Cardiac-generated sympathetic stress alters heart-brain communication, reduces EEG-theta activity, and increases locomotor behavior

Jacopo Agrimi [a, b], *Danilo Menicucci* [c], *Marco Laurino* [d], *Chelsea D Mackey* [b], *Laila Hasnain* [b], *Sneha Dodaballapur* [b], *Ross A McDevitt* [h], *Donald B Hoover* [e, g], *Angelo Gemignani** [c], *Nazareno Paolocci** [a, f], *Edward G Lakatta** [b]

a) *Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA.*
b) *Laboratory of Cardiovascular Sciences, National Institute on Aging, National Institutes of Health Biomedical Research Center (BRC), Baltimore, MD, USA*
c) *Department of Surgical, Medical, Molecular Pathology, and Critical Care Medicine, University of Pisa, Pisa, Italy*
d) *Institute of Clinical Physiology, National Research Council, Pisa, Italy*
e) *Department of Biomedical Sciences, James H. Quillen College of Medicine, East Tennessee State University, Johnson City TN.*
f) *Department of Biomedical Sciences, University of Padova, Padova, Italy*
g) *Center of Excellence in Inflammation, Infectious Disease and Immunity, East Tennessee State University, Johnson City, TN*
h) *The Comparative Medicine Section, National Institute on Aging, National Institutes of Health, Baltimore, MD, USA.*

**Contributed equally*

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Correspondence to:

Nazareno Paolocci, M.D., Ph.D.

Division of Cardiology, Traylor 911
Johns Hopkins Hospital
720 Rutland Avenue
Baltimore, MD, 21212
npaoloc1@jhmi.edu

Edward G. Lakatta, M.D.

Laboratory of Cardiovascular Sciences,
National Institute on Aging,
National Institutes of Health Biomedical Research Center (BRC),
Baltimore, MD, 21224
lakattae@grc.nia.nih.gov

Abstract

Brain modulation of myocardial activity via the autonomic nervous system is increasingly well characterized. Conversely, how primary alterations in cardiac function, such as an intrinsic increase in heart rate or contractility, reverberate on brain signaling/adaptive behaviors - *in a bottom-up modality* - remains largely unclear. Mice with cardiac-selective overexpression of adenylyl cyclase type 8 (TGAC8) display increased heart rate and reduced heart rhythm complexity associated with a nearly abolished response to external sympathetic inputs. Here, we tested whether chronically elevated intrinsic cardiac performance alters the heart-brain informational flow, affecting brain signaling and, thus, behavior. To this end, we employed dual lead telemetry for simultaneous recording of EEG and EKG time series in awake, freely behaving TGAC8 mice and wild-type (WT) littermates. We recorded EEG and EKG signals, while monitoring mouse behavior with established tests. Using heart rate variability (HRV) *in vivo* and isolated atria response to sympathomimetic agents, we first confirmed that the TGAC8 murine heart evades autonomic control. The EEG analysis revealed a substantial drop in theta-2 (4-7 Hz) activity in these transgenic mice. Next, we traced the informational flow between EKG and EEG in the theta-2 frequency band via the Granger causality statistical approach and we found a substantial decrement in the extent of heart/brain bidirectional communication. Finally, TGAC8 mice displayed heightened locomotor activity in terms of behavior, with higher total time mobile, distance traveled, and movement speed while freezing behavior was reduced. Increased locomotion correlated negatively with theta-2 waves count and amplitude. Our study shows that cardiac-born persistent sympathetic stress disrupts the information flow between the heart and brain while influencing central physiological patterns, such as theta activity that controls locomotion. Thus, cardiac-initiated disorders, such as persistently elevated cardiac performance that escapes autonomic control, are penetrant enough to alter brain functions and, thus, primary adaptive behavioral responses.

Introduction

According to the somatic marker theory postulated by Antonio Damasio (following the thoughts of one of the fathers of psychophysiology, William James), afferent somatic signals arising from the body's peripheral districts are integrated into higher brain regions, particularly the ventromedial prefrontal cortex (VMPFC), and they can influence complex behaviors, such as decision making[1,2]. The bidirectional relationship between the heart and the brain falls entirely within this loop. Indeed, besides the autonomic nervous system's descending control on cardiac function, information is processed by the intrinsic cardiac nervous system (the so-called "little brain of the heart") that communicates back to the brain through ascending fibers located in the spinal cord and vagus nerve[3]. These afferent impulses reach relay stations such as the medulla, hypothalamus, thalamus, and, ultimately, the cerebral cortex[3], carrying sensory information that can initiate centrally-directed behaviors[4].

Cardiac rhythmicity is under continuous surveillance and control of the two branches of the autonomic nervous system (ANS)[5]. A balanced influence of sympathetic and parasympathetic efferent fiber discharge over cardiac pacemaker cells located in the sinoatrial and atrioventricular nodes is ultimately needed to generate the final heart beating frequency (HR)[5]. However, there are clinical situations in which the heart escapes this autonomic control. In this sense, the transplanted heart that harbors severed efferent and afferent autonomic fibers is emblematic in that it evades both sympathetic and parasympathetic surveillance almost entirely[6]. These individuals experience persistently elevated HR at rest and cannot adjust their myocardial performance to an increased workload, such as during exercise[7]. What is more, after heart transplantation, 63% of heart recipients face anxiety and depression, especially during the first post-transplant year[8]. Despite numerous observational clues suggesting that peripheral changes in cardiac activity can influence behavior, definitive experimental evidence proving this point is lacking, along with the nature of the mechanism(s) eventually underlying this *bottom-up* phenomenon and most behavioral patterns that are affected[9].

Transgenic mice that overexpress adenylyl cyclase (AC) type 8 (TGAC8) in a cardiac-selective manner display persistent elevated HR, reduced HR variability (HRV), and increased

92 contractility owing to enduringly high intrinsic cardiac cAMP-PKA-$Ca2^+$ signaling[10,11]. The TGAC8
93 heart flees top-down autonomic surveillance by blocking beta-adrenergic signaling and
94 catecholamine production to escape harmful additional sympathetic stress. In the present study,
95 we sought to determine whether persistent cardiac-initiated stress also disrupts bottom-up
96 signaling from the heart to the brain, leading to altered brain activity and behavioral sequelae.

97 **2. Materials and Methods**

98 **2.1 Animals**

99 All studies were executed in agreement with the Guide for the Care and Use of Laboratory Animals
100 published by the National Institutes of Health (NIH Publication no. 85-23, revised 1996). The
101 experimental procedures were approved by the Animal Care and Use Committee of the National
102 Institutes of Health (protocol #441-LCS-2016). A breeder pair of TG^{AC8} mice, generated by ligating
103 the murine α-myosin heavy chain promoter to a cDNA coding for human AC8[11], were a gift from
104 Nicole Defer/Jacques Hanoune, Unite de Recherches, INSERM U-99, Hôpital Henri Mondor, F-
105 94010 Créteil, France. Wild type (WT) littermates, bred from the C57BL/6 background, were used
106 as controls.

