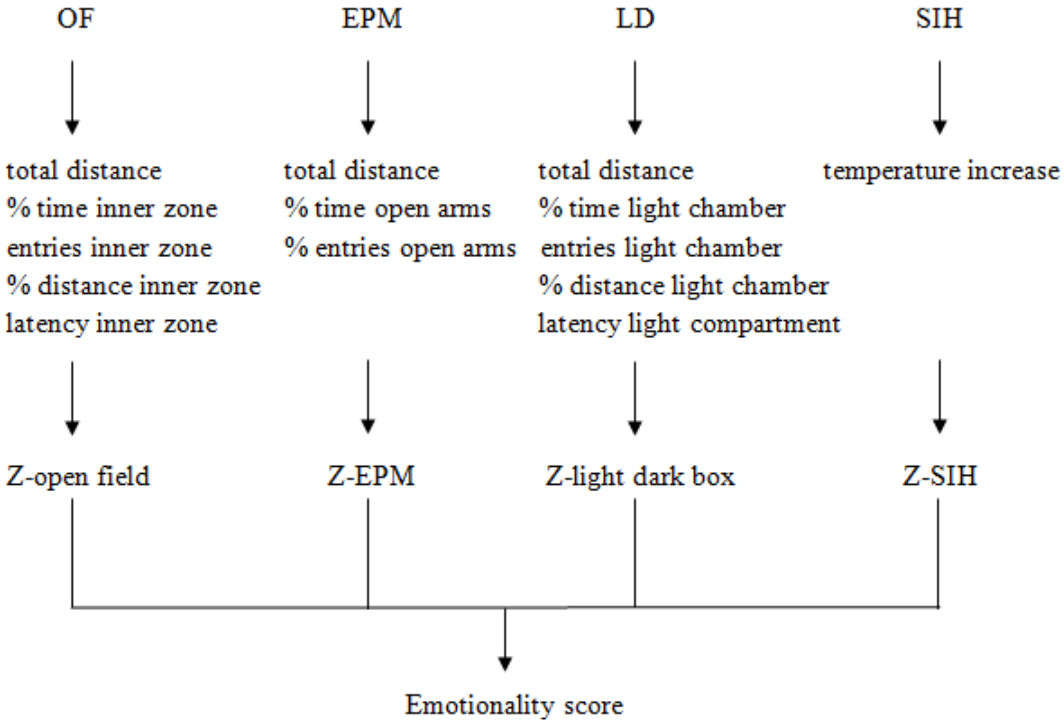


Fig. 22: Emotionality score is an average of the z-scores from open-field, elevated plus maze, light-dark box and stress-induced hyperthermia test. It represents a possibility to assess the overall effect of enriched environment on anxiety-related behavior.

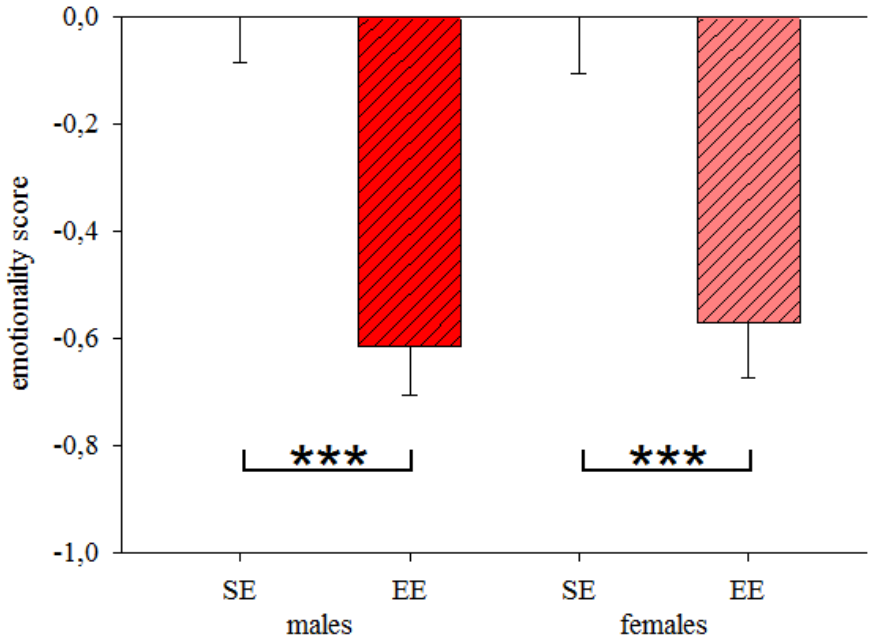


Fig. 23: Anxiety-related behavior is significantly reduced by EE. Emotionality score of males and females is 0.61 and 0.56 standard deviations below the mean of the respective housed SE mice. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

Emotionality score reveals a robust anxiolytic effects of EE ($F_{(1,79)} = 37.29$; $p \leq 0.001$). Even after taking all measured parameters into account (including the ones, which are not significantly different), males show a reduction of 0.61 and females of 0.56 standard deviations below the mean of their SE housed controls (fig. 23; $p \leq 0.001$ for both). Importantly, all z scores used to generate the emotionality score were obtained from behavioral tests performed during a single experiment.

4.1.2. Effects of EE on exploratory behavior and locomotion

Enriched environment increases exploration independent of locomotion. Instead, home cage activity is slightly decreased compared to SE housed controls.

To assess exploration independent of locomotion, we performed the EP test. Indeed, EE increased the number of head dips as indicated by an effect of housing ($F_{(1,79)} = 19.74$; $p \leq 0.001$). EE Males ($p \leq 0.05$) and females ($p \leq 0.01$) performed head dips more often compared to SE controls as demonstrated via Tukey's *post-hoc* test (fig. 24).

Factors	P-value for main effect:
housing	≤0.001
sex	0.761
housing x sex	0.891

Due to the absence of freezing behavior, we couldn't assess anxiety-related behavior.

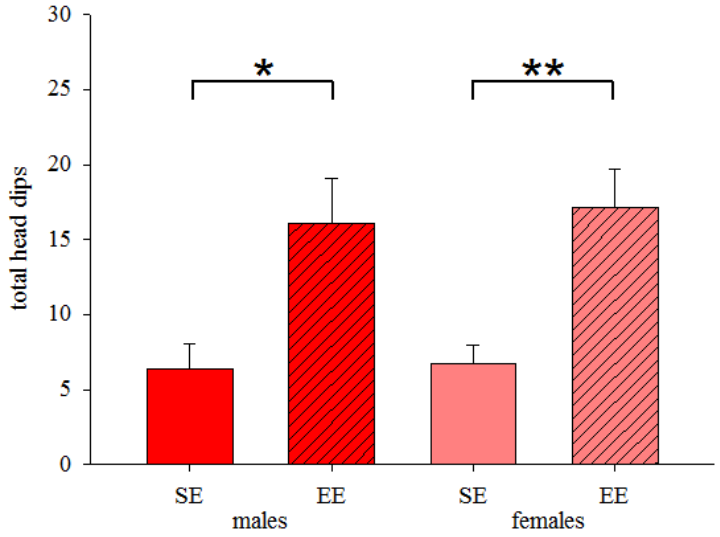


Fig. 24: Exploratory behavior significantly increased for both sexes, indicated by a higher number of head dips of EE animals compared to SE mice. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

To assess further aspects of exploratory behavior, we counted number of rearings performed during OF. Our data corroborate an increased exploratory behavior for EE mice in relation to their SE housed counterparts seen as an effect of housing ($F_{(1,79)}$ = 7.75; $p \leq 0.01$).

Factors	**P-value for main effect:**
housing	0.007
sex	0.953
housing x sex	0.764

Fisher's *post-hoc* test revealed a significantly higher number of rearings for females ($p \leq 0.05$) and a trend for males ($p = 0.081$) (fig. 25).

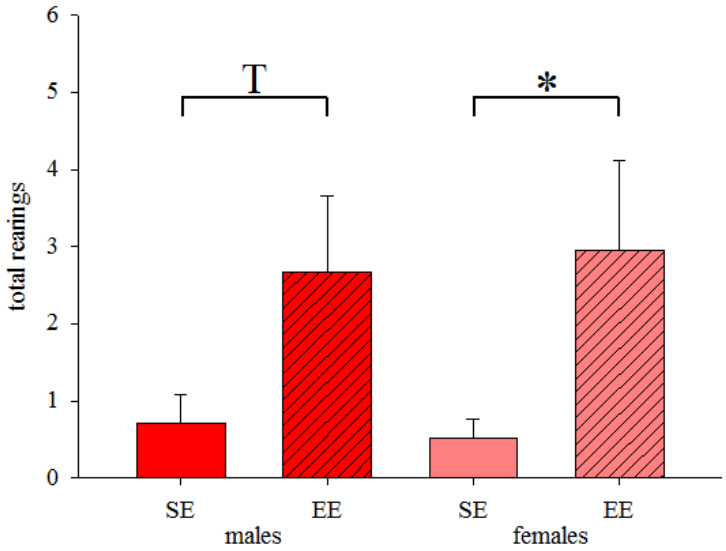


Fig. 25: As shown for EP, OF points towards an increased exploratory behavior for EE compared to SE animals. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

Finally, we counted rearings performed during LD test. 2-way ANOVA revealed no significant difference between EE and SE mice. Neither housing ($F_{(1,79)}$ = 0.03; p=0.957) or sex ($F_{(1,79)}$ = 1.08; p=0.302), nor an interaction of housing x sex ($F_{(1,79)}$ = 0.001.; p=0.982) were significantly different between the groups.

To evaluate the overall effect of EE on exploration, we calculated an "exploration score" (fig. 26) by averaging the z-scores from EP, OF and LD (same procedure as for emotionality score). This included the parameters head dips performed during EP, rearings performed during OF and "rearings performed during LD". Indeed, EE increases exploratory behavior as shown by an effect of housing ($F_{(1,79)}$ = 14.379; p≤0.001).

Factors	**P-value for main effect:**
housing	≤0.001
sex	0.338
housing x sex	0.338

Fisher *post-hoc* tests revealed a significant increase of exploratory behavior for males (p≤0.05) and females (p≤0.01) with 0.80 and 1.35 SDs above the mean of SE controls (fig. 26).

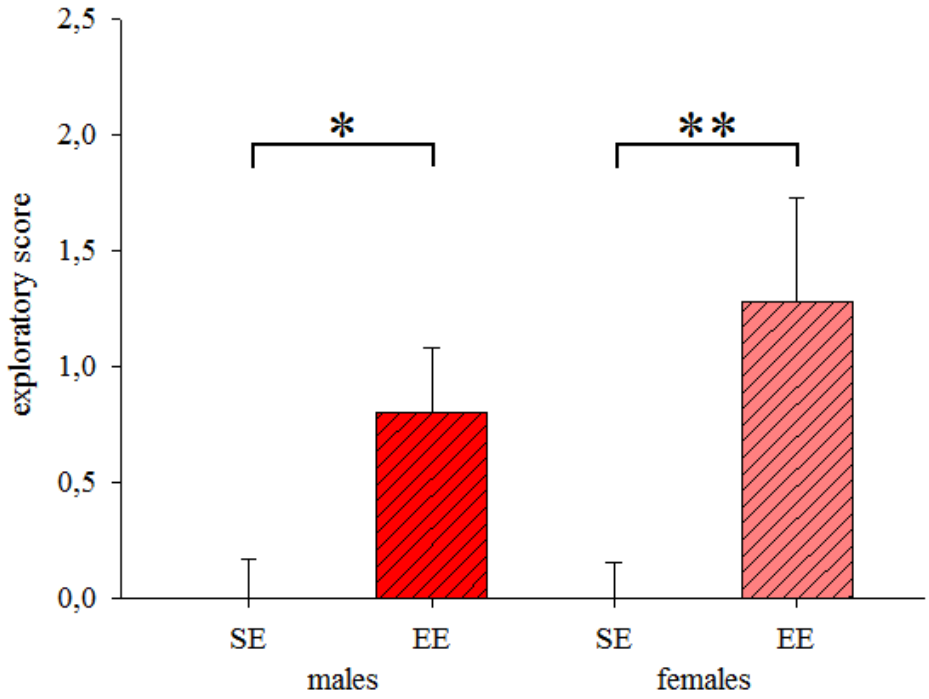


Fig. 26: EE indeed increases exploration for both sexes significantly compared to SE housed animals. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

Locomotion is a cross-test dimension of anxiety and thus, we analyzed locomotor activity during behavioral tests including OF, EPM, LD and in a more natural-like situation, namely HCA. There was no significant effect of housing in any of the behavioral tests:

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
open field	0.321	0.802	0.233
elevated plus maze	0.387	0.720	0.685
light-dark box	0.638	0.157	0.315

Activity, including locomotion can be very different between an artificial test situation and within the home cage. Repeated measures ANOVA revealed no difference in activity for males ($F_{(1,16)}$ = 0.317; p=0.581) and a trend indicating lower activity for females ($F_{(1,16)}$ = 4.23; p=0.057) (fig. 27).

home cage activity	**p-value for main effect of:**		
	housing	time	housing x time
males	0.581	≤0.001	≤0.001
females	0.057	≤0.001	≤0.001

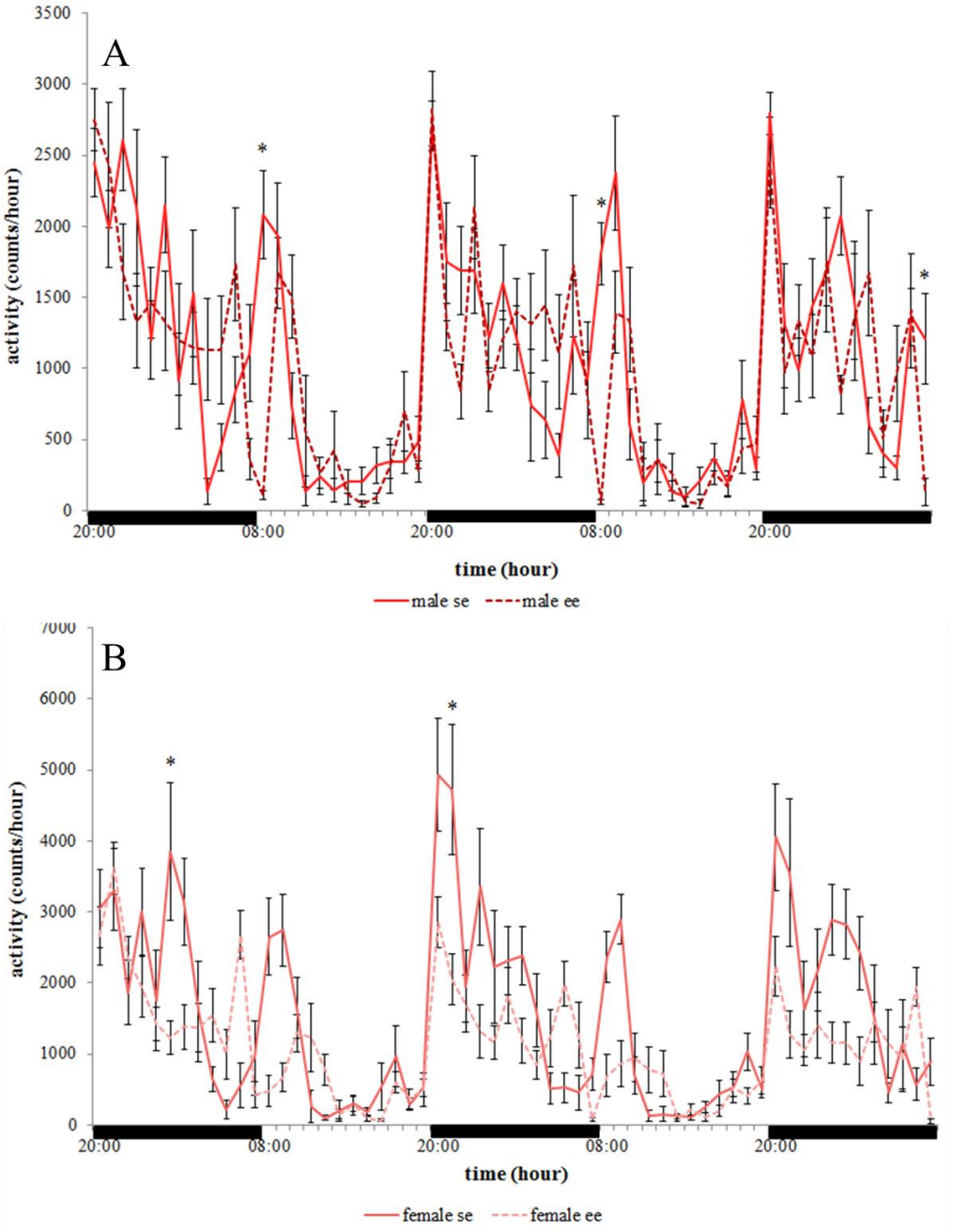


Fig. 27: EE does not increase activity, instead a trend for females indicates even less activity compared to standard controls. Black bars indicate dark phases. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

There was a significant effect of time for both sexes, showing higher activity during the dark compared to the light phase ($F_{(1,16)}$ = 13.028; p≤0.001 for males and $F_{(1,16)}$ = 12.510.; p≤0.001 for females). The significant interaction of housing x time points towards a lower activity for both, EE males ($F_{(1,16)}$ = 2.802; p≤0.001) and females ($F_{(1,16)}$ = 4.221; p≤0.001) in relation to SE mice.

In summary, we observed no differences concerning locomotor activity in behavioral tests and a significantly decreased activity in the home cage for EE animals.

4.1.3. Effects of EE on coping style and stress reactivity

EE does not alter coping style but stress reactivity in a mouse model of pathological anxiety and comorbid depression.

There was a significant effect of housing on latency to first immobile episode ($F_{(1,71)}$ = 22.134; p≤0.001), but no effects of housing, sex or an interaction of housing x sex on percent time spent immobile and number of immobile episodes.

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
latency to first immobile episode	≤0.001	0.273	0.163
number of immobile episodes	0.660	0.510	0.190
percent time spent immobile	0.166	0.755	0.223

Tukey's *post-hoc* confirmed a significant short latency for EE females (p≤0.001) and a trend for males (p=0.091) compared to their respective controls (fig. 28).

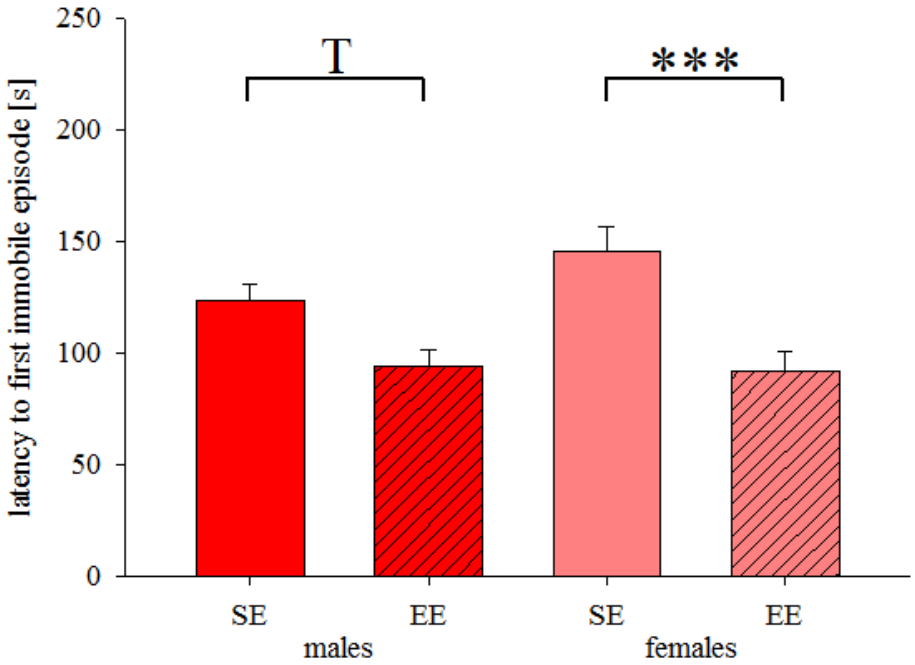


Fig. 28: Latency to first immobile episode during TST is significantly reduced for females after EE, with males showing a trend. N (males) = 21 SE and EE; N (females) = 21 SE and 20 EE

FST was performed only for males. A MWU test revealed a significant decrease for EE males in latency to first struggling episode, which did not survive Dunn-Šidák correction for multiple testing (α=0.017).

To assess stress reactivity, we performed a SRT for male HABs with either a mild (15min. immobilization) or severe stressor (6min. FST; please refer to material and methods). Indeed, we found a significant effect of housing ($F_{(1,54)}$ = 5.996; p≤0.05), time (basal or stress; $F_{(1,54)}$ = 643.722; p≤0.001) and paradigm (mild or severe; $F_{(1,54)}$ = 58.472; p≤0.001) as well as an interaction of housing x time ($F_{(1,54)}$ = 6.974; p≤0.05) and time x paradigm ($F_{(1,54)}$ = 64.699; p≤0.001). Tukey *post-hoc* tests reveal a significant increase of CORT after application of a stressor for both paradigms (p≤0.001 for all). EE males show a strong trend to release less CORT after a mild stressor (p=0.063). Moreover, EE males that performed the mild paradigm show a significant reduction (p≤0.01) of CORT release when we do not compare the mild and severe paradigm (i.e. experiments) but every experiment separately (fig. 29).

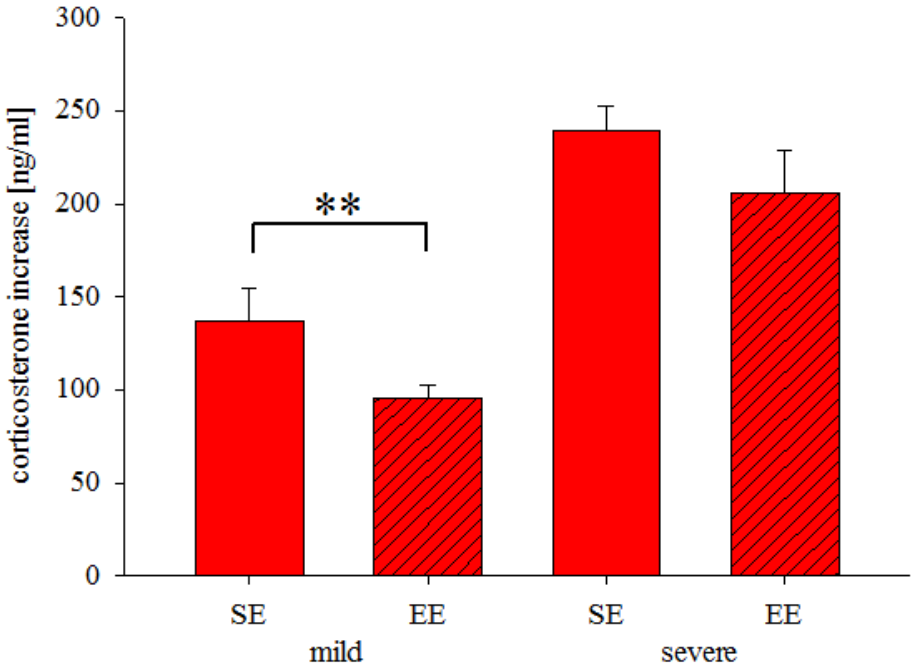


Fig. 29: EE significantly reduces the released CORT after application of a mild stressor. N (mild) = 6 SE and 10 EE; N (severe) = 7 SE and EE

Moreover, we observe that the HPA-axis reactivity of EE males in intact. When a severe stressor is applied, the protecting neuroendocrine effect of EE disappears and CORT levels are indistinguishable between EE and SE males. This indicates that the significant reduction of CORT release after a mild stressor in EE males is not due to ceiling effects.

4.2 Variability and reproducibility of EE: a meta-analysis of beneficial effects

4.2.1. Meta-analysis of anxiolytic effects

The anxiolytic effect on a mouse model of pathological anxiety is stable, reproducible and exhibits a small to strong effect size.

Meta-analysis of anxiety-related behavior was performed by averaging the emotionality scores of all conducted experiments. Thus, we averaged the z-scores of OF, EPM and LD for every experiment to obtain the respective emotionality score. These emotionality scores were then averaged to receive a "final emotional score" comprising the effect sizes of all performed experiments. In total, 4 experiments for males and 3 experiments for females were conducted (fig. 30).

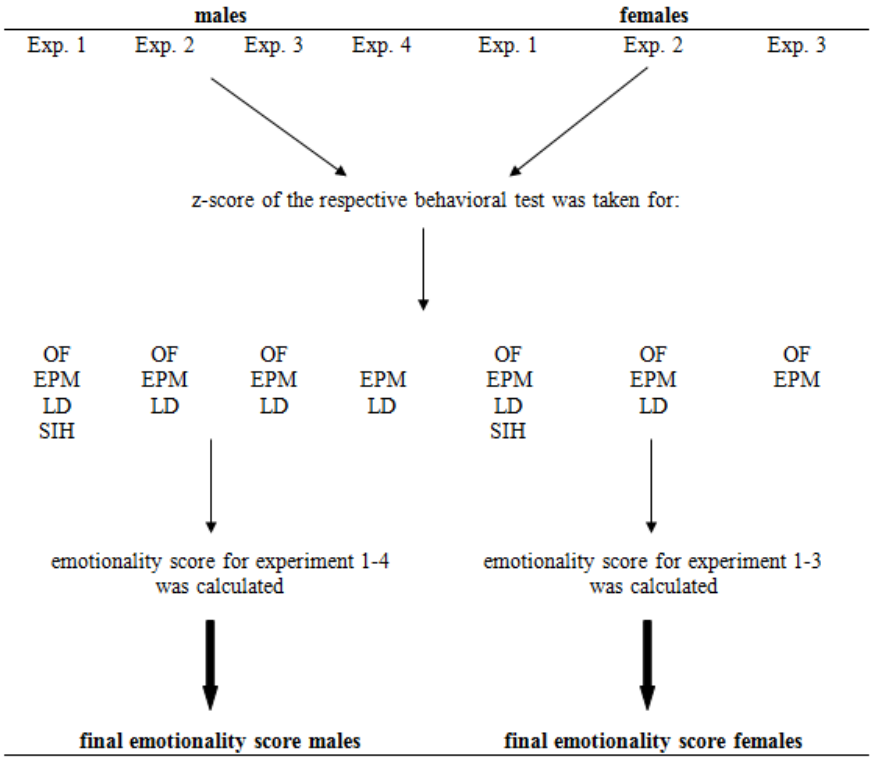


Fig. 30: Workflow to obtain a "final emotionality score". This meta-analysis allows an overall assessment of EE on anxiety-related behavior.

Z-score of LD test from female experiment 3 and OF from male experiment 4 were excluded from emotionality score and thus from meta-analysis because merely few SE mice entered the light or inner zone at all, thereby creating a standard deviation (σ) close to zero and finally a z-score ≥10, which is much higher in relation to all other conducted experiments ($Z = \frac{X-\mu}{\sigma}$).

Meta-analysis revealed an effect of housing ($F_{(1,125)} = 86.23$; $p \leq 0.001$), sex ($F_{(1,125)} = 12.27$; $p \leq 0.001$) and an interaction effect of housing x sex ($F_{(1,125)} = 13.37$; $p \leq 0.001$). Tukey's *post-hoc* test showed that EE significantly reduced anxiety-related behavior for males ($p \leq 0.001$) and females ($p \leq 0.001$) by 1.04 and 0.44 SDs below the mean of respective standard housed controls (fig. 31).

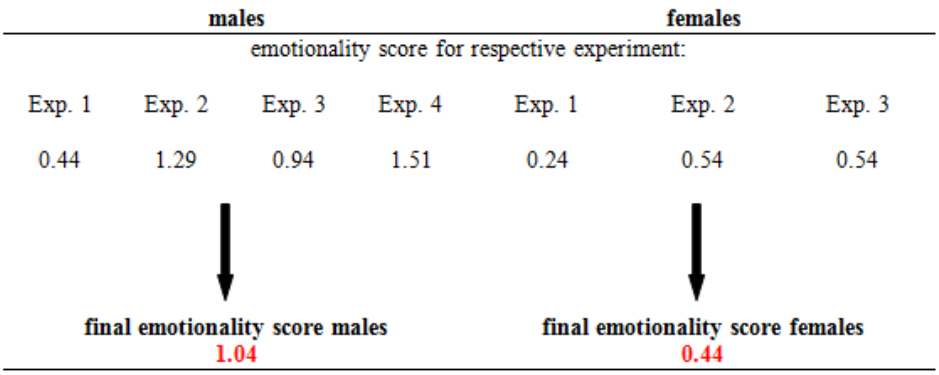


Fig. 31: Overall assessment of anxiolysis by emotionality score reveals a strong and small effect size for males and females, respectively. N (males) = 61 SE and 68 EE; N (females) = 48 SE and 52 EE

Guilloux (2011) proposed the following ranges for observed effect sizes:

effect size	strength
≤0.5	small
0.5 - 1.0	medium
≥1.0	strong

Thus, we performed a χ^2-test with a 2x3 factorial design: 2 housing conditions (SE or EE) and 3 effect sizes (small, medium and strong). It revealed a significantly increased number of EE animals showing a medium or strong effect size compared to standard housed controls. This is true for both sexes ($p \leq 0.001$) with EE males showing an even more skewed distribution to a higher effect size compared to EE females ($p \leq 0.001$). Interestingly, we observed no difference between SE males and females ($p=0.209$).

comparison	χ^2	degree of freedom	p-value	Cramer's V
male SE vs. EE	58.36	2	p≤0.001	0.5402
female SE vs. EE	15.17	2	p≤0.001	0.2754
male vs. female EE	30.15	2	p≤0.001	0.3883
male vs. female. SE	3.13	2	p=0.2091	0.1251

Fig. 32 depicts a significant decrease of small as well as a significant increase of strong effect size for both sexes reflecting an anxiolytic effect. Here, the distribution of males is almost exclusively shifted from a small to strong effect size, whereas effect size of females is shifted to equal parts from small to medium and strong.

