Behavioral and autonomic dynamics during contextual fear conditioning in mice

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Abstract

Aversive conditioning to contextual stimulation was performed in mice implanted with ECG transmitters to investigate heart rate (HR) and behavioral responses during contextual retention. The dynamics of HR were analyzed by advanced nonlinear techniques to uncover central neuroautonomic outflow inferred from its sympathetic (SNS) and parasympathetic (PNS) projection onto the sinus node of the heart. Mice experienced a single foot shock (US, unconditioned stimulus) either immediately (USi) or late (USl) after placement in the conditioning context. Contextual memory was tested 24 h after training by reexposure to the conditioning context for 32 min. Only mice that experienced the USl exhibited a pronounced and sustained behavioral suppression (immobility) indicative of conditioned contextual fear. In contrast, HR was initially close to its maximal physiological limit (~800 bpm) in all groups, and recovery towards baseline levels was sluggish, the most pronounced delay observed in the USl group. The results demonstrate that behavioral immobility was associated with maximum activation of autonomic system output in response to contextual reexposure. However, advanced complexity analysis of the variability of HR revealed uniform or stereotyped dynamical properties that were interpreted to reflect a generalized state of anticipatory emotional arousal experienced during reexposure to contextual stimuli. It is concluded that the dynamics of HR is a highly sensitive index of the autonomic nervous system response and emotional state elicited by sensory stimulation of an unfamiliar environment.
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1. Introduction

The neurocircuitries underlying conditioned context- and tone-dependent fear have been investigated in some detail (cf., Fendt and Fanselow, 1999). Contextual fear conditioning involves multimodal sensory information processing of persistent environmental or extrinsic stimuli and is assumed to depend on hippocampal function. In contrast, auditory fear conditioning involves discrete unisensory information processing that is independent of hippocampal function. Conventionally, in fear-conditioning studies in rodents, the level of conditioned fear has been inferred from alteration of activity-related measures, e.g., freezing or exploratory behavior (cf., Stiedl et al., 1999b, 2000). However, strain-specific coping strategies differentially affect the expression of fear (Koolhaas et al., 1999) and may thus confound the assessment of the individual's emotional state if restricted to a single activity measure, such as freezing. Hence, there is a compelling need for novel or multiple measures indicative of the emotional state in animals (cf., Antoniadis and McDonald, 1999, 2000; Lee et al., 2001).

Emotional states, such as anxiety or fear, are not only reflected on the behavioral level but also involve con-

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comitant neuroautonic adjustment resulting in alteration of heart rate (HR) and blood pressure dynamics (Berntson et al., 1998). The neurocircuitry involved in the expression of fear overlaps in part with the neuroautonomic circuitry that projects onto the heart (Loewy, 1990; Ter Horst et al., 1996). Retention of conditioned tone-dependent fear has been demonstrated to elicit a pronounced tachycardia under otherwise stress-free baseline conditions in the home cage of unrestrained mice (Stiedl and Spiess, 1997). Depending on the magnitude of its change, HR is generally taken as an indicator for attention (and auditory competence) and associative learning (Stiedl and Spiess, 1997; Stiedl et al., 1999b). The conditioned tone-dependent tachycardia is linked to a protein synthesis-dependent long-term memory function (Stiedl et al., 1999a). In contrast to the experimental conditions in auditory fear conditioning, the investigation of conditioned contextual fear by behavioral parameters, such as freezing, requires handling of mice prior to any actual measurement. Handling by itself constitutes a potentially aversive procedure that induces a strong tachycardia in mice (Kramer et al., 1993). Aversive experimental conditions are therefore expected to put the animal on the strain, which may interfere with cognitive functions (Kim and Diamond, 2002). In order to avoid confusion about the use of terms, strain is used here to signify the animals' response to some acute mental burden or challenge associated with emotional behavioral-neuroendocrine expressions and is not to be confused with the most used, misused, misunderstood, and confusing lay notion of stress. A state of enhanced generalized emotional strain may have contributed to the earlier findings of differential extinction of behavioral but not autonomic indices of conditioned tone-dependent fear in C57BL/6N or C57BL/6J mice (Stiedl et al., 1999b).

The evaluation of conditioned contextual fear in mice is typically restricted to behavioral measures with particular emphasis leveled upon the assessment of freezing. Given the limitations encountered in the assessment of freezing (cf., McNish and Gewirtz, 2000), we hypothesized that neuroautonomic measures would provide more reliable indicators of conditioned contextual fear-induced neuroautonomic adjustments. While the activity of the autonomous nervous system and its countervailing components, i.e., the sympathetic (SNS) and parasympathetic (PNS) branches, is not directly accessible for quantitative assessment, the complex interaction of neuroautonomic outflow may be studied from its control of cardiac sinus node activity. Hence, dynamical analysis of HR utilizing advanced techniques of nonlinear time series analysis presents an investigative tool providing for a handle towards uncovering the combined interaction of the neuroautonomic system components. Ultimately, the significance of HR-derived measures for identifying the animal's neuroautonomic response or readjustment to conditioned contextual fear presents the major emphasis of this study.

2. Materials and methods

2.1. Animals

The experiments were performed on 35 male C57BL/6J@Rj mice (Centre D'Elevage, Le Genest St. Isle, France) obtained at an age of 8 weeks. They were individually housed in standard Macrolon cages with free access to food and water and were kept on a 12-h dark–light cycle with lights switched on at 7 a.m. At the time of testing (performed during the light phase), mice were 11–13 weeks of age. All experiments were in accordance with the European Council Directive (86/609/EEC) by permission of the Animal Protection Law enforced by the District Government of Lower Saxony, Germany.

2.2. ECG acquisition and data processing

The ECG was continuously monitored by telemetry using miniature ECG transmitters (Data Sciences, TA10EA-F20, St. Paul, MN, USA) implanted into the abdominal cavity as described previously (cf., Stiedl and Spiess, 1997). The experiments were performed 14–21 days after surgery. The ECG was continuously recorded during the contextual memory test (see below), and 32-min epochs were collected ($\sim 2 \times 10^4$ beats). The digitized ECG (sampling rate 4 kHz, resolution 0.25 ms) was automatically analyzed and annotated to obtain discrete time points corresponding to the successive R-wave maxima using an adaptive QRS template pattern-matching algorithm (Fig. 1). Ectopic beats, typically 1 in 10^4 beats, were identified by fitting a third-order autoregressive model to the beat interval data stream using multiples of the interquartile distance as detection threshold and replaced by linear-spline interpolation. The analytical framework for detecting and identifying the complex dynamical structure of the cardiac time series (see Section 2.5.) was applied to the full-length 32-min data sets (range: from ~16460 to ~23820 beats).