107 **2.2 Evaluation of isolated atrial function and response to adrenergic agents**

108 To evaluate adrenergic responses of the sinoatrial node (SAN) in the absence of autonomic inputs,
109 we isolated the cardiac atria leaving the SAN intact. TGAC8 and control mice were deeply
110 anesthetized with isoflurane and euthanized by decapitation. Hearts were removed rapidly and
111 transferred to oxygenated (95% O_2, 5% CO_2), cold (4°C) Krebs-Ringer bicarbonate buffer (pH 7.35
112 to 7.4) of the following composition (mM): 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$,
113 1.2 $MgCl_2$, and 11.1 D-glucose. The entire atrium was dissected from the ventricles, and each side
114 was impaled with a small metal hook (#28 trout hooks) with attached 5-0 sutures. The left atrium
115 was anchored to the bottom of a vertical support rod in a 15 mL tissue bath, and the right atrium
116 was attached to a 25-g force transducer (World Precision Instruments, Sarasota, FL). Krebs-Ringer
117 buffer in the tissue bath was oxygenated continuously and maintained at 37°C. Spontaneous
118 atrial contractions were recorded at a resting tension of 0.3 to 0.5 g using a ML224 Bridge
119 Amplifier (ADInstruments, Colorado Springs, CO), a PowerLab/8SP, and a computer running Lab

Chart version 7.3.8. Buffer was replaced at 10 min intervals, and three times after recovery from drug treatment. Baseline atrial rate and force of contractions were recorded after a 30 min stabilization period. Then responses to a maximally effective concentration of the tyramine (10-4 M final concentration), which stimulates release of norepinephrine from noradrenergic nerves, and the directly acting sympathomimetic, L-isoproterenol, were recorded[12]. Treatments were separated by at least 30 min to preclude desensitization.

2.2.1 Drugs

L-isoproterenol hydrochloride and tyramine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO).

2.3 Telemetry double implant to simultaneously monitor the EEG and EKG

Telemetric radio transmitters (F20-EET; Data Sciences International (DSI), St. Paul, MN) were surgically implanted in young (3-4 months) WT and TG^{AC8} mice as described[13]. Briefly two surface electrodes, a positive electrode (parietal cortex; AP, -2.0 mm; L, 2.0 mm) and a reference electrode (cerebellum; AP, -6.0 mm; L, 2.0 mm), were passed subcutaneously to the cranial base and placed directly on the dura mater. Two additional bipotential electrodes were routed subcutaneously via a vertical midline incision overlying the abdomen with leads situated in the right upper chest and

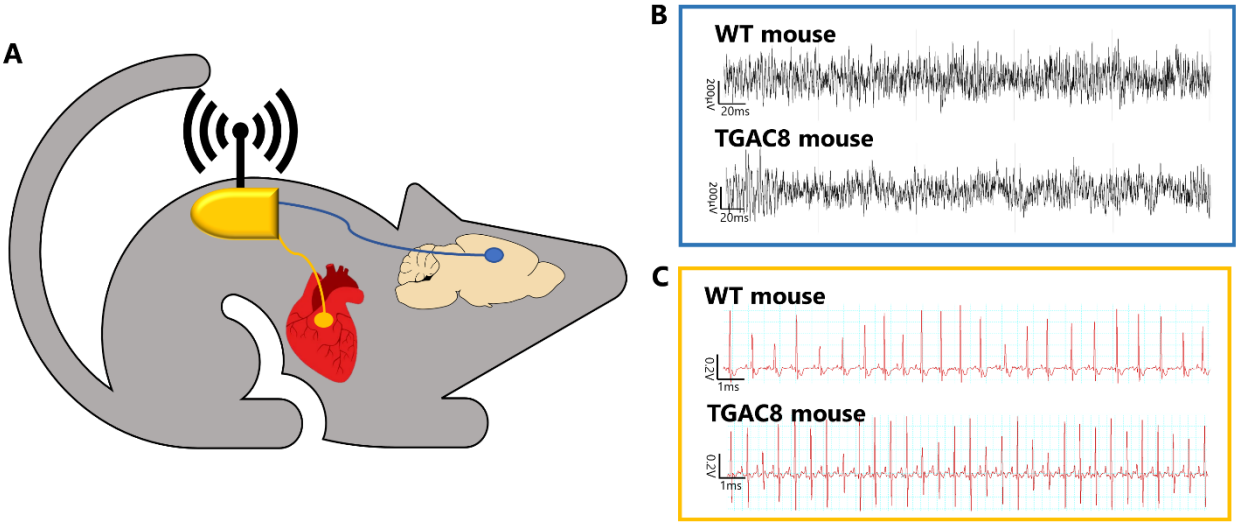

Figure 1 (A) Schematic of F20-EET double implant for telemetry recording in mice. (B) Representative images of EEG recording in a WT and TGAC8 mouse. (C) Representative images of EKG recording in a WT and TGAC8 mouse.

lower left abdomen below the heart to monitor continuous heart rate (Fig.1). Following a two-weeks recovery period, 24-hr free behaving electrocardiogram (EKG) and electroencephalogram (EEG) were recorded at a sampling rate of 1000 Hz and 500 Hz, respectively, with simultaneous activity recording using the Dataquest A.R.T acquisition system (DSI, version 4.36). Average activity counts were obtained every 10s.

2.4 Signal analysis

2.4.1 EKG analysis

Scoring of wake-sleep states (NeuroScore, DSI, version 3.2.0) allowed selecting all 10 seconds wake epochs and extracting signals.

After appropriate pre-processing (see Supplemental Information), the series of heart beat time intervals (RR series) was processed to derive HRV features. All features were signal-length independent[14] estimated on 10 seconds epochs to account for highly frequent wake/sleep switches, thus describing the short-time heartbeat dynamics.

More in detail, we considered: mean value of RR intervals and, from frequency spectrum analysis (Hamming-windowed FFT), the power in low frequency (LF: 0.15–1.5 Hz) and high frequency (HF: 1.5–4 Hz) bands. We also estimated some nonlinear features that provide additional information about cardiac dynamics. According to previous works studying non-linearity in a heartbeat series[10], we characterize the RR series auto similarity through the Detrended Fluctuation Analysis (DFA).

2.4.2 EEG Spectral analysis

For each 10 seconds wake epoch, a spectral analysis was performed on the EEG signal. The mean power spectrum density characterizing wake state of each animal was evaluated by applying a Hamming-windowed Fast Fourier Transform (FFT) and averaging them. Power values were measured in dB, thus data from FFT were log-transformed. The activity in the theta-2 band (4-7 Hz) was estimated by averaging the bins belonging to the band.

2.4.3 Theta wave recognition

The theta wave recognition was carried out following the approach proposed and validated by Ferrarelli and colleagues[15] for sleep spindles, with some adaptations, as detailed in the SI. Three parameters were derived from all detected events: the rate, peak amplitude, and dominant frequency. The rate corresponded to the number of events per time unit, and the peak amplitude was defined as the local maximum of each theta wave event. The dominant frequency is the average of 4-7 *Hz* frequencies weighted by the associated squared coefficients of the discrete Fourier transform. Measures characterizing each animal were derived as the average overall theta wave events detected during its wake epochs.

