**Experimental Physiology**

# Heart rate variability in mice: a theoretical and practical guide

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**The mouse is the animal model principally used to study biological processes in mammals. The mutation, overexpression or knockout of one or several genes can provide insight into human disease. In cardiovascular research, evaluation of autonomic nervous function is an essential tool for a better understanding of the pathophysiological conditions in which cardiomyopathy arises and develops. Analysis of heart rate variability is the least invasive method to evaluate the sympathovagal balance on the sino-atrial level. The need to perform this technique on freely moving mice emerged in the 1990s, but despite previous studies it has been difficult to set up and standardize a common protocol. The multitudes of techniques used, plus subtle differences in methodology, impede the comparison and clear interpretation of results. This article aims to make a survey of heart rate variability analysis and to establish a standardized protocol for the assessment of the autonomic neural regulation of heart rate in mice.**

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Life requires fine variations, allowing a cell, an organ or an individual to adapt their physiological constants to their specific environment. The cardiovascular system permanently undergoes reflex circulatory adjustments by the autonomic nervous system (ANS) to maintain homeostasis. Heart rate variability (HRV) analysis, developed to assess orthosympathetic and parasympathetic influences in humans (Sayers, 1973; Katona & Jih, 1975), has become a useful tool in clinical studies carried out to adapt patient care (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Malliani, 2005). Decreased HRV in humans is an independent predictor of cardiac morbidity and mortality in patients suffering from various forms of heart disease, including myocardial infarction (Bigger *et al.* 1992), congestive heart failure (Sandercock & Brodie, 2006) and sudden cardiac death (Chiou & Zipes, 1998). Although various methods are used to assess ANS status (biochemical assays, pharmacological and cardiovascular reflex tests), the one most routinely used has been HRV analysis in the time and frequency domains (Akselrod *et al.* 1981; Bigger *et al.* 1992).

The development of genomic technologies has made it possible to generalize the use of engineered animals, especially mice, to elucidate the roles of genes. Concurrently, the equipment has been miniaturized, and recording/analysis techniques have been adapted from humans to mice. Thus, in electrophysiological laboratories, HRV analysis is used to study the cardiac phenotype of transgenic mice (Gehrmann *et al.* 2000), the role of cardiac mediators and signalling pathways in heart rhythm (Uechi *et al.* 1998; Witte *et al.* 2004) and the effects of pharmacological substances on cardiac frequency (Elghozi *et al.* 2001; Tank *et al.* 2004). This method has also proved its capacity in assessment of the arrhythmic risk of new drugs (Thomsen *et al.* 2006).

While, for humans, the standard analysis techniques were established and recommendations published in 1996 by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996), it has proved difficult to standardize HRV analysis in laboratory animals. Heart rate variability analysis can be done in the time and frequency domains, but the special characteristics of heart rate in mice, such as high heart rate and frequent artifacts, make it worthwhile to standardize the ECG recording protocol and to adapt the method of analysis. In fact, there do exist some basic reports on mice, but it is not easy to compare the results between the different studies because of the

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variety of methods used, the different adjustments for analysis parameters and the multiple conditions for ECG recording. The purpose of this paper is to study the limits and difficulties of HRV analysis in mice and to set up a practical guide for HRV analysis through experiments and in accordance with previous studies. Simple principles are proposed to perform HRV studies quickly and effectively using commercial software.

## Methods

### Animal preparation and ECG recordings

Experiments were performed on male FVB/N mice (Charles Rivers laboratory; Centre de Distribution, Typage, Archivage Animal CDTA, France), 12 months old, in accordance with ECC directive 86/609/ECC and French Government animal protection laws.

### Telemetry

Heart rate variability analysis requires continuous 24 h ECG recording in mice housed in single cages in a room with regulated temperature and hygroscopic conditions ($23 \pm 1^{\circ}$C, $45 \pm 10$% relative humidity). The animals must be synchronized to a light–dark schedule of 12 h–12 h to conserve circadian rhythm. Telemetry implies surgery under general anaesthesia (2% inhaled isoflurane in $O_2$, Aerrane®, Baxter, France) for subcutaneous implantation of the teletransmitter (Data Sciences International, Saint Paul, MN, USA) in the animal's back (less invasive than intraperitoneal implantation, thus ensuring a higher survival rate in fragile transgenic mice). An analog signal from the telemetric receiver, digitalized at a sampling rate of 2 kHz with 12-bit precision without a signal filter, was applied to catch all ANS modulations of HR. Figure 1 shows an example of a mouse after implantation of the teletransmitter with the recording set-up. Studies of ECG were performed from 2 to 26 days after device implantation to gauge the effects of the implantation on HRV and to estimate the minimal recovery period necessary to perform HRV analyses.

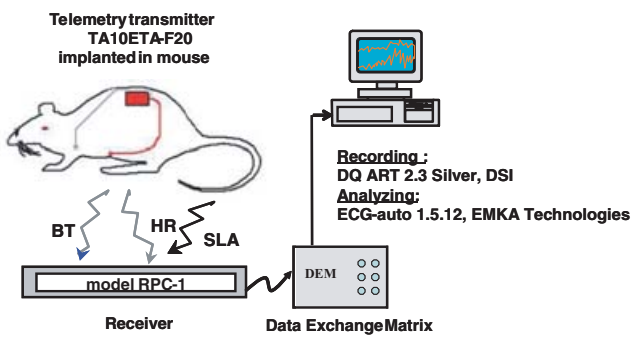


**Figure 1. Heart rate variability set-up**
A teletransmitter, allowing simultaneous recording of body temperature (BT), spontaneous locomotor activity (SLA) and heart rate (HR), is implanted subcutaneously in the mouse's back. It sends radiofrequency signals to a receiver located below the mouse cage. A data exchange matrix converts the analog signal into a digital signal before storage in the microcomputer. Data were collected by DQ ART software and analysed off-line with ECG-auto.

