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Differential Impact of Type-1 and Type-2 Diabetes on Control of Heart Rate in Mice

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Abstract

Aims—Cardiac autonomic dysfunction is a serious complication of diabetes. One consequence is disruption of the normal beat-to-beat regulation of heart rate (HR), i.e. HR variability (HRV). However, our understanding of the disease process has been limited by inconsistent HR/HRV data from previous animal studies. We hypothesized that differences in the method of measurement, time of day, and level of stress account for the differing results across studies. Thus, our aim was to systematically assess HR and HRV in two common diabetic mouse models.

Methods—ECG radiotelemetry devices were implanted into *db/db* (type-2 diabetic), STZ-treated *db/+* (type-1 diabetic), and control *db/+* mice (n=4 per group). HR and HRV were analyzed over 24 hours and during treadmill testing.

Results—24 hour analysis revealed that *db/db* mice had an altered pattern of circadian HR changes, and STZ-treated mice had reduced HR throughout. HRV measures linked to sympathetic control were reduced in *db/db* mice in the early morning and early afternoon, and partially reduced in STZ-treated mice. HR response to treadmill testing was blunted in both models.

Conclusions—It is important to consider both time of day and level of stress when assessing HR and HRV in diabetic mice. *db/db* mice may have altered circadian rhythm of sympathetic

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Duality of Interest

The authors declare that there is no duality of interest associated with this manuscript.

Contribution Statement

All authors contributed to the conception and design of the experiments. In addition, CLS performed experiments, analyzed and interpreted the results, prepared the figures, and drafted the manuscript. LDA and SW performed experiments. DA advised on data analysis and edited the manuscript. All authors contributed to critical revision of the manuscript and approved the final version.

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control of HR, whereas STZ-treated mice have a relative reduction. This study provides baseline data and a framework for HR analysis that may guide future investigations.

Keywords

Heart Rate; Autonomic Nervous System; Diabetes Complications; Disease Models; Animal

1 Introduction

Diabetes affects almost 400 million people worldwide (Nathan, 2015) and is associated with increased risk of both cardiovascular disease (>2-fold increased risk (Kannel et al., 1979)) and autonomic dysfunction (approximately 20% incidence of abnormal cardiovascular autonomic function in diabetic patients (Vinik et al., 2003)). Cardiovascular autonomic dysfunction is linked to increased mortality: a meta-analysis in 2,900 patients revealed a relative risk of 2.14, with a 95% confidence interval of 1.83–2.15 ($p < 0.0001$) (Vinik et al., 2003). Various aspects of cardiovascular physiology are under autonomic control, including heart rate (HR). Large changes in HR occur as part of the normal physiological response to stress and exercise, but even during periods of rest HR is tightly controlled; this control is evident as HR variability (HRV). Diabetes-related cardiac autonomic dysfunction increases resting HR (by an average of 6–10% (Ewing et al., 1981)) and decreases HRV (Pop-Busui, 2010). For example, a study by Spallone et al. (Spallone et al., 1993) showed an 8% increase in HR and a 10–20% decrease in HRV measures in diabetic patients vs. control subjects ($n = 25$ per group). These changes are associated with worse prognosis in both type-1 and type-2 diabetic populations (Hillis et al., 2012a; Hillis et al., 2012b; Singh, 2002; Vinik et al., 2003). Dysfunctional HR regulation also contributes to exercise intolerance, which is common in these patients (Pop-Busui, 2010; Vinik et al., 2007). In a study of 170 type-2 diabetic patients vs. 56 control subjects, exercise capacity (measured in metabolic equivalents) was reduced by 28% (Fang et al., 2005). In later stages of disease, severe cardiac autonomic neuropathy results in almost no HRV and a fixed HR unresponsive to sleep, stress, or moderate exercise (Pop-Busui, 2010). Thus, cardiac autonomic dysfunction is a serious and potentially devastating complication of diabetes; however, there is no effective treatment and it is currently understudied (Vinik et al., 2007).

Better understanding of diabetes-induced autonomic dysfunction requires further research that will rely on well-characterized animal models. The most commonly-used mouse models are *db/db* mutant mice (type-2 diabetes; secondary to hyperphagia and obesity caused by a mutation in the leptin receptor gene) and streptozotocin (STZ)-treated mice (type-1 diabetes; destruction of pancreatic islets by STZ). Published studies using these models, however, contain variable and sometimes conflicting results (Stables et al., 2013). Specifically, HR has been reported to be increased (Goncalves et al., 2009), decreased (Park et al., 2008; Semeniuk et al., 2002; Senador et al., 2009), or unaltered (Su et al., 2008) in *db/db* mice, and increased (Kellogg et al., 2009) or decreased (Lin et al., 2010; Mabe et al., 2011) in STZ-treated mice. Papers reporting HRV also show mixed results both in *db/db* (Goncalves et al., 2009; Senador et al., 2009) and STZ-treated (Mabe et al., 2011) mice. Our hypothesis was that differences in method of measurement, time of day, and level of stress account for these inconsistent results.

