International Journal of Neuropsychopharmacology (2011), **14**, 1341–1355. © CINP and Cambridge University Press 2011
doi:10.1017/S1461145711000058

ARTICLE

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A mouse model of high trait anxiety shows reduced heart rate variability that can be reversed by anxiolytic drug treatment

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Abstract

Increasing evidence suggests that specific physiological measures may serve as biomarkers for successful treatment to alleviate symptoms of pathological anxiety. Studies of autonomic function investigating parameters such as heart rate (HR), HR variability and blood pressure (BP) indicated that HR variability is consistently reduced in anxious patients, whereas HR and BP data show inconsistent results. Therefore, HR and HR variability were measured under various emotionally challenging conditions in a mouse model of high innate anxiety (high anxiety behaviour; HAB) *vs.* control normal anxiety-like behaviour (NAB) mice. Baseline HR, HR variability and activity did not differ between mouse lines. However, after cued Pavlovian fear conditioning, both elevated tachycardia and increased fear responses were observed in HAB mice compared to NAB mice upon re-exposure to the conditioning stimulus serving as the emotional stressor. When retention of conditioned fear was tested in the home cage, HAB mice again displayed higher fear responses than NAB mice, while the HR responses were similar. Conversely, in both experimental settings HAB mice consistently exhibited reduced HR variability. Repeated administration of the anxiolytic NK1 receptor antagonist L-822429 lowered the conditioned fear response and shifted HR dynamics in HAB mice to a more regular pattern, similar to that in NAB mice. Additional receiver-operating characteristic (ROC) analysis demonstrated the high specificity and sensitivity of HR variability to distinguish between normal and high anxiety trait. These findings indicate that assessment of autonomic response in addition to freezing might be a useful indicator of the efficacy of novel anxiolytic treatments.

Received 12 June 2010; Reviewed 31 August 2010; Revised 7 December 2010; Accepted 6 January 2011; First published online 15 February 2011

Key words: Anxiety, autonomic system, biomarker, fear conditioning.

Introduction

Psychiatric disorders are a leading cause of disability worldwide. However, a considerable part of these disorders are not adequately diagnosed because classification of the different pathologies is based on symptoms specified in DSM-IV (APA, 2000) that are often common to various disorders. Therefore, defining biological or physiological markers would aid a more precise diagnosis of psychiatric disorders including anxiety disorders.

The high degree of correlation between the severity of anxiety and perturbations of the cardiovascular system has motivated researchers to analyse autonomic parameters in these pathologies (Goodwin *et al*. 2009; Vogelzangs *et al*. 2010). Autonomic function can be studied in humans by means of various parameters such as heart rate (HR), blood pressure (BP) and body

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temperature (BT). Whereas those markers have been widely characterized as potential predictors for developing different cardiovascular disorders and associated increased risk of death (Casscells *et al*. 2005; Harnik, 2005; Pocock *et al*. 2001), in psychiatric disorders these markers are not considered consistently reliable predictors (for review see Berntson *et al*. 1998). For example, different basal HR and BP regulation has been observed in anxious patients in different laboratories (for review see Berntson *et al*. 1998).

Therefore, the field has moved towards assessing other autonomic markers such as HR variability analysis (for review see Cohen & Benjamin, 2006; Friedman, 2007). This method provides a measure of the swings of HR over a certain time period and has become an important assay to assess the autonomic nervous system regulation. Specifically, HR variability reflects qualitatively and quantitatively the cardiovascular control mediated by the two components of the autonomic system, the sympathetic and parasympathetic (vagal) system (Pieper & Hammill, 1995). Linear HR variability analysis is generally performed using time-domain and frequency-spectrum analyses. Frequency-spectrum analysis has been used for selective frequency band to determine high- *vs.* low-frequency ratios as an index of sympathovagal balance in humans (Montano *et al*. 2009). However, this approach is less validated in mice and hampered by the fact that the energy contributing to the analysis in selected frequency bands represents only a small portion of the signal (~20%) that depends on harmonics, whereas the data stream has been shown to exert nonlinear properties (Meyer & Stiedl, 2003). Therefore, we used the root mean square of successive RR interval differences of the ECG signal, the root mean square of successive RR interval differences (RMSSD) measure of time-domain analysis, since it appears to be most stable with regard to drift-like behaviour of non-stationary data (Meyer & Stiedl, 2003), which is frequently and reliably used in rodents as an index of short-term variability or vagal activity.

To date, reduction in HR variability is clinically associated with an increased risk of cardiac death in patients with anxiety and/or comorbid mood-disorder patients (Bleil *et al*. 2008; Lavoie *et al*. 2004; Martens *et al*. 2008). Such patients show evidence of altered brain activity in regions involved in emotional/autonomic control (Aihara *et al*. 2007; Mayberg, 1997; Tolkunov *et al*. 2010). A reduction in HR variability is also observed in studies involving animal models of various psychiatric disorders (Cohen *et al*. 2007; Grippo *et al*. 2007; Pattij *et al*. 2002). However, so far it is unknown whether assessment of HR variability may be a valid biological marker for predicting and/or diagnosing the development, course and outcome of a specific psychiatric disorder. According to definition, biomarkers are biological indicators that objectively describe normal biological or pathogenic processes, or responses to a therapeutic intervention. Therefore, we first investigated whether HR and/or HR variability can be used as indicator of pathological trait anxiety in a validated psychopathological mouse model of innate high anxiety-related behaviour termed 'HAB' mouse (Kromer *et al*. 2005) *vs*. the corresponding NAB mouse line with normal anxiety-related behaviour. Supporting the validity of this model, HAB mice have been shown to display altered cortico-limbic activation upon challenge (Muigg *et al*. 2009), similar to that observed in patients with anxiety disorders (Mujica-Parodi *et al*. 2009). Female HAB and NAB mice, which also show the pronounced difference in anxiety-related behaviour (Kromer *et al*. 2005) were used in the present study since investigation of anxiety-related mechanisms in female subjects is particularly interesting, given the higher prevalence of anxiety disorders in women than in men (Bekker & van Mens-Verhulst, 2007; Johnson & Stewart, 2010).

Radio-telemetry devices allow the recording of autonomic markers in freely moving animals (Kramer & Kinter, 2003; Sgoifo *et al*. 1996; Stiedl & Spiess, 1997; Stiedl *et al*. 1999). This technique was used in combination with fear conditioning to assess the full range of HR dynamics from baseline to maximal activation upon conditioned emotional challenge. Fear conditioning was used as challenge for three main reasons. First, HR responses by radio-telemetry have been established to be an index of fear in rodents. Second, radio-telemetric measurements offers the invaluable advantage of providing an independent concomitant measure to behavioural measures under home-cage conditions devoid of experimenter-based interference with proper assessment of undisturbed baseline values before phasic stimulation by the conditioned stimulus (CS). Third, exaggerated fear responses have been observed in anxious patients (for review see Shin & Liberzon, 2010) that can be modelled in rodents using fear conditioning.

