Supplementary Information for:

Low vagal modulation of resting heart rate in rat lines bred for extremes in corticosterone responsiveness to stress

Damien Huzard[1], Sriparna Ghosal[1], Jocelyn Grosse[1], Luca Carnevali[2], Andrea Sgoifo[2] and Carmen Sandi[1,#]

[1] Laboratory of behavioral genetics, Brain Mind Institute, Ecole polytechnique fédérale de Lausanne, Lausanne, Switzerland

[2] Stress Physiology Lab, Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parma, Italy

[#] Corresponding author: carmen.sandi@epfl.ch

Supplementary Material and Methods

CAST protocol

During the CAST protocol, rats were stressed with different stressors between P28-30 (Supplementary Figure 1A). The stress results from previous generations were presented in a previous report by Walker et al., (2017), we illustrated here the results from the following CAST generations for males and females breeders (Supplementary Figure 1B and 1C).

Statistical analysis of corticosterone data

It should be noted that whereas within-lines values from Figure 1A and 1E were normally distributed, this was not the case for CORT and RMSSD data when all subjects were considered together. Moreover, for CORT values the standard deviations of the three lines differed. Thus, we applied a log-normalization to CORT data and computed a one-way ANOVA on the Log(CORT) values. We also computed Log(RMSSD) in order to determine the correlation between Log(CORT) and Log(RMSSD). See Supplementary Figures 2A and 2B.

Elevated plus maze (EPM)

Anxiety-like behaviors were evaluated using the EPM test (del Paso et al., 2013). The apparatus consisted of two opposite open-arms (50 x 10 cm) perpendicular to two closed-arms (50 x 10 x 50 cm), extending from a central platform (10 x 10 cm) and elevated 65 cm above the floor. Light levels were maintained between

14-16 lux on the open-arms and 5-7 lux in the closed-arms. At the start of the test, the rat was placed on the central platform facing a closed-arm and allowed to explore the maze for 5 minutes. Behavior was monitored using a ceiling mounted video camera and analyzed with a computerized tracking system (Ethovision 10; Noldus IT, Netherlands). The distance traveled, the time spent in the open- and closed-arms were analyzed. In between animals, the apparatus was cleaned with 5% ethanol solution and dried.

Light/Dark box (LD box)

In order to assess anxiety-like behaviors, the natural aversion of rats against bright environments was used in the LD box. The maze (30 x 60 x 40 cm) consisted of 2 connected equal-sized chambers, one covered and dark (approx. 0 lux) and one open and bright (approx. 100 lux). During the test, the rat was placed in the open chamber facing the wall and allowed to explore for 10 min. Behavior of the rat in the bright compartment was video-recorded. An experimenter blind to group assignment, scored the videos and determined the time spent in the dark or bright chambers. The more the rats spent time in the lit chamber was used as an index of lower anxiety-like behaviors.

Quantification of HRV

The relevance of the low over high frequency ratio (LF/HF) is currently a matter of debate (Task Force, 1996). It has been previously used as a synthetic measure of sympathovagal balance (Moak et al., 2007) but it has also been suggested to be an indicator of baroreflex sensitivity instead of sympathetic innervation (Moak et al., 2007). For this reason, LF/HF ratio values are reported with HRV results but no conclusions concerning the sympathovagal balance or baroreflex sensitivity will be drawn.

Supplementary Results

Analysis of log(CORT) data

When CORT data were Log-transformed, the equal variance test was passed for the data from the three lines. One-way ANOVA indicated a main line effect on log(CORT) values ($F_{2,57} = 34.42$, $p < 0.001$). Post hoc Holm-Sidak's multiple comparison indicated that the three lines differed between each other ($t_{57} > 3.52$, $p < 0.001$), as indicated in Supplementary Figure 2A.

In addition, a Log-transformation was applied to RMSSD data, followed by correlational analyses between log(CORT) and log(RMSSD) data. As shown in Supplementary Figure 2B, these analyses yielded a significant negative correlation.