2.5 Brain/heart communication

The brain/heart communication study was based on a Granger Causality (GC) analysis. $GC_{x->y}$ is a measure of the contribution of the past of the x(t) time-series to the prediction of the present value of y(t), compared to the contribution of the past of the *y(t) time series* in the prediction of its own present value. In our application (Fig. 5) x(t) and y(t) are signals related to brain and heart functioning and the GC has allowed estimating heart rhythm influence on brain EEG rhythms and vice versa (by switching x and y). Specifically, our analysis takes advantage of the variability expressed over time by both the brain theta rhythm and heart rhythm and checks whether amplitude variations of the former are predictive of changes in the latter or vice versa. For the variability over time of the theta rhythm, we used the amplitude of the envelope of the theta-filtered signal (see supplementary data, steps 1 and 2 in the theta wave recognition procedure), whereas the heart rhythm variability was directly expressed in the tachogram. To perform the GC analysis, both series were interpolated at the same frequency (10 Hz). The GC analysis was conducted using the Causal Connectivity Toolbox[16], which assumes a linear model and order within the model, estimated using the Akaike Information Criterion. Herein, in unison with the GC applicability criteria, EEG epochs satisfying the stationarity test[17]were retained for the analysis (more than 95% retained). For each mouse, the median of their epochs model orders was chosen as the representative order. The 95th percentile of 'animals' model orders was taken as the overall order [18]: from data collected in this experiment, this corresponded to 20 samples, equivalent to 2 seconds. The overall model order was applied to the GC estimates of all wake epochs for all mice (both groups). Finally, the validity of the model order was verified by estimating the model

consistency (percentage of data correlation structure explained by the model)[19]. Consistency values higher than 75% were considered satisfactory and all periods had mean consistencies of 90% or above.

2.6 Behavioral tests

2.6.1 Open field

Open field (OF) is a gold standard behavioral test used to study locomotor activity, exploratory behavior, and anxiety-like behavior in rodents [20]. The animal is free to explore a circumscribed environment (50cm x 50cm arena surrounded by walls), and its locomotor activity and its tendency to explore open spaces or hide in the peripheral corners of the arena is evaluated. The analysis of the behavioral parameters has been performed using ANY-maze software (Stoelting Co., IL, USA).

2.6.2 Elevated plus maze

Elevated plus maze (EPM) is another consolidated test to study anxiety-like behavior and locomotor activity in rodents. Elevated Plus Maze test exploits the rodent's conflict between aversion to open spaces (i.e., hides in closed arms of a labyrinth) and instinct to explore new environments (e.g., exploration of open arms of the same labyrinth) [20]. The analysis of the behavioral parameters has been performed employing ANY-maze.

2.6.3 Light-Dark Box

The light/dark box (LDB) test is based on the innate aversion of mice to illuminated spaces and the spontaneous exploratory activity in response to minor stressors, such as a new environment and light. This experimental task allows for evaluation of the level of anxiety-like behavior experienced by the animals. The test apparatus consists of a small dark compartment and a larger illuminated compartment[21]. The analysis of the behavioral parameters has been performed through ANY-maze software.

2.6.4 Fear Conditioning Test

The fear conditioning (FC) test measures the capacity of rodents to learn and recall an association between environmental cues and aversive experiences. In this paradigm, mice are placed into a

conditioning chamber and are given pairs of a neutral auditory stimulus and an aversive stimulus (electric foot shock). After the conditioning acquisition, 24 hours later, the mice are exposed to the identical chamber and a differently shaped space with the presentation of the auditory cue. Freezing behavior during the test is recorded as an index of associative learning[22].

2.6.5 Y-Maze

Y-Maze allows studying spatial memory in rodents. The test stems from the tendency the rodents have to explore new environments, preferring a labyrinth arm that is not yet explored to an already known one[23]. The analysis of the behavioral parameters has been performed using ANY-maze software.

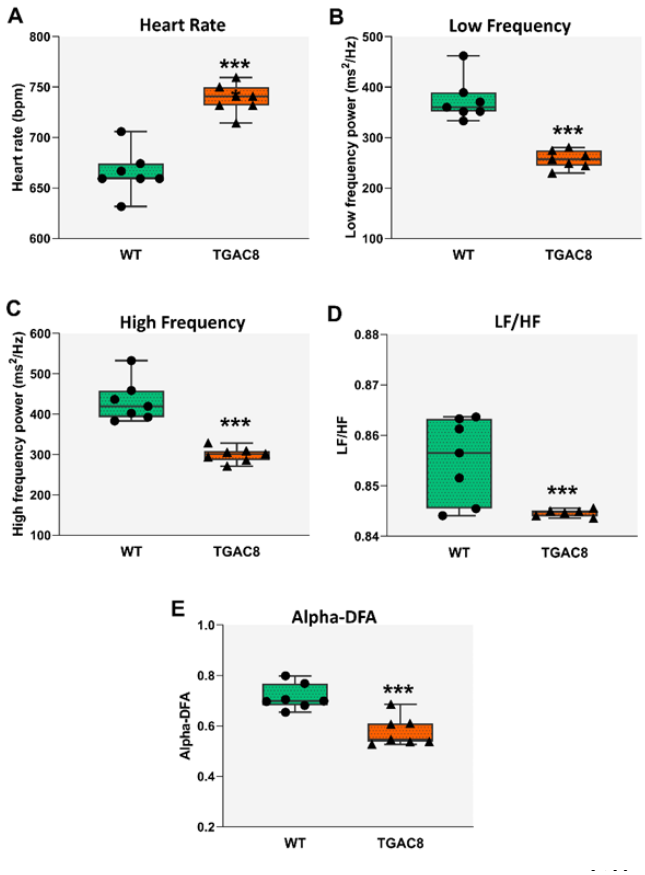

Figure 2 *Heart rate variability is impaired in TGAC8 mice. (A-E) Heart Rate Variability measured in vivo from telemetric EKG recording. (A) Heart Rate; (B) Low Frequency; (C) High Frequency; (D) Low Frequency/High Frequency ratio; (E) Alpha-Detrended Fluctuation Analysis. Results are shown as box and whiskers plot; N=7, unpaired t-test; ***p<0.001*

2.7 Statistical analysis procedures

Results are presented as box-and-whisker plots (the box extends from the 25th to 75th percentiles, the median value is depicted by the line in the middle of each box, the whiskers go down to the 1thpercentile and up to the 99th). All parametric data were analyzed by unpaired t-test between the WT and TGAC8 groups. Correlations between groups of values were evaluated through Pearson's coefficient, P-value <0.05 was considered statistically significant.