In addition, we wanted to evaluate the cardiovascular and behavioural response to anxiolytic drug treatment. Although benzodiazepines (BDZs) are the most frequently clinically used anxiolytic drugs, sedation and cognitive impairment are well known problems limiting the use of conventional BDZs in anxiety patients (reviewed in Atack, 2003; Stewart, 2005). Indeed, when we tested the effect of diazepam

at different doses in HAB and NAB mice in a preliminary experiment, it was not possible to distinguish the anxiolytic from the sedative effects in HAB mice (R. Landgraf, personal communication). Moreover, BDZs are known to induce memory impairments both in rodents and humans (Harris & Westbrook, 1998; Sanger *et al*. 1995). Therefore, to avoid pronounced sedative effects and confounding effects on fear learning (disrupted learning) and subsequent fear expression (e.g. immobility/activity), we used the well characterized neurokinin 1 (NK1) receptor antagonist L-822429 (Ebner *et al*. 2004, 2008; Ebner & Singewald, 2007; Singewald *et al*. 2008). This and other NK1 receptor antagonists have been shown to exert anxiolytic effects in animals and humans (reviewed in Czeh *et al*. 2006; Ebner *et al*. 2009). We investigated the sensitivity and specificity of the RMSSD biomarker. This was achieved by utilization of receiver-operating characteristic (ROC) analysis. This procedure has been used to clinically evaluate the quality of biological biomarkers in the classification of patients with and without disease states.

Material and methods

Animals

Female HAB and NAB mice (age 8–12 wk, weight 25–30 g, bred on a CD-1 background) that were used in first two experiments were bred in the animal facilities of the Max Plank Institute of Psychiatry in Munich, as described previously (Kromer *et al*. 2005; Landgraf & Wigger, 2002). For the chronic NK1 receptor antagonist treatment, mice were bred in the animal facility of the University Innsbruck. All experiments were approved by the local Ethical Committee for Animal Care and Use (Bundesministerium für Wissenschaft and Verkehr, Kommission für Tierversuchsangelegenheiten, Austria) and were in compliance with international laws and policies and European Council Directive 86/609/EEC.

Implantation of radio-telemetry transmitters

Mice were individually housed starting 24 h before the onset of surgery until the completion of the behavioural experiments. TA10ETAF20 transmitters (Data Sciences, USA) were implanted in the animals as described previously (Stiedl & Spiess, 1997; Stiedl *et al*. 1999). For anaesthesia a mixture of sodium pentobarbital (40 mg/kg i.p.) and ketamine (50 mg/kg i.p.) was used. Post-operatively, mice were treated with buprenorphine (0.2 mg/kg i.p.) for 3 d. Following surgery, mice were allowed to recover for 3 wk before the onset of behavioural testing.

Locomotion and ECG data acquisition and analysis

Locomotor activity and ECG were acquired by the use of a radio-telemetry system (Data Sciences). Data were collected at 64 Hz (activity counts) and 2000 Hz (ECG) using the Dataquest A.R.T. 4.1 software (Data Sciences). The ECG signal was exported as a text file using Dataquest Reader (Data Sciences) and imported into Chart software (Chart 5.0, AD Instruments, Germany) for ECG data editing. Briefly, recordings were edited offline for the correction of unrecognized beats and exclusion of artifacts via the HR variability module of the Chart software as described previously (Stiedl *et al*. 2005). Pairs of successive R waves of the ECG signal were used to derive instantaneous readings of HR, measured in beats per min (bpm). HR variability was determined as the RMSSD in milliseconds.

Basal circadian measurement (home cage)

Three weeks after surgery, baseline measurements ($n = 6$/line) were continuously acquired in the home cage for a 48-h observation period. Radio-telemetrically transmitted data for locomotor activity and HR were averaged in 1-h intervals. To determine the full cardiovascular range, basal HR variability expressed in RMSSD was measured between 09:00 and 11:00 hours for the light phase, when basal HR levels should be the lowest, and between 23:00 and 01:00 hours for the night phase, when basal HR levels should be highest (Li *et al*. 1999). Intervals of 30 s were used to calculate HR variability.

Novel cage exposure

To assess the effects of novelty on autonomic responses, naive HAB and NAB mice ($n = 8$/line) were placed in a clean standard Makrolon cage ($22 \times 16 \times 14$ cm) without bedding. Recordings of ECG and locomotor activity were started in the home cage and lasted for 30 min. The mice were then placed into the middle of a new cage and ECG recordings continued for 30 min. Data for HR and HR variability were averaged in 30-s intervals and divided into pre-exposure and novelty.

Fear conditioning

Quantification of fear and autonomic responses

Behavioural experiments were video-recorded and freezing behaviour was assessed from the recording.

Freezing was defined as absence of all movements except those associated with breathing (Blanchard & Blanchard, 1969). Freezing data were averaged in 2-min intervals (duration of CS exposure) and divided into baseline measurement (pre-CS) and fear response (CS exposure). Data for locomotor activity, HR and HR variability were averaged in 30-s intervals and divided into pre-CS and CS exposure in the fear-conditioning experiments.

Cued fear conditioning with an air stream as unconditioned stimulus (US)

After basal measurement, the animals used for novel cage exposure were fear-conditioned via five CS (light intensity 600 lx, 2-min duration) each co-terminating with a US (air stream, intensity 50 dB, 30-s duration; Salchner *et al*. 2006) which has been previously used as US for aversive conditioning (Landers & Sullivan, 1999). The US was delivered via a plastic tube directed to the nose of the mouse by the experimenter, who followed the mouse in its movements for the entire duration of the US. Stimulus-free periods (2 min) separated and followed the CS/US pairings. Freezing during conditioning session was evaluated during the last CS/US pairings. This paradigm was chosen as it does not interfere with the radio-telemetry assessment such as electric shock which is classically used as US. Conditioning took place in a novel $26 \times 30 \times 32$ cm chamber with transparent walls and a metal grid floor (Coulbourn Instruments, USA). Fear retention was tested 24 h following conditioning by exposing mice to one CS (light intensity, 2 min, 600 lx) in a new context (empty cage).

Home-cage cued-fear conditioning

This paradigm was chosen as a possibility of avoiding non-specific autonomic effect due to context changes. Furthermore, we used mild footshock as US since it has already been successfully employed to induce cardiovascular and fear responses in this specific setting (Stiedl *et al*. 2009).