Anxiety-like behaviors

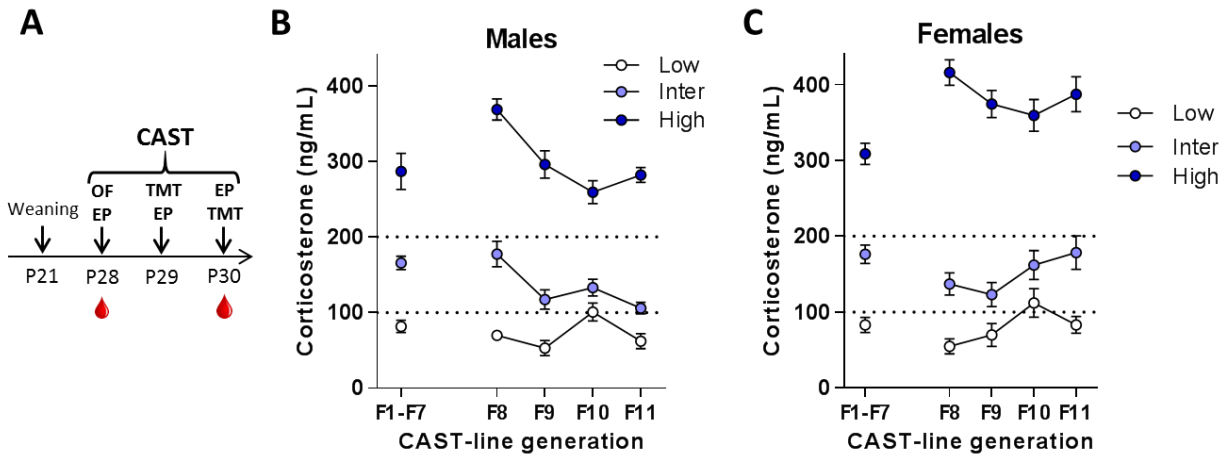
Before (P93) and after (P110) radiotelemetric implants surgery, anxiety-like behaviors of the rats were assessed rats were in and EPM and LD box respectively (Supplementary Figure 2A). During EPM (Supplementary Figure 2B), there was a line effect ($p = 0.012$) on the time spent in the open arms. High-line

rats showed higher anxiety-like behaviors than the Low- ($p = 0.003$) and Inter-lines ($p = 0.046$). During the LD box (Supplementary Figure 2C), there was a line effect ($p = 0.003$) on the time spent in the bright part. High-line rats showed higher anxiety-like behaviors than the Inter-line ($p = 0.005$), but there were no differences with Low-line rats ($p > 0.151$) in the time spent in the bright part.

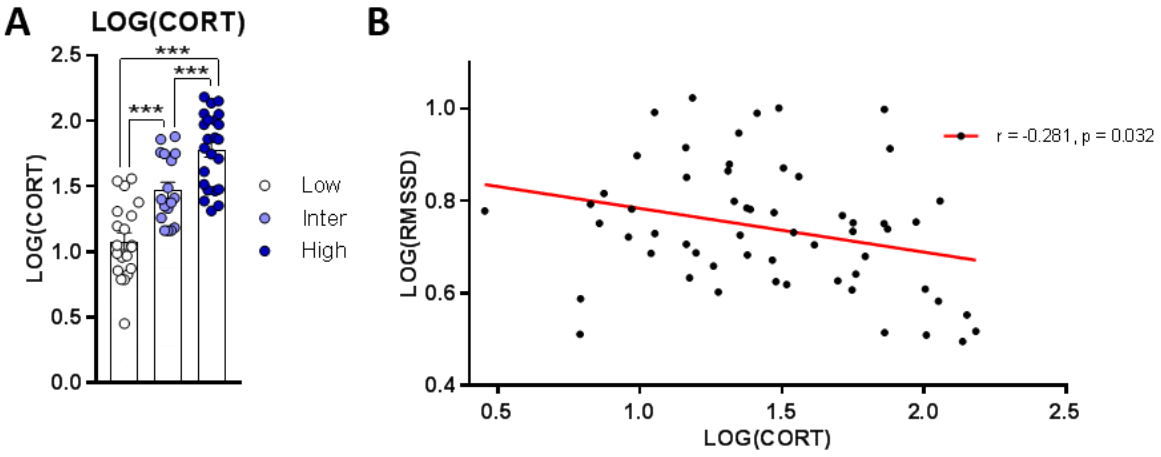
Baseline recordings of ECG

During baseline recording, Inter-line rats exhibited higher values of total power than Low- ($p < 0.008$) and High-lines ($p < 0.027$) during light- and dark-phases (Supplementary Table 1). During the dark-phase, there was a tendency for a higher LF power in rats from the Inter-line compared to Low- ($p = 0.05$) and High-lines ($p = 0.033$) (Supplementary Table 1). There were no differences in the LF/HF ratio, T° and LOC between the lines (Supplementary Table 1).

