Errata corrige Tesi Dr. Stefano Gaburro

Titolo: ***Autonomic function in mice with altered anxiety/depression related behavior***

Pagina	**Riga**	**Parola**	
		Originale	**Modificata**
32	7	none	any
38	9	shown	showed
38	10	not	no
40	4	in	(eliminato)
40	16	showe	show
40	19	increased	increase
41	10	significance	significant
41	22	was observed	(eliminato)
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52	5	observed is	is observed



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DOCTORAL PROGRAMME
XXI CICLE

Autonomic function in mice with altered anxiety/depression related behavior

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Abstract English

Autonomic dysregulation has been described in patients suffering from anxiety and depression disorders. The vast majority of animal studies investigating autonomic function in anxiety and depression involve “non pathological” models. Thus, the aim of the present study was to investigate parameters of autonomic regulation (e.g. body temperature, heart rate and heart rate variability) in a mouse model selectively bred for high innate anxiety (HAB) versus normal anxiety like behaving mice (NAB).

Basal (home cage) and mild unconditioned stressor conditions (e.g. novel cage, open field) did not reveal any difference in heart rate or body temperature between HAB and NAB mice. Surprisingly, when we investigated heart rate variability during conditioned stressors (e.g. fear conditioning), we revealed reduced heart rate variability (HRV) associated with impaired fear extinction in HAB versus NAB mice. This finding of reduced HRV suggests a strong resemblance to what is observed in patients suffering from anxiety and depressive disorders. Moreover, the enhanced anxiety- and depression-related behavior was attenuated after successful chronic NK1 receptor antagonist treatment in HAB mice. The behavioral effect of the NK1 receptor antagonist was associated with normalization of the reduced HRV. These findings demonstrate that heart rate variability is a useful additional diagnostic marker, sensitive to successful drug treatment, characterizing high anxiety and depression trait. Thus, futures studies will elucidate if on the basis of HRV it will be possible to predict successful drug treatment in high anxiety and depression patients.

Riassunto Italiano

Disturbi psico-affettivi, come sintomatologie ansioso-depressive, sono state spesso associate con alterazioni del sistema cardiovascolare, che a lungo andare può dare luogo all´insorgenza di malattie cardiovascolari croniche e quindi ad un incremento del rischio di associata morbilità e mortalità. Purtroppo, la maggior parte degli studi animali che analizzano la stretta incidenza tra disturbi psico-affettivi e il sistema cardiovascolare, utilizzano modelli animali cosiddetti non patologici. Di conseguenza, tali studi mancano di informazioni sulle strette analogie che intercorrono nella regolazione neuro-vascolare in uno stato patologico come ansia e depressione. Quindi oggetto della presente tesi è l´analisi di parametri cardiovascolari (temperature corporea, frequenza cardiaca e variabilità della frequenza cardiaca) in un modello animale selezionato per innato comportamento ansioso-depresso HAB (molto ansioso) e normal ansioso (NAB), quindi mimando la vasta eterogeneità presente negli studi clinici in disturbi psico-affettivi.

In condizioni basali e in una serie di test comportamentali considerati mediamente stressanti (novel cage, open field), non sono state rilevate differenze nella frequenza cardiaca e nella temperatura corporea tra le due linee di topo. Quando invece è stata studiata la variabilità della frequenza cardiaca (HRV), in un test comportamentale in cui viene misurata la paura dei topi (fear conditioning), associata ad un'esagerata espressione della paura nella linea di topi HAB è stata trovata una ridotta HRV. Questo riflette quanto osservato in pazienti con sintomatologia ansioso-depressiva in cui una ridotta HRV è correlata ad un'aumentata espressione della paura.

Poi, con l´ausilio di un antidepressivo sperimentale ancora in fase di sviluppo, appartenente alla famiglia degli antagonisti del recettore NK1, si è riusciti a normalizzare HRV con coincidente riduzione del comportamento molto ansioso e depresso nella linea di topi HAB. Questi risultati dimostrano che HRV può essere utilizzata come marker per caratterizzare individui con alti livelli di ansia e depressione. Inoltre, studi futuri chiariranno se sulla base di HRV sia possibile identificare effetti positivi di un trattamento farmacologico per ansia e depressione ancor prima di osservare gli effetti comportamentali.

1. Introduction

Mental health problem are quite common. Around 450 million people worldwide currently suffer from such conditions, placing mental disorders among the leading causes of ill health. Moreover the spread of mental illnesses is increasing from being 12% of the total burden of disease, projections for 2020 reach 15%.The most eloquent example is depressive disorder, the fourth leading cause of disease and disability, which are expected to rank second in 2020 (WHO, 2003).

In patients suffering from mental diseases, there is also an increased risk of mortality for cardiovascular diseases (i.e. coronary heart disease) which has been shown to be strictly correlated with the severity of the psycho-pathology. Furthermore, the probability of developing a mental disorder is twice higher in women than in men. However, the cardiovascular associated risk is not different among the sex as hormones in female appear to play a protective role.

The current drug treatment in anxiety and depression display a delayed onset of efficacy and induce severe side effects (e.g. sexual dysfunction, nausea, headaches). Therefore there is an urgent need of novel compounds development possessing anxiolytic and/or antidepressant potential that could also possibly reduce the incidence of morbidity related to these pathologies.

Although anxiety and depression are different clinical pathologies and they have to be treated as different, some evidences in literature suggest that there is a high degree of comorbidity among these pathologies and the autonomic markers generally used suggest a perturbation in both pathologies (Hirschfeld, 2001; Zimmerman et al., 2002). Therefore, it is important to develop a new drug that could successfully act as "panacea" against these diseases as well as identify novel physiological marker aiming at improving therapy. However, to reach this goal a better understanding in the neurobiology and underlying neurochemistry behind anxiety/depression and autonomic regulation is needed.

1.1 Anxiety and Fear

Anxiety is considered a general long lasting state of distress, involving behavioral and physiological responses including avoidance, vigilance and arousal, which evolved to

protect the individual from danger. In its non-pathological form anxiety-related responses have been evolutionary developed as response to a threatening stimulus (fear response) and the brain mechanisms are highly conserved across the species.
Fear can be distinguished from anxiety. For example, the perceived possibility of the occurrence of negative consequences produces anxiety, whereas the immediate presence of an obviously harmful stimulus elicits fear (Cannistraro & Rauch, 2003; Stiedl *et al.*, 2009). Although there are proved differences between fear and anxiety, they are processed in greatly overlapping neurocircuitries (Hyman, 1998; Cannistraro & Rauch, 2003; Kent & Rauch, 2003; Bremner, 2004; Singewald, 2007).
In its pathological form, anxiety can severely affect daily life and according to DSM-IV (Diagnostic and Statistical Manual of mental Disorders, Forth Edition, 1994), anxiety disorders have been classified in six main categories: Phobias, Panic Disorder, Generalized Anxiety Disorder (GAD), Obsessive-Compulsive Disorder (OCD), Post-Traumatic Stress Disorder (PTSD), and Acute Stress Disorder.

1.2 Depression

Depression is a common mental disorder that presents with depressed mood, loss of interest or pleasure (anhedonic behavior), feelings of guilt or low self-worth, disturbed sleep or appetite, low energy, and poor concentration. In addition to mortality associated with suicide, depressed patients are more likely to develop coronary artery disease and type-2 diabetes. Many evidences have correlated depressive disorders with an increased incidence of cardiovascular diseases.
According to modern classification systems such as the DSM-IV (American Psychiatry Association, 1994) and ICD-10 (World Health Organisation, 1992) depressive disorders are generally distinguished between unipolar and bipolar forms, although these are not completely distinct. On one hand, the bipolar affective disorder (also known as maniac depressive illness) cannot be diagnosed until an individual has suffered at least one episode of mania, mixed mania, or hypomania. On the other hand the unipolar disorders (also known as unipolar major depression or simply unipolar depression) patients suffer one or more episodes of depression without ever experiencing episodes of pathologically elevated mood. In both cases patients cannot deal with normal daily life.

1.3 Anxiety and Comorbid depression

Both depression and anxiety are considered independent markers for higher risk of cardiovascular diseases. Although clinically these are considered as two different classes of psychiatric disorders, there are studies showing that anxiety and depression share a high degree of comorbidity.

The most common combination is comorbidity of GAD and depression and its prevalence has been reported in as high as 60% to 90% of patients with GAD in community populations.

Unfortunately, the majority of these patients are not adequately diagnosed or treated (Rickels & Schweizer, 1998), because this comorbidity poses a complicated diagnostic and treatment challenge. Therefore, there is an urgent need to find reliable markers for successful individual treatment based on the severity of the pathology.

1.4 Pharmacological treatments

As anxiety and depression are clinically considered different in the last years treatments were also different for both pathologies.

Among the drugs used in anxiety, benzodiazepines are the most used, with a wide variety of choice, as at least 20 different compounds commercially are available. Those drugs are positive allosteric modulator on benzodiazepine- sensitive GABAa receptor complex. The consequence of this binding is a neuronal inhibition leading to a sedative and relaxant effect (for review see (de Mooij-van Malsen *et al.*, 2008; Hoffman & Mathew, 2008). These treatments have low side effects but they act both on patients and normal controls showing no pathological specificity (Murphy et al., 2008a). Furthermore, some clinical effective doses display several side effects as sedative effect and physiological dependence with long term use making these drugs not really specific for patients suffering from anxiety disorders (Hoffman & Mathew, 2008) .

In depressive patients the most common treatments are moamine oxydase inhibitors, tricyclic antidepressant, selective serotonin reuptake inhibitors (SSRI, i.e. fluoxetine), selective noradrenaline reuptake inhibitors (NRIs, i.e. reboxetine) and combination of both (SNRIs, i.e. duloxetine). With regard to tricyclics such as nortriptaline, an antidepressant effect has been demonstrated but generally this is followed by alterations in the autonomic system such as hypotension and in long terms increased

risk for myocardial infarction (Glassman & Preud'homme, 1993) when compared to SSRI. In this respect, SSRI are preferred because they exert fewer side effects, especially on the cholinergic system. Studies confirm that treatment wit tricyclic show higher antidepressant efficacy compared to SSRI but more side effects as modifications in the autonomic system (Roose et al., 1998a; Roose et al., 1998b). Furthermore, studies has shown that non-responders to SSRI, can be treated with the double inhibitors of serotonin and nor-adrenaline such as duloxetine or tianeptine showing an antidepressant efficacy but hypotension and tachycardia as side effects (Hudson et al., 2005). However SSRI display a delayed onset of efficacy and other minor side effects (nausea, sexual dysfunction) (Murphy et al., 2008b) and almost 30% of the patients have shown remission by the treatment (Berger et al., 2008). In this respect the function of novel receptors in the brain has been studies and identified as potential target for a pro-depressive phenotype.

Apart from these, studies have been done on oxytocin (Neumann, 2008), vasopressin (Simon et al., 2008) and the substance P receptor (neurokinin) inhibitor namely neurokinin 1 (NK1) receptor antagonist. The localization of substance P and its preferred receptor (NK1 receptor), in brain regions involved in regulation of affective behavior and the neurochemical response to stress, represents the basis of involvement of substance P/NK1 receptor pathway in the modulation of affective behavior (Herpfer & Lieb, 2005; Ebner & Singewald, 2006).

Moreover anxiety and depression occur in the context of some form of stress (Kendler *et al.*, 2001; Esch *et al.*, 2002; van Praag, 2004; Nemeroff & Vale, 2005; Kruger *et al.*, 2006) and it has been widely reported an altered stress-induce neuronal processing in depressed patients (Drevets, 2003; Phillips et al., 2003; Malhi et al., 2004; Strakowski et al., 2005). Thus, the stress-related model can be use to study the neurobiology of depression and antidepressant actions.

In this respect, antagonism of NK1 receptor has been shown to produce behavioral effect under stress situation (Ebner et al., 2004). In line to this study, NK1 receptor antagonist has been proposed as a novel class of compounds exerting anxiolytic (Varty et al., 2002) and antidepressant (Kramer *et al.*, 1998; Ranga & Krishnan, 2002; Varty *et al.*, 2003; Kramer *et al.*, 2004) actions in human and rodents. As NK1 receptors are expressed in the brain and also in the cardiovascular system, it has been shown that activation of the receptors lead to produce also bradycardic responses in isolated guinea pig rats (Hoover et al., 1998). However, the integrated and complex

relationship between behavioral and autonomic responses mediated by NK1 receptor antagonist treatment is still largely unknown (Culman et al., 1997).

In the last years, many NK1 receptor antagonist compound have been produced displaying different binding affinities to its receptors, differential pharmacokinetics and potency to produce an anxiolytic and antidepressant effect (Herpfer & Lieb, 2005; Ebner & Singewald, 2006).

1.5 Physiological markers in anxiety/depression

Patients suffering from anxiety and depression and or having comorbidity display other physiological alterations that in long term could drive to increased mortality (e.g. coronary heart disease) (Dickens et al., 2004; Stiedl et al., 2009). Many studies have shown that anxiety and depression can occur with respiratory and hormonal changes as well as autonomic dysfunctions (for review see (Roth, 2005). Many of the physiological variables previously mentioned appear to be concomitant correlates of both anxiety and depression states, in that a person reporting a distress state manifests these physiological change and vice versa. Yet other studies demonstrate that physiological activation is not specific to anxiety/depression, but appear in an identical or closely related form with emotions verbalized as excitement or anger.

Among all the dysfunction that could occur, Friedmann (Friedman, 2007) reported the autonomic alteration that could occur in psychopathologies such as increased blood pressure and increased heart rate appears to be closely correlated to specific anxiety state (specific phobia) and major depression. However there is a mixed literature on psychopthatology and cardiovascular parameters. Whereas several investigations have reported that depressed patients have lowered levels of cardiovascular markers (e.g heart rate, blood pressure) than do non-depressed controls (e.g. (Rottenberg, 2007) others report no differences in cardiovascular parameters between depressed subjects and non-depressed controls.

Cohen (Cohen & Benjamin, 2006) has proposed heart rate variability could serve as useful marker in patients suffering from anxiety/depression. Indeed patients suffering from different anxiety disorders share reduced heart rate variability (HRV). In table 1 some examples of studies linking anxiety and depression with alteration in autonomic parameters.

Table1.1. Studies linking different anxiety and depression disorders to autonomic nervous system dysregulation . Modified from (Cohen & Benjamin, 2006)

TYPE OF ANXIETY DISORDER	AUTONOMIC RESPONSE	STRESSOR TYPE	REFERENCE
GENERAL ANXIETY DISORDER (GAD)	≈HR ↓HRV	Trauma recalled	(Thayer et al., 1996)
PANIC DISORDERS (PD)	↓HR ↓HRV	Resting state	(Yeragani et al., 1993)
SOCIAL PHOBIA	↓HR in females not in males ↓HRV	Speech presentation	(Grossman et al., 2001)
POST TRAUMATIC STRESS DISORDERS (PTSD)	≈HR ↓HRV	Trauma recalled	(Cohen et al., 2000; Blechert et al., 2007)
OBSESSIVE COMPULSIVE DISORDER (OCD)	≈HR ≈HRV	Resting state	(Slaap et al., 2004)
DEPRESSION	≈HR ↓HRV	-	(Gotthardt et al., 1995)

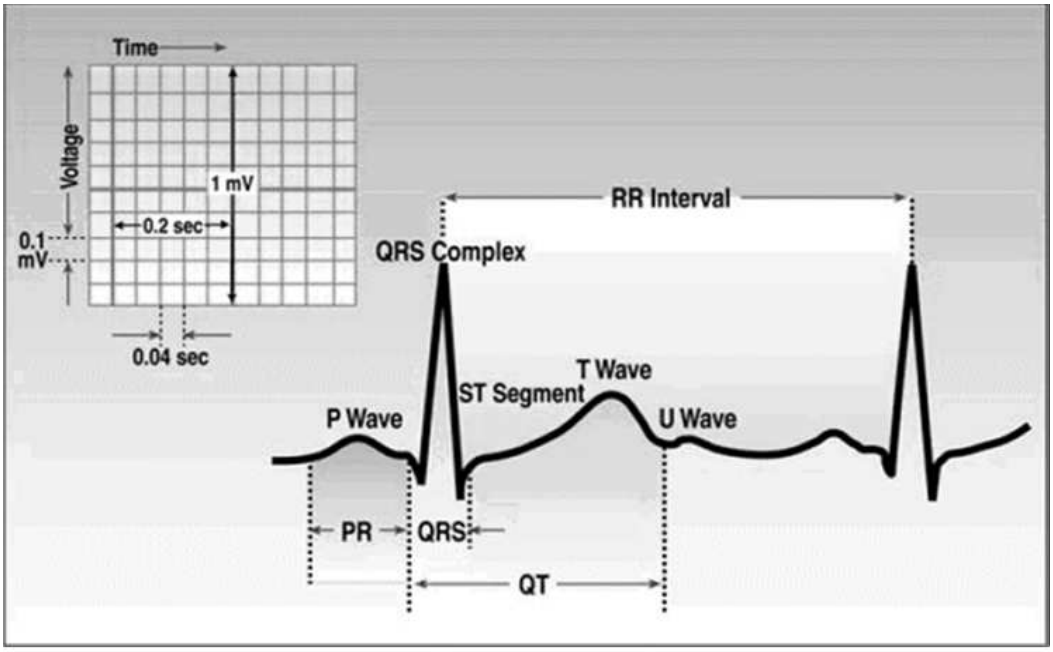
As cardiovascular responses here reported **HR** and **HRV** which correspond to heart rate and heart rate variability, respectively

Moreover a reduced HRV has been validated as indicator of pathological states in human as well as rodents (Friedman, 2007; Rowan et al., 2007). Indeed a normalized HRV, but not other physiological markers, has been correlated with a recovered depression after chronic fluoxetine treatment. Furthermore, fMRI studies correlating HRV and brain area activity (i.e. amygdala and pre-frontal cortex) in patients suffering from pathological anxiety, have shown a correlation between normalized HRV, restored brain activity, and a behavioral anxiolytic effect after chronic antidepressant treatment. In addition, first evidence has shown that autonomic reactivity (i.e. HRV) can be a predictor of successful antidepressant treatment just when HRV was measured in emotional states (Fraguas et al., 2007). Thereby, there is a need of studying HRV under basal and emotions conditions also in animal model for anxiety and depression related behavior.