3. Results

3.1 The heart escapes autonomic surveillance in mice over-expressing AC8

Overexpressing AC8 in the SA node cells renders the heart unresponsive to ANS surveillance[10]. Here, we validated and expanded this evidence. Indeed, we first observed that the persistently elevated *in vivo* HR found in TGAC8 mice (**Fig. 2A**) goes hand-in-hand with a marked drop in both time domains and high- and low-frequency domain HR variability and their ratio recorded by the EKG branch of the double telemetry implant (p<0.001*** TGAC8 vs. WT) (**Fig. 2B-D**). The Detrended Fluctuation Analysis alpha (alpha-DFA) confirmed such evidence. Indeed, HR in TGAC8 mice is less influenced by external systems compared to the WT counterparts (p<0.001** TGAC8 vs. WT) (**Fig.2E**).

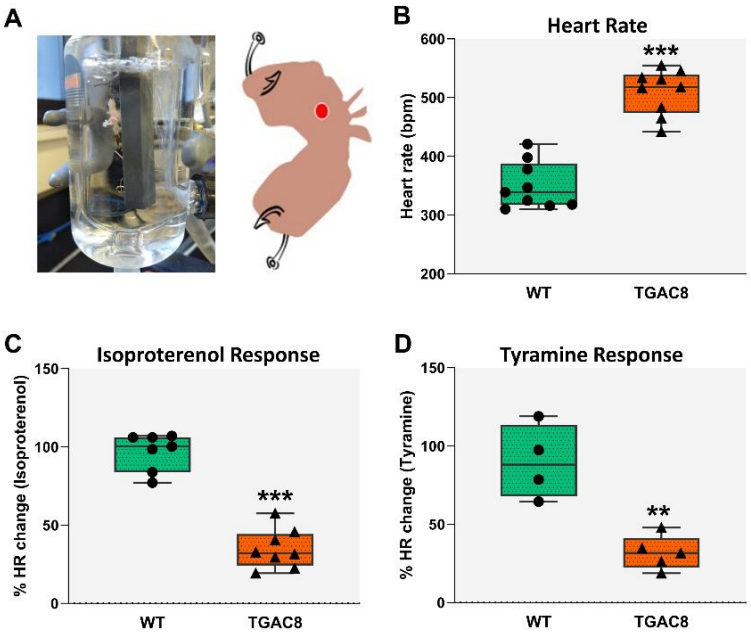

*Figure 3 TGAC8 mice show a blunted response to sympathomimetic agents. Results from studies conducted in isolated mouse atria. (A) Representative picture and diagram of isolated atria preparation; (B) Heart Rate, n=9; (C) Change (in percentage) of heart rate after Isoproterenol administration n=7-8; (D) Change (in percentage) of heart rate after Tyramine administration, n=4-5. Results are shown as box and whiskers plot; unpaired t-test has been performed between WT and TGAC8 groups, ***p<0.001.*

We assessed the intrinsic response of the SAN to external sympathetic input in isolated atria, having an intact SAN. The basal beating rate of isolated atria from TGAC8 was markedly increased (**Fig.3B**) and there was a substantially decreased response to sympathomimetic agents, namely isoproterenol and tyramine (**Fig.3 C, D**). In aggregate, these data confirm that the cardiac pacemaker activity in TGAC8 escapes external sympathetic control. Therefore, this mouse strain represents a valuable model to evaluate the functional repercussions of possible alterations in heart-brain communication.

3.2 TGAC8 mice show lower EEG theta-2 power and decreased theta waves count and amplitude

Next, we analyzed the EEG recordings to address the core hypothesis of the work, i.e., persistent cardiac-initiated stress can influence brain signaling in a bottom-up modality. We first analyzed all EEG power spectrum, comparing each *hertz* (1-50) in WT and TGAC8 mice (**Fig. 4A**). Cardiac-selective AC8 overexpression significantly decreased the power in the range of theta-2 frequency

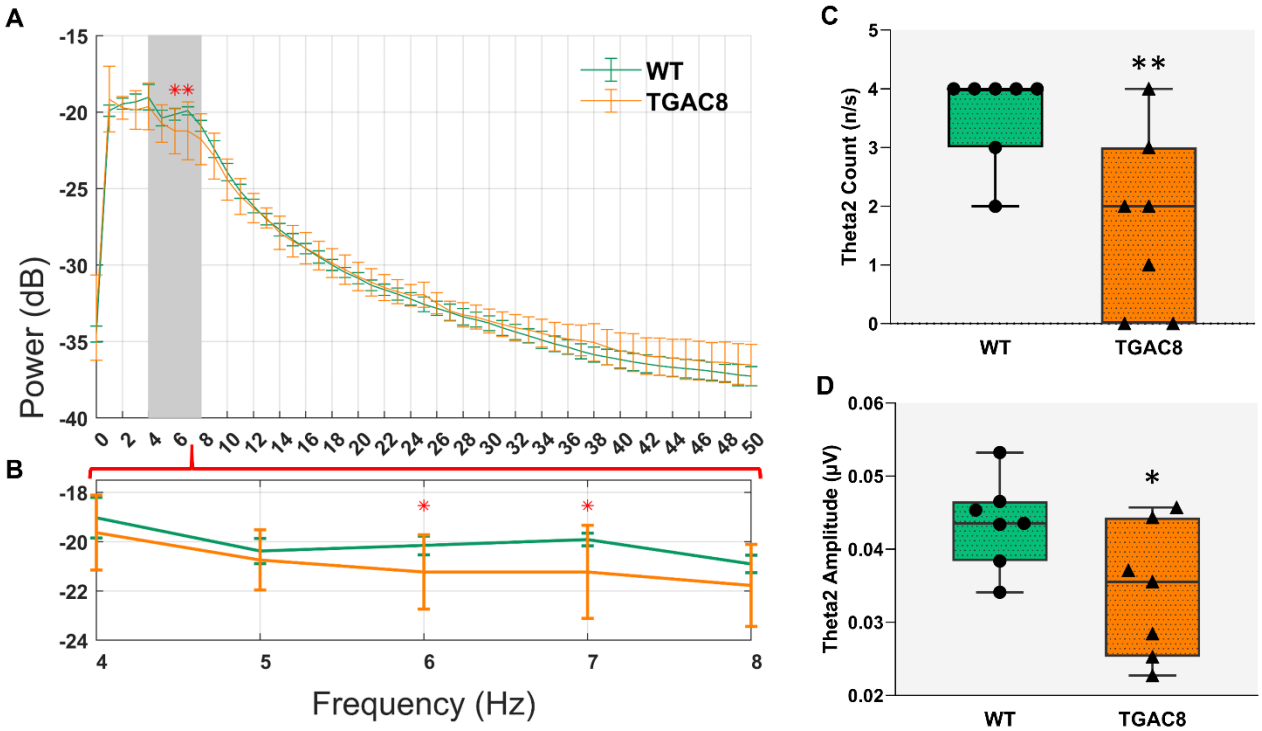

*Figure 4 TGAC8 mice display an overall reduction in theta-2 activity. (A) Power spectrum 1-50 Hz; (B) Power spectrum, zoom on the theta-2 range; (C) Theta-2 waves count; (D) Theta-2 waves amplitude. Results in A and B are shown as mean and SD, in C and D as box and whiskers plot, N=7, unpaired t-test has been performed between WT and TGAC8 groups, *p<0.05, **p<0.01.*

(6-7 *Hz*) (**Fig. 4A, B**). These changes in theta-2 activity were not limited to the power spectrum but also involved waves counts (-50% **p=0.01) and amplitude (*p<0.05), all markedly reduced in the TGAC8 mice (**Fig. 4C, D**). Thus, AC8 myocardial overexpression leads to a pronounced downregulation of the theta-2 activity.