### Analysis of ECG recordings

Heart rate variability analysis is usually performed off-line with computerized techniques. From a continuous ECG recording, each R peak due to sinus depolarization is detected, and the normal R–R intervals (NN intervals) are determined. All telemetric data should be scanned by software to verify the quality of the ECG signals. Since settings and analysis can be standardized with commercial software, we chose ECG-auto by EMKA Technology (EMKA Technologies SA, Paris, France). This software performs fast, reliable, field-tested, in-depth ECG analysis with full user control. It allows fast Fourier transform (FFT)-based time-domain analysis and frequency-domain study with beat-by-beat control and review. Ectopic and artifact beats should be identified and quantified on the tachogram to gauge R–R interval stationarity. The ECG signals were digitally bandpass-filtered between 0.1 and 1000 Hz. Separate studies were carried out using ECG recordings taken over 12 h days and 12 h nights. Similarly, HRV was simultaneously analysed in the time and frequency domains. Since the excluded beats were not replaced by any averaged or interpolated beats, ECG recordings should be excluded if they show a number of abnormal beats exceeding 5% of the total.

### Time-domain analysis

**Selection of sinus heart beats.** Arrhythmias, ectopic beats and artifacts must be discarded before HRV analysis. Since manual beat-to-beat correction is impossible in mice owing to the high number of cardiac beats per 24 h (about $1 \times 10^6$ beats day$^{-1}$), we excluded from a 12 h ECG the R–R values not contained between mean R–R interval $\pm$ 2 s.D. (95.5% confidence intervals) when calculating time-domain indices and we present examples of analyses performed without and after selection of sinus heart beats.

**Definitions of HRV parameters in time-domain analysis.** The time-domain parameters calculated over 12 h light and 12 h dark periods were: (1) mean R–R intervals (in ms); (2) standard deviation of all normal R–R intervals (SDNN, in ms), reflecting total autonomic variability; (3) square root of the mean square successive differences between successive normal intervals (RMSSD, in ms),

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reflecting short-term variations in HR; and (4) percentage of normal consecutive R–R intervals differing by $> x$ ms (pNNx,%), reflecting cardiac parasympathetic activity.

The pNN calculated for mice is derived from the pNN50 ($x = 50$ ms) determined for humans, in whom 50 ms corresponds to about 10% of the mean R–R interval. We empirically tested different values (NN ranging from 4 to 10 ms and the corresponding pNN) under different conditions (pharmacological tests and circadian differences) to determine the best value with which to evaluate parasympathetic activity.

### Frequency-domain analysis

**Selection of ECG recordings.** Since the signal is non-stationary in mice, averaging multiple short-term analyses is recommended for HRV analysis in the frequency domain (Task Force of the European Society of Cardiology, 1996). We therefore looked for the optimal duration of analysed ECGs and the best sampling of these ECG fragments on which to perform HRV analysis. We selected and analysed one ECG period lasting from 1 to 5 min with no erratic fluctuations every 30, 60, 120 or 180 min during a phase of inactivity (according to the spontaneous telemetry recording of motor activity). Thus, 6–24 analyses (segment length of 2048 beats with linear interpolation and resampling to a 20 Hz interbeat time series, together with Hamming windowing) were averaged from data recorded during the light period.

**Definition of frequency ranges.** The typical cut-off frequency ranges for low-frequency (LF) and high-frequency (HF) powers (LF, 0.15–1.5 Hz; HF, 1.5–5 Hz) seem a good compromise to gauge the sympathetic and parasympathetic components of HRV (Table 1). Nevertheless, for the HF band we tested our own range (1.5–5 Hz) and the one recommended by Baudrie's group (2.5–5 Hz; Baudrie *et al.* 2007) in baseline conditions and after atropine injection, and then compared the results.

### Replicability

To verify the replicability of our protocol, we performed HRV analysis in the time and frequency domains on implanted FVB/N mice for 2 months.

### Pharmacological agent testing and short-term ECG analysis

To test the effects of pharmacological agents on HRV, we made short ECG recordings on conscious animals. We looked for the best time to perform the HRV analysis on these ECG recordings before and after the injection of atropine (atropine sulphate, Laboratory Renaudin, Saint Cloud, France, 1 mg kg$^{-1}$, i.p.). We averaged at least three stable 3 min ECG analyses 15 min before injection and three analyses at least 30 min after injection during a steady-state period to determine the pharmacological effects.

**Table 1. List of authors and the frequency ranges they have used in conscious mice**

| Authors | LF (ms$^2$) | HF (ms$^2$) | Variable |
|---|---|---|---|
| Ishii *et al.* (1996) | 0.1–1.0 | 1.0–5.0 | RR |
| Uechi *et al.* (1998) | 0.1–1.75 | 1.75–5.0 | HR |
| Wickman *et al.* (1998) | 0.4–1.5 | 1.75–5.0 | RR |
| Gehrmann *et al.* (2000) | 0.4–1.5 | 1.5–5.0 | RR |
| Just *et al.* (2000) | 0.15–1.5 | 1.5–5.0 | MBP, PI |
| Pelat *et al.* (2003) | 0.4–1.5 | 1.5–5.0 | BP |
| Williams *et al.* (2003) | 0.4–1.5 | 1.5–4.0 | BP, PI |
| Joaquim *et al.* (2004) | 0.1–1.0 | 1.0–5.0 | MBP, PI |
| Tankersley *et al.* (2004) | 0.2–1.5 | 1.5–5.0 | RR |
| Witte *et al.* (2004) | 0.08–1.5 | 1.5–5.0 | BP, PI |
| Xue *et al.* (2004) | 0.1–1.75 | 1.75–5.0 | PI |
| Campen *et al.* (2005) | 0.2–1.5 | 1.5–5.0 | BP |
| Fazan *et al.* (2005) | 0.1–1.0 | 1.0–5.0 | BP, PI |
| Adachi *et al.* (2006) | 0.15–1.5 | 1.5–5.5 | RR |
| Farah *et al.* (2006) | 0.1–1.0 | 1.0–5.0 | BP |
| Baudrie *et al.* (2007) | 0.15–0.6 | 2.5–5.0 | BP, PI |
| Duan *et al.* (2007) | 0.4–1.5 | 1.5–4.0 | RR |

Abbreviations: RR, R–R interval; BP, blood pressure; MBP, mean BP; and PI, pulse interval.