After a 2-min acclimation period, conditioning took place in a novel $26 \times 30 \times 32$ cm chamber with transparent walls and a metal grid floor (Coulbourn Instruments) under bright light condition (300 lx). HAB and NAB mice ($n = 8$/line) were fear-conditioned via five CS (2-min duration, intensity 80 dB, white noise) each co-terminating with an US (2 s, 0.7 mA scrambled footshock). The CS/US pairings were separated by 2 min without phasic stimulation. Twenty four hours later, after a 2-min baseline period without phasic stimulation, mice received a single CS presentation (2-min duration, intensity 80 dB, white noise) in the home cage as fear retention test at low illumination (red light, 5–10 lx). The freezing response could not be evaluated during the fear retention in the home-cage settings, since the cage lid prevented its assessment. However, correlation analysis between freezing levels and radio-telemetrically transmitted locomotor activity after light–CS + air–US retention testing indicated that the telemetrically detected activity can serve as an indirect measure of the fear response (Supplementary Fig. S1, available online).

Drug treatment

Auditory fear conditioning in HAB mice ($n = 8$/treatment group) was performed as described above using white noise as CS and footshock as US. Freezing during the conditioning session was evaluated during the last of five CS/US pairings. In the fear-retention test 24 h later, a single CS (2-min duration, intensity 80 dB, white noise) was presented in a new context (empty cage) under dim red-light illumination (5–10 lx). The NK1 receptor antagonist L-822 429 [(2S,3S)-*N*-[2-cyclopropoxy–5-(5-trifluoromethyl)-tetrazol-1-yl] benzyl-2-phenylpiperidin-3-amine-dihydrochloride] (Singewald *et al*. 2008) was dissolved and provided to mice via drinking water at a dose of 30 mg/kg for 30 d until the end of the fear-conditioning experiment. The control group received drinking water only. This dose was chosen according to preliminary experiments indicating that L-822 429 exerted an anxiolytic effect in HAB mice in the light/dark test (S. B. Sartori *et al.* unpublished data).

Statistical analysis

Statistical analysis was performed using Statistica 8.0 (StatSoft, USA). Analyses of variance (ANOVA) of repeated measures were performed with the factor line (HAB or NAB mice) and repeated measure for time (light or dark phase of circadian cycle). Two-factor ANOVAs were performed with factor line (see above) and second factor light or dark for circadian HR variability, baseline and novelty for novelty exposure and pre-CS (before CS presentation, baseline) and CS (during CS presentation, fear response) for fear conditioning. Significant effects were further analysed by Bonferroni *post-hoc* comparisons. The ROC curves were estimated by calculating the area under the curve (AUC) for HR variability in each of the three fear-conditioning experiments. A Z score was used to compare the differences in areas and standard errors between the ROC curves according to the method described previously (Hanley & McNeil, 1982). For

analysis of results shown in Supplementary Figs S1 and S3 Student's *t* test was used. For Supplementary Fig. S2, Pearson product-moment correlation coefficient *R* was calculated between freezing and telemetric locomotor activity. The threshold for statistical significance was set to $p<0.05$.

Results

Circadian changes in locomotor activity, HR and HR variability

In the home cage, circadian rhythmicity was observed in both HAB and NAB mice as indicated by higher locomotor activity ($F_{48,480}=4.18$, $p<0.001$, Fig. 1*a*), higher HR ($F_{48,480}=4.74$, $p<0.001$; Fig. 1*b*) and reduced HR variability ($F_{1,104}=74.76$, $p<0.001$, Fig. 1*c*) during the dark phase. No significant line × time effect was found for HR ($F_{48,480}=1.37$, $p>0.05$) or for locomotor activity ($F_{48,480}=0.82$, $p>0.05$). Moreover, HR variability (RMSSD) did not differ between the lines during the light or dark phases ($F_{1,104}=0.343$, $p>0.05$).

Novelty exposure

Novelty exposure was used to determine the effects of an unconditioned challenge on locomotor activity and autonomic parameters in HAB and NAB mice. There was no line × novelty interaction for locomotor activity ($F_{1,60}=3.57$, $p>0.05$; Fig. 2*a*), HR ($F_{1,60}=0.21$, $p>0.05$; Fig. 2*b*) or HR variability ($F_{1,60}=1.25$, $p>0.05$; Fig. 2*c*). ANOVA yielded a significant novelty effect for locomotor activity ($F_{1,60}=566.99$, $p<0.001$; Fig. 2*a*) indicating a significant increase in locomotor activity in both lines during novelty exposure compared to baseline values. A significant novelty effect was also found for HR ($F_{1,60}=692.38$, $p<0.001$, Fig. 2*b*) and HR variability ($F_{1,60}=168.28$, $p<0.001$; Fig. 2*c*). While HR levels were increased by novelty in both lines compared to baseline values, HR variability was reduced.

Freezing, HR and HR variability in conditioned fear retention tested with air stream as US

Next, we tested whether there was a difference in the autonomic/behavioural responses in a cued fear-conditioning experiment using air stream as US. At the end of the conditioning session, HAB and NAB mice showed similar levels of freezing and activity levels (Supplementary Figs S1A and S1B). However, increased HR and reduced HR variability (Supplementary Figs S1B and S1C) was observed in HAB mice suggesting that the enhanced autonomic response observed in HAB mice might be due to the anxiety trait rather than induced by the challenge *per se*.

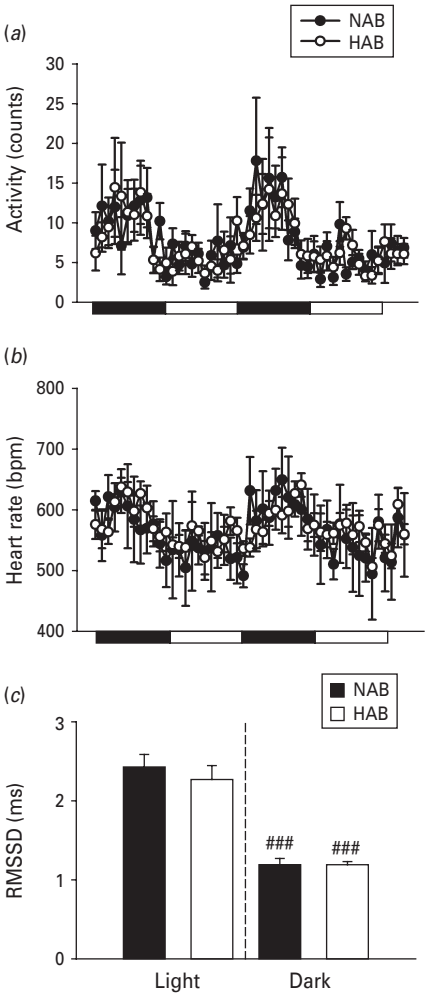

Fig. 1. High anxiety behaviour (HAB) and normal anxiety-like behaviour (NAB) mice displayed similar locomotor activity and heart rate (HR) patterns in the home cage. Data collected over 48 h of recordings showed comparable patterns in (*a*) spontaneous locomotor activity and (*b*) HR [expressed as beats per min (bpm)]; changes in HAB (white symbols) and NAB mice (black symbols) during the dark and light (indicated by black and white bars, respectively, below the x axis) phases. (*c*) HAB and NAB mice also exhibited similar basal HR variability (root mean square of successive RR interval differences; RMSSD) in the dark and light phases. Data are means ± S.E.M. ($n=6$/line). ### $p<0.001$ dark *vs.* light phase.