1.6 Quantification of Heart Rate Variability (HRV)

In the last decade reduced HRV variability has been validated as one of the most promising markers in anxiety and depression (Yeragani *et al.*, 1991; Friedman & Thayer, 1998; Yeragani *et al.*, 1998; Yeragani *et al.*, 2000; Yeragani & Rao, 2003; Cohen & Benjamin, 2006; Friedman, 2007). However, the dynamics of sympathovagal balance is still unknown. It is important to understand how affective pathology and successful drug treatment can perturb and normalize the autonomic balance respectively. Studies in the early 90s on HRV were difficult to interpret because there were no clear guide-lines (for review see Task Force (1996). Therefore in 1996, a task force made of mathematicians and cardiologist defined standard measurement and physiological interpretation of heart rate variability in humans. Since then, the most commonly used methods are time and frequency domain analysis of HRV.

The time domain analysis is perhaps the easiest to perform. With these methods the heart rate at any time-point or the intervals between successive normal complexes are determined. In other words, in a continuous electrocardiographic (ECG) record, each QRS complex is detected, and the so-called normal-to-normal (NN) intervals (corresponding to each sinus node depolarization), or the instantaneous heart rate are determined.



(i) The normal ECG

Fig.1.1.Taken from (Gonçalves & Guizzardi, 2005)

From the statistical analysis of consecutive NN intervals, validated markers for the two components sympathetic and parasympathetic (vagal) of the autonomic systems (ANS) are extrapolated. The commonly used and simple to calculate parameter is the standard deviation of two consecutive NN intervals. This variable, when lower compared to control, indicates an altered autonomic state. However, it is of limited importance as it fails to provide any information as to which of the two branches of the ANS are affected. A validated measure of the vagal (parasympathetic) component of the ANS is the variable called squared root of the mean square differences of successive NN intervals (RMSSD).

Table 1.2. Selected Time Domain Measures of HRV, Table adapted from Task force (1996)

Variable	Units	Description
Statistical Measures		
SDNN	ms	Standard deviation of all NN intervals
SDANN	ms	Standard deviation of the averages of NN intervals in all 5-minute segments of the entire recording
RMSSD	ms	The square root of the mean of the sum of the squares of differences between adjacent NN intervals
SDNN index	ms	Mean of the standard deviations of all NN intervals for all 5-minute segments of the entire recording
SDSD	ms	Standard deviation of differences between adjacent NN intervals
NN50 count		Number of pairs of adjacent NN intervals differing by more than 50 ms in the entire recording; three variants are possible counting all such NN intervals pairs or only pairs in which the first or the second interval is longer
pNN50	%	NN50 count divided by the total number of all NN intervals

Many others variable considered in table 1.2 can be generated from the study of HRV, but the present thesis will focus mainly on the RMSSD variable.

The frequency domain analysis, instead, provides information of how power (i.e. variance) is distributed as a function of frequency. There are many mathematical

algorithms based on the assumption that ECG can be approximated to a sinusoidal distribution. On this basis, complex calculation can be executed (i.e. fast fourier transformation or periodogram analysis) and frequency extrapolated.

On the basis of pharmacological studies using either cholinergic blockers for parasympathetic system or adrenergic blockers for the sympathetic system the physiological relevance of the frequencies have been identified for humans and non humans including rodents(for review seeTask Force(1996; Elghozi & Julien, 2007; Rowan *et al.*, 2007).

The very low frequency (VLF) component (≤ 0.04 Hz) has not yet been given a precise physiological meaning and is subject to considerable debate, having been attributed variously to thermoregulatory processes, peripheral vasomotor activity and the renin–angiotensin system. It is considered to be a predominately sympathetic indicator.(Akselrod et al., 1981)

The high frequency band (HF) ranging from (0.4-1.5 Hz) has respiration component as the primary rhythmic stimulus ("sinus arrhythmia") and is mainly mediated by changing levels of parasympathetic tone.

The low frequency band (LF) (1.5- 4.0 Hz) is affected by the oscillatory rhythm of the baroreceptor system and is mediated from both sympathetic and parasympathetic system.

The ratio LF/HF indicates how the sympathovagal balance is driven: on one hand if the ratio is high that means there is more sympathetic activity. On the other hand, if the ratio is low that means there is more parasympathetic control.

Table 1.3. Selected Frequency Domain Measures of HRV. Table modified from Task Force (1996)

Variable	Units	Description	Frequency Range	Physiological Relevance
VLF	Power ms^2	Power in VLF range	≤ 0.04 Hz	Unknow influence
LF	Powertms^2	Power in LF range	0.04-0.15 Hz	Sympathetic and Parasympathetic control
HF	Power ms^2	Power in HF range	0.15-0.4 Hz	Respiratory (Parasympathetic contorl
LF/HF		Ratio LF/HF	Ratio	Sympathovagal balance. If high more sympathetic control, if low more parasympathetic control

Very low frequency (VLF), low frequency (LF), high frequency (HF)

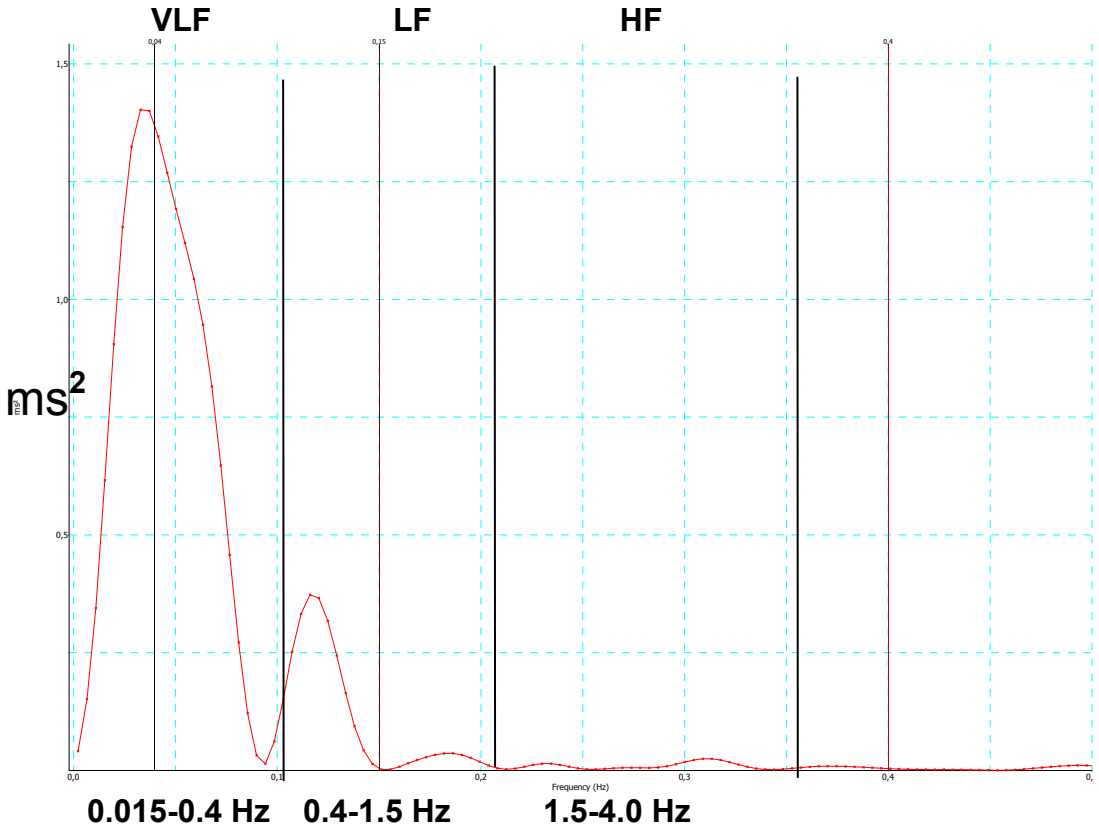


Figure 2. Graphical representation of frequency domain analysis of HRV in mouse. Here reported all the components of HRV. Very low frequency (VLF), low frequency (LF) and high frequency (HF) in a time resolution of 30 seconds.

This kind of analysis requires fast computation and short term variability (5 minutes ECG) because it is based on the assumption of stationarity (stability) of the signal. In other words, if the ECG is a very long stretch (24 hours), the loss of sinusoidal rythmicity can occur producing an error in the interpretation of the frequencies. Especially, this could be the case in rodent where the basal heart rate is 300 beats per minute in rats and 500 beats per minute in mice. In this respects other guidelines has been written and the length of the ECG recording adjusted to the species (i.e. 30 seconds for mice)(Rowan et al., 2007). HF and RMSSD are highly correlated and according to pharmacological validation they both correspond to the vagal (parasympathetic) component of ANS. In the recent past, some studies in mice have evaluated the autonomic response comparing the entire dynamic range of heart rate versus its variability (RMSSD) from basal to stress conditions. This analysis permits a better understanding of the symphatovagal balance in different mice lines or strains. (for review see (Stiedl et al., 2009).

Apart from the analyses mentioned above, some other analysis can also be performed using HRV, for instance, the non linear dynamics which is based on the principle that the ECG is not stationary and produce variance (chaos) thus, this study permit to

identify differences not evaluable with conventional autonomic markers as HR (Gross et al., 2005). However, this kind of analysis requires much more computation, experience and time. Therefore this kind of analysis will not be the object of the thesis.

1.7 Autonomic measurement in rodents: radio-telemetry device

Scientists have adapted available technologies to study animals in their quests to unravel and understand the biological functions and processes for several centuries; in the most recent of these endeavors, scientists have applied radiotelemetry technology. Radiotelemetry combines miniature sensors and transmitters to detect and broadcast biological signals in animals to remote receiver. The receiver converts the analog frequency signal into a digital signal to be imputed into a computerized data.

Acquisition system. The acquisition system can store, manipulate, format, tabulate, and output the data in accord with the instructions of the user. Currently, radiotelemetry systems can collect blood pressure (BP), heart rate (HR), blood flow (BF), electrocardiogram (ECG) and other biopotentials (EEG, EMG), respiratory rate (RR), pH, body temperature (BT), and activity indices. The advantages of this technique are enormous: i) data can be collected from freely moving, unrestrained animals avoiding then artifacts, ii) reduce the number of animals used for a single experiment.

During the last decade there has been a large increase in using transgenic models, and specifically mouse models, to investigate the causes of, and potential treatments for pathological autonomic functions as in cardiovascular disease and in affective disorders (Berul et al., 1996; Fewell et al., 1997; James et al., 1998). Recent measurements of ECG, HR, and BP using telemetry in inbred strains as well as transgenic mouse models have resulted in qualitative and quantitative changes in definitions of ECG waveforms, in baseline values of HR and BP, and in responses to autonomic agents, compared with non-transgenic control mice (Wickman et al., 1998; Itokawa et al., 1999; Gehrmann et al., 2000; Just et al., 2000; Nguyen-Tran et al., 2000; Davisson et al., 2002; Gross et al., 2002; Hoit et al., 2002; Shusterman et al., 2002). Indeed with the use of HRV (above described) it has been possible to discriminate the sympathovagal components in animal models with altered autonomic functions. Since radiotelemetry permits investigation of drug effects in freely moving, unrestrained animals, it facilitates the studies of autonomic functions in pathological animal models mimicking the clinical conditions for new compound investigation.

1.8 Animal model of enhanced anxiety- and depression-like behavior

Even if HRV is nowadays well studied in humans and rodents, new insights on autonomic regulation in affective disorders are needed. In particular an important information missing is whether it is possible to restore the high heart rate variability that is generally reduced in anxiety/depression by means of successful drug treatment. Literature provides differences in views of the effects of clinically effect antidepressants: i) further reducing HRV with an efficient behavioral effect (i.e. tricyclic, antidepressant) ii) restoring HRV with an effective behavioral effect. For all these consideration animal models for enhanced anxiety/depression are needed, beside the ethical reasons, animal can be bred, reared, maintained under specific laboratory condition controlling many variables not possible to control in human daily life Thus. It is possible to address the questions mentioned above.

Ideally an animal model should mimic the specific condition in humans regarding disease etiology, symptomatology, treatment and biological basis (McKinney, 2001). As the emotional complexity and cognitive abilities of rodents are much less defined than in humans, three validated criteria for animal model has been developed. The causal conditions (construct validity) and the diagnosed symptoms (face validity) should be similar to those found in humans and pharmacological treatments of the animals should result comparable to those observed in patients (predictive validity).

Achieving all of the three points is difficult, because symptoms such as suicide instinct, which is assessed by verbal report in humans, cannot be assessed in rodents. However, the other key-feature regarding anxiety and depression such as weight loss, loss of appetite, anhedonia and so forth can be mimicked.

One of the strategies of studying affective disorders in rodents is to use the rodents strain with enhanced anxiety- and depression-like behavior. In rats different lines have been described demonstrating increased emotionality including Fischer-344 vs. Wistar (e.g. Sudakov et al., 2001), fawn-Hooded vs. Wistar or Sprague–Dawley rats (e.g. Kantor et al., 2000; Neophytou et al., 2000) and further examples (reviewed in Ramos and Mormede, 1998). In mice, inbred lines as BALB/cByJ mice can be used as model for enhanced anxiety- and depression-like behavior when compared to C57BL/6J, DBA/2, C3 H, CBA (Anisman et al., 2008). However BALB/cByJ mice have a lower mobility in general and therefore, their state anxiety is difficult to interpret in anxiety-related tasks. Another strategy for modeling anxiety is the use of transgenic models by targeting receptor involved in anxiety and depression (i.e. 5-HT1A, CRF2 receptor

or the $GABA_B$ receptor (Holmes et al., 2003; Risbrough et al., 2004; Mombereau et al., 2005) can produce enhanced anxiety- and depression-related behavior in these transgenic mice when compared to control. However, as many of these receptors are not just located in the brain but they are expressed across the body mediating several functions, some behavioral effects as fear response can be driven by peripheral effects rather than a central effects (Busquet et al., 2008). To circumvent this problem new technologies arose as the brain specific silencing of a specific gene with siRNA technique (Kumar et al., 2007) or the so-called conditional knockout where genes can be switch on/off by means of particular compound (Casper et al., 2007)(i.e. Cre-recombinant mice TET ON/OFF systems). However one of the drawbacks relative to the siRNA techniques is the efficacy of transfecting neuron (30-40 % of the neurons can be silenced)(Casper et al., 2007). Moreover, the high viral infections used to carry the siRNA, could induce an inflammatory state resulting into an altered behavioral response.

Animal models for psychiatric illnesses based on chronic stress (chronic restraint stress) have been shown to display an increased anxiety- and depression-like behavior. However, total durations of repeated restraint stress and the evaluation time points used after the last restraint application vary from experiment to experiment. One reason for these methodological heterogeneities is related to considerable ambiguity concerning the definition of chronic stress, particularly in animal models (Kim & Han, 2006).