### Statistical analysis

All data are reported as means ± S.E.M. Statistical analysis was performed using Prism version 4 (GraphPad Software Inc., San Diego, CA, USA). The effects of the pharmacological compound were analysed using a Wilcoxon matched pairs test to compare HRV measurements taken before and after drug injection. The effects of computing treatment by selecting normal sinus beats in time-domain analyses were tested by a Wilcoxon matched pairs test. The effects of recovery time on heart rate and SDNN after surgical implantation, the effects of time on HRV replicability and the determination of the best duration and sampling time for frequency-domain analysis were analysed by a one-way ANOVA for multiple comparisons, followed by Dunnett's *post hoc* tests. The minimal significant probability value was set at 0.05.

## Results

### Telemetry and recovery period

Figure 2 displays the evolution of heart rate and HRV (represented by SDNN) after the implantation of the teletransmitter on FVB/N mice. While heart rate was stable 4 days after instrumentation, HRV was increased by the surgery, becoming stable only from day 10.

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### Selection of sinus heart beats

**In the time domain.** For HRV analysis, assuming a sinus cardiac rhythm and having discarded ectopic beats, we proposed removing the R–R values not included between R–R interval ± 2 s.d. (95.5% confidence intervals). Indeed, owing to the high number of heart beats in the mouse, manual exclusion of abnormal beats is impossible, and we suggested retaining only the beats within this confidence interval. This selection proved effective in getting rid of artifacts, false R detections and ectopic beats. Selection of normal beats modified the SDNN, RMSSD and pNN6 values. Before the selection of normal beats, parasympathetic parameters were overestimated (Table 2).

**In the frequency domain.** Unless a stable period of ECG is selected, it is difficult to use fast Fourier transformation, which assumes a stationary signal, to perform HRV. Therefore, several stable, regular periods were chosen. Stable ECG period was defined by the absence of artifact, premature ventricular contraction, sinus cardiac arrest and atrioventricular block. A stationary isoelectric line was required. Spontaneous motor activity conjunctively analysed with ECG must exhibit no mouse activity. Figure 3*A* displays an example of an HRV spectrum obtained from all recorded beats, and Fig. 3*B* shows the spectrum obtained after selection of a stable short period of the same recording. In the first spectrum, no peak is distinguished but in the second spectrum, two conspicuous peaks appear, corresponding to LF and HF activity.

We thus tested different durations for the stable period and different values of the interval between two analyses.

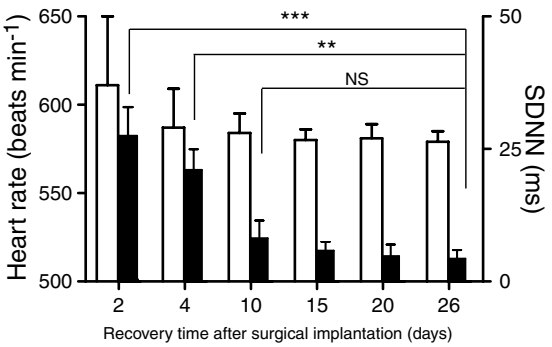


**Figure 2. Influence of recovery time after surgical implantation of a teletransmitter in FVB/N mice**
$n = 8$. The heart rate, determined from ECG redordings with ectopic beats removed, was monitored from day 2 to day 24 after surgery. Note that the heart rate (open bars; means + s.e.m.) was stable 4 days after implantation, but heart rate variability (SDNN, shown as filled bars; means + s.e.m.) required at least 10 days to stabilize (one-way ANOVA followed by Dunnett's test; NS, not significantly different; $^{**}P < 0.01$ and $^{***}P < 0.001$).

**Table 2. Selection of ECG period for time-domain analyses**

| | All R–R intervals | Normal sinus interval |
|---|---|---|
| R–R interval (ms) | 99.26 ± 4.33 | 94.13 ± 2.21* |
| SDNN (ms) | 10.21 ± 2.77 | 7.89 ± 2.33* |
| RMSSD (ms) | 7.86 ± 0.86 | 4.67 ± 0.22** |
| pNN6 | 4.91 ± 0.62 | 3.22 ± 0.23* |

Time-domain analyses were performed on FVB/N mice ($n = 8$). The values of R–R interval, SDNN, RMSSD and pNN6 were calculated from 12 h ECGs over all the R–R intervals and after excluding beats not found between R–R interval ± 2 s.d. in order to analyse normal sinus beats exclusively. Removed points correspond to paroxysmal atrioventricular blocks, premature ventricular contractions or artifacts. Note the evident change in SDNN, RMSSD and pNN6 values. ($^{*}P < 0.05$, $^{**}P < 0.01$, all R–R interval *versus* normal sinus interval, Wilcoxon matched pairs test).

The results are summarized in Table 3. A period of 3 min every 2 h appears to be valid. For this value, the standard error of the mean for LF and HF parameters and the mean of the standard error for the eight animals (mean N.s.e.m. ± N.s.e.m.) were lowest, suggesting a low interindividual variability. Furthermore, 3 min chosen every 2 h is a good compromise between the results and the time cost of the ECG analyses. In contrast, a period of 5 min is not appropriate for analysis of HRV in mice because it is difficult to find several stable periods as long as 5 min. We excluded two animals (out of 8) because the signal showed rhythmic disorders and was not stable enough during 5 min.

### Definitions of HRV parameters

**In the time domain.** Owing to the high heart rate in mice, we have to redefine the pNN value for time-domain analysis. Table 4 displays the evaluation of pNN for various differences between two consecutive R–R intervals. This table shows the dependence of pNN on the cut-off point. Six milliseconds is the optimal value because pNN6 (needed to reflect parasympathetic tone) is completely abolished by atropine administration. Moreover, pNN6 reflects circadian variations when mice enter a period of activity (dark period).