During the fear retention test, there was a significant line × CS interaction for freezing behaviour ($F_{1,28}=111.97$, $p<0.001$; Fig. 3*a*) *Post-hoc* tests showed that the fear response was higher in HAB than in NAB mice during CS exposure (Fig. 3*a*). In both lines,

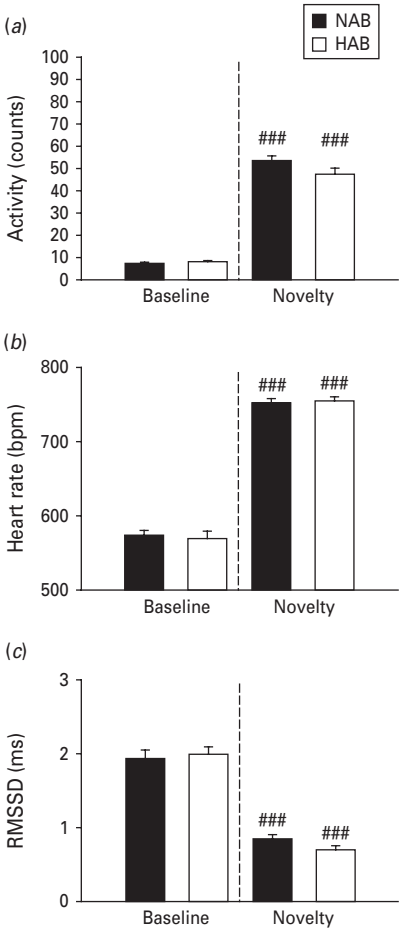

Fig. 2. High anxiety behaviour (HAB) and normal anxiety-like behaviour (NAB) mice displayed similar basal and stress-induced locomotor activity and heart rate (HR) patterns upon novel cage exposure. Compared to baseline values, exposure to a novel environment in both lines caused an increase in (*a*) locomotor activity and (*b*) HR [expressed as beats per min (bpm)]; whereas (*c*) HR variability (root mean square of successive RR interval differences; RMSSD) was reduced. Locomotor activity and autonomic responses did not differ between HAB (□) and NAB mice (■) under both basal and novelty conditions. Data are means ± S.E.M. ($n = 8$/line). $^{###}p < 0.001$ novelty *vs.* baseline.

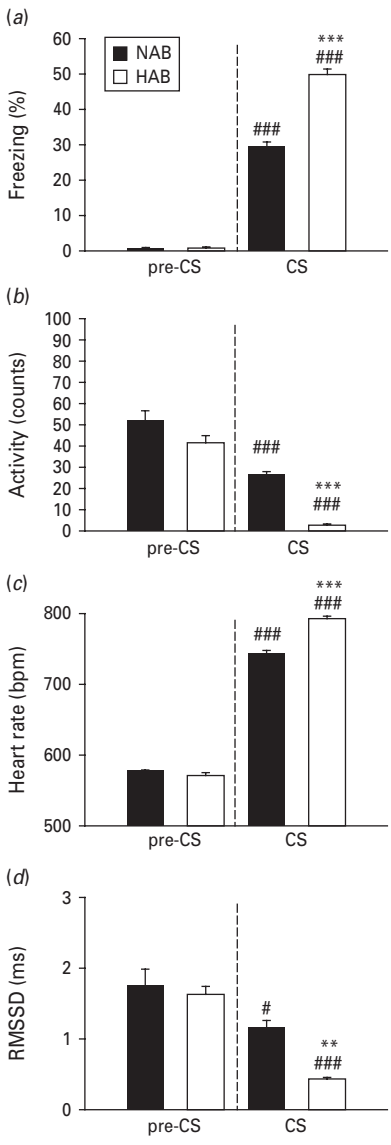

Fig. 3. High anxiety behaviour (HAB) mice showed increased fear expression, pronounced tachycardia, and reduced heart rate (HR) variability in retention of cued-conditioned fear tested using air stream as unconditioned stimulus. (*a*) Upon conditioned stimulus (CS) presentation, fear expression as indicated by the percentage of freezing was elevated in both lines but significantly more in HAB (□) than in normal anxiety-like behaviour (NAB) (■) mice. (*b*) Both, NAB and HAB mice displayed a reduction in locomotor activity upon CS exposure. The decrease in locomotor activity was more pronounced in HAB mice than in NAB mice. (*c*) CS induced a tachycardic response in both lines with an exacerbated increase of HR in beats per min (bpm) in HAB mice. (*d*) In contrast, CS presentation reduced HR variability (root mean square of successive RR interval differences; RMSSD) in both lines. Data are means ± S.E.M. ($n = 8$/line). $^{**}p < 0.01$, $^{***}p < 0.001$ HAB *vs.* NAB; $^{\#}p < 0.05$, $^{###}p < 0.001$ CS *vs.* pre-CS values.

freezing was higher during the CS phase than during pre-CS phase (Fig. 3*a*). Furthermore, a significant line × CS interaction was found for locomotor activity ($F_{1,60} = 5.54$, $p < 0.001$; Fig. 3*b*). *Post-hoc* test revealed reduced locomotor activity of HAB mice upon CS exposure compared to NAB mice (Fig. 3*b*). A significant line × CS interaction was found for HR ($F_{1,60} = 59.50$, $p < 0.001$; Fig. 3*c*). *Post-hoc* tests revealed higher tachycardic responses in HAB mice than in NAB mice (Fig. 3*c*). Indeed, upon CS exposure HAB mice

reached maximum physiological HR levels of up to 820 bpm that are higher than those previously reported for other mouse lines (Stiedl *et al*. 1999). Furthermore, there was a significant line × CS interaction for HR variability ($F_{1,60}=4.78$, $p<0.05$; Fig. 3*d*). *Post-hoc* tests yielded a reduced HR variability (RMSSD) in HAB mice compared to NAB mice (Fig. 3*d*). Finally, Pearson coefficient demonstrated a highly significant negative correlation (Supplementary Fig. S2) between freezing responses and locomotor activity during the retention test indicating that telemetrically determined locomotor activity can also serve as an index of fear response.