2.3. Conditioning paradigm

For habituation to handling and novelty, mice were initially exposed for 32 min to three different contexts (context 1–3) on three successive days (Fig. 2). The actual conditioning experiments followed the procedures outlined previously (cf., Stiedl et al., 1999a,b, 2000), except that the overall time course of the contextual memory test was followed for 32 min. After habituation, fear conditioning (acquisition) was performed in an acrylic cage mounted into a constantly illuminated (100–500 lx) fear-conditioning box (day 4, context 4). Mice were assigned to three different experimental groups. In the first group (conditioning group, US*l* group), mice were given 180 s for exploration followed by 30 s tone exposure (10 kHz, 75 dB SPL, pulsed 5 Hz). The tone was terminated by a foot shock (US; 0.7 mA, 2 s, constant current) delivered
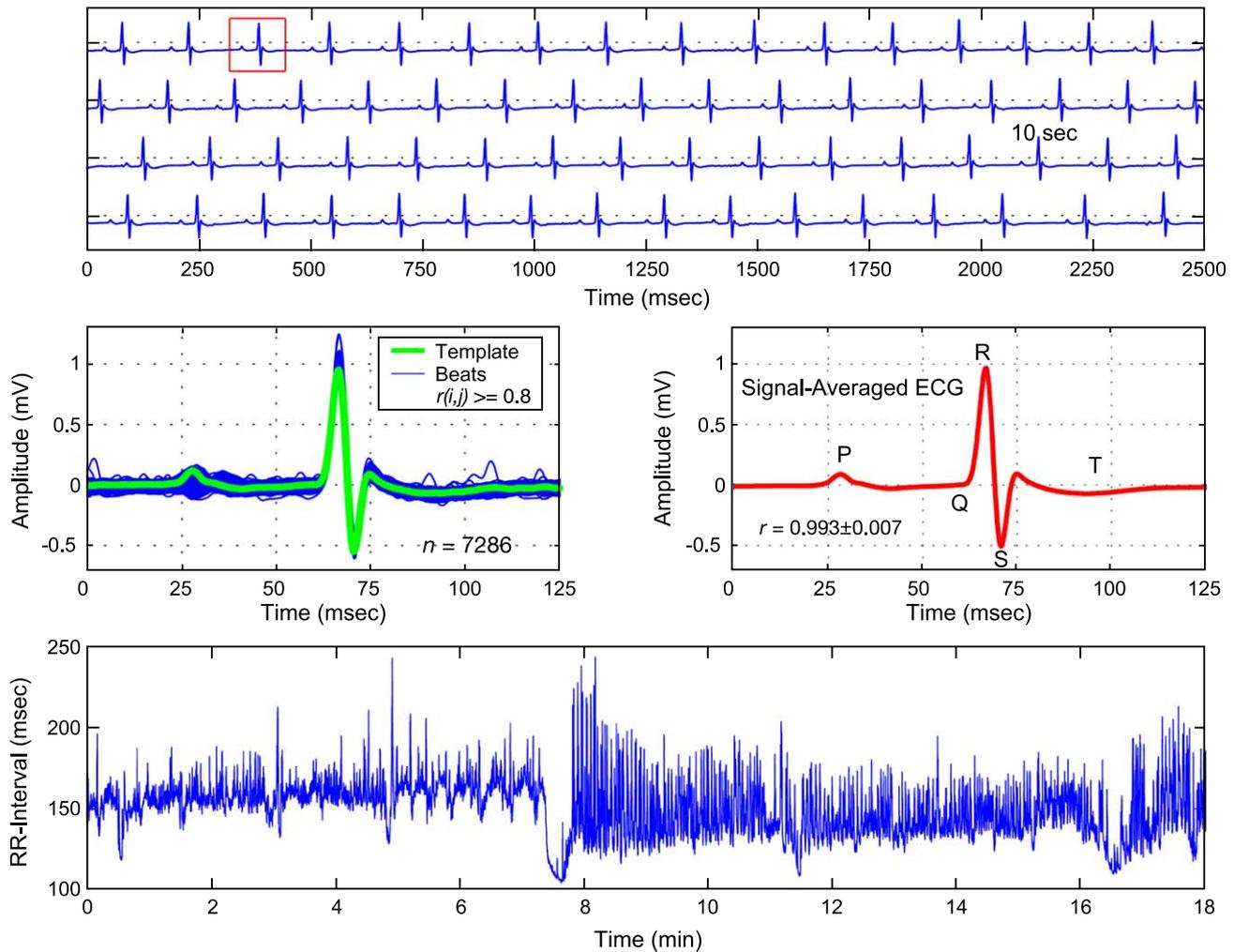
Fig. 1. Processing of ECG signals. Upper: strip-chart recording (10 s window) of awake unrestrained mouse. The beat template indicated by red rectangle serves for screening the ECG signal using a pattern-matching algorithm. Middle-left: superposition of template beat and matching individual beats (n=7286; correlation coefficient $\geqq 0.8$) identified from 18-min ECG recording. Beats are centered on the peak of the R-wave of the ECG. Middle right: signal-averaged ECG waves and peaks are labeled by conventional ECG terminology. Lower: the heartbeat interval (RR interval) time series is obtained from the discrete time points corresponding to the successive R-wave maxima identified by the template-matching algorithm.

through a metal floor grid. Mice were returned to their home cages 30 s after shock termination. The second group of mice (USi group) experienced the US right after placement into the fear-conditioning box. The third group of mice (USn group) experienced the same training sequence but no US exposure. Behavioral measurements were performed in all groups during acquisition to determine locomotor activity, exploratory behavior, and US responses.

The memory test (retention) was performed by contextual reexposure to the conditioning box (context 4) on day 5. Continuous ECG recording was performed in parallel with automatic activity monitoring by a fear-conditioning system (TSE, 303410, Bad Homburg, Germany). Activity was detected by photobeam sensors (sampling rate 10 Hz) and averaged over short windows of time as described previously (cf., Stiedl et al., 1999a,b, 2000). Inactivity was defined as the percentage

of time during which the activity was≤1 cm/s and was averaged over 2-min subepochs. Furthermore, the area (expressed as percentage of total cross-sectional area) covered by an exploring mouse during a given subepoch was calculated.

2.4. Stress-free state and handling

For comparison of HR dynamics in the three groups of mice undergoing contextual conditioning (see above) with the more general effects on HR dynamics elicited by unspecific external stimuli related to the animal's environment, two additional groups of mice were included in this study. HR dynamics was additionally analyzed for 32 min during undisturbed conditions in the animal's home cage (UNT group) or exposed to the handling procedure only (HAN group). Handling included the transportation of the animal in its home
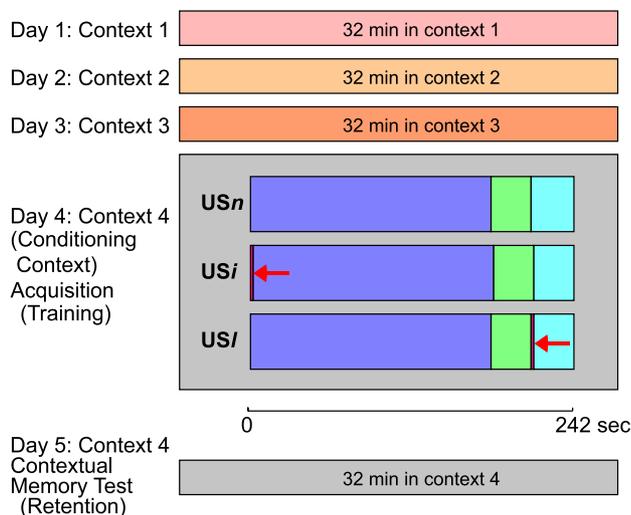
Fig. 2. Conditioning paradigm and experimental protocol. US, uncon-ditioned stimulus (2-s foot shock) indicated by left-arrows. US suffices *i*, *l*, or *n* refer to immediate, late, or no US, respectively. Colored fields: blue, 180-s exploration; green, 30-s tone exposure; cyan, 30-s recovery. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cage to the experimental room, brief placement (by hand) into the conditioning box, followed by removal to its home cage and return to the housing room (~60 s total time). Upon completion of the operational 'sham' procedures, HR was monitored for 32 min. The addi-tional groups of mice did not experience any other context exposure or adverse stimulation by US.

