Autonomic changes induced by provocative motion in rats bred for high (HAB) and low (LAB) anxiety-related behaviour: paradoxical responses in LAB animals.

Luca Carnevali[a], Paul L. Andrews[b], Inga Neumann[c], Eugene Nalivaiko[d] and Andrea Sgoifo[a]

[a]Department of Neuroscience, University of Parma, Italy; [b]Division of Biomedical Science, St George's University of London, UK; [c]Department of Behavioural and Molecular Neurobiology, University of Regensburg, Germany; and [d]University of Newcastle, Australia.

Corresponding author: A/Prof Eugene Nalivaiko
School of Biomedical Sciences and Pharmacy
University of Newcastle
Callaghan NSW 2308
Australia
Tel.: +61 2 4921 5620
Email: eugene.nalivaiko@newcastle.edu.au

ABSTRACT

In humans, associations between anxiety and nausea are reported but the underlying mechanisms are not known. Hypothermia is proposed to be an index of nausea-like behavior in rats, and utilising this and heart rate as outcome measures we investigated the response to provocative motion in rats selectively bred for high (HAB) and low (LAB) anxiety-related behaviors and in non-selected (NAB) rats. Core temperature and electrocardiogram were monitored in each group (N=10 per group) using telemetry, with or without circular motion (40min; 0.75Hz) and vehicle or diazepam (2mg/kg, i.p.) pre-treatment. Heart rate and time- and frequency-domain parameters of heart rate variability were derived from the electrocardiogram.There was no baseline difference in core temperature between the three groups (mean 38.0±0.1^{o}C), but HAB animals had a significantly lower resting heart rate (330±7 bpm) compared to LAB (402±5 bpm) and NAB (401±9 bpm). Animals in all groups exhibited hypothermia during motion (HAB: 36.3±0.1^{o}C; NAB: 36.4±0.1^{o}C; LAB: 34.9±0.2^{o}C) with the magnitude (area under the curve, AUC) of the response during 40-min motion being greater in LAB compared to NAB and HAB rats, and this was also the case for the motion-induced bradycardia. Diazepam had minimal effects on baseline temperature and heart rate in all groups, but significantly reduced the hypothermia response (AUC) to motion in all groups by ~30%. Breeding for extremes in anxiety-related behavior unexpectedly selects animals with low trait anxiety that have enhanced bradycardia and hypothermic responses to motion. Thermal and cardiovascular responses to motion were little different between HAB and NAB rats indicating that either hypothermia is not an index of a nausea-like state in rats, or that the positive correlation between anxiety and nausea demonstrated in humans does not exist in rats. The mechanism underlying the enhanced physiological responses in LAB requires more detailed study and may provide a novel model to investigate factors modulating motion sensitivity.

Key words: Anxiety, diazepam, hypothermia, motion sickness, nausea, rat.

1. INTRODUCTION

The neurobiology of nausea is relatively poorly understood, in contrast to the well-defined neural pathways for vomiting (emesis) [1]. Whilst vomiting is a reflex motor response evoked by activation of brainstem pathways primarily by vestibular, area postrema and abdominal vagal afferent inputs, nausea is the sensory experience induced by activation of the same inputs (usually at a lower intensity) but requiring mid- and fore-brain pathways (see [48] for review). The relatively poor understanding of nausea mechanisms is to a large extent due to the deficiency of preclinical models, as nausea is a subjective, self-reported experience in humans. Research on nausea (or a functionally equivalent sensation) in experimental animals is based on indirect measurements that are of arguable specificity and validity (e.g. modified behavior pattern [18, 27]; reduced food consumption and gastric stasis [4, 30]; pica, indicated by kaolin consumption [45, 51] or require conditioning (e.g. conditioned taste/food aversion [14, 16] and conditioned gaping [38, 39]). In addition, such measures are often species-specific (for review see [48]).

It is well documented, but not widely known, that in humans nausea is commonly associated with a fall in the core body temperature that is caused, at least in part, by local vasodilation at specific cutaneous sites (reviewed by [33]); this phenomenon is proposed to represent an evolutionary beneficial adaptive response to protect body metabolism during presence of a toxin [33]. We have recently demonstrated that similar hypothermic responses are present in rats (lacking an emetic reflex [19]) and in house musk shrews (*Suncus murinus,* an insectivore possessing emetic reflex) [9, 35]. We have proposed, based on several arguments, that the drop in body temperature might represent a biomarker of a nausea-like state in laboratory animals. Firstly, these responses are provoked by both centrally and peripherally acting emetic stimuli. Stimuli for which a characteristic thermoregulatory profile has been demonstrated include motion [9, 35], lithium chloride, copper sulphate [17], opiates [3] and apomorphine [8]. Secondly, differential pharmacological sensitivity of these responses in rats mirrors sensitivity in humans, such that lithium chloride-induced hypothermia is prevented by the histamine$_1$ receptor antagonist promethazine, whereas motion-induced hypothermia is sensitive to the muscarinic receptor antagonist scopolamine [17]. Thirdly, in house musk shrews motion-induced hypothermia precedes emetic episodes [35]. Fourthly, there is a similarity in the hypothermic responses of humans and rats in both timing and underlying mechanism [33], with an observed fall in body temperature, which occurs, at least in part, due to regionally specific cutaneous vasodilatation that favors heat loss [35].

In humans, there are associations between anxiety and nausea. Nausea and vomiting are among the key symptoms of generalized anxiety disorder and of panic attacks [31]. Anxious subjects are more susceptible to anticipatory and therapeutic-induced nausea and vomiting resulting from chemotherapy

[2, 21], and the level of nausea and vomiting following surgery is positively correlated with preoperative anxiety magnitude [28, 44]. Anxiety is considered to be a sensitising factor for air and motion sickness [23, 37, 42], and subjects with a better ability to handle stress have a lower risk of being motion sick [20]. In addition, when anxiety is conditioned to motion, it is considered to contribute indirectly [15], which is supported by studies in the cat, where naive animals exposed to a motion stimulus were relatively resistant, but responded on subsequent exposure [7]. An alternative explanation suggested by the authors was that the acute fear response, stress or anxiety associated with the first exposure in naive animals actually suppressed emesis [7]. It must be acknowledged that the evidence for associations between anxiety and nausea is not uniform. No correlations with the Big Five personality inventory were found with sensitivity to motion sickness [15]. In support, in a study of visually-induced motion sickness "state anxiety" was not significantly different between nausea sensitive and resistant subjects, although "trait anxiety" tended to show higher scores in the sensitive *vs.* resistant groups [12]. Interestingly, in patients with anxiety disorders there is evidence for a higher prevalence of peripheral vestibular dysfunction suggesting an increased vestibular sensitivity [13].

The principal aim of the current study was to determine, if there is an association between trait anxiety and nausea as reflected by hypothermia in rats. If so, this would further support the hypothesis that hypothermic responses to emetic stimuli are an index of a nausea-like state in animals, and also open the way to the mechanistic study of this association. Therefore, our study was conducted in three lines of Wistar rats selectively bred for high (HAB) or low (LAB), or non-selected for (NAB) anxiety-related behavior. The HAB/LAB rats have been proven to represent the extremes in trait anxiety, as revealed in a variety of behavioral tests [24, 25]. The behavioural differences are robust and reliable [46, 50]. Therefore, the use of these psychogenetically selected rats represents, in our view, a valid methodological approach for investigating the nausea-like state in animal populations that possess genetically determined differences in the level of baseline anxiety. Our principal hypothesis was that HAB rats would have significantly larger hypothermic responses to provocative motion compared to the two other lines, and that this difference could be reduced or prevented by anxiolytic treatment, such as diazepam.