3.3 AC8 cardiac-selective overexpression impairs the brain-heart flow of information

To further prove that the alterations in EEG theta-2 frequency and patterns are related to the cardiac-initiated sympathetic stress experienced by the TGAC8 mice, we finally applied the GC analysis, a statistical approach that estimates causality between two time series, to evaluate the bidirectional flow of information between the heart and the brain[24]. Herein, it enabled us to predict how cardiac rhythm can influence EEG frequencies and vice versa, thus providing a metric for the bidirectional flow of information between the two systems (**Fig. 5A**). In the range of theta-2, we

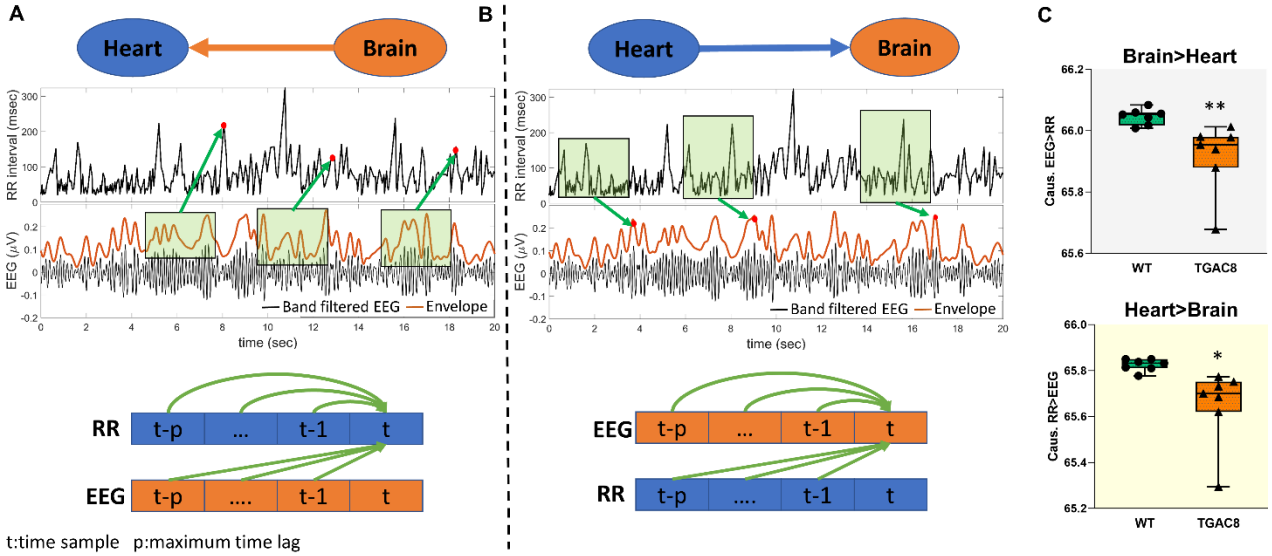

Figure 5. *TGAC8 mice show an impairment in the heart-brain flow of information.* ***A. GC brain->heart estimate.*** *Amplitude of the envelope of the theta-filtered EEG signal (local amplitude) was extracted and used to estimate its contribution in the prediction of the heart rate variability (HRV). The box over the envelope signal depicts the length of past values in the time series used in the GC analysis to predict current HRV series. To derive the GC brain->heart, this predictive contribution was compared with that made by past values of the HRV series on its own current value.* ***B. GC heart->brain estimate.*** *HRV time-series was extracted and used to estimate its contribution in the prediction of the local amplitude of theta-filtered EEG signal. The box over the HRV time series depicts the length of past values in the time series used in the GC analysis to predict current theta amplitude. To derive the GC heart->brain, this predictive contribution was compared with that made by past values of the theta amplitude series on its own current value.* ***C.*** *dispersion within groups of GC estimates both for heart->brain and brain->heart directions. Results in C are shown as box and whiskers plot, N=7, unpaired t-test has been performed between WT and TGAC8 groups, *p<0.05, **p<0.01.*

found significant impairment of the flow of information between the heart and the brain, and this effect was evident in both directions (**Fig. 5B, C**). Of relevance, to validate that this impairment in communication specifically involves theta activity, we then ran the Granger analysis for all main EEG frequency domains (**Sup. Fig 1**). We observed that the flow of information between the tachogram and the EEG traces was affected only in the range of theta and unaltered in the other

windows. Thusr, this data set suggests that the impairment of informational flow between heart and brain, caused by chronic and marked cardiac stress, can underlie the decrease of the theta rhythm at the central level.

3.4 TGAC8 mice display increased locomotor activity

Hippocampal theta activity is a crucial determinant of overall locomotor activity in species, such as mice[25]. In particular, theta-1 (8-12 Hz) seems to be related to active movement and theta-2 (4-7 Hz) to alertness and immobility[26]. Theta frequency is directly proportional to the animal velocity: slower oscillatory rhythms mean slower speed. Moreover, a regular theta rhythm also underpins a more stable and slower running speed during exploration[27]. Thus, it is plausible that the decrease