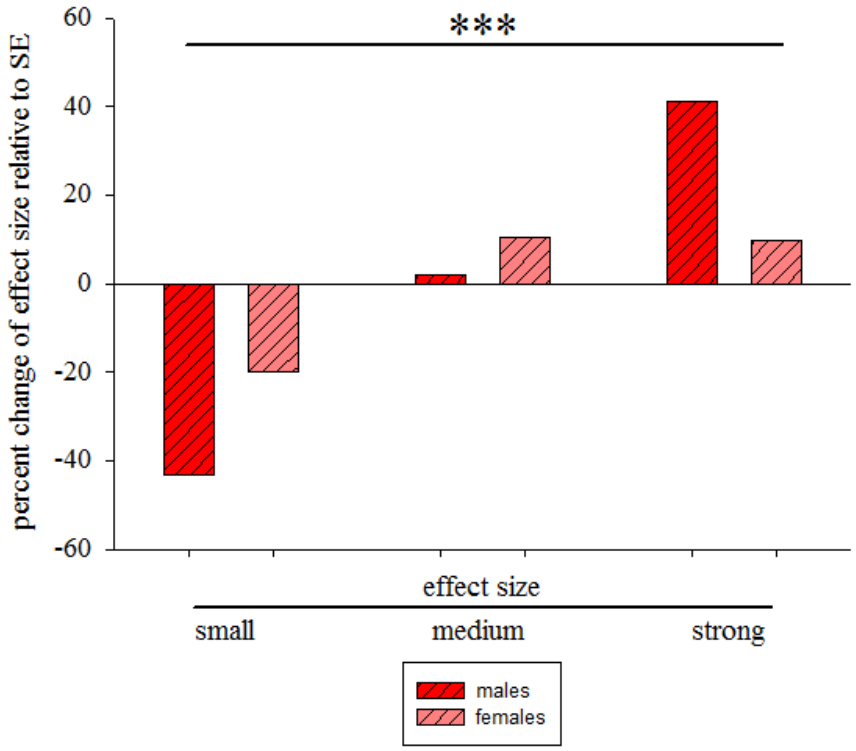


Fig. 32: EE causes a shift in effect size, which reflects an anxiolytic effect. It is shifted from small to strong for males, whereas for females, effect size is shifted from small to medium and strong. N (males) = 61 SE and 68 EE; N (females) = 48 SE and 52 EE

To interpret the strength of the anxiolytic effect elicited by EE we used Cramer's V. It is a

contingency coefficient, independent of sample size, which expresses the strength of an observed effect. It ranges from $0 \leq x \leq 1$, whereat 1 is the highest effect size possible. It reveals a strong effect for EE compared to SE males (0.54), in contrast to females, which offer a medium effect strength (0.28) corroborating a stronger anxiolytic effect for males in relation to females.

4.2.2. Meta-analysis of locomotion

Enrichment does not alter locomotion of females, whereas merely a trend was observed for males. This indicates that locomotion, a cross test parameter of anxiety, is not masking the observed anxiolytic effects elicited by EE.

To evaluate the effect of locomotion on anxiety-related behavior, we averaged the z-scores of "total distance travelled in OF" for all performed experiments, i.e. 4 for males and 3 for females. This parameter is the most intensively used index of assessing confounding locomotor effects (fig. 33).

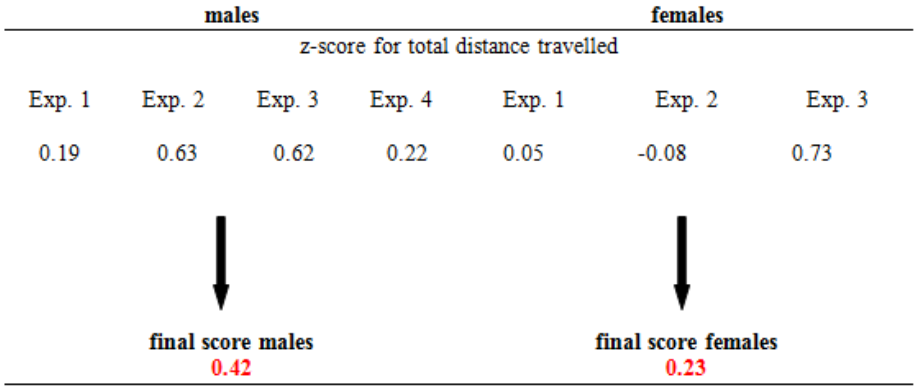


Fig. 33: We observe a trend for EE males to travel more distance compared to controls, which exhibit an increase of 0.42 standard deviations above the mean of SE animals.

2-way ANOVA (fig. 34) revealed a significant effect of housing ($F_{(1,222)} = 2.080$; $p \leq 0.05$), which did not survive significance after a Tukey *post-hoc* test for females (p=0.785) and showed a trend for males (p=0.070).

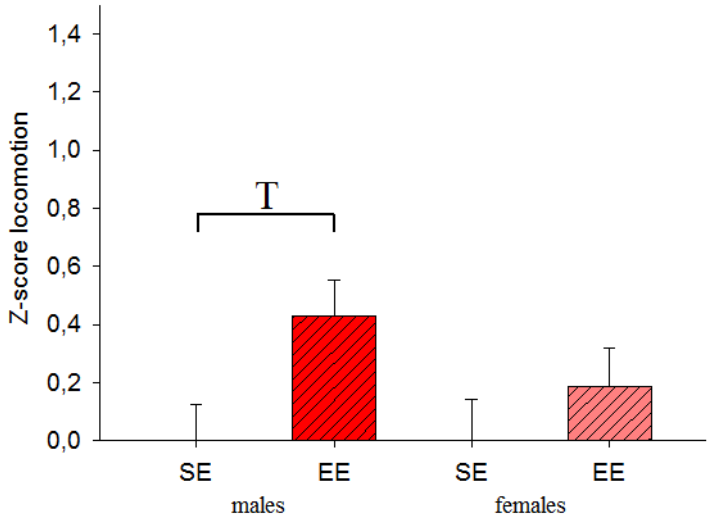


Fig. 34: EE increases locomotion by 0.42 and 0.23 SDs for males and females compared to respective controls. N (males) = 61 SE and 68 EE; N (females) = 48 SE and 52 EE

4.2.3 Meta-analysis of coping style

Enrichment does not improve depression-like behavior or alter coping style, respectively.

To assess the overall effect of EE on coping style, we averaged the z-scores from TST and FST to obtain a "depression score" (fig. 35) comprising nine parameters in total: latency to first immobile episode, number of immobile episodes and percent time spent immobile for both, TST and FST, as well as number of struggling episodes, latency to first struggling and percent time struggling for FST. There was a significant effect of housing on depression score ($F_{(1,71)} = 6.697$; $p \leq 0.05$), without an effect of sex ($F_{(1,71)} = 1.23$; $p=0.269$) or an interaction effect of housing x sex ($F_{(1,71)} = 1.23$; $p=0.269$). Fig. 34. shows that the significant effect of housing did not survive Tukey's *post-hoc* test for males ($p=0.840$) and females ($p=0.145$), respectively.

factors	p-value for main effect:
housing	≤0.05
sex	0.146
housing x sex	0.146

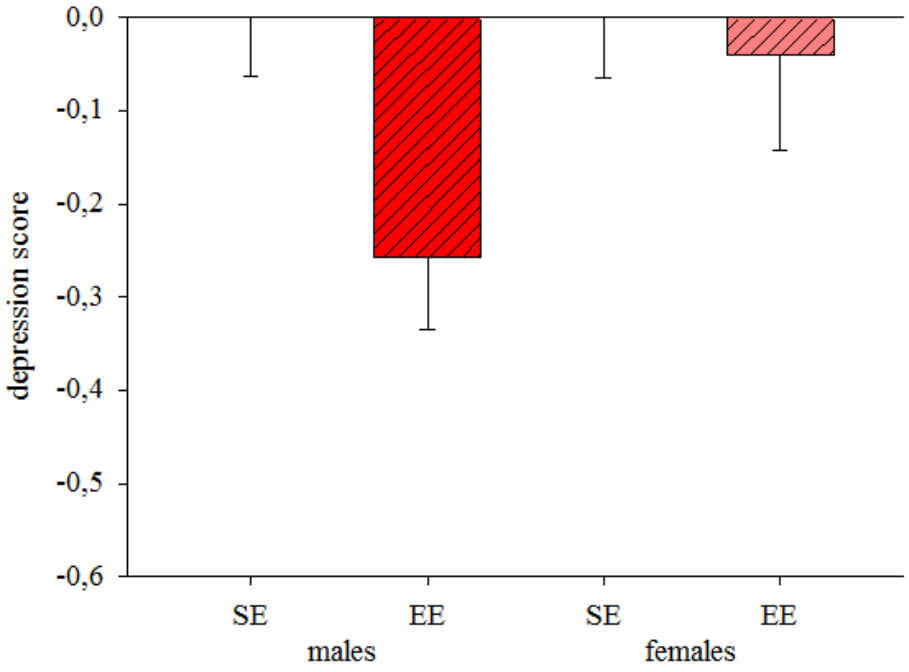


Fig. 35: Enrichment does not ameliorate depression-like behavior or alter coping style, respectively. N (males) = 47 SE and 44 EE; N (females) = 32 SE and 30 EE

Males showed a reduction of 0.24 and females of 0.05 SDs below the mean of controls indicating a small effect size.

4.3 Impact of enrichment on "normal" anxiety-related behavior animals

4.3.1. Effects of enrichment on anxiety-related behavior

Anxiety-related behavior is significantly reduced in males with minor effects in females entailing a small effect size for NABs compared to HABs.

To assess anxiety-related behavior, we performed OF, EPM and LD. For OF, 2-way ANOVA reveals a significant effect of housing on total distance travelled ($F_{(1,35)} = 17.424$;

p≤0.001), latency to enter inner zone ($F_{(1, 35)}$ = 7.387; p≤0.01), percent time spent in inner zone ($F_{(1, 35)}$ = 6.097; p≤0.05), percent distance travelled in inner zone ($F_{(1, 35)}$ = 6.333; p≤0.05) and total entries inner zone ($F_{(1, 35)}$ = 11.584; p≤0.01) without an effect of sex or an interaction of housing x sex.

parameter measured	**p-value for main effect of:**		
	housing	sex	housing x sex
total distance travelled	≤0.001	0.592	0.833
latency to enter inner zone	0.010	0.723	0.904
percent time spent in inner zone	0.019	0.271	0.623
percent distance travelled in inner zone	0.017	0.240	0.484
total entries inner zone	0.002	0.481	0.696

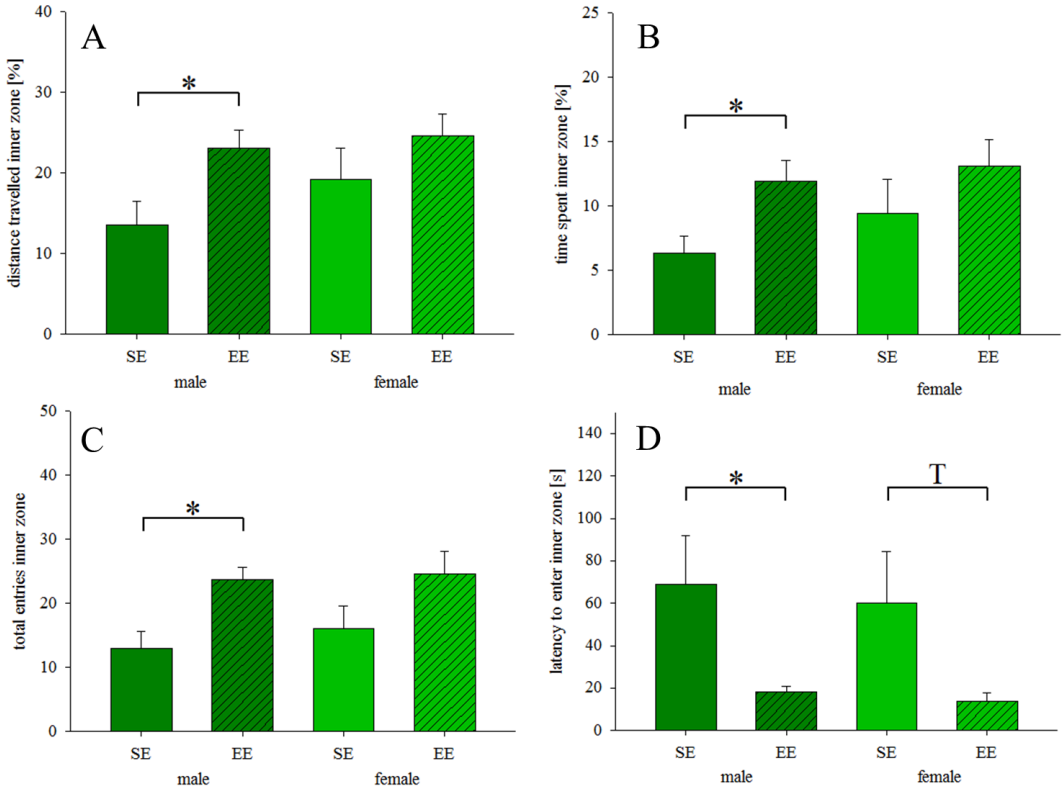


Fig. 36: OF points towards an anxiolytic effect of EE for males but not females with significant increases in percent distance travelled (A) and percent time spent inner zone (B), total entries into (C) and a reduced "latency to enter inner zone" (D). N (males) = 12 SE and 11 EE; N (females) = 8 SE and EE

Fisher *post-hoc* tests reveal for males a significant increase in percent distance travelled and percent time spent in inner zone as well as shorter latency to enter (p≤0.05 for all) and "more entries" (p≤0.01) into it (fig. 36). EE Females merely show a trend (p=0.10) to enter the inner zone with a shorter latency in relation to SE controls. These parameters indicate a significant anxiolytic effect for males but not females.

To assess the overall anxiolytic effect of EE, we calculated z-open field exactly as described before for HAB mice. 2-way ANOVA shows a significant effect of housing ($F_{(1, 35)}$ = 4.484; p≤0.05) without effects of sex or an interaction of both.

Factors	P-value for main effect:
housing	0.041
sex	0.427
housing x sex	0.427

Fisher *post-hoc* test confirmed a significant anxiolytic effect for males ($p \leq 0.05$) but not females ($p=0.398$) with 0.53 and 0.24 SDs below SE housed controls (fig. 37).

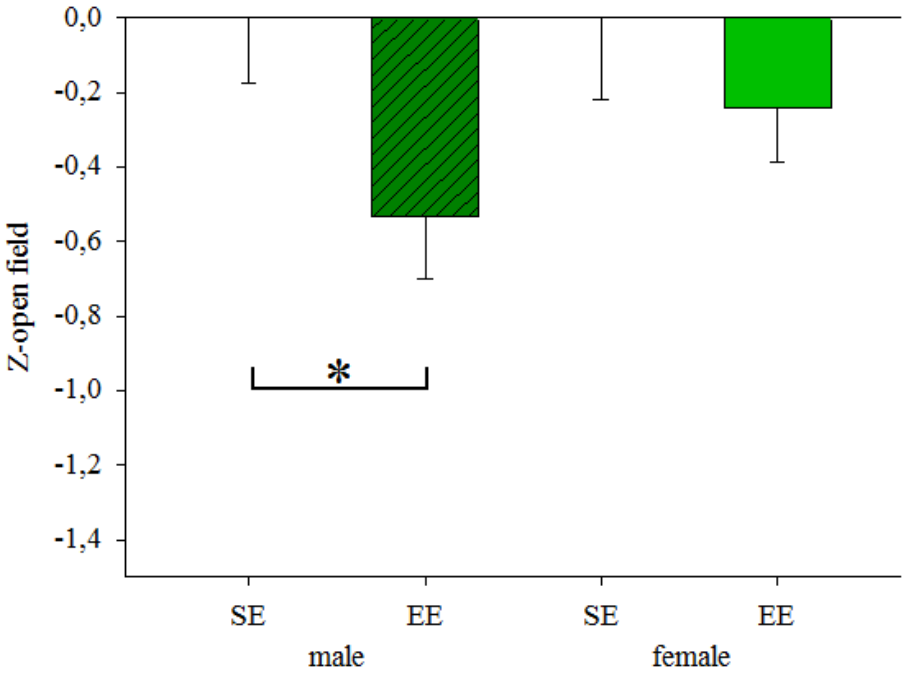


Fig. 37: Z-open field corroborates the anxiolytic effect of enrichment for males without decreasing anxiety-related behavior for females. N (males) = 12 SE and 11 EE; N (females) = 8 SE and EE

Importantly, we still observe an anxiolytic effect though z-open field corrects for existing differences in total distance travelled, thereby excluding confounding effects of locomotion on anxiety-related behavior.

For EPM, 2-way ANOVA detected a trend of housing ($p=0.076$) with EE animals entering percent open arms more often compared to controls. This trend did not survive Fisher *post-hoc* test for males ($p=0.258$) and females ($p=0.161$). There is neither an effect of housing, sex or interaction of both for percent time spent on open arms, nor total distance travelled.

Parameter measured	P-value for main effect of:		
	Housing	Sex	Housing x sex
total distance travelled	0.811	0.402	0.741
percent entries open arms	0.076	0.871	0.712
percent time spent on open arms	0.115	0.957	0.533

As expected, there is no overall effect of housing ($F_{(1, 35)} = 0.01$; $p=0.115$), sex ($F_{(1, 35)} = 0.444$; $p=0.511$) or housing x sex ($F_{(1, 35)} = 0.444$; $p=0.511$) on z-EPM indicating no anxiolytic effect of enrichment captured by parameters of this behavioral test (fig. 38). EE males are 0.1 SDs above, whereas females are 0.03 SDs below their respective SE controls.

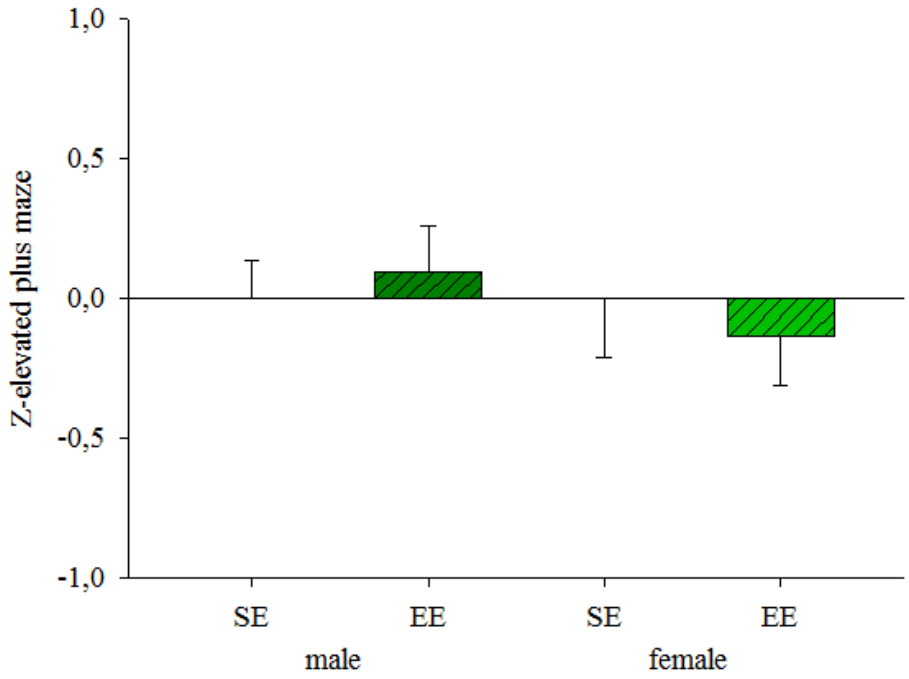


Fig. 38: Z-elevated plus maze depicts no difference between enriched and standard housed mice. N (males) = 12 SE and 11 EE; N (females) = 8 SE and EE

LD corroborates the anxiolytic effect of OF by an effect of housing on percent time spent in light compartment ($F_{(1, 35)} = 34.75$; $p \leq 0.001$) and entries into light compartment ($F_{(1, 35)} = 7.842$; $p \leq 0.01$) as well as a trend for percent distance travelled in light compartment ($F_{(1, 35)} = 3.722$; $p = 0.062$) and total distance travelled ($F_{(1, 35)} = 3.726$; $p = 0.062$).

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
total distance travelled	0.062	0.084	0.356
latency to enter light compartment	0.841	≤0.001	≤0.001
entries into light compartment	0.008	0.304	0.279
percent distance travelled in light compartment	0.062	0.122	0.059
percent time spent in light compartment	≤0.001	0.356	0.020

Fisher *post-hoc* tests reveal a significant increase in percent time spent in light compartment for both, EE males ($p \leq 0.01$) and females ($p \leq 0.001$) as well as decreased latency to enter the light compartment for both sexes ($p \leq 0.01$ and $p \leq 0.05$ for males and females, respectively). Enriched compared to standard housed females performed significantly more entries into the light compartment and travelled more percent distance in the light compartment ($p \leq 0.05$ for both), whereas males do not exhibit a difference for both ($p = 0.193$ and $p = 0.988$, respectively) (fig. 39).

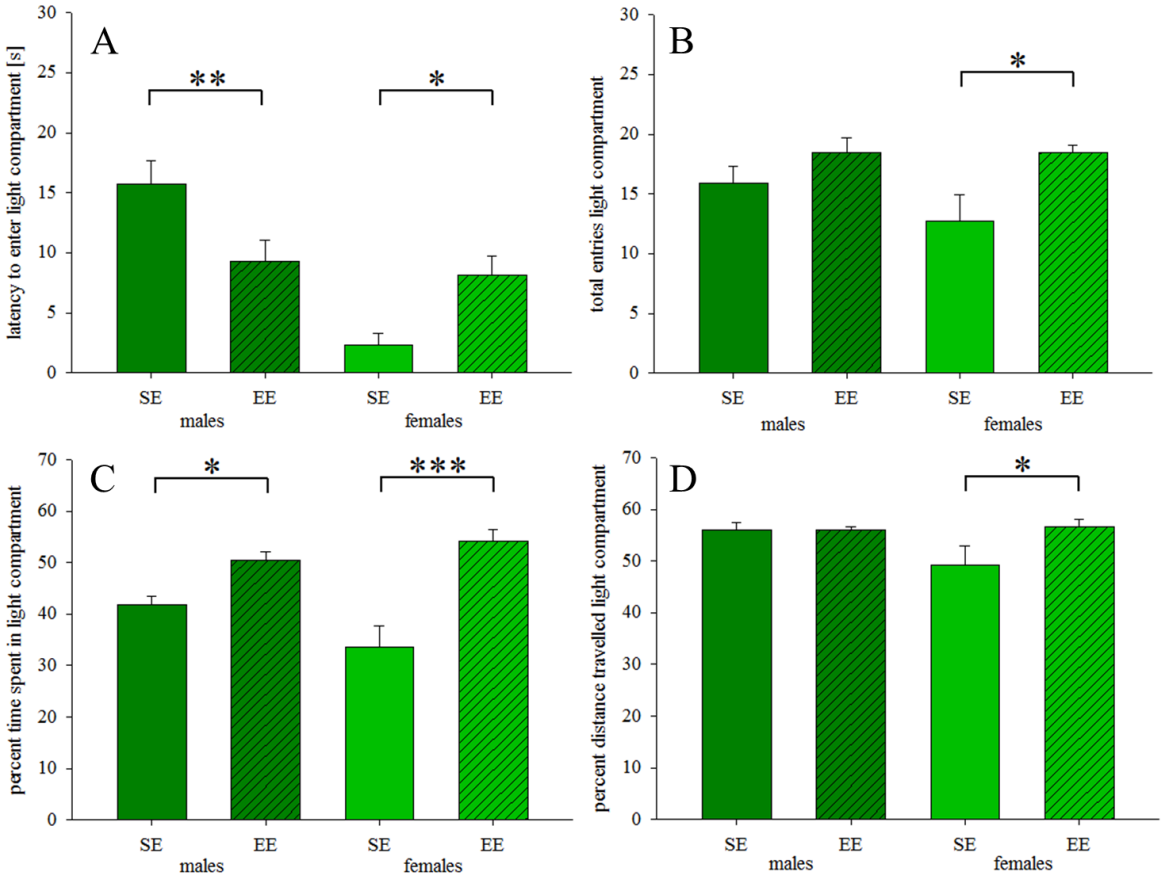


Fig. 39: EE reduces anxiety-related behavior significantly for males with females exhibiting an ever stronger anxiolytic effect compared to males. Enrichment reduces latency to enter light compartment (A) and increases entries (B), percent time (C) and percent distance travelled (D) into light compartment. N (males) = 12 SE and 11 EE; N (females) = 8 SE and EE

To evaluate the overall anxiolytic effect of enrichment, we calculated the "emotionality score", as described earlier, by averaging z-scores of OF, EPM and LD. 2-way ANOVA revealed a trend of housing ($F_{(1, 36)} = 3.726$; $p \leq 0.05$), indeed confirming an anxiolytic effect of enrichment for "normal" anxiety-related mice, too.

Factors	**P-value for main effect:**
housing	0.062
sex	0.084
housing x sex	0.356

Tukey *post-hoc* revealed a significant anxiolytic effect of enrichment by 0.38 SDs below the mean of SE males ($p \leq 0.05$). Females do not differ significantly, but EE mice are 0.16 SDs below the mean of their controls (fig. 40).

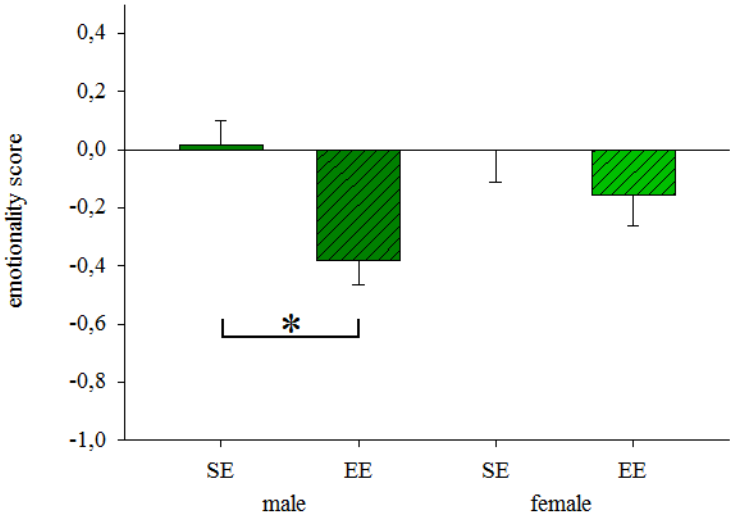


Fig. 40: An anxiolytic effect for males, not females seems to be elicited by EE. N (males) = 12 SE and 11 EE; N (females) = 8 SE and EE

To analyze whether the distribution is shifted towards a medium or strong effect size, we performed a χ^2-test with a 2x3 factorial design as described before. Indeed, males show a shift of effect size, i.e. an anxiolytic effect, from a small to a medium effect size (χ^2 = 19.17; $p \leq 0.001$). Thus, effect size of enriched males is shifted by 25% from small to medium.

Comparison	χ^2	Degree of freedom	P-value	Cramer's V
male SE vs. EE	19.17	2	$p \leq 0.001$	0.3096
female SE vs. EE	0	2	p=1.000	0
male vs. female EE	12.65	2	p=0.002	0.2515
male vs. female. SE	0.89	2	p=0.641	0.0667

Cramer's V indicates a medium effect size for EE in relation to SE males. Standard housed groups do not differ significantly, whereas EE males in contrast to females show an anxiolytic effect (fig. 41).

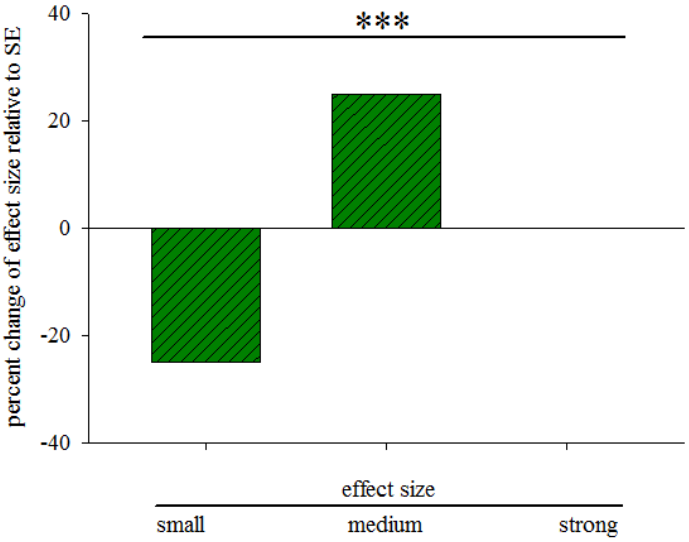


Fig. 41: Male NABs show an anxiolytic effect after EE depicted as a shift of effect size from small to medium. N (males) = 12 SE and 11 EE; N (females) = 8 SE and EE

Similar to HABs, the overall anxiolytic effect of NABs seems to be more pronounced for enriched-housed males compared to females. This entails that EE in relation to SE females do not differ significantly anymore and the shift of effect size observed for male EE NABs is medium in contrast to a high shift seen merely for male EE HABs.

4.4.2. Impact of EE on exploratory behavior

Enrichment increases exploratory behavior as indicated by a significantly increased number of rearings.

Differences in exploration might entail altered locomotion, which in turn is a confounding factor of anxiety. Thus, we counted rearings performed during OF and LD to assess the impact of exploration on locomotion and thus anxiety-related behavior. We do not observe a significant effect of housing ($F_{(1, 35)} = 2.679$; $p=0.111$), sex ($F_{(1, 35)} = 1.176$; $p=0.286$) or an interaction of housing x sex ($F_{(1, 35)} = 0.184$; $p=0.670$) on rearings performed during OF. In contrast, 2-way ANOVA reveals a significant effect of housing ($F_{(1, 35)} = 16.137$; $p \leq 0.001$) for LD, with EE mice performing significantly more rearings.