One of the most effective ways of producing an animal model is to select animals according to their phenotype, resulting in behavioral extremes. Many examples of animal models induced by selected breeding are reported in table 4

Table 1.4. Differential anxiety-like behavior in rodents by selective breeding approaches based on anxiety-related tests and parameters

Model name	Selection criterion	Enhanced anxiety verified in	Reference
Rats			
Maudsley Reactive vs. Nonreactive lines	OFD	OFD, OF, L/D, staircase test, EPM (inconsistent)	Review: (Blizard and Adams, 2002)
Roman low vs. high avoidance	Avoidance (shuttle box)	EPM, OF, L/D, L/D-OF	Review (Steimer and Driscoll, 2003; Steimer and Driscoll, 2005)
		FPS	(Yilmazer-Hanke et al., 2002)
Syracuse low- vs. high avoidance rats	Avoidance (shuttle box)	OFD, CTA, PAV CER, and others	Review (Brush, 2003):
Tsukuba high vs. low emotional line	Runway test	Defecation, OF, I-maze	Review: (Fujita et al., 1994; Kitaoka and Fujita, 1991)
		USV (of pups)	(Naito et al., 2000)
HAB vs. LAB	EPM	EPM, OF, L/D, SD, mod. HB, SI, USV(pups)	Review (Landgraf and Wigger, 2002)
		OA	Salome et al., 2004)
Floripa L vs. Floripa H rat line	Locomotion in the central area of an OF	OF, EPM, L/D	(Ramos et al., 2003)
Infantile high- vs. low USV	USV	USV, EPM (inconsistent)	Review (Brunelli, 2005; Dichter et al., 1996)
		SI, OF, emergence	(Zimmerberg et al., 2005)
Mice			
HAB-M vs. LAB-M	EPM	EPM, OA, USV, L/D	(Kromer et al., 2005)

Table taken from (Singewald, 2007) CER, conditioned suppression; CTA, conditioned taste aversion; EPM, elevated plus maze; FPS, fear-potentiated start test; L/D, light dark test; L/D-OF; light dark test—open field; mod. HB, modified holebord test; OF, open field; OFD, open field defecation; PAV, passive avoidance learning; SD, social defeat; SI, social interaction test; USV, ultrasonic vocalization

1.9 HAB/NAB rodent model for enhanced anxiety- and depression- related behavior

From 1993, three Wistar rat lines were selectively bred for high (HAB) and low (LAB) and normal (NAB) anxiety-related behavior on the EPM at the Max Planck Institute of Psychiatry in Munich (for review, see (Landgraf & Wigger, 2002). These lines are extremely different in their innate anxiety levels as revealed in different behavioral tests in addition to the EPM, including the open field, light/dark, hole board, social interaction and maternal separation-associated neonate ultrasound vocalization tests (Liebsch *et al.*, 1998a; Ohl *et al.*, 2001; Wigger *et al.*, 2001). Since the beginning of 2000 three CD1 mice line were selectively bred to give rise to HAB, NAB and LAB using the same breeding strategy used for the rats model(Liebsch *et al.*, 1998b). As recently shown HAB mice display different neuronal pattern activation and enhanced anxiety-related behavior when compared to NABs and LABs (Muigg et al., 2008). These findings are in line with the rat model (Singewald, 2007), suggesting that this is a reliable model for enhanced emotionality response. Although the behavioral, neurobiological and neuroendocrine parameters in HAB/NAB/LAB have been characterized, the autonomic regulation is still to be investigated. Recent results have shown that the HAB and LAB do not differ in heart rate and body temperaure under basal conditions, but under a particular challenge conditions (new cage, open field) where LABs display an increased HR response compared to HABs (Muigg et al. , unpublished data). This outcome is in contrast to that observed in humans because it is generally reported that patients suffering from

anxiety/depression display an exaggerated HR response. However pharmacological autonomic characterization of normal SD rats have shown that under stress conditions there are both sympathetic and parasympathetic system active leading to the BP and HR responses (Carrive, 2006). However, in humans it is generally observed that under stress conditions there is a parasympathetic withdrawal and sympathetic activation (Grippo & Johnson, 2009). Yet, when different mice strains were characterized no significant differences under basal conditions were observed, while under stress conditions a parasympathetic withdrawal was followed by a sympathetic activation (Swynghedauw et al., 1997; Rowan et al., 2007) reflecting more closely the human situation. Furthermore, as reported previously females have twice the probability to develop an anxiety disorder after a traumatic event in comparison to men. HAB and NAB female mice have been already characterized on their anxiety levels not differing so much from their male counterpart (Landgraf & Wigger, 2002). Yet the number of behavioral experiments with female rodents are limited due to their estrous cycle effects as estrogen can induce a behavioral effect (enhanced arousal) in mild stress (Morgan & Pfaff, 2001). Nevertheless, it would important to know how HAB/NAB male and female mice differ on the basis of the autonomic responses under basal and challenge conditions.

1.10 Test for anxiety in rodents

Various tests to assess anxiety like behavior have been developed in the last decades. There is a main difference among these tests: some are based on a conditional response others on unconditional response. The latter ones are based on the natural exploratory behavior of the rodents and its inhibition for the new aversive environment, thus inducing a compromise between approach and escape.

Table 1.5. Commonly used tests of anxiety adapted from (Rodgers, 1997; Millan, 2003; Fuchs & Flügge, 2004)

Models based on unconditioned responses	**Models based on conditioned responses**
Models based on exploration	***Conflict tests***
Elevated plus maze	Geller-Seifter
Free exploration test	Vogel
Light/dark test	Pigeons and monkeys conflict
Open-field test	Conditioned suppression
Hole board	Safety-signal withdrawal
Zero-maze	Conditioned place aversion
Novelty suppressed feedin	
Models based on social behavior	***Miscellaneous***
Social interaction test	Active/passive avoidance
Social competition	Conditioned emotional response
Human threat	Conditioned taste aversion
Ultrasonic distress vocalisation	Fear-potentiated startle reflex
	Shock-probe burying test
Stress-induced modification of behavioral or physiological responses	
Consummatory behavior	
Thermic response	
Corticostrone response	
Defecation/micturition	
Miscellaneous	
Anxiety/fear test battery	
Marble burying	

The best example of test based on conditioned responses is Pavlovian fear conditioning, which is commonly utilized to study the neural basis of memory, learning and emotional cognition (Fendt & Fanselow, 1999). In this paradigm, fear-

related behaviors are induced by exposure to a neutral stimulus (CS: conditioned stimulus; e.g. tone, light) which is paired (single or multiple) with an innately aversive stimulus (US: unconditioned stimulus; e.g. foot shock, air jet). After a number of pairings the neutral stimulus becomes a conditioned stimulus sufficient enough to elicit fear responses by itself. Fear-related behavior quanfied as percentage of time spent at freezing in mice can be defined as a complete suppression of spontaneous locomotor activity, and of all movements except those needed for respiration (Fanselow, 1980). An increasing body of evidence suggests that conditioned fear responses in rodents involve neural mechanisms and pathways implicated in human depression and anxiety (Anderson & Insel, 2006), including amygdala, hippocampus and prefrontal cortex. However, scoring freezing in rodents can be subjective to single observer (Nielsen & Crnic, 2002).Thus, the assessment of autonomic response and freezing behavior can generate a more integrate view of neural processing of emotional stimuli as there is a close connection of neuro-cardiac function in cognitive/emotional responses.

Through the study of the dynamic range of HR versus its variability it is possible to gain more information regarding affective/emotional state of the brain in animal model.

In this respect, gender specific autonomic regulation can be studied in fear conditioning as conditioned animals can be left in their home cage and by exposure to the CS only (Stiedl & Spiess, 1997), freezing behavior and autonomic responses can be assessed without effect of autonomic artifacts induced by manipulating the animals presented in other tests (Van Bogaert et al., 2006).

1.11 Test for depression in rodents

As mentioned above, the main feature of depression cannot be measure in rodents (suicidal intention or feeling of worthlessness). Nevertheless loss of appetite, body weight loss, anhedonic behavior and so forth can be easily assessed. Among the various test for depression listed in the table (for review see Cryan, 2007) one of the most used is the Tail Suspension Test (TST). In this test after initial escape oriented movements animals developed an immobile posture when placed in an inescapable stressful situation. In contrast in the forced swim test rodents after an initial escape-oriented strategy (struggling and swimming) develop and immobile posture when placed in an inescapable cylinder filled with water. Immobility (form of passive stress coping), is considered to be a symptom of “behavioral despair” and is generally

described as an indicator of depression-like behavior (Porsolt et al., 1977). Both tests have been validated using a variety of antidepressant drugs which restore the activity of the animals. However, the TST has several advantages in comparison to the Forced swim test for autonomic measurement i) the apparatus for TST can be placed close to the receiver while in the FST water reduces the transmission of the radio-telemetry device to its receiver ii) the weight of the transmitter (3.2g) placed intraperitoneally might sink the rodent in water.

1.12 Automated system of scoring freezing behavior

Freezing behavior is easily quantified, for instance by human observers using a stopwatch (Phillips and Ledoux, 1994) or time sampling (e.g. Fanselow, 1980; Westbrook et al., 1997). With time sampling, several animals may be simultaneously monitored, but this still involves lengthy observation time and the possibility of observer biases (subjectivity, fatigue, etc.). Several automated techniques have been recently developed to save experimenter time and increase reproducibility (Marchand et al., 2003; Kopec et al., 2007). However, even if automated system can give some cost- time benefits, it is important to take into account then sensitivity of the automated system. Indeed, in (Marchand et al., 2003) automated system the two automated scoring methods (SUB vs. RAW) produced different results and made different types of errors, classifying some bouts of walking, rearing, sniffing, grooming and moving behavior as “freezing”(Brown, 2008). Thereby there is a need in the fear conditioning field of development of a time saving, reliable and costless methods for scoring freezing behavior.

1.13 Specific aims of this thesis

The main aim of the present study was to investigate the complex relationship between anxiety- and depression-related behavior to autonomic regulation, also in relation to possible gender differences, in a selectively bred HAB and NAB mice. By investigating autonomic parameters (e.g. body temperature, heart rate and heart rate variability) under resting conditions and in anxiety/fear/depression paradigms we wanted to investigate the use of autonomic parameters as additional and reliable markers for evaluation of successful drug treatment in anxiety and depression research. In parallel to this research line, novel methods of scoring fear behavior were evaluated. Specifically, the following aims were addressed:

1) To investigate how body temperature, heart rate and heart rate variability are regulated under resting conditions (home cage) in HAB and NAB mice (both male and female)

2) To monitor the autonomic parameters in response to various unconditioned anxiety provoking challenges in male HAB and NAB mice

3) To reveal differences autonomic regulation in response to fear conditioning in both male and female HAB and NAB mice

4) To assess the response of autonomic parameters to a successful anxiolytic and antidepressant treatment in HAB mice.

5) To develop fast, sensitive and automated systems for scoring freezing behavior

2 Methods

2.1 Animals

Male and HAB and NAB female mice tested were bred in the animal facilities of the Max Plank Institute of Psychiatry in Munich, as described previously (Landgraf and Wigger 2002; Kromer et al 2005). In brief, CD1 mice were selected and mated according to the results of an elevated plus maze test, to establish the lines termed HAB and NAB. Mice that spend less than 5% or more than 20% in the open arms are considered as HABs and NABs, respectively. All offsprings (including those used for the present thesis) are tested routinely at an age of 7 weeks in Munich to ensure assignment to the HAB or NAB line, respectively. All experiments were approved by the local Ethical Committee on Animal Care and Use (Bundesministerium für Wissenschaft und Verkehr, Kommission für Tierversuchsangelegenheiten, Austria) and were in compliance with international laws and policies.

2.2 Treatment

The NK1 receptor antagonist L822429 ([2-cyclopropoxy-5-(5-(trifluoromethyl) tetrazol-1-yl) benzyl] - (2-phenylpiperidin-3-yl)amine) (Singewald et al., 2008) was dissolved in water and was given at a dose of 30mg/kg in drinking water for 24 days (HAB female drug n=8, HAB control n=8) .

2.3 Surgery

Mice were individually separated 24 hours before the onset of the surgery. All biopotential transmitters were implanted under sodium pentobarbital (40 mg/kg i.p.) and ketamine (50 mg/kg i.p.) anaesthesia. The TA10ETA-F-20 transmitters (Data Sciences International, St. Paul, MN,USA), which are made of a device body (electronics module battery) with two flexible leads extending from it, were implanted in HAB and NAB mice into the peritoneal cavity through a subxyphoid incision. The two biopotential leads exit the peritoneal cavity through stab incisions lateral to a midline incision and travel subcutaneously to the desired placement site. For proper

ECG signals, the negative lead was placed in the area of the right shoulder and the positive lead immediately to the left of the xyphoid space and caudal to the ribcage (as recommended, see device surgical manual, Datasciences) Postoperatively, mice were treated with a one-time injection of gentamycine (5 mg/kg i.p.) and buprenorphine (0.1 mg/kg i.m.) for 2 days. Following implantation animals were kept single housed and allowed to recover 14-21 days, before the testing procedures started.

2.4 Radio telemetry

The radio-telemetry system is composed of the earlier mentioned transmitter, measuring body temprerature and locomotor activity, with two flexible leads which measure ECG/HR, a telemetry receiver (model RLA 1020), a data exchange matrix collecting input from receivers, all connected to a computer running Dataquest Art version 4.1. The transmitters are equipped with magnetically activated switches, to turn on/off the device. All equipment and software were obtained from (Data Sciences International, St. Paul, MN, USA).

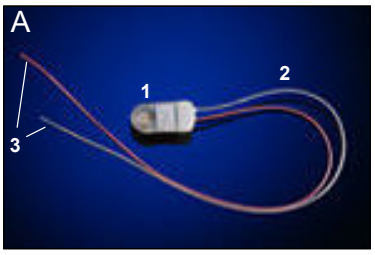


Figure 2.1 Photographs of the biocompatible transmitters. A) TA10ETA-F201) device body (electronic module, battery), 2) biopotential leads (silicone tubing), 3) helix of wire.

2.5 Behavioral testing

24 hours before the onset of the experiments, animals were taken in their home cages to the experimental room and allowed to habituate. All experiments were carried out between 9:00 and 12:00. HAB and NAB mice HR, BT and activity were monitored before (pre-challenge in home cage), during and after the challenge (post-challenge in home cage) For anxiety and depression tests the HR, BT and activity were averaged on 5 minutes intervals. For fear conditioning HR, BT and activity were averaged on 30 seconds intervals. HR was expressed as in beats per minute, body temperature in Celsius and locomotor activity as number of movements per minute

Anxiety tests

Novel cage

To investigate the cardiovascular effects on novelty, animals were individually placed in an odourless, empty mouse cage (22x37.5x15 cm) for 20 min. The illumination at floor level was 100 lux. Immediately after the novelty exposure animals were returned to their familiar home cages. The novel cage was cleaned with water between each test session.

Open field

Mice were placed the animals in the open field for 30 min, consisting of a plastic box (41cm × 41 cm × 41 cm). The illumination was 150 lux. Immediately after open field exposure animals were returned to their home cages. The open field arena was cleaned with water between each test session.

Handling stress

Handling consisted of removing mice from the home cage and placing them directly on the flat receiver. There, mice were kept in motion with the experimenters hands for 30 seconds. The illumination was 100 lux.

Elevated plus maze

The elevated plus maze (EPM) test was performed as described previously(Tschenett et al., 2003). It consisted of two opposing open and two opposing closed arms of same size (30 × 5 × 15 cm) which extended from a central platform (5 × 5 cm). The closed arms were made of nontransparent walls (14 cm height). The apparatus was elevated 73 cm above the floor and exposed to the illumination was 100 lux. At the beginning of each trial, a mouse was placed onto the central area of the maze, facing a closed arm. Mice were tested for a period of 5 min and their movements on the maze were tracked and subsequently analyzed by the TSE VideoMot2 system (TSE Technical & Scientific Equipment GmbH – Bad Homburg, Germany). Mice were followed by the throughout the experiments with ECG receiver to monitor HR and BT. In addition to

the conventional parameter (time spent in the open arms), the number of head dips and stretched-attend postures (SAPs) (Blanchard & Blanchard, 1989; Blanchard *et al.*, 1990; Rodgers *et al.*, 1992) were assessed.

Stress induced hyperthermia

The test procedure of stress-induced hyperthermia (SIH) was performed as described previously (Van der Heyden et al., 1997). Rectal temperature was measured in each mouse twice, i.e. at t = 0 min (T1) and 10 min later (T2). After the first measurement of temperature the mouse was reallocated to its cage. The difference in temperature (T2-T1) is considered as SIH. Rectal temperature was measured with an accuracy of 0.1°C using a digital thermometer DM 852 (Ellab, Copenhagen, Denmark) by inserting a glycerol lubricated thermistor probe into the rectum till a stable temperature was measured for 20 s.

Light/Dark test

Mice were subjected to the light/dark test as previously described(Singewald et al., 2004). The fully automated light/dark test apparatus consisted of a top-open square box separated into a brightly illuminated white (20.5x 9.41x 9.41 cm high, 400 lux) compartment and a covered black compartment (20.5x 9.41x 9.41 cm high, 10 lux) (Tru Scan, Coulbourn Instruments, Allentown, USA). The compartments were connected by a small opening (7 9 7 cm wide) located in the centre of the partition at floor level. Animals were individually placed into the dark compartment facing away from the opening and allowed to freely explore the apparatus for 10 min. Behaviour of each mouse was tracked by the computer-assisted scanning system. The following parameters were quantified: (1) latency to the first entry into the lit compartment, (2) time spent in the lit compartment, (3) number of shuttle crossings between the two compartments (entries into the lit arena), (4) number of rearings and (5) the overall distance travelled by the mice.

Depression test

Tail suspension test

The tail suspension test (TST) was carried out essentially as described previously (Steru et al., 1985). Mice were individually suspended by securely fastening them with medical adhesive tape by the tip (approximately 1.0-1.5 cm) of the tail to a steel bar placed horizontally at the height of 30 cm above a table. The illumination on the floor of the table was about 100 lux. The activity of the mice was videotaped and immobility, defined as when the animal hung passively without limb movement, was subsequently scored using Eventlog 1.0 (EMCO Software) over a 6 min test session by a trained observer blind to the genotype of the mice.