The aim of this study was to assess changes in HR and HRV in *db/db* and STZ-treated mice in a more systematic and comprehensive manner than previously published studies. We used both echocardiography and the gold-standard method of implantable ECG radiotelemetry in conscious, unrestrained mice. This enabled analysis of both circadian changes and progression of disease over several weeks. To study response to both stress and exercise, we subjected the telemetered mice to treadmill testing.

2 Methods

2.1 Mice

Male BKS *db/db* (type-2 diabetic) and *db/+* (control) mice (BKS.Cg-Dock7m *+/+* Lepr^{db}/J; stock number 000642) were obtained from Jackson Labs (Bar Harbor, Maine, USA). Principles of laboratory animal care (NIH publication no. 85–23, revised 1985; <http://grants1.nih.gov/grants/olaw/references/phspol.htm>) were followed. Mice were cared for following the University of Michigan Committee on the Care and Use of Animals guidelines.

2.2 Echocardiography

For the echocardiography (echo) study, 24-week-old *db/db* and *db/+* mice (*n* = 6 per group) were lightly anesthetized using isoflurane and cardiac echo was conducted as previously described (Zolov et al., 2012). Left ventricular (LV) ejection fraction was measured from the two-dimensional long axis view. Systolic and diastolic dimensions and wall thickness were measured by M-mode in the parasternal short axis view at the level of the papillary muscles. Diastolic function was assessed by conventional pulsed-wave spectral Doppler analysis of mitral valve inflow patterns (early [E] and late [A] filling waves). For further details see supplemental methods.

2.3 ECG Telemetry

For the ECG telemetry study, three groups of mice were telemetered (*n*=4 per group): STZ-treated *db/+* (type-1 diabetes); vehicle-treated *db/db* (type-2 diabetes); and vehicle-treated *db/+* as a control. An overview of the experimental protocol is shown in figure 1. STZ or vehicle was administered at 5 weeks of age, and ECG devices (Data Sciences International (DSI), St Paul, MN, USA; ETA-F-20) were implanted at 7 weeks of age in approximately lead II configuration. Animals were housed in individual cages with a receiver below each cage. ECG, temperature, and activity were recorded. For clarity, most results are displayed with *db/db* vs. control and STZ-treated vs. control on separate graphs. STZ was administered as a single i.p. injection (150 mg/kg in sodium citrate solution); mice were given access to 10% sucrose water for the first 3 days post-STZ and DietGel (ClearH2O, Maine, USA) in their cages thereafter, with cage bedding changed regularly due to increased urination. The room had a 12-hour light/dark cycle, with lights on/off at 6am/6pm. Animals were fed on normal chow *ad libitum*, plus DietGel for the STZ-treated mice.

For weekly measurements, recordings were made for 5 minutes every 10 minutes (i.e. 5 min on, 5 min off) for 72 hours each week for 16 weeks (age 8 to 24 weeks). Average values for HR, temperature, and activity were calculated using DSI's Analysis software. To reduce

variability due to noise or human presence we averaged the hourly values over 3 days to obtain an average 24-hour dataset for each mouse each week.

HRV was analyzed at 10 weeks of age. To calculate HRV, we first used DSI's Ponemah software to generate RR interval data for one 5-minute ECG segment per hour, over 3 days. Next, using the R programming language and environment, we split this data into separate files (one per 5-minute interval), and for each file we then excluded RR intervals greater than 3 standard deviations from the mean to remove artifacts. Files for which >5% of RR intervals were excluded in this way were removed from further analysis (only one file was removed for this reason). Next, HRV analysis was conducted using the RHRV package. Time-domain (standard deviation of the RR intervals, SDRR; root mean square of successive differences, RMSSD), frequency-domain (by fast fourier transform (FFT); high frequency (HF) power, low frequency (LF) power, and LF/HF ratio), and Poincare plot parameters (SD1 and SD2) were calculated. For full details and a link to R code, see supplemental methods. Finally, values were averaged over the 3 days to obtain 24-hour values for each animal.