2. MATERIAL AND METHODS

2.1. Animals and regulatory approval

Experimental procedures were carried out on 5-month-old male Wistar rats (350-450 g) selectively bred for high (HAB) and low (LAB) anxiety-related behavior non-selected rats (NAB). The animals were obtained from the animal facilities of the University of Regensburg (Germany), where they were tested at the age of 9 weeks on the EPM to confirm the selection criteria, and then retested prior to

implantation of telemetric transmitters at the start of the motion study. After arrival in the laboratory at the University of Parma, they were kept in groups of two according to anxiety status in standard home cages (39 cm x 24 cm) in rooms with controlled temperature (22±2°C) and a reversed light-dark cycle (light on from 19:00 to 07:00 h), with free access to food (standard diet 4RF21, Mucedola, Italy) and water. All experimental procedures followed the guidelines established by the Italian Council on Animal Care (Legislative Decree no. 26, March 2014) and European regulations (2010/63/UE) and were approved by the Veterinarian Animal Care and Use Committee of Parma University and the Italian Ministry of Health.

2.2. Preliminary behavioral testing for anxiety

Anxiety-related behavior was assessed in HAB, NAB and LAB rats using the EPM test [40]. The EPM consisted of 4 elevated arms (100 cm above the floor, 50 cm long and 10 cm wide) arranged in a cross-like position, with two opposite arms being enclosed (by means of 40 cm high walls), and two being open, including at their intersection a central square platform (10×10 cm) which gave access to the four arms. Each rat was initially placed on the central platform facing one closed arm and behaved freely for 5 min. The behavior during the test was recorded using a video camera mounted above the maze. The percentage of time spent on the open arms during the 5-min test and the number of entries in the open arms were assessed as anxiety-related behaviors [50].

2.3. Surgery: Radiotelemetry transmitter implantation

One week after the behavioral testing, animals were anesthetized with tiletamine hydrochloride + zolazepam hydrochloride (Zoletil, Virbac, France), 20 mg/kg, s.c.). Radiotelemetry transmitters (TA11CTA-F40, Data Sciences International, St. Paul, MN, USA) for electrocardiogram (ECG) and core body temperature (BT, °C) recordings were implanted in the abdominal cavity according to a procedure described in detail by Sgoifo and colleagues [47]. Post-operative analgesia was provided by buprenorphine (0.1 mg/kg s.c.). Immediately after surgery, rats were individually housed, injected for 2 days with gentamicin sulfate (Aagent, Fatro, 0.2ml/kg, s.c.) and allowed to recover for 10 days before the start of experimental recordings. Recovery from surgery was uneventful.

2.4. Provocative motion protocol

After recovery from surgery, HAB, NAB and LAB rats (n=10 for each group) were exposed to four experimental sessions conducted on different days, at least 72 h apart: (1) vehicle injection + rotation, (2) vehicle injection + no-rotation, (3) diazepam injection + rotation, and (4) diazepam injection + no-rotation. Experiments were conducted between 9 am and 3 pm. The sequence of experimental sessions was counterbalanced for every rat. On the day of the experiment, the home cage with an animal was positioned at the center of a custom-built turntable. Ten minutes later, rats received an i.p.

injection of either vehicle (1% Tween-80 saline solution) or 2 mg/kg diazepam (Sigma, USA; dose and time course of administration were chosen based on [10]). Thirty minutes after the injection, rats were either rotated on the turntable at 0.75 Hz (45 rpm) or not rotated for 40 min, according to our previous protocols [35] for induction of rotation-induced hypothermia. Animals were monitored for an additional 40min after the rotation/non-rotation period without removing the cage from the turntable.

2.5. Data acquisition and analysis

Body temperature (BT) and electrocardiographic (ECG; sampling frequency 1000 Hz) signals were picked up by platform receivers located under the experimental cages and continuously recorded throughout each experimental phase by means of ART-Gold 4.2 data acquisition system (Data Sciences Int., St. Paul, MN, USA). BT values were automatically generated for each 1-min time period and then further averaged as means of 2-min epochs. Subsequently, we calculated the area under the response curve (AUC) of BT values during the 40-min rotation period relative to the respective pre-rotation value (i.e., the mean of the last 10 min prior to rotation). ECG signal was visually inspected to ensure that all R-waves were correctly detected. Subsequently, we split each recording period into 2-min epochs. For each epoch, heart rate (HR) and time- and frequency-domain parameters of heart rate variability (HRV) were then quantified using ChartPro 5.0 software (ADInstruments, Sydney, Australia). HR was calculated by plotting the number of R waves per unit time (reported in beats per minute; bpm). For time-domain analysis of HRV, we calculated the root mean square of successive R-R ECG intervals (RMSSD, ms), which reflects vagal input to the heart [41]. For frequency-domain analysis of HRV (fast-Fourier transformation), we measured (i) the power of the low-frequency (LF; 0.2-0.75 Hz) and the high-frequency (HF; 0.75-2.5 Hz) bands, the latter reflecting respiratory-related vagal influences [43], and (ii) the LF to HF ratio, which is taken as a synthetic measure of sympatho-vagal balance, where smaller values indicate lower relative sympathetic dominance [41]. Finally, we calculated the AUC of HR and HRV parameters during the 40-min rotation period relative to the respective pre-rotation values (i.e., the mean of the last 10 min prior to rotation).

2.6. Statistical analysis

All statistical analyses were performed using the software package SPSS (version 22). For repeated measures data on BT, an average value was calculated for each 10-min recording period (min 0-10, min 10-20, etc.). Two-way ANOVAs were then applied, with time as within-subject factor and between-subject factors being either (i) the factor “rotation condition” (2 levels: rotation and no-rotation), (ii) the factor “group” (3 levels: HAB, NAB and LAB), or (iii) the factor “treatment” (2 levels: vehicle and diazepam). Data obtained from the elevated plus maze test and “area under the curve” (AUC) data were analyzed with one-way ANOVA. Follow-up analyses were conducted using Student’s t-tests, with a Bonferroni correction for multiple comparisons for each outcome variable separately. Correlations

between behavioral and physiological parameters were assessed using linear regression analysis (95% confidence interval). Data are presented as means ± standard error of the mean (S.E.M.). Statistical significance was set at $p<0.05$.

3. RESULTS

3.1. Behavior on the elevated plus-maze

The behavioral performance of HAB, NAB and LAB rats on the EPM prior to transmitter implantation is summarized in Table 1. As expected, HAB rats spent less time on the open arms of the EPM and entered them less frequently compared to LAB rats (% time open arms: $t=-21.3$, $p<0.01$; n° of entries: $t=-9.1$, $p<0.01$ versus LAB and NAB). NAB rats displayed intermediate anxiety-related behavior (% time open arms: $t=5.1$, $p<0.01$ vs. HAB; $t=-4.0$, $p<0.01$ vs. LAB) (n° of entries: $t=4.3$, $p<0.01$ vs. HAB; $t=-2.7$, $p<0.05$ vs. LAB).