**In the frequency domain.** The value of HF power depends on the predefined range: 1.5–5 or 2.5–5 Hz. The first band looks more interesting because the baseline peak is below 2.5 Hz, as is shown in Fig. 3*B*. Also, injection of a parasympatholytic agent (atropine) dramatically decreased the first frequency band, giving less variability between individuals than in the second one (HF, 1.5–5 Hz, from $2.82 \pm 0.34$ to $0.94 \pm 0.11$ ms$^2$, $P < 0.01$; and HF, 2.5–5Hz, from $1.98 \pm 0.41$ to $1.12 \pm 0.32$ ms$^2$, $P < 0.05$, $n = 8$)

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**Table 3. Determination of the best duration and sampling time for frequency domain analyses**

| | Parameters | 1 min | 2 min | 3 min | 4 min | 5 min |
|---|---|---|---|---|---|---|
| LF ($ms^2$) | | | | | | |
| Every 30 min (24 analyses per mouse) | Mean ± S.E.M. | 1.46 ± 0.69* | 1.39 ± 0.48* | 1.81 ± 0.41 | 1.84 ± 0.33 | NE |
| | Mean N.S.E.M. ± N.S.E.M. | 1.11 ± 0.35** | 0.90 ± 0.31** | 0.88 ± 0.29* | 0.80 ± 0.34* | NE |
| Every 60 min (12 analyses per mouse) | Mean ± S.E.M. | 1.84 ± 0.73 | 1.82 ± 0.48 | 1.86 ± 0.55 | 1.92 ± 0.36 | 1.98 ± 0.45 |
| | Mean N.S.E.M. ± N.S.E.M. | 1.33 ± 0.49** | 0.91 ± 0.29** | 0.97 ± 0.36** | 0.84 ± 0.33* | 0.88 ± 0.27* |
| Every 120 min (6 analyses per mouse) | Mean ± S.E.M. | 1.79 ± 0.64 | 1.82 ± 0.37 | 1.89 ± 0.18 | 1.98 ± 0.45 | 1.78 ± 0.87 |
| | Mean N.S.E.M. ± N.S.E.M. | 0.99 ± 0.35** | 0.85 ± 0.32* | 0.35 ± 0.12 | 0.87 ± 0.29* | 1.54 ± 0.64*** |
| Every 180 min (4 analyses per mouse) | Mean ± S.E.M. | 1.98 ± 1.10 | 1.93 ± 0.99 | 2.10 ± 0.97 | 2.00 ± 1.01 | 1.89 ± 0.89 |
| | Mean N.S.E.M. ± N.S.E.M. | 1.36 ± 0.68*** | 1.31 ± 0.65** | 1.27 ± 0.63** | 1.10 ± 0.38** | 1.49 ± 0.53*** |
| HF ($ms^2$) | | | | | | |
| Every 30 min (24 analyses per mouse) | Mean ± S.E.M. | 2.98 ± 0.77 | 2.71 ± 0.52 | 2.45 ± 0.39 | 2.51 ± 0.61 | NE |
| | Mean N.S.E.M. ± N.S.E.M. | 0.97 ± 0.48** | 0.69 ± 0.27* | 0.48 ± 0.19 | 0.77 ± 0.32* | NE |
| Every 60 min (12 analyses per mouse) | Mean ± S.E.M. | 2.53 ± 0. 42 | 2.53 ± 0.37 | 2.29 ± 0.42 | 2.22 ± 0.40 | 2.33 ± 0.69 |
| | Mean N.S.E.M. ± N.S.E.M. | 0.39 ± 0.12 | 0.41 ± 0.19 | 0.57 ± 0.32 | 0.64 ± 0.23* | 0.84 ± 0.33* |
| Every 120 min (6 analyses per mouse) | Mean ± S.E.M. | 2.62 ± 1.21 | 2.67 ± 1.12 | 2.54 ± 0.37 | 2.49 ± 0.59 | 2.57 ± 0.72 |
| | Mean N.S.E.M. ± N.S.E.M. | 1.33 ± 0.51*** | 1.24 ± 0.49** | 0.42 ± 0.11 | 0.84 ± 0.27* | 0.95 ± 0.34* |
| Every 180 min (4 analyses per mouse) | Mean ± S.E.M. | 2.94 ± 1.69 | 2.59 ± 1.11 | 3.05 ± 0.92 | 3.08 ± 0.89 | 4.83 ± 1.45* |
| | Mean N.S.E.M. ± N.S.E.M. | 1.47 ± 0.43*** | 1.22 ± 0.46** | 1.01 ± 0.46** | 0.98 ± 0.42** | 1.38 ± 0.41*** |

Low-frequency (LF, in $ms^2$) and high-frequency powers (HF, in $ms^2$) were analysed in FVB/N mice ($n = 8$). The mean ± S.E.M. values of LF and HF powers for $n$ mice are shown and the mean ± S.E.M. values of N.S.E.M. are presented. NE, non-effective analysis. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, one-way ANOVA followed by Dunnett's test to compare all values with measure obtained with one 3 min ECG sampling every 120 min.

## Replicability

Long ECG analysis or multiple averaging of short-term analyses yield results that can be replicated and avoid the errors that show up in single short-term analyses (Task Force of the European Society of Cardiology, 1996). Time- and frequency-domain results are replicable, as is shown in Fig. 4. The values of NN interval and