Factors	P-value for main effect:
housing	≤0.001
sex	0.127
housing x sex	0.867

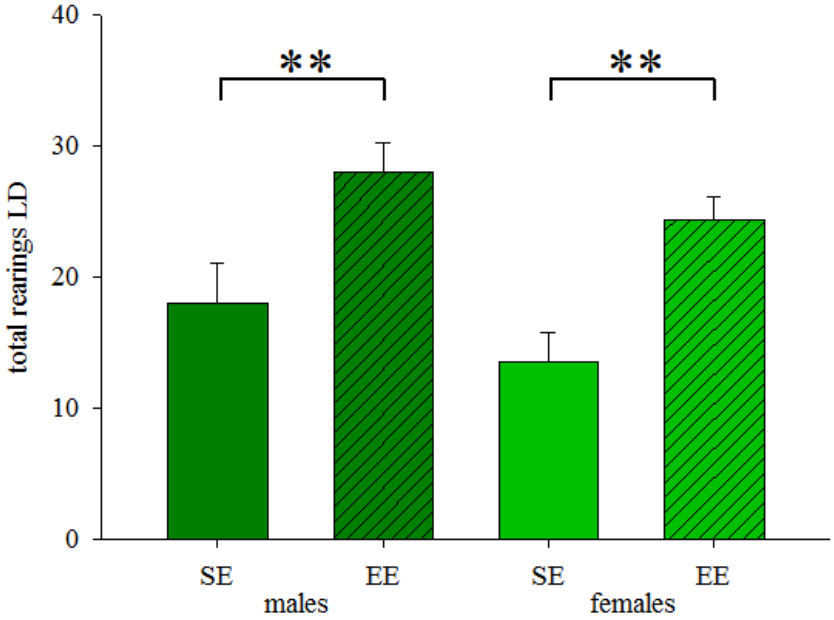


Fig. 42: Exploratory behavior is significantly increased for both sexes after environmental enrichment. N (males) = 12 SE and 11 EE; N (females) = 8 SE and EE

Fisher *post-hoc* test confirmed an increased number of rearings for males and females (fig. 42; $p \leq 0.01$ for both). To finally assess the effect of EE on exploration, we calculated "exploratory score" as described before for HABs by averaging z-scores of rearings for OF and LD. 2-way ANOVA revealed a significant effect of housing ($F_{(1, 36)} = 13.675$; $p \leq 0.001$) without effects of sex or an interaction of both factors.

Factors	P-value for main effect:
housing	≤0.001
sex	0.648
housing x sex	0.766

Tukey *post-hoc* tests corroborated a significant increase in rearings for enriched-housed males ($p \leq 0.05$) and a trend for females ($p=0.065$) compared to controls. Males and females were 0.82 and 1.01 SDs above the mean of their respective standard-housed counterparts (fig. 43), therewith pointing toward an increased exploratory behavior after EE.

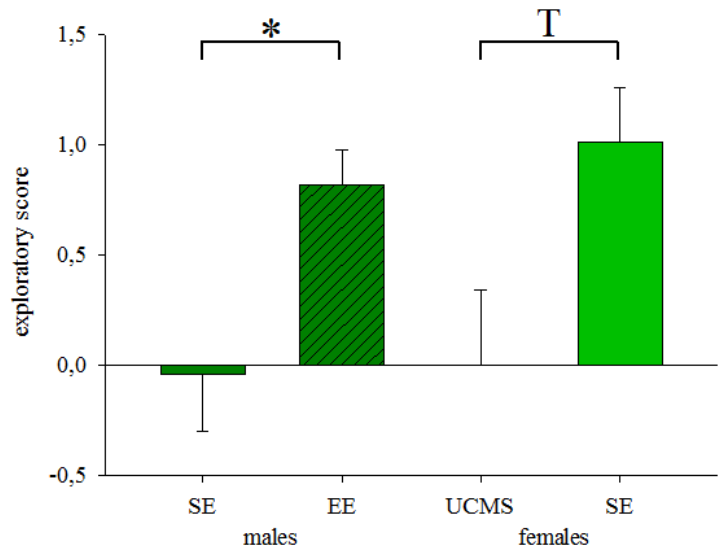


Fig. 43: Exploratory score confirms an increased exploration for both sexes, with males performing 0.82 and females 1.01 SDs more rearings related to respective controls. N (males) = 12 SE and 11 EE; N (females) = 8 SE and EE

4.3.3 Effect of environmental enrichment on coping style and anhedonia

EE does not alter coping style in NABs.

We performed TST to evaluate the impact of EE on coping style or depression-like behavior, respectively. We observe a trend of housing ($F_{(1, 34)} = 4.089$; $p=0.051$), but no significant effect of sex ($F_{(1, 34)} = 1.718$; $p=0.199$) or housing x sex ($F_{(1, 34)} = 0.377$; $p=0.544$) on latency to first immobile episode. This trend does not survive Fisher *post-hoc* test for both sexes ($p=0.251$ and $p=0.108$ for males and females, respectively). Thus, EE does not ameliorate depression-like behavior or alter coping style, respectively (fig. 44).

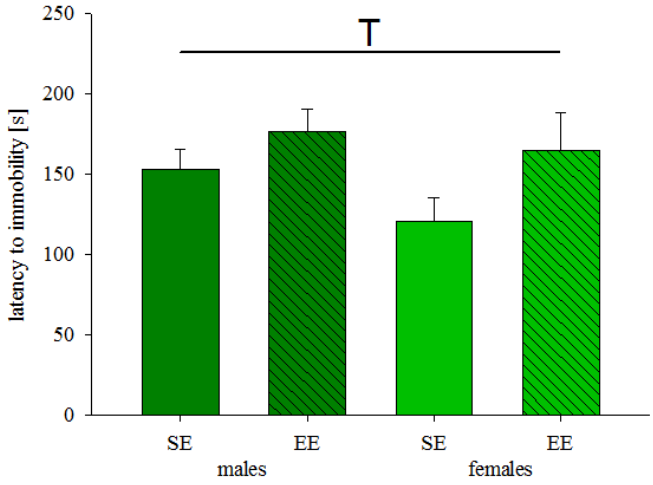


Fig. 44: Coping style is not significantly altered when NABs were housed in an EE (B). N (males) = 12 SE and EE; N (females) = 6 SE and 8 EE

4.4 Impact of environmental enrichment on anxiety-related behavior of outbred CD1 mice.

EE does not cause anxiolysis in "normal" anxiety-related, genetically heterogeneous CD1 mice.

We performed OF, EPM and LD to evaluate whether enrichment causes an anxiolytic effect in outbred CD1 mice:

Test	Parameter measured	P-value for main effect of:		
		Housing	Sex	Housing x sex
OF	total distance travelled	0.849	0.631	0.389
	latency to enter inner zone	0.996	0.130	0.624
	total entries inner zone	0.114	0.343	0.367
	percent time spent in inner zone	0.081	0.222	0.195
	percent distance travelled in inner zone	0.185	0.082	0.207
EPM	total distance travelled	0.176	0.828	0.383
	percent time spent on open arms	0.933	0.039	0.683
	percent entries in open arms	0.312	0.306	0.202
LD	total distance travelled	0.559	0.214	0.003
	percent distance travelled in light compartment	0.863	0.627	0.005
	percent time spent in light compartment	0.739	0.289	0.009

We observe a trend of housing to spent more percent time in the inner zone in the OF ($F_{(1, 30)}$ = 3.272; p=0.081). Fisher *post-hoc* tests reveal a significant difference for females (p≤0.05) but not males (p=0.737) for percent time spent in the inner zone indicating a minor anxiolytic effect (fig. 45).

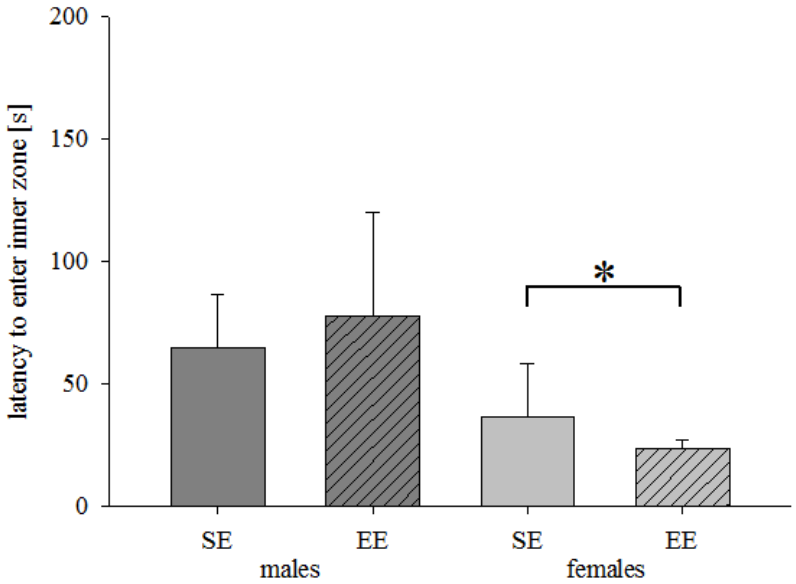


Fig. 45: Latency to enter the inner zone is significantly decreased for females. N (males) = 6 SE and 9 EE; N (females) = 5 SE and 14 EE

In all other parameters measured during OF, EPM and LD, we do not see an effect of housing. Fisher *post-hoc* tests reveal a significant difference or trend for the following parameters measured during LD (fig. 46): total distance travelled for males (p=0.067) and females (p≤0.05), percent time spent in light compartment for males (p≤0.05) and females (p=0.096) and percent distance travelled in light compartment for males (p≤0.05) and females (p=0.052).

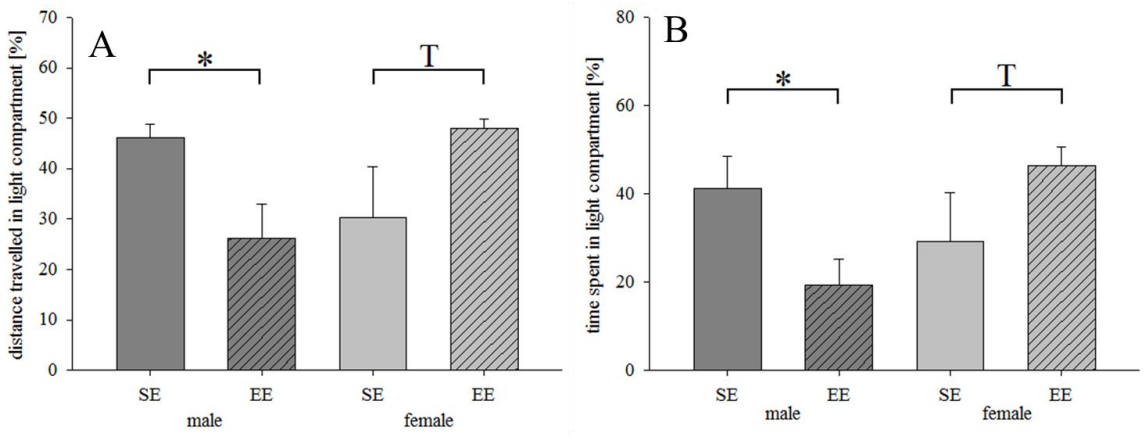


Fig. 46: Enrichment entails a significant anxiogenic effect for males and a minor anxiolytic effect for females indicated by a higher or lower percentage of time (A) or distance (B) travelled in the light compartment, respectively. N (males) = 7 SE and 11 EE; N (females) = 7 SE and 10 EE

To evaluate the anxiolytic effect of OF and anxiogenic effect of LD, we calculated the "emotionality score" to assess the overall impact of EE on CD1 mice. Therefore, we averaged the z-scores of OF, EPM and LD. 2-way ANOVA shows no significant effect of housing on anxiety-related behavior ($F_{(1, 37)} = 0.018$; p=0.895).

Factors	**P-value for main effect:**
housing	0.895
sex	0.053
housing x sex	0.027

No significant difference survived Tukey's *post hoc* test for both groups and sexes, indicating no impact of enriched environment on genetically heterogeneous and "normal" anxiety-related mice.

Due to the already missing effect of EE on coping style of NABs, we didn't perform behavioral tests to asses coping style in CD1 animals.

4.5 Impact of duration on the effects elicited by environmental enrichment

4.5.1 Effects on prolonged enrichment on anxiety-related behavior

Ten instead of four weeks of enrichment further improve the anxiolytic effects observed in females but not males.

We performed OF, EPM and LD to evaluate anxiety-related behavior in HAB mice. Instead of four weeks, HAB mice were housed for ten weeks in EE to investigate the effect of duration on anxiety. For OF, 2-way ANOVA revealed a significant effect of housing for total distance travelled ($F_{(1, 53)} = 30.21$; $p \leq 0.001$), total entries inner zone ($F_{(1, 53)} = 31.89$; $p \leq 0.001$), percent time spent in inner zone ($F_{(1, 53)} = 30.04$; $p \leq 0.001$), percent distance travelled in inner zone ($F_{(1, 53)} = 35.39$; $p \leq 0.001$) and latency to enter inner zone ($F_{(1, 53)} = 38.53$; $p \leq 0.001$). Tukey *post-hoc* tests show a significant increase in total distance travelled comparing EE males ($p \leq 0.05$) and females ($p \leq 0.001$) to its respective controls. Likewise, there is a significant increase for total entries into inner zone, percent distance travelled in inner zone, percent time spent in inner zone and latency to enter inner zone for females ($p \leq 0.001$ for all) but not males (p=0.144, p=0.142, p=0.205 and p=0.241, respectively) (fig. 47A-D).

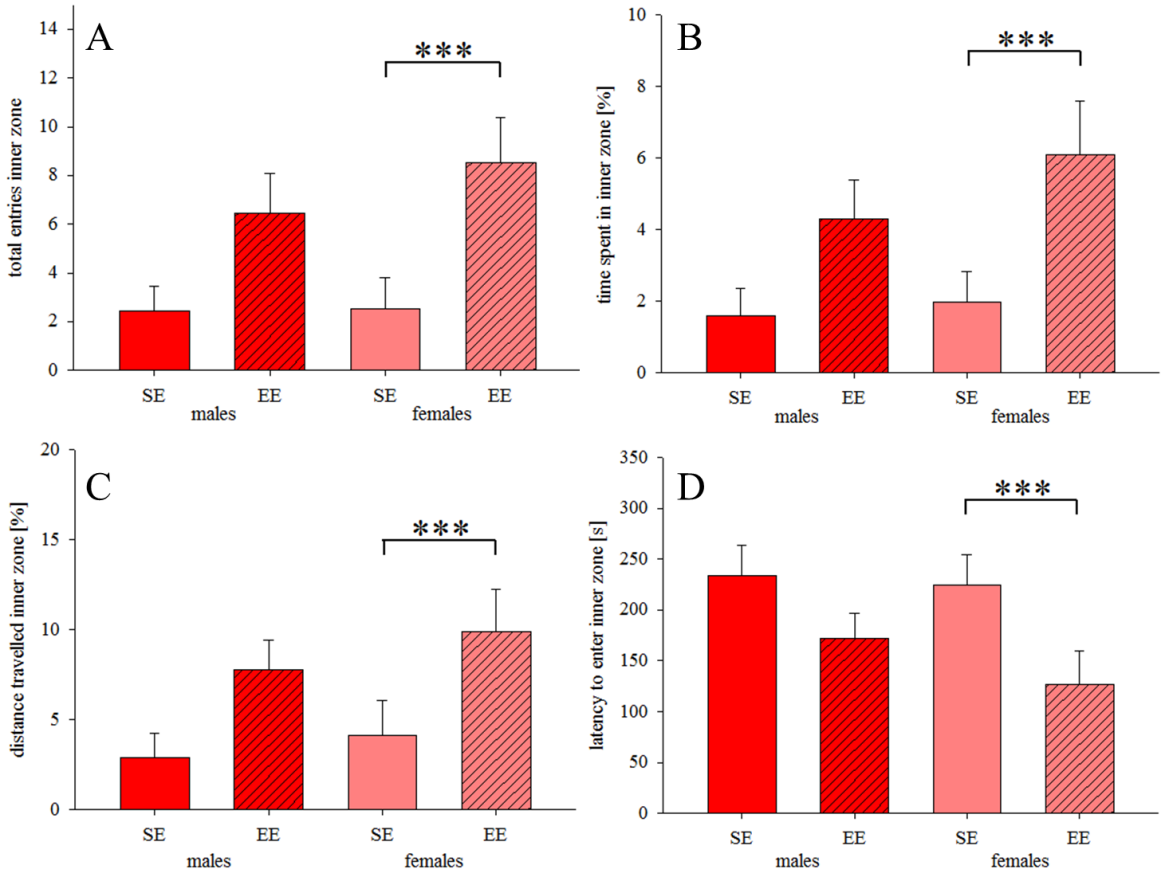


Fig. 47: ten instead of four weeks cause a significant anxiolysis in female HABs, seen in more "total entries inner zone" (A), "percent time" (B) and "percent distance travelled in inner zone" (C) as well as a decreased "latency to enter the inner zone" (D). N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
total distance travelled	≤0.001	0.455	0.177
latency to enter inner zone	≤0.001	0.230	≤0.001
total entries inner zone	≤0.001	0.397	0.160
percent time spent in inner zone	≤0.001	0.244	0.013
percent distance travelled in inner zone	≤0.001	0.275	0.008

We calculated Z-open field to assess the overall anxiolytic effect for OF by correcting for locomotor differences and averaging z-scores of every measured parameter. 2-way ANOVA confirmed a significant effect of housing ($F_{(1, 53)}$ = 6.199; p≤0.05) without an effect of sex ($F_{(1, 53)}$ = 0.012; p=0.912) or an interaction of sex x housing ($F_{(1, 53)}$ = 0.012; p=0.912). Tukey *post-hoc* tests show a trend for an effect of housing for males (p=0.080) and females (p=0.089) indicating an anxiolytic effect of prolonged enrichment for both sexes (fig. 48). Males are 0.58 and females 0.53 SDs below the mean of their respective housed controls indicating a medium effect size.

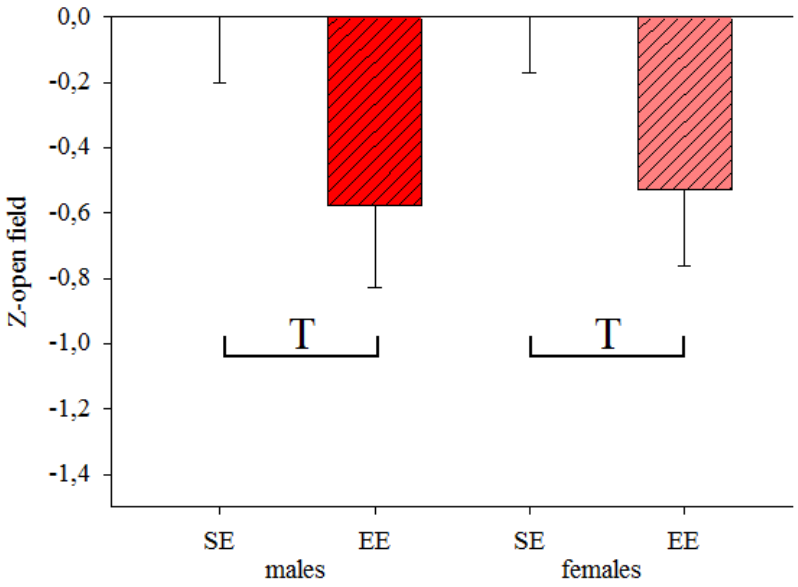


Fig. 48: Z-score for open field corroborates the anxiolytic effects observed for single parameters by integrating and averaging their z-scores and correcting for locomotion. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

The anxiolytic effect of OF is corroborated by EPM (fig. 49). 2-way ANOVA shows a significant effect of housing, i.e. significantly more percent entries into open arms ($F_{(1, 53)}$ = 19.76; p≤0.001) and percent time spent on open arms ($F_{(1, 53)}$ = 48.91.; p≤0.001) without any difference related to locomotion ($F_{(1, 53)}$ = 0.124.; p=0.726).

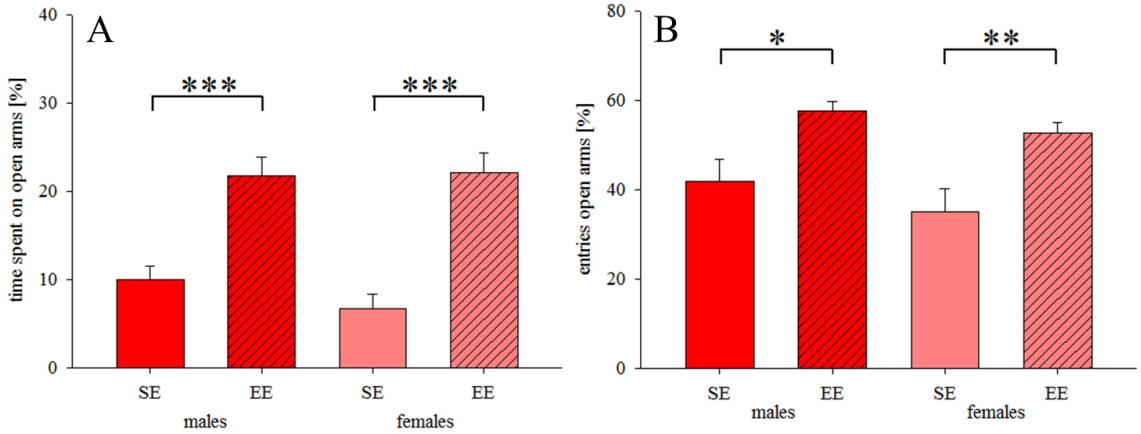


Fig. 49: Both sexes show an anxiolytic effect after EE apparent as increased percent time (A) and entries (B) spent on the open arms. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

We evaluated the overall impact of prolonged EE on anxiety-related behavior measured by EPM via Z-EPM as described earlier. 2-way ANOVA shows a highly significant effect of housing ($F_{(1, 53)}$ = 68.86.; p≤0.001) suggesting a strong anxiolytic effect confirmed by Tukey *post-hoc* tests for males and females (p≤0.001 for both). Males are 1.25 and female 0.91 SDs below the mean of their respective standard-housed controls, representing a strong and medium to strong effect size, respectively (fig. 50).

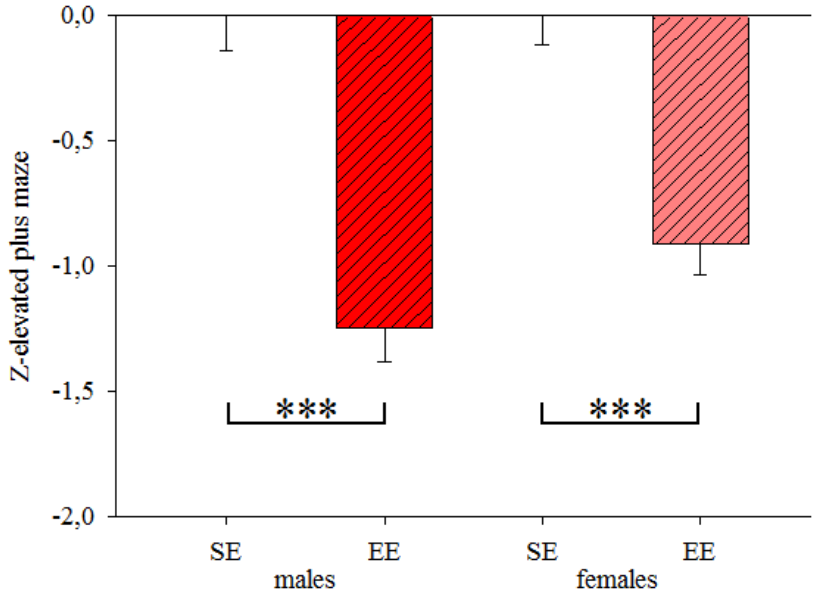


Fig. 50: Z-EPM implies a strong anxiolytic effect for both sexes as indicated by a reduction of 1.25 and 0.91 SDs below the mean compared to controls. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

For LD, we observe significant effects of housing implied by a decrease of latency to enter light compartment ($F_{(1, 53)} = 21.04$; $p \leq 0.001$), more entries into light compartment ($F_{(1, 53)} = 30.17$; $p \leq 0.001$), percent time ($F_{(1, 53)} = 23.68$; $p \leq 0.001$) and percent distance travelled in light compartment ($F_{(1, 53)} = 25.5$; $p \leq 0.001$) for EE mice. 2-way ANOVA also reveals a significant increase in total distance travelled ($F_{(1, 53)} = 25.5$; $p \leq 0.01$) for animals housed in EE.

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
total distance travelled	0.002	0.891	0.003
latency to enter light compartment	≤0.001	0.496	0.09
total entries light compartment	≤0.001	0.711	≤0.001
percent time spent in light compartment	≤0.001	0.460	≤0.001
percent distance travelled in light compartment	≤0.001	0.284	≤0.001

Tukey *post-hoc* tests reveal a significant anxiolytic effect for females but not males indicated by an increased time spent and distance travelled in light compartment as well as a shorter latency to enter light compartment and more entries into it ($p \leq 0.001$ for all) ($p = 0.965$, $p = 0.900$, $p = 0.214$ and $p = 0.643$ for males, respectively) (fig. 51).

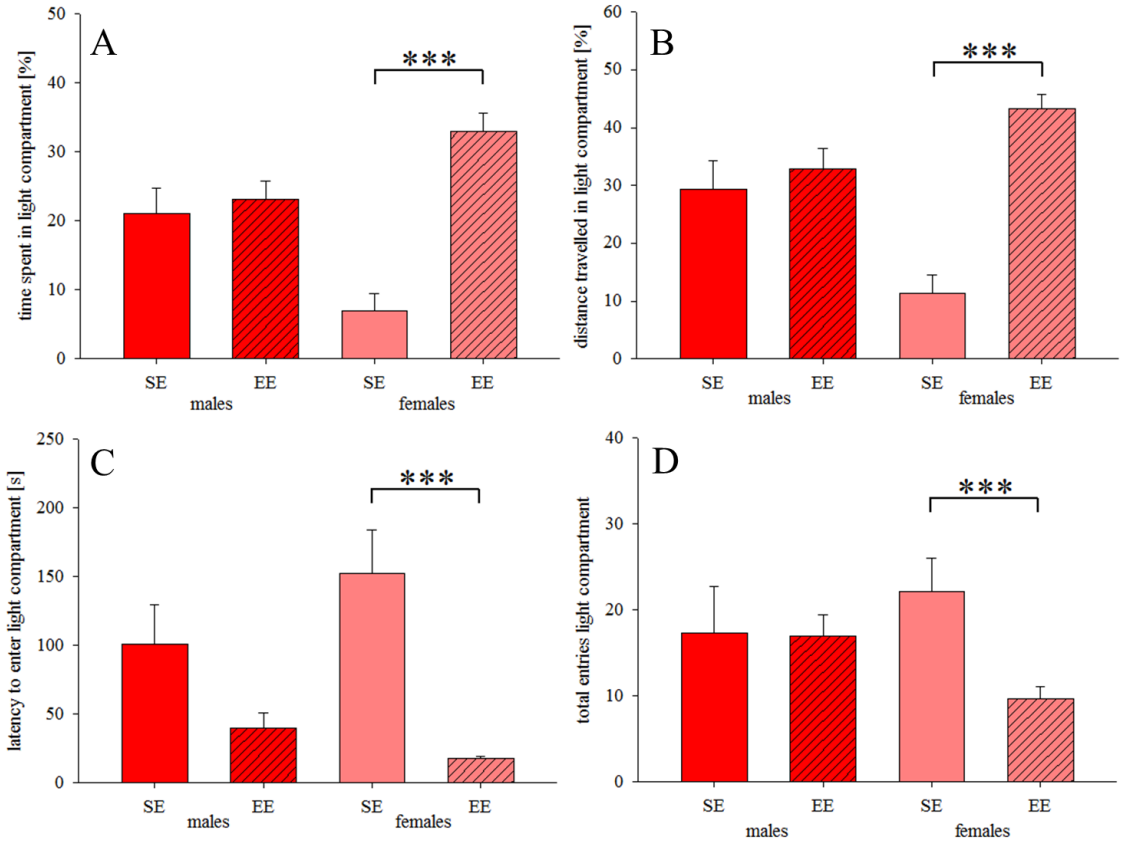


Fig. 51: Females but not males exhibit a strong anxiolytic effect after prolonged EE as seen by more percent time spent (A), distance travelled (B) and more entries (D) as well as a shorter latency to enter the light compartment (C) related to SE controls. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

Z-light dark box was calculated (fig. 52) for evaluation of the overall effect of prolonged EE on parameters measuring anxiety-related behavior via LD. 2-way ANOVA suggests strong anxiolytic effect for females but not males, indicated by an effect of housing ($F_{(1, 53)} = 43.08.$; $p \leq 0.001$), being 1.56 (0.24 for males) SDs below the mean of SE mice.