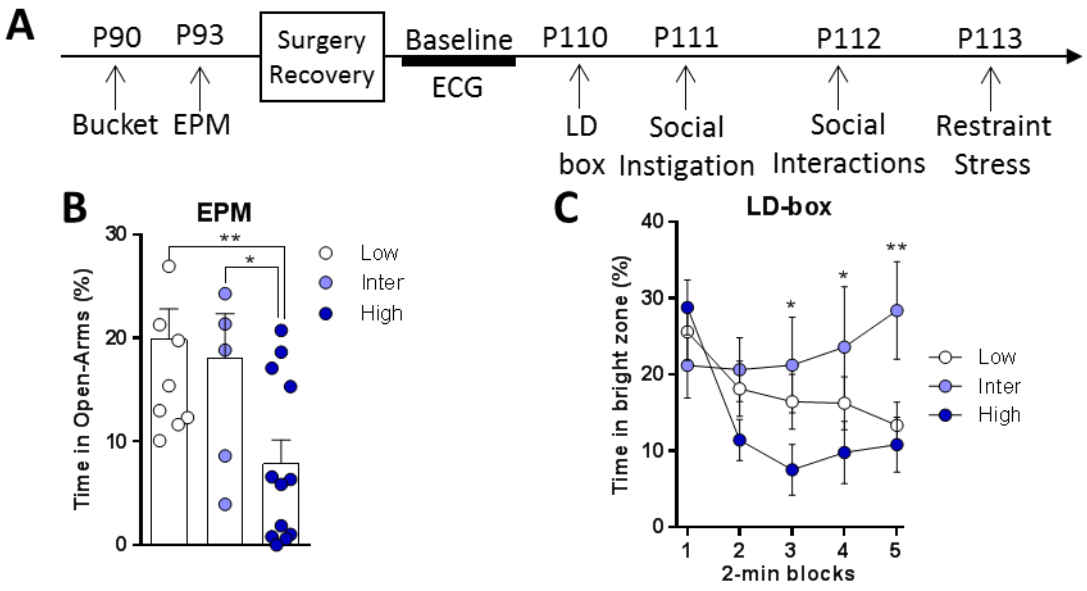
Supplementary Figures



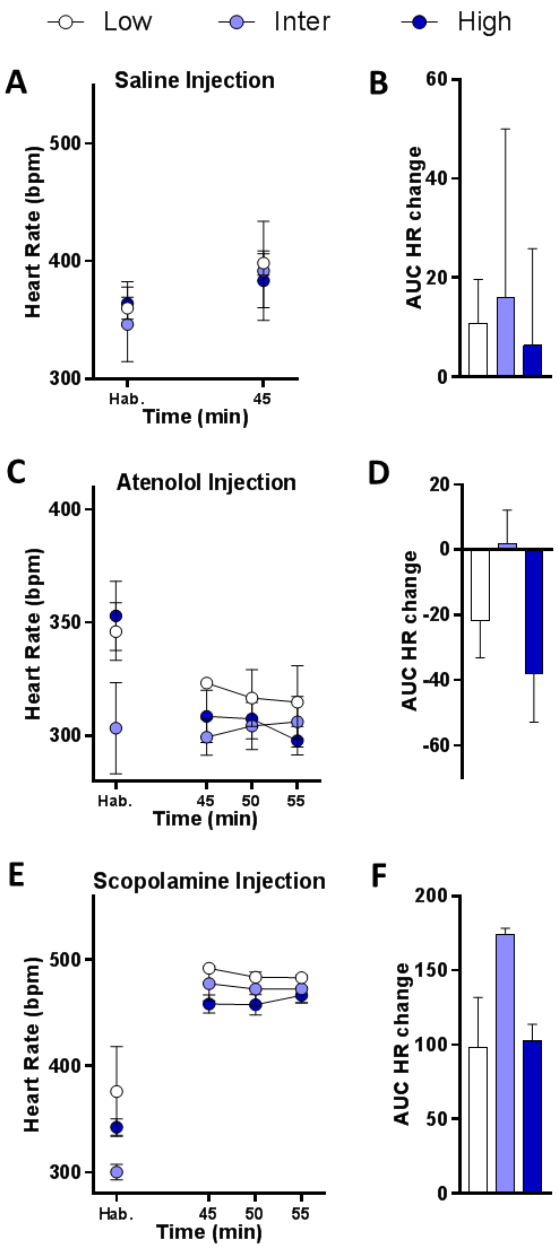
Supplementary Figure 1: The corticosterone-adaptation-stress-test (CAST) and selection of Low-, Inter- and High-lines according to their CORT responsiveness at P30. A, Experimental timeline of the CAST protocol during the juvenile period. After weaning (P21), rats were submitted to 3 days of stress (P28-30) with tail-blood sampled at P28 and P30. After assessment of locomotor activity with an open-field (OF), stressors included EP (Elevated-platforms) and TMT (Trimethylthiazoline: predator scent) for 25 min each. **B**, **C,** Representations of plasma CORT from eleven generations of the lines in males (**B**) and female breeders (**C**). The selection of rats from lines was based on CORT concentration at P30 and classified as `Low-' ([CORT] < 100ng/ml), `Inter-' ([CORT] between 100-200 ng/ml) and `High-'lines ([CORT] > 200ng/ml). **D,** Averaged CORT response at P30 of the breeding pairs selected to generate the 12th generation of CAST animals.