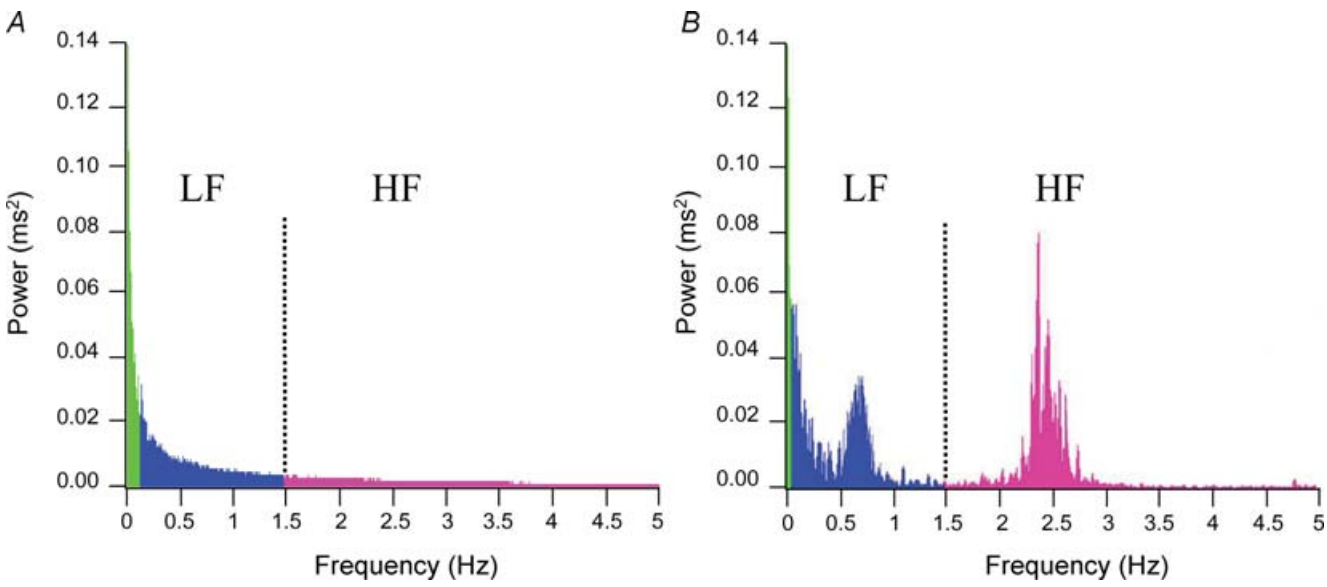


**Figure 3. Spectral analysis**
Analysis was performed on FVB/N mice during the waking period (night). Low-frequency (LF; 0.15–1.5 Hz, in $ms^2$) and high-frequency powers (HF; 1.5–5 Hz, in $ms^2$) were calculated by FFT. In *A*, all R–R intervals from 12 h ECG recordings were included in HRV analysis. The spectrum was uninterpretable, owing to the high number and complexity of the harmonics used during fast Fourier transformation. In *B*, spectral analysis was performed after selection of a stable 3 min period once every 2 h during 12 h. (One example is given.) The spectrum in *B* shows two highly defined power peaks.

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**Table 4. Determination of the best *x* value for the pNN index in mice**

| | Light | | Dark | |
|---|---|---|---|---|
| *x* (ms) | NNx (events) | pNNx (%) | NNx | pNNx (%) |
| A. | | | | |
| 4 | 20725 | 6.66 | 20271 | 5.44 |
| 5 | 12506 | 4.01 | 11794 | 3.17 |
| 6 | 7109 | 2.68 | 6884 | 1.84 |
| 7 | 3647 | 1.17 | 3851 | 1.02 |
| 8 | 1637 | 0.53 | 2114 | 0.57 |
| 9 | 682 | 0.22 | 1186 | 0.32 |
| 10 | 311 | 0.10 | 713 | 0.19 |
| | Before Atropine | | After Atropine | |
| B. | | | | |
| 4 | 138 | 6.9 | 100 | 5.1 |
| 5 | 76 | 3.8 | 61 | 2.9 |
| 6 | 62 | 3.1 | 10 | 0.5 |
| 7 | 20 | 1.0 | 1 | 0.0 |
| 8 | 8 | 0.4 | 0 | 0.0 |
| 9 | 4 | 0.2 | 0 | 0.0 |
| 10 | 0 | 0.0 | 0 | 0.0 |

Values of pNN of 4–10 ms were tested in a FVB/N mouse with a 12 h analysis in light and dark periods with no erratic fluctuations (A) and before and after atropine injection (1 mg kg$^{-1}$, I.P.; B). Values of pNN of 10, 9, 8 and 7 ms showed no circadian variations, as observed by a typical decrease in pNNx when the mouse enters a phase of activity (dark period). Likewise, after atropine injection pNN4 and pNN5 diminished less than pNN6.

SDNN exhibit no significant differences between analyses (one-way ANOVA) performed over a period of 2 months. Likewise, LF and HF powers were reproducible over time.

### Short-term ECG analysis

The effects of pharmacological agents appear difficult to study in mice because of the non-stationarity and great variability of the heart rate. Choosing the moment and duration for baseline determination was the major problem in comparing the effects of drug administration on HRV. Figure 5 illustrates this dilemma. Three successive HRV analyses (represented by the boxes numbered 1, 2 and 3) are carried out in the time and frequency domains on FVB/N mice (left panel). Each of them is run for 1 h, overlapping 50% of the previous analysis. In the time domain, while there is little variation in the mean R–R interval, a 30 min time shift may increase the SDNN by a factor of 10 and the pNN6 by a factor of 270. In the frequency domain, the LF and HF powers may be modified by a factor of 4–6. Thus, single short-term analyses may introduce error if they are made in blind conditions without manual verification. Some precautions should be taken before using HRV analysis in short-term recordings. Thus, after testing different conditions (data not shown), we suggest averaging at least three stable 3 min ECG analyses around 15 min before injection and three analyses at least 30 min after injection during a steady-state period to determine the pharmacological effects. As shown in the right panel of Fig. 5, the values of HRV parameters that were obtained with three 3 min ECG recordings taken with 5 min intervals between the groups, were more replicable than parameters obtained with single ECG recordings of longer duration (1 h). This difference results from the more drastic selection of stable ECG fragments during the analyses of the three 3 minute periods. With this protocol applied during pharmacological testing, LF and HF were significantly diminished by atropine (LF, $2.48 \pm 0.23$ *versus* $0.89 \pm 0.12$ ms$^2$; and HF, $2.82 \pm 0.34$ *versus* $0.94 \pm 0.11$ ms$^2$, $P < 0.01$, before and after injection, respectively). Nevertheless, the timing should depend on the lag phase (the time it takes to reach a peak) and on the duration of effects of the drug.