2.5. Assessment of heart rate dynamics

Physiological signals, such as the heartbeat interval time series studied herein, are typically generated by complex self-regulating systems that process inputs with a broad range of characteristics. The beat-to-beat fluctua-tions in the HR of humans and other mammals display inhomogeneous, nonstationary extremely irregular tempo-ral organization, and the statistics of these fluctuations exhibit self-affine or fractal properties. The complex dynamical properties of heartbeat interval time series, i.e., the correlated structure where each event is statisti-cally dependent on all past ones, present a manifestation of a *nonlinear fractal random process* with many interacting components ultimately determining the cardiac rhythm. Recent studies of the cardiac beat-to-beat variability have shown that the inherent fluctuations of heartbeat interval time series are not uncorrelated, structureless random error but rather exhibit a strong long-range correlated structure that is generally referred to as *1/f*-noise and results from scale invariance and self-similarity, which are characteristic features of fractal dynamics. Moreover, it has been established that heartbeat intervals, rather than being monofractal (or uniscaling), exhibit multifractal properties in healthy humans that are

lost in a life-threatening condition known as congestive heart failure. The fractal properties of heartbeat dynamics of normal man, cardiac patients, and experimental mice along with an extensive allusion to advanced nonlinear methods of time series analysis have been presented elsewhere, and no comprehensive review will be given here (cf., Ivanov et al., 1999, 2001; Goldberger et al., 2002; Stiedl and Meyer, 2002, 2003a,b; Meyer and Stiedl, 2003; Meyer et al., 2003; see also references therein). The choice of methods developed in most advanced statistical physics and nonlinear sciences and introduced here in cardiac time series analysis of mice is based on objective mathematical criteria, but there is a strong bias towards methods that we have found either conceptionally interesting or useful in practical biomedical signal processing or both. To our best knowledge, the analytical methods (see below) used herein present the first-time application to cardiac beat-to-beat time series of mice.

2.5.1. Bayesian adaptive multiresolution wavelet denoising

Intuitively, the observational highly irregular ('noisy') heartbeat interval time series may be visualized as a merely regular original signal corrupted by noise. The regular signal may undergo transient shifts ('trends'), but the precise nature of the noise is a priori unknown. In order to recover the 'baseline' trend or unknown function of the original, nonparametric regression (or denoising) was applied using a wavelet-based approach. The method is to expand the noisy data in wavelet series, extract the 'significant' wavelet coefficients by thresholding, and then inverting the wavelet transform of the denoised coeffi-cients. The choice of thresholding rule is improved by data-adaptive Bayesian wavelet shrinkage and thresh-olding estimators. Extensive reviews and descriptions are presented elsewhere (cf., Abramovich et al., 1998; Antoniadis et al., 2001). We emphasize here that the pieces of the bursty original signal that are removed are not noise in the traditional sense! The essence of wavelet denoising lies in the ability to represent locally non-smooth phenomena with a few significant wavelet coefficients. Fig. 3 (upper left) illustrates the wavelet denoising analysis. The persistent fluctuations after denoising reveal that the original time series is nonsta-tionary. After preprocessing, the denoised signal was resampled at 12 Hz to adjust all 32-min heartbeat interval time series for an equal number of data points (length). Means of 1-min subepochs were calculated and averaged over the number of animals in each experimental group (group-means±S.E.).

2.5.2. 2-Microlocal regularity exponents

In the analysis of time series, the fine local regularity of a function contains essential information for further processing. Heartbeat interval time series presents a signal that is a nondifferentiable ('singular') function at almost all points in time. A singularity in the signal occurs
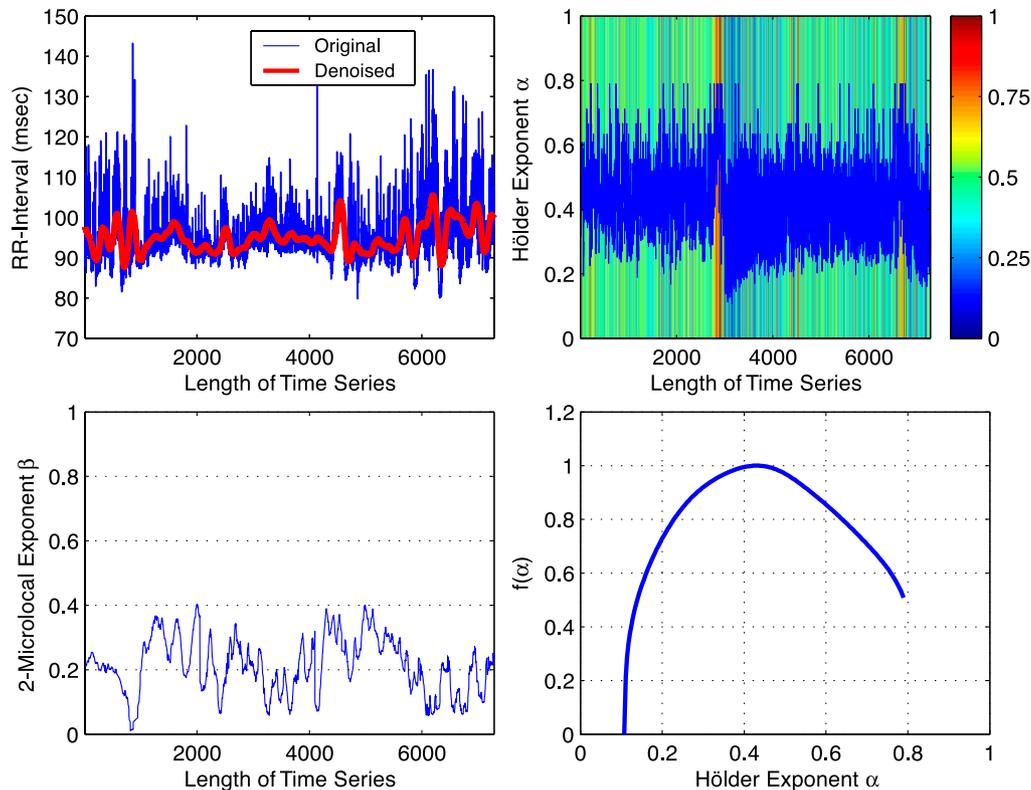
Fig. 3. Assessment of heart rate dynamics. Upper left: original heartbeat interval time series (18 min) and extraction of 'baseline' trend by Bayesian adaptive multiresolution wavelet denoising. Lower left: 2-microlocal regularity exponents of original cardiac time series. Large exponents correspond to low dynamic in the change of heartbeat interval duration, while low values of the exponent indicate bursty and frequent changes. Upper right: estimated local Hölder exponents of original signal (blue trace) indicating its local 'roughness'. The irregularity of a function (e.g., time series) can be represented differently as a colored 'barcode' where regions where it is smooth (red) may be visually distinguished from regions where it is rough or irregular (blue). The range of colors shows the range of local irregularity and the distribution of the colors within the signal illustrates the distribution in time of the local irregularities. Lower right: the complex multifractal pattern is characterized by the large deviation multifractal spectrum (LDMS). The multifractal spectrum yields statistical information as to which singularities occur in the time series. Abscissa represents Hölder coefficients α, and ordinate is related to the amount of points $f(\alpha)$ where a given regularity is encountered. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

whenever the heartbeat interval undergoes an abrupt change, and various singularities are embedded in the variability of HR. In the nonlinear analysis of irregular time series, the strength of a singularity, indicating the local regularity/irregularity property of the signal, is characterized by its point-wise Hölder exponents with dimensionless values defined in a compact of [0,1]. The basic principle is to associate to a signal $f(t)$ a function $\alpha(t)$, the Hölder function of f, which measures the regularity of f at each point t. However, this exponent, although a powerful measure of the local regularity, is insensitive to oscillatory behavior. A further generalization of time-dependent Hölder regularity that yields a more precise and complete description of the local regularity properties is provided by 2-microlocal analysis (cf., Guiheneuf and Véhel, 1998; Kolwankar and Véhel, 2002; Seurat and Véhel, 2003). In the case of sampled nonstationary signals, no preprocessing of the data is required, and reasonable accuracy is obtained for 'complicated' continuous but nowhere differentiable functions. The 2-microlocal exponents (β) designate the local smoothness of the time series, lower exponents corre-

sponding to more irregular parts of the signal. The estimated exponents' function that characterizes the local 'roughness' at a given date is displayed in Fig. 3 (lower left).