Table 1. *Behavior of HAB, NAB and LAB rats (n=10 per group) on the elevated plus maze.*

	HAB	NAB	LAB
% time spent on open arms	$4.2 \pm 1.4^{a,b}$	32.0 ± 5.3	54.8 ± 1.9^{a}
n° of entries in open arms	$1.5 \pm 0.5^{a,b}$	5.8 ± 0.9	8.7 ± 0.6^{a}

Data are reported as means ± SEM. Significant differences (Bonferroni test, p values are reported in the text): ^{a} = vs. NAB value; ^{b} = vs. LAB value.

3.2. Provocative motion-induced changes in body temperature

Provocative motion-induced changes in BT of HAB, LAB and NAB rats are depicted in Figure 1A. Two-way ANOVAs for repeated measures yielded (i) a significant effect of “rotation” for BT in HAB ($F=101.7$, $p<0.01$), NAB ($F=56.6$, $p<0.01$) and LAB ($F=123.5$, $p<0.01$) rats, and (ii) a significant effect of “group” ($F=22.1$, $p<0.01$) for BT values during “rotation” condition. Follow-up analyses revealed that HAB, NAB and LAB rats had a similar BT during the pre-rotation phase. Provocative motion caused a consistent fall in BT, with absolute values being significantly lower during the last 10 min of rotation compared to the respective pre-rotation values in the three groups (HAB: $t=-10.9$, $p<0.01$; NAB: $t=-13.6$, $p<0.01$; LAB: $t=-14.6$, $p<0.01$) (Figure 1A). The magnitude (AUC values) of this hypothermic response was significantly larger in LAB compared to HAB ($t=6.4$, $p<0.01$) and NAB ($t=6.2$, $p<0.01$) rats, whereas no differences were observed between HAB and NAB animals (Figure 1B). There were no group differences in BT during “vehicle + no-rotation” condition (Figure 1C). There was no correlation between motion-induced hypothermia and anxiety score in individual groups; however when all animals were grouped together, a significant inverse correlation was found ($r^2=0.429$, $F=21.2$, $p<0.01$) as illustrated in Fig. 1D.

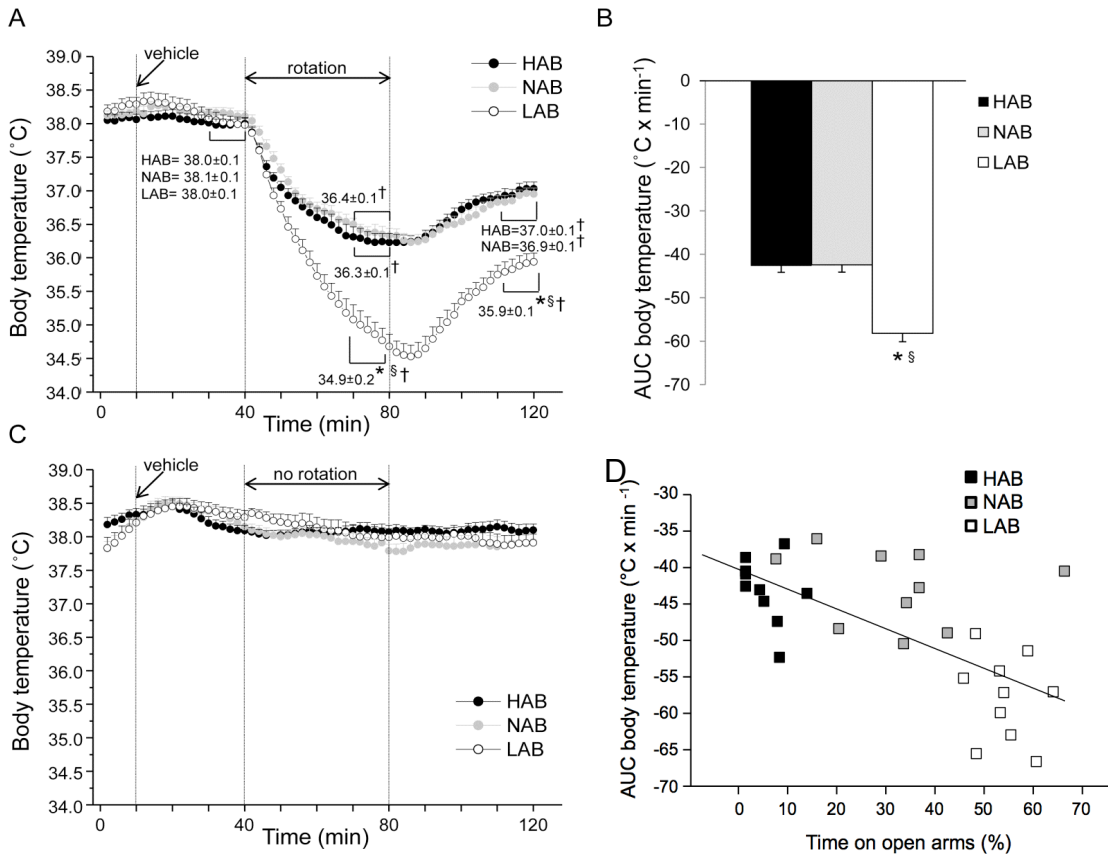

Figure 1. Time course of body temperature changes during "vehicle + rotation" (Panel A) and "vehicle + no-rotation" conditions in HAB, NAB and LAB rats (Panel C; n=10). Statistical analysis was conducted on 10-min epochs (i.e., 5 time-points). Mean values ± SEM are presented near corresponding traces. Panel B represents the area under the response curve (AUC) of body temperature during rotation relative to the respective pre-rotation value (i.e., the mean of the last 10 min prior to rotation). Data are shown as means ± SEM. Significant differences (Bonferroni test, p values are reported in the text): * vs. NAB value, § vs. HAB value, † vs. respective pre-rotation value. Panel D shows correlation between the AUC for body temperature and time on open arms (r^2=0.429, F=21.2, p<0.01).

3.3. Provocative motion-induced changes in heart rate and heart rate variability.

Provocative motion-induced changes in HR in HAB, LAB and NAB rats are depicted in Figure 2A. Two-way ANOVAs for repeated measures yielded (i) a significant effect of "rotation" for HR values in HAB (F= 46.1, p<0.01) and LAB (F= 7.8, p<0.05) rats, and (ii) a significant effect of "group" for HR values during "rotation" (F= 31.7, p<0.01) and "no-rotation" (F= 5.2, p<0.05) conditions. Follow-up analyses revealed that HAB rats had lower HR values compared to NAB (t=-8.4, p<0.01) and LAB (t=-8.4, p<0.01) rats during the 10 min prior to rotation. Similar differences were observed during "vehicle + no-rotation" condition (t=-3.9, p<0.01 vs. NAB; t=-3.4, p<0.01 vs. LAB) (Figure 2C). During the first 10 min of rotation, HAB, NAB and LAB rats showed significantly lower HR values compared to the respective pre-rotation values (HAB: t=-3.1, p<0.01; NAB: t=-2.3, p<0.05; LAB: t=-8.1, p<0.01) (Figure

2A). This difference persisted until the end of the rotation period (Figure 2A). The magnitude (AUC values) of this bradycardic response was significantly larger in LAB rats compared to HAB (t=3.3, p<0.01) and NAB (t=4.5, p<0.01) counterparts, whereas no differences were observed between HAB and NAB rats (Figure 2B). There was no correlations between changes in HR (assessed as AUC) and anxiety score (Fig. 2D).