Factors	**P-value for main effect:**
housing	≤0.001
sex	≤0.001
housing x sex	≤0.001

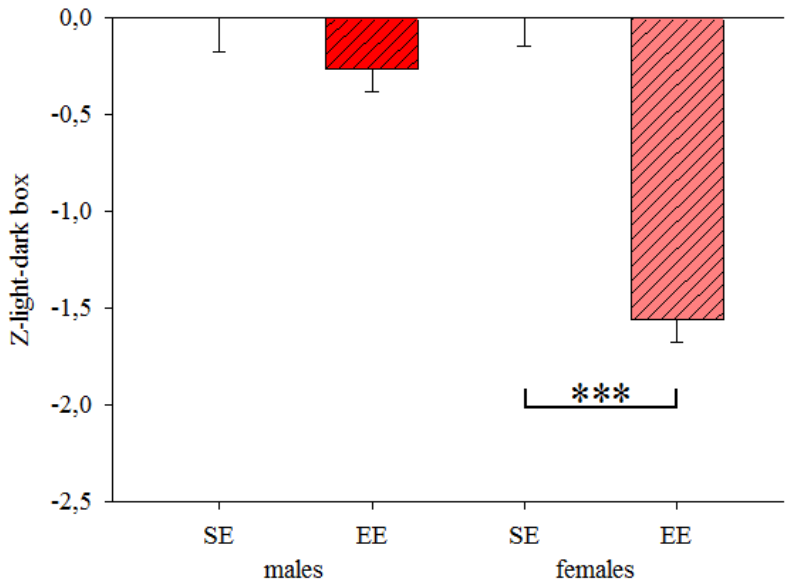


Fig. 52: As indicated by single LD parameters, merely females show in LD an anxiolytic effect after prolonged EE. They are 1.56 SDs below the mean of SE controls, in contrast to 0.24 SDs for EE males. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

Finally, we averaged z-scores of OF, EPM and LD to calculate "emotionality score" for prolonged EE lasting ten weeks. 2-way ANOVA indicates a significant reduction of anxiety-related-behavior shown by an effect of housing ($F_{(1, 53)}$ = 71.38.; p≤0.001), without an effect of sex ($F_{(1, 53)}$ = 2.27.; p≤0.138) or housing x sex ($F_{(1, 53)}$ = 2.27.; p≤0.138) (fig. 53).

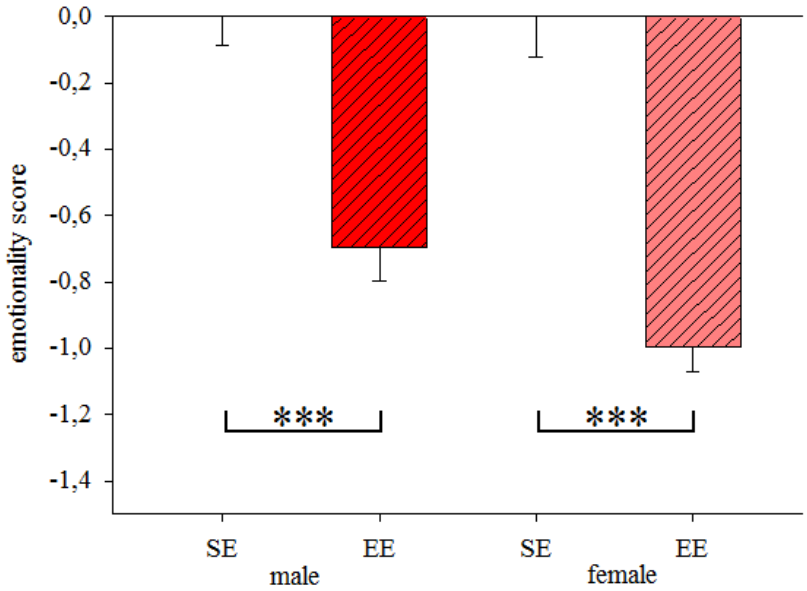


Fig. 53: Prolonged EE entails a significant anxiolytic effect for both sexes. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

Tukey *post-hoc* tests show a significant anxiolytic effect for both males and females (p≤0.001 for both) by 0.7 and 1.0 SDs below the mean of their respective controls, respectively. This implies a medium effect for males and a strong effect for females.

To analyze whether the distribution is shifted towards a medium or strong effect size, we performed a χ^2-test with a 2x3 factorial design as described before. Indeed, males show a shift of effect size almost exclusively from a small to a medium effect size (χ^2 = 88.27; p≤0.001). Females show an even stronger shift from a small equally to a medium and strong effect size (χ^2 = 102.9; p≤0.001). Thus, shift of effect size, i.e. an anxiolytic effect is clearly observed after prolonged enrichment for both sexes with females exhibiting an even more pronounced shift (fig. 54).

Comparison	χ^2	Degree of freedom	P-value	Cramer's V
male SE vs. EE	88.27	2	p≤0.001	0.6643
female SE vs. EE	102.9	2	p≤0.001	0.7173
male vs. female EE	25.95	2	p≤0.001	0.3602
male vs. female SE	12.5	2	p=0.019	0.2500

Cramer's V indicates a strong effect size for EE in relation to SE males (0.66) and females (0.72). Interestingly, both standard and enriched females show a shift towards a medium and strong effect size compared to respective male HABs.

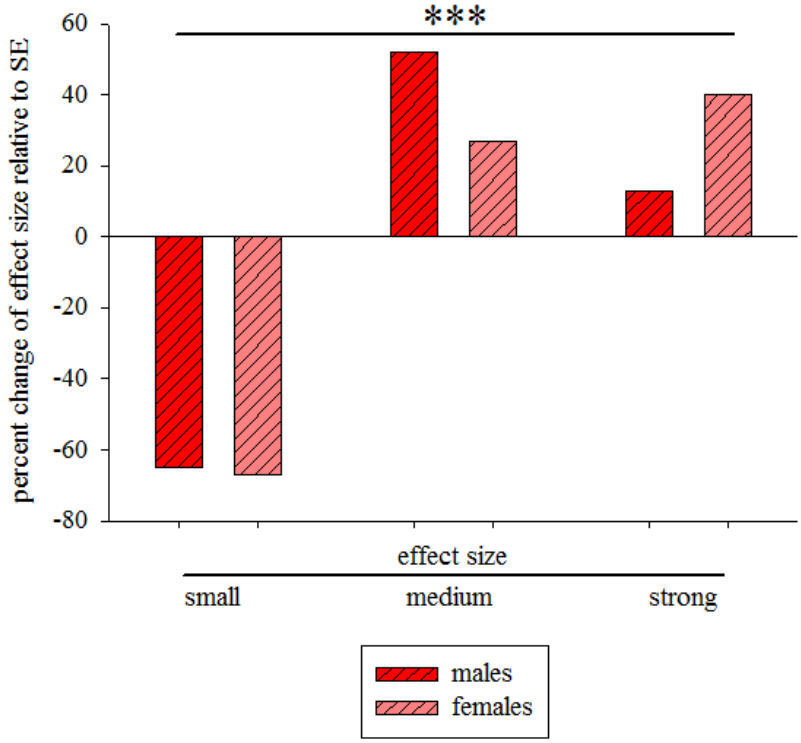


Fig. 54: EE Males shift their effect size mostly from small to medium in contrast to females, which show a shift to medium and predominantly strong effect size compared to respective controls. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

4.5.2 Impact of prolonged enrichment on coping style

Ten instead of four weeks of environmental enrichment do not alter the coping style of males, whereas females seem to switch their coping style from active to passive.

We performed TST and FST to assess coping style of prolonged enrichment. For TST, there is no significant effect of housing on percent time spent immobile ($F_{(1, 53)}$ = 2.27; p≤0.138), number of immobile episodes ($F_{(1, 53)}$ = 1.97; p=0.166) and latency to first immobile episode ($F_{(1, 53)}$ = 1.228; p=0.273) suggesting no difference of coping style after prolonged enrichment.

Parameter measured	P-value for main effect of:		
	Housing	Sex	Housing x sex
percent time spent immobile	0.138	0.047	0.993
number of immobile episodes	0.166	0.466	≤0.001
latency to first immobile episode	0.273	0.316	0.531

A Tukey *post-hoc* test indicates significantly more immobile episodes of EE compared to SE females ($p \leq 0.01$). To evaluate the overall impact of prolonged enrichment on coping style, we calculated Z-TST and indeed, we observe a significant effect of housing ($F_{(1, 53)} = 5.59$; $p \leq 0.05$), sex and interaction of housing x sex ($F_{(1, 53)} = 9.216$; $p \leq 0.01$ for both) via 2-way ANOVA (fig. 55). Enriched housed females are 1.45 SDs above the mean of their SE counterparts, pointing towards a shift of coping style from active to passive. There is no significant difference between male groups seen as a decrease of 0.18 SDs of EE compared to SE males.

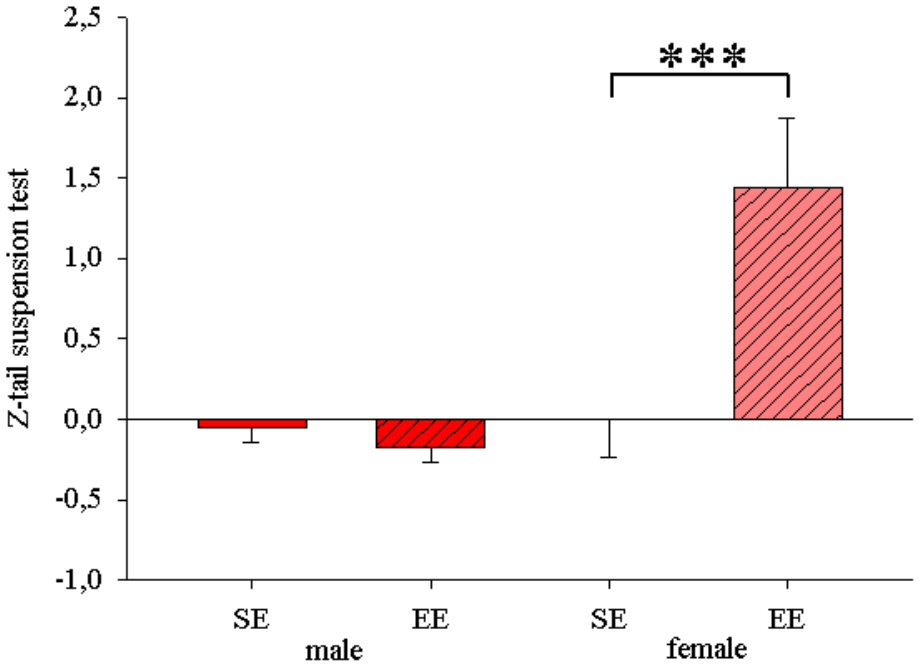


Fig. 55: Prolonged enrichment seems to shift the coping style of females from active to passive. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

For FST, 2-way ANOVA reveals a significant effect of housing on number of immobile episodes ($F_{(1, 53)} = 4.995$; $p \leq 0.05$) and latency to first immobile episode ($F_{(1, 53)} = 5.626$; $p \leq 0.05$).

Parameter measured	P-value for main effect of:		
	Housing	Sex	Housing x sex
number of immobile episodes	0.030	0.442	0.003
latency to first immobile episode	0.021	0.573	0.432

A Tukey *post-hoc* test shows a significant higher number of immobile episodes ($p \leq 0.01$) for EE in relation to SE females. Latency to first immobile episode does not survive Tukey's *post-hoc* test ($p = 0.110$). 2-way ANOVA for Z-FST reveals no significant effect of housing ($F_{(1, 53)} = 2.44$; $p = 0.124$) but an effect of sex and an interaction of housing x sex ($F_{(1, 53)} = 10.98$; $p \leq 0.01$ for both). Similar to Z-TST, EE females show a significant shift from active to passive coping compared to SE females by 0.52 SDs above their mean (fig. 56). In

contrast, there is no significant difference of EE related to SE males with the former being 0.18 SDs below the mean of controls.

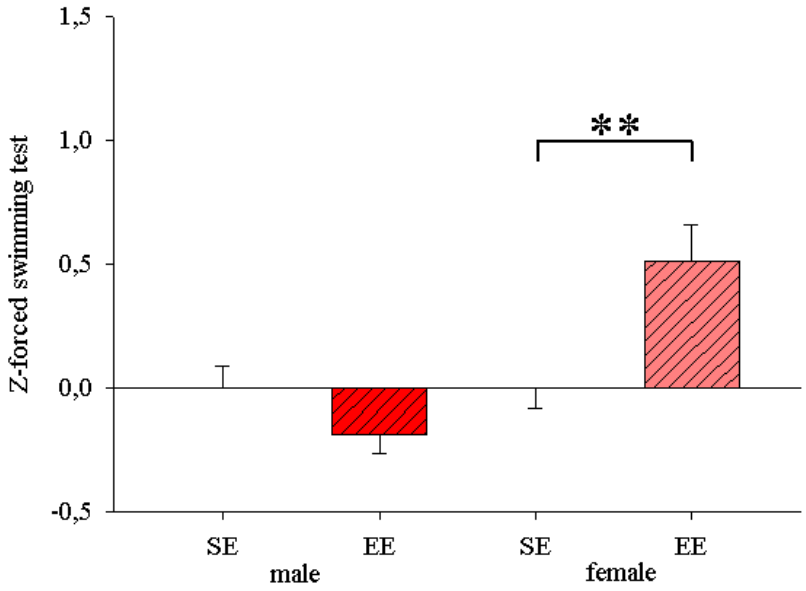


Fig. 56: Similar to Z-TST, FST indicates that prolonged enrichment seems to shift the coping style of females from active to passive. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

To assess the overall effect of prolonged enrichment on the coping style of HABs, we calculated "depression score" by averaging z-scores of FST and TST. A 2-way ANOVA followed by a Tukey *post-hoc* test ($p \leq 0.001$) for depression score corroborates a significant shift from active to passive coping for EE related to SE females ($F_{(1, 53)} = 6.41$; $p \leq 0.05$) (fig. 57).

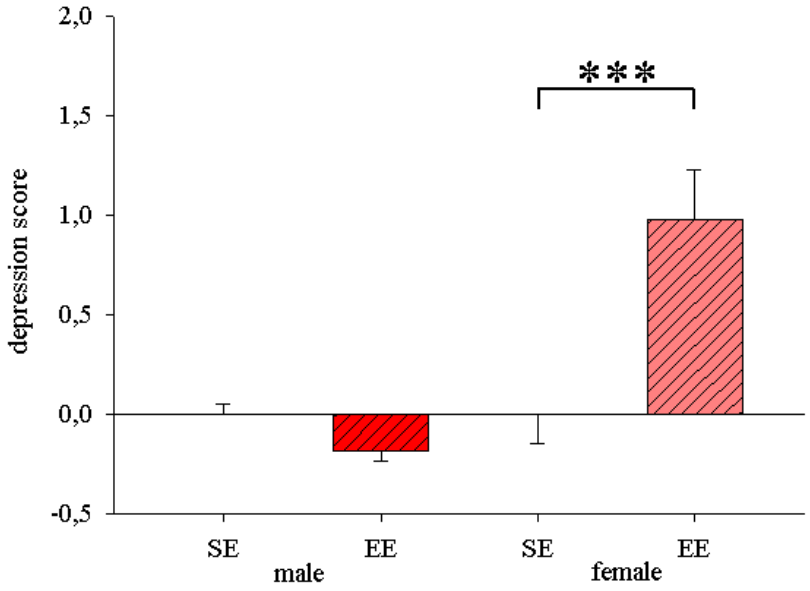


Fig. 57: Depression score depicts a significant shift from active to passive coping for EE related to SE females, whereas there is no difference between male groups. N (males) = 12 SE and 15 EE; N (females) = 15 SE and EE

EE females and males are 0.98 and 0.18 SDs above and below the mean of respective

control groups. Depression score indicates a medium to strong effect for females and a weak effect for males.

4.6 Comparison of the impact of environmental enrichment between NABs and HABs receiving either 4 or 10 weeks of EE.

HABs compared to NABs show a stronger anxiolytic effect after enrichment, which can be further increased for females when housed for 10 instead of 4 weeks in an EE. In contrast, the anxiolytic effect observed of males does not seem to be increased any further by prolonged housing in an EE.

We performed a 2-way ANOVA to compare the anxiolytic effect size, i.e. emotionality score, between NABs and HABs receiving either 4 or 10 weeks of enrichment. Indeed, there is a significant effect of subline (NAB, HAB 4 weeks of EE, HAB 10 weeks of EE) and an trend for the interaction of subline x sex.

Factors	P-value for main effect:
housing	≤0.001
sex	0.898
housing x sex	0.052

Fisher *post-hoc* tests indicate a significant increase of effect size comparing NABs with HABs receiving four (p=0.100 and p≤0.05 for males and females, respectively) or ten weeks (p≤0.05 and p≤0.001 for males and females, respectively) of EE (fig. 58).

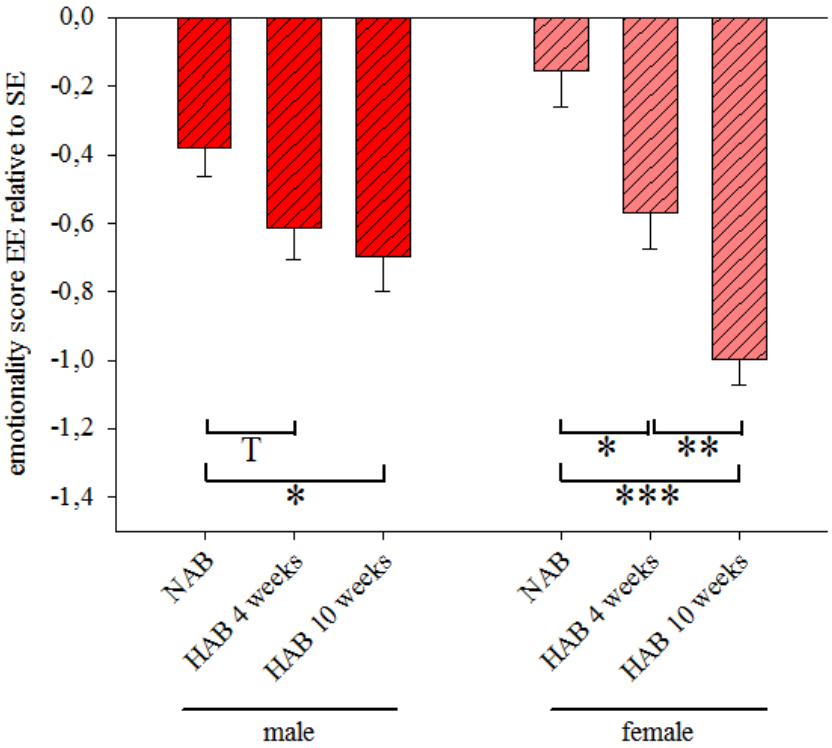


Fig. 58: HABs related to NABs show a stronger anxiolytic effect after EE, which can be further increased for females by prolonging EE from 4 to 10 weeks. N (males) = 12 (NAB), 21 (4 weeks EE) and 15 (10 weeks EE); N (females) = 8 (NAB), 20 (4 weeks EE) and 15 (10 weeks EE)

The anxiolytic effect observed in females can be in increased significantly by prolonging housing in an EE for 10 weeks, whereas males seem not to benefit from 10 instead of 4

weeks of enrichment. The difference of effect size for females between the groups seems to be more pronounced compared to males.

4.7 Contribution of maternal, pup and adolescent behavior to the anxiolytic effect elicited by environmental enrichment in HABs.

4.7.1 Impact of maternal behavior on anxiety-related behavior during enrichment.

Maternal behavior does not contribute to the anxiolytic effect elicited by EE. Instead, SE compared to EE dams seem to take more care of their pups.

All data of section 3.7 were gained in close collaboration with Sergey V. Sotnikov who contributed invaluably to ensure successful completion. We performed 2-way ANOVA to investigate whether maternal care contributes to the anxiolytic effect observed after EE in HABs. This analysis enables us to identify differences between the groups on the respective days of observation (PND 15 and 17) and whether the behavior of the EE or SE mothers differs significantly between these days. There is no effect of housing on licking/grooming or nursing ($F_{(1,\ 14)}$ = 0.354; p=0.562 for both), but we detected a significant impact of housing on sitting together with body contact on ($F_{(1,\ 14)}$ = 7.955; p≤0.05). Tukey *post-hoc* test identifies that SE mothers spent more time sitting together with body contact with their pups compared to EE dams on PND 17 (p≤0.05) (fig. 59A). Moreover, we monitor a significant effect of housing on locomotion on PND 15 ($F_{(1,\ 14)}$ = 8.134; p≤0.05), time mother spent with pups on PND 15 ($F_{(1,\ 14)}$ = 5.536; p≤0.05) and digging on PND 17($F_{(1,\ 14)}$ = 21.86; p≤0.001). Tukey *post-hoc* tests reveal that there is a trend for SE mothers to spent more time with their pups (p=0.064), whereas we observe a trend of increased locomotion (p≤0.064) and significant increase in digging (p≤0.001) for EE in relation to SE dams (fig. 59 B-D).

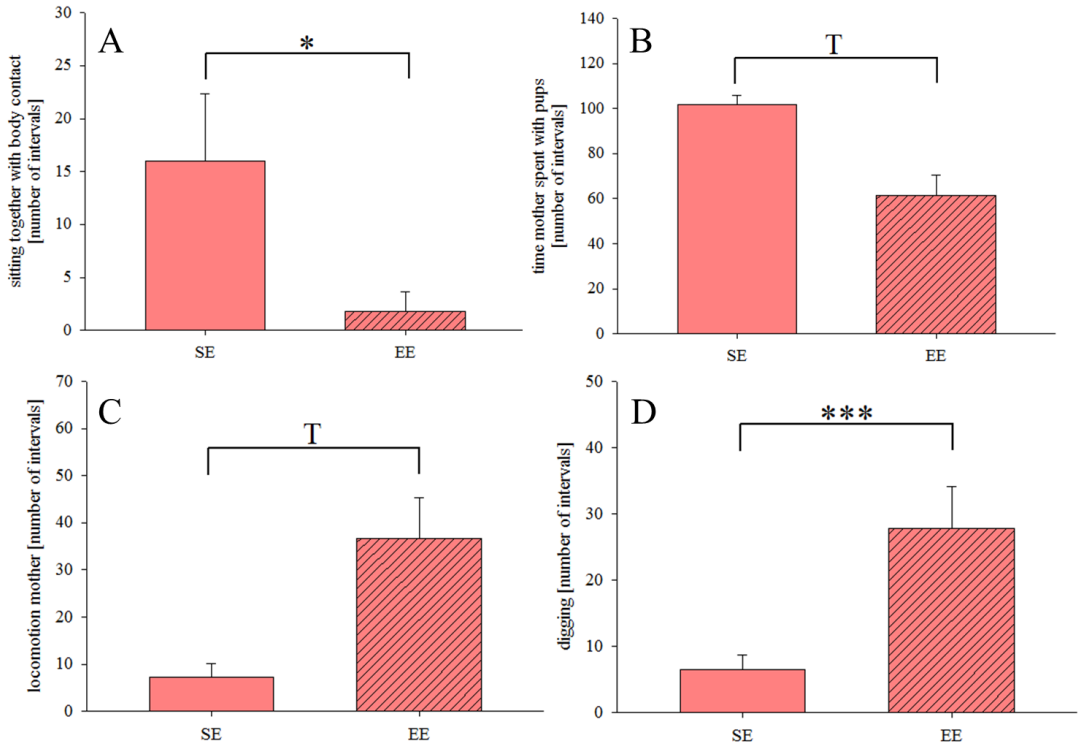


Fig. 59: SE related to EE dams spent more time sitting together with body contact (A) and more time with pups in total (B), whereas EE mothers spent more time to interact with their environment indicated by an increased locomotion (C) and more frequent digging (D). N (cages) = 4 SE and 5 EE

In total, SE dams spent more time with their pups by actively taking care of them (PND 15 and 17) contrary to EE mothers, which spent more time to interact with their environment indicated by increased locomotor (PND 15) and digging (PND 17) activity. There is no significant difference for both housing conditions for any of the monitored behaviors comparing PND 15 and 17.

We monitored the behavior of the dams during the night of PND 15 to 16. During that time, mothers and pups are in their home cage, which is identical for EE and SE groups during partial enrichment lasting from PND 15-28. A MWU test reveals a significant increase of locomotion for EE related to SE dams ($p \leq 0.05$) without any difference for all other parameters monitored. Importantly, we observed very low levels of locomotion and digging during the night (≤10 time intervals) since dams spent a lot of time with eating/drinking as well as grooming and nursing their pups.

4.7.2 Effect of EE on pup behavior

Pups raised in EE actively use the provided toys and manipulate their microenvironment, whereas SE pups engage significantly more in running possibly due to a lack of alternatives.

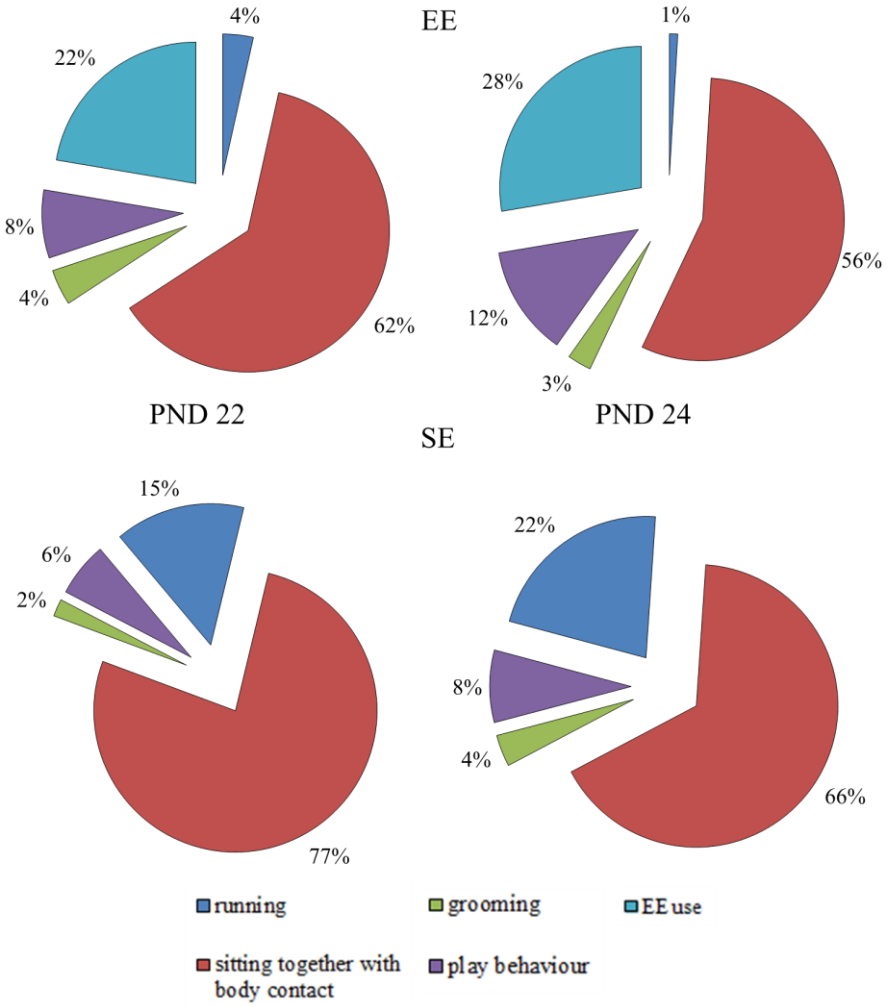


Fig. 60: EE pups actively use toys and manipulate their microenvironment by spending almost all of their active time with “play behavior” and “EE use” on both days of observations. Contrary, SE pups significantly spent more time with “running”, possibly trying to compensate a lack of alternatives. N (cages) = 4 SE and 5 EE

Fig. 60 depicts the percentage that animals spent to perform all monitored behaviors. EE pups invest 30% (PND 22) and 40% (PND 24) of their total activity for active behaviors like interaction with EE and play behavior. At the same time, they spend 62% (PND 22) and 56% (PND 24) of their total activity for passive activities like sitting together with body contact. Contrary, SE pups spent more time for passive behaviors indicated by spending 77% (PND 22) and 66% (PND 24) of their total activity for sitting together with body contact. In parallel, SE pups spend more time, i.e. 15% (PND 22) and 22% (PND24) of their total activity for "running" around.

2-way ANOVA revealed a significant effect of housing on running ($F_{(1,\ 13)}$ = 80.33; $p \leq 0.001$) and for sitting together with body contact ($F_{(1,\ 13)}$ = 4.816; $p \leq 0.05$), but no significant effect of housing on sociopositive behavior comprising grooming ($F_{(1,\ 13)}$ = 0.409; p=0.533) and play behavior ($F_{(1,\ 13)}$ = 1.873; p=0.194). A Tukey *post-hoc* test reveals a significant increase of running in the home cage for SE compared to EE pups on PND 22 ($p \leq 0.01$) and PND 24 ($p \leq 0.001$) (fig. 61).

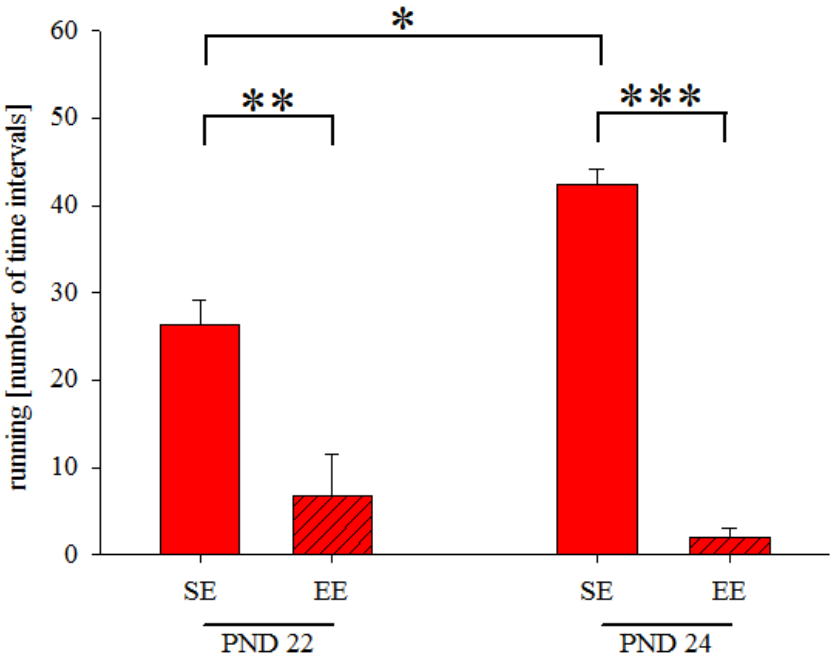


Fig. 61: SE related to EE pups significantly engage more in "running around" in their home cage on both days of observation. N (cages) = 4 SE and 5 EE

It seems like SE pups shift their behavior: on the one hand the are significantly more inactive indicated by an increased time sitting together with body contact ($F_{(1,\ 13)}$ = 4.816; $p \leq 0.05$) and on the other hand their active behavior is predominantly reduced to running due to a lack of alternatives. This is corroborated by the fact that the significant increase of running gets lost when EE and SE pups are housed in an identical home cage during the dark period of partial enrichment (fig. 62; MWU; U=3, p=0.111).