2.5.3. Large deviation multifractal spectrum

Multifractal structures have been found in a variety of physical systems, and more recently in the heartbeat interval time series of normal subjects and patients with cardiac disease (Ivanov et al., 1999, 2001; Goldberger et al., 2002; Meyer et al., 1998a, 2003; Meyer and Stiedl, 2003). At this time, there exist some different mathematical methods for describing multifractal scaling behavior in a signal, but they are, in general, difficult to interpret and delicate to calculate. One of the most commonly used methods is based on the *multifractal spectrum*, a global summary statistic that provides the frequency with which different local scaling coefficients occur in the underlying signal. In practice, it is approximated and estimated via the Legendre transformation of the structure function, where the latter for singular signals may be constructed by wavelet-based scaling analysis tools (Ivanov et al., 1999;

Gilbert, 2001). Here, we employ an alternative method that can be naturally incorporated into conventional time series and that describes multifractal scaling or local irregularities in a signal.

The theory of multifractals and Cramer's Large Deviation theory provides a further generalization and convenient representation of the distribution viz. inhomogeneity of local Hölder exponents. The large deviation multifractal spectrum (LDMS) presents a global statistical description of the set of singularities present in the time series and is characterized by the spectrum of the Hölder singularity exponents α and probabilities of occurrence $f(\alpha)$. More precisely, $f(\alpha)$ estimates the exponential speed of the probability to encounter a singularity equal to α when the resolution tends to infinity. The graph of $f(\alpha)$ is a smooth parabolic function shaped like the symbol "∩", usually leaning to one side (Fig. 3, right panels). Evidence for the multifractal nature of cardiac time series using the technique of LDMS has recently been provided, and a full account of the theory and application is given elsewhere (Meyer et al., 2003; Meyer and Stiedl, 2003).

2.6. Statistics

Statistical evaluation was performed by analysis of variance (ANOVA) and ANOVA for repeated measures. The post hoc comparison was performed using Fisher's protected least significant difference test (PLSD) at a significance level of $p<0.05$.

3. Results

3.1. Behavioral responses

The locomotor activity during the 180-s exposure to context 4 (before tone presentation and excluding the 2-s subepoch of electric stimulation from analysis) in the training session (day 4) is displayed in a box plot format in Fig. 4 (left panel). While the mean activity in the USn and USl groups was close to ~6 cm/s (and similar to that of mice that were not provided with an ECG transmitter; cf., Stiedl et al., 1999b), post hoc statistical analysis reveals that mice receiving the immediate shock (USi group) were less active (~4 cm/s, $p<0.05$). The enhanced activity (~24 cm/s) elicited by US exposure was not different between the USi and USl groups, indicating that the instantaneous response due to the 2-s foot shock was the same no matter whether the stimulus was applied early or late in the training sequence (Fig. 4, right panel). The relative inactivity during contextual retention testing (day 5) was markedly enhanced in the USl group as compared to the USn ($p<0.01$) or USi ($p<0.02$) groups, whereas no significant differences of the inactivity profiles were detectable between the USn and USi groups ($p>0.27$; Fig. 5, upper panel). The results indicate that the foot shock (US) was equally perceived, but association with the contextual stimulus was dissociated. The formation of an aversive association of context with the US, as reflected by the suppression of locomotor activity, would occur only if the US was applied at a late instant relative to context exposure. Linear regression of group data
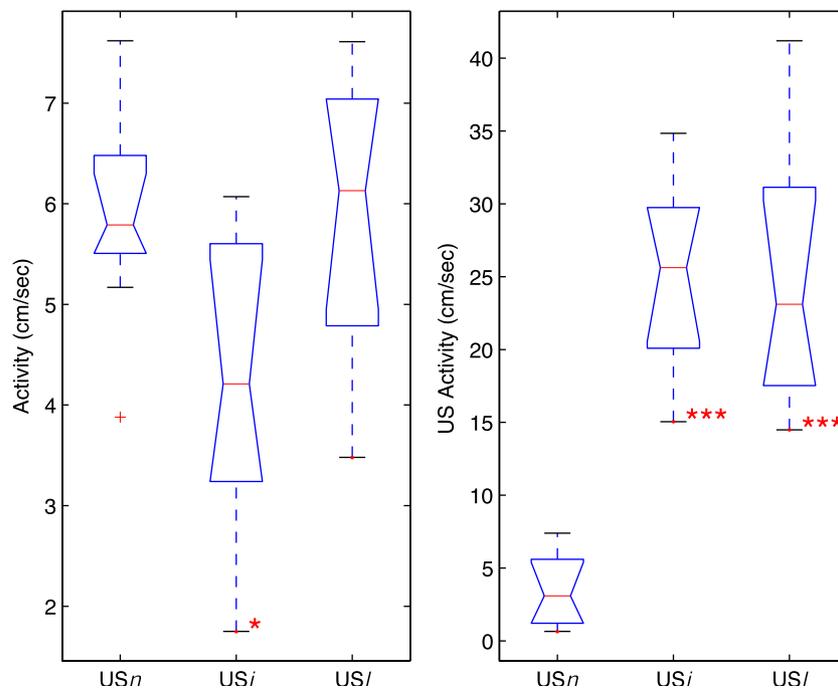