HR and HR variability in auditory fear retention in the home cage

Next, we investigated HR and HR variability responses to a fear retention test performed in the home cage. Locomotion, as indicated from the previous correlation analysis, was taken as an indirect measure of fear response because the lid of the cage prevented direct visual assessment of freezing. There was a significant line × CS interaction for locomotor activity ($F_{1,60}=8.31$, $p<0.01$; Fig. 4*a*). *Post-hoc* comparison indicated reduced locomotor activity upon CS presentation compared to baseline conditions. HAB mice displayed lower activity than NAB mice upon CS exposure (Fig. 4*a*). There was no line × CS interaction for HR ($F_{1,60}=0.24$, $p>0.05$; Fig. 4*b*). A main significant CS effect was found indicating increased HR levels in both lines during CS presentation ($F_{1,60}=834.58$, $p<0.001$; Fig. 4*b*) compared to baseline values. There was a significant CS phase × line interaction for HR variability (RMSSD) ($F_{1,60}=5.20$, $p<0.05$; Fig. 4*c*). Compared to baseline values, *post-hoc* comparison showed reduced HR variability (RMSSD) in both lines during CS presentation. HR variability (RMSSD) was lower in HAB mice than in NAB mice during CS exposure.

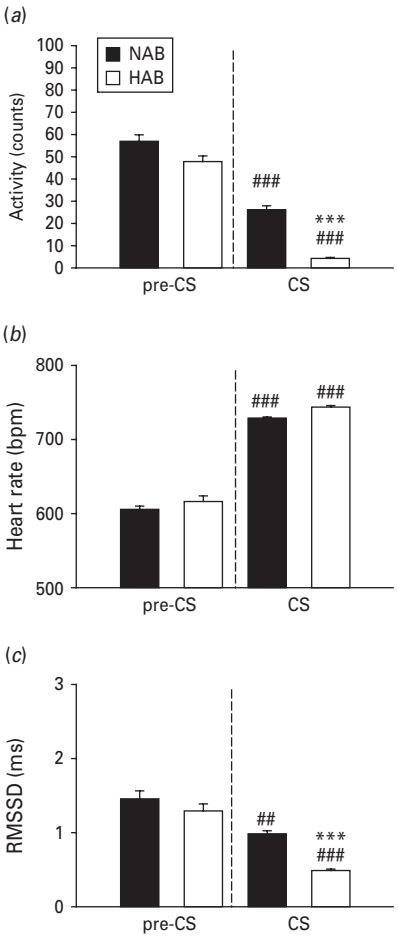

Fig. 4. High anxiety behaviour (HAB) mice exhibited reduced locomotor activity and heart rate (HR) variability to conditioned auditory fear in the home cage. (*a*) Presentation of conditioned stimulus (CS) induced a significant reduction in locomotor activity in HAB (□) and normal anxiety-like behaviour (NAB) (■) mice. The reduction in activity levels was more pronounced in HAB mice compared to NAB mice. (*b*) The CS presentation induced an increase in HR rate in beats per min (bpm) along with a decline in (*c*) HR variability (root mean square of successive RR interval differences; RMSSD) in both lines. However, significantly lower HR variability (RMSSD) was detected in HAB mice than in NAB mice. Data are means ± S.E.M. ($n=8$/line). *** $p<0.001$ HAB *vs*. NAB; ## $p<0.01$, ### $p<0.001$ CS *vs*. pre-CS values.

Effects of the NK1 receptor antagonist L-822429 on conditioned fear retention

Finally, we tested the effects of chronic NK1 receptor antagonist treatment on altered autonomic responses in HAB mice and investigated in particular whether HR variability is sensitive to behaviourally successful anxiolytic treatment. This was studied by means of a classical fear-conditioning paradigm with footshock/sound as the CS/US pairing. During fear conditioning both mouse lines expressed similar levels of fear arguing against an influence of possible analgesic actions of the NK1 receptor antagonist in this experiment (Supplementary Fig. S3).

A significant CS × treatment interaction was observed for the freezing response ($F_{1,24}=29.47$, $p<0.001$; Fig. 5*a*). *Post-hoc* comparison showed lower freezing during CS exposure in drug-treated compared to untreated HAB mice. Furthermore, no significant CS × treatment interaction for locomotor

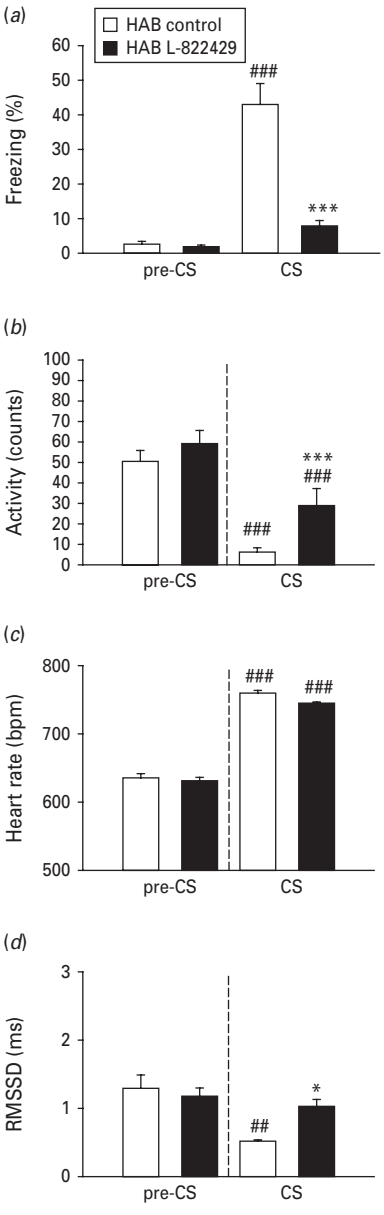

Fig. 5. Chronic treatment with the NK1 receptor antagonist L-822429 caused a reduction in fear expression and a normalization of heart rate (HR) variability in high anxiety behaviour (HAB) mice. (*a*) The conditioned stimulus (CS) presentation produced significant increased fear expression in untreated (□) but not drug-treated (■) HAB mice. (*b*) Both control and L-822429-treated HAB mice displayed a reduction in locomotor activity upon CS exposure, which was less pronounced in the treatment group. (*c*) CS presentation elicited elevated HR [beats per min (bpm)] in both groups. (*d*) Compared to HAB controls, L-822429-treated HAB mice displayed an increased HR variability (root mean square of successive RR interval differences; RMSSD) upon CS exposure. Data are means ± S.E.M. ($n = 7$/group). * $p < 0.05$, *** $p < 0.001$ L-822429-treated *vs.* untreated HAB groups; ## $p < 0.01$, ### $p < 0.001$ CS *vs.* pre-CS values.

activity was observed ($F_{1,32} = 1.32$, $p > 0.05$; Fig. 5*b*). However, a main treatment effect was found ($F_{1,52} = 6.96$, $p < 0.05$; Fig. 5*b*) indicating that NK1 receptor antagonist-treated HAB mice displayed higher locomotor activity than HAB control mice. Furthermore, Student's *t* test revealed similar locomotor pre-CS activity between drug-treated compared to untreated HAB mice ($t_{14} = -1.06$, $p > 0.05$).