Cued Fear conditioning

Air jet as unconditioned stimulus

For acquisition, mice were placed in the conditioning box (context A). Fear acquisition was elicited by presenting audible cues (CS: light, 600 lux, 2 min) that co-terminated with the US (pressured air, 30 sec). Two minute stimulus-free periods preceded, separated, and followed the pairings. Extinction was carried out in context B after 24 h of memory consolidation. Mice were placed in a new cage without saw dust (context B), habituated for 2 min and were subsequently presented 15 CS presentations with an intertrial interval of 5 sec.

Extinction in Home cage

For acquisition, mice were placed in the conditioning box (context A). Fear acquisition was elicited by presenting audible cues (CS: sound, 80 db, 2 min) that co-terminated with the US (footschock, 0.7 mA, 2 sec). Two minute stimulus-free periods preceded, separated, and followed the pairings. Extinction was carried out in their home cage after 24 h of memory consolidation. Mice in their home cage (context B) were subsequently presented to 15 CS presentations with an intertrial interval of 5 sec.

Classical fear Conditioning

For acquisition, mice were placed in the conditioning box (context A). Fear acquisition was elicited by presenting audible cues (CS: sound, 80 db, 2 min) that co-terminated with the US (footschock, 0.7 mA, 2 sec). Two minute stimulus-free periods preceded, separated, and followed the pairings. Extinction was carried out in context B after 24 h of memory consolidation. Mice were placed in a new cage without saw dust (context B), habituated for 2 min and were subsequently presented 15 CS presentations with an intertrial interval of 5 sec.

2.6 Heart Rate Variability

Telemetry recordings of ECG were done using Dataquest A.R.T. 4.0 software (Data Sciences International, St. Paul, MN). Data of ECG were then imported and analyzed with Chart v5.2.2 and the HRV Module v1.0 (ADInstruments, Colorado Springs, CO). Heart rate variability was determined from 30-sec segments of ECG data, recorded at 1 kHz. The segments chosen for analysis were based on the absence of movement and arrhythmia artifacts. Around 10% of the total beats were arrhythmic beats (beats not initiated in the atria, defined as artifacts). Then, after visual inspection of ECG, these artifacts were manually removed.

Frequency domain analysis

For frequency domain analysis of HRV Fast Fourier transformation (FFT) of the same 30 sec- segments of ECG data was performed after removal of linear trend and application of Welch window with an FFT setting $n = 1024$ points with 50% overlap. Spectral power was quantified within the following frequency bands: very low frequency (VLF) power 0 to 0.04 Hz; low frequency power (LF), 0.04-0.15 Hz; and high frequency power (HF), 0.015 to 4.00 Hz.

Time domain analysis

In the time domain analysis HR variability (HRV) was determined by the square root of the mean of the sum of successive NN interval differences (RMSSD) over 30 sec-segments of ECG during extinction. Unlike conventional statistical time-domain measures of the variability of a given time series such as variance, the analysis of RMSSD is based on time increments between consecutive beats and has been demonstrated to converge rapidly to stable values in the presence of nonlinearity and/or nonstationarity of the data stream (Stiedl & Meyer, 2003)

2.7 Automated system of scoring freezing behavior

Comparison of scoring freezing behavior between topowatch, a commercially available software called TSE and manual (observer) evaluation were done on videos of mice during extinction training.

Specifically, a group termed CS+ (n=4) that underwent classical conditioning (CS paired to US) and mice CS- group (n=4) that did not undergo to classical conditioning (CS only) were compared.

Videos were first converted from VOB (DVD format) to mpeg 1 or mpeg 2 format and fed into topowatch. After opening topowatch three parameters (represented with scrolling bars) were set:

1) *Background .to mouse threshold*: this parameter indicates what is the difference, in pixels, between the color of the background and the color of the fur of the mouse.

2) *Noise level*: this parameter indicates the background noise that can be generated in the recording of the video. The higher is the video quality the lower will be this value.

3) *Freezing threshold*: this variable indicates the differences in pixels from one frame to another one. Considering that standard quality videos have a frame speed of 30 frames/second speed, the algorithm calculates the intervals time spent at freezing of the mouse object of evaluation.

Lastly, the color of the fur of the mouse was set in order to facilitate the software the recognition of the mouse.

To test the accuracy of the software, correlation analyses in scoring freezing behavior between manual-topowatch and manual-TSE were performed and Pearson correlation coefficient r was given.

Lastly, an error index of sensitivity of the software, based on the difference in the standard deviation of manual to topowatch freezing evaluation and manual to TSE manual evaluation, was calculated

2.8 Statistical analysis

Data are represented as mean ± SEM. Numbers of animals per group are given in figure legends. Statistical analysis of heart rate, body temperature, activity and behavior of tests dependent on time was performed using repeated measures ANOVA following post-hoc Bonferroni´s test where appropriate. Statistical analysis of the light dark, elevated plus maze, stress-induced hyperthermia, tail suspension and grouped heart rate variability were done using unpaired t-test was used for comparisons between two groups. When more than two groups were compared, statistical analysis was performed using ANOVA followed by post-hoc Bonferroni´s P levels < 0.05 were considered statistically significant.

3 Results

3.1 No differences in baseline heart rate, body temperature and heart rate variability between female and male HAB and NAB mice

Baseline HR and BT did not differ between both male and female HAB and NAB mice ($p>0.05$) (Figure 3.1) over 60 hours of recordings. As for HR and BT, frequency domain analysis of basal HRV did not reveal any difference between both male and female HAB and NAB mice ($p>0.05$) (Figure 3.2).

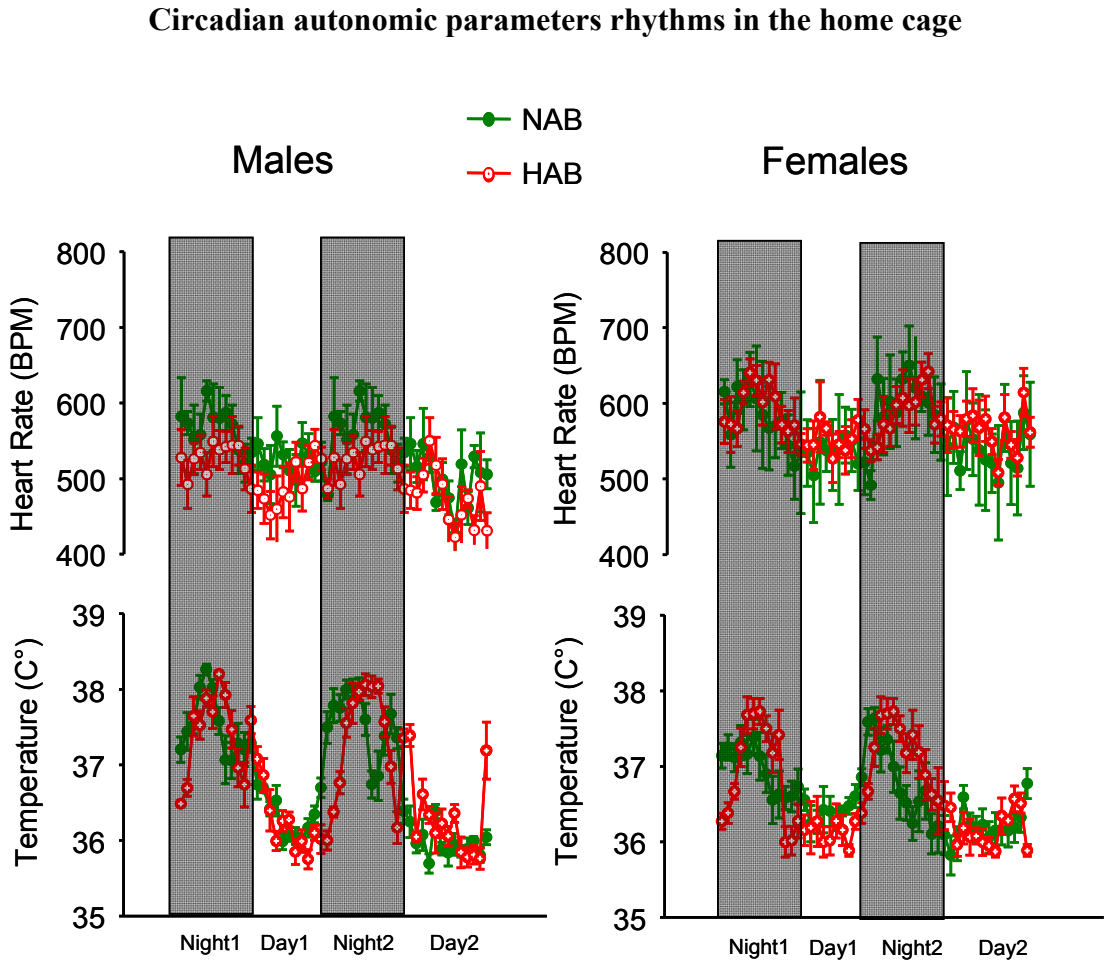


Figure 3.1 No difference in cardiovascular parameters of male and female HAB and NAB mice. Basal circadian measurement of heart rate (upper panel) and body temperature (lower panel). Dark shading represents lights off. HAB mice (red open circles) n=8, NAB mice (green filled circles) n=8.Data are means ± SEM.

Heart Rate Variability Frequency Domain during day (Home Cage)

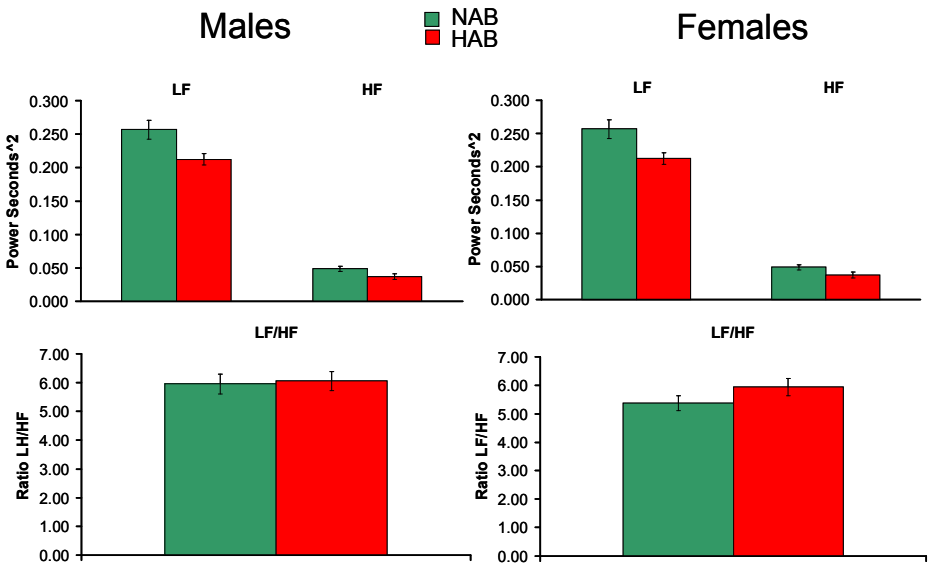


Figure 3.2 Frequency domain analysis of HRV in HAB and NAB male and female. In the upper panel LF (low frequency), HF (high frequency) and in the lower panel the ratio of LF/HF are measured over 30 seconds intervals in the day period. HAB mice (red rectangles) n=8, NAB mice (green rectangles) n=8.Data are means ± SEM

3.2 Similar autonomic responses to unconditioned stressors in male HABs and NABs

Successive exposure to Novel Cage and Open Field elicited a similar marked increase in HR, BT and activity followed by a gradual decrease in both, HAB and NAB mice. However, in none of these tests there were not significant differences between the two lines in HR, BT and activity ($p>0.05$) (Figure 3.3).

To test whether the increase in HR and BT responses were not just related to increased locomotor activity, handling exposure, a test where exploratory behavior is not affected, was investigated. Indeed, after handling the animals, there was a smaller ($p<0.05$) rise in HR and BT in both HABs and NABs compare to open field and novel cage challenge, while the activity was comparable for both lines to the other tests ($p>0.05$).

Locomotor and autonomic parameters in male HAB and NAB mice during unconditioned stressors

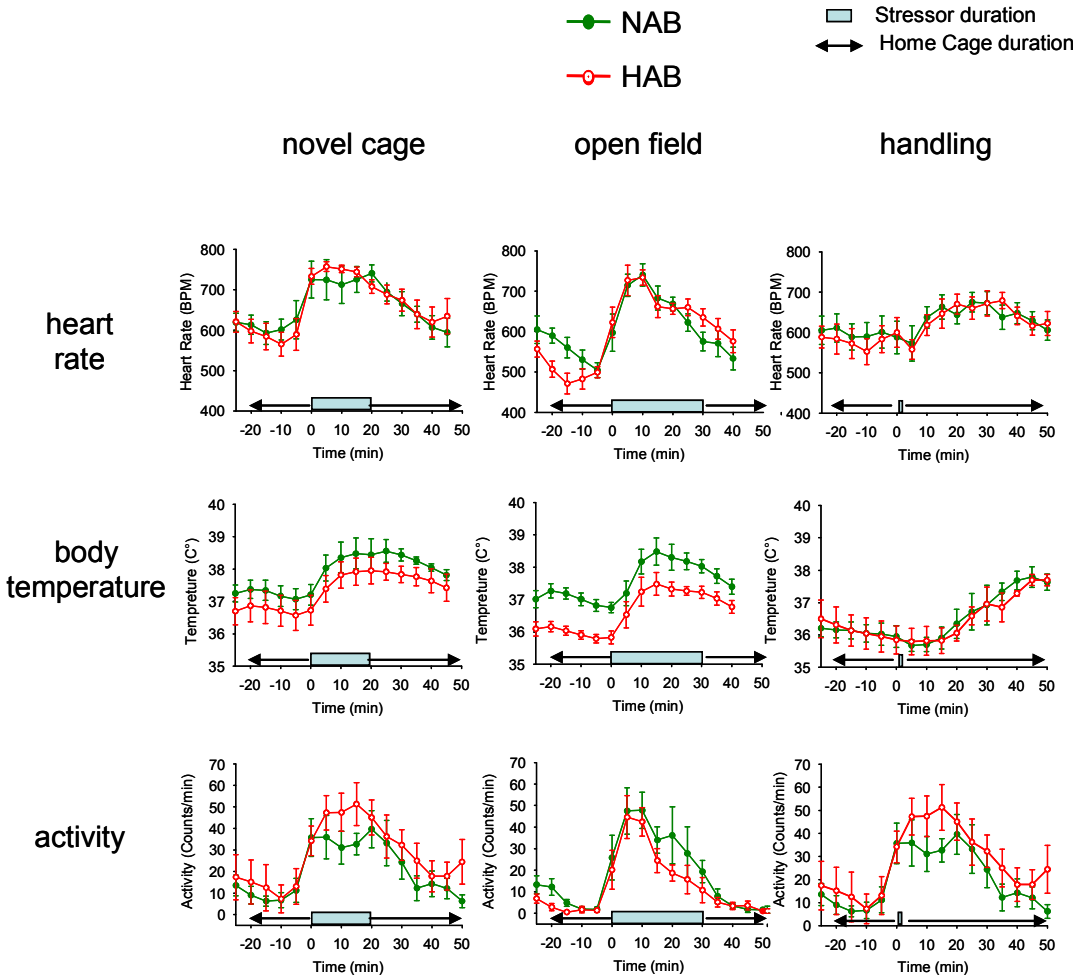


Figure 3.3 Effect of unconditioned stimuli on cardiovascular and activity parameters in HAB and NAB male mice. Time course changes in heart rate (upper panel), body temperature (middle panel) and activity (lower panel) induced by exposure to novel cage exposure (20 min), open field exposure (30 minutes) and handling (0.5 minutes). Blue rectangles represent the duration of each challenge and arrows represent the duration of pre and post stress in home cage. HAB mice (red open circles) n=8, NAB mice (green filled circles) n=8.Data are means ± SEM.

3.3 Impaired extinction accompany the reduced heart rate variability in female HAB mice with air jet paradigm.

When exposed to fear acquisition, both HAB and NAB male mice, did not display any sign of freezing behavior (Figure 3.4). No differences in HR or BT were observed ($p>0.05$). As both lines did not display freezing behavior on acquisition day, there were no statistical differences between HABs and NABs in freezing behavior in the extinction phase as well as no differences in HR or BT were observed ($p>0.05$) (Figure 3.5).