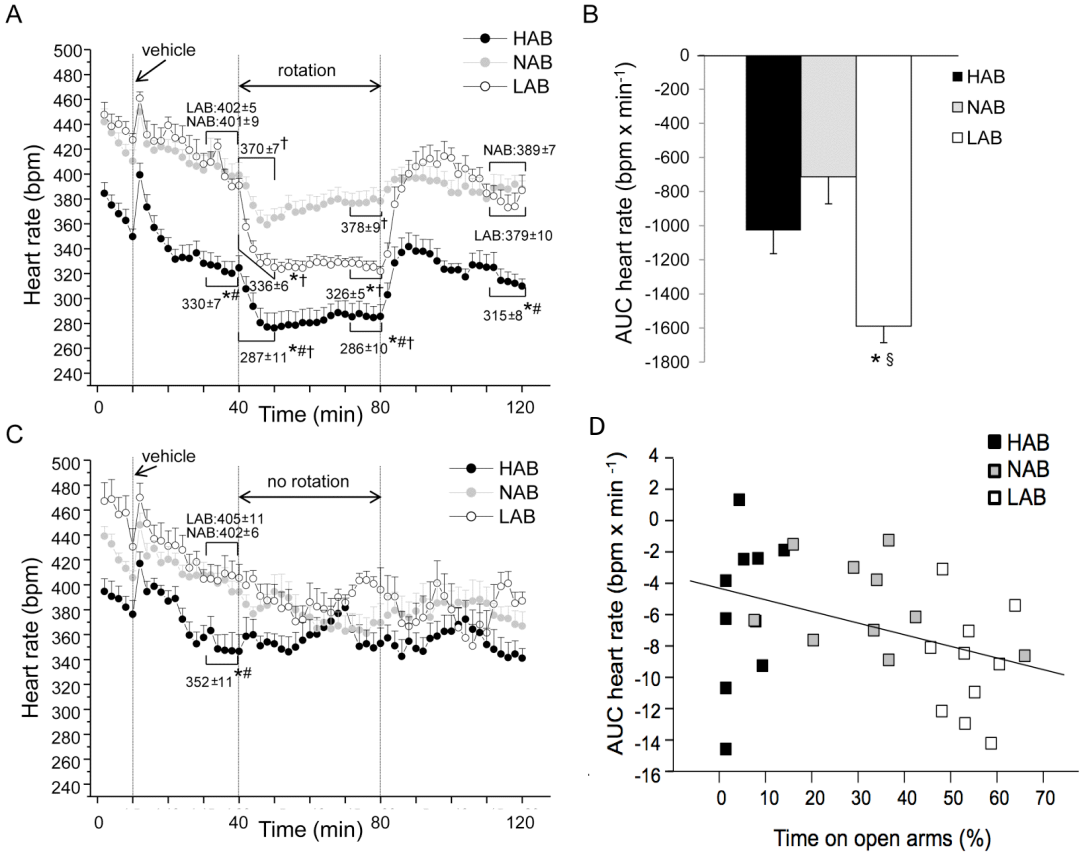

Figure 2. Time course of heart rate changes during “vehicle + rotation” (Panel A) and “vehicle + no-rotation” conditions in HAB, NAB and LAB rats (n=10). Statistical analysis was conducted on 10-min epochs (i.e., 5 time-points). Mean values ± SEM are presented near corresponding traces. Panel B represents the area under the response curve (AUC) of heart rate during rotation relative to the respective pre-rotation value (i.e., the mean of the last 10 min prior to rotation). Data are shown as means ± SEM. Significant differences (Bonferroni test, p values are reported in the text): * vs. NAB value, # vs. LAB value, § vs. HAB value, † vs. respective pre-rotation value. Panel D shows correlation between the AUC for HR and time on open arms.

Provocative motion-induced changes in HRV parameters are summarized in Table 2. Two-way ANOVA for repeated measures yielded a significant effect of “rotation” for RMSSD and HF values in HAB (F_{RMSSD}= 4.6, p<0.05; F_{HF}= 4.6, p<0.05) and LAB (F_{RMSSD}= 4.4, p<0.05; F_{HF}= 4.9, p<0.05) rats. Follow-up analyses revealed that during the pre-rotation phase, HAB, NAB and LAB rats showed similar values of RMSSD, HF power, and LF to HF ratio (Table 2). During the 40-min rotation period,

HAB and LAB rats showed significantly higher RMSSD and HF mean values compared to the respective pre-rotation values (HAB_{RMSSD}: t=2.2, p<0.05; HAB_{HF}: t=2.2, p<0.05; LAB_{RMSSD}: t=3.1, p<0.01; LAB_{HF}: t=3.6, p<0.01) (Table 2), which is indicative of a potent vagal activation. This effect was not observed in NAB rats (Table 2). However, HAB, NAB and LAB rats showed a significantly lower LF to HF mean ratio during rotation compared to the respective pre-rotation value (HAB: t=2.1, p<0.05; NAB: t=2.1, p<0.05; LAB: t=2.2, p<0.05), which is suggestive of a shift of the sympathovagal balance toward a lower sympathetic prevalence in all the three groups. There were no group differences in HRV parameters during "vehicle + no-rotation" condition. There were also no correlations between individual measures of HR and anxiety scores (Fig. 2D) or HRV and anxiety scores (data not shown).

Table 2. Heart rate variability parameters in HAB, NAB and LAB rats (n=10 per group) during "vehicle + rotation" and "diazepam + rotation" conditions.

	Vehicle condition	Pre-rotation	Rotation	Diazepam condition	Pre-rotation	Rotation
RMSSD (ms)	HAB	3.6±0.5	6.1±1.2[a,c]	HAB	3.8±0.4	4.7±0.6[c]
	NAB	3.3±0.3	3.5±0.4	NAB	3.2±0.3	3.4±0.3
	LAB	4.0±0.5	6.4±0.6[a,c]	LAB	3.6±0.4	5.1±0.6[c]
HF (ms^2)	HAB	4.7±1.2	15.3±4.9[a,c]	HAB	5.3±1.0	10.0±2.3[c]
	NAB	3.5±0.6	4.6±0.9	NAB	3.8±0.8	4.6±0.9
	LAB	5.5±1.2	14.4±1.8[a,c]	LAB	4.0±0.8	10.8±2.4[c]
LF/HF	HAB	0.8±0.1	0.5±0.1[c]	HAB	0.8±0.1	0.5±0.1[c]
	NAB	0.8±0.1	0.5±0.1[c]	NAB	0.6±0.1	0.4±0.1
	LAB	1.0±0.1	0.5±0.1[c]	LAB	0.8±0.2	0.4±0.1[c]

Data are reported as means ± SEM. Pre-rotation value is the mean of the 10 min prior to rotation. Rotation value is the mean of the 40-min rotation period. Significant differences (Bonferroni test, p values are reported in the text): [a] vs. NAB value; [b] vs. LAB value; [c] vs. respective pre-rotation value. Abbreviations: RMSSD = root mean square of successive RR intervals; HF= high frequency; LF = low frequency.