Fig. 4. Activity during training session. Mean activity during 180 s of contextual exploration (left) and US exposure (right) during training in the conditioning context (context 4, day 4). Means±S.E. ($n=9$/group), *$p<0.05$ USi vs. USn,l, ***$p<0.001$ USn vs. USi,l. US, unconditioned stimulus (2-s foot shock).
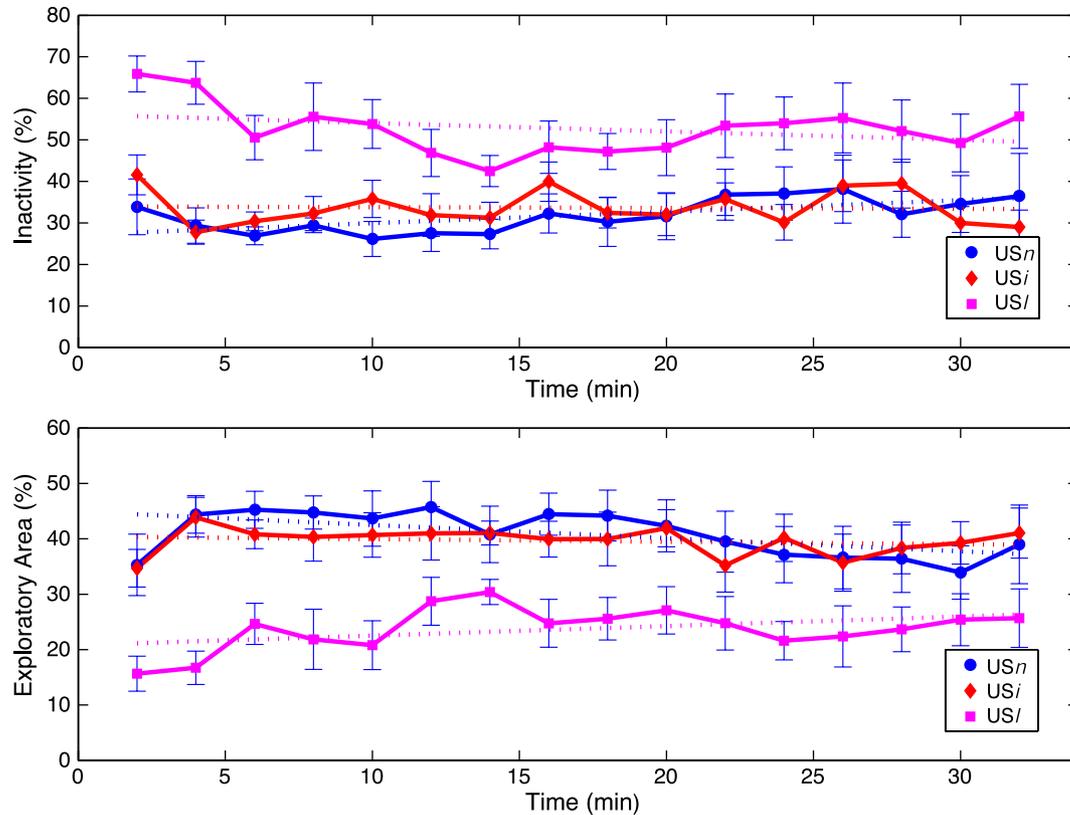
Fig. 5. Behavioral measures during contextual conditioning. Inactivity (upper) and exploratory area (lower) in US$n,i,l$ groups of mice (see legend to Fig. 2) during contextual retention test (day 5). Means±S.E. of 2-min subepochs, n=9/group. Dotted lines, linear regression of data points through observation period.

reveals that convergence of regression lines would require extended periods of time (well beyond the 32-min window of observation); hence, recovery to normal activity in response to the aversive experience appears to be extremely sluggish. In line with the results for relative inactivity, the results for relative exploratory area display a mirror image demonstrating that the relative exploratory area experienced by animal groups was markedly reduced in the USl group (Fig. 5, lower panel).

3.2. Heart rate dynamics

3.2.1. Temporal kinetics of heart rate

The time course of heartbeat interval duration during contextual retention test for the three experimental groups is compiled in Fig. 6 (left panels). Consistently, all groups demonstrated a uniform tachycardic response (mean RR interval ~78 ms=770 bpm) in the early stage of contextual retention testing. The HR achieved approaches the maximum physiological limit, which is determined by the AV conduction time (~80 ms; cf., Hagendorff et al., 1999). While the initial tachycardia is maintained for ~5 min in both USn and USi groups, USl group animals display a sustained tachycardia for ~15 min. In all experimental groups, the initial tachycardia is followed by a sluggish (almost linear) recovery towards baseline levels (~122 ms=492 bpm, see below). The halftime ($T1/2$) of recovery

is estimated at ~50 min for the USn or USi groups, whereas ~60 min is expected for the USl group. Statistical evaluation reveals that USn and USi mice demonstrated the same temporal pattern of response to contextual retention testing, which in turn was clearly different from that of the USl group, reflecting the aversive association established by the training session. Heuristically, USn and USi group animals felt the novel environment arousing and quite interesting for further exploration although they had seen it before briefly (for ~4 min during training on day 4; cf., Fig. 2), whereas the USl group had bad memories of the past. The complementary data of unrestrained mice maintained essentially untreated in their home cage and thus being familiar with the environment (UNT group) or subjected to short-term alteration of environment by (HAN group) are summarized in Fig. 6 (right panels). While unrestrained mice demonstrated a relatively stable baseline of HR viz. RR interval duration, mice subjected to handling exhibited marked tachycardic exertion (much like that in US$n,i,l$ group mice) but $T1/2$ of recovery was much faster (~ 15 min).

3.2.2. Local regularity of heart rate

The local regularity/irregularity of heartbeat interval time series assessed from 2-microlocal exponents estimation for all experimental groups is compiled in Fig. 7. Here the RR variability is mapped onto the [0,1] interval indicating the
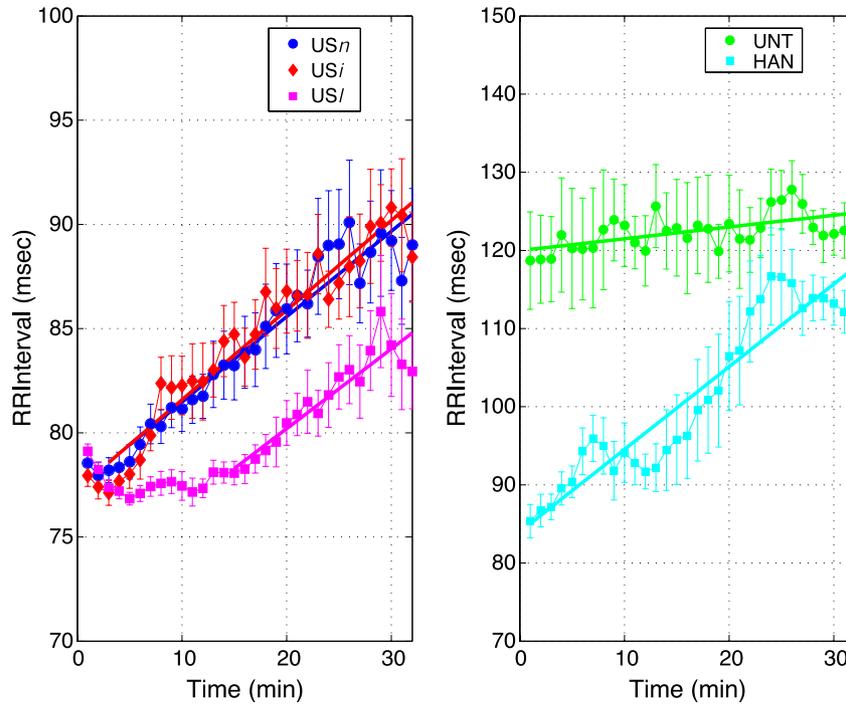
Fig. 6. Heartbeat interval patterns during contextual conditioning. 'Baseline trend' of RR intervals after wavelet-based denoising in US$n,i,l$ group mice (left) and untreated (UNT) mice or mice undergoing gentle short-term handling (HAN; right). Means±S.E. of 1-min subepochs, $n=9$/group. Solid lines, linear regression of data points through recovery periods. Note the different ordinate scaling in left and right panels.