Supplementary Figure 2: Analysis of CORT data with a logarithmic transformation. A, There is a main effect of lines. Post hoc analyses validated that lower CORT for Low line compared to the two other lines, and higher CORT for the High line than the Inter line. **B,** There is a negative linear correlation between Log(CORT) and LOG(RMSSD) when all subjects from the three lines are analyzed together.



Supplementary Figure 3: Increased anxiety-like behaviors in the High-rats. A, experimental timeline of Experiment 2. **B**, During 5 minutes on the EPM, there was a line effect on the time spent on the open arms ($F_{2,26} = 5.3$, $p = 0.012$). High-line rats spent less time in the open arms than Low- ($p = 0.003$) and Inter-line rats ($p = 0.046$). **C**, During 10 minutes in the LD-box test, results were analyzed using 2-min blocks and a repeated measure ANOVA. There was a line effect on the time spent in the bright part ($F_{2,115} = 6.12$, $p = 0.003$). High-line rats spent less time in the bright part compared to Inter-line rats ($t_{92} = 2.89$, $p = 0.005$), but there were no differences between Low-line rats and the other lines ($p > 0.151$). During the two first blocks all rats had similar exploration of the bright part (blocks 1 & 2: $p > 0.152$), but then, High-line rats spent less time than Inter-line rats in the light (blocks 3, 4 & 5: $p < 0.035$). Due to technical issues, two animals (1 Inter and 1 High) were not video-recorded during LD-box testing.



Supplementary Figure 4. Analysis of heart rate response to pharmacological treatments 45 minutes after drug injections. **A,** 45 min after saline injection the heart rate (HR) was not different compared to baseline recording (time effect: $F_{1, 6} = 1.692$, $p = 0.241$). **B,** The area under the curve (AUC) of the change of HR between baseline and 45 min after saline injection did not differ between the lines ($F_{2, 6} = 0.044$, $p = 0.957$). The AUC of HR change did not statistically differ from 0 for the three lines (t-tests: $p = 0.17$, $p = 0.5$ and $p = 0.63$). **A, B,** Those results illustrate that 45 min after saline injection HR values were similar to baseline level. **C,** 45 min after atenolol injection there was no line effect in HR values ($F_{2, 6} = 0.7781$, $p = 0,501$). **D,** Similarly, the AUC of HR changes 45 min after atenolol injection did not differ between the three lines ($F_{2, 6} = 2.731$, $p = 0.144$). One sample t-tests (against 0) were performed and we obtained $p = 0.1878$, $p = 0.8708$ and $p = 0.1217$ for the Low, Inter and High lines respectively. **E,** 45 min after scopolamine injection there was no line effect in HR values ($F_{2, 6} = 1.59$, $p = 0.28$). **F,** the AUC of HR changes 45 min after scopolamine injection did not statistically differ between the three lines ($F_{2, 6} = 4.35$, $p = 0.068$).