HRV that occurs on a short-term (high frequency) scale (largely due to respiratory sinus arrhythmia) is an indication of parasympathetic/vagal control, and HRV that occurs on a slightly longer-term (low frequency) scale is generally considered to be more of an indication of sympathetic control (Task-Force, 1996), though the exact physiological interpretation is somewhat controversial (Task-Force, 1996). We take the view that longer-term measures of HRV are influenced by both sympathetic and parasympathetic fluctuations, but by comparing differences in short-term and long-term HRV measurements, as well as HR, we can make inferences about both types of autonomic control of HR in these animals. Short-term HRV parameters in this study = RMSSD, SD1, and HF power (Guzik et al., 2007; Task-Force, 1996). Longer-term HRV parameters = SDNN (a measure of total variability), SD2, LF power, and LF/HF ratio (Guzik et al., 2007; Task-Force, 1996).

Treadmill testing was conducted at 9 and 16 weeks of age. The protocol used is shown in figure 1B. In an attempt to separate the effects of stress from those of exercise, mice were initially placed on the non-moving treadmill for 10 min. The treadmill was then started at the slowest setting of 8 m/min for 10 min. Control and STZ-treated mice were then run at 10, 12 and 14 m/min for 2 min each. *db/db* mice were unable to run at speeds faster than 8 m/min. If a mouse moved too close to the rear of the testing area they were encouraged forward using a vertical cylinder a maximum of three times; this was done to avoid using the standard electric shock method on the telemetered mice. All mice were able to maintain the speeds outlined above. ECG was recorded continuously, via a receiver placed alongside the treadmill, from 10 min pre-test until 10 min after stopping. 30s intervals, at time points shown in figure 1B, were analyzed using DSI's Ponemah software. All R wave detections were checked for accuracy and repositioned or excluded manually as necessary. HR and three time-domain measures of HRV were calculated: SDRR, RMSSD, and % of successive RR intervals differing by >6 ms (pNN_6). Due to the short 30-second period of analysis, it was not appropriate to conduct frequency domain analysis.

2.4 Statistics

For results in which control, *db/db*, and STZ-treated mice are presented in the same graph, we used one-way ANOVA with Dunnett's test for multiple comparisons. For all other results, since we are not directly comparing *db/db* vs. STZ groups but are comparing each to control, we conducted statistical analysis separately using unpaired t-tests. Investigators were blinded to experimental groups during analysis. All data are presented as mean $\pm$ SEM.

3 Results

3.1 Echocardiography revealed a reduced HR in *db/db* mice

Echocardiography of 24-week-old *db/db* mice revealed that HR was decreased vs. control, and several other functional parameters were altered: LV internal diameter during systole (LVIDs) was reduced, fractional shortening (FS) and LV ejection time were increased, and cardiac output (CO) was decreased (table 1). LVIDs, FS, LV ejection time, and CO are all dependent on HR (Hasenfuss et al., 2000), so the primary dysfunction may have been the reduction in HR.

3.2 24-hour ECG telemetry revealed a change in circadian rhythm of HR in *db/db* mice and a reduced HR in STZ-treated mice

To further investigate and characterize HR changes in mouse models of both type-1 (STZ) and type-2 (*db/db*) diabetes, we conducted an ECG telemetry study. Three groups (STZ-treated, *db/db*, and control) were implanted with radiotelemetry devices, with ECG, temperature, and activity being measured over 16 weeks (age 8–24 weeks). The telemetered mice developed diabetic characteristics as expected (figure 2): *db/db* mice became overweight (47 ± 1 g vs. 29 ± 1 g in control at 16 weeks of age), STZ-treated mice lost weight (20 ± 0.3 g vs. 25 ± 1 g in control at 10 weeks of age), and both developed severe hyperglycemia (average fasting blood glucose of 331–773 mg/dL in *db/db* and 319–750 mg/dL in STZ-treated mice vs. 136–190 mg/dL in control after week 10). STZ-treated mice are fragile, and don't usually survive beyond 16 weeks of age in this model (without insulin treatment), but in combination with the telemetry implant their survival was further decreased; in this study the average survival of STZ-treated mice was just 12 weeks of age (figure 2C). There was also some unexpected mortality in the control group, with mice dying at weeks 15 and 17, presumed to be due to infection. For this reason, although we include data out to 24 weeks of age, we limit the majority of our analysis for this study to week 16 and earlier for the *db/db* group and week 10 for the STZ-treated group.