relative smoothness of the dynamics. The results consistently demonstrate in the US$n,i,l$ group animals that heart rate is more regular during the early tachycardic episodes gradually declining to more irregular levels within the first 10 min (left panels). The lower regularity exponents observed in the UNT group (upper right) throughout the window of observation is a reflection of the more complex and highly irregular pattern of beat-to-beat fluctuations that
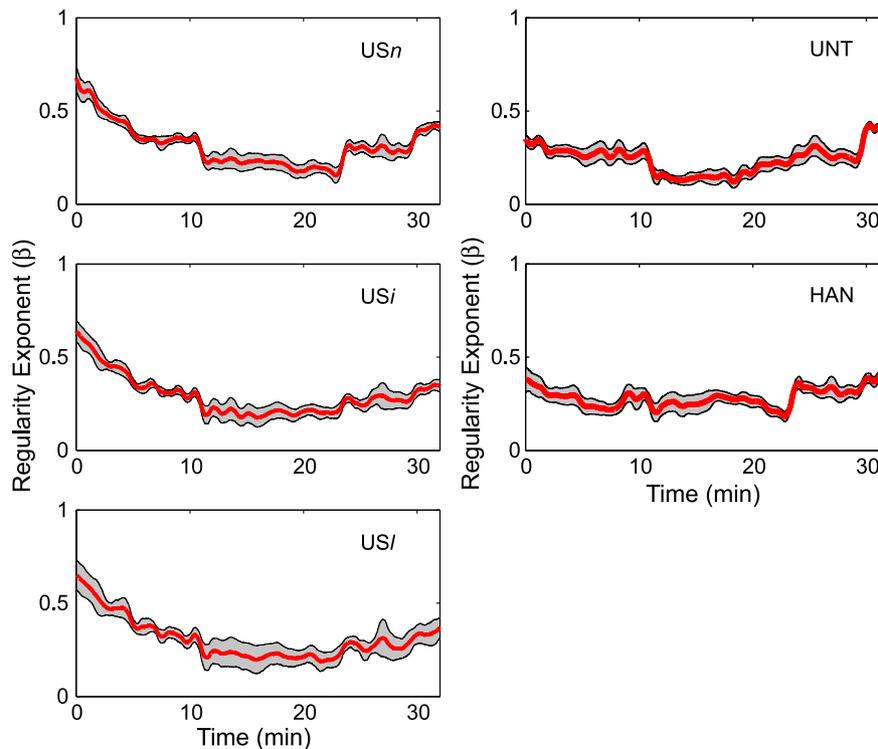

Fig. 7. Regularity of heartbeat interval time series determined by 2-microlocal exponents (β) analysis. Left panels: US$n,i,l$ group animals; right panels: UNT and HAN group animals. Means±S.E., $n=9$/group. See text for further details.

is characteristic for mice maintained in a familiar environment (home cage) that is free from external stimuli and is similar to what is observed in resting humans. Interestingly, short-term handling (HAN group, lower right) has little if any effects on local variability of HR, notwithstanding the fact that handling prompted a transient tachycardic response.

3.2.3. Large deviation multifractal spectrum of heartbeat interval fluctuations

The multifractal spectra reflecting the inhomogeneity of local Hölder exponents present in a given cardiac time series are summarized in Fig. 8. For untreated mice (UNT group) that were not subjected to any extrinsic stimuli, the spectrum $f(\alpha)$ is a smooth concave function over a broad range of Hölder exponents α (upper right panel). The broad range spectrum indicates that HR of unrestrained mice (much like in normal resting man) exhibits multifractal dynamics; that is, the normal cardiac rhythm displays self-affine multifractal variability (cf., Meyer et al., 2003; Meyer and Stiedl, 2003). Notably, the $f(\alpha)$ spectrum of cardiac dynamics in mice undergoing gentle handling (HAN group, lower right panel) was not materially affected as compared to mice left essentially untreated. In contrast, the $f(\alpha)$ spectrum of mice undergoing reexposure to context 4 (US$n,i,l$ groups) consistently displayed a marked departure from strict concavity reflected in the left-sided shape of the $f(\alpha)$ curve

(left panels). The 'removal' of high singularity strength, i.e., cutoff of the upper-range Hölder exponents ($\alpha > 0.5$), indicates a diminution of multifractality strength, which is given by the $\alpha_{max} - \alpha_{min}$ difference. The pattern of $f(\alpha)$ was statistically indistinguishable among the three groups of primary interest suggesting that the mechanisms giving rise to altered cardiac dynamics were uniform in the US$n,i,l$ groups.

4. Discussion

In mammals and lower vertebrates, the perception of threat in a hostile environment mediated via the sensory system (vision, hearing, olfaction, nociception) is conveyed to the central nervous system that elicits the execution of response patterns ultimately serving to protect organisms from harmful events. The response patterns typically encompass preformed triads with specific classified expressions of (i) the somatomotor (behavioral) system, (ii) the visceromotor (neuroautonomic) system, and (iii) the endocrine system. The animal's response to noxious physical or mental challenges is therefore fully characterized by three elements, the latter two serving to optimize the efficacy of the former. Hence, we studied the response patterns in mice exposed to exterioceptive sensory stimulation, i.e., contextual fear conditioning. While the assessment of the
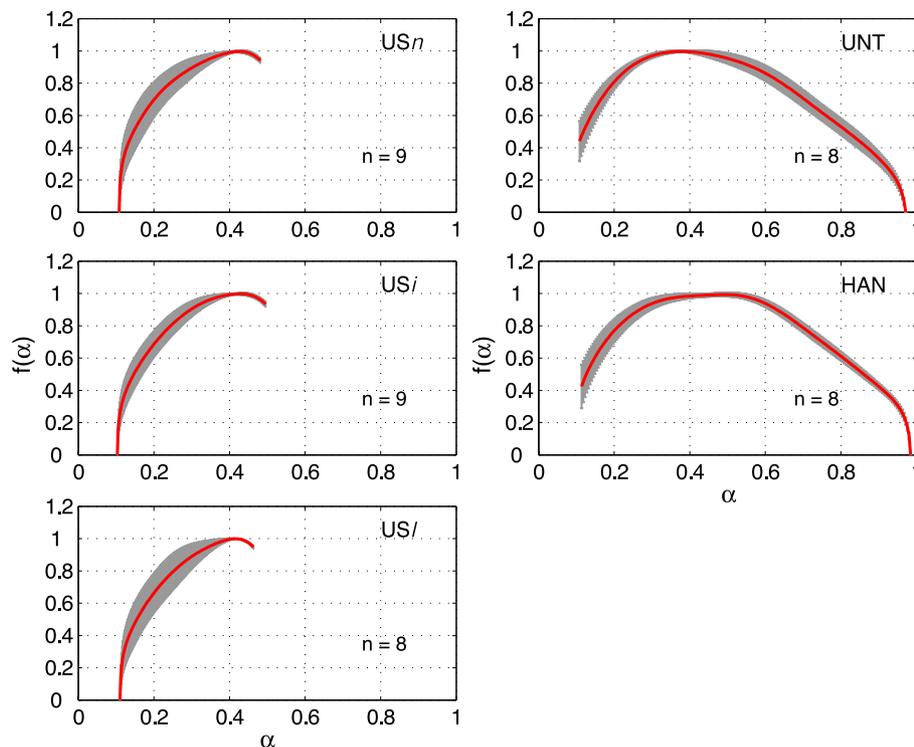

Fig. 8. Multifractality and neuroautonomic cardiac control. Multifractal spectra [$f(\alpha)$ vs. α] of US$n,i,l$ group animals (left panels) and UNT and HAN group animals (right panels). The concave shape of the spectra exemplifies multifractal properties of the cardiac interbeat rhythm. The different patterns of $f(\alpha)$ reveal the importance of neuroautonomic cardiac control in generating the broad-range multifractal spectrum of cardiac dynamics in control groups (UNT, HAN). The spectra of the US$n,i,l$ groups display a markedly left-sided shape which reflects the uniform or stereotyped adjustment of the neuroautonomic system in response to contextual stimulation.