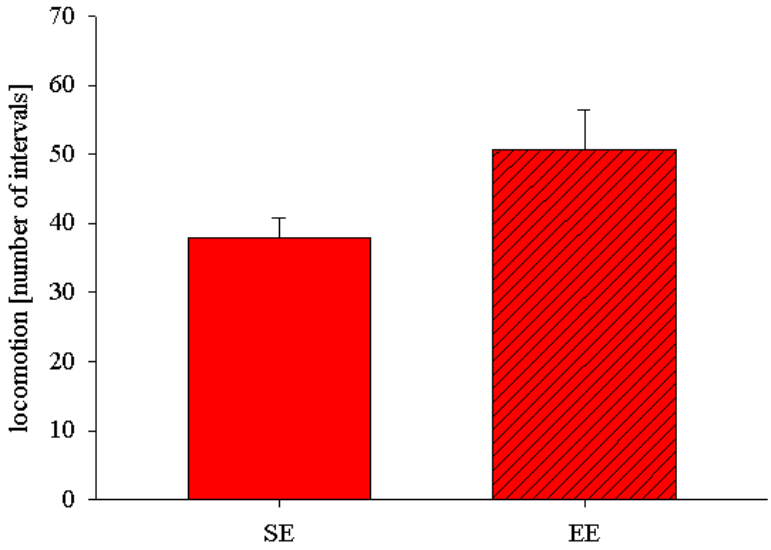


Fig. 62: When EE and SE pups are housed in identical home cages during the dark period, the increase of running of SE pups disappears. N (cages) = 4 SE and 5 EE

A Tukey *post-hoc* test shows that SE pups significantly increase running from PND 22 to PND 24 ($p \leq 0.05$). This might indicate that, with increased time, housing in SE not only thwarts highly motivated behaviors, which might be compensated by running - the thwarting seems to be further increased causing even higher levels of running (fig. 61).

4.7.3 Impact of EE on adolescent behavior

Enrichment increases the number of actively performed behaviors like social interactions contrary to SE adolescents, which significantly sleep more during the day and spent more time with body contact during the nighttime compared to the daytime. Moreover, EE adolescents intensively use the provided toys and manipulate their microenvironment, whereas SE adolescents perform significantly more rearings during day and night, maybe reflecting a lack of alternatives.

SE in relation to EE adolescent mice show more passive behaviors during the day- and nighttime of PND 35. 2-way ANOVAs reveal an effect of time ($F_{(1, 15)} = 25.94$; $p \leq 0.001$) and an interaction of time x housing ($F_{(1, 15)} = 25.94$; $p \leq 0.001$) for sitting together with body contact. A Tukey *post-hoc* test surprisingly reveals that SE adolescents spend even more time sitting together with body contact during nighttime in relation to daytime ($p \leq 0.001$) though mice are nocturnal mammals being mostly active during the night (fig. 63A). However, there is no significant difference between EE and SE adolescents for sitting together with body contact during day- and nighttime ($p=0.891$ and $p=0.296$, respectively). Furthermore, we observe an effect of housing on sleeping ($F_{(1, 15)} = 25.94$; $p \leq 0.001$) with SE compared to EE adolescents sleeping significantly more during daytime ($p \leq 0.001$). Not surprisingly, a Tukey *post*-hoc test shows that SE animals sleep less during night- related to daytime ($p \leq 0.001$). On contrary, this is not true for EE adolescents ($p=0153$) corroborating a shift to more active behaviors during daytime. During nighttime, SE adolescents invest the time gained due to less sleeping predominantly in "sitting together with body contact", indicated by an increase from 11% during daytime to 23% during nighttime (increase of ca. 110%).

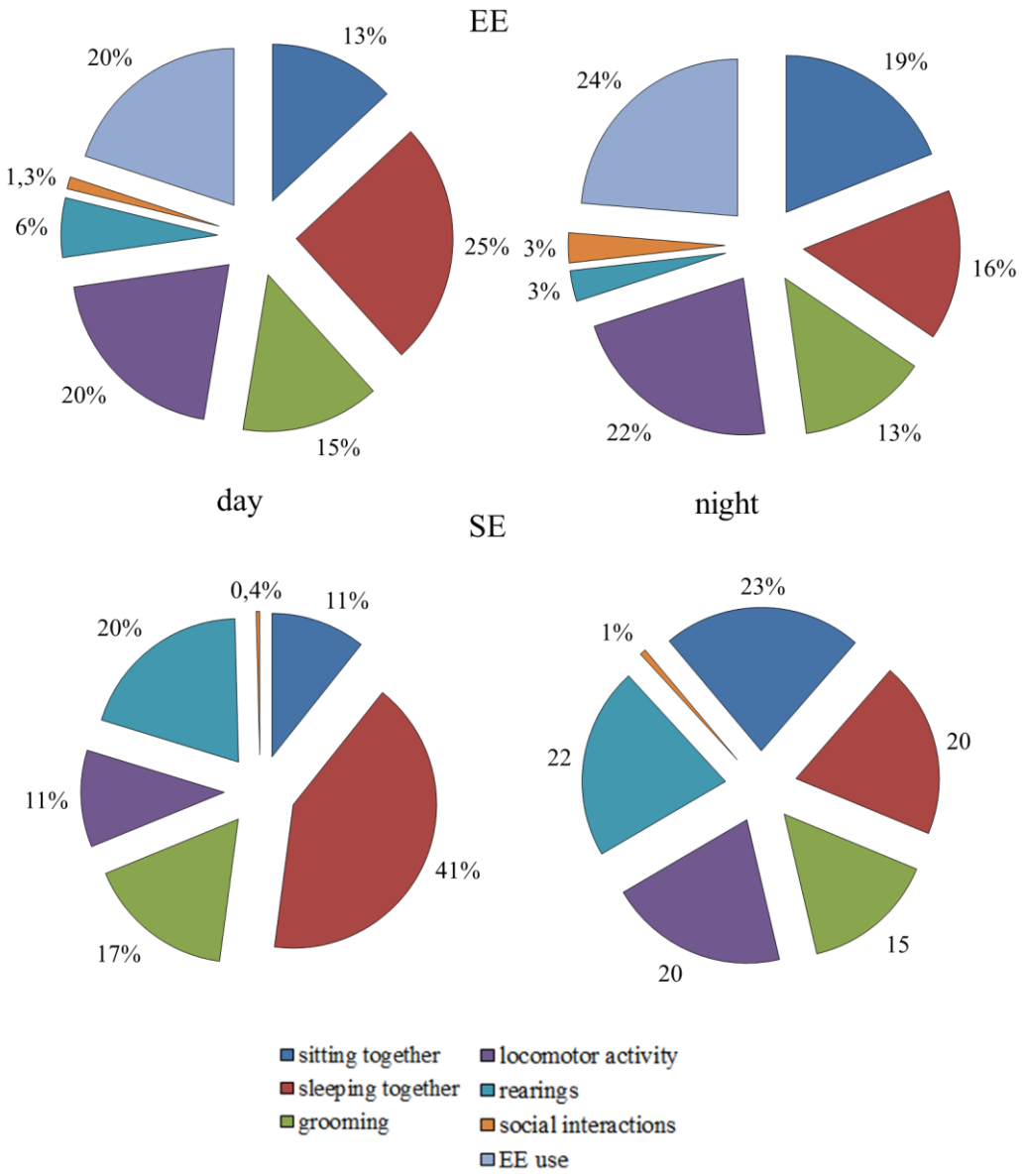


Fig. 63: SE compared to EE adolescents engage more in passive behaviors indicated by sleeping significantly more during daytime and by sitting significantly more together with body contact during night- in relation to daytime. SE mice sleep less during nighttime and invest the gained time predominantly in sitting together with body contact and to a less amount in running and rearing. N (cages) = 5 SE and 6 EE

A trend of housing for "running" ($F_{(1,\ 15)} = 3.79$; $p \leq 0.071$) and a significant effect of housing for "rearings" ($F_{(1,\ 15)} = 20.63$; $p \leq 0.001$) indicates that SE adolescents invest the remaining time in "rearing" and "running". A Tukey *post-hoc* confirms a significant increase of locomotion for SE adolescents from day- to nighttime ($p \leq 0.001$) from 11% to 20% (increase of ca. 80%), thereby reaching levels of EE mice. Though a significant effect of housing, "rearings" of SE mice do not increase significantly from day- to nighttime as shown by a Tukey *post-hoc* test ($p = 0.829$), but SE in relation to EE adolescents show a trend to perform more rearings during day- ($p = 0.063$) and significantly perform more rearings during nighttime ($p \leq 0.05$). EE related to SE animals show a trend to perform more social interactions during the nighttime ($p = 0.065$)

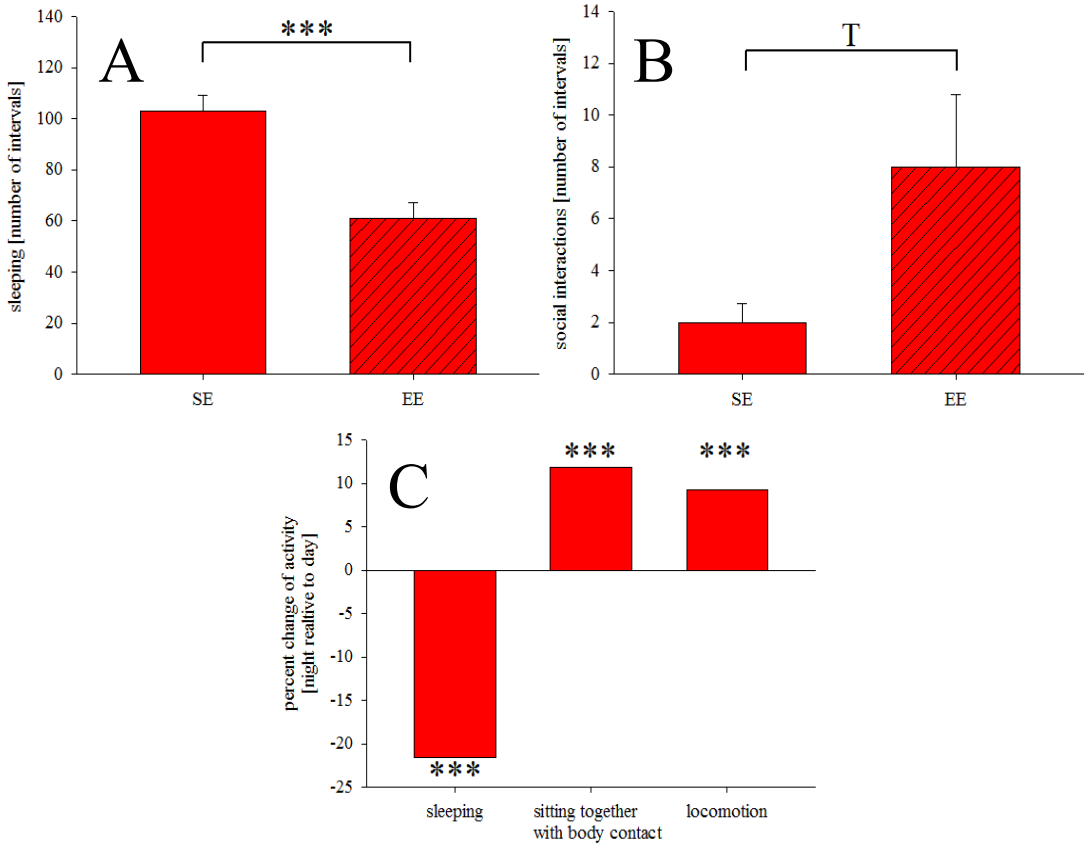


Fig. 64: EE causes a significant decrease of sleeping during daytime (A) and a significant increase of social interactions during nighttime (B). SE adolescents invest the time gained due to less sleeping during nighttime in sitting together with body contact and locomotion. N (cages) = 5 SE and 6 EE

As a summary, EE adolescents are more active during the daytime (less sleeping) and show significantly more social interactions during nighttime. SE mice split the time gained due to less sleeping almost equally to sit together with body contact and for running (fig. 64). Interestingly, locomotion of EE and SE adolescents is not significantly different anymore compared to EE and SE pups but the latter perform more rearings during day- and nighttime. We would like to emphasize two differences related to housing conditions: i) SE adolescents in contrast to SE pups were housed in same sex groups of three instead of mixed sex groups of eight pups and ii) and SE adolescents were housed in a smaller cage comprising 363cm² in place of 825cm², thereby reducing the possibility to run *per se*.

4.8 Identification of candidate genes related to anxiety-related behavior.

4.8.1 Quantitative real-time PCR

We identified Ucn, DBH, Tmem132d and Crhr1 as genes likely to be associated with the anxiolytic effect observed after EE. Thereby, Crhr1 emerges as the major gene of interest.

We used qPCR to identify gene expression differences in brain regions known to be associated with anxiety-related behavior. Thus, we combined literature research with a list of candidate genes (tab. 11) identified by Czibere (2008) to obtain brain regions and genes of interest:

Gene name	Gene symbol	Brain region
arginine vasopressin	Avp	PVN
corticotropin-releasing hormone receptor 1	Crhr1	BLA
corticotropin-releasing hormone	Crh	BLA
dopamine beta hydroxylase	Dbh	LC
neuropeptide S receptor 1	Npsr1	LC
transmembrane protein 132d	Tmem132d	CG
urocortin 1	Ucn	BLA

Tab. 11: Depicted genes were analyzed by qPCR after extraction of RNA from the corresponding brain regions.

We observe a significant difference of relative gene expression comparing SE and EE mice for the following genes by performing MWU-tests:

Gene symbol	Significance
Tmem132d	$p \leq 0.01$
Dbh	$p \leq 0.01$
Crhr1	$p \leq 0.05$
Ucn	$p \leq 0.01$

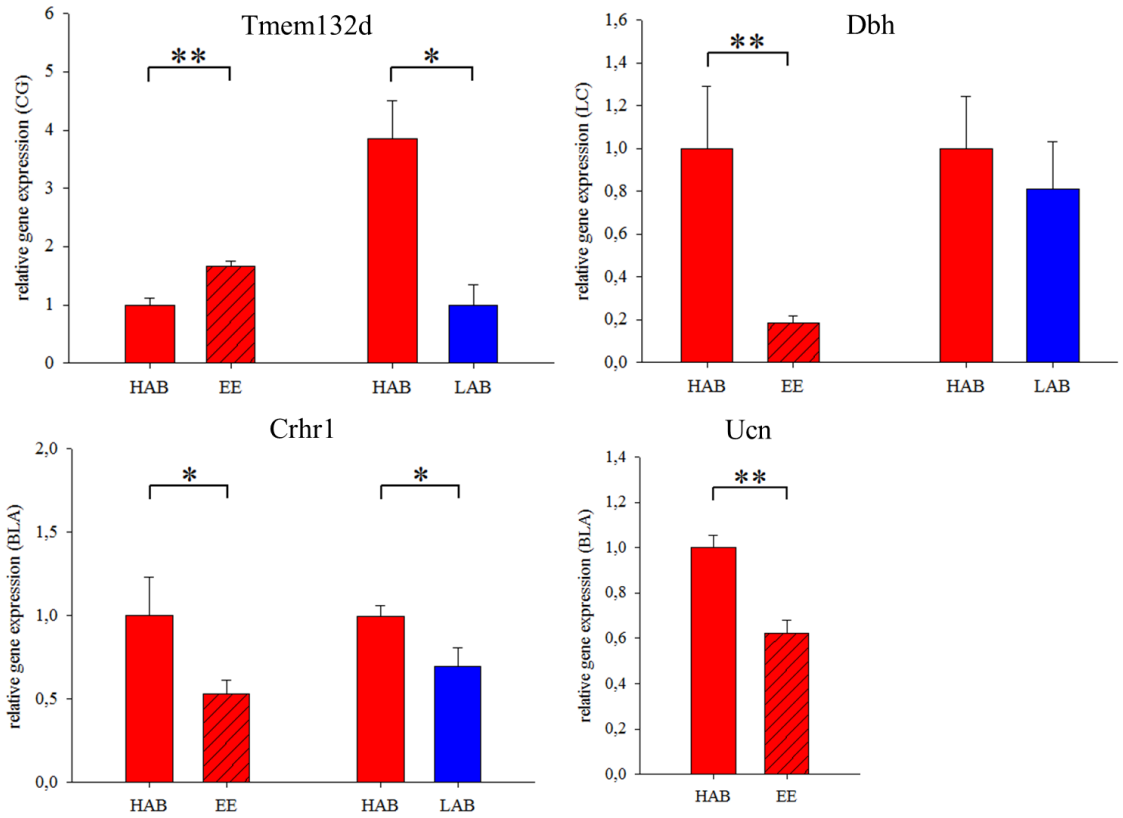


Fig. 65: EE significantly increases Tmem132d, Dbh and Crhr1 mRNA levels. Contrary to EE, Tmem132d expression is higher in HABs related to LABs, thereby highlighting Crhr1 as the gene of major interest since EE is able to reduce overexpression of HABs. Ucn can bind to Crhr1 and rather potentiates than masks the anxiogenic effect of Crhr1 because it is significantly less expressed after EE too. Data of *Tmem132d* mRNA expression comparing HAB vs. LAB were kindly provided by Dr. Czibere from Erhardt et al. (2011). N = 5-9 for HAB, 4-5 for LAB and 6-7 for EE

Fig. 65 depicts a significant increase of Tmem132d expression in CG after EE, which is surprisingly in the opposite direction we expected: HAB mice show higher levels of Tmem132d expression compared to LABs, pointing towards an anxiogenic effect of Tmem132d in SE HAB, whereas our data indicate an anxiolytic effect in EE HAB mice. Moreover, EE causes a highly significant reduction of Dbh within LC to ca. 20% of HAB expression indicating an important contribution of this gene to the observed anxiolytic effect elicited by enrichment. However, a MWU-test shows no difference comparing HAB and LAB mice ($p=0.537$). Crhr1 emerges as the gene of major interest since EE entails a significant reduction of gene expression and HAB compared to LAB animals show higher expression of Crhr1 in the BLA. Our data indicate an anxiogenic role of Crhr1, which seems to be determined by genetic predisposition and is amenable for environmental stimuli as provided by EE. Because Ucn can bind to Crhr1 and mask possible effects caused by EE, we compared EE and SE animals for gene expression differences and found a significant decrease of UCN after enrichment. Thus, Ucn rather potentiates than masks the anxiolytic effect of EE since we observe less Crhr1 and less Ucn expression in EE related to SE mice.

Importantly, we do not find a significant difference of Crh expression within the BLA comparing EE and SE HABs, thereby minimizing possible masking effects of the ligand ($p>0.1$). Though, our group identified Npsr1 (Slattery, Naik et al, in preparation) and AVP (Bunck et al. 2009) as candidate genes likely to be associated with anxiety-related behavior due to significantly different expression levels between HAB and LAB, we were not able to verify a significant difference for Npsr1 in LC ($p=0.818$) and AVP within the PVN ($p>0.1$).

4.8.2 Western Blot

Low protein levels within the amygdala of both, CRHR1 and GR within the nucleus prevented verification of significant differences on protein level as indicated on mRNA level by qPCR.

Though a multitude of different protocols for protein extraction and pooling of up to five animals per group were used, GR concentration was still under the detection limit of the antibody. Similar, CRHR1 antibodies used were not specific, i.e. bands appeared at unexpected positions (ca. 95kd instead of 47kD) or were not specific enough and bound to CRHR1 and 2 (fig. 66). This problem has been recently described by Refojo et al. (2011).

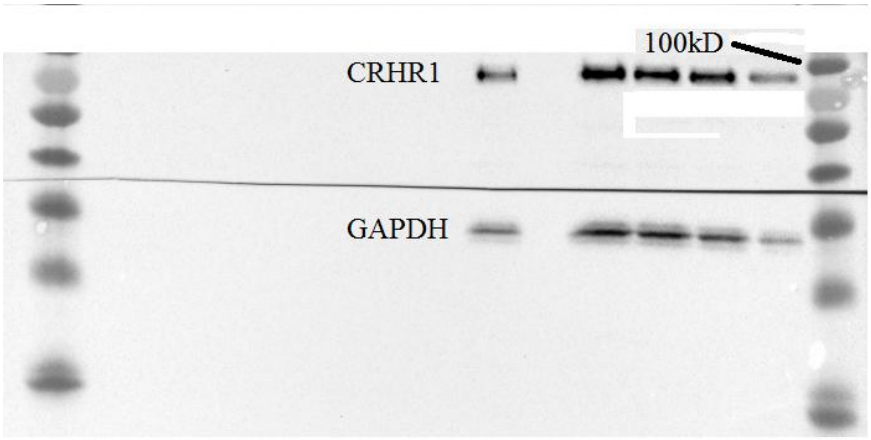


Fig. 66: Due to lacking antibody specificity of CRHR1, observed differences on mRNA level couldn't be confirmed on protein level. Corticotropin-releasing hormone receptor 1 (CRHR1), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). N = 5 per sample

4.9 Pharmacological validation of CRHR1 via an α-helical antagonist

Bilateral injection of the α-helical CRF (9-41) antagonist within the BLA confirms the involvement and anxiogenic role of Crhr1.

We injected an α-helical CRF (9-41) antagonist bilaterally into the BLA of HAB animals to verify whether CRHR1 indeed contributes to the anxiolytic effect of EE. We excluded 5 and 6 animals from the vehicle and treatment group, respectively since histology revealed that these animals did not met the criteria to guarantee an injection into the BLA. We performed OF, EPM and LD to assess the effect of the α-helical antagonist after one and three bilateral injections of the antagonist. We do not observe a significant behavioral change after one injection, but, indeed, a MWU test shows a significant increase of percent time spent in the inner zone ($p \leq 0.05$) and a trend for more entries into the inner zone ($p \leq 0.1$) of the OF after three injections (fig. 67 A and B). Moreover, we observe a significant reduction of 1.91 SDs of EE below the mean of SE mice ($p \leq 0.05$) corroborating an anxiogenic effect of CRHR1 utilizing Z-open field as an indicator (fig. 67C). Thus, we averaged z-score of latency to enter inner zone, total entries inner zone and percent time spent in inner zone but not total distance travelled and percent distance travelled inner zone due to technical problems, which ensued loss of these parameters.

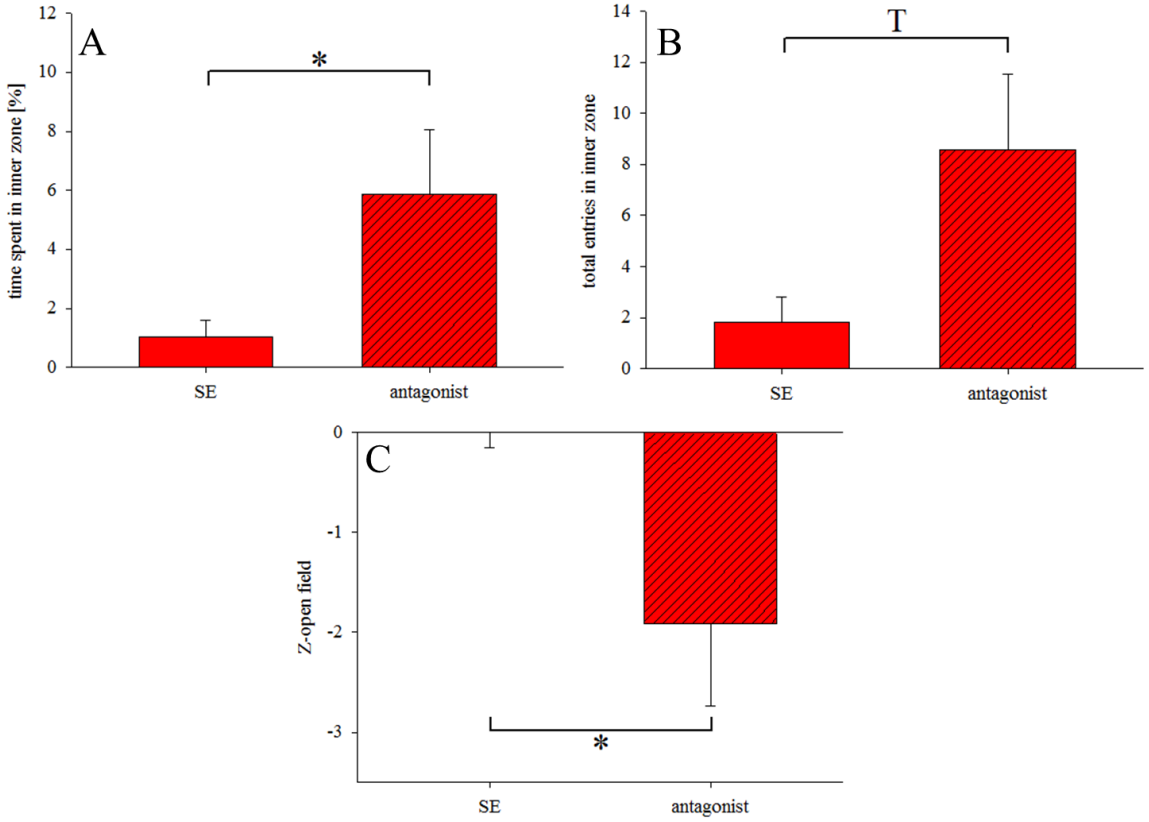


Fig. 67: A significant reduction of "percent time spent in inner zone" (A), a trend for "total entries inner zone" (B) and a significant reduction of "Z-open field" (C) indeed point toward an involvement and anxiogenic effect of CRHR1. N = 6 SE, 7 EE

We do not observe a significant difference in EPM and LD test between vehicle and treatment group.

4.10 Assessment of Crhr1 promoter methylation by pyrosequencing
EE significantly increases methylation of a CpG site located -1348bp before the start codon.

Pyrosequencing was performed to identify methylation levels of all 186 CpG sites located within the CpGi of Crhr1. However, merely methylation of 174 out of 186 CpG sites could be assessed due to low signal intensity for the respective missing sites. Low signal intensity can be caused by different reasons with an adverse secondary structure of DNA, sequencing primers or both being the most likely. We were interested whether total promoter methylation differs between HAB and LAB on the one and EE compared to SE HABs on the other hand. Kruskal-Wallis ANOVA reveals no significant difference between the groups ($H(2, N=522) = 1.586$; $p=0.452$). As expected, total methylation of the Crhr1 promoter is low, i.e. ≤5% and thereby under the detection limit of the pyrosequencing technique. Thus, we must assume that the promoter of EE and SE HABs as well as LABs is overall unmethylated exhibiting an average methylation of 2.54%, 2.28% and 2.48%, respectively.

A vast body of literature suggests that DMRs might play an important role in regulating gene expression. Though total methylation is not significantly different, DMRs can be. Therefore, we analyzed single CpGs for significantly different methylation comparing HAB to LAB and EE to SE mice. We observe no significant differences relating HAB to LAB CpG sites, suggesting a different mechanism than methylation to regulate the increased expression of Crhr1 in HABs related to LABs. Contrary, a MWU test identified a trend for a differentially methylated CpG site, i.e. a DMR comparing EE to SE mice ($p=0.083$) (fig. 68). This DMR is located -1348bp before TSS and constitutes the first CpG site of the CpGi whereat EE increases methylation, which could explain the reduced Crhr1 levels.

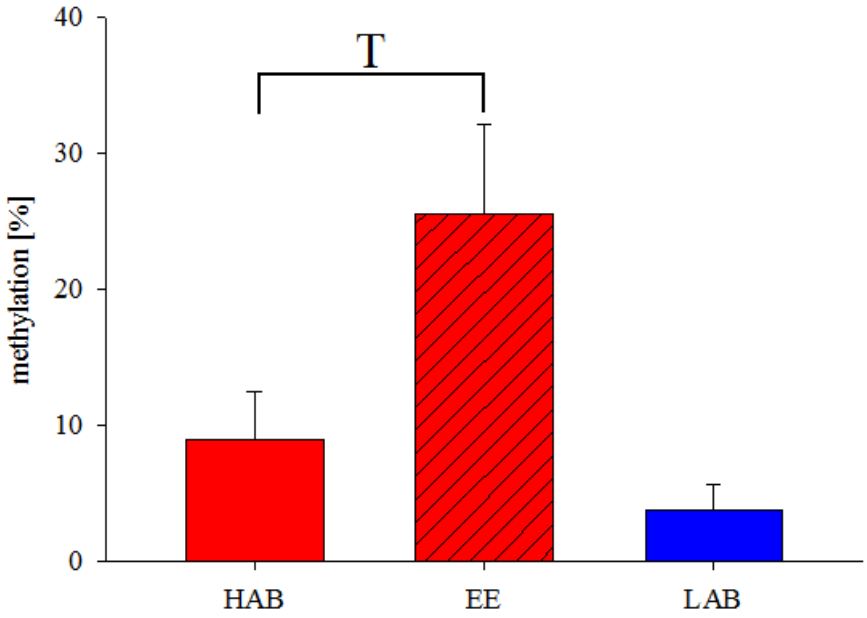


Fig. 68: HAB and LAB do not significantly differ, whereas EE shows a trend to increase methylation at the identified DMR located -1348bp before TSS. N = 4 for HAB, LAB and EE

4.11 Identification of transcriptional regulators by *in silico* analysis

In silico analysis with subsequent qPCR identified the transcription factor Yin Yang 1 (YY1) as a potential regulator of Crhr1. Moreover, Nr4a1 and D3Ertd300e together with Crh1 and YY1 might constitute a small interacting network.