24-hour measurements of HR, temperature, and activity in STZ-treated mice vs. control (10 weeks of age) and *db/db* vs. control (12 and 16 weeks of age) are shown in figure 3. Similar results were found across all time points (supplemental figures 1 and 2). Control mice showed clear circadian rhythmicity, with all three parameters having higher values at night (during the awake, active period). *db/db* mice also displayed circadian rhythmicity in HR and temperature, but the timing and pattern of this was different than that of control mice. This was particularly evident in the early morning (2–5am), when HR was higher in *db/db* mice (statistically significant at 2am and 4am time points in week 12, and 2am, 3am, and 4am in week 16; HR higher by an average of 46–96 bpm at these time points). It was also

evident during the transition from dark to light periods (lights on at 6am), with both HR and temperature decreasing markedly between 4am and 9am in *db/db* mice but not in controls (HR reduced by 122 ± 34 bpm (week 12) and 122 ± 30 bpm (week 16) in *db/db* vs. -11 ± 21 bpm (week 12) and 29 ± 27 bpm (week 16) in control, $p<0.05$ for both; Temperature reduced by 2.4 ± 0.4 °C (week 12) and 2.5 ± 0.3 °C (week 16) in *db/db* vs. 0.5 ± 0.3 °C (week 12) and 0.9 ± 0.3 °C (week 16) in control, $p<0.05$ for both). Thus, our data suggest that *db/db* mice may have altered circadian rhythms in comparison to controls. STZ-treated mice, on the other hand, had a reduced HR across most time points (statistically significant at 1am, 2am, 8am, 11am, 12pm, 6pm, 7pm, 8pm, 9pm, 10pm, and 11pm in week 10; HR reduced by an average of 38–98 bpm). A lower HR in STZ-treated mice was seen relatively consistently from weeks 8 to 11 (supplemental figure 2). Temperature was not consistently reduced in parallel to HR. *db/db* and STZ-treated animals showed very low activity levels over the entire 24 hours.

3.3 HRV measures linked to sympathetic control of HR are reduced in *db/db* mice in the early morning and early afternoon, and partially reduced in STZ-treated mice

Figure 4 shows HRV data for 10-week-old animals. RMSSD and SD1, which are measures of short-term variability, differed little between groups, suggesting that parasympathetic control of HR may not be affected in these diabetic animals. *db/db* mice had reduced SDNN and SD2, which are measures of longer-term variability; together with the lack of difference in parasympathetic control, this indicates reduced sympathetic control of HR in these mice. This was particularly evident during the early morning (2am–5am) and early afternoon (2pm–5pm): SDNN was significantly lower in *db/db* animals at 3am, 5am, 9am, 2pm, 3pm, 5pm by 4.9–8.6 ms ($p<0.05$); SD2 was significantly lower at 2am, 3am, 9am, 5pm by 18–47 units ($p<0.05$). STZ-treated mice showed reduced HRV in the SD2 measure (statistically significant at 6am, 9am, 12pm, 5pm, 7pm, 8pm; reduced by 13–54 units), but not in SDNN. Frequency domain HRV parameters were also calculated (supplemental figure 3) and the results were generally consistent with the other HRV data, but inherent limitations of our HRV analysis method means that the FFT data must be interpreted with caution. This, along with other difficulties and limitations associated with HRV analysis in mouse models, is discussed in further detail below.

3.4 HR response to stress and exercise is blunted in *db/db* and STZ-treated mice

HR and HRV parameters measured during treadmill testing at week 9 are shown in figure 5. Transferring control mice to the treadmill caused a large increase in HR (from 456 ± 35 to 740 ± 9 bpm), which dropped only slightly (to 710 ± 3 bpm) during the 10 min acclimatization period (treadmill stationary). When the treadmill started the HR increased again to 771 ± 13 bpm, and remained high throughout the 10-minute period. Increasing the speed of the treadmill did not increase HR further. 10 minutes after returning to their home cages HR had recovered somewhat, to 589 ± 28 bpm, but was still not down to original levels. *db/db* and STZ-treated mice both had similar HR to control at baseline, but the increase in HR upon placement on the treadmill was significantly blunted in both groups (HR after 2 min was 665 ± 8 bpm in *db/db* and 619 ± 21 bpm in STZ-treated; both $p<0.05$ vs. control), and in STZ-treated mice the HR was significantly lower than control throughout the test (by 65–127 bpm). There was no decrease in HR due to acclimatization in either of the diabetic groups,

and no further increase in HR upon treadmill running. HR recovery after the test was similar to control, with *db/db* HR reduced to 609 ± 17 bpm and STZ-treated to 537 ± 17 . The different measures of HRV (SD of HR, RMSSD, and pNN₆) all showed fairly consistent results: initially, upon placement on the treadmill, HRV decreased (particularly for the short-term variability measures; RMSSD reduced from 7.0 ± 1.6 to 1.6 ± 0.2 ms, pNN₆ reduced from 34 ± 12 to 0.4 ± 0.2 %). HRV then increased as control mice became accustomed to the treadmill environment (for example, S.D. of HR increased from 19 ± 7 to an average of 52–61 bpm), but this did not occur in either *db/db* or STZ-treated mice (statistically significant for S.D. of HR for both *db/db* and STZ-treated after 4, 6, and 8 min). Upon treadmill running, all mice had relatively low HRV. The results were similar in *db/db* mice at week 16 (supplemental figure 4). In summary, it appears that the diabetic mice had a reduced maximum HR, with the STZ group being most affected, but the HR remained close to this maximum throughout the treadmill test.