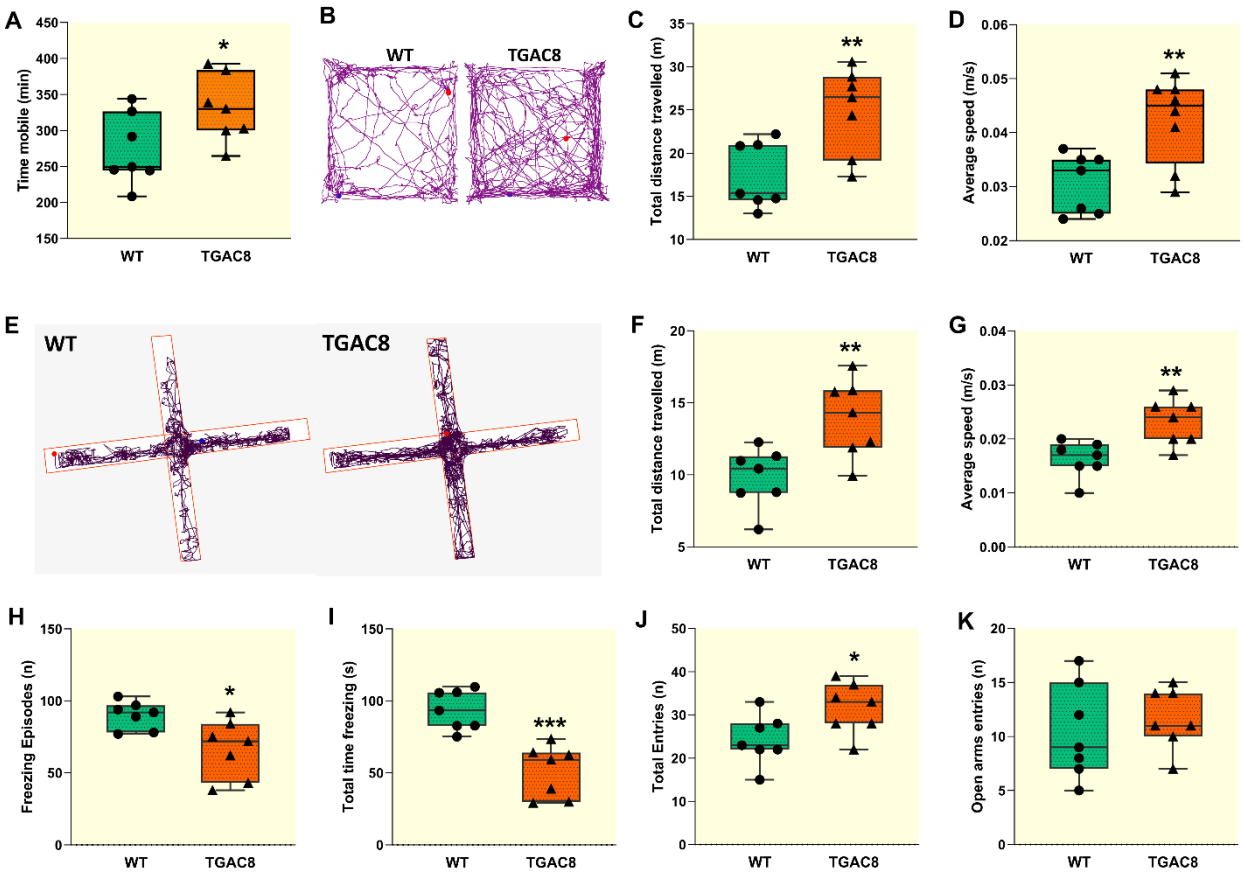

Figure 6 *TGAC8 mice display a general increase in locomotor behavior. (A) Time mobile recorded trough actigraph; (B) Plot of the animals movements during OF test, WT vs TGAC8 mouse; (C) OF test, total distance travelled; (D) OF test, average speed; (E) Plot of the animals movements during EPM test, WT vs TGAC8 mouse; (F) EPM test, total distance travelled; (G) EPM test, average speed; EPM test, freezing episodes; (I) EPM test, total time freezing; (J) EPM test, total entries in the arms of the labyrinth; (K) EPM test, number of entries in the open arms of the labyrinth. Results are shown as box and whiskers plot, N=7, unpaired t-test has been performed between WT and TGAC8 groups, *p<0.05, **p<0.01, ***p<0.001.*

we observed in theta-2 activity in TGAC8s leads to changes in the behavioral phenotype of this mouse. First, we assessed locomotor activity through a Holter actigraphy recording, open field, and elevated plus maze test. We found a significant increase in all parameters linked to locomotion in TGAC8 mice compared to age-matched littermates. For instance, compared to WT, TGAC8 mice had heightened total time mobile in 24 hrs actigraphy (*p<0.05, **Fig. 6A**). Consistent with this finding, the open field (OF) test revealed that the transgenic mice traveled more and at a higher average speed than WT ones (+43% and +38%, respectively, p<0.01) (**Fig. 6 C, D**). The elevated plus-maze (EPM) test also yielded consistent evidence, showing a significant difference in total distance traveled and average speed is present between TGAC8 and WT mice (**p<0.01) (**Fig. 6F, G**). Of relevance, the transgenic strain also displayed a considerable decrease in episodes and time of freezing behavior (-25%, p<0.05 and -45%, p<0.001) (**Fig. 6H, I**). In keeping with the heightened locomotor activity, the total entries in the arms of the labyrinth were significantly increased in TGAC8, in the absence of changes in open arms entries (**Fig. 6J, K**). In aggregate, these findings indicate that under persistent chronotropic/inotropic stress and cardiac autonomic control escape, such as that occurring in TGAC8 mice, heightened locomotor activity occurs. To further validate this new notion, i.e., that in TGAC8 mice, locomotor behavior changes associate with theta-2 activity depletion, we correlated the time spent by the mice in mobile status with their theta pattern. We observed that theta waves count was inversely proportional to the mobile time (r=-0.61, p<0.05) (**Fig. 7A**). We observed the same trend concerning theta amplitude, i.e., negative

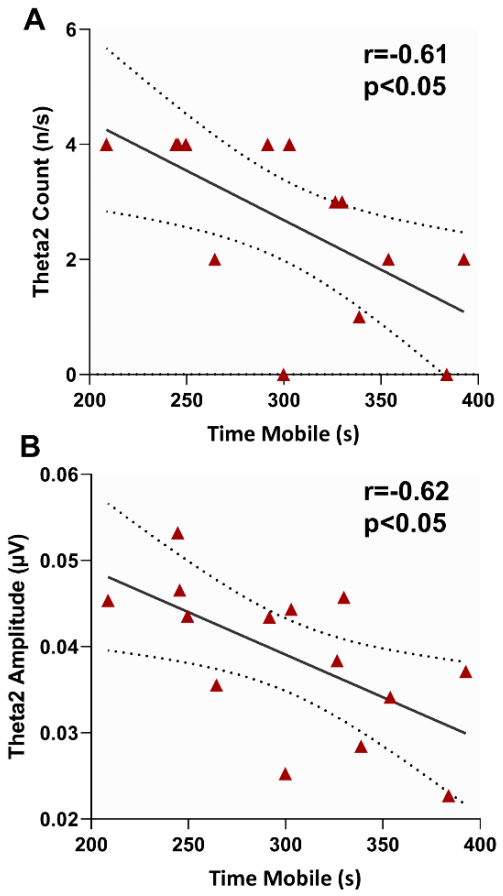

Figure 7 *Time spent mobile correlates with theta-2 patterns. (A) Correlation between 24hrs time mobile and theta-2 waves count; (B) Correlation between 24hrs time mobile and theta-2 waves amplitude. Data are displayed in scatterplots; thick black lines represent the best linear fit through data; dotted lines represent two-tailed 95% confidence interval; text inset reports Pearson's Rho values and associated p-values.*

correlation with time mobile (r=-0.62, p<0.05) (**Fig. 7B**). Hence, our data show that the decrease in theta-2 oscillatory rhythm is correlated to an overall increase in locomotor activity.