No significant CS × treatment interaction was observed for HR ($F_{1,52} = 0.45$, $p > 0.05$; Fig. 5*c*). However, a main CS effect ($F_{1,52} = 668.79$, $p < 0.001$) but not a treatment effect ($F_{1,52} = 2.60$, $p > 0.05$) was observed indicating similar elevation in HR during CS exposure in both groups.

There was a significant CS × treatment interaction for HR variability ($F_{1,52} = 3.85$, $p < 0.05$; Fig. 5*d*). *Post-hoc* comparison indicated enhanced HR variability in NK1 receptor antagonist-treated compared to untreated HAB mice during CS presentation. HR variability did not differ under baseline conditions (pre-CS values) between treated and control HAB mice.

HR/HR variability curve analysis

The relationship between HR (RR interval) and its variability (HR variability, quantified by the RMSSD measure) was compared in HAB and NAB mice (Fig. 6). The linear relationship between HR *vs.* RMSSD was determined in HAB and NAB mice along the whole dynamical range of HR encountered during baseline measurements and after HR adjustments in response to CS. The results indicated that HR variability is inversely related to absolute HR. Significant correlations between HR and HR variability were determined in NAB mice ($F_{1,406} = 416.86$, $p < 0.001$) and HAB mice without treatment ($F_{1,228} = 56.44$, $p < 0.001$) and after chronic NK1 receptor antagonist treatment ($F_{1,91} = 8.75$, $p < 0.01$). The linear relationship approached minimum RMSSD values at maximum HR (generally ~800 bpm; RR interval ~75 ms). However, maximum HR values approached 850 bpm (RR interval ~70 ms) in HAB mice. Chronic treatment with the NK1 antagonist L-822429 reduced the slope of the RR/RMMSD relationship below the slope observed in NAB mice, i.e. resulted in a higher regularity of HR dynamics, and appeared to have limited maximum HR to values below 800 bpm.

Sensitivity, specificity and ROC curves

Analysis of the AUC assessed the diagnostic accuracy distinguishing between normal and high anxiety trait in the three sets of fear-conditioning experiments (Fig. 7). The AUCs for HR variability in the three sets

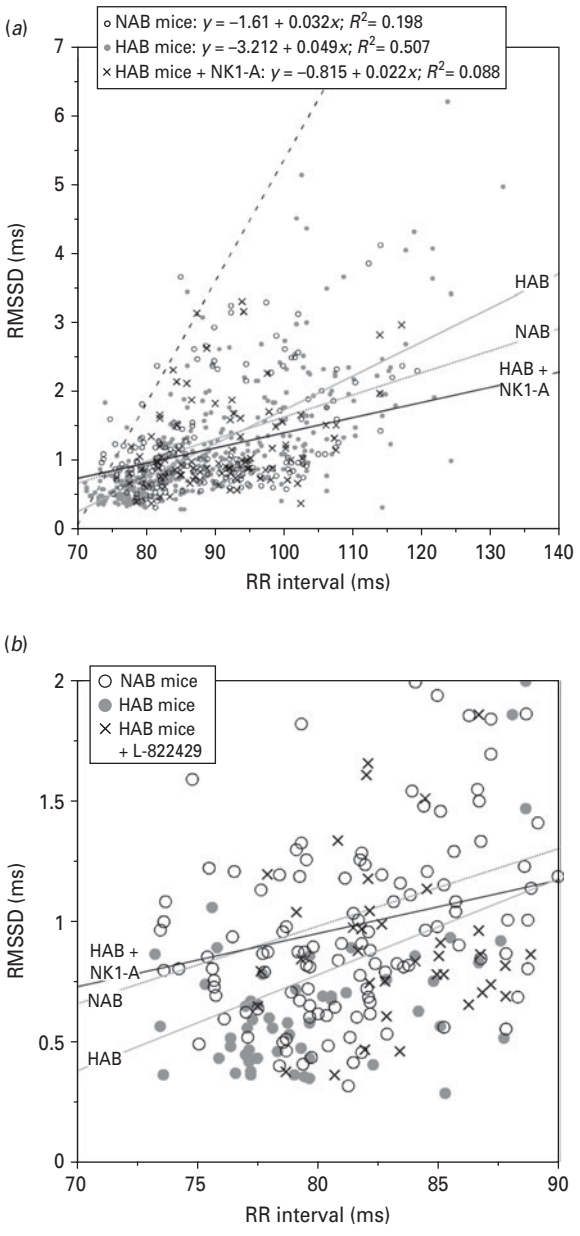

Fig. 6. RR *vs.* RMSSD correlation analysis of high anxiety behaviour (HAB) and normal anxiety-like behaviour (NAB) mice across the different fear-conditioning experiments. (*a*) The correlation analysis shows that RR intervals are negatively correlated with root mean square of successive RR interval difference (RMSSD) values. Moreover, the lines show a different steepness of slope of the linear HR/RMSSD correlation function that is further shifted to reduced increase of heart rate (HR) variability with decreasing HR (increasing RR interval) upon treatment with the NK1 antagonist L-822429. (*b*) The zoom depicts the range of maximum HR/minimum HR variability in NAB mice and untreated *vs.* L-822429-treated HAB mice. The dashed line in panel (*a*) denotes the RR/RMSSD correlation in C57BL/6N mice for comparison (data as reported by Tovote *et al.* 2004).