behavioral response is pretty straightforward, the activity of the sympathetic (SNS) and parasympathetic (PNS) nervous system, the two main divisions of the autonomic nervous system (ANS), is not open to direct evaluation. However, the cardiac beat-to-beat intervals, which are basically determined by excitation of the sinoatrial node as cardiac pacemaker, show significant variability through the sympathetic and parasympathetic branches of the ANS and thus contain useful information to understand how the ANS controls the cardiocirculatory system. Strictly, autonomic output to the heart is by way of (i) projections to cardiac pacemakers, and (ii) by way of direct neural projections into the myocardium itself to provide the source of release of bioactive agents that have receptors located on myocardial cells themselves. Both types of neural projections ultimately determine the complex nonlinear dynamics of the heartbeat interval pattern. The hormonal link is not addressed in this study as it requires inadvert invasive intervention by blood sampling. Sympathoadrenal activation associated with the release of catecholamines from the adrenal medulla and engagement of the renin–angiotensin–aldosteron axis is well described in any textbook of medical physiology.

The major results from this study are summarized as follows:

– Exposure to the conditioning environment (context 4) is characterized by enhanced strain imposed onto the animal, which is reflected by increased locomotor activity associated with transient maximum tachycardia (USn, USi groups).
– Contextual fear conditioning, i.e., the aversive association of the conditioning context with the foot shock is characterized by vigilance (freezing) associated with a sustained tachycardic response (USl group).
– The execution of emotional response patterns, both in terms of the behavioral and neuroautonomic adjustment (based on linear HR means), appears to depend on the precise nature of the stimulus (nonaversive viz. neutral vs. negative, short-term vs. sustained).
– However, a uniform stereotyped trait of neuroautonomic expression (based on nonlinear HR dynamics) is entrained in the adaptation of autonomic responses to an unfamiliar environment (US$n,i,l$ groups) which is substantially different from that of a merely trivial (in part proprioceptive) stimulus, such as gentle short-term handling (HAN group).

4.1. Behavioral responses

In behavioral research, well-known response patterns are typically induced when animals are exposed to potentially harmful environmental challenges. The typical transient reaction to sudden unexpected stimuli of a few minutes duration is generally referred to as *defense* (*alarm*) reaction (DR) and locomotor activity is that of 'flight or fight' type,

although a graded fashion of enhanced activity may be induced by various confrontations. Likewise, both USn and USi group animals engaged DR by enhanced locomotor activity and exploration when exposed to the novel context. The DR is always marginally engaged whenever animals are mentally alert and active in exploring a novel environment. Reexposure to context 4 of the experimental setup, irrespective of three times of preexposure to different contexts provokes a similar behavioral response in either group. We have demonstrated previously that the USi group presents a shock-exposed control group that does not form an aversive association of US with the context (Milanovic et al., 1998). Hence, the USi group serves as a control group for the control of other unspecific stimuli that subsequently may affect the behavioral reaction in the absence of associative learning (Fanselow, 1990; Wiltgen et al., 2001). The relatively high activity displayed during training by the USi group confirms our previous observations of lack of post-US freezing in mice (Stiedl et al., 1999a), which is at variance with what is generally observed in rats (Fanselow, 1980).

Unlike in the USn and USi group mice, the behavioral response to an aversely conditioned stimulus is substantially different in the USl group animals in that animals demonstrated a *vigilance* (*freezing*) reaction (VR) that is characterized by complete immobility in alert position (not to be confused with the inhibitory playing-dead reaction; whether the animals were in fact alert depends on the associated neuroendocrine response, see below). It is well known that animals can instantaneously shift from VR to DR with flight or fight, and rapid shifts between the two options seem to be common. The reason why USl group animals 'opted' for VR may be related to the specific experimental conditions of the setup. In experimental fear conditioning, mice are typically maintained in an experimental box, and upon exposure to an aversively conditioned stimulus, may suddenly experience being 'cornered with no way out'. Hence, animals may not engage a DR with 2 degrees of freedom (flight or fight), because their degrees of freedom are reduced by $2-1=1$ as the option for flight may not be realized in the given surrounding. It may be conceivable that animals remained more or less 'frozen' in intense 'anticipatory' alertness and eventually may have engaged DR if indicated or possible. Engagement of DR or VR or rapid changeover between the two is unlikely to result from voluntary control, which would require experience-based learning and memory recall from repetitive aversive stimulation in the past.

4.2. Neuroautonomic responses

Cardiovascular reactivity peaks early during the initial exposure to contextual novelty when the demanding task and uncertainty are greatest. The entrainment of the ANS by instantaneous withdrawal of parasympathetic activity and activation of sympathetic activity is reflected by the

expression of a massive tachycardia. The tachycardia associated with an increase in blood pressure (unpublished observations) leads to 'anticipatory' increased cardiac output favoring skeletal muscle, myocardial, and cerebral blood flow suited to support sudden physical exertions. The pattern of cardiovascular adjustment is characteristic of a DR and serves to mobilize all resources on virtually all points in preparation for all-out flight or fight. Notably, all groups (US$n,i,l$) displayed the same initial tachycardic response (cf., Fig. 5), but the behavioral pattern was markedly different, USn and USi mice demonstrating enhanced physical exertion, whereas USl mice were completely immobile (cf., Fig. 4). In other words, in terms of neuroautonomic adjustment, all animal groups displayed a clear DR, but cardiovascular adjustment was inappropriate for the VR displayed by the USl group animals that were exposed to aversive stimulation. Thus, neuroautonomic mobilization was more or less 'in vain' and presents a strong burden onto the cardiovascular system, because the initial rise of HR (and blood pressure) is no longer balanced-off by exercise-induced skeletal muscle vasodilatation and decreasing peripheral resistance. The inadvert neuroautonomic HR response in the USl group is further enhanced by the sustained maximum tachycardia, which was maintained for ~15 min, suggesting that the quality of the exterioceptive stimulus elicited by context 4 and by its aversive association was different and/or stronger. While the initial increase of HR results from combined parasympathetic withdrawal and sympathetic activation, the fall of HR (recovery to baseline) until the animals were fully familiar with context 4 (which is not achieved within the 32 min of observation) is primarily a function of reactivation of parasympathetic activity, sympathetic withdrawal playing a minor role. Interestingly, the rate of recovery mediated by vagal rebound was similar in all groups exposed to contextual challenge and is expected to reflect an active mechanism that promotes restitution to baseline levels. It is conceivable that mental alertness cannot be maintained endlessly without being properly interspaced by regular recovery periods. However, full relaxation from mental arousal is expected to require extended periods of ~1-h duration. It is important to realize that gentle short-term handling (HAN group) prompted neuroautonomic activation and a tachycardic response with almost similar strength as compared to the US$n,i,l$ group animals, but the rate of recovery was much faster ($T1/2$ ~15 min) as exterioceptive stimulation was relatively short.