4 Discussion

We performed a detailed analysis of HR and HRV in two major mouse models of type-2 and type-1 diabetes (*db/db* and STZ-treated mice). *db/db* mice had reduced HR as measured by echocardiography (table 1), but implantable ECG telemetry revealed altered HR and temperature fluctuations over 24 hours, in particular a higher HR in the early morning (2–4am; increased by an average of 46–96 bpm) and a steeper decrease in both HR and temperature from 4am to 9am (HR reduced by 122 ± 34 bpm in *db/db* vs. -11 ± 21 bpm in control; temperature reduced by 2.4 ± 0.4 °C in *db/db* vs. 0.5 ± 0.3 °C in control; week 12; $p < 0.05$ for both; figure 3). These findings indicate an alteration in circadian control. STZ-treated mice had lower HR across the entire 24 hour cycle (reduced by an average of 38–98 bpm; figure 3). Short-term HRV measures (related to parasympathetic control of HR) were not altered in either *db/db* or STZ-treated mice, suggesting that parasympathetic control is intact (figure 4). *db/db* mice had reduced HRV by longer-term variability measures (more influenced by sympathetic control), particularly during the early morning (2–5am) and early afternoon (2–5pm) (SDNN reduced by an average of 4.9–8.6 ms, SD2 reduced by 18–47 units; figure 4). This, together with the lack of differences in parasympathetic control, indicates alteration in circadian patterns of sympathetic HR control in *db/db* mice, whereas in STZ-treated mice this longer-term variability was partially reduced throughout the 24-hour period (reduced in SD2 (statistically significant at 6am, 9am, 12pm, 5pm, 7pm, 8pm), but not in SDNN; figure 4). Both models had blunted HR response to stress and exercise (HR in upon placement on the stationary treadmill was 665 ± 8 bpm in *db/db* and 619 ± 21 in STZ-treated mice: significantly lower than the 740 ± 9 bpm in control; $p < 0.05$ for both; figure 5). Overall our data suggest impaired sympathetic control of HR in both diabetic mouse models, but in different ways: in *db/db* mice there may be an alteration in the circadian patterns, whereas in STZ-treated mice the data point to a relative reduction in sympathetic control.

One caveat to these conclusions is that, because of practical considerations, we were able to perform these detailed studies using only four mice per group; hence, it will be important for future studies to build on our research and thereby better establish the reproducibility of our observations. Nevertheless, our conclusions, which are supported by the statistical analyses,

may help to clarify a field in which the existing literature presents extremely mixed results. Thus, we believe that the results of the present study provide important insights for future research in this field.

The current studies utilized two major mouse models of type-2 and type-1 diabetes (*db/db* and STZ-treated mice). The *db/db* mouse model has been extensively utilized in diabetes research because it develops an early and robust diabetes and consistently develops significant nerve deficits. The main limitation is that the mutation significantly disrupts leptin signaling which may impact the interpretation of some behavioural and physiological measures (O'Brien et al., 2014). The STZ-treated mouse is the most commonly used model of type-1 diabetes due to the ease of induction and the ability to consistently synchronize diabetes in a cohort of animals. The single high dose protocol (as opposed to multiple lower doses) was used because it gives more pronounced diabetic phenotypes, with neuropathy in particular being only mild in the multiple-low dose paradigm. However, a high mortality rate (in our case exacerbated by major abdominal surgery to fit the ECG telemetry device) is a considerable disadvantage (O'Brien et al., 2014).

As previously described, there are mixed reports in the literature on HR and HRV changes in *db/db* mice (Stables et al., 2013). The results of our ECG telemetry study, which showed altered circadian HR and HRV, as well as a reduction in stress-induced maximum HR, may help to explain some of the apparently contradictory results. For example, the difference in HR between groups may depend upon the time of day at which the measurements were taken, and/or the level of stress that the animal was under at the time of measurement. Based on these results, we recommend that the time of day at which HR recordings are taken should be considered, and the amount of stress to the animal minimized (largely dependent on the method used). Care should also be taken when measuring HR in anesthetized mice, since our echo study showed a reduction in HR in *db/db* mice that was not replicated in the ECG telemetry study. Notably, anesthesia can reduce HR, particularly when high doses are used (Wu et al., 2010), and since dosing for obese individuals is challenging (Casati et al., 2005) it is likely that *db/db* mice require a higher dose to achieve the same level of anesthesia.