We performed an *in silico* analyses of transcription factor binding sites using the "Jaspar Core Vertebrate" database and selecting M. musculus as organism (fig. 69). We submitted the sequence "GATGGAGACCCGGACCTGAGAG" comprising 10bp up- and downstream of the identified DMR highlighted in red. Analysis yielded the following transcription factors as potential regulators of the DMR:

Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0095.1	YY1	7.389	0.949226261010955	1	6	-1	TCCATC
MA0130.1	ZNF354C	4.723	0.816487152026638	2	7	-1	CTCCAT
MA0056.1	MZF1_1-4	4.916	0.809768936774344	3	8	1	TGGAGA
MA0146.1	Zfx	6.607	0.807942518484635	5	18	1	GAGACCCGGACCTG
MA0103.1	ZEB1	5.487	0.850143937200851	13	18	1	GACCTG
MA0037.1	GATA3	4.990	0.857443706407993	17	22	1	TGAGAG

Fig. 69: *in silico* analysis using the "Jaspar Core Vertebrate" database identified YY1 as the TF with the highest probability to bind the DMR of Crhr1.

YY1 emerges as the TF with the highest probability to bind in the near proximity (4bp) of the Crhr1 DMR indicated by a relative score of 0.949 with 1.0 being the maximum score to be achieved (fig. 70).

Fig. 70: YY1 binds next to the DMR of Crhr1 rendering it very likely to block the TF and thereby reducing gene expression. Binding site of YY1 is highlighted in red.

We performed qPCR to verify whether YY1 is really involved in transcriptional regulation of Crhr1 as indicated by *in silico* analyses. Indeed, a MWU test reveals a strong trend for YY1 to be expressed less in EE compared to SE animals (p=0.053) (fig. 71).

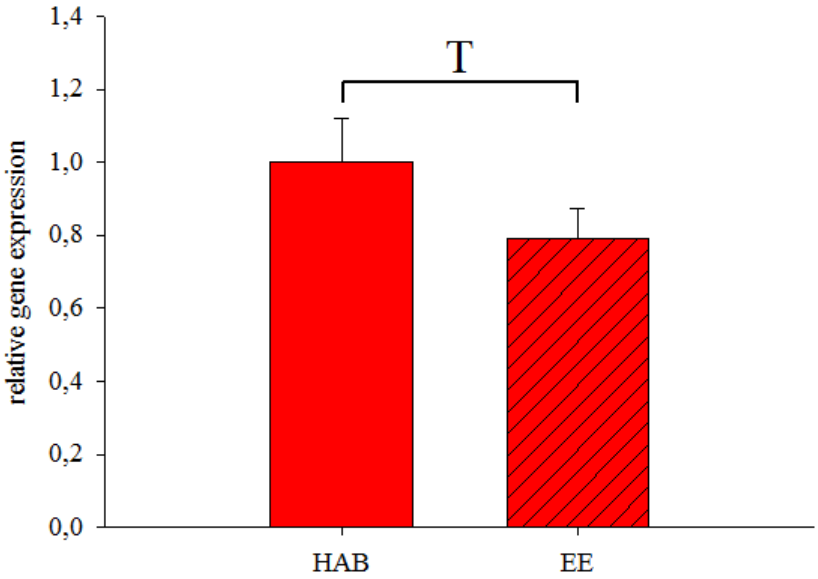


Fig. 71: YY1 expression is indeed reduced after EE in HABs. N = 5 for HAB and EE

To elucidate the potential connection between YY1 and Crhr1, we performed a gene network analysis by "Pubgene" (www.pubgene.org) utilizing a literature network search to identify genes, which co-occur in scientific publications. Subsequently, we verified connections illustrated by Pubgene by reading the respective literature. By doing so, we identified two factors, which interact with Crhr1 or YY1 in a small network (fig. 72).

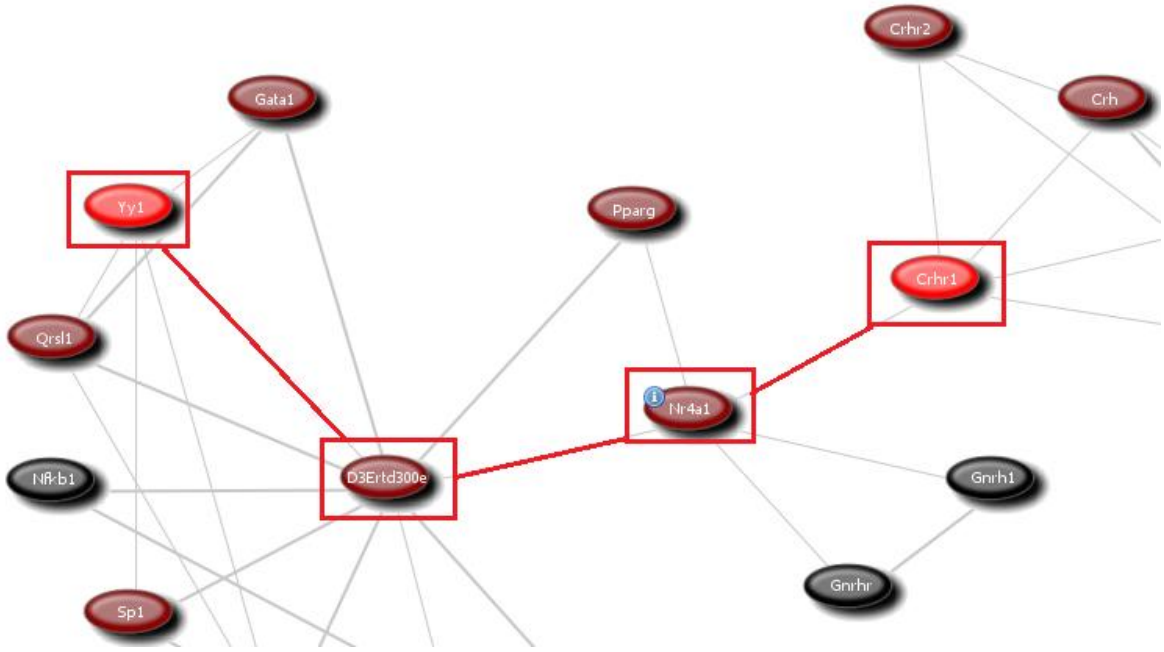


Fig. 72: Pubgene with subsequent literature search identified D3Ertd300e and Nr4a1 as factors interacting with Crhr1 and YY1 in a small network.

We used the gene ontology database (Amigo v1.8) to verify the functions of the identified factors. Nr4a1, also known as "nur77" is a nuclear receptor and belongs to a large superfamily of transcription factors. We found a functional association for "steroid hormone receptor" activity, namely "Nr3c1" also referred as "GR" ($p = 3x10^{-6}$) together with Fkbp4 (1.5×10^{-74}) and Fkbp5 (1.4×10^{-119}). Moreover, we found an association with "adrenocorticotropin-releasing hormone activity" ($p = 1.23 \times 10^{-6}$).

D3Ertd300e also known as "p38IP" is strongly associated with "transcription factor complex" ($p = 1.31 \times 10^{-122}$) and seems to exhibit a positive regulatory role for YY1 ($p = 2.8 \times 10^{-5}$), which in turn is associated with a "negative epigenetic role for gene expression" ($p = 1.16 \times 10^{-6}$). Thus, YY1, D3Ertd300e and Nr4a1 exhibit the potential to from a transcriptional complex in the nucleus and to exert regulatory influence on each other and finally Crhr1.

4.12 Modulation of behavior by epigenetic drugs

The DNMTi 5-Aza-2'-deoxyuridine seems to exert an anxiogenic effect on SE and EE animals contrary to the HDACi valproic acid, which might exert an anxiolytic effect in SE and anxiogenic effect in EE animals, respectively. We do not observe an additive effect of EE and (epigenetic) drug treatment to ameliorate depression-like behavior.

To investigate whether the anxiolytic effect of EE on HABs is (partially) of epigenetic nature, we tried to shift the phenotypes of both groups by injecting drugs, known to exert an effect via an epigenetic mechanism. Thus, we performed OF, EPM and LD to assess anxiety-related *behavior*. A 2-way ANOVA for OF reveals no significant effect of treatment, but a significant effect of housing on latency to enter inner zone ($F_{(1, 47)} = 9.438$; $p \leq 0.01$), total entries inner zone ($F_{(1, 47)} = 10.35$; $p \leq 0.01$), percent time spent in inner zone ($F_{(1, 47)} = 4.227$; $p \leq 0.05$), percent distance travelled inner zone ($F_{(1, 47)} = 4.271$; $p \leq 0.05$) and total distance travelled ($F_{(1, 47)} = 4.59$; $p \leq 0.05$).

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
latency to enter inner zone	0.004	0.141	0.141
total entries inner zone	0.002	0.279	0.279
percent time spent in inner zone	0.045	0.169	0.169
percent distance travelled in inner zone	0.044	0.229	0.229
total distance travelled	0.037	0.770	0.314

Importantly, EE again exerts an anxiolytic effect on HABs comparing EE and SE animals receiving saline. Tukey *post-hoc* tests depict that EE compared to SE mice exhibit a significantly lower latency to enter the inner zone, enter the inner zone more often, spent more percent time and travel more percent distance in it (all $p \leq 0.01$).

Moreover, Fisher *post-hoc* tests show a significantly higher latency to enter the inner zone of EE HABs, which received the HDACi compared to DNMTi ($p \leq 0.05$) and saline ($p \leq 0.01$) (fig. 73B). Similarly, EE mice receiving HDACi enter the inner zone significantly less (fig. 73C) and spent less percent time in it (fig. 73D) in relation to saline controls ($p \leq 0.05$ and $p \leq 0.01$, respectively) indicating an anxiogenic effect of the used HDACi 5-Aza-2'-deoxyuridine on EE mice, though we did not observe an effect of treatment. In addition, DNMTi compared to saline treated EE mice spent significantly less percent time in the inner zone ($p \leq 0.05$) pointing towards a mild anxiogenic effects of the utilizes DNMTI valproic acid.

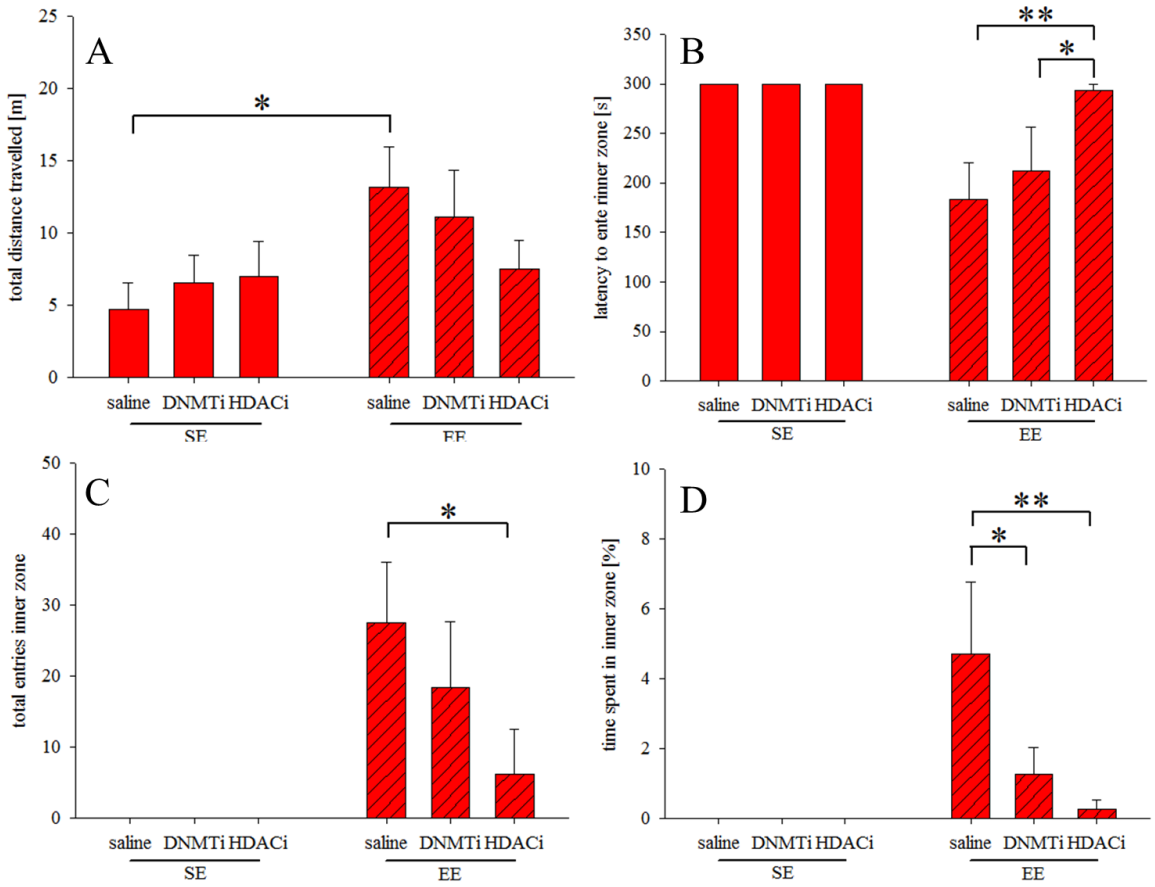


Fig. 73: The HDACi 5-Aza-2'-deoxyuridine seems to exhibit anxiogenic properties indicated by an increased latency (B), less entries (C) and less percent time spent in the inner zone (D) related to saline EE controls. Merely saline EE animals travel significantly more total distance compared to their SE counterparts (A). N = 8 for all SE groups and 9 for all EE groups

A 2-way ANOVA of EPM indicates an effect of housing and treatment for percent entries open arms ($p \leq 0.01$ and $p \leq 0.05$, respectively) and percent time spent on open arms (both $p \leq 0.05$) demonstrating that i) EE again elicited an anxiolytic effect and that ii) application of drugs indeed had an effect on the behavior of SE and EE HABs. Fisher *post-hoc* tests reveal a significant increase of percent time spent on open arms ($p \leq 0.05$) and a strong trend for entering the open arms more often ($p=0.055$) for SE HABs, which received HDACi related to saline controls. Contrary, we do not observe any difference comparing saline to DNMTi SE mice (fig. 74). Surprisingly, DNMTi had the complete opposite effect on EE mice as HDACi had on SE animals: we monitor an anxiogenic effect revealed by Fisher *post-hoc* tests showing a significant decrease of entries in ($p \leq 0.05$) and a trend for percent time spent on open arms ($p=0.093$) of EE mice, which received DNMTi in relation to saline controls. These results are corroborated by the fact that SE compared to EE mice, which received DNMTi as well as HDACi do not differ significantly anymore in regard to entries into ($p=0.182$ and $p=0.514$, respectively) and percent time spent on open arms ($p=0.303$ and $p=0.700$, respectively).

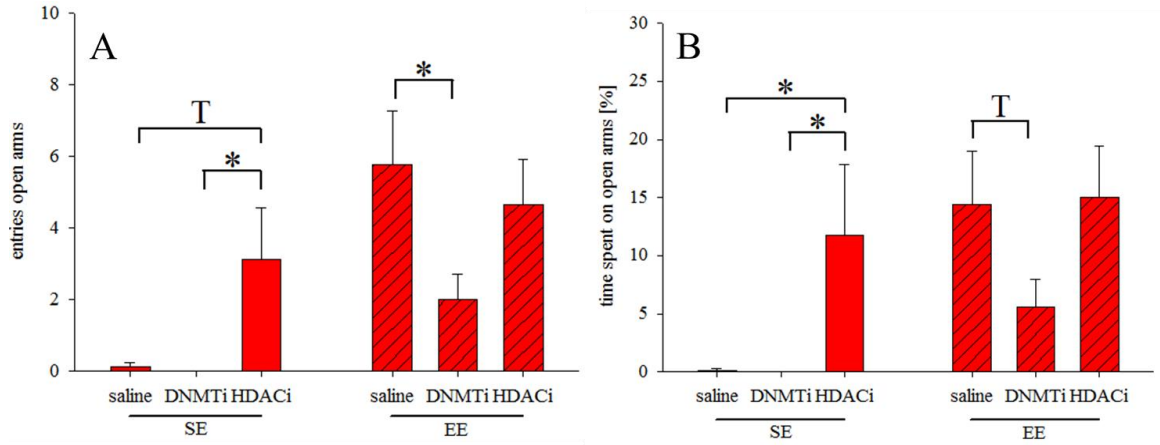


Fig. 74: The DNMTi valproic acid exhibits an anxiogenic effect on EE mice, whereas the HDACi 5-Aza-2'-deoxyuridine possesses an anxiolytic effect on SE mice. This is indicated by a respective significant in- or decrease of entries in (A) and percent time spent on open arms (B) for SE and EE mice receiving HDACi or DNMTi, respectively compared to saline controls. N = 8 for all SE groups and 9 for all EE groups

Finally, a 2-way ANOVA for LD revealed a significant effect of treatment on Latency to enter the light compartment, total entries into (both $p \leq 0.01$) and percent time spent in it ($p \leq 0.05$).

Parameter measured	**P-value for main effect of:**		
	Housing	Sex	Housing x sex
latency to enter light compartment	≤0.001	0.008	0.801
total entries light compartment	≤0.001	0.004	0.423
percent time spent in light compartment	≤0.001	0.018	0.567

Fisher *post-hoc* tests reveal an anxiogenic effect of DNMTi on both, SE and EE animals compared to their respective saline controls. Treatment with valproic acid reduced entries into ($p \leq 0.05$ for SE and $p = 0.053$ for EE, respectively) and increased the latency to enter the light compartment ($p \leq 0.01$ for SE). These results are corroborated by Z-light-dark box, which shows an anxiogenic effect of DNMTi by an increase of 1.03 SDs above the mean of SE mice. Likewise, the anxiolytic effect of EE on HABs was reduced from 1.42 to 0.56 SDs below the mean of EE mice by treatment with DNMTi (fig. 75).

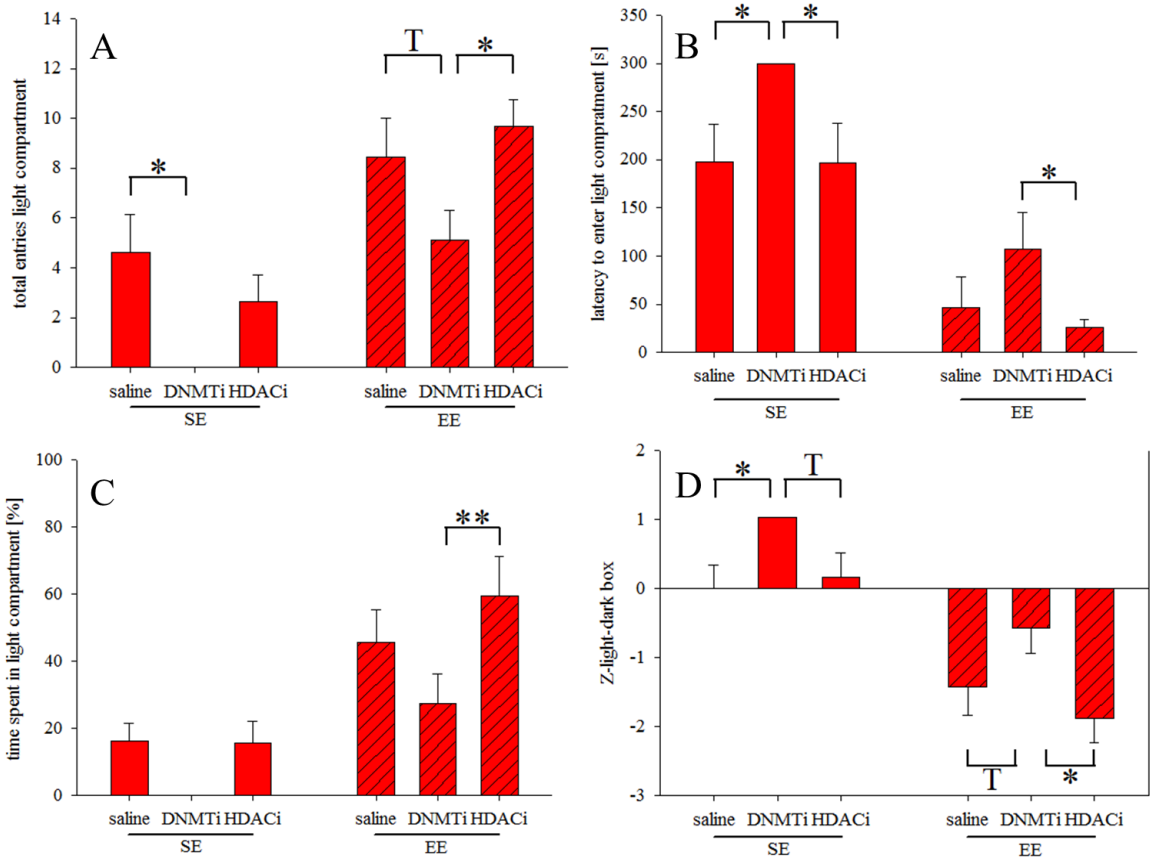


Fig. 75: The DNMTi valproic acid exhibits an anxiogenic impact on SE and EE mice, depicted as less entries into (A), an increased latency (B) to enter the light compartment and Z-light-dark box (D) showing an increase of 1.03 and a decrease from 1.42 to 0.56 standard deviations compared to the mean of their SE and EE controls, respectively. N = 8 for all SE groups and 9 for all EE groups

In total, there seems to be a clear cut anxiogenic effect of the DNMTi valproic acid on both, SE and EE animals indicated by all three tests. The HDACi 5-Aza-2'-deoxyuridine might possess an antidromic effect on SE and EE mice: an anxiolytic effect on SE and an anxiogenic effect of EE HABs.

4.13 Evaluation of transgenerational effects

There might be a transgenerational anxiolytic effect priming some male individuals, denoted as responders, to future environmental conditions. Moreover, two successive generations of EE seem to increase the anxiolytic effect for both sexes.

To investigate transgenerational effects of anxiety-related behavior, we performed EE as described earlier and again, we observe a significant reduction of anxiety-related behavior without masking effects of locomotion for the parental generation (fig. 76)

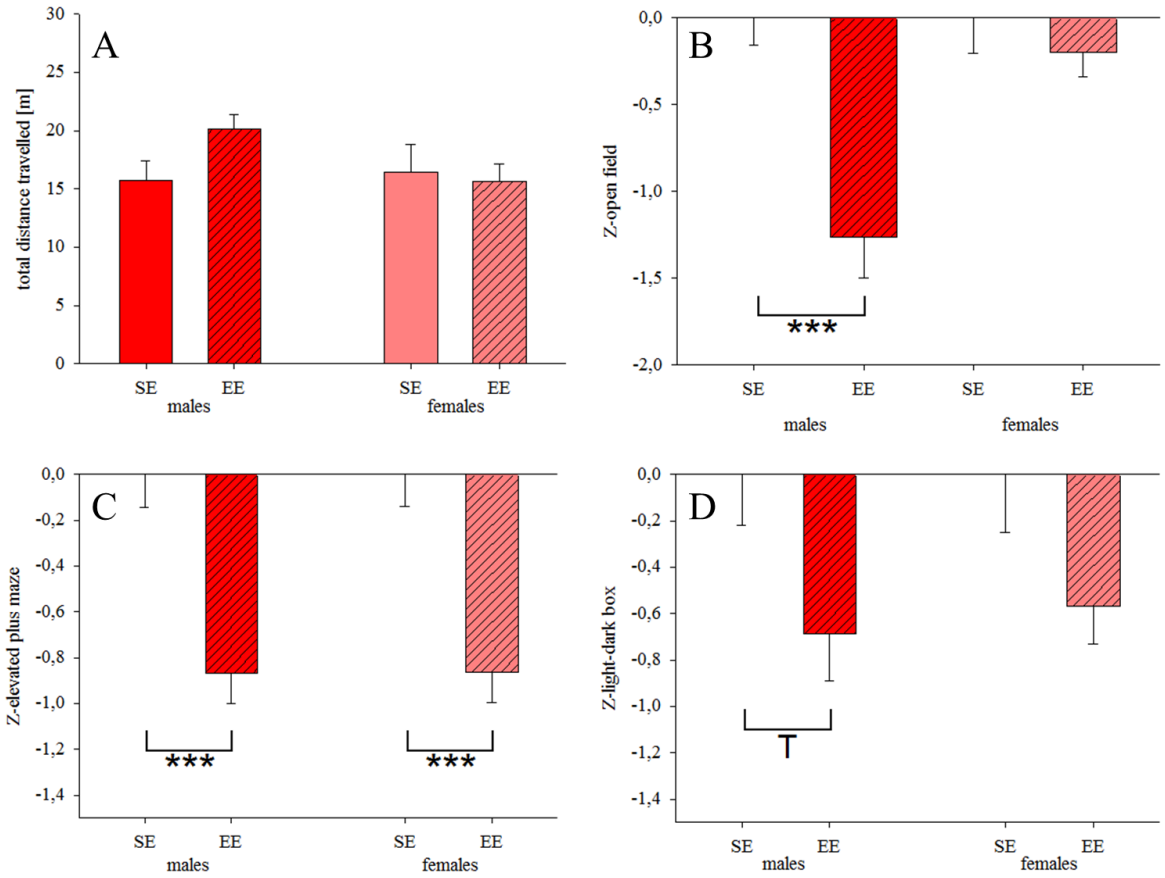


Fig. 76: EE ensues a significant anxiolytic effect indicated by z-scores of OF (B), EPM (C) and LD (D) without a masking effect of locomotion (A). N (males, females) = 18 SE, 21 EE

2-way ANOVA reveals a significant effect of housing for Z-open field ($F_{(1, 67)}$ = 9.298; p≤0.001), Z-elevated plus maze ($F_{(1, 64)}$ = 13.25; p≤0.001) and Z-light-dark box ($F_{(1, 66)}$ = 4.59; p≤0.05). Tukey *post-hoc* tests show a significant anxiolytic effect for females depicted as a reduction of 0.86 SDs below the mean of SE controls in EPM (p≤0.001), whereas males show a significant reduction in all tests: 1.26 SDs in OF (p≤0.001), 0.87 SDs in EPM (p≤0.001) and 0.69 SDs in LD (p=0.082) below the mean of respective controls.

By performing this experiment, we would like evaluate whether: i) the anxiolytic effect of EE be passed on to the next generation (i.e. transgenerational inheritance), an additive effect of EE exist, which can be seen as ii) an increase of the anxiolytic effect size and/or iii) an antidepressive effect, which can't be observed after a singular EE. To verify whether a transgenerational effect exists, we mated three males each with two females for both housing conditions to generate F1. We performed OF, EPM and LD to assess anxiety-related behavior. 2-way ANOVAs show the following effects:

Test	**Parameter measured**	**P-value for main effect of:**		
		Housing	Sex	Housing x sex
OF	total distance travelled	0.011	0.123	0.994
	latency to enter inner zone	0.009	0.584	0.957
	total entries inner zone	0.008	0.855	0.635
	percent time spent in inner zone	0.446	0.483	0.727
	percent distance travelled in inner zone	0.239	0.727	0.692
	Z-open field	≤0.001	0.006	0.001

EPM	percent time spent on open arms	0.279	0.937	0.008
	percent entries in open arms	0.764	0.150	0.731
	Z-elevated plus maze	0.026	0.003	0.013
LD	latency to enter light compartment	0.011	0.123	0.994
	percent time spent in light compartment	≤0.001	0.138	0.699
	total entries light compartment	≤0.001	0.224	0.904
	Z-light-dark box	≤0.001	0.610	0.333

For OF, Z-open field was the only parameter surviving a Tukey *post-hoc* test with EE females showing a reduction of 4.41 SDs below the mean of SE controls ($p \leq 0.001$) revealing the expected anxiolytic effect of EE for females.

Surprisingly, females whose parents had been housed in EE but mice themselves were raised in SE (EESE) show a strong trend to spent more time on open arms compared to SE mice ($p=0.059$) and do not significantly differ from EE females ($p=0.619$) indicating a transgenerational effect for females. We would like to emphasize that SE females exhibited a mean value close to zero, thus increasing Z-open field to levels that might not be representing real effect size (please refer to materials and methods). A fact that is corroborated by the absence of transgenerational effects in all other measured parameters. For LD, we observe a significant effect of housing for all measured parameters, thereby confirming the anxiolytic effect of EE on both sexes. We monitor significantly more entries into and time spent within the light compartment for EE males ($p \leq 0.001$ for both) and females ($p \leq 0.05$ for both) compared to their SE counterparts, but we do not observe a transgenerational effect by comparing any of the measured parameters between SE and EESE animals. These data are corroborated by Z-light-dark box showing a significant reduction for EE mice of 2.57 and 2.63 SDs below the mean of male ($p \leq 0.05$) and female SE controls ($p \leq 0.01$), respectively (fig. 77).