A rough estimate of the excess energy expenditure of heart following neuroautonomic stimulation may be obtained by the following reasoning. Assuming (i) that pre- and afterload of heart had remained unchanged (i.e., peripheral resistance and venous return were constant), and (ii) that the mechanical efficiency of cardiac contraction was constant, the excess energy expenditure of heart is a linear function of excess HR with reference to baseline levels in unstimulated 'resting' state (UNT group). Hence, the average number of heartbeats (actually the number of points) within a given window of observation (32 min) in excess of that of the reference group is proportional to the excess cost (e.g., in terms of O_2 uptake) of the cardiac response. While the assumptions may not be completely true for USn and USi group animals, they appear to be more realistic for the USl group as a result of lack of physical activity in this group. From the average number of points in file (USn ~22,990; USi ~23,000; USl ~23,820; HAN ~20,180; UNT ~16,460), the excess energy expenditure of the cardiac response is ~40% (USn, USi), ~45% (USl), and ~23% (HAN), respectively. In fact, the extra cost of the cardiac response would be greater if the window of observation had followed full recovery of HR to baseline levels. Anyway, the numbers designate a sizable cost of cardiac mobilization associated with the animal's neuroautonomic engagement. Indeed, repetitive contextual conditioning associated with aversive stimulation over some extended periods (weeks) may lead to attenuation of the autonomic response or may leave the animal in a state of chronic strain that may result in mental disorders (e.g., major depression) potentially associated with the evolution of cardiac disease. However, we are not aware of any approach along these lines, but the concept may touch upon the learned helplessness model (cf. Vollmayr and Henn, 2003).

4.3. Dynamical neuroautonomic responses

In neurobiological research, the quantitative analysis of irregular physiological signals typically starts and often ends with the calculation of some linear statistics. The fluctuations of the signal are usually ignored in conventional studies focusing on averaged quantities. In fact, the fluctuations are often labeled as 'noise' to distinguish them from the 'true' signal of interest. Generally, in the conventional approach, it is assumed that there is no meaningful structure in the apparent noise, and therefore one does not expect to gain any understanding about the underlying system through the study of these fluctuations. Here, we reiterate that the most commonly used linear time or frequency-domain statistical measures (mean, variance, power spectrum) would not provide for an adequate characterization of the HR data when the data stream is nonstationary, nonlinear, and long-range correlated (i.e., self-affine or fractal). The pitfalls and limitations of linear analysis have previously been discussed in detail (see Stiedl and Meyer, 2002, 2003a,b). It is just for convenience that the conventional approach was included in the first part of our analysis (except for the fact that the 'baseline trend' in the cardiac time series was extracted by an advanced wavelet-based denoising technique).

In contrast, nonlinear or dynamical analysis is basically concerned with an analysis of the structure of the 'noise', assuming that there is some albeit unknown structure in the point-by-point differences in the signal. Previous analyses using detrended fluctuation analysis (DFA; Peng et al.,

1995) have quantified intrinsic long-range power–law correlations in noisy heartbeat fluctuations indicating the presence of scale-invariant, *unifractal* structures (*1/f*-scaling) in the heartbeat of humans and animals (Meyer, 1998; Meyer et al., 1998a,b; Stiedl and Meyer, 2002, 2003a,b). Recently, by adapting and extending methods developed in modern statistical physics and nonlinear dynamics, we and others have demonstrated that human heartbeat dynamics exhibit even higher complexity than inferred previously from the finding of monofractal *1/f* scaling, which is characterized by a broad *multifractal* spectrum (Ivanov et al., 1999, 2001; Goldberger et al., 2002; Meyer et al., 2003; Meyer and Stiedl, 2003). These findings provide for the possibility that understanding the origin of the temporal structure and the alterations in response to a given intervention may have potential for better identification of the neurocardiac response to contextual conditioning. The results from nonlinear analysis have revealed a different picture of the pattern of the cardiac response that would have remained unrecognized by conventional linear measures. One-dimensional Hölder regularity exponents estimation (cf., Fig. 7) demonstrates that the initial tachycardic response associated with more smoothness of the dynamics was essentially the same in all US$n,i,l$ group animals. The efferent response of the ANS is generally alluded to sympathetic and parasympathetic activities (SNA, PNA). Efferent vagal impulses are very short and discrete, and their intrinsic frequency is much faster than that of its sympathetic counterpart. Withdrawal of PNA along with potential sympathetic overstimulated SNA causing HR to approach its maximum necessitates the loss of potential of HR being 'modulated', which is ultimately reflected in a wiggly line of the cardiac interbeat time series. This effect was seen to dominate over the first 10 min of exposure to contextual novelty (context 4), followed by increasing vagal efferences to the heart throughout the later stages. The present findings that would remain unrecognized by conventional linear techniques of analysis and furthermore demonstrate that the neuroautonomic response of heart is *uniform* or *stereotyped* and hence would not allow for a posteriori conclusions as to the precise nature of its cause.

A global picture of neuroautonomic flow to the heart is obtained from the multifractal spectrum of cardiac interbeat time series. Narrowing of the spectrum and the dominance of lower-range Hölder exponents ($\alpha < 0.5$) indicates that active vagal flow to the heart as reflected by prevailing high variability (low α values) rapidly sets in and promotes restitution to baseline levels. Again, the multifractal spectrum seen in the US$n,i,l$ groups is a reflection of a generalized trait of expression of neuroautonomic regulation in response to contextual stimulation no matter whether the nature of the contextual stimuli was 'neutral' or 'aversively associated'.

The multifractal spectrum may be taken as indicative of the animal's emotional state. Here, we emphasize that animals exhibiting DR or VR display the same neuro-autonomic multifractal profile of anticipatory arousal suggesting that the emotional state was alike but behavioral responses may be substantially different. 'Negative' emotions, like fear, or emotions elicited from facing challenges foreign to its nature happen to elicit stereotyped neuro-autonomic adjustments. Alternatively, given different findings of the behavioral response of two different experimental groups of animals, any inference from linear HR analysis as to concomitant differences of emotional state or mental arousal has no solid basis. Hence, the allusion from linear analysis of HR patterns as to animals expressing more or less fear or anxiety (cf., Antoniadis and McDonald, 1999, 2000; Carrive, 2000; Nijsen et al., 1998) remains ambiguous or just pointless. This study also renders easy cause–effect conclusions drawn from individual or group differences of averaged HR patterns in response to extrinsic stimulation (e.g., drug administration, exposure to acute or chronic strain, phenotypic screening of targeted mutations) unwarranted unless the impact of the ensuing strain on the animals' emotional state is known or controlled. Emotional exertion may have contributed to the relatively high baseline HR reported in some previous studies (cf., Gehrmann et al., 2000; Mitchell et al., 1998).

This study in mice has demonstrated that mice in terms of their neuroautonomic response are extremely sensitive to a fluctuating environment. We do not claim for a novel finding here but reiterate on this notion as the interaction of experimental mice with the environment appears to have been largely ignored. We furthermore argue that the emotional susceptibility of standard laboratory mice is likely to result from lack of adaptation to a fluctuating environment, i.e., from being maintained for life in impoverished housing conditions. Indeed, all the experience encountered by experimental mice is typically that of every now and then being placed into a newly cleaned cage for maintenance. It would seem that experimental contextual conditioning was the first experience of a mutually fluctuating environment encountered by the animals of this study. To that end, laboratory mice may present a model of 'unexperienced' mice that were unadapted to a fluctuating environment. Hence, the behavioral or neuroautonomic responses to a given strain may not reflect a 'normal' response that otherwise would be demonstrated by animals that were experienced in a fluctuating environment and were utilizing memories of the past. The translational impact of these findings in mice for the psychophysiology of normal man remains unclear. However, an analogy reminiscent of the kind of exertion observed in patients suffering from panic disorders may be discussed.

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