Our finding of altered circadian patterns of HR in *db/db* mice agrees with previously published data. Two blood pressure telemetry studies have shown a reduction in the amplitude of circadian oscillation in HR in parallel to a “non-dipping” blood pressure (BP) phenotype (Goncalves et al., 2009; Su et al., 2008), although another study found no change (Senador et al., 2009). These papers have relatively low temporal resolution, however, with data from between two and six time points per 24 hours. Circadian variation in vascular smooth muscle contractility (Su et al., 2012) and liver function (Matsumoto et al., 2010) has also been shown to be disrupted in *db/db* mice. A study in rabbits showed that a high fat diet causes a reduction in circadian oscillations of HR (Burke et al., 2013), though the timing was largely related to the time of feeding (whereas in the current study the mice had unlimited access to food). Our HRV results also suggest a circadian aspect to the changes in sympathetic control of HR in *db/db* mice.

The underlying causes of the circadian changes in HR and sympathetic control of HR in *db/db* mice are unknown, but may be related to leptin signaling and altered patterns of eating (Kudo et al., 2004) and sleeping (Laposky et al., 2008). Leptin acts via the hypothalamus to inhibit the sensation of hunger, and due to their lack of functional leptin receptors *db/db* mice fed *ad libitum* eat almost constantly, night and day. Kudo et al. (Kudo et al., 2004) showed that the oscillation of expression of known circadian genes in liver is disrupted in *db/db* mice, and further demonstrated that this could be normalized by restricting feeding to night-time only. It is possible that restricted feeding would also normalize circadian rhythmicity of sympathetic control of HR in these mice; further studies are required to determine this. The role of leptin in energy homeostasis is far more complicated than just its role in satiety, and it has also been shown to regulate sleep (Sinton et al., 1999). *db/db* mice have disrupted circadian patterns of sleep (Laposky et al., 2008), which is also related to autonomic function (Trinder et al., 2001). Similarly to *db/db* mice, there is evidence that humans with metabolic syndrome can benefit from restricted timing of eating (Rothschild et al., 2014) and disrupted sleep has been linked to type-2 diabetes (Cappuccio et al., 2010).

Although it would be inappropriate to extrapolate data from this study to patients, there are certain comparisons that can be made. While most studies in type-2 diabetic patients have shown an increase in HR and a reduction in HRV (Pop-Busui, 2010; Vinik et al., 2003), very few studies have examined these parameters over 24 hours. Two studies in which HRV analysis was conducted on 24-hour Holter ECG recordings in type-2 diabetic patients with autonomic impairment showed altered circadian patterns of HRV and sympathovagal balance (Spallone et al., 1993; Yamamoto et al., 1999). Yamamoto et al. measured LF and HF power and present the results as average 6-hour values. They found that control individuals had diurnal variations/circadian oscillations in these measures, with LF power peaking at 12–6pm and HF peaking at 12–6am. Patients with symptomatic diabetic autonomic neuropathy (n=11) had significantly lower HRV and showed complete loss of circadian rhythmicity of these measures compared to age-matched controls. Spallone et al., analyzing every hour, also showed circadian oscillations in HF power (peak at 4am) but a less obvious oscillation in LF power (with peak at 6am), and an *enhanced* diurnal variation (greater difference between peak and nadir) of LF power in diabetic patients vs. controls. They also showed a dampened oscillation in HF power. This resulted in a relative sympathetic prevalence during the night so that the LF/HF ratio day-night change (i.e. diurnal variation) was significantly reduced in the diabetic group (-13.4 ± 109.9 % in diabetic patients vs. 28.7 ± 29.7 % in controls; $n=25/37$; $p<0.05$). The main point to take from these studies is that the effect of diabetic autonomic dysfunction on circadian oscillations of autonomic control, and how this progresses with the disease, is not well established. Our data show that *db/db* mice have altered circadian patterns of autonomic control of HR and HRV, and may therefore be a useful experimental model. More research is clearly needed, but we nevertheless suggest that more consideration should be given to the time of day at which autonomic testing is conducted in type-2 diabetic patients.