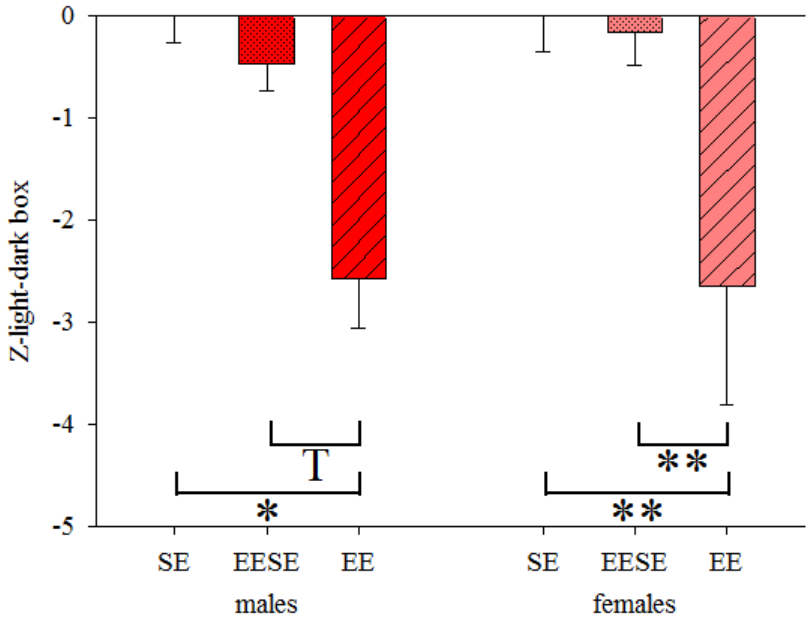


Fig. 77: As expected, EE ensues a significant anxiolytic effect for both sexes depicted as a reduction of z-score of 2.57 and 2.63 SDs for males and females, respectively related to controls. Like in all other tests, LD does not point towards a transgenerational anxiolytic effect apparent as a missing difference between SE and EESE mice. N (males) = 14 SE, 13 EESE and 8 EE; N (females) = 8 SE and EESE and 6 EE

Though we do not monitor transgenerational inheritance of the anxiolytic effect for all mice, we further analyzed our data similar to Elliot et al., (2010) who showed that some animals might respond to a certain treatment (denoted as responders), whereas others do not (referred to as non-responders, NR). Indeed, we found a bimodal distribution of male F1 animals exhibiting and not exhibiting an anxiolytic effect. Out of 13 male EESE mice, 5 did not enter the light compartment at all, thereby resembling the behavior and phenotype of SE mice contrary to EESE females, which do not show this bimodal distribution. We took out these 5 male possible non-responders and analyzed them as a separated group when performing Kruskal-Wallis ANOVA (KWA) followed by MWU as *post-hoc* test and Dunn-Šidák correction for multiple testing (significance at $p \leq 0.013$ due to 4 simultaneous tests). This action did not alter the results for OF and EPM, but had a dramatic effect on LD analysis – the test which yielded the most significant results of EE before separating responders and NR (fig. 78). We observe a significant difference ($p \leq 0.001$ for all) for latency to enter light compartment ($H=20.71$), percent time spent in light compartment ($H=21.01$), total entries light compartment ($H=18.56$) and Z-light-dark box ($H=17.57$).

Parameter measured	**SE vs. EESE**	**SE vs. EE**	**EESE vs. EE**	**SE vs. NR**
latency to enter light comp.	0.006	p≤0.001	0.279	0.391
% time spent in light comp.	0.029		0.007	0.391
total entries light comp.	0.005		0.050	0.391
Z-light-dark box	0.008		0.021	0,186

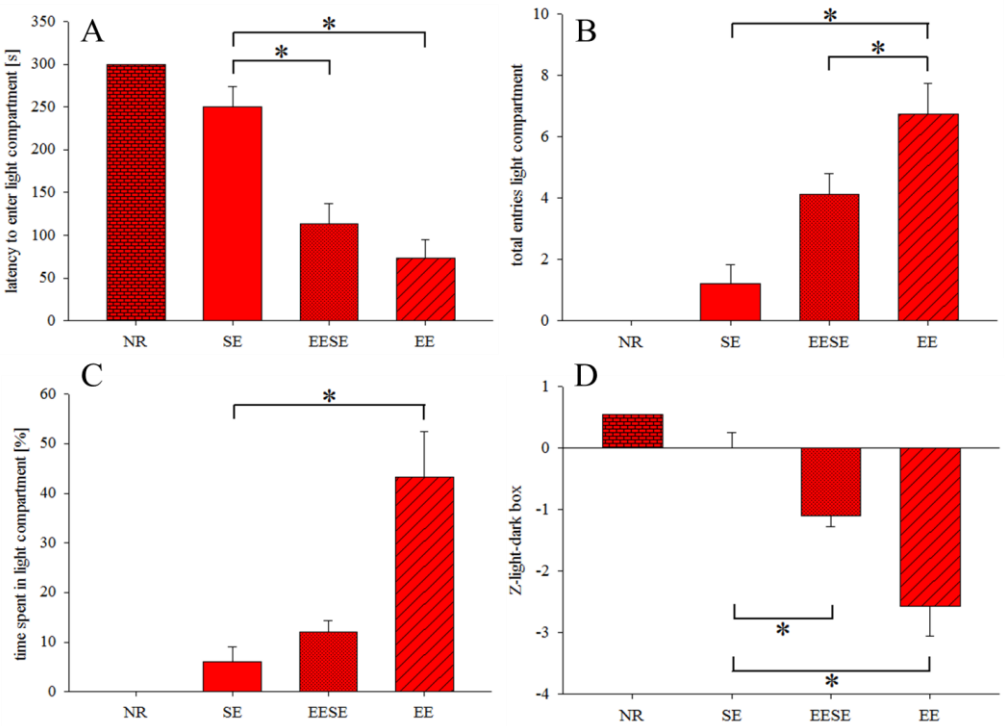


Fig. 78: A subgroup of males indeed might show an inherited anxiolytic effect originating from their EE parents. They have a shorter latency to enter (A) and more entries into the light compartment (B) as well as a significantly reduced Z-score of 1.1 SDs compared to SE controls (D). Importantly, on the one hand, EESE males do not significantly differ from EE males except by percent time spent in light compartment (C), on the other hand SE and NR do not differ in any of the measured parameters indicting a similar phenotype. N = 5 NR, 14 SE, 8 EESE and 8 EE

These results suggest that male F1 indeed can be separated in responders and non-responders and that the first differ significantly from SE mice by having a shorter latency to enter and more entries into the light compartment as well as a strong reduction of 1.10 SDs below the mean compared to controls. At the same time, EESE and EE F1 males do not differ significantly anymore in all parameters except percent time spent in light compartment (p=0.007). We emphasize that F1 SE and NR do not significantly differ in any of the parameters suggesting that these two groups are indeed similar.

Research suggests that a transgenerational effect should be inherited to F3 since dams expose their embryos, which in turn expose their gametes to the environmental conditions, which elicit a certain effect. Thus, a true transgenerational effect should be inherited to F3 to exclude any confounding effects (Skinner, 2011). Therefore, we tested F2 and F3 for an inherited anxiolytic effect originating from the parental generation. F2 SE animals of both sexes exhibited means close to zero or were equal to zero, thereby increasing z-scores to values not representing real effect sizes. Due to this reason we were not able to perform z-scores for F2. The anxiolytic effect of EE was again observed in F2 corroborating the robustness and reproducibility of our paradigm but we did not monitor transgenerational inheritance of the anxiolytic phenotype (fig. 79).

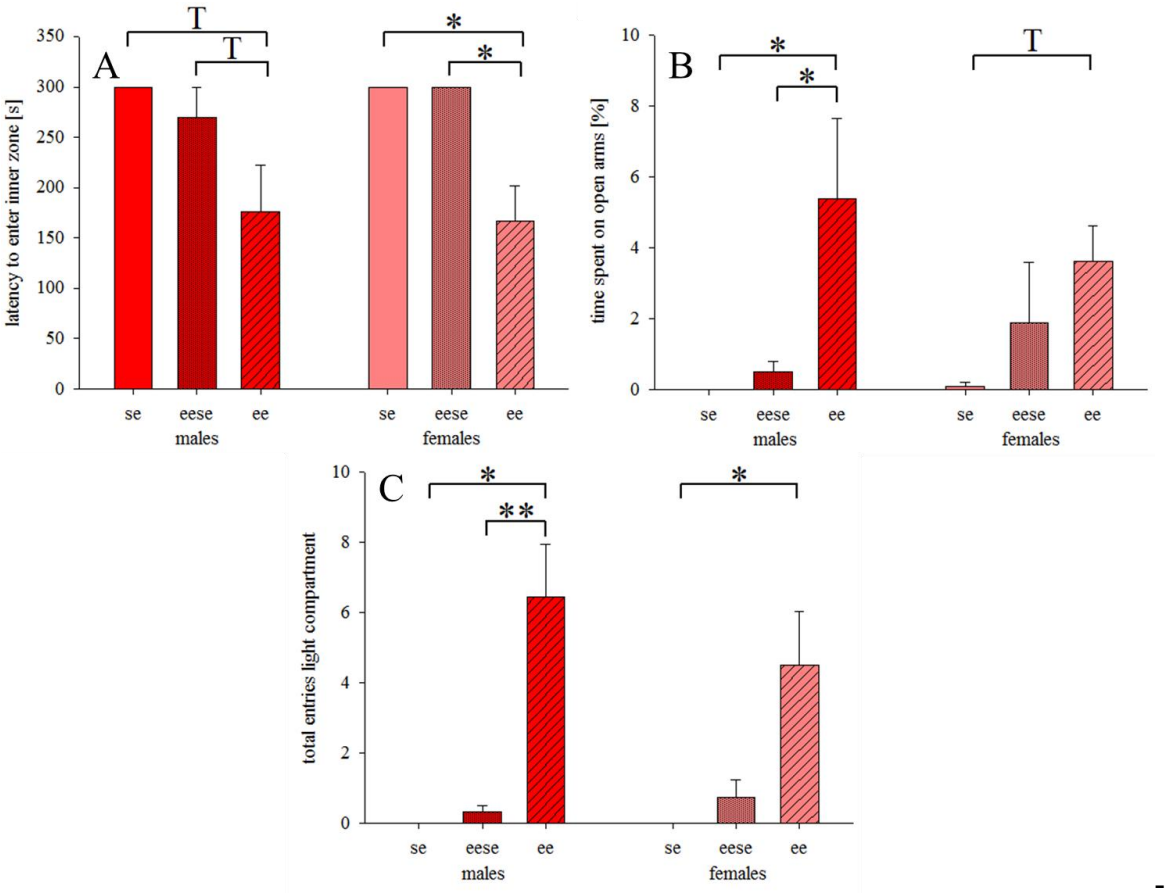


Fig. 79: As expected, EE again caused an anxiolytic effect but we do not observe an inherited anxiolytic phenotype in any of the measured parameters of OF, EPM and LD. A reduced latency to enter the inner zone of OF (A), an increased percent time spent on open arms of EPM (B) and more entries in the light compartment of LD (C) are examples of the anxiolytic effect elicited by EE. N (males) = 4 SE, 8 EESE, 9 EE; N (females) = 5 SE, 4 EESE and 15 EE

A 2-way ANOVA revealed significant effects of the following parameters:

Test	Parameter measured	P-value for main effect of:		
		Housing	Sex	Housing x sex
OF	total distance travelled	0.018	0.099	0.998
	latency to enter inner zone	0.004	0.843	0.889
	total entries inner zone	0.019	0.713	0.858
	percent time spent in inner zone	0.038	0.902	0.982
	percent distance travelled in inner zone	0.038	0.992	1.000
EPM	percent time spent on open arms	0.033	0.780	0.523
	percent entries in open arms	0.010	0.950	0.537
LD	latency to enter light compartment	≤0.001	0.244	0.491
	percent time spent in light compartment	≤0.001	0.397	0.359
	total entries light compartment	≤0.001	0.718	0.701

Fisher *post-hoc* tests for OF reveal that EE males and females compared to:

- respective EESE animals show a trend to travel more total distance (p=0.081 for males and p= 0.059 for females),
- have a shorter latency to enter the inner zone compared to SE (p=0.065 for males and p≤0.05 for females) and EESE mice (p=0.085 for males and p≤0.05 for females),
- enter the inner zone more often than male SE (p=0.075) and EESE (p≤0.05) HABs,
- spent more percent time in the inner zone than EESE males (p=0.077) and
- travel more percent distance in the inner zone related to EESE males (p=0.096).

For EPM, EE related to SE males enter the open arms more often (p≤0.05) and spent more percent time on the open arms compared to male SE and EESE mice (p≤0.05 for both), whereas EE in relation to SE females show a trend for the latter (p=0.090). Fisher *pots-hoc* tests for LD reveal a significantly shorter latency for both EE sexes to enter the light compartment compared to SE (p≤0.01 for males and p≤0.05 for females) and EESE HABs (p≤0.01 for males and p=0.087 for females). Moreover, male but not female EE HABs spent significantly more percent time in the light compartment in relation to SE (p≤0.01) and EESE counterparts (p≤0.001). EE males and females enter the light compartment significantly more often than SE mice (p≤0.05 for both), which is also true comparing EE and EESE males (p≤0.01).

Finally, we assessed inheritance of anxiety-related behavior in F3 mice. As expected, EE compared to SE F3 animals - like F2 HABs – show an anxiolytic effect after EE but this effect was not passed from (F2) parents to (F3) offspring. Fig. 79 shows z-scores of OF, EPM and LD illustrating the anxiolytic effect of EE and the absence of transgenerational inheritance. 2-way ANOVAs show a significant effect of housing on Z-open field ($F_{(1, 78)}$ = 5.736; p≤0.001), Z-elevated plus maze ($F_{(1, 79)}$ = 6.971; p≤0.001) and Z-light-dark box ($F_{(1, 79)}$ = 14.4; p≤0.001).

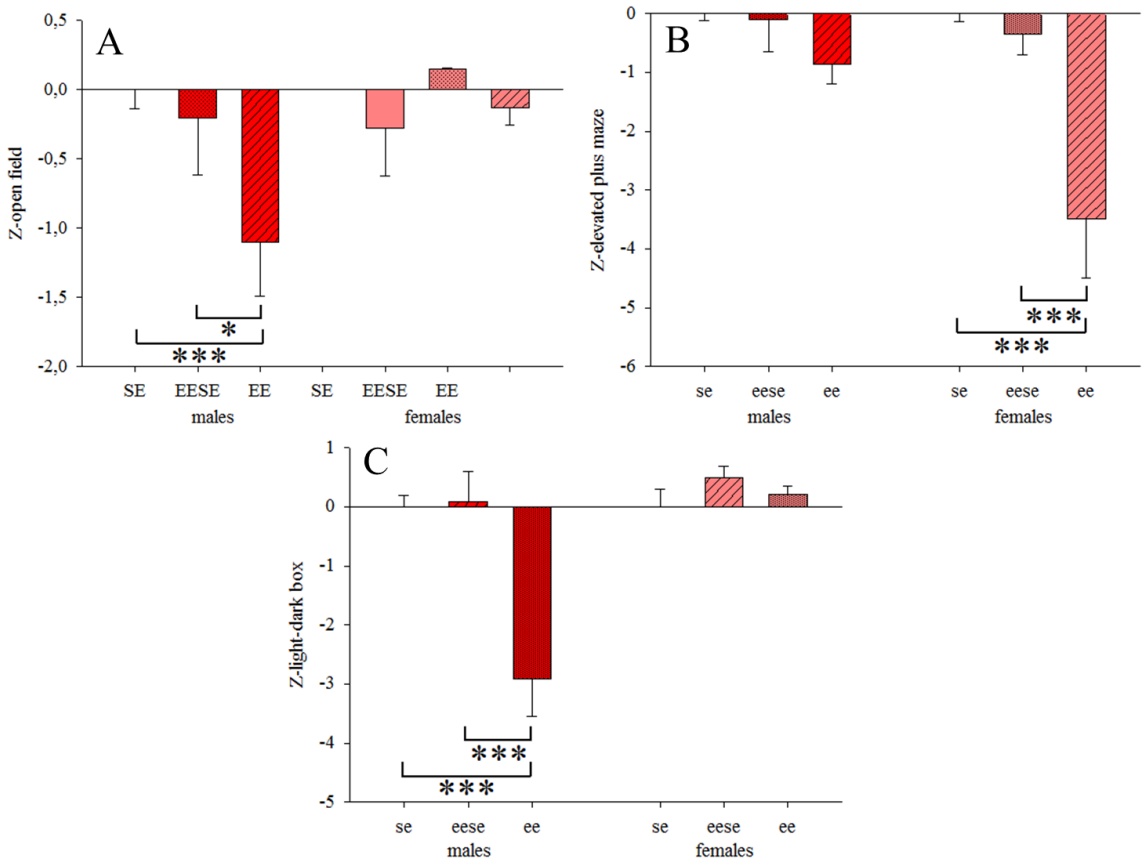


Fig. 80: Like in F1 and F2, we observe a significant anxiolytic effect of EE without transgenerational transmission indicated by significant decrease of z-score for OF (A), EPM (B) and LD (C). N (males) = 20 SE, 5 EESE, 13 EE; N (females) = 9 SE, 17 EESE and 20 EE

Fisher *post-hoc* tests depict for EE males a significant decrease of 1.10 SDs in OF and 2.92 SDs in LD below the mean of respective SE controls indicating a strong effect size, whereas EE in relation to SE females show a reduction of 3.49 SDs below the mean in EPM (fig. 80).

We were interested whether successive generations of EE might cause an anti-depressive effect, which is not seen after single EE. Thus, we conducted FST for F3 mice whereby a 2-way ANOVA reveals a significant effect of housing ($F_{(1, 76)}$ = 8.311; $p \leq 0.001$) and sex ($F_{(1, 76)}$ = 6.89; $p \leq 0.01$) for time spent floating but no significant effect of housing for number of immobile episodes ($F_{(1, 76)}$ = 1.091; p=0.341) and latency to first immobile episode ($F_{(1, 76)}$ = 1.458; p=0.239). A Tukey *post-hoc* test indicates a trend for female EE related to EESE HABs to spent more percent time floating (p=0.065). To our surprise, this results points towards a mild pro-depressive or passive coping phenotype for EE females and opposes our hypothesis of a beneficial additive effect of EE but resembles the pro-depressive phenotype of females housed for 10 weeks in EE (fig. 81).

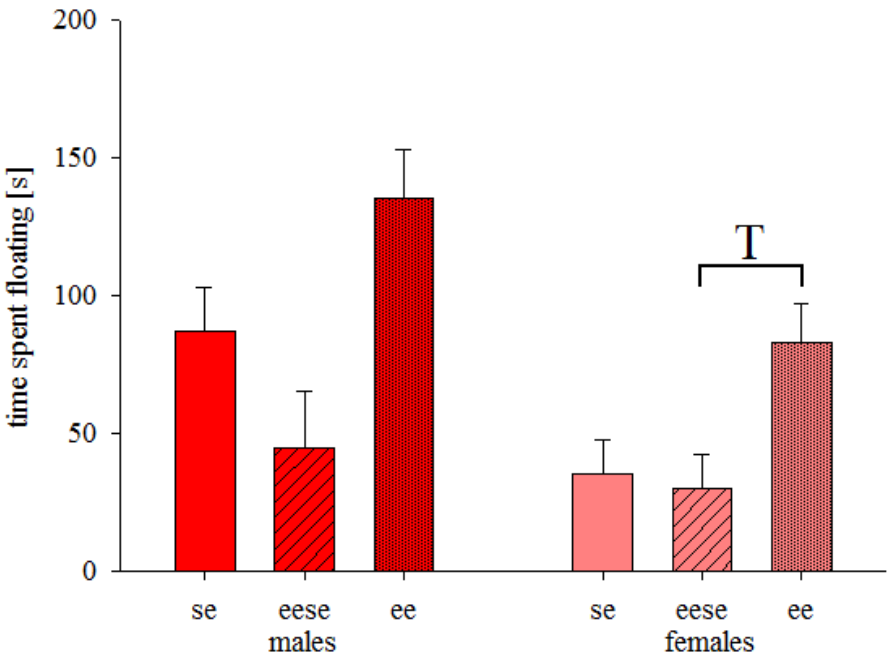


Fig. 81: Surprisingly, EE females show a mild pro-depressive or passive coping style after 3 generations of EE compared to EESE mice. N (males) = 22 SE, 5 EESE, 12 EE; N (females) = 9 SE, 14 EESE and 20 EE

Multiple generations of enrichment might increase the anxiolytic effect size and thereby show a preferable additive effect. Since F2 SE mice possess mean values close to zero or equal to zero, we compared parental, F1 and F3 for differences in effect size by comparing z-scores of OF, EPM and LD wherever possible. 2-Way ANOVAs reveal a significant effect of generation for Z-OF ($F_{(1, 80)} = 18.2$; $p \leq 0.001$) and Z-LD ($F_{(1, 77)} = 10.66$; $p \leq 0.001$) as well as a strong trend for Z-EPM ($F_{(1, 79)} = 3.103$; $p = 0.051$).

Parameter measured	**P-value for main effect of:**		
	Generation	Sex	Generation x sex
Z-OF	0.002	0.053	≤0.001
Z-EPM	0.051	0.042	0.078
Z-LD	≤0.001	0.002	≤0.001

Tukey *post-hoc* tests depict that EE females indeed increase the anxiolytic effect size from the parental generation to F1 by a significant decrease of Z-OF from -0.20 SDs to -4.41 SDs ($p \leq 0.001$) and of Z-LD from -0.57 SDs to -2.64 SDs ($p \leq 0.001$) below the mean of respective SE controls. EE Males too, show a significant decrease of Z-LD from -0.69 SDs to -2.57SDs below the mean of SE HABs ($p \leq 0.05$), thereby achieving a level very similar to that of females. These results might be a hint for a beneficial additive effect of EE when successively applied to 2 generations.

Comparing F1 to F3, we observe an antidromic shift of effect size: EE females show a further increase of anxiolytic effect size in Z-EPM from -1.22 SDs to -3.49 SDs ($p \leq 0.001$) but a significant decrease of the same in Z-OF from -4.41SDs to -0.13SDs in OF ($p \leq 0.001$) and from -2.64 SDs to 0.21 SDS in LD ($p \leq 0.001$) below and above the mean of respective SE animals. Contrary to females, EE males maintain the anxiolytic effect size in all tests, i.e. they do not significantly differ between F1 and F3 indicating no further in- or decrease of the anxiolytic effect size (fig. 82).

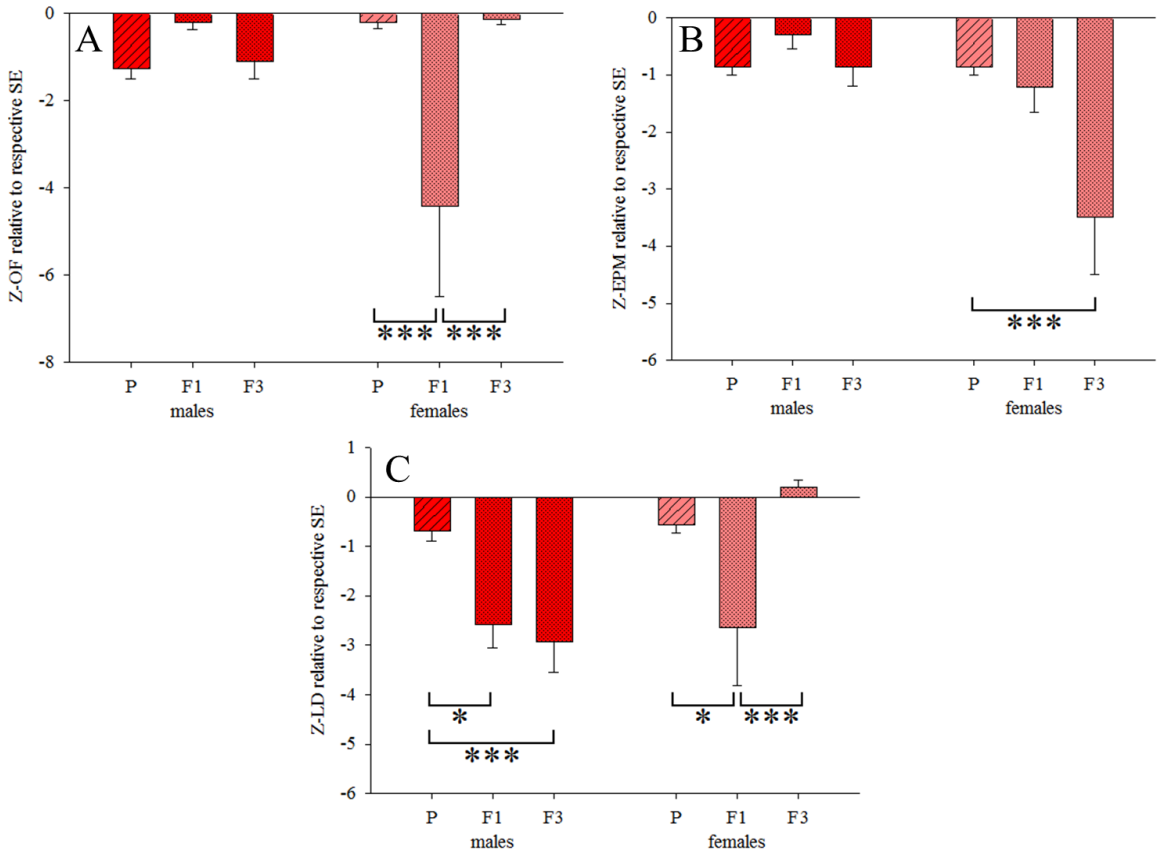


Fig. 82: 2 successive generations of EE might ensue a beneficial additive increase of anxiolytic effect size for both sexes. Males do not show a change of effect size when housed for further generations in EE, whereas females show an antidromic shift preventing an unambiguous statement whether further generations of EE have a beneficial or detrimental effect. Shift of effect size is depicted for OF (A), EPM (B) and LD (C). N (males) = 21 P, 8 F1, 13 F2; N (females) = 18 P, 6 F1 and 20 F3

In total, 2 generations of EE seem to entail a beneficial additive effect, which further increases the anxiolytic effect size for both sexes. Females compared to males might benefit more since 2 out of 3 tests suggest an additive effect, whereas 1 out of 3 tests does so for males. Males maintain effect size until F3, whereas females show an antidromic shift preventing an unambiguous statement about a further beneficial or a detrimental effect of EE when applied for 2 more generations.

5 Discussion and future experiments

A beneficial environmental manipulation such as EE can indeed mitigate even extreme trait anxiety - but not depression-like behavior - in a mouse model of pathological anxiety.

The process of selective bidirectional breeding focusing on anxiety-related behavior created a highly valid mouse model of pathological anxiety (i.e. HABs), which has proven its extreme trait anxiety in behavioral tests assessing anxiety-related behavior either via an approach-avoidance conflict (e.g. OF, EPM, LD; Kromer et al, 2005; Markt unpublished data, 2009; Markt and Sotnikov, in preparation; Muigg et al, 2009) or conditioned paradigms (fear conditioning; Gaburro et al, 2011; Sartori et al, 2011b). Our main focus

applied to whether an environmental manipulation such as EE is capable of rescuing the rigid genetic drive of high anxiety in this mouse model via GxE or epigenetic processes.

Indeed, EE is able to mitigate even extreme trait anxiety of HABs throughout all behavioral tests assessing anxiety-related behavior, apparent as a shift from high towards normal anxiety-related behavior for both sexes, though this is difficult to achieve using classical pharmacological treatment, at least in male mice (Sah, in preparation; Landgraf, personal communication). Importantly, home cage locomotion - a cross test dimension of anxiety (Henderson et al, 2004) - is indistinguishable from SE housed controls indicating no masking or confounding effect of locomotion on anxiety-related behavior. In addition, a significantly increased exploration activity in EE HABs may further corroborate anxiolytic effects since the behavioral tests performed exploited an approach-avoidance conflict (i.e. exploration vs. anxiety). A meta-analysis using z-score to evaluate the overall effect of EE on anxiety, locomotion and exploration confirms our data obtained from single experiments - a strong anxiolytic effect for EE males (≥1 SD) and a small for EE females (≤0.5 SD), which is not masked by locomotion and ensued a significantly increased exploration. A χ^2-test accentuates the sex-specific differences of anxiolysis: females showed a shift of effect size predominantly from small to medium, whereas males exhibited a shift mainly from small to strong. Our data are in line with the majority of previous studies indicating an anxiolytic effect of EE (Arai et al, 2009; Benaroya-Milshtein et al, 2004; Kuzumaki et al, 2011; Markt and Sotnikov, in preparation) and extend them by demonstrating that even a rigid genetic predisposition modeling pathological anxiety can be mitigated or even rescued by EE. Importantly, our meta-analysis illustrates that a well-chosen and appropriate design of EE indeed increases reproducibility and decreases variability, whereas the use of different mouse strains and/or designs (Chapillon et al, 1999; Nevison et al, 1999; Van de Weerd et al, 1994) might lead to controversial results with few studies even demonstrating an anxiogenic effect of EE (Pietropaolo et al, 2006).

Surprisingly, EE did not alter the coping style (indicative of depression-like behavior) in HABs, though many studies were able to show an antidepressive effect in "normal" rodents (Brenes et al, 2009; Hattori et al, 2007). A study by Xu et al. (2009) indicates that an elevation of glucocorticoid levels seems to be required for the antidepressive effects of EE. They monitored a significant reduction of depression-like behavior associated with increased serum CORT levels in animals, which have been exposed for two months to EE. Contrary, EE animals that received a low dose of CORT supplement after adrenalectomy did neither exhibit increased serum CORT levels, nor a significant decrease of depression-like behavior. Our data show that male HABs housed in EE relative to SE seem to exhibit lower serum levels of CORT after application of a mild stressor. Moreover, CORT levels are indistinguishable between male groups when facing a severe stressor. It might be speculated that both, an attenuated CORT release after a mild stressor and a missing increase of CORT after facing a severe stressor, may be accounted for the missing antidepressive phenotype in male EE HABs. Another possibility for the missing antidepressive phenotype might be duration of exposure to EE. Xu et al. (2009) exposed their animals in comparison to ours 8 instead of 4 weeks to EE. Our results indicate that duration of EE exposure seems to be of minor importance for coping style since a

prolongation of EE exposure from 4 to 10 weeks did not alter the phenotype in our male HABs, thus pointing towards elevated glucocorticoid levels as one major factor. Contrary, EE females showed a shift to a pro-depressive phenotype after 10 weeks of EE. We hypothesize that their CORT levels might be further decreased after prolonged EE, thereby causing the monitored shift. Depression-like behavior in NABs was neither attenuated by EE. We did not assess HPA axis reactivity in NABs, thus it may be concluded that either EE NABs i) lack an increase of glucocorticoids after application of a stressor similar to EE HABs and/or ii) do not exhibit the genetic predisposition modeling pathological anxiety and comorbid depression and, thus, do no exhibit a depressive phenotype, which can be attenuated. The latter possibility may be more likely since it is known that antidepressive treatment does not cause an effect in healthy individuals.

In essence, our model clearly suggests that anxiety and depression-like behaviors are not necessarily associated with each other, providing a unique opportunity to focus on the former.