Autonomic and behavioral responses of HAB and NAB mice during cued fear conditioning

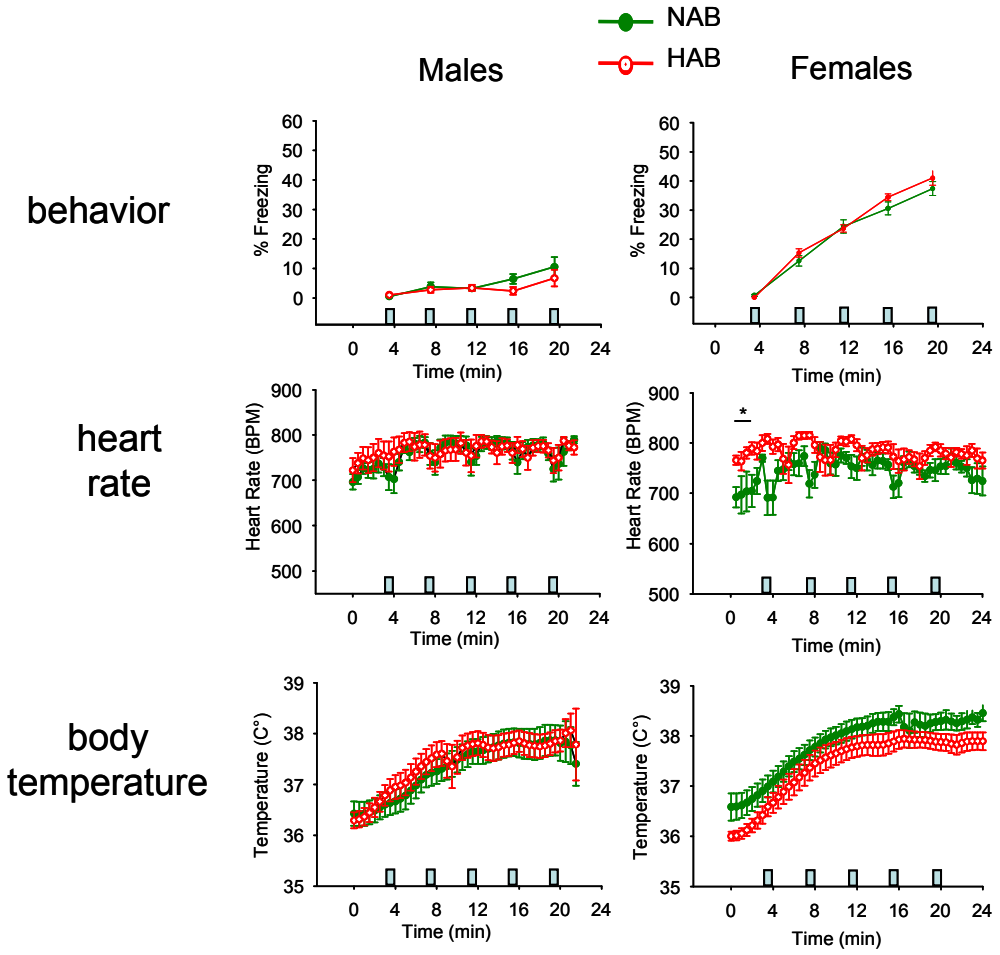


Figure 3.4 Autonomic and behavioral readouts of HAB and NAB male and female mice in fear conditioning Time course of Behavior (upper panel), heart rate (middle panel) and body temperature (lower panel) induced by exposure to fear conditioning . Blue rectangles represent 5 CS-US pairings (CS=light, US=pressured air). HAB mice (red open circles) n=8, NAB mice (green filled circles) n=8.Data are means ± SEM, * $p<0.05$, ** $p<0.01$ where statistical difference was reached.

In contrast, HAB and NAB female mice, displayed acquisition of conditioned fear and difference in initial higher HR for HAB mice but not BT were observed compared to NAB mice (Figure 3.4). In the extinction phase, HAB female mice showed impaired extinction accompanied with initial higher HR levels but not BT compared to NAB mice (Figure 3.5).

Autonomic and behavioral responses of HAB and NAB mice during extinction phase of fear cued conditioning

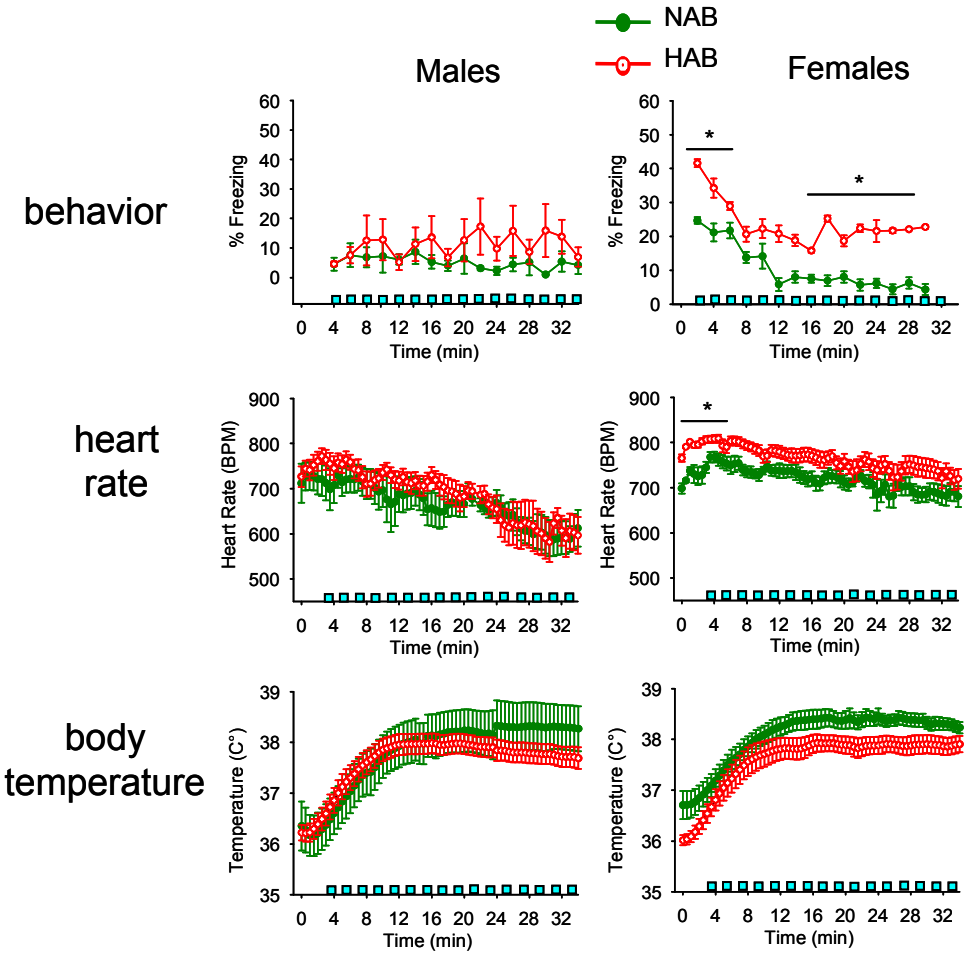


Figure 3.5 Autonomic and behavioral readouts of HAB and NAB male and female mice in extinction of fear conditioning Time course of Behavior (upper panel), heart rate (middle panel) and body temperature (lower panel) induced by exposure to repeated CS (blue rectangles) presentation in extinction of fear conditioning (CS=light). HAB mice (red open circles) n=8, NAB mice (green filled circles) n=8.Data are means ± SEM, * $p<0.05$, ** $p<0.01$ where statistical difference was reached.

As no basal HRV were observed different for both male and female HAB and NAB mice, frequency and time domain analysis of HRV were conducted in females HAB and NAB mice where a statistical behavioral difference in fear extinction was observed.

Frequency domain analysis in extinction phase indicated a tendency (p=0.053) to a reduced HF influence (parasympathetic) in HAB mice compared NAB mice and increased LF/HF ratio (sympathetic) in HAB mice (Figure 3.6).

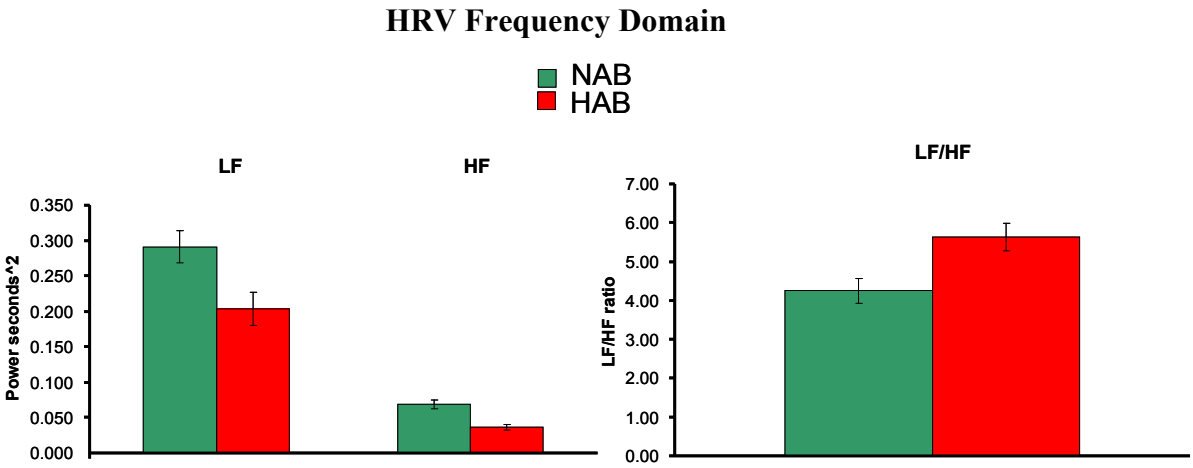


Figure 3.6. Frequency domain analysis of HRV in HAB and NAB female mice in extinction phase. In the part left of panel l LF and HF and in the right part of the panel the ratio of LF/HF are measured over 30 seconds intervals in the extinction phase. HAB mice (red rectangles) n=8, NAB mice (green rectangles) n=8.Data are means ± SEM

Indeed, the study of the time domain of HR against HRV confirmed the reduced HRV in extinction phase only (stress) ($p<0.05$) (Figure 3.7) in HAB mice compared to NAB mice giving indication of reduced parasympathetic control and increased sympathetic tone.

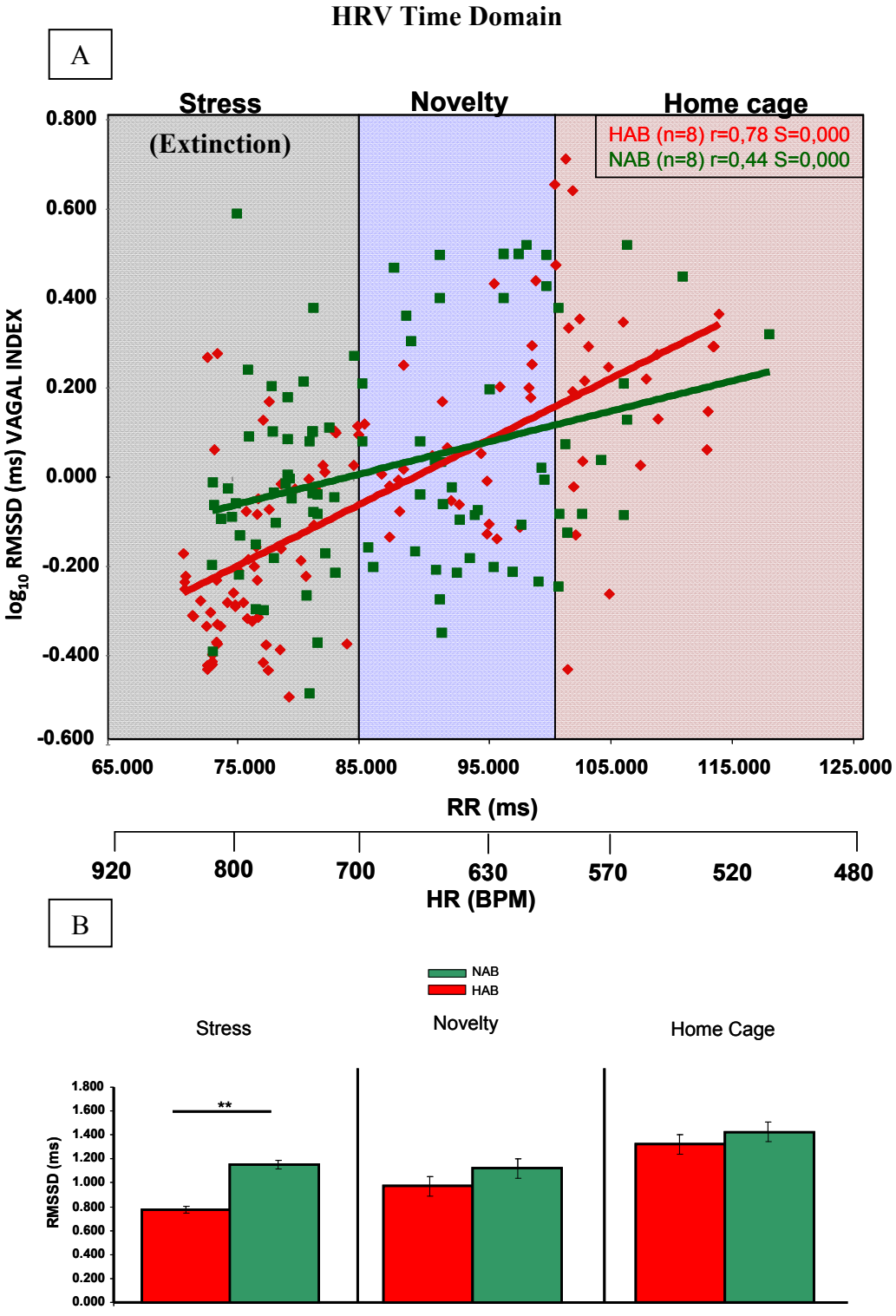


Figure 3.7. Time domain analysis of HRV (log_{10} RMSSD) versus HR (BPM) in HAB and NAB female mice during extinction. **A,** The graph represents correlation analysis of all the dynamic range of heart rate values (BPM) versus HRV (log_{10} RMSSD) from home cage (basal, right, pink shaded area), novelty (mice in a new environment, middle, t blue shaded area) to stress condition (extinction, left, gray shaded area) Correlation analysis showed higher degree of correlation coefficient (r) in HAB compared to NAB mice. **B**, Grouped HRV (pooled individual HRV data is depicted in bar graphs) corresponding to home cage, novelty and stress (right to left) indicated that in stress situation there was a reduced HRV HAB mice compared to NAB mice. HAB mice (red diamonds, red correlation line and red bar graphs) n=8, NAB mice (green squares, green correlation line and green bar graphs) n=8. .Data are means ± SEM, * $p<0.05$, **$p<0.01$ where statistical difference was reached.

3.4 Confirmation of reduced heart rate variability in HAB female mice by means of different fear conditioning paradigms

To reduce the impact of handling on the autonomic parameters measured (increased in HR and BT) in HAB and NAB female, mice were tested during extinction phase in their home cage. During acquisition phase (conditioning phase), there was slight increase in HR, BT ($p>0.05$) and significantly reduced locomotor activity in HAB mice.(Figure 3.8).

In extinction phase in their home cage, HAB and NAB female shown a reduced locomotor activity compared to home cage ($p<0.05$), but not differences in HR and BT were observed ($p>0.05$).(Figure 3.8). However, time domain analysis of HR vs. its variability in extinction phase confirmed the reduced HRV in HABs compared to NABs. (Figure 3.9) ($p<0.05$).