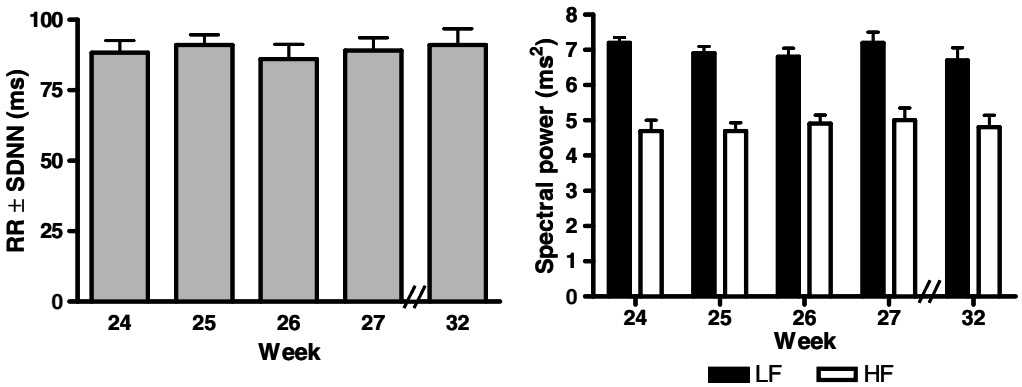


**Figure 4. Long-term HRV replicability**
On the left, R–R interval ± SDNN was calculated over a 12 h light period in one FVB/N mouse at the 24th, 25th, 26th, 27th and 32nd weeks. On the right, LF and HF powers + s.e.m. were determined over short-term periods selected from the same recordings. All measurements were replicable (no significant difference was found between measurements effected on different dates by one-way ANOVA test).

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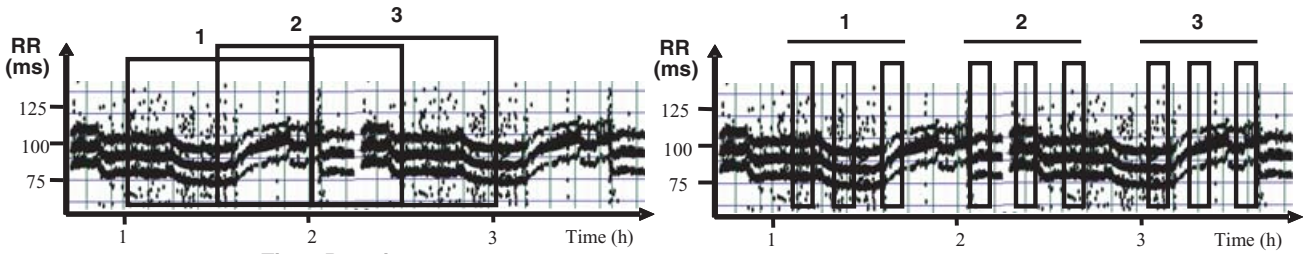
## Discussion

The contractile activity of the heart adapts blood flow to the needs of the organism. Contraction is preceded and triggered by electrical activity that modulates and synchronizes the rhythm and intensity of each beat. This beat-to-beat regulation is largely modulated by the ANS, which controls the electrical properties of pacemaker cells by acting on the ion channels which modify the automatic sinus activity (Couette *et al.* 2006).

This neural control is affected by a complex interplay of the sympathetic and vagal outflows. The sympathetic system enhances automaticity (via the rostral ventrolateral medulla), the parasympathetic system inhibits it (via the ambiguus and vagal dorsal motor nuclei), and reflex feedbacks constantly adjust the impact of each system in a push–pull interplay (Montano *et al.* 1994; Malliani, 2000).

Analysis of HRV can detect ANS-caused rhythm modifications that are not necessarily reflected by changes in the mean value of the heart rate. Time- (Task Force of the European Society of Cardiology, 1996) and frequency-domain indices (Akselrod *et al.* 1981; Malliani *et al.* 1991) are currently the standard parameters. Time-domain analysis measures changes in R–R intervals between successive normal cardiac cycles over time. All HRV indices in the time domain are based on descriptive statistical calculations. Power spectral analysis can be performed in two ways: (1) by autoregressive model estimation, a parametric method resulting in a continuous smooth spectrum of activity (Farah *et al.* 2006); or (2) by FFT, a non-parametric method characterized by discrete peaks for the several frequency components (Akselrod *et al.* 1981; Malliani *et al.* 1991, Task Force of the European Society of Cardiology, 1996). Its computational facility, with *a priori* selection of how many and which frequency bands are of interest (Akselrod *et al.* 1981), and the fact that the majority of the software available is provided with an FFT algorithm, make FFT the most widely used method for assessment of the ANS (Mansier *et al.* 1996). The FFT converts time information into frequency information by




Time Domain

| Periods | 1 | 2 | 3 |
|---|---|---|---|
| $RR_m$ (ms) | 89.3 | 93.9 | 91.8 |
| SDNN (ms) | 0.99 | 9.40 | 3.28 |
| pNN6 | 0.09 | 24.3 | 9.21 |

Time Domain

| Periods | 1 | 2 | 3 |
|---|---|---|---|
| $RR_m$ (ms) | 90 ± 3 | 89 ± 2 | 92 ± 2 |
| SDNN (ms) | 7.21 ± 2.12 | 6.98 ± 1.87 | 7.49 ± 2.42 |
| pNN6 | 3.28 ± 0.31 | 3.16 ± 0.20 | 3.45 ± 0.44 |

Frequency Domain

| Periods | 1 | 2 | 3 |
|---|---|---|---|
| LF ($ms^2$) | 0.481 | 2.8 | 0.11 |
| HF ($ms^2$) | 0.15 | 0.66 | 0.16 |
| LF/HF ratio | 3.10 | 4.21 | 0.69 |

Frequency Domain

| Periods | 1 | 2 | 3 |
|---|---|---|---|
| LF ($ms^2$) | 1.87 ± 0.51 | 2.31 ± 0.24 | 1.68 ± 0.36 |
| HF ($ms^2$) | 1.10 ± 0.28 | 0. 87 ± 0.17 | 1. 22 ± 0.19 |
| LF/HF ratio | 1.70 ± 0.36 | 1.97 ± 0.24 | 1.37 ± 0.25 |

**Figure 5. Non-stationarity and non-replicability of single short-term HRV analysis**
Three successive 1 h analyses in the time and frequency domains of R–R intervals, overlapped by 50%, obtained from one conscious FVB/N mouse, showed that single short-term HRV analysis was difficult and subject to criticism (left panel). Between the first (1), second (2) and third (3) periods analysed, SDNN (standard deviation), pNN6 (the percentage of normal consecutive R–R intervals differing by > 6 ms), LF and HF powers and the LF/HF ratio can vary drastically without significant variation in the mean R–R interval ($RR_m$), obscuring results and interpretations (left panel). In contrast, three 3 min ECG analyses with a 5 min sampling were performed three times (1, 2 and 3) over the same ECG (right panel). Results indicate that the ECG parameters are replicable for the three 3 min periods.