Genetic background, duration of exposure and behavioral differences between EE and SE HABs observed during the juvenile and early adolescent phase seem to be factors that contribute to the anxiolytic effect elicited by EE. Thereby, sex steroids might affect anxiety-related behavior in a sex-specific manner.

After confirming that even a rigid genetic predisposition can be rescued by EE, we were interested to identify processes and behaviors that contribute to the observed anxiolysis in HABs.

The onset of pathological anxiety may be attributed to a maladaption to environmental demands resulting from an interaction of genetic predisposition and environmental influences (Svrakic et al, 2011). To assess the contribution of a rigid genetic predisposition modeling pathological anxiety to the anxiolytic effect elicited by EE, we subjected NABs instead of HABs to EE. As expected, EE induced in male NABs only a small anxiolytic effect (≤0.5 SD) compared to HABs (≥1 SD), whereas the anxiolytic effect got almost completely lost in NAB females. They still show a trend or a significant reduction in some of the monitored anxiety-related parameters but lost the overall anxiolytic effect using emotionality score as a comprehensive anxiolytic index. Again, we observed sex-specific differences related to the anxiolytic effect mimicking those observed in HABs. Moreover, the genetic background (outbred CD1 animals vs. inbred HABs) of an animal may exert a critical influence on the impact of environmental stimuli. CD1 mice of both sexes compared to inbred HABs and NABs did not show an anxiolytic effect at all. Our result may highlight the importance of inbred mouse models to eliminate the "noise" of genetic heterogeneity. Anxiety is a complex, multigenic disease comprising a multitude of genes likely to be involved in the onset of pathological anxiety. Therefore, it seems plausible that every single gene contributes to a small extent, which might be revealed only at the behavioral level if masking effects including genetic heterogeneity are minimized.

In addition to the genetic background, our data might illustrate that the accumulation of risk factors in HABs (due to selective inbreeding) seems to be an important factor influencing the strength of the anxiolytic effect observed after EE. It might be speculated

that these results not only show that a rigid genetic predisposition modeling pathological anxiety might be mitigated by EE, but suggests an increased amenability to beneficial environmental stimuli. It seems conceivable that HABs exhibit different (epi-) genetic, neuronal and physiological mechanisms that contribute to their high anxiety phenotype, and exactly this multiplicity of altered processes increases the likelihood that one or several of these processes might be amenable to beneficial environmental manipulation.

To evaluate the contribution of exposure duration, we increased the time HABs were housed in EE from 4 to 10 weeks. Solely females showed a further significant increase of the observed anxiolytic effect, whereas males do not seem to benefit from prolonged EE. These results further corroborate that EE may have a sex-specific impact on anxiety-related behavior, a phenomenon that has been described earlier in the literature (Lin et al, 2011). Our data extend earlier findings by showing that even mitigation of extreme trait anxiety by EE in mice with a rigid genetic predisposition seems to exhibit sex-specific differences. Which mechanism might be accounted for the observed differences? Experimental evidence suggests that male and female brains are differing on structural, molecular and cellular levels (Lebron-Milad and Milad, 2012). Sexual dimorphisms are eminent in amygdala, HIP and PFC (Goldstein et al, 1999; 2001), brain regions that are important for the regulation of anxiety and the stress response. Thereby, the hypothalamic-pituitary-gonadal (HPG) axis plays a major role in the regulation and release of sexual hormones.

Interestingly, Bakos et al. (2009) showed that EE increased testosterone levels in male rats, which is likely to reduce the cortisol response and HPA axis reactivity (Hermans et al, 2007; Rubinow et al, 2005). Moreover, EE seems to increase the synthesis of allopregnanolone from progesterone in male rats (Munetsuna et al, 2011), with allopregnanolone being known to exert anxiolytic effects when infused in the amygdala or mPFC (Akwa et al, 1999; Engin et al, 2007). Further experiments determining testosterone and allopregnanolone levels via RIA will be performed to verify a contribution of these sex steroids to the observed sex-specific differences in anxiety-related behavior of HABs.

Importantly, both estrogen receptor types are expressed within the amygdala with estrogen receptor α offering obviously anxiogenic (Weiser et al, 2008) and estrogen receptor β exhibiting anxiolytic properties (Imwalle et al, 2005; Krezel et al, 2001). Up to now, no studies assessing the impact of EE on these receptors exist. It might be speculated that these receptors contribute to and/or attenuate the anxiolytic effect of EE on female HABs. A qPCR and/or WB to assess the gene and/or protein expression of these receptors will unravel a possible sex-specific effect. Taken together, sexual hormones offer the potential to critically contribute to anxiety-related behavior. Interestingly, male sex steroids seem to be beneficially influenced by EE, whereas data for female sex hormones are lacking and/or suggest that they do not profit, thereby maybe explaining the weaker anxiolytic effect of EE on female HABs. Nevertheless, females are affected twice as much by psychiatric disorders as males (Lebron-Milad and Milad, 2012) emphasizing the necessity to increase treatment efficacy in women. It is tempting to speculate these findings raise the possibility that there might be indeed treatment strategies with a higher efficacy in women. Studies in humans investigating the impact of environmental influences (e.g. social) on the treatment outcome of women are needed to validate this hypothesis.

In addition to genetic background, accumulation of risk factors and exposure duration, Meaney and Champagne (2007) highlighted maternal care as an important factor contributing to anxiety. To our surprise, we did not observe any differences in nursing style comparing EE and SE HAB mothers. Instead, EE mothers spent less time with their pups and invested more time in behaviors to explore (e.g. running around) and manipulate (e.g. digging) their environment (fig. 59). These findings indicate that maternal care comprising nursing style is unlikely to contribute to the anxiolytic effect of EE on HABs.

Besides the contribution of maternal care to the anxiety phenotype, the juvenile and early adolescent phases seem to be important for the development and refinement of a species-specific behavioral repertoire including motor, cognitive and social skills. This seems to be necessary to foster a general disposition for neurobehavioral plasticity (Martin and Caro, 1985; Terranova and Laviola, 2005), and it is conceivable that these skills contribute to the regulation of anxiety. Indeed, EE in relation to SE juveniles seem to invest more time in behaviors that are thought to be important for establishing the aforementioned skills. They actively used their provided EE (fig. 60, 63), whereas SE juveniles spent most of their time with passive behaviors. Moreover, EE in relation to SE adolescents engage more in social interactions (fig. 63). Many studies describe that EE indeed increases prosocial (e.g. mutual grooming, vocalizations, social exploration) and play behavior (Schindler et al, 2010), whereas aggressive behavior seems to be reduced (Schindler et al, 2010), perhaps because animals have the opportunity to escape from aggressive encounters by seeking protection in a shelter and seem to engage in a more affiliative and less aggressive social interaction strategy (FELASA working group standardization of enrichment; Pietropaolo et al, 2004). This highlights the possibility that EE facilitates cognitive, sensory and motor stimulation and leads to the expression of a much wider range of social interactions and a more naturalistic behavioral pattern (Kempermann et al, 2010).

Thus, it might be speculated that a broader and more naturalistic repertoire might be acquired by our animals, though we did not explicitly monitor all of the parameters mentioned in the literature. Importantly, behaviors falling in exactly these categories show the expected shift (e.g. increased social interactions during adolescence in EE HABs). It is tempting to conclude that a bigger behavioral repertoire increases the possibility that animals can indeed perform a behavior suited to reduce anxiety and the physiological consequences associated with it, when facing a stressor, since stressors are characterized by unpredictability and uncontrollability (Joels et al, 2009). A bigger behavioral repertoire might therefore reduce uncontrollability and thus facilitate behavioral and physiological adaptation.

EE seems to reduce expression of *Crhr1 by* increasing site-specific methylation, which may be linked to reduced HPA axis activity and noradrenaline release.

After identification of behaviors that likely contribute to the monitored anxiolytic effect of EE on HABs, we were interested to unravel particularly epigenetic processes that might be accounted to cause the respective behavioral changes.

The HPA axis and the central noradrenergic system are thought to play a major role in the regulation of the stress response and in the etiology of psychiatric disorders (Itoi et al,

2009; Reul and Holsboer, 2002). Our results indicate that EE decreases the release of CORT in male HABs after application of a mild stressor. They are in line with previous human (Abelson et al, 2007) and animal studies (Flandreau et al, 2012; Mora et al, 2012) highlighting the role of a dysregulated HPA axis for the onset of anxiety disorders. Abelson et al. (2007) were able to show that a hyperreactive HPA axis seems to contribute to PD. Interestingly they identified novelty cues as a major factor contributing to the dysregulation of the HPA axis. Van Praag et al. (2000) suggested that reduced fear to novelty constitutes an important mechanism that contributes to the observed anxiolytic effect of EE. Indeed, EE offers animals an environment with more complexity, *inter alia* due to the offered variety of novel stimuli (e.g. toys, shelter) and the possibility to create novelty by manipulation of the microenvironment (e.g. rearrangement of bedding material to demarcate territories). Thus, it seems likely that EE HABs exhibit reduced neophobia, whereat a behavioral test assessing neophobia (e.g. modified hole board) may be used to definitely verify the aforementioned hypothesis. Taken together, these results might illustrate that a combination of both, mitigation of neophobia and the increased behavioral repertoire thought to reduce uncontrollability when facing a stressor, could be important to prevent or attenuate high anxiety, which on the other hand might reduce the release of CORT and normalize HPA axis activity.

Therefore, it might be desirable to perform studies evaluating whether treatment of neophobia might be beneficial beyond the treatment of neophobia itself. This would raise the possibility that anxiety disorders like PD could benefit from the same or a similar treatment and whether it can be taken into account as a part of future treatment options for psychotherapies. Importantly, a lot of actual psychotherapies indeed try to alter or create new behavioral patterns to enable affected persons to cope with an unpleasant situation. Novel environments would thus represent a detrimental environment for affected persons, and as a logical consequence altered or new behavioral patterns would be referred to as an "enrichment" compensating maladaptive behavioral strategies. It might be speculated that EE for humans might comprise environmental situations or stimuli were preventive strategies or behavioral patterns are learned. Though research has just begun to explore this possibility, education in school might be a promising candidate not merely to treat, but also to prevent anxiety disorders in youth (e.g. the coping CAT program; Podell et al, 2010).

Our results show that reduced neophobia and an increased behavioral repertoire contribute to a reduced HPA axis activity in male EE HABs, which, in turn, might cause a decreased release of NA from LC. This decrease might further attenuate CORT release by changing the activity of a neuronal circuit regulating emotionality as follows: when animals face a stressor, the mPFC disinhibits the amygdala, which in turn activates the HPA axis and noradrenaline release from LC (Belujon and Grace, 2011; Kröner et al., 2005; Rosenkranz and Grace, 1999). Importantly, PFC can regulate its catecholamine input via direct and indirect connections to LC (NA), substantia nigra and ventral tegmental area, where dopamine projections originate. Optimal levels of catecholamines ensue a "delicious cycle" enhancing PFC regulation, whereas stress causes an increased release of NA and dopamine linked to impaired PFC regulation and strengthened amygdala function causing a "vicious cycle" (Arnsten, 2009). This might be interpreted as a switch from task-relevant

"top-down" regulation via PFC to "bottom-up" regulation by sensory cortices, whereby the salience of the stimulus captures our attention (Buschmann and Miller, 2007).

As a consequence, this would imply that a decreased NA release from LC in EE HABs predominantly activates the delicious cycle, whereas an increased release of NA from LC in SE HABs initiates the vicious cycle causing increased activity of the amygdala.

Indeed, Avrabos (2012) was able to show that neuronal activity flow through the amygdala seems to be correlated with anxiety-related behavior, apparent by decreased activity propagation through the amygdalar network of male EE in relation to SE HABs (Avrabos, Markt et al, in preparation). In other words, SE compared to EE HABs exhibit a hyperreactive amygdala, which inherently propagates neuronal signals stronger, independent of stimulus origin. This feature can also be seen in patients suffering from anxiety disorders (Shin and Liberzon, 2010) and might underlie the bias to interpret neutral stimuli as dangerous. Based on the results by Avrabos (2012), we performed qPCR with earlier identified candidate genes likely to be involved in anxiety-related behavior (Czibere, 2008) to further elucidate the origin of decreased activity propagation through the amygdalar network.

Thereby, we identified *Crhr1* as a promising candidate: the expression of *Crhr1* is increased under basal conditions in male HABs compared to LABs and, importantly, this increased expression can be reduced by exposing HABs to EE. Thus, it might be concluded that EE decreases *Crhr1* expression, which in turn causes decreased activity propagation through the amygdala. Corroborating evidence comes from Avrabos (2012), who was able to show that non-responding HABs housed in EE do not exhibit decreased activity propagation through the amygdala, thereby establishing a direct connection between neuronal activity flow and EE. Further studies assessing the expression of *Crhr1* in the BLA of non-responding EE HABs will be performed in the future to be able to verify a causal link. Moreover, EE significantly increases mRNA levels of *Tmem132d* in the mPFC, similar to individuals suffering from PD compared to controls, thereby contrasting earlier findings from Erhard et al. (2011). *Tmem132d*, also known as mature OL transmembrane protein, seems to be an important marker for the maturation from oligodendrocyte precursor cells to mature oligodendrocytes (Nomoto et al, 2003), which insulate axons in the CNS. When viewed in the light of Arnsten's (2009) hypothesis, it is tempting to speculate that an increased expression of *Tmem132d* constitutes a mechanism to counteract the attenuated PFC control over the amygdala in individuals suffering from PD (to circumvent a vicious cycle), whereas an increased expression in EE HABs might strengthen the delicious cycle. Though both results seem to contradict each other, this is not necessarily the case because the increased expression observed in both studies might have the same aim - to increase top-down PFC control over the amygdala. These data are in line with the vicious cycle hypothesis from Arnsten (2009), whereat an impaired regulation of the mPFC could be verified by running a microdialysis study in the PFC of EE vs. SE HABs or by using immunohistochemistry to visualize the presence of NA in this brain region.

We identified reduced mRNA levels of *Crhr1* in the BLA of EE HABs as an important process that contributes to reduced anxiety-related behavior in EE HABs. Since EE and SE HABs are genetically identical, we speculated that epigenetic processes participated in the reduction of *Crhr1* expression. To follow our main aim and test the possibility of an epigenetic regulation of *Crhr1* in BLA, we performed an *in silico* analysis to verify whether a CGi exists in this gene, which would render it amenable to epigenetic regulation via DNA methylation. Indeed, subsequent pyrosequencing of the *in silico* identified CGi was performed and identified a DMR in the promoter region of *Crhr1*. EE seems to increase the methylation of a CpG site -1348bp before TSS in male HABs, whereas we did not observe this or any other difference in DNA methylation comparing HABs and LABs, indicating that there might exist a different regulatory mechanism. We performed an *in silico* analysis to identify potential TFs that might interact with the aforementioned DMR and identified *YY1* as a candidate, with subsequent qPCR in fact revealing a reduction of *YY1* mRNA levels in male EE compared to SE HABs (fig. 71). This result is in line with the literature, since it is generally accepted that increased methylation causes gene repression or reduced TF binding (Mazzio and Solimann, 2012).

To assess whether and how reduced CORT release and increased methylation of the DMR in EE HABs might be connected, we performed an additional *in silico* analysis and identified two co-TFs - Nr4a1 and D3Ertd300e - that seem to interact with YY1 (fig. 72). Interestingly, D3Ertd300e appears to have a positive regulatory role for YY1, which in turn is associated with a negative epigenetic role for gene expression. In other words, higher availability of D3Ertd300e seems to be associated with higher availability of YY1, which on the other hand might prevent or cause lower methylation of the *Crhr1* DMR. Noteworthy, Nr4a1 seems to be associated and regulated by the quantity and availability of a TF complex of GR and Fkbp4/5, which raises the possibility that reduced CORT release - as observed in EE HABs (fig. 29) - entails reduced formation of this TF complex. Recently, Breuillaud et al. (2012) were able to show that mice lacking CREB-regulated transcription coactivator 1 - a TF important for *Bdnf* expression - indeed entailed lower levels of Nr4a1 in PFC and HIP and an increased anxiety-related behavior strengthening the importance of this TF in the regulation of anxiety.

Our main findings discussed before could lead to the hypothesis that a reduced CORT release decreases the availability and thus binding of CORT to GR in the BLA. This, in turn decreases the binding affinity of Nr4a1 to D3Ertd300e, which then cannot positively regulate YY1 to decrease or even prevent methylation at the identified CpG site of *Crhr1*. This would finally result in a DMR with higher methylation levels in EE HABs, which underlies the observed gene expression differences. The DMR might therefore be used as a biomarker for high or pathological anxiety (fig. 83). Our findings support Belsky's concept of plasticity genes: he suggested the existence of genes that "[…] make individuals more susceptible to environmental influences - for the better and the worse" (Belsky et al, 2009), thereby increasing phenotypic plasticity, which in turn is likely to increase adaptive capacity to environmental demands and finally survival. We would like to emphasize that our proposed epigenetic chain of events might have shifted the genetically driven

phenotype of high towards normal anxiety or vice versa, i.e. the behavioral profile was altered by EE and caused this epigenetic chain of events.

A vast body of literature supports the dysregulation of *Crhr1* in the etiology of psychiatric disorders (Arborelious et al, 1999; Heinrichs et al, 1997; Nemeroff, 2009; Reul and Holsboer, 2002). Our data indicate that *Crhr1* might be regulated bidirectionally, depending on the quality of environmental stimuli. They are in line with earlier findings (Sztainberg et al, 2010b) and extend them by suggesting an epigenetic mechanism that is likely to contribute to the anxiolytic effect of EE. Altogether, it might be speculated - to the best of our knowledge - that *Crhr1* seems to be first identified plasticity gene of anxiety, which is involved in the regulation of the stress response by an epigenetic mechanism.

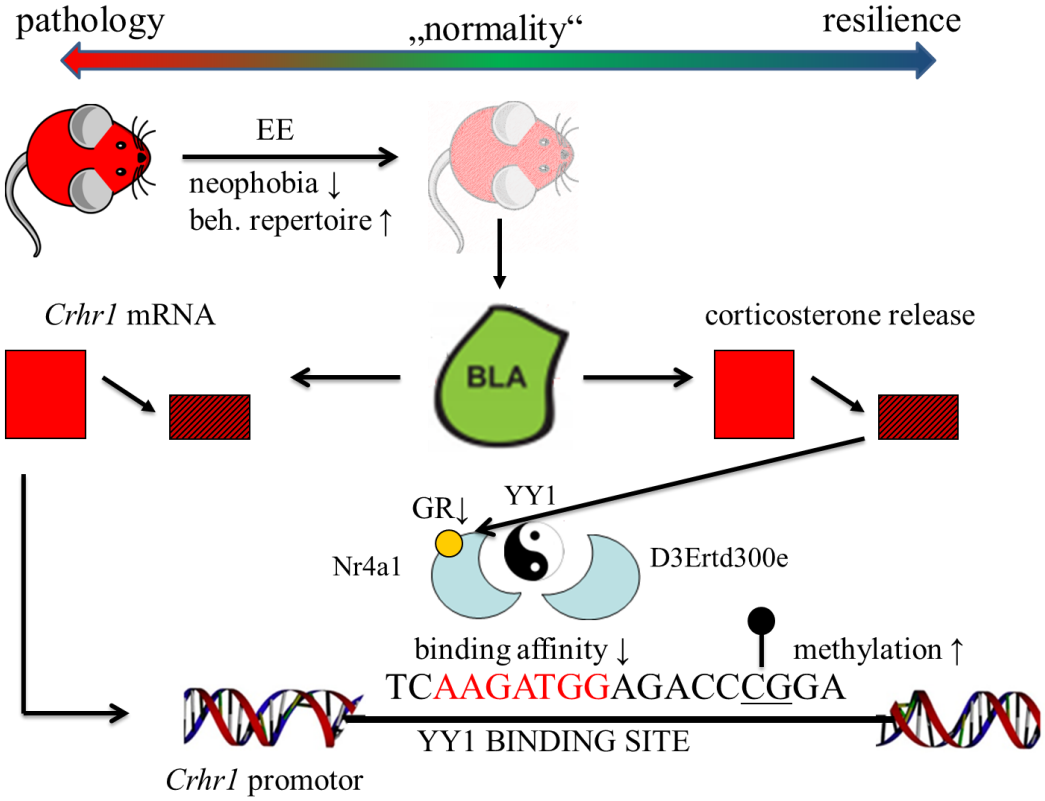


Fig. 83: Epigenetic chain of events. EE is likely to reduce neophobia and increase the behavioral repertoire of HABs, which is thought to minimize uncontrollability of stressful situations, thereby shifting their phenotype from high towards normal anxiety. This reduction causes a decreased expression of *Crhr1* in the BLA, which in turn might reduce the neuronal activity flow through the amygdala. Thereby, a reduced release of CORT after a stressor decreases its availability and binding to GR and subsequently Nr4a1 in the BLA. This decrease reduces binding affinity of the TF complex comprising YY1, Nr4a1 and D3Ertd300e to the YY1 TFB, finally increasing methylation of a DMR in the promoter of *Crhr1*. Either, the epigenetic chain of events shifts the high anxiety related behavior towards normality or the altered behavior causes the epigenetic chain of events. Basolateral amygdala (BLA), corticotropin releasing hormone receptor 1 (*Crhr1*), enriched environment EE, transcription factor (TF), transcription factor binding site (TFB), Yin Yang 1 (YY1)

Though speculative, the possibility exists that the identified DMR is part of a CpG shore - a region ca. 2000bp before the TSS but without being part of a CGi. It is conceivable that the first CpG of the *Crhr1* CGi might be the tip of the iceberg with several other CpGs being differentially methylated upstream of our identified DMR raising the necessity to perform pyrosequencing for these CpGs too. Indeed, several recent papers suggest that genes seem to be regulated by CpG shores rather than CGis, which are associated with tissue-specific gene expression of housekeeping genes (Doi et al, 2009; Irizarry et al, 2009). In fact, upstream of our identified DMR are 15 more CpGs which could be part of a *Crhr1* CpG shore (Ensembl version: ENSMUSG00000018634.10, date of accession 12.09.2012). Importantly, TFs can have the size of several dozens to hundreds of amino acids leading to the assumption that increased methylation of exactly these CpGs might contribute to the observed decreased expression of *Crhr1*.

Further evidence for the contribution of epigenetic mechanisms in the high anxiety phenotype of HABs might be provided by our results showing that the anxiety-related behavior of HABs is influenced by epigenetic drugs. There seems to be a robust anxiogenic effect of the DNMTi valproic acid on both, SE and EE animals (fig. 74, 75). These results allow two conclusions to be drawn: first, DNMTs would no longer be able to transfer a methyl group to the identified DMR in *Crhr1*, which in turn would mimic an SE expression phenotype that would entail an increased anxiety-related behavior - this is exactly what we monitor in EE HABs, which received valproic acid. Second, other epigenetically regulated genes contribute to the high anxiety phenotype of HABs and are associated with the anxiolytic effect of EE since DNMTis are known to exert effects on all genes amenable for DNA methylation. This might explain why valproic acid induced an anxiogenic effect in SE HABs too, though they exhibit low methylation of the *Crhr1* DMR. HDACi 5-Aza-2'-deoxyuridine might possess an antidromic effect with an anxiolytic impact on SE and an anxiogenic effect on EE HABs (fig. 73-75). Similar to DNMTIs, HDACis influence acetylation levels of all histones rendering it likely to alter the expression of genes with anxiolytic and anxiogenic properties. Thus, the overall effect is likely to be different between SE and EE HABs because it depends on environmental factors - which are likely to alter histone modifications itself - acting on the genetic predisposition. This emphasizes the potential of epigenetic regulation to contribute to or shape the final phenotypic outcome and to identify epigenetic factors and/or processes that may act as or represent novel therapeutic targets.

To verify our suggested epigenetic chain of events, it is necessary to perform a chromatin immunoprecipitation, which could reveal whether YY1, Nr4a1 and D3Ertd300e together with GR and Fkbp4/5 really form a complex at *Crhr1* to regulate its expression. Our data give rise to the proposed hypothesis, but do not show a direct association of the identified factors with *Crhr1*. It has to be mentioned that there is the possibility that YY1 can regulate one or several different genes than *Crhr1* because qPCR can merely compare expression levels independent of the truly associated gene. Moreover, it is possible to alter the methylation of single CpGs, which raises the possibility to mimic the expression phenotype we observed in EE HABs to verify whether methylation is truly involved in the regulation of gene expression. Our findings are a first hint that beneficial environmental

conditions or stimuli indeed can mitigate or rescue even a rigid genetic predisposition of extreme trait anxiety obviously by triggering an anxiolytic epigenetic mechanism. This emphasizes the necessity to identify the influence of beneficial environmental stimuli as preventive or attenuating strategies in humans. The author is fully aware that mice can model merely some aspects of how beneficial environments can modify genetic regulation. The behavioral repertoire, brain architecture and genetic regulation in humans is likely to be far more complex compared to mice as corroborated very recently by the ENCODE project (http://www.nature.com/encode/#/threads), but our findings might indicate that also humans could benefit from enrichment strategies compensating maladaptive behavioral patterns or a detrimental genetic predisposition. This hypothesis is strengthened by an increasing number of papers trying to emphasize the importance of beneficial stimuli for human well-being (Kobau et al, 2012; Pedrals et al, 2012; Proyer et al, 2012) or to alleviate psychopathology by positive activity interventions like positive thinking, affect and behaviors, with at least the last parameter likely contributing to the anxiolytic effect of EE as well (Layous et al, 2011).

A gene x environment correlation might transmit the anxiolytic effect of EE in a mouse model of pathological anxiety to a subgroup of male F1 individuals.

The possibility to transmit acquired traits from one generation to the next has gathered increasing attention and might explain the rapidly growing body of literature dealing with this topic (Arai et al, 2009; Champagne and Meaney, 2007; Leshem and Schulkin, 2011). Many researchers argue that transgenerational effects must be transmitted at least to F3 since the effect acts on the mother carrying the F1 generation, which on the other hand has already established germ line cells for F2, on which the effect might act. Our data suggest that the anxiolytic effect elicited by EE might be transmitted to a subgroup of male F1 HABs without affecting animals from F2 and F3 and thus, excludes a transgenerational effect *per definitionem*. Interestingly, we observe a phenomenon earlier described by Elliott et al. (2010): obviously it is possible that some animals do and others do not respond to a treatment - a phenomenon well known from humans (Lanouette and Stein, 2010). It has to be mentioned that our study tried to assess transgenerational transmission, whereas Elliott et al. (2010) monitored the behavior of adult mice.

The majority of our male HABs housed in EE seems to offer an anxiolytic effect, which we can observe in LD but not OF and EPM (fig. 78). It is thought that different behavioral tests monitor different aspects of anxiety-related behavior (Bouwknecht and Paylor, 2008; Sartori et al., 2011) and, thus, it might be speculated that the anxiolytic effect of responding F1 HABs might be "diluted" compared to the parental generation since merely 1 out of 3 tests suggests an anxiolytic effect. Our first hint is in line with recent studies (Curley et al, 2009; Walker et al, 2012) indicating that this anxiolytic transmission in fact might exist. Further studies with a higher number of animals are planned to pursue our first hint and to definitely verify whether such a mechanism might exist.

The crucial question is whether such a mechanism would make sense in the light of evolution. The author would like to highlight one conceivable scenario: gene x environment correlation (rGxE) suggests that certain genetic variants influence

environmental exposure indirectly via behavior (Rutter et al, 2006). This mechanism is different from transgenerational transmission, but can be viewed complementary to prepare the animals in the best way possible: individuals exhibit a genetic predisposition, which causes them to seek certain environments, and epigenetic mechanisms may change these behaviors (by changing underlying gene expression) to fine-tune organisms to actual or future environmental demands. It seems plausible that selective breeding of HABs accumulated genes contributing to high anxiety entailing a behavioral phenotype, which actively avoids dangerous situations and stressful stimuli. This explains why SE HABs rarely enter the aversive zone of tests assessing anxiety-related behavior; but it might be beneficial to be able to change that phenotype if environmental changes demand it. If rodents life in an environment with rapidly changing conditions, this might in turn exert an influence on their emotionality (e.g. low food availability might entail decreased anxiety to forage for food). We have suggested one epigenetic mechanism that could contribute to the shift from high towards normal anxiety in HABs, but it is likely that several additional mechanisms contribute to anxiolysis. Now, two speculative scenarios are conceivable: (i) some of these mechanisms were passed on to a subset of male F1 individuals (therefore, the effect was merely seen in 1 out of 3 tests) and/or (ii) the associated epigenetic changes cause the offspring to seek the environment of their parents, where the environmental conditions again can modulate their anxiety phenotype in the same manner it did for their parents (i.e. rGxE). To the best of our knowledge, this highlights for the first time the exciting possibility that an effect causing anxiolysis can be transmitted to the offspring but cannot be measured *per se* in exactly this offspring. This hypothesis is difficult to verify since rodents favor EE over SE housing, thereby excluding preference studies (van de Weerd, 2001). Maybe, field studies in a big semi-natural set-up enclosure can shed light on this hypothesis at a behavioral level. In addition, whole genome methylation studies assessing methylation levels in parents and offspring could aid to verify this hypothesis at a molecular level by identifying transmitted epigenetic states that might cause the proposed rGxE. The author is fully aware that new studies trying to verify this hypothesis have to be performed.

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8 Curriculum vitae

Curriculum Vitae

personal data

name:	Patrick Markt
date of birth:	02.03.1983 in Munich
marital status:	engaged, no children
nationality:	German

academic career

04/2009 – 09/2012	*PhD student at the Max Plack Institute of Psychiatry* Graduation: „Interaction of genetic predisposition and epigenetic factors at the development of anxiety“
10/2004 - 03/2009	*TU München/ Ludwig-Maximilians-Universität München* Field of study: biology diploma Main subject: zoology, minor subjects: genetics, neurobiology

Publications:

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Avrabos C, Markt PO, Sotnikov SV, Eder M, Landgraf R. Environmental enrichment decreases anxiety in a pathological mouse model with high trait anxiety by reducing neuronal propagation within the amygdala. *Manuscript in preparation*