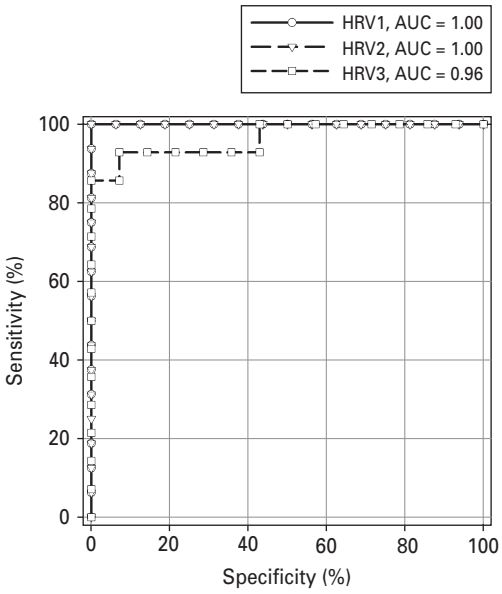

Fig. 7. ROC curves of heart rate (HR) variability during the different fear-conditioning paradigms for discriminating mice with high anxiety behaviour (HAB) from those with normal anxiety-like behaviour (NAB). –○–, HR variability analysis (HRV1) during the first conditioned fear experiment; –▽–, HR variability analysis (HRV2) during home-cage conditioned fear; and –□–, HR variability analysis (HRV3) for the NK1 receptor antagonist fear-conditioning treatment.

of fear-conditioning experiments were 1.00, 1.00 and 0.964 ± 0.033. The Z scores did not reveal any statistical difference between the AUC of the three experiments. The optimal cut-off of HR variability levels determined from the ROC curves were as follows: 0.587 ms for the first conditioned fear experiment, 0.723 ms for the home-cage fear conditioning and 0.633 ms for the experiment with NK1 receptor antagonist treatment. The sensitivity and specificity for the cut-off values of >0.587 ms and >0.723 ms demonstrated 100% specificity and sensitivity, whereas the cut-off value of >0.633 ms demonstrated a sensitivity and specificity of 92.9%.

Discussion

The main finding of the present study is that HR variability is reduced in HAB mice compared to NAB mice as consistently revealed in fear retention tests using different (auditory and visual) fear-conditioning paradigms. This altered autonomic state was behaviourally associated with an enhanced fear response. NK1 receptor antagonist treatment reduced the fear response and altered autonomic response (i.e. increased HR variability at high HR but decreased HR

variability at low HR) to NAB levels. These findings further demonstrate that changes observed in the behavioural response are strongly associated with changes in the cardiovascular system and thereby alteration to the sympathovagal balance may account for the pathological high anxiety of HAB mice. ROC curve analysis validated RMSSD, as a measure of HR variability, with high specificity and sensitivity in distinguishing between normal *vs.* high trait anxiety. Finally, the altered autonomic function of HR dynamics that was observed in HAB further validated the mouse model, as it resembles the altered autonomic regulation observed in patients suffering from anxiety disorders (Cohen & Benjamin, 2006; Friedman, 2007).

Basal autonomic regulation between HAB and NAB mice

In the present study, HAB and NAB mice exhibited similar circadian locomotor activity recorded by the radio-transmitter supporting a previous study using a different technique (Kromer *et al*. 2005). Comparable locomotor activity levels were observed in another study in C57BL/6J mice using the same device (Van Bogaert *et al*. 2006). Unaltered locomotor activity observed in different mouse lines demonstrates that the ECG transmitter does not appear to affect the behavioural performance of mice after appropriate recovery period (Stiedl *et al*. 2004).

Resting HR and HR variability did not differ between HAB and NAB mice. These findings parallel those of a study which reported that a rat model selectively bred for high ultrasonic vocalization (USV), exhibiting enhanced anxiety and depression-related behaviour, displayed similar HR and HR variability to low USV-emitting rats under resting conditions but not under emotionally challenging conditions (Brunelli & Hofer, 2007). Finally, a number of studies in patients suffering from anxiety disorders failed to find differences in autonomic regulation during resting conditions (for review see Friedman, 2007). Thus, in HAB and NAB mice the breeding strategy based on a selection criterion of behavioural difference (i.e. open arm time on the elevated plus maze), did not significantly affect basal homeostatic mechanisms involved in locomotor activity and HR regulation.

Autonomic and behavioural responses during novelty exposure

There is evidence that changes in autonomic regulation might be detected, particularly when the system is challenged (for review see Berntson *et al*. 1998). Exposure of rodents to a novel environment produces autonomic adjustments (e.g. tachycardia) as a result of unpredictability and unfamiliarity as well as physical activity during exploration. Therefore, following a baseline recording period in the home cage, behavioural and autonomic responses of HAB and NAB mice were determined to novelty exposure to a new cage. Novelty exposure produced an increase in locomotor activity and was associated with increased HR and decreased HR variability in both lines. However, no differences between lines were found. High tachycardic responses recorded in both HAB and NAB mice reached similar levels to those reported in C57BL/6N mice (Tovote *et al*. 2004) indicating that the general dynamical range of cardiovascular regulation was not altered in both lines. Hence, a challenge of increased aversiveness such as auditory fear conditioning was employed to test whether a perturbed autonomic system might be present in the case of learned fear.

Fear and autonomic response during visual fear conditioning

As outlined in the Introduction, most fear-conditioning protocols use footshock to elicit freezing behaviour. Alternatively, an air stream used as tactile US in aversive conditioning protocols (Landers & Sullivan, 1999) which has been shown to activate fear-related brain areas (Salchner *et al*. 2006), might be considered as less pain-related compared to footshock yet generating similar behavioural responses (e.g. similar USV levels) (Knapp & Pohorecky, 1995). Hence, we evaluated cardiovascular and fear responses provoked by this non-painful yet aversive US paired with light as CS. The behavioural fear response after five CS/US pairings indicated similar levels of fear ($\sim$40% freezing) in both lines. Locomotor activity was also similar in both lines. However, the behavioural response was associated with higher HR and reduced HR variability in HAB mice compared to NAB mice.

Although it was possible to quantify HR variability, movement-related artifacts confounded the ECG recording during air-stream stimulation and required precise manual editing for quantification of HR variability. RMSSD is highly sensitive to artifacts and ectopics produced to the ECG signal if unrecognized. Nevertheless, this kind of analysis was necessary to determine the cardiovascular responses concomitantly with the behavioural responses.

Despite similar fear levels during conditioning in both mouse lines, HAB mice showed higher fear response and lower locomotor activity than NAB mice during the retention test. The behavioural response

was associated with higher HR and reduced HR variability responses indicating that physical activity was not the main determinant of the tachycardia. Comparable cardiovascular responses have been observed in other C57BL/6 substrains tested in auditory fear conditioning (Stiedl *et al*. 1999). Since mice are highly sensitive to any interference by an experimenter (Stiedl *et al*. 2004), it cannot be excluded that the HR and HR variability responses recorded during our experiments were in part affected by the human interaction with the mice. The findings of reduced HR variability after handling or novelty exposure are in line with studies employing animal models of increased anxiety upon phasic challenges (e.g. involving chronic mild stress, or social isolation, or gene knockout) (Cohen *et al*. 2007; Costoli *et al*. 2004; Grippo *et al*. 2007; Groenink *et al*. 2003; Spani *et al*. 2003).