3.4. Effects of diazepam on provocative motion-induced changes in body temperature

The effect of diazepam-pretreatment on provocative motion-induced changes in BT in HAB, LAB and NAB rats is represented in Figure 3A, 3C and 3E, respectively. Two-way ANOVAs for repeated measures yielded a significant "treatment x time" interaction for BT values in HAB (F= 5.2, p<0.05), NAB (F= 12.2, p<0.01) and LAB (F= 10.4, p<0.01) rats. Follow-up analyses revealed that diazepam

administration provoked a significant reduction in pre-rotation BT values in HAB ($t=-2.9$, $p<0.05$) and LAB ($t= -2.2$, $p<0.05$) rats compared to the respective vehicle values (Figure 3A, 3C, 3E). Similarly to what was observed in vehicle-injected rats, provocative motion provoked a significant reduction in BT values in diazepam-injected rats compared to their respective pre-rotation values (HAB: $t=-7.4$, $p<0.01$; NAB: $t=-5.3$, $p<0.01$; LAB: $t=-7.4$, $p<0.01$). However, during the last 10 min of rotation BT values were significantly higher in diazepam-treated rats compared to vehicle-treated rats in the three groups (HAB: $t=2.2$, $p<0.05$; NAB: $t=2.3$, $p<0.05$; LAB: $t=1.9$, $p=0.08$). Consequently, the magnitude (AUC values) of rotation-induced hypothermia was significantly smaller in diazepam-treated rats compared to the respective control (vehicle) condition (HAB: $t=4.8$, $p<0.01$; NAB: $t=4.6$, $p<0.01$; LAB: $t= 3.5$, $p<0.05$) (Fig. 3B, 3D & 3F).

3.5. Effects of diazepam on provocative motion-induced changes in heart rate and heart rate variability parameters

The effect of diazepam-pretreatment on provocative motion-induced changes in HR is represented in Figure 4. Pre-rotation mean HR values did not differ between diazepam and vehicle-treatment in any of the three groups (Figure 4A, 4C & 4E). Similarly to what was observed in vehicle-injected rats, provocative motion provoked a significant reduction in HR values in diazepam-injected rats compared to their respective pre-rotation values (HAB: $t=-2.2$, $p<0.05$; NAB: $t=-2.2$, $p<0.05$; LAB: $t=-2.5$, $p<0.05$), but during the first 10 min of rotation, HR values were significantly higher in NAB ($t=2.5$, $p<0.05$) and LAB ($t=2.3$, $p<0.05$) rats, treated with diazepam compared to the respective vehicle condition, an drug-effect which was not found in HAB rats,. However, the magnitude (AUC values) of this rotation-induced bradycardia was similar between diazepam-treated and vehicle-treated rats in the three groups (Figure 4B, 4D & 4F). In addition, there were no differences in HRV parameters between “diazepam + rotation” and “vehicle + rotation” conditions in any of the three groups (Table 2).

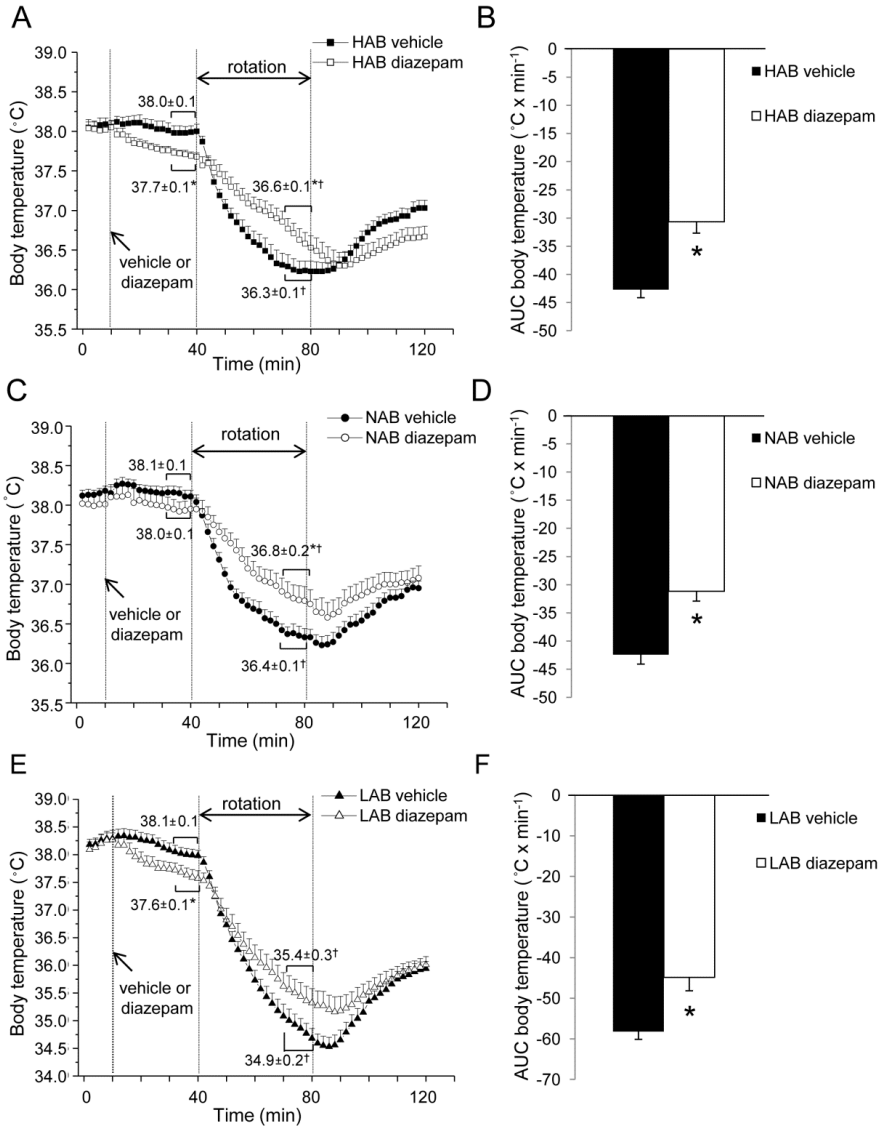

Figure 3. Panels on the left show the time course of body temperature changes during "vehicle + rotation" and "diazepam + rotation" conditions in HAB, NAB and LAB rats (n=10). Statistical analysis was conducted on 10-min epochs (i.e., 5 time-points). Mean values ± SEM are presented near corresponding traces. Panels on the right represent the area under the response curve (AUC) of body temperature during rotation relative to the respective pre-rotation value (i.e., the mean of the last 10 min prior to rotation). Data are shown as means ± SEM. Significant differences (Bonferroni test, p values are reported in the text): * vs. vehicle value, † vs. respective pre-rotation value.