Supplementary Tables

parameters	Light/dark phase	Lines			ANOVA main effects		Post hoc (Fischer's LSD p-values)		
		Low (n=22)	Inter (n=13)	High (n=21)	Line	Line * time	Low vs. Inter	High vs. Inter	Low vs. High
Heart-Rate (bpm)	Dark	414.7 ± 3.1	392.4 ± 4.1	411.4 ± 5.2	0.002	0.32	.001	.006	.56
	Light	356 ± 3.9	336.1 ± 4.4	353 ± 4.3			.003	.012	.059
RMSSD (ms)	Dark	4.2 ± 0.2	5.3 ± 0.7	4 ± 0.2	0.003	0.021	.035	.014	.65
	Light	5.6 ± 0.4	7.4 ± 0.9	5.1 ± 0.3			.019	.003	.44
HF Power (ms2)	Dark	7.2 ± 0.8	14.1 ± 4.5	6.4 ± 0.7	0.003	< 0.001	.021	.011	.75
	Light	13.5 ± 1.8	26.7 ± 7.8	10.8 ± 1.3			.009	.002	.53
Total Power (ms2)	Dark	69.2 ± 4.3	97.2 ± 8.6	67.6 ± 5	0.002	0.32	.002	.001	.83
	Light	99.5 ± 6.5	142.7 ± 15	107 ± 10.9			.008	.027	.58
LF Power (ms2)	Dark	7.9 ± 0.9	8.7 ± 0.7	6.1 ± 0.6	0.183	0.99	.050	.033	.086
	Light	13.4 ± 1.5	13.8 ± 1.1	10.6 ± 1.3			.85	.14	.135
LF/HF	Dark	1.26 ± 0.09	1.11 ± 0.13	1.16 ± 0.08	0.368	0.18	.3	.73	.43
	Light	1.24 ± 0.1	1.01 ± 0.17	1.2 ± 0.09			.18	.27	.79
Temperature (°C)	Dark	37.9 ± 0.06	37.9 ± 0.05	37.9 ± 0.045	0.99	0.5	.99	.99	.99
	Light	37.3 ± 0.07	37.3 ± 0.09	37.2 ± 0.04			.99	.32	.25
Locomotion (count)	Dark	3.63 ± 0.33	4.04 ± 0.41	3.69 ± 0.19	0.34	0.19	.38	.45	.88
	Light	1.66 ± 0.16	1.26 ± 0.11	1.33 ± 0.15			.09	.76	.11

Supplementary Table 1: Daily rhythms of radiotelemetric and HRV parameters during basal recording. For the 12 hours dark- and light-phases, values of 4 min segments recorded every hour, were averaged and are reported as 'means + SEM' for Low- (n = 23), Inter- (n = 16) and High- lines (n = 26). Abbreviations: HRV = heart rate variability; RMSSD = square root of the mean squared differences of successive RR intervals; HF = high frequency; LF = low frequency; Statistical comparisons are represented as p-values of post hoc LSD's Fischer comparisons.