3.5 Cardiac AC8 overexpression does not affect anxiety, associative learning, and spatial memory

Anxiety responses evoke irregular variations in the cardiac rhythm that can be perceived centrally as a state of danger[28,29]. Conversely, here, we set out to determine whether in the context of chronically elevated heart-born sympathetic stress anxiety-like behavior is present in TGAC8. To

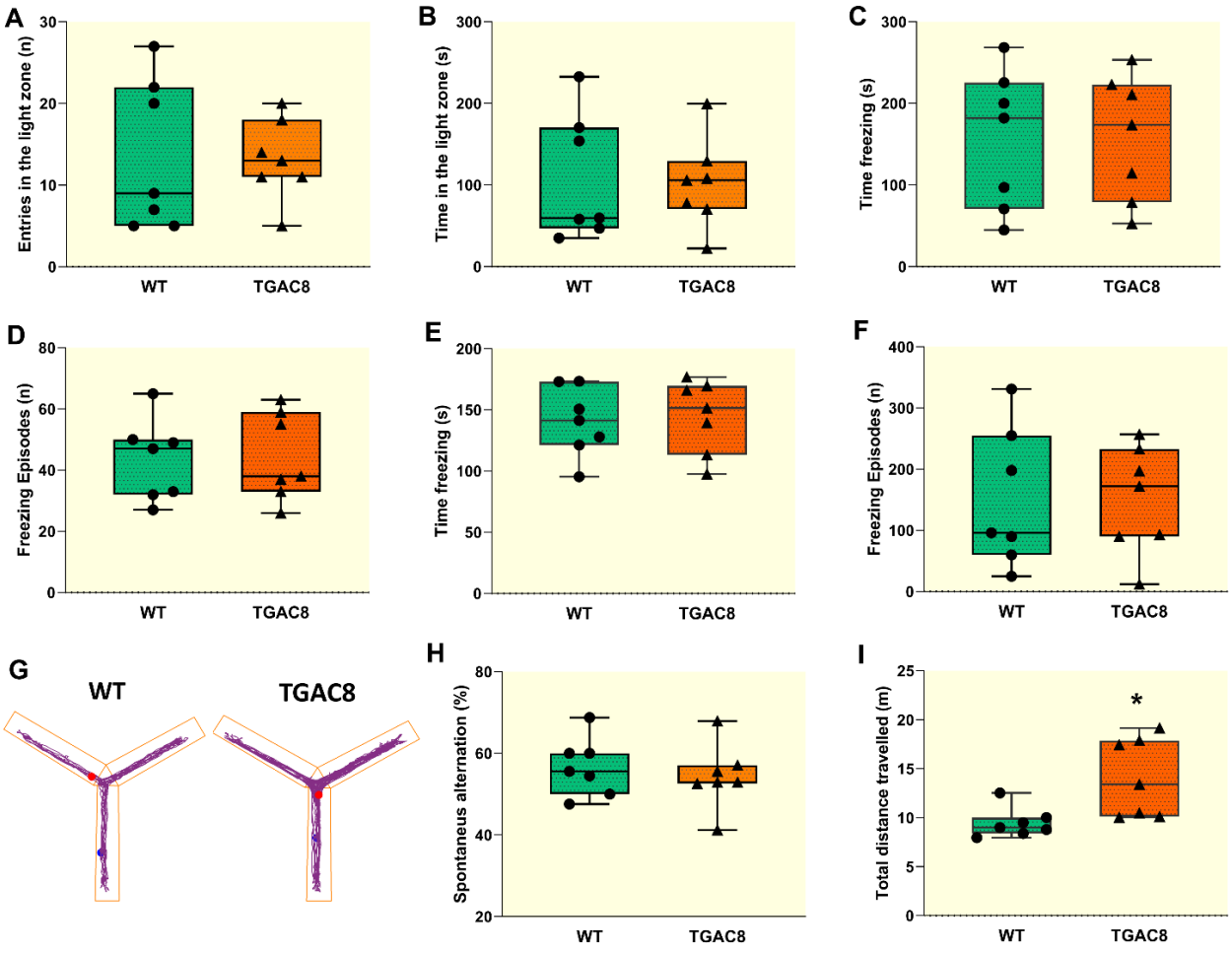

Figure 8. *TGAC8 mice display a general increase in locomotor behavior not related to emotional or cognitive domains. (A) LDB test, number of entries in the light zone; (B) LDB test, time spent in the light zone; (C) Contextual FC test, time freezing; (D) Contextual FC, freezing episodes; (E) Cued FC, time freezing; (F) Cued FC, freezing episodes; (G) Plot of the animals movements during Y-maze test, WT vs TGAC8 mouse; (H) Y-maze test, percentage of spontaneous alternations; (I) Y-maze test, total distance travelled. Results are shown as box and whiskers plot, N=7, unpaired t-test has been performed between WT and TGAC8 groups, *p<0.05*

sort this out, we first performed the EPM test that revealed no difference in entries in open arms (**Fig. 6K**). This is a primary parameter that could be extrapolated from the EPM test to measure fear of open spaces. However, the EPM test can be biased by possible changes in locomotor activity. Hence, we next performed the light-dark Box test (LDB) to evaluate this possibility. Again, no differences were evident between WT and TGAC8 mice when considering the two main parameters of the trial, i.e., entries in the light zone and time in the light zone, thus confirming lack of heightened anxiety-like behavior (**Fig. 8A, B**). Since TGAC8 mice displayed a marked decrease in freezing behavior, we evaluated the reasons for such alteration, namely whether this effect is dependent on abnormal locomotor activity or other behavioral domains. To answer this question, we subjected mice to the fear conditioning test, in which the motor component is minimal. Under these circumstances, there was no difference between the two groups (**Fig. 8 C-F**). Finally, to exclude the possibility of enhancing locomotor activity in TGAC8 mice related to perturbation of spatial memory and orientation in this mouse strain, we performed the Y-Maze test that showed no differences in terms of spatial memory (**Fig. 8H**) but confirmed a substantial increase in total distance traveled by the transgenic mice **(Fig. 8I**). Altogether, these data suggest that the increase in locomotor activity in TGAC8 mice is not secondary to emotional or cognitive abnormalities.

Discussion

Anxiety and mood disorders can affect the heart rhythm and its variability[30] chronically, and loss of the top-down control of the heart rhythm is an emerging risk factor for adverse outcomes in several forms of cardiovascular disease[31]. Less clear is whether and how altered heart rhythm, contractility, and lack of autonomic control on cardiac function affects brain functioning/signaling in a bottom-up modality. For the first time, our study shows that TGAC8 mice harboring persistent cardiac-initiated (chronotropic/inotropic) stress display altered heart-brain communication as well as EEG patterns modifications and behavioral changes, i.e., locomotor activity.