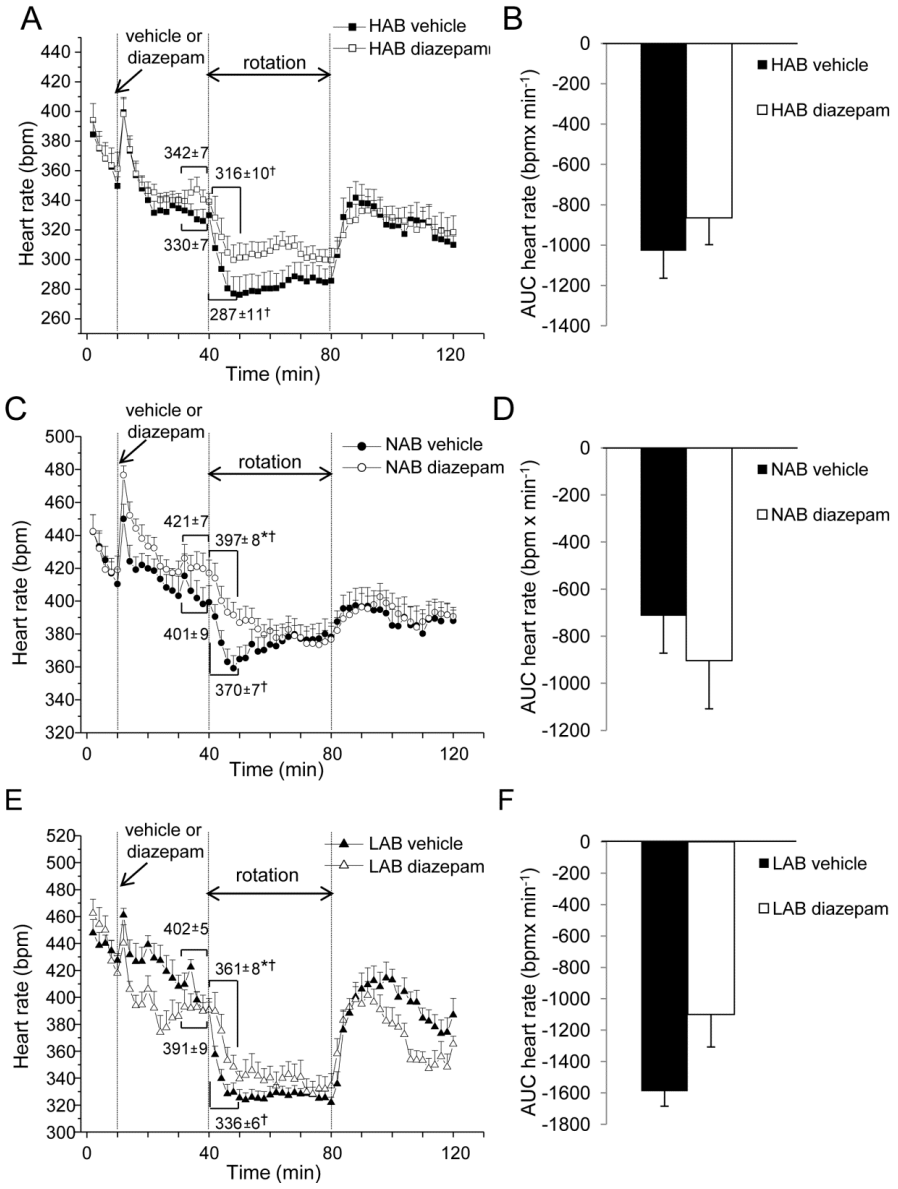

Figure 4. Panels on the left show the time course of heart rate changes during "vehicle + rotation" and "diazepam + rotation" conditions in HAB, NAB and LAB rats (n=10). Statistical analysis was conducted on 10-min epochs (i.e., 5 time-points). Mean values ± SEM are presented near corresponding traces. Panels on the right represent the area under the response curve (AUC) of heart rate during rotation relative to the respective pre-rotation value (i.e., the mean of the last 10 min prior to rotation). Data are shown as means ± SEM. Significant differences (Bonferroni test, p values are reported in the text): * vs. vehicle value, † vs. respective pre-rotation value).

3.6. Effects of diazepam on body temperature and heart rate

Diazepam administration reduced BT values compared to vehicle between 30 and 40 min of "no-rotation" condition, the difference being statistically significant only in HAB rats ($t=2.2$, $p<0.05$) (Figure 5). No HR changes were observed after diazepam administration compared to vehicle during "no-rotation" condition in any of the three groups (Fig. 5).