parameters	Line	Habituation	Social Stressors	Recovery (0-15 min)	Recovery (15-30 min)	Repeated measures ANOVA LSD Fischer's (p-values)
Heart rate (bpm)	**Low**	438.1 ± 10.4	459.5 ± 7.2	443.4 ± 8.4	420.3 ± 10.5	Line (0.01), time (0.001), int. (0.34) LvI (0.03), LvH (0.23), IvH (0.003)
	Inter	417.3 ± 9.1	430.2 ± 13.2	413.1 ± 10.2	395.7 ± 11.2	
	High	459.2 ± 8.5	467..4 ± 7.7	453.5 ± 7.7	428 ± 10.7	
RMSSD (ms)	**Low**	3.71 ± 0.38	3.1 ± 0.24	3.38 ± 0.25	3.94 ± 0.29	Line (0.50), time (0.001), int. (0.35) LvI (0.74), LvH (0.37), IvH (0.28)
	Inter	3.85 ± 0.52	3.25 ± 0.34	3.49 ± 0.41	4.22 ± 0.5	
	High	3.14 ± 0.17	2.90 ± 0.2	3.05 ± 0.22	3.34 ± 0.31	
Total Power (ms^2)	**Low**	74.3 ± 10.4	60.2 ± 6.5	59.7 ± 8.8	69.3 ± 7.2	Line (0.21), time (0.001), int. (0.06) LvI (0.39), LvH (0.08), IvH (0.5)
	Inter	61.6 ± 11.7	58 ± 6.7	48.1 ± 8.3	59.3 ± 8.4	
	High	49.1 ± 4.8	51.6 ± 6.3	46.3 ± 6.7	61.5 ± 10.2	
HF power (ms^2)	**Low**	7.75 ± 1.55	5.3 ± 1.01	6.35 ± 1.19	6.71 ± 0.92	Line (0.22), time (0.001), int. (0.21) LvI (0.82), LvH (0.14), IvH (0.14)
	Inter	7.76 ± 1.94	5.63 ± 1.12	6.34 ± 1.57	7.64 ± 1.8	
	High	4.24 ± 0.59	4.06 ± 0.69	4.41 ± 0.75	6.15 ± 0.98	
LF Power (ms^2)	**Low**	12.1 ± 2.07	9.65 ± 1.68	10.55 ± 2.12	9.7 ± 1.35	Line (0.09), time (0.55), int. (0.11) LvI (0.16), LvH (0.03), IvH (0.65)
	Inter	9.11 ± 1.2	6.87 ± 1.25	12.6 ± 1.8	7.32 ± 1.09	
	High	6.02 ± 0.66	7.59 ± 1.51	6.66 ± 1.32	7.99 ± 1.54	
LF/HF	**Low**	1.73 ± 0.14	1.94 ± 0.17	1.86 ± 0.21	1.56 ± 0.13	Line (0.16), time (0.001), int. (0.16) LvI (0.06), LvH (0.31), IvH (0.29)
	Inter	1.39 ± 0.17	1.34 ± 0.15	1.4 ± 0.21	1.22 ± 0.22	
	High	1.5 ± 0.12	1.88 ± 0.18	1.53 ± 0.09	1.42 ± 0.21	
Temperature (°C)	**Low**	38.62 ± 0.13	38.68 ± 0.09	38.68 ± 0.09	38.35 ± 0.09	Line (0.28), time (0.001), int. (0.94) LvI (0.15), LvH (0.23), IvH (0.65)
	Inter	38.81 ± 0.2	38.88 ± 0.16	38.83 ± 0.17	38.55 ± 0.16	
	High	38.72 ± 0.14	38.82 ± 0.08	38.73 ± 0.07	38.57 ± 0.12	
Locomotion (count)	**Low**	9.91 ± 0.86	14.1 ± 0.82	7.09 ± 1.01	4.77 ± 0.81	Line (0.35), time (0.001), int. (0.19) LvI (0.61), LvH (0.16), IvH (0.48)
	Inter	10.02 ± 1.38	14.35 ± 1.36	7.52 ± 2.1	5.36 ± 1.52	
	High	9.45 ± 1.15	15.46 ± 1.31	9.55 ± 1.11	5.3 ± 0.89	

Supplementary Table 2: Radiotelemetric and HRV parameters during the social stressors. Values are reported as means ± SEM of data obtained by averaging multiple 180s segments acquired during habituation (30min), during the social instigation (30min) and the recovery phase (0-15min and 15-30min), in Low-line (n = 11), Inter-line (n = 6) and High-line (n = 11) animals. Abbreviations: HRV = heart rate variability, bpm = beat-per-minute, Hab = Habituation, Instig = instigation, Recov = recovery, LvH = Low vs. High, LvI = Low vs. Inter, HvI = High vs. Inter, RMSSD = square root of the mean squared differences of successive RR intervals, LF = Low Frequency, HF = High Frequency.