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decomposing the periodic oscillations of heart rate into harmonics of different frequencies and amplitudes. By this method, it has been shown that in wild-type mice, the sympathetic nervous system is the one major factor which controls heart rate (Mansier *et al.* 1996; Uechi *et al.* 1998) and that the power spectrum of HRV in mice resembles those derived from humans, dogs and rats, showing two principal components: the LF and HF frequencies (Gehrmann *et al.* 2000). The exact significance of each frequency band was not fully elucidated and has been subject to controversy (Parati *et al.* 2006). It appears that they cannot be used as exclusive markers of sympathetic and parasympathetic activity, respectively. Nevertheless, a long list of well-documented published articles strongly argues that HRV analysis seems to be more useful as a qualitative method for assessment of autonomic change in cardiac rhythm than as a quantitative method for assessment of autonomic tone (see for overview Parati *et al.* 2006).

While in humans HRV analysis is well defined, in mice some adjustments are necessary because the characteristics of heart rate (high heart rate and non-stationary signal) are quite different. In this study, we showed that it was important to respect certain rules in recording and analysing the ECG if diffusion of HRV results between laboratories for purposes of comparison is desired. We thus proposed a quick, efficient protocol, based on our experiments and experience, to standardize HRV analysis in mice.

## Suggested protocol for HRV analysis in mice

**Recording method.** Recordings on anaesthetized animals obtained with surface electrodes are scarcely pertinent and of little interest in comprehending the physiological regulation of heart rate through the study of HRV. Pharmacological anaesthetic agents are known to interfere with respiration depth, sinus rhythm and atrioventricular conduction (Galletly *et al.* 1994). Therefore, telemetry is the only method by which ECG can be measured for HRV analysis under physiological conditions over long periods.

Telemetric instrumentation can influence HRV in the days following teletransmitter implantation. Animal manipulation, changes in environmental conditions, surgery-related stress and anaesthesia lead to heart rate disturbances that require a convalescence period. Two studies have shown that circadian rhythms were recuperated 4 days after surgical implantation of a telemetric transmitter (Clement *et al.* 1989; Johansson & Thoren, 1997), but it took 2 weeks to stabilize weight (Clement *et al.* 1989). In addition, HRV clearly depends on time of day. Circadian variations make it necessary to perform measurements and analyses in both light and dark conditions separately in order to assess heart rate regulation. In line with Leon *et al.* (2004), we recommend 10 days of recovery time to exclude all surgical and anaesthetic interferences with heart rate. Before this length of time, the heart rate and SDNN are not stable, and HRV could be overestimated. To our knowledge, telemetry does not modify heart rate after the recovery period. This approach thus appears advisable (Wickman *et al.* 1998; Gehrmann *et al.* 2000; Janssen *et al.* 2000; Stauss, 2006).

**Selection of sinus beats and stationary period.** In the same way, it appears very important to perform HRV analysis from sinus cardiac beats only. This is emphasized in the guidelines for humans (Task Force of the European Society of Cardiology, 1996) and must be respected in mice as well. Standard methods for calculating HRV require the detection of ECG R waves. It is seldom possible to acquire a long-term heart rate time series without artifacts, ectopic beats or beats that cannot be detected reliably. Therefore, before HRV indices can be calculated, R waves corresponding to non-sinus beats (artifacts and ectopic beats) must be eliminated because they are not generated by the regulatory system responsible for HRV. In the time domain, we propose a simple method to exclude all abnormal beats or artifacts by excluding the beats not found between mean R–R intervals $\pm$ 2 S.D. Without this preselection, HRV is overestimated.

Similarly, spectral analysis has intrinsic limitations, especially concerning the possible non-stationarity of R–R intervals due to high ECG signal variability and the very high number of cardiac beats in mice. Heart rate variability analysis by FFT requires a stationary signal defined by its statistical properties (mean and variance), which should be the same when estimated at different time intervals within the same recording session. This problem with long-term recording has been frequently discussed because the LF and HF power components cannot be considered stationary over a 24 h period (Furlan *et al.* 1990). Consequently, HRV analysis cannot be applied blindly; manual corrections and verifications are indispensable. Three methods are used to avoid this problem. First, some investigators delete the interruptions in the stationary signal and concatenate the surrounding data. This method can disturb the long-term correlations in the time series and introduce errors (Lippman *et al.* 1994). Second, other researchers eliminate non-stationary signals and interpolate all missing beats with a mathematical algorithm (Malik, 1995; Wickman *et al.* 1998). Third, analyses can be performed on only a selection of short, uninterrupted sequences of stationary signals, introducing a possible selection bias that can be reduced by averaging several short-term calculations.

Many resampling schemes have been proposed to replace these removed beats by phantom points (Clifford & Tarassenko, 2005). Fast Fourier transform analyses with linear or cubic interpolation are considered standard methods even though they cannot describe the

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relationships between all R waves. This method rests on algorithms that resample R–R intervals using a given frequency and a user-specific interpolation method onto a regular time axis. Theoretically, time series should be resampled using a frequency at least twice the maximal frequency of the signal given by the Nyquist–Shannon sampling theorem (Nyquist, 1928; Malliani, 2005). Harris has shown that after this resampling by interpolation, a Hamming window provides high-performance FFT analysis in terms of spectral leakage, side lobe amplitude and width of the central peak (Harris, 1978). Nevertheless, no method is perfect, and they all show both advantages and drawbacks (Clifford & Tarassenko, 2005). Objectively, the choice among these methods depends on user preference and the suitability and limitations of the software used to perform HRV analysis. For spectral analyses, we retain only repeated short, uninterrupted 3 min sequences of stationary signals every 2 h (segment length of 2048 beats with linear interpolation and resampling to a 20 Hz interbeat time series, together with Hamming windowing), which is a good compromise between the time cost and interindividual variability.

In addition, many HRV studies exclusively based on R–R interval computation have been done with 'home-made' software. Generally, little information is provided on the design of these tools, which can affect the measurements of parameters through different possibilities for settings and different data processing approaches to R–R interval analysis. We thus advise using commercial software that allows for a broad range of ECG analysis parameters (segment length, resampling rate, interpolation and windowing type).