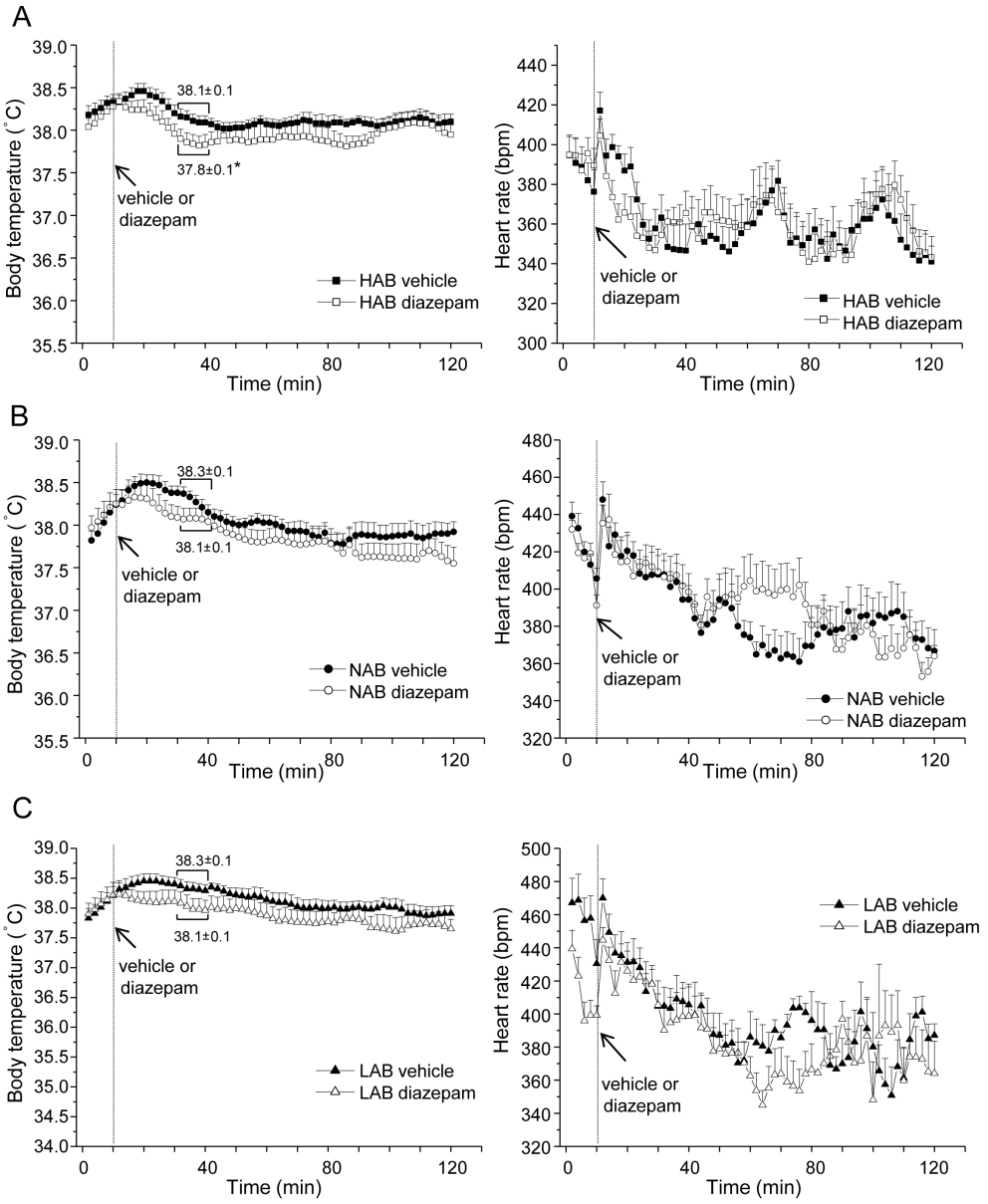

Figure 5. Time course of changes in body temperature (left panels) and heart rate during "vehicle + no-rotation" and "diazepam + no-rotation" conditions in HAB, NAB and LAB rats (n=10). Data are shown as means ± SEM. Significant differences: * vs. vehicle value.

4. DISCUSSION

The results of the present study have confirmed previous findings [9, 17, 35] showing that a fall in core temperature is a robust physiological response to a provocative motion stimulus in rats. The novel finding is that selective breeding for low anxiety as confirmed on the EPM identifies a LAB phenotype that clearly shows enhanced autonomic responses to motion. The typical thermoregulatory response to stress in rodents is hyperthermia [36], and thus it appears that the hypothermic response is highly specific for the motion stimulus and not a response to the generally stressful nature of sustained provocative motion. The mechanism underlying the *hypothermic* response is proposed to include vestibular system modulation of hypothalamic sympathetic outflow resulting in a decreased drive to brown adipose tissue and tail vasculature [35]. The hypothermic response observed in HAB, LAB and NAB rats was accompanied by bradycardia, thus confirming a previous study [9], with changes in heart rate variability parameters demonstrating that this fall in heart rate was a consequence of a shift of sympatho-vagal balance (reflected by LF:HF ratio), likely due to an increase in vagal drive (reflected by RMSSD and HF power).

In humans, the link between anxiety and nausea is well established (see Introduction). Therefore, and based on the assumption that hypothermia is a biomarker of a nausea-like state in rats, we hypothesised that rats selected for high (HAB) or low (LAB) anxiety states should show differences in the thermoregulatory response to motion. We further hypothesized that HAB rats should generally display a greater hypothermia response. Indeed, we could show that the hypothermic response to motion-induced nausea is dependent on the innate level of anxiety-related behavior. However, contrary to our expectations, the LAB rats had the largest magnitude of hypothermic and cardiovascular responses to motion. The basis for this unexpected response and the implications for the “hypothermia nausea hypothesis” [33] are discussed below.

4.1. Baseline differences between NAB, HAB and LAB rats.

As the relative magnitude of the hypothermic responses to motion in NAB, HAB and LAB animals was reverse to our expectation, the evidence for differences in the anxiety state of the animals must be considered. The animals originated from an established selective breeding colony and were classified as NAB, HAB or LAB based on their behavioural performance on the EPM. Significant differences between the breeding lines are consistent on the EPM as reflected, for example, by the percentage of time spent on the open arms and the percentage of number of open arm entries, among others, and have been repeatedly confirmed also in other tests for anxiety-related behavior such as the light-dark box and open field [6, 50]. The lower baseline heart rate in HAB rats of the present study is consistent with a previous study of heart rate in HAB and LAB rats [6], but as no significant differences were found in the baseline heart rate variability parameters, we have no insight into the underlying

mechanism, although a higher vagal tone appears likely. Whilst the rats are selected for high *vs.* low anxiety-related behaviour, our results suggest that this selection is linked to a number of underlying baseline physiological differences and unexpected variations regarding the thermoregulatory and cardiovascular responses to motion. We have in fact previously reported genetically driven differences in other physiological parameter such as an increased hypothalamic expression and release of vasopressin [32], differences in pain sensitivity [22], increased hormonal response of the HPA axis to novelty [26] and elevated frequency of breathing [5] in HAB rats.

The validity of the EPM as a method for the assessment of anxiety and hence anxiolytic drugs has been challenged, and it is suggested that it may be more appropriate to view this (and other tests such as zero-maze, light-dark box and open field) as a test of "preference for unlit and/or enclosed spaces" [11]. Here we discuss the results based upon the assumption that the elevated plus-maze test is stratifying the animals into high, normal and low anxiety phenotypes, but in view of concerns regarding the relevance of rodent studies in the EPM to anxiety disorders caution needs to be exercised in extrapolating findings to humans.

4.2. Responses of NAB, HAB and LAB rats to motion

All three lines showed a clear hypothermic response to motion, but the magnitude was substantially larger in LAB compared to both HAB and NAB, which did not differ from each other. The temperature fall was ~3^{0}C in LAB compared to ~2^{0}C in HAB and NAB, with the latter being identical to that reported in a comparable motion study in adult male Wistar rats (2.0±0.2^{0}C; [17]). If it is assumed that there is no fundamental difference in the vestibular sensitivity to circular motion in the three groups of animals (but see below), the hypothermic response to motion is exaggerated in LAB animals. Evidence for modified thermoregulatory responses in LAB *vs* HAB rats comes from studies showing an enhanced hyperthermic response to a novel environment and social defeat, with the latter stimulus also producing more pronounced hypothalamic neuronal activation and ACTH responses in LABs *vs* HAB and NAB [29, 49]. The enhanced *hypothermic* response to motion in LAB would appear to mirror the similarly enhanced *hyperthermic* response to other stressors compared to responses in HAB and NAB. A difference in the sensitivity of the hypothalamic thermoregulatory pathways to inputs from the brainstem vestibular nucleus activated by motion would appear to be the most likely origin of the differences between LAB and HAB/NAB.

The cardiac responses to motion reflected by AUC show that the response in LAB animals is greater than NAB=HAB, but using heart rate as a criterion, the groups separate with the rank order of bradycardia responses being LAB>HAB>NAB. A previous study in adult male Sprague-Dawley rats also reported bradycardia in response to motion [9]. The bradycardia response to rotation shows a

similar temporal profile in all groups, with a dynamic phase over the initial 10 min which then sustains for the remaining 30 min. This larger *bradycardia* response to motion in LAB animals mirrors their enhanced *tachycardia* response to restraint stress compared to HAB animals [6] (cf. temperature responses described above). The bradycardia is not secondary to baroreflex activation as rotation stimuli do not affect blood pressure in rats [9]. The increase in RMSSD and HF power in response to motion indicate that the mechanism of the bradycardia is a centrally mediated increase in vagal drive to the heart. Changes in respiration which can be induced by emetic stimuli in rats [34] may further modify cardiac autonomic outflow.

Taking the temperature and cardiovascular responses to motion together with the literature data, we propose that the LAB phenotype also selects for animals with altered hypothalamic-autonomic control leading to enhanced tachycardia and hyperthermic responses to a range of external stressors, but to enhanced bradycardia and hypothermia to the circular motion. The studies with diazepam discussed below provide further support that the magnitude of the bradycardia and the hypothermia in the three lines is unrelated to the behavioural phenotype of the underlying genotype.