Fear conditioning using home cage for extinction phase

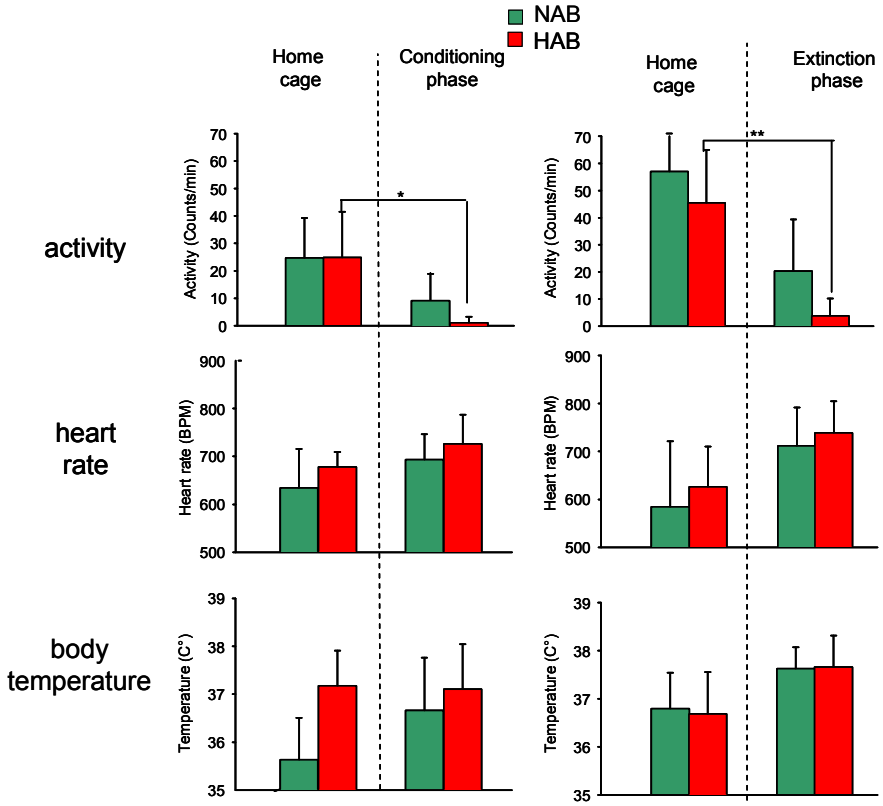
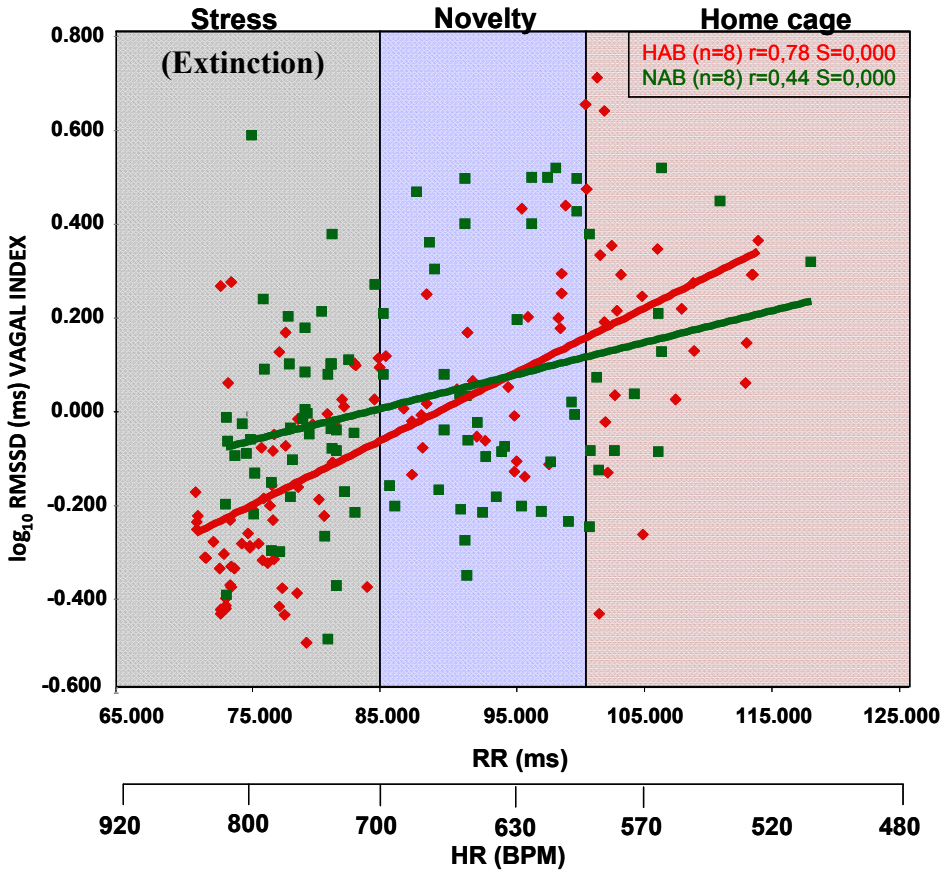


Figure 3.8 Representation of averaged autonomic response and locomotor activity during home cage, conditioning phase and extinction in HAB and NAB female mice over 32 minutes of recordings. Mice were conditioned with classical protocol (5 CS-US presentations) , but extinction was conducted in the home cage (15 CS presentations). In the left panel, activity (upper panel), heart rate (middle panel) and body temperature (lower panel) induced by exposure to repeated CS-US in the fear conditioning day (CS=sound, US=footshock). In the right panel, the same locomotor and autonomic readouts measured in repeated exposure to CS in extinction day (CS=sound). HAB mice (red bars) n=8, NAB mice (green bars) n=8.Data are means ± SEM, * $p<0.05$, ** $p<0.01$ where statistical difference was reached

A



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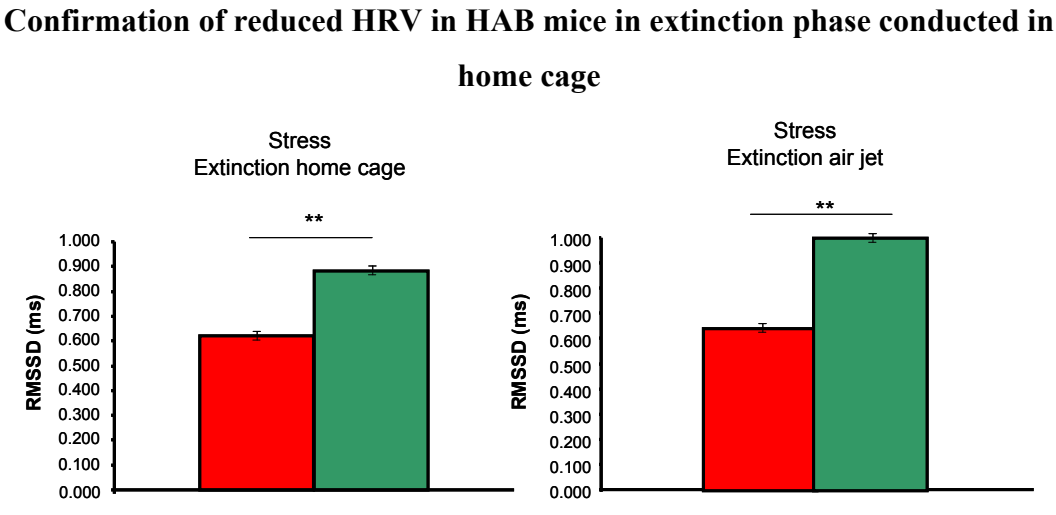

Figure 3.9. Time domain analysis of HRV (log_{10} RMSSD) versus HR (BPM) in HAB and NAB female mice during extinction in their home cage. **A,** the graph represents correlation analysis of all the dynamic range of heart rate values (BPM) versus HRV (log_{10} RMSSD) from home cage (basal, right, pink shaded area), novelty (mice in a new environment, middle, t blue shaded area) to stress condition (extinction, left, gray shaded area) Correlation analysis showed higher degree of correlation coefficient (r) in HAB compared to NAB mice. **B**, comparisons of grouped HRV (pooled individual HRV data is depicted in bar graphs) corresponding to stress (extinction in home cage) and stress (extinction in air jet) (right to left) This analysis indicated that in both stress conditions there was a reduced HRV in HAB mice compared to NAB mice. HAB mice (red diamonds, red correlation line and red bar graphs) n=8, NAB mice (green squares, green correlation line and green bar graphs) n=8. .Data are means ± SEM, * $p<0.05$, ** $p<0.01$ where statistical difference was reached.

3.5 Reversed HRV accompanied by anxiolytic and antidepressant effects of chronic L822429 (NK1 receptor antagonist) in female HAB mice.

No effect under in basal autonomic parameters

Chronic application NK1 receptor antagonist did not affect basal circadian HR and BT rythmicity in HAB female mice (Figure 3.10).

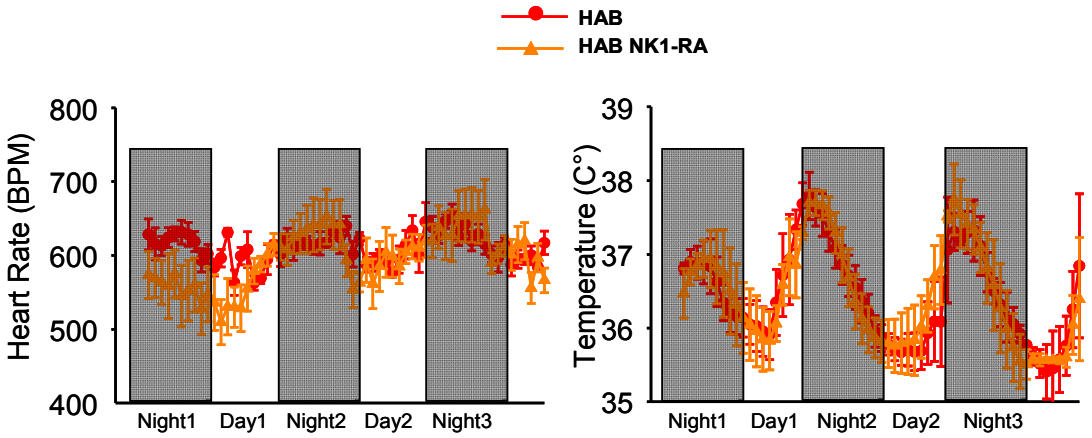

Figure 3.10. Cardiovascular characterizations of female HAB NK1 antagonist treated and HAB control mice. Basal circadian measurement of heart rate (left side of the panel) and body temperature (right side of panel). Dark shadings represent lights off. HAB treated mice (red closed circles) n=8, HAB control mice (orange filled triangles) n=8.Data are means ± SEM.

Chronic NK1 receptor antagonist in anxiety tests

In stressed induced hyperthermia test (SIH), chronic NK1 antagonist did not showe any effect on basal and stress induced increased BT measured with both telemetry device and rectal probe of SIH in HAB treated and HAB control ($p>0.05$) (Figure 3.11). Interestingly, there was a an increased in basal temperature and reduced stress induced BT difference measured with telemetry device in contrast to SIH probe in both treated and untreated HAB mice ($p<0.05$) (Figure 3.11).

Stress induced hyperthermia

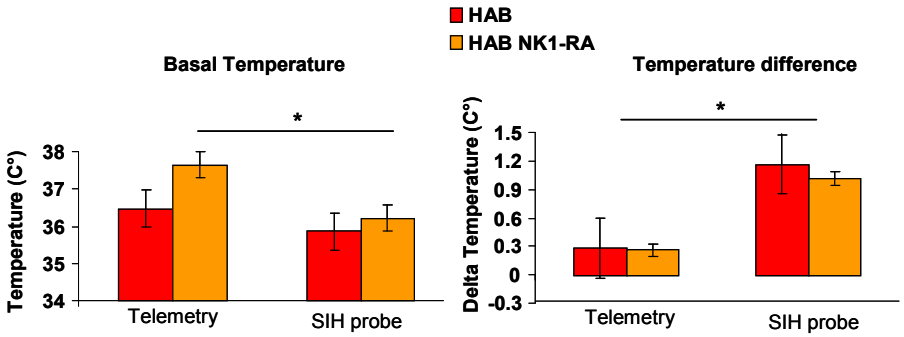

Figure 3.11. Core body temperature measurement with telemetry device and stress induce hypethermia test in HAB NK1 antagonist treated and HAB control mice. On the left panel basal core temperature measured at time (T1=0 min) and on the right panel the difference in core body temperature given at (T2=10 min). From which Delta T (T1-T2). In red bars HAB control n=8, and in orange bar HAB NK1 treated n=8. Data are means ± SEM, * p<0.05, **p<0.01 where statistical difference was reached.

Furthermore, chronic treated HAB mice showed anxiolytic behavior in the Light Dark test, significance differences were observed in time spent in the lit compartment and latency to lit compartment when compared to HAB control mice ($p<0.05$) (Figure 3.12). However, in light dark test, no autonomic measurements were feasible because of thee set-up of this test.

Light Dark Test

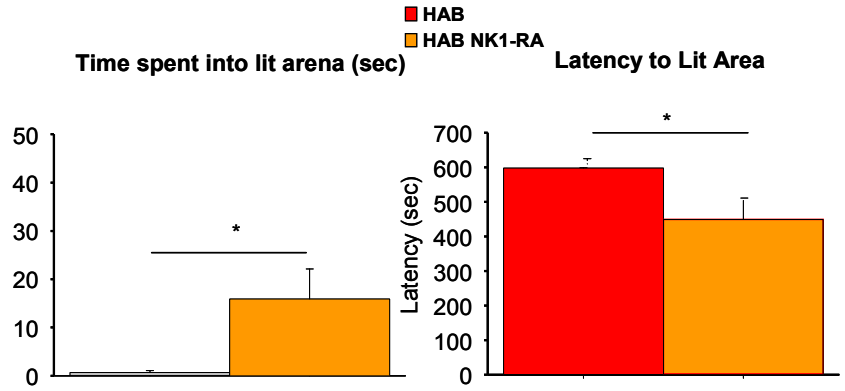

Figure 3.12. Anxiolytic effect of NK1receptor antagonist in light dark test. As anxiety readout time spent into lit arena (left panel) and latency to lit arena (right panel) were taken. In red bars HAB control n=8, and in orange bar HAB NK1 treated n=8. Data are means ± SEM, * p<0.05, **p<0.01 where statistical difference was reached.

In addition, on the elevated plus maze a markedly reduced HR ($p<0.05$) was observed in NK1 receptor treated HAB mice, but not anxiolytic was observed or BT effects were observed ($p>0.05$) (Figure 3.13).

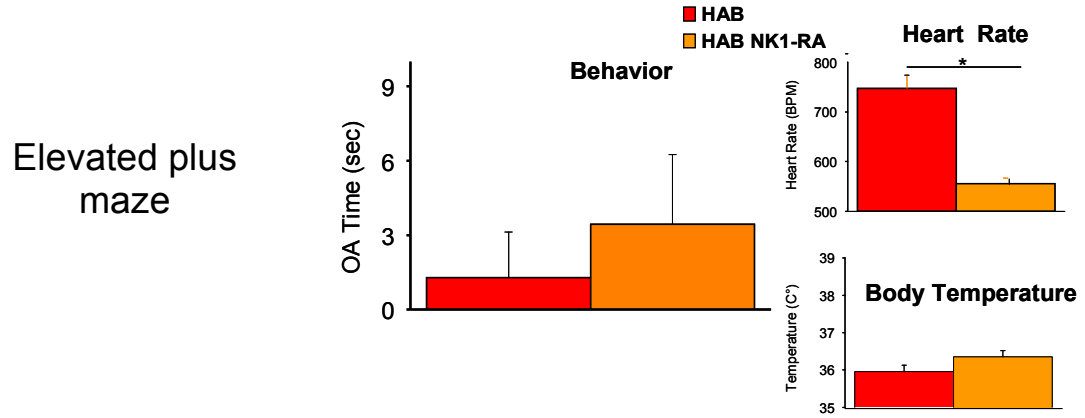

Figure 3.13. Non significant anxiolytic effect of NK1receptor antagonist in elevated plus maze. Open arm time (anxiety redaout) on the left panel, on the right panel the autonomic readout (heart rate and body temperature were quantified. In red bars HAB control n=8, and in orange bar HAB NK1 treated n=8. Data are means ± SEM, * p<0.05, **p<0.01 where statistical difference was reached.

Antidepressant effect of chronic NK1 receptor antagonist in tail suspension test

An antidepressant effect was observed in tail suspension test (TST) where treated HAB mice displayed decraesed immobility, while there were no effects on stress induced HR or BT compared to HAB control (Figure 3.14).

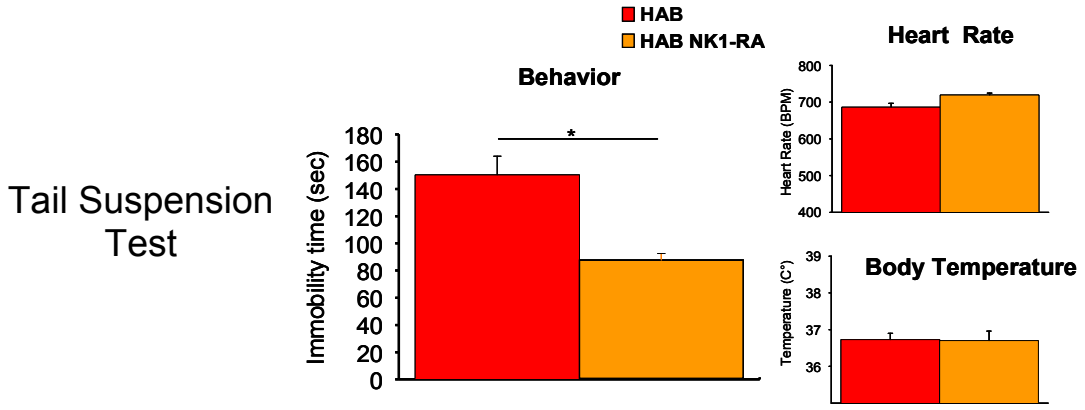

Figure 3.14. Antidepressant effect of NK1receptor antagonist in tail suspension test. Immobility time (antidepressant effects) on the left panel, on the right panel the autonomic readout (heart rate and body temperature were quantified. In red bars HAB control n=8, and in orange bar HAB NK1 treated n=8. Data are means ± SEM, * p<0.05, **p<0.01 where statistical difference was reached.

Finally, to a reduce fear response in extinction in HAB treated mice ($p<0.05$) there was also an increased HRV in extinction phase when compared to HAB control ($p<0.05$) (Figure 3.15).