To isolate the CS-induced cardiovascular response from unspecific stressors, we performed a retention test of conditioned fear in the home cage.

Reduced HR variability in auditory fear retention in the home cage

Retention tests of cued auditory conditioned fear can be performed in the home cage thereby exploiting autonomic readouts undisturbed from any unspecific interference (for review see Stiedl *et al*. 2009). Therefore, we used this successful approach in our experiments. During CS presentation, both HAB and NAB mice showed a clearly reduced locomotor activity indicative, as shown by previous correlation analysis, of enhanced fear. The activity suppression was more pronounced in HAB mice than in NAB mice. During the fear response, lower HR variability was observed in HAB than in NAB mice. In contrast, HR did not differ between the lines indicating that the difference in HR responses observed in the air-stream fear-conditioning experiment was indeed the outcome of emotional (CS-induced) plus physical (experimenter handling) challenge.

Few studies have attempted to address the impact of emotional challenge on the cardiovascular response in inbred mouse strains using a familiar environment (reviewed in Stiedl *et al*. 2009). The authors of that series of studies conclude that environmental challenge, such as novelty exposure, increases physical activity (as shown also in the present study) and thus may easily elevate baseline HR to the level of aversive challenge such as fear retention test. Thereby, future studies should try to determine whether autonomic effects are attributable to anxiety, or simply to increased locomotor activity.

In light of the findings presented here, it appears that reduced HR variability, but not HR, is the only reliable marker of enhanced fear/anxiety response in our mouse model. Thus, modulation of the autonomic response in HAB mice was studied in response to effective anxiolytic drug treatment.

Effects of anxiolytic drug treatment on HR variability

NK1 receptor antagonists have been shown to elicit anxiolytic effects in animals and humans (see Introduction). Previous experiments using the light/dark test demonstrated reduction of anxiety-like behaviour in HAB mice by chronic treatment with the NK1 receptor antagonist L-822429 (S. B. Sartori *et al.* unpublished observations). We have now revealed that chronic treatment with this NK1 receptor antagonist greatly attenuated the freezing response and increased the locomotor activity in the fear retention test, indicating a profound fear-reducing effect in HAB mice. Interestingly, fear acquisition was not affected, as freezing responses were similar in control and L-822429-treated mice following five sound/footshock pairings. Additionally, pre-CS freezing and activity levels were similar between the lines excluding sedative effects often described with BDZ treatment (see Introduction). The fear-reducing effect is consistent with that observed using other NK1 receptor antagonists in unselected rodents (Rupniak *et al*. 2003; van der Hart *et al*. 2005).

NK1 receptor antagonist treatment increased HR variability during CS presentation to the level of NAB mice, while basal and CS-induced HR responses remained unaffected, despite somewhat reduced steepness of the slope of the HR/HR variability function. Interestingly, acute intracerebroventricular administration of the NK1 receptor antagonists was shown to reduce HR (Culman *et al.* 1997, 2010).

For a better understanding of modification to the sympathovagal balance mediated by the drug treatment the HR/HR variability correlation analysis was conducted incorporating all experimental data. This correlation analysis was previously used for a qualitative description of effects of pharmacological intervention (Tovote *et al*. 2004) indicating a reduced steepness of slope by intracerebroventricular injection of an NPY1 receptor agonist that is attributed to blunted sympathetic activity. The steepness of the slope of the HR/HR variability correlation was much lower in both HAB and NAB mice than previously reported in C57BL/6N mice (Tovote *et al*. 2004). This result indicates an altered sympathovagal antagonism

compared to C57BL/6N and suggests that the steepness of slopes is under genetic control as previously shown in humans (Kupper *et al*. 2004) but opposed to recent claims on HR variability of different strains of mice (Howden *et al*. 2008). Furthermore, the NK1 antagonist L-822429 reduced the steepness of the RR/RMMSD relationship, almost to the level of NAB mice, i.e. resulted in a more regular HR dynamics. In line with the present findings, successful anxiolytic/antidepressant treatment is associated with increased HR variability upon challenge but not under resting condition (Cohen *et al*. 2000; Fraguas *et al*. 2007). BDZs, such has diazepam, have been reported to increase HR variability (Ikeda *et al*. 1994) but results are inconsistent (Agelink *et al*. 2002). In contrast, reduced basal HR variability has also been reported after long-term antidepressant treatment in humans (Licht *et al*. 2010). Conceptually, an increase in HR variability is thought to be physiologically favourable and successful anxiolytic treatment should restore the potentially altered cardiovascular dynamics towards an enhanced parasympathetic tone combined with attenuated sympathetic activity by decreasing altered limbic (amygdala) activity previously observed in our mouse model (Muigg *et al*. 2009), resulting in normalization of dysfunctional brain–heart interaction.

RMSSD – from HR variability to biomarker?

The experiments suggest that RMSSD may serve as indicator to distinguish normal from pathological trait anxiety. Specifically, optimal cut-off calculated indicate a range or RMSSD values (between 0.587 and 0.723 ms) above which HR variability is considered 'non-pathological' and may be used as reference values for future testing of new anxiolytic drugs in HAB mice.

A recent study has provided evidence of the usefulness of integrating HR variability assessment into the context of a broader analysis spectrum that takes into consideration the interaction of dysfunctional physiological systems of the whole organism (Cerutti *et al*. 2009). This holistic approach considers the disorder-related dysfunction occurring concomitantly at several organs (i.e. central and autonomic nervous system, respiratory system, immune system, endocrine–metabolic systems). The approach combined with multivariate statistical analysis and nonlinear associations between different physiological signals could provide fundamental novel information, which might not only contribute to improved risk stratification in humans but also to a better understanding of the symptom progression with earlier diagnosis. Therefore, this integral approach to monitor different physiological domains needs to be applied in the different rodent models of psychiatric disorders to more closely mirror the human pathology and improve its translational value.

Conclusion

Taken together the present study shows that elevated fear responses in HAB mice are extended to altered autonomic function indicating the importance of an integral characterization of anxiety-like behaviour involving physiological responses. The observed blunting of the hyperemotional responses in HAB mice by the NK1 receptor antagonist concomitant with normalization of the reduced HR variability may further indicate that anxiolytic effects of drug treatments in hyperanxiety might be studied by regularization of HR dynamics.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

Acknowledgements

This work was supported by a research grant from the Austrian Science Fund (FWF): NFN-S102 (to N.S.). The authors thank Christina Bauchmann for excellent animal care.

Statement of Interest

None.

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