parameters	Line	Habituation	Restraint Stress (0-15 min)	Recovery (0-15 min)	Recovery (15-30 min)	Repeated measures ANOVA LSD Fischer's (p-values)
Heart rate (bpm)	**Low**	409.1 ± 9.8	478.2 ± 9.5	431.3 ± 9.4	396.3 ± 8.9	Line (0.27), time (0.001), int. (0.49) LvI (0.83), LvH (0.17), IvH (0.18)
	Inter	412.9 ± 9.8	468.7 ± 9.8	423.3 ± 8	399.7 ± 8.7	
	High	437.7 ± 9.9	485.1 ± 5.8	438.4 ± 8.6	408.4 ± 9.2	
RMSSD (ms)	**Low**	3.8 ± 0.39	2.71 ± 0.18	3.71 ± 0.40	4.3 ± 0.38	Line (0.67), time (0.001), int. (0.21) LvI (0.63), LvH (0.35), IvH (0.12)
	Inter	3.86 ± 0.59	2.83 ± 0.55	2.97 ± 0.32	3.94 ± 0.6	
	High	3.44 ± 0.25	2.81 ± 0.23	3.21 ± 0.19	3.66 ± 0.24	
Total Power (ms^2)	**Low**	61.6 ± 7.9	34.0 ± 3.7	54 ± 8.2	62.8 ± 8	Line (0.24), time (0.001), int. (0.58) LvI (0.1), LvH (0.4), IvH (0.32)
	Inter	51.2 ± 8.7	27.7 ± 2.1	34.3 ± 4.9	44.8 ± 6.1	
	High	53.4 ± 5.8	37.1 ± 4.9	44.3 ± 3.6	55.0 ± 4.9	
HF power (ms^2)	**Low**	5.71 ± 1.16	2.81 ± 0.33	5.12 ± 1.06	6.73 ± 1.11	Line (0.59), time (0.001), int. (0.24) LvI (0.77), LvH (0.31), IvH (0.58)
	Inter	5.91 ± 2.1	3.42 ± 1.47	3.22 ± 0.72	6.25 ± 1.95	
	High	4.2 ± 0.57	3.14 ± 0.75	3.77 ± 0.48	4.75 ± 0.76	
LF Power (ms^2)	**Low**	9.2 ± 1.68	4.2 ± 0.73	7.8 ± 1.52	10.3 ± 1.69	Line (0.07), time (0.001), int. (0.76) LvI (0.046), LvH (0.06), IvH (0.67)
	Inter	6.35 ± 0.86	2.26 ± 0.36	4.1 ± 0.34	6.54 ± 0.6	
	High	6.49 ± 0.64	2.96 ± 0.46	5.42 ± 0.84	6.87 ± 0.86	
LF/HF	**Low**	1.84 ± 0.17	1.32 ± 0.10	1.71 ± 0.12	1.69 ± 0.17	Line (0.54), time (0.001), int. (0.57) LvI (0.86), LvH (0.69), IvH (0.60)
	Inter	1.46 ± 0.22	0.98 ± 0.18	1.53 ± 0.2	1.41 ± 0.24	
	High	1.83 ± 0.25	1.16 ± 0.13	1.68 ± 0.24	1.91 ± 0.32	
Temperature (°C)	**Low**	38.3 ± 0.12	38.71 ± 0.09	38.84 ± 0.06	38.53 ± 0.065	Line (0.85), time (0.001), int. (0.001) LvI (0.03), LvH (0.23), IvH (0.003) Hab: LvI (0.07), LvH (0.55), IvH (0.02)
	Inter	38.57 ± 0.08	38.82 ± 0.08	38.73 ± 0.1	38.34 ± 0.08	
	High	38.22 ± 01	38.7 ± 0.09	38.84 ± 0.09	38.43 ± 0.11	
Locomotion (count)	**Low**	7.94 ± 0.74	3.27 ± 0.38	7.85 ± 0.88	3.54 ± 0.59	Line (0.83), time (0.001), int. (0.98) LvI (0.61), LvH (0.92), IvH (0.55)
	Inter	8.69 ± 1.16	3.14 ± 1.02	8.6 ± 0.83	4.13 ± 0.91	
	High	8.18 ± 1.07	2.4 ± 0.55	7.91 ± 1.11	3.77 ± 0.88	

Supplementary Table 3: Radiotelemetric and HRV parameters during the restraint stress. Values are reported as means ± SEM of data obtained by averaging multiple 180s segments acquired during habituation (30min), during the restraint stress (30min) and the recovery phases (0-15min and 15-30min), in Low-line (n = 11), Inter-line (n = 6) and High-line (n = 10) animals. Abbreviations: HRV = heart rate variability, bpm = beat-per-minute, Hab = Habituation, LvH = Low vs. High, LvI = Low vs. Inter, HvI = High vs. Inter, N.S. = not significant, RMSSD = square root of the mean squared differences of successive RR intervals, LF = Low Frequency, HF = High Frequency.

Supplementary References

Walf, A. A., and Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* 2, 322–8.