Effect of chronic NK1 receptor administration antagonist during extinction phase of cued fear conditioning

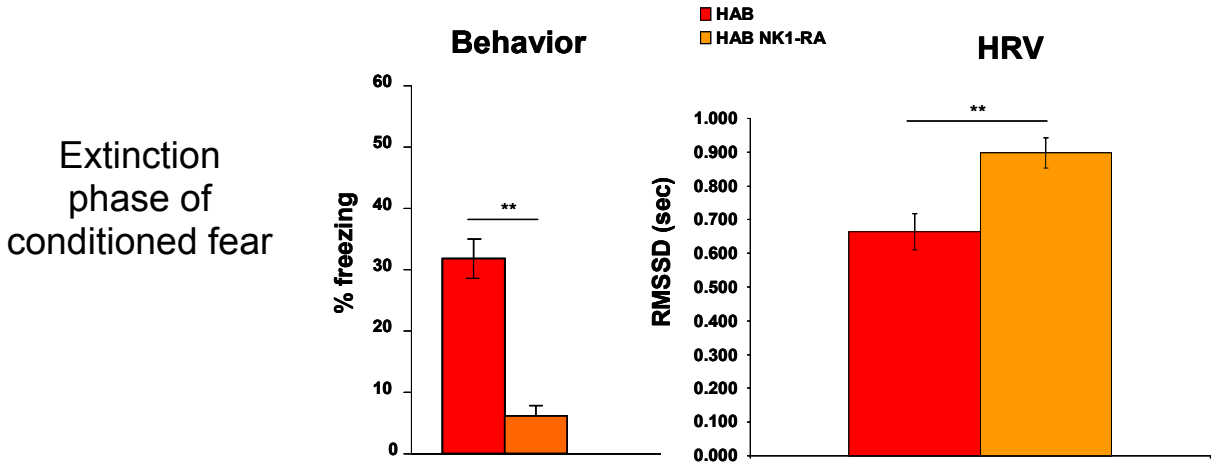

Figure 3.15 Average of 32 minutes of fear response during extinction session. In detailed, reduced fear and normalization of HRV in HAB NK1 treated mice. Fear response (average of total freezing, right panel) on and HRV (time domain analysis, right panel) were evaluated in extinction phase. In red bars HAB control n=8, and in orange bar HAB NK1 treated n=8. Data are means ± SEM, * $p<0.05$, ** $p<0.01$ where statistical difference was reached.

3.6 Successful automated scoring of freezing behavior: comparisons between topowatch, manual (observer) evaluation and a non-sensitive commercially available automated system (TSE system)

Comparison of manual, topowatch and TSE evaluation on extinction was assessed. Similar extinction profiles were observed between topowacth, TSE and manual evaluation in condition animals (CS-US pairings in the conditioning day, CS+) ($p>0.05$). However, in mice that were not conditioned (CS only in the conditioning day, CS-), topowatch and manual evaluation were almost comparable while TSE system, detected high freezing levels ($p<0.05$) (Figure 3.16)

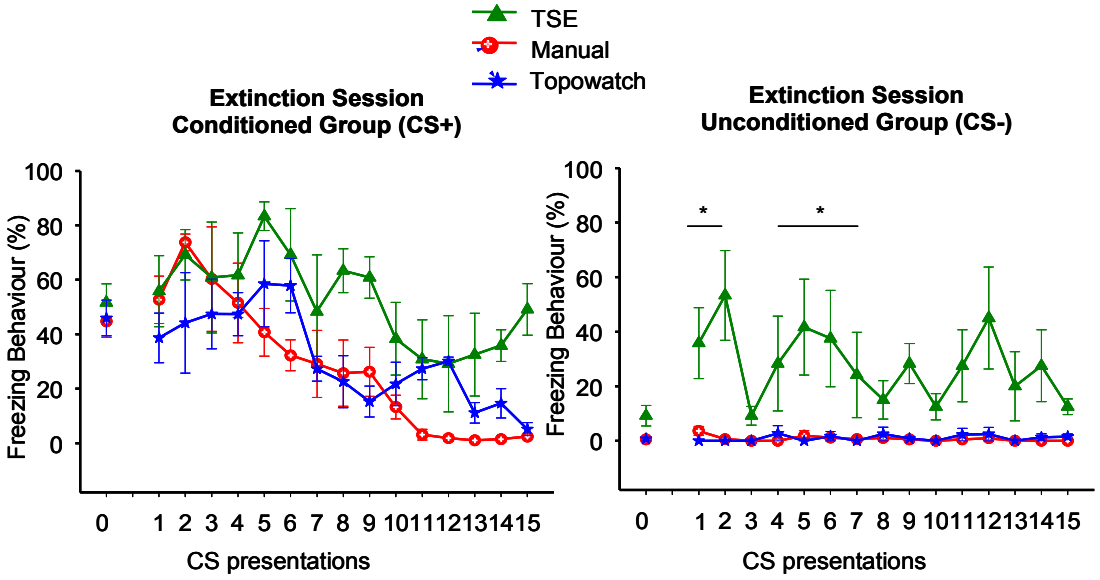

Figure 3.16. Manual and automatized scoring of freezing behavior in extinction phase in conditioned (CS-US exposure on conditioning day) (right panel) mice and unconditioned (CS only on conditioning day) mice (left panel). Topowatch (blue star) TSE (green filled triangles), manual evaluation (red open circles). Data are means ± SEM, * $p<0.05$, **$p<0.01$ where statistical difference was reached.

Moreover, correlation analysis between manual-topowatch displayed and higher correlation coefficient (Pearson correlation coefficient=0.89) when compared to correlation analysis TSE-manual (r=Pearson correlation coefficient=0.78) (Figure 3.17)

Correlation software-manual

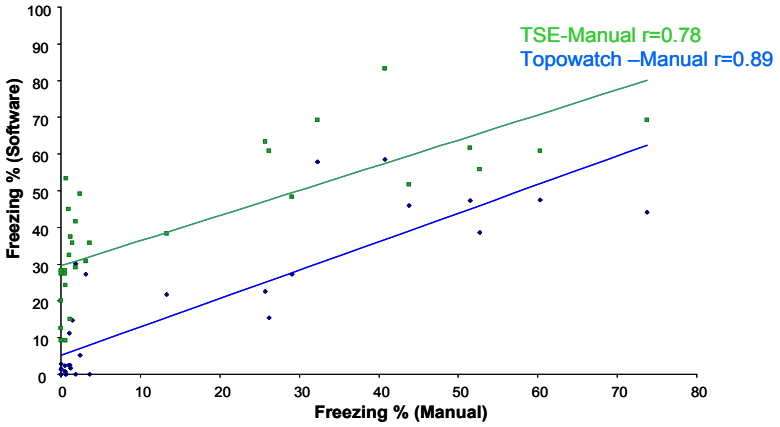

Figure 3.17. Correlation analysis (Pearson coefficient r) of TSE vs. manual (green) and topowatch vs. manual on scoring freezing behavior). TSE (green diamonds and green correlation line) and topowatch blue diamonds and blue correlation line are represented.

Furthermore, error rate of the softwares considered index of sensitivity (Marchand et al., 2003), calculated upon the difference of the standard deviation of scored freezing behavior between manual-topowatch and manual-TSE, revealed an error rate of 10-15 % on average for topowatch, while 30-40% on average for TSE ($p<0.05$) (Figure 3.18)

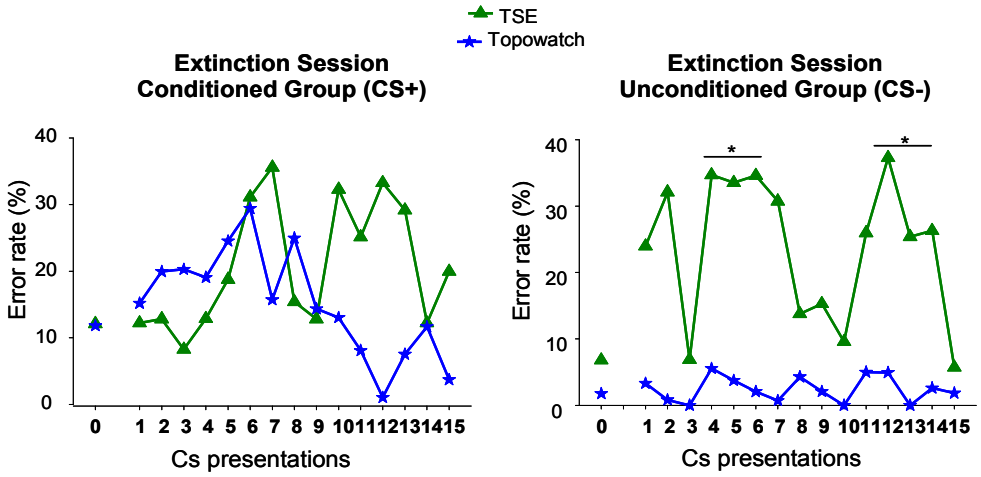

Figure 3.18. Different sensitivity based on error rate of topowatch and TSE software on scoring freezing behavior in conditioned (left, CS+) and unconditioned (right, CS-) animals during extinction. Topowatch (blue star) and TSE (green triangles) are represented.). Data are means,* $p<0.05$, **$p<0.01$ where statistical difference was reached.

4 Discussion

The aim of the present study was to assess the autonomic regulation under basal and challenge conditions in the HAB/NAB mouse model. Results of the autonomic parameters are summarized in Table 4.1

Table 4.1. Comparison of the autonomic parameters measured under basal (circadian rhythm) and challenge conditions (unconditioned stimulus and conditioned stimulus) for HAB and NAB mice.

Untreated HABs vs. NABs					**Chronic NK1RA- treated vs. untreated HABs**			
Sex	Challenge	Heart Rate	Body Temperature	Heart Rate Variability	Challenge	Heart Rate	Body Temperature	Heart Rate Variability
Basal (Home cage)								
Male		~	~	~		Not performed#		
Female		~	~	~		~	~	Not performed#
Unconditioned Stimulus								
Male	Novel cage	~	~	Not performed#		Not performed#		
	Open Field	~	~	Not performed#		Not performed #		
	Handling	~	~	Not performed#		Not performed #		
Female	Elevated Plus Maze		Not performed #			↓	~	Not performed
	Stress induced hypethermia		Not performed #			~	~	Not performed#
Conditioned Stimulus								
Male	Air Jet paradigm	~	~	~		Not performed#		
Female	CS (light) US (air jet)	↑	-	↓		Not performed#		
Female	Modified home cage CS (sound) US(footshock)	~	~	↓		~	~	↑

not performed either due to limited number of biopotential transmitters or use of tests not suited for autonomic recordings (see text for full details).

Although, some drawbacks (e.g. limited number of expensive biopotential transmitters, non suited behavioral test for autonomic recordings) were experienced in establishing the autonomic measurement in this animal model, it was still possible to gain important insights in neuro-cardiac regulation in animal model selected for trait-

anxiety correspondent to pathological anxiety in humans. Thus, only selected tests that revealed alterations in autonomic responses could be repeated

4.1 Autonomic characterization of HAB NAB mice under basal conditions

Autonomic recordings under basal conditions revealed no alterations in heart rate, body temperature and heart rate variability circadian rhythmicity for both gender and lines. The circadian regulation of HAB and NAB mice was similar as this in the range reported for other mice strains. (Van Bogaert et al., 2006). In support of this finding, patients suffering from anxiety and depression do not display alterations in basal heart rate and body temperature. (Frank et al., 2000). However, as mentioned in the introduction, conditions under which baseline autonomic measurements in psychopathology are obtained can vary widely across studies as the experimental context and stimuli challenges are different from one study to another. Thereby, the outcome is often confounding as some studies have shown that anxiety with comorbid depression is often accompanied with reduced basal HRV and others do not (Friedman, 2007). Additionally, Te at al. didn't find differences in the HRV between BalB/c and C57J mice (Depino & Gross, 2007). Notably, in the rat HAB/LAB model (see introduction), HABs displayed similar c-fos expression under resting conditions in many brain regions known to be involved in the autonomic regulation of cardiovascular activity including brainstem/midbrain areas (e.g. locus coeruleus, periaqueductal gray), medial prefrontal cortex, hypothalamus and amygdala (Salome et al., 2004; Frank et al., 2006; Salchner et al., 2006). Moreover, in medullary regions such as the nucleus of the solitary tract, the rostroventrolateral reticular nucleus and the caudoventrolateral reticular nucleus, no differences in basal c-Fos expression between the lines could were observed (Salchner and Singewald, unpublished data). (locomotor activity). Collectively, when compared to the rat model, the cuf off criteria used in the breeding strategy (i.e. Open Arm Time) that allowed to differentiate among HAB and NAB mice, did not affect the basal autonomic regulation or brain activity of regions controlling the neuro-cardiac outputs.

4.2 Autonomic responses of HAB and NAB mice in a battery of mild unconditioned stressors

It is widely reported that various unconditioned and conditioned psychological stressors lead to increase in autonomic parameters (e.g. heart rate and body

temperature) in rodents (Van Bogaert et al., 2006). The magnitude of this increase strictly depends on the nature and the intensity of the stimulus given (Hoehn-Saric & McLeod, 2000). The autonomic system, via both sympathetic component and parasympathetic component, is considered to be responsible for the homeostatic regulation of physiologic functions and for fine changes of this homeostasis during psychological stress. However, there is a species differences in regulation of the autonomic system during anxiety provoking stimuli. In fact, (healthy) humans upon stressful stimulus display first higher parasympathetic withdrawal, and to a lesser extent, successive sympathetic activation (Friedman, 2007) comparable to mice but different than in rats (Rowan et al., 2007). In pathological anxiety, however, this autonomic balance seems to be perturbed.

Our current results showed a specific tachycardic response and stress induced hyperthermia relative to the magnitude of the different stressors in both HAB and NAB mice. Nevertheless, we failed to observe differences in heart rate or body temperature in HAB and NAB male mice. Furthermore, locomotor activity was not affected in both lines across the tests. Interestingly, mice known to be high anxious 129S6 showed no differences in heart rate and body temperature compared to C57Bl6J mice after novelty exposure (Van Bogaert et al., 2006). Along with these findings, no differences have been found in open field exposure between another model of anxiety BalbCj mice against C57Bl6J mice. Furthermore, corticotropin relasing factor (CRF) overexpressing mice, which display enhanced anxiety like behavior (Stenzel-Poore et al., 1994), did not show any difference in heart rate during novelty exposure, when compared to the less anxious wildtype mice. However, there exists also one study in which 5-HT1A receptor knockout mice, displaying a more anxious like-phenotype, showed exaggerated tachycardic response to novelty stress, as compared to the less anxious wildtype mice (Pattij et al., 2002). Presumably, the nature and magnitude of the stressor seems to play an important role in autonomic regulation

To prove the species specificity of autonomic regulation, in the more anxious Roman low avoidance rats showed more passive coping responses to various challenges by typically displaying behavioral inhibition accompanied with bradycardia. (Bohus, eta el 1987, Bohus 1991, Roozendall 1992). Moreover, Tsukuba high emotional rats, compared to their low emotional counterparts, showed at least a tendency toward reduced heart rate in the runway test, which went along with reduced locomotor activity (Yayou eta ll 1993).

There are several reasons for these inconsistent findings :i) the environment in which animals are tested vary from lab to lab ii) the time resolution, in which ECG recordings are performed in behavioral tests (e.g. 5 minute intervals), is not accurate enough to identify possible autonomic differences . In our case we averaged 5 minutes time period to quantify autonomic response in HAB and NAB mice. This time period was chosen because we wanted to quantify autonomic responses from basal to stress. Increasing the resolution would have generated a large data set, analysis of which was beyond the computational capacity of the software (Rowan et al., 2007) . In fact, heart rate of rodents under basal conditions is almost 10 times higher than humans and correlates with body mass (Stiedl & Spiess, 1997) Thus, considering that flight or fight response in anxiety provoking is induced in a scale of seconds or milliseconds it could be that averaged heart rate or body temperature in 5 minute intervals leads to loss of sensitivity to detecting fast autonomic changes.

Furthermore, during handling a test where behavioral inhibition is not displayed, we did not find any relation between the rise in autonomic parameters and locomotor activity in handling test that was observed in novelty and open field. Indeed, it has also been shown that in rodents with moderate activity and normal heart rate (Van Bogaert et al., 2006) thereby indicating a partial dissociation between autonomic and locomotor activity.

4.3 Altered fear and autonomic responses in HAB mice

Conditioned fear in mice has been widely studied and characterized in rodents on the basis of behavioral (e.g. freezing behavior), activated neuronal pathways (e.g. amygdala, prefrontal cortex) and physiological changes (e.g. CORT, ACTH and autonomic functions). Especially a shock paired discrete stimulus (e.g. conditioned stimulus) has consistently been shown to produce freezing behavior accompanied by a marked increase in blood pressure and delayed increase in heart rate in both rats and mice (Tovote *et al.*, 2005a). However, in few other studies freezing is often accompanied by hypotension, bradycardia, and increased respiration (Keller et al., 2006). All of these findings indicate a great heterogeneity in fear and autonomic responses evaluated in different studies.

In the present study, male HAB and NAB mice did not display any sign of freezing behavior in the first day of conditioned fear when compared to female mice. This difference could be explained by several reasons: i) alternative protocol to induce fear

conditioning (i.e. air jet) ii) hormonal differences. With regard to the first point few evidence in human and rodents has proposed that air jet be used as an alternative non-painful stimulus in fear conditioning as similar as to other painless stimuli such as predator odour exposure (Endres et al., 2005; Fendt et al., 2005).