4.3. Differential effects of diazepam on the physiological responses to motion in NAB, HAB and LAB rats

The dose of diazepam used is in the range that has previously been shown to have behavioural effects in HAB animals consistent with an anxiolytic effect [10, 29]. While diazepam had minor hypothermic effect shortly after its administration, it did not affect body temperature at the time corresponding to provocative motion in the no-rotation experiments. In all three phenotypes diazepam reduced the magnitude of the hypothermic response assessed by AUC, with the magnitude of the reduction (~30%) comparable in each case. If the magnitude of the thermoregulatory response to motion was linked to the anxiety phenotype, a differential response to diazepam would have been expected, with HAB>NAB>LAB. However, although there was no phenotype-related effect of diazepam, it is possible that rotation induces anxiety in all three phenotypes, and the ~30% reduction in the response by diazepam reflects this component. An alternative explanation is that diazepam has an effect on the thermoregulatory control mechanisms and/or an “anti-emetic” effect reducing the activation of the vestibular pathways. The equivalent efficacy of diazepam on hypothermia is consistent with the hypothesis that hypothermia is an indicator of activation of pathways inducing a nausea-like state in rats and that the diazepam is having an “anti-emetic” effect possibly by its anxiolytic effect reducing the animals’ conscious perception of the motion.

Analysis of the AUC of cardiac responses failed to show any significant effect of diazepam on rotation-induced bradycardia, although for LAB animals there is a clear trend towards a reduction of the

response by diazepam. This is consistent with the significant reduction in the magnitude of the bradycardia in diazepam-treated LAB animals after 10 min rotation. Diazepam also had an effect on rotation-induced bradycardia after 10 min in NAB, but not in HAB animals, although there is a trend towards a similar decrease. Similar to the effect on hypothermia, the effects of diazepam on heart rate again show that the response is not inextricably linked to the anxiety phenotype but, in contrast to hypothermia, the effect of diazepam on heart rate may be confined to the "dynamic phase" of the bradycardia in the first 10 min after the onset of rotation. This transient effect of diazepam might suggest that the initial bradycardia could be related to motion-induced anxiety and that mechanisms unrelated to anxiety operate after 10 min motion.

4.4. Do our findings challenge the validity of the "hypothermia nausea hypothesis"?

The initial premise of this study was that we expected that if the hypothermic response to motion is an index of a nausea-like state in rats (10, 18), then as anxiety is a risk factor for nausea in humans (see Introduction), the hypothermic response to rotation in HAB rats should be greater than in LAB rats. Contrary to our expectations, the LAB animals had the largest hypothermic and bradycardia responses to motion, with no major differences between HAB and NAB. From these results, we can take two opposite views outlined below:

1.) If the view is taken that HAB animals selected on the basis of the EPM test model human patients with an anxiety disorder or individuals experiencing elevated anxiety, then HAB and not LAB animals should have the largest hypothermic (and bradycardia) response to motion, and this would challenge the hypothesis that hypothermia is an index of a nausea-like state in rats. However, it should be noted that the relationship between anxiety and nausea is based primarily on the higher probability of occurrence of nausea in subjects with higher anxiety levels [2, 21, 23, 37, 42], and correlations between degree of anxiety and magnitude of nausea are weak [27, 44]; the present study only models the latter situation. Taking the results at face value, motion clearly produced hypothermia in all three lines, and this is consistent with previous studies of hypothermia induced by a number of emetic stimuli in both rats (lacking an emetic reflex [19]) and *Suncus murinus* (with an emetic reflex) [9, 17, 35]. However, the magnitude of the hypothermia response differed between the three lines suggesting that LAB animals had a more intense nausea-like response to motion compared to HAB or NAB rats. If the effect of diazepam is used as a measure of the component contributed by anxiogenic pathways, and excluding any indirect (sedative) or direct anti-emetic effect, then in each line the effect is similar, with ~30% of the hypothermic ("nausea") response contributed by this pathway. Taking this approach, we would conclude that: i) motion induces a nausea-like state in all lines as indicated by hypothermia; ii) the contribution of anxiety (diazepam-sensitive component) to this response is the same in all three lines; iii) the greater magnitude of the

hypothermia (and bradycardia) response in LAB compared to HAB and NAB is due to an increased sensitivity in the vestibular-thermoregulatory/cardiovascular pathways that are psychogenetically selected by the elevated plus-maze test. Previous studies [5, 6] have identified phenotypic differences in the cardio-respiratory control in LAB compared to HAB/NAB animals, and this study identifies a novel unpredicted phenotypic difference. In addition, it is also possible that selection of the LAB phenotype included selection for animals with enhanced vestibular sensitivity. A human genome wide array study has shown significant associations between motion sickness sensitivity and single nucleotide polymorphisms in regions involved in balance, inner ear and eye development [20]. An enhanced vestibular sensitivity in LAB animals would be expected to lead to improved balance, and this could provide a greater sense of security to spend more time on the open arms of the elevated plus-maze compared to HAB and NAB rats. Whether vestibular sensitivity is higher in LAB animals could be tested experimentally.

2.) If it is accepted that the magnitude of hypothermia in rats reflects a nausea-like state, then we would conclude that the LAB animals were experiencing more severe motion sickness than HAB or NAB. Furthermore, if in humans there really is a robust relationship between degree of anxiety and magnitude of nausea (or vomiting) then the elevated plus-maze is not selecting for the same anxiety characteristics or mechanisms in rats as in anxious subjects or patients with anxiety disorders. Whilst acute administration of diazepam increases the time spent on open arms of the elevated plus-maze [10] and this behavioural effect is substantially larger in HAB compared to LAB rats [29], the diazepam-sensitive component of the hypothermia response does not differentiate between any of the three lines. This potentially indicates that the magnitude of anxiety associated with circular motion does not differ between HAB, NAB or LAB animals. In other words, it may be that “anxiogenic” potential of provocative motion is fundamentally different from that of common behavioural anxiety tests. In view of these considerations, it appears likely that the high-anxiety phenotype selected by the elevated plus-maze does not provide a rat model for investigating interactions between anxiety levels and induction of motion sickness. The elevated plus-maze does however identify a novel phenotype that has an enhanced response to motion and may provide a novel model for investigating factors regulating sensitivity to motion. Further studies using other biomarkers of the response to motion (e.g. defecation, locomotor activity, gastric myoelectrical activity, plasma oxytocin; see [1] for review) are required to fully characterise the phenotype followed by studies using other emetic challenges (e.g. lithium chloride) also known to induce a hypothermic response [17].

5. Conclusion.

Breeding for extremes in anxiety-related behavior unexpectedly selects animals with low trait anxiety that have enhanced bradycardia and hypothermic responses to motion. Thermal and cardiovascular responses to motion were little different between HAB and NAB rats indicating that either hypothermia is not an index of a nausea-like state in rats, or that the positive correlation between anxiety and nausea demonstrated in humans does not exist in rats. The mechanism underlying the enhanced physiological responses in LAB requires more detailed study and may provide a novel model to investigate factors modulating motion sensitivity.

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