STZ-treated mice showed similar circadian rhythmicity of HR to that in control mice, but HR was reduced overall (by an average of 38–98 bpm; statistically significant at 1am, 2am, 8am, 11am, 12pm, 6pm, 7pm, 8pm, 9pm, 10pm, and 11pm in week 10; figure 3). At week

10, STZ-treated mice had relatively consistently lower levels of longer-term HRV as measured by SD2 (reduced by 13–54 units; statistically significant at 6am, 9am, 12pm, 5pm, 7pm, 8pm; figure 4), but no change in short-term HRV measures. This indicates that there is a relative reduction in sympathetic control in these mice, which fits with their overall reduction in HR and agrees with most of the previously published studies in type-1 diabetic animal models (Stables et al., 2013). However, the SDNN data (which reflects a combination of parasympathetic and sympathetic control (Task-Force, 1996)) did not show the same reduction. Type-1 diabetic patients with cardiac autonomic neuropathy have *higher* HR and reduced HRV versus control. This is thought to be due to a predominance of sympathetic tone, but a reduced capacity for increase of this sympathetic activation in response to stimuli (Pop-Busui, 2010). This is one area in which the STZ-treated mouse does not appear to be a good model of the human disease.

The treadmill tests showed impaired HR response to stress and exercise in both *db/db* and STZ-treated mice, though in different ways. *db/db* mice were able to increase HR in response to stress, but the initial response was blunted compared to control (HR after placement on the stationary treadmill was 665 ± 8 bpm in *db/db* vs. 740 ± 9 bpm in control; $p < 0.05$; figure 5). Once they reached maximum HR they maintained it without much variation throughout (665 – 718 bpm on average), whereas in control mice HR dropped to 710 ± 3 bpm during the acclimatization period and increased to 771 ± 13 bpm once the treadmill started moving). STZ-treated mice had a lower maximum HR (619 ± 21 bpm after placement on treadmill; $p < 0.05$ vs. control), but, like *db/db* mice, once they reached this maximum they maintained it without much variation (619 – 650 bpm on average), regardless of the speed of the treadmill. The effects of exercise are impossible to separate from those of stress under these conditions (this would require substantial pre-training to acclimatize animals to both the stationary and the moving treadmill). Diabetic patients with cardiac autonomic dysfunction often find exercise difficult, though in type-2 diabetes there are many underlying causes (for example, impairments in systemic oxygen tissue transport, arteriovenous oxygen difference, and skeletal muscle metabolism) and an inability to regulate HR correctly is thought to be a relatively minor factor (Voulgari et al., 2013). The *db/db* mouse appears to be a good model in this regard, since the reduced HR response to stress and exercise was relatively minor, but the mice were unable to run at the faster speeds on the treadmill.

HRV analysis is challenging in mice, and we had to change from our initial approach to arrive at the method we used. A full description of the initial method chosen (and the reasoning behind it) is included in the supplemental methods. Briefly, we selected stable HR segments and correct R wave detection was carefully checked beat-by-beat. However, after extensive analysis it became clear that selecting for stable signals was biasing our results towards lower heart rates, particularly in the control animals, and since HR is known to be inversely related to HRV (Zaza et al., 2001), this was also likely to bias the HRV results. Therefore, we changed our approach. To counteract the increase in variability inherent in less-stringent selection of stable periods, we decided to take averages across multiple days at multiple time points. However, this required automation of certain aspects of the analysis, and it was not feasible to check correct positioning of all R wave detections in such large

datasets. Although this automation hugely increased the amount of data we could analyze, one negative consequence is a reduction in the reliability of HRV measures that are particularly sensitive to artifacts and non-stationarity of signal (Thireau et al., 2008) (i.e. frequency domain analysis by FFT). Thus, the FFT results (supplemental figure 3) should only be considered in combination with the more robust (Kamen et al., 1995; Thireau et al., 2008) time-domain and Poincare plot data (figure 4).

A major limitation is not knowing when the animals were at rest vs. active (Kim et al., 2008). The “activity” count from the telemetry devices tells us at what times they were moving around the cage, but cannot capture information about activity in-place such as grooming or eating. This makes it difficult to compare the HR and HRV data to that in human studies in which the patient’s ECG is recorded at rest, but is somewhat comparable to 24-hour Holter monitoring. We are also unable to account for the effect of stress due to human presence in the room; in particular the frequent cage changes required for STZ-treated mice due to excessive urination.

In the treadmill test, diabetic mice had significantly lower HRV than control during the acclimatization period while the treadmill was stationary (S.D. of HR increased from 19 ± 7 to an average of 52–61 bpm in controls, but this did not occur in either *db/db* or STZ-treated mice; statistically significant for both groups after 4, 6, and 8 min on the stationary treadmill; figure 5). One possible explanation is variation in mouse activity: the control mice may have been more actively exploring within the treadmill enclosure, causing larger variations in HR. Once the treadmill started, all mice had to run at around the same speed, so variability in activity would have decreased, corresponding to a reduction in HRV. Unfortunately, it was not possible to measure this type of variation in activity while on the treadmill.