Human psycho-physiological studies use air puff as an aversive stimulus to document abnormal fear conditioning in children of parents with anxiety disorders (Pine et al., 2001). In rodents it has been shown that pressurised air can be used as a procedure for presentation of an aversive but non-painful stimulus for the elicitation of ultrasonic vocalisation from rats (Knapp & Pohorecky, 1995)

Secondly, literature regarding estrous cycle phases and its relation to fear conditioning is highly inconsistent. In one of the few studies, gender differences have not been found comparing "normal anxious like behavior" males and female mice in fear conditioning (Bolivar et al., 2001). There are some indications of correlation between pro-estrus and anxiogenic effects in mild stressors (e.g. elevated plus maze) (Lund et al., 2005), thereby most of studies avoid females to reduce the number of variables to control in behavioral research.

Indeed, a study has shown, looking at sex differences in response to observational fear conditioning procedure, that women were more likely to report greater distress and dislike over the conditioned stimulus compared to men (Kelly & Forsyth, 2007).

Additionally, the interactions of multiple vulnerabilities (i.e., social roles, genetic predispositions, hormonal factors, emotion regulation strategies) may play a substantial role in how women and men differentially respond to fear conditioning procedures (Mineka & Zinbarg, 2006). Accordingly to the human study previously mentioned (Kelly & Forsyth, 2007), during extinction training female HAB mice displayed impaired fear extinction when compared to female NAB mice suggesting that HAB female mice could be a possible model for enhanced fear vulnerability.

With regard to autonomic responses, in both conditioning day and extinction day, HAB female mice displayed similar stress-induced hyperthermia and initial exaggerated heart rate response that went even higher after the first CS presentation compared to NAB mice. This initial increased heart rate observed in HAB mice was due to placing the animals from their home cage to the new conditioning context. Therefore, as for the unconditioned stimulus heart rate and body temperature seem to meet the criteria for reliable markers in anxiety and fear research in rodents. In this respect time and frequency domain analysis of heart rate variability were performed as it appears to be a more reliable indicator of fear/anxiety state (see Introduction).

Frequency domain analysis, in female mice during extinction day, revealed a tendency to reduce parasympathetic tone indicated by reduced HF values in HAB mice and increased sympathetic activation indicated by the increased LF/HF ratio in HAB mice compared to NAB mice. Frequency domain analysis is one of the oldest fashioned analyses of heart rate variability in humans and until recently the correct band spectrum (see Introduction) for rodents was not identified (Stauss, 2007). Also, this kind of analysis is based on approximation of ECG to a sinusoid signal followed by transformations (see Introduction). Therefore, it could possibly be that this system is not powerful enough to identify small autonomic changes in heart rate variability.
Indeed, the tendency provided by the time domain analysis became more pronounced statistically different in the time domain analysis of heart rate variability during extinction. In this analysis, the whole spectrum of heart rate is plotted versus its variability and it has been already described as valuable model to describe differences in sympathovagal balance during extinction day (Stiedl & Spiess, 1997; Milanovic *et al.*, 1998; Stiedl *et al.*, 1999; Stiedl *et al.*, 2000; Tovote *et al.*, 2005b). In fact, HAB mice displayed reduced heart rate variability compared to NAB mice under stress (extinction) condition. In other words increased parasympathetic withdrawal and increased sympathetic activation. These findings are in parallel to what has been observed in anxiety patients under stress conditions in which heart rate variability is reduced compared to control (Cohen & Benjamin, 2006; Friedman, 2007; Mujica-Parodi *et al.*, 2009) indicating that HRV

4.4 Home cage as test environment for extinction session to reduce unspecific autonomic responses

Most of the studies looking at autonomic regulation in rodents in fear/anxiety field have investigated the increase in heart rate or body temperature in home cage and induce by specific challenges without taking into account that mouse autonomic system is affected by simple handling (Van Bogaert et al., 2006). Indeed, what we observed with the previous experiment was a marked increase in initial heart rate response in HAB mice in the extinction phase and that was primarily due to transport effect (handling and novelty components). Indeed, in this respects a few studies have been conducted in fear conditioning in mice investigating autonomic responses induced by CS presentations in fear conditioning in their home cage in C57Bl6N and

C57Bl6J mice, thus avoiding cardiovascular transports artifacts (Tovote *et al.*, 2004; Tovote *et al.*, 2005a).

As CD1 mice outbred strain, from which HAB and NAB selection started from, display low freezing levels in cued fear conditioning (Adams et al., 2002), we decided to couple the conditioned stimulus (CS) to a stronger unconditioned stimulus (US) as mild footshock. We choose another protocol because though air jet induces fear conditioning in female mice, however familiar environment such as home cage where extinction is performed can fail to induce autonomic or behavioral responses (Depino & Gross, 2007).

As mice were in their home cage during extinction phase, freezing behavior was not possible to quantify. Instead, as indirect measure of fear response we used the activity of the mice (expressed as counts/min) as reduced locomotor activity in other laboratories is considered a measure of fear response (Keller *et al.*, 2006). Indeed, in both acquisition and extinction phase, HAB mice displayed reduced activity compared do NAB mice but no differences in heart rate or body temperature were observed in conditioning or extinction day. Presumably the familiar environment produced from the home cage played a positive role inducing a reduced increase tachycardic response due to CS effects. However, when time domain analysis of heart rate variability was performed we found that under stress conditions there was marked reduced heart rate variability in HAB mice compared to NAB mice. Thus, that independent from transport effects, we could reproduce the reduce heart rate variability and it could indeed be proposed as an additional marker in fear research.

4.5 Changes in heart rate variability accompanied by anxiolytic and antidepressant drug effect in HAB mice

Behavioral effect of chronic application of NK1 receptor antagonist

In pharmacotherapy of stress-related psychiatric disorders such as depression and anxiety disorders there is still an urgent need for drugs with improved efficacy, faster onset of action and better long term tolerability. As outlined in the introduction, there is evidence that NK1 receptor antagonism represents a promising novel treatment strategy. In contrast to SSRIs, NK1 receptor antagonists elicit anxiolytic effects already after acute treatment. This was found in rodents and humans (Michelgard et al., 2007; Ebner et al., 2008). However, whether acute treatment also induces

antidepressant-like effects is still unclear since a decrease in depressive symptoms was found only after chronic treatment. In contrast, most animal models related to depression are sensitive to acutely administered NK1 receptor antagonists making them less comparable to the clinical situation. Therefore, the use of other models such as the HAB model might be more useful as these animals show unique sensitivity to chronic and not acute antidepressant treatment (Keck et al., 2005) In the present study, chronic NK1 receptor antagonist (L-822249) treated HAB mice display a reduced immobility time during the tail suspension test suggesting an antidepressant-like effect. This is in line with previous studies demonstrating antidepressant-like effects after chronic treatment with other NK1 receptor antagonists in chronically stressed rats or tree shrews (Papp et al., 2000; van der Hart et al., 2005).

Accompanied to the antidepressant-like effect, chronic administration of the NK1 receptor antagonist induced an anxiolytic effect in HAB treated mice during the light/dark but not elevated plus maze test. As the elevated plus maze is the criterion on which HAB animals are selected (Kromer et al., 2005), a re-exposure to the same stressor changes the behavioral readout. Indeed, one study have shown that re-exposure to elevated plus maze does not lead to anxiolytic effect mediated by benzodiazepines (Calzavara et al., 2005). Thus, our findings are in agreement to a number of other studies demonstrating anxiolytic effects after chronic as well as acute NK1 receptor antagonist treatment (for review see (McLean, 2005; Ebner & Singewald, 2006). Further studies investigating the brain sites mediating these behavioral effects indicate that brain areas such as amygdala, septum, and PAG are critically involved (Ebner & Singewald, 2006).

Lastly we observed a reduced fear expression in chronically treated NK1 receptor HAB mice during extinction. This could be explained by the anxiolytic/antidepressant effect produced by NK1 receptor antagonist, as some studies have shown a correlation between chronic treatment with anxiolytic/antidepressant and the reduction in fear response (Kitaichi et al., 2006; Hashimoto et al., 2009).

Autonomic effects of chronic NK1 receptor antagonist treatment

As antidepressant drugs (e.g. imipamine) modulate autonomic function in depressed patients (Tulen et al., 1996), we investigated the effects of chronic NK1 receptor antagonist administration on cardiovascular function. Notably, chronic NK1 receptor antagonist treated HAB mice did not show any changes on circadian rhythm of heart

rate or body temperature (resting conditions) compared to control animals. It is well known that modulation of NK1 receptor function via selective agonists/antagonists leads to changes in the autonomic system (for review see (Hoover *et al.*, 2000; Walsh & McWilliams, 2006). However, the exact role of the SP/NK1 receptor system in cardiovascular function is not well understood. Different ways of applying NK1 receptor ligands such as chronic vs. acute, different administration routes such as intracerebrally or systemically as well as species differences might be responsible for discrepant results.

Under stress conditions during elevated plus-maze exposure, we observed a blunted tachycardic response in chronic NK1 receptor antagonist treated HAB mice when compared to controls. Interestingly, a reduced tachycardia in response to a physical stressor was also found in rats acutely treated with NK1 receptor antagonist (Culman et al., 1997) .

In regard to body temperature, no effects of chronic NK1 treatment were observed in another validated test evaluating the effect of anxiolytic drugs (Vinkers et al., 2009) as stress-induced hyperthermia. Moreover, there was no relation between basal temperature and stress induced hyperthermia measured with telemetry device and stress induce hyperthermia probe. The lack of correspondence of temperature measurements between with the two systems might be due to the different placement of the sensor. In the case of telemetry device the sensor that reveals the temperature was placed intraperitoneally, while in the case of stress induced hyperthermia probe, the sensor was placed rectally.. Differences in vascularization of the two parts of the body could explain these differences in temperature measurements.

However, in contrast to the elevated plus maze, the heart rate activity during tail suspension test was not affected by chronic NK1 receptor antagonist treatment. One explanation for this finding might be that the vertical position of the mouse during the tail suspension test changes blood circulation towards mouse head and counter regulatory mechanisms driven by baroreflex (Powers & Bernstein, 2004) hide any possible bradycardic effects induced by NK1 receptor antagonist.

The more striking results after chronic NK1 receptor antagonist treatment was the normalization of heart rate variability followed by reduced fear expression in HAB mice. This is in line with findings in patients suffering from depression in which heart rate variability was found to be a potential predictor in response to antidepressant treatment during emotional state and not under resting condition (Fraguas et al.,

2007). Although preliminary, these results are encouraging and need to be replicated in future studies with other mouse model.

The clear action mechanism of anxiolytic, antidepressant and normalization of heart rate variability after of chronic NK1 receptor antagonist pose futher questions that have to be still further investigated, starting from the brain activity regions controlling autonomic functions by means of immediate gene (e.g. c-fos). This technique will reveal which are the specific brain pattern activation induced by chronic NK1 receptor treatment.

4.6 Development of reliable automated assessment of quantification of freezing behavior

We recently developed a software namely "topowatch" for scoring freezing behavior which is based on the same principle of (Kopec et al., 2007). In brief a simple algorithm calculates the difference of rodent to background in pixel, and simultaneously the pixel to pixel difference per frame, indicating then in a separate window the time events in which the animal was "freezing". The advantages of this novel method were evaluated against an established system used in fear conditioning field.

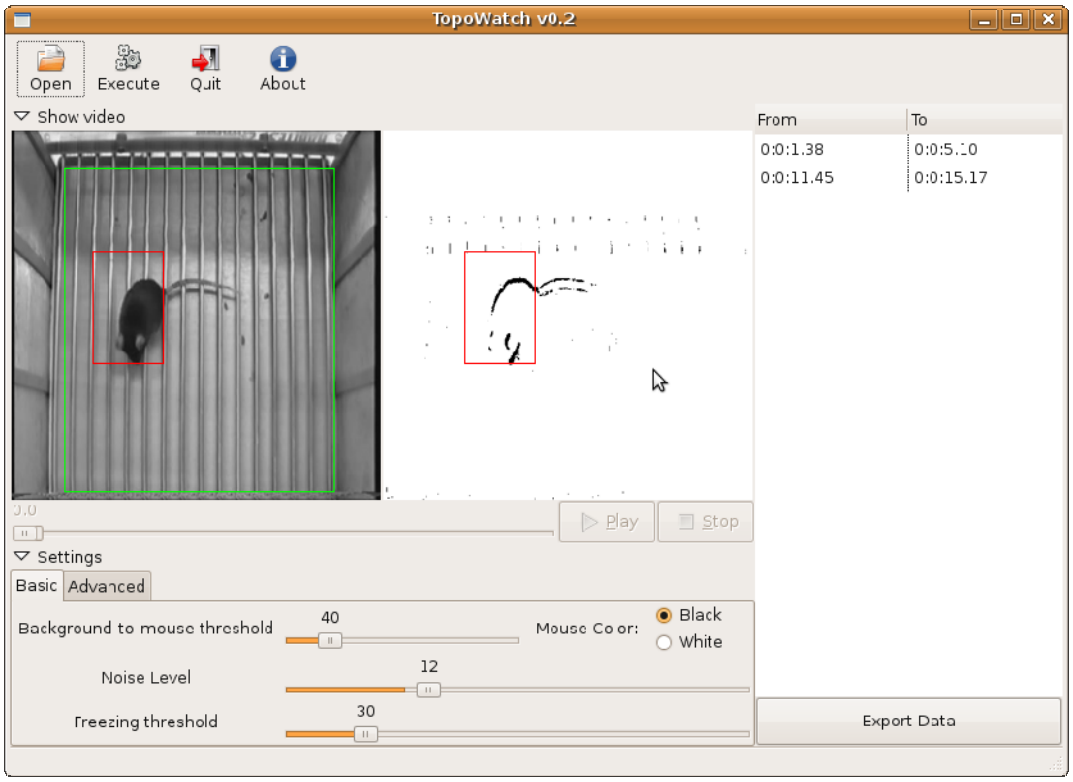

Figure.4.1. Representative Photograph of a novel automated system for scoring freezing behavior called topowatch

We assessed the sensitivity of our system for scoring freezing behavior in extinction phase against manual (observer) and commercially available software TSE that calculates the immobility/freezing based on laser beams. When the animal interrupts these laser beams by moving in the conditioning chamber the number of interruptions is calculated into excel. Thus by setting the threshold of number of movements and so forth freezing levels can be extrapolated. When we evaluated the freezing behavior in a group of animals who underwent to classical fear conditioning (CS and US pairing, CS+) no differences were observed between manual, TSE and topowatch during extinction. However when animals were exposed to only CS- in the conditioning day (CS no US, CS-), TSE detected 30-40% of "freezing" on average while manual and topowatch scored nearly 0% during extinction. Further correlation analysis and evaluation of error rate for both TSE-manual and topowatch-manual evaluation topowatch resulted to be more sensitive software in detecting freezing behavior. Furthermore, topowatch can read almost any type of video format and the faster is the processor of the computer the faster is the computation of the image. On average topowatch can evaluate 50 minutes video in 10 minutes.

Ideally, adjusting the background bar and the freezing level in terms of pixel (which is possible to do real time as the analysis is run) also the immobility in test for depression as TST and FST can be evaluated.

This new technique has extremely potential applications in fear conditioning as the commercially available software are not so sensitive (see results) and involve costly apparatus.

5 Conclusion

In the present study we have shown for the first time reduced HRV in a psychopathological mouse model .This result corresponds to what observed is in human suffering from anxiety and depression.

Validation of HRV as a marker for enhanced (pathological) anxiety and depression was pharmacologically assessed by chronic NK1 receptor treatment in HAB mice. As normalization of HRV is observed in anxiety and depressive disorders after successful drug treatment, the normalization of HRV, coincident with reduced anxiety- and depression- related behavior in treated HAB mice, confirmed the importance of HRV as potential marker in translational studies in anxiety and depression research.

Future time course studies will determine whether quantification of HRV can be used as predictive marker of a successful anxiolytic /antidepressant treatments.

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7 Appendix

ANS	autonomic system
BP	blood pressure
US	unconditioned stimulus
TST	tail suspension test
SSRI	selective serotonin reuptake inhibitors
SNRIs	selective serotoni and noradrenaline reuptake inhibitors
SIH	stress induced hypethermia
RR	respiratory rate
RMSSD	root of the mean square differences of successive NN intervals
PTSD	post traumatic stress disorders
OCD	obsessive compulsive disorders
OA	open arm
NRIs	selective noradrenaline reuptake inhibitors
NAB	normal anxiety like behavior mice
LF	low frequency
LAB	low anxiety-relatedbehavior
HRV	heart rate variability
HR	heart rate
HF	high frequency
HAB	high anxiety-related behavior mice
GAD	generalized anxiety disorders
FST	forced swim test
EPM	elevated plus maze
EMG	electromiography
EEG	electroencephalography
ECG	electrocardiography
DMS-IV	diagnostic and statistical manual for mental disorder IV
CS	conditioned stimulus
CRF	corticotropin-releasing factor
BT	body temperature
5-HT	5-hhydrixytryptamine

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