**Selection of HRV parameters.** In the time domain, we defined the optimal pNN value in mice, an important parameter in evaluating the parasympathetic modulation of heart rate. As shown in previous studies, we found that pNN6 is the best value for mice (Shusterman *et al.* 2002).

In the frequency domain, it is commonly accepted that in mice the low-frequency bands are situated around 0.6–0.7 Hz and the high-frequency bands around 2.4 Hz, and this corresponds to our observations (Mansier *et al.* 1996). This has led many authors to select a LF range between 0.1 and 1.5 Hz, while setting the HF range between 1.5 and 5.0 Hz. Recently, Baudrie *et al.* (2007) have tried to determine the most accurate frequency ranges affected by the sympathetic and parasympathetic nervous system in accordance with the best fit of significant variations during drug injections. They restricted the LF band to between 0.15 and 0.60 Hz of the systolic blood pressure spectrum and HF to between 2.5 and 5.0 Hz of the pulse interval spectrum (Baudrie *et al.* 2007). The use by Baudrie *et al.* (2007) of a cross between the pulse interval spectrum and blood pressure variability is helpful because it employs both cardiac and vascular components (such as vasodilatation or vasoconstriction by $\alpha_1$- or $\beta_2$-adrenergic receptors) to gauge the effects of autonomic control on the circulation. Their parameters require verification, however, since HRV depends on the duration of the ECG analysed (Task Force of the European Society of Cardiology, 1996), on the mouse strains tested (Campen *et al.* 2005) and on the recording method (mean blood pressure, pulse interval or ECG; Janssen *et al.* 2000). It is thus important to take these parameters into consideration before comparing the HRV results between different studies. Thus, we showed with our protocol that the peak of HF power is close to 2.5 Hz and that a broad HF range (1.5–5 Hz) allows for a better evaluation of parasympathetic modulation than a restricted HF range (2.5–5 Hz) during muscarinic blockade.

**Analysis of pharmacological effects.** One major point with short-term analysis is the choice of the baseline period, especially if the acute effects of drugs on HRV are studied. We showed that a small variation in the baseline time can significantly modify the value of the HRV parameters. The selection of three periods of 3 min before and after drug injection looks like a good choice to limit errors.

The final considerations pertain to tests involving successive pharmacological administration.

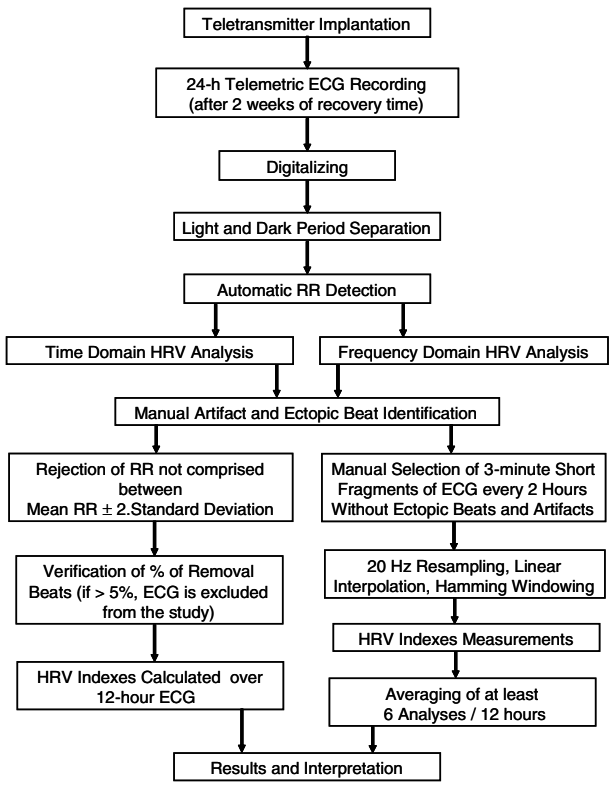


**Figure 6. Practical protocol to standardize HRV analysis in mice**

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Pharmacological tests should be performed on the same mice with at least a 2 day latency period to prevent interferences among drugs that may modify HRV without necessarily altering mean heart rate.

Moreover, for the sake of rigorous practice and with the aim of comparing pharmacological effects, drug administration should be effected at the same times for each animal to limit time-dependent variations of the pharmacological response.

### Applications and limits

Heart rate variability analysis requires a serious investment in telemetry equipment and an efficient microcomputer. Nevertheless, with regard to information gain, this technique is not prohibitively expensive compared with other electrophysiological set-ups (such as patch clamp or intracardiac electrophysiological testing). In addition, the method provides quantities of data which may be long and difficult to analyse and interpret, thus justifying a simple, standardized protocol. Concerning the value of genetic manipulation, the mouse may not be the most relevant model for directly extrapolating human clinical disease, especially because of the high heart rate, low cardiac mass and differences in the expression and distribution of gap junctions and ionic channels. Nevertheless, transgenic mice have become an indispensable tool for cardiovascular research. Several authors have successfully applied HRV analysis to genetically engineered mice to gain an understanding of pathophysiological issues related to human diseases, such as heart failure (Shusterman *et al.* 2002), Chagas cardiomyopathy (Peter *et al.* 2005), myotonic dystrophy (Wakimoto *et al.* 2002) and congenital heart disease (Mangoni *et al.* 2006).

### Conclusion

Cardiovascular autonomic assessment plays an important role in elucidating autonomic nervous system function and dysfunction. The number of techniques that can be used to test cardiovascular function is increasing, which is helpful in the comprehension of physiological adaptation or modification during disease development. The feasibility of using HRV analyses to assess ANS modulation of heart rate in mice has been demonstrated by several authors, but a standardized protocol still needed to be determined. With our protocol (Fig. 6), we have shown that HRV measurements can be made quickly and efficiently in mice. Autonomic nervous system assessment through HRV analyses obtained with a homogeneous, standardized protocol, combined with other techniques, such as invasive *in vivo* cardiac electrophysiological testing on whole mice by the closed-chest approach, the practice of cardiac electrophysiological mapping of conduction pathways and patch-clamp technique in cardiomyocytes, could open new horizons in cardiovascular research.

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