Beat-to-beat heart variability, determined by both intrinsic SAN pacemaker cell activity and its autonomic modulation, is crucial for optimal heart function[32]. Indeed, loss of proper top-down brain controls, taken as a whole, exerted on the cardiovascular system is implicated in CVD progression. For instance, after myocardial infarction (MI), or other cardiac disorders, loss of HRV

364 denotes a lack of vagal control and represents a significant predictor of death in MI subjects[33].
365 Indeed, downsizing parasympathetic influence on heart function implies unremitted sympathetic
366 activation, eventually exacerbating myocardial dysfunction and remodeling[34,35]. This autonomic
367 imbalance is also present in patients with severe neuropathies or transplanted heart[6]. It is also
368 worth noting that individuals with anxiety or depressive disorders display a reduction in HRV,
369 manifested as a lack of complexity on heart rhythm that is the signature of increased sympathetic
370 signaling, in this instance initiated in the brain, predisposing to a higher risk of cardiovascular
371 death[31]. Furthermore, after MI, patients become more prone to depression. In turn, this eventuality
372 further fuels post-MI mortality and chronic LV dysfunction in these subjects[36]. Experimentally,
373 inducing depression via chronic mild stress[37] in rats heightens sympathetic activity and aggravates
374 LV remodeling after MI[38]. In the same vein, in a murine model of arrhythmogenic cardiomyopathy,
375 anxiety induced by psychosocial stress increases mortality and remodeling[39].

376 The present study confirms that in TGAC8 mice, heart rate is increased, while LF and HF
377 power bands are reduced, according to reduced parasympathetic and sympathetic autonomic
378 controls acting on heart function[10]. What is more, the alpha coefficient from the DFA, a measure
379 of the RR time series autosimilarity, is significantly reduced in TGAC8 mice. Low alpha value is an
380 adverse prognostic index in patients with implantable cardioverter-defibrillators[40]. This measure
381 is in line with the loss of complexity within the TGAC8 heart rhythm as highlighted with the
382 reduced multiscale entropy[10], and it suggests accelerated cardiac aging or pathology[41] .

383 Brain-heart communication is bidirectional in nature [42,43]. For this reason, we inquired
384 whether the marked cardiac-initiated heart rhythm abnormalities in TGAC8 are associated with
385 concurrent EEG changes in a bottom-up modality[4]. Here, we report that mice with persistently
386 elevated intrinsic heart rate associated with a markedly coherent rhythm, show altered EEG
387 patterns. In mammals, including humans, the theta oscillations are among the most solid and
388 regular rhythms in the brain[44]. According to the dualistic theory, two pairs of theta rhythms can
389 be recognized in rodents, ranging from 4 to 12 *Hz*, theta 1 oscillations (in the 8-12 Hz band), and
390 theta 2 oscillations (in the 4-7 Hz band)[26,45]. This distinction stems from the dependence on
391 cholinergic signaling and sensitivity to anesthetics, such as urethane. Theta-1 is atropine-resistant
392 and urethane-sensitive, whereas theta-2 is atropine-sensitive but urethane-resistant[26]. Here, we

393 observed a marked reduction in the EEG power spectrum in the range of theta-2, associated with
394 a depletion of the wave's count and amplitude.

395 To further validate that the observed changes in EEG patterns derived from an alteration
396 in the signaling between the heart and the brain, we assessed the heart/brain bidirectional
397 communication using the Granger Causality approach. Past functional magnetic resonance
398 imaging studies have identified several structures, including the amygdala, hypothalamus, and the
399 areas belonging to the prefrontal cortex that causally interact with autonomic nervous system
400 outflow at rest[46,47]. This study is critical because it highlights pivotal structures in the top-down
401 control of cardiac output; among these is the hippocampus, the main generator of the theta
402 activity we studied[26]. However, the same study failed at identifying causal interactions in the
403 direction from heart to brain, probably due to the study limitation on the rest condition.
404 Notwithstanding, previous work on the heart-brain coupling evaluating the impact of different
405 levels of sedation on the heart-brain coupling indicated that activity in the thalamocortical circuits
406 appears to be influenced by variations in cardiac rhythm and that this influence increases with the
407 level of sedation. This finding is in line with evidence on the importance of visceral rhythms[9],
408 including the cardiac one, in modulating brain activity during sleep[48]. At variance, in this work, we
409 show for the first time that this coupling is bidirectionally effective also during active wakefulness
410 and that it tends to be less effective in the TGAC8 model in which the heart is genetically
411 uncoupled from the brain since birth.

412 To assess the physiological relevance of the altered heart-brain communication, we
413 evaluated possible behavioral adaptations in TGAC8s. In rodents, theta rhythms have been linked
414 to locomotor activity because it plays a pivotal role in controlling initiation of the movement[49] and
415 regulating locomotion speed[50,27,51]. Bellesi and coworkers reported that reduced EEG theta power,
416 expressed in the frontal and parietal cortical areas of rats, increased their motor activity, especially
417 when EEG changes were more prominent[52]. Consistent with this view, theta-2 has been related to
418 a state of immobility and alertness, with a possible role in inhibiting voluntary movement.[53] On
419 this basis, we anticipated that locomotor behavior in TGAC8 could be affected by theta-2
420 reduction. Moreover, we speculate that the overall rise in locomotor activity is "functionally
421 justified" and congruent with the chronic peripheral physiological modification, such as the

increased heart rate. Indeed, the TGAC8 mouse heart experiences and must handle a status akin to "perpetual exercise". At the same time, however, the brain needs to adapt its outflow, switching the animal behavior from a state of rest to an active one, such as running, thus congruent with the substantial increase of cardiac work experienced by TGAC8 mice. This phenomenon fits perfectly with Damasio's theory of the somatic marker[1]: the brain adapts and responds befittingly to a stimulus detected in the periphery. Moreover, at least in our opinion, it is not surprising that the behavioral phenotype observed in TGAC8 is characterized by an increase in locomotor activity and not by a state of anxiety. Indeed, it is worth recalling that the cardiac chronotropic stress experienced by the TGAC8 mice is regular and continuous, i.e., similar to a state of "perpetual exercise". Conversely, the rhythm alterations that subtend anxiety typically come in sudden and irregular bouts, often characterized by palpitations and arrhythmias[54,55]. Actually, similar changes in heart-brain communication occur in anticipation of exercise performance. Therefore, in this instance, the brain signals to the heart to increase its beating rate prior to the actual start of the physical effort[56]. In the case of TGAC8 the situation is reverted and it is increased heart rate and reduced HRV, born within the heart itself, that signal to the brain to start locomotor activity.

Persistent AC8 overexpression can be perceived as a limitation of the present study due to the difficulty of pairing this genetic manipulation to specific clinical settings. However, such an extreme mouse model served well to the purpose of disconnecting (at least partially) the heart from the brain while subjecting the heart of the TGAC8s to perpetual intrinsic stress *in situ*. And testing the latter's effects on central functions/behavioral patterns was precisely the primary goal of the present research.

Conclusions

Our study shows that cardiac-born persistent sympathetic stress bidirectionally alters the information flow between the heart and brain, indicating heart-initiated signaling to the brain, which influences central physiological patterns associated with locomotion. These results provide proof of concept that pathophysiology arising within the heart is penetrant enough to alter brain functions, thus primary adaptive behavioral responses.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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