4.1 Conclusions

Both *db/db* and STZ-treated mice displayed dysfunction in autonomic control of HR and may be useful models in which to study the mechanisms underlying diabetes-induced cardiac autonomic dysfunction and links to morbidity and mortality. *db/db* mice showed altered circadian patterns of both HR and sympathetic control of HRV. This has implications for future experiments in these mice; the time of day of experiments should be considered, recorded, and if necessary controlled in the experimental design. *db/db* mice also displayed a blunted HR response to stress and exercise. This should be considered when measuring HR using any method that may induce stress. STZ-treated mice had reduced HR, reduced HRV measurements that are indicative of reduced sympathetic control, and reduced maximum HR in response to stress and exercise. Through this study we have provided baseline data and a framework for ECG analysis (particularly HRV) that we hope will provide guidance for future investigations using these mouse models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BP	Blood pressure
CO	Cardiac output
FFT	Fast fourier transform
FS	Fractional shortening
HF	High frequency
HR	Heart rate
HRV	Heart rate variability
IVS	Inter-ventricular septum
LF	Low frequency
LV	Left ventricle
LVIDd	LV internal diameter in diastole
LVIDs	LV internal diameter in systole
LVPW	LV posterior wall
MV A	Mitral valve A velocity
MV E	Mitral valve E velocity
pNN₆	Percentage of RR intervals differing by more than 6 milliseconds
PW	Pulse wave
RMSSD	Root mean square of successive differences
SDRR	Standard deviation of RR intervals
STZ	Streptozotocin
SV	Stroke volume

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Highlights

- Heart rate (HR) autonomic control assessed in T1 (STZ) + T2 (db/db) diabetic mice
- db/db mice had altered circadian pattern of both HR and sympathetic control of HR
- STZ mice had reduced HR and reduced sympathetic control of HR
- HR response to stress and exercise was blunted in both models
- Consider time of day and stress levels when assessing autonomic control of HR

A) Experimental Design

B) Treadmill Test

Figure 1. ECG telemetry experiment design

A) Experiment overview. Streptozotocin (STZ) or vehicle were injected at 5 weeks. Telemetry devices were implanted surgically at 7 weeks. ECG, temperature, and activity were recorded for three days each week. B) Treadmill test protocol. ECG traces (30 seconds duration) were analyzed at the time points indicated.

Figure 2. Characteristics of mice in the ECG telemetry study

(A) Weekly body weight. (B) Fasting blood glucose levels. (C) Survival curves. * $p < 0.05$

db/db vs. control; # $p < 0.05$ STZ vs. control.

Figure 3. 24-hour telemetry parameters

24-hour heart rate, temperature, and activity levels measured via implantable telemetry device. X-axis = time, with 0=midnight; lights on/off at 6am/6pm as indicated by dashed lines. * p<0.05

Figure 4. 24-hour Heart Rate Variability (HRV) at week 10

Measures of short-term HRV (RMSSD and SD1) and long-term HRV (SDNN and SD2). X-axis = time, with 0=midnight. * $P < 0.05$.

Figure 5. Treadmill test at week 9

Treadmill tests conducted on telemetered mice at 9 weeks of age. All mice were placed on the stationary treadmill for 10 min acclimatization, then ran at 8 m/min for 10 min. For control and STZ mice this was followed by 10, 12 and 14 m/min for 2 minutes each. 30 second ECG traces were analyzed for HR and HRV at the time-points shown. * $P < 0.05$.

Table 1

Echocardiography measures in 24-week-old *db/db* versus *db/+* mice.

	<i>db/+</i>		<i>db/db</i>		
	Mean	SEM	Mean	SEM	
Body Weight, g	31.0	0.6	50.9	2.2	***
Heart Rate, beats/min	472	15	367	18	**
IVS, mm	0.837	0.054	0.883	0.047	ns
LVPW, mm	0.747	0.047	0.738	0.052	ns
LVIDD, mm	4.41	0.09	4.11	0.16	ns
LVIDs, mm	3.15	0.12	2.69	0.14	*
FS, %	28.7	1.6	34.6	1.0	*
SV, μl	44.5	2.2	40.2	4.3	ns
CO, ml/min	21.0	1.2	15.0	2.2	*
LV ejection time, ms	43.0	0.8	62.4	1.6	***
MV E, mm/s	1031	27	959	39	ns
MV A, mm/s	680	71	556	57	ns
E/A	1.57	0.14	1.78	0.11	ns

IVS = inter-ventricular septum; LVPW = left ventricular (LV) posterior wall; LVIDD and LVIDs = LV internal diameter in diastole and systole; FS = fractional shortening; SV = stroke volume; CO = cardiac output; MV E = mitral valve E velocity; MV A = mitral valve A velocity; PW = pulse wave. N = 6 per group.

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$.