Journal of Diabetes Science and Technology
2014, Vol. 8(6) 1157–1167

DOI: 10.1177/1932296814545669
dst.sagepub.com
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A Novel Quantitative Method for Diabetic Cardiac Autonomic Neuropathy Assessment in Type 1 Diabetic Mice

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Abstract

In this work, we used a sensitive and noninvasive computational method to assess diabetic cardiovascular autonomic neuropathy (DCAN) from pulse oximeter (photoplethysmographic; PPG) recordings from mice. The method, which could be easily applied to humans, is based on principal dynamic mode (PDM) analysis of heart rate variability (HRV). Unlike the power spectral density, PDM has been shown to be able to separately identify the activities of the parasympathetic and sympathetic nervous systems without pharmacological intervention. HRV parameters were measured by processing PPG signals from conscious 1.5- to 5-month-old C57/BL6 control mice and in Akita mice, a model of insulin-dependent type 1 diabetes, and compared with the gold-standard Western blot and immunohistochemical analyses. The PDM results indicate significant cardiac autonomic impairment in the diabetic mice in comparison to the controls. When tail-cuff PPG recordings were collected and analyzed starting from 1.5 months of age in both C57/Bl6 controls and Akita mice, onset of DCAN was seen at 3 months in the Akita mice, which persisted up to the termination of the recording at 5 months. Western blot and immunohistochemical analyses also showed a reduction in nerve density in Akita mice at 3 and 4 months as compared to the control mice, thus, corroborating our PDM data analysis of HRV records. Western blot analysis of autonomic nerve proteins corroborated the PPG-based HRV analysis via the PDM approach. In contrast, traditional HRV analysis (based on either the power spectral density or time-domain measures) failed to detect the nerve rarefaction.

Keywords

autonomic nervous system, diabetic cardiac autonomic neuropathy, heart rate variability, photoplethysmogram, power spectral density, principal dynamic modes

Diabetic cardiovascular autonomic neuropathy (DCAN) is one of the most overlooked of all serious diabetes complications, and it can cause abnormalities in heart rate (HR) control as well as central and peripheral vascular dynamics.[1-6] Consequences of DCAN include exercise intolerance, intraoperative cardiovascular instability, orthostatic hypotension, myocardial ischemia, increased risk of mortality and morbidity, and reduced quality of life for persons with diabetes.[2-6] One useful noninvasive method to assess autonomic function in various physiological and pathophysiological conditions, including evaluation of the autonomic dysfunction in diabetic subjects, is the use of heart rate variability (HRV).[7-9] HRV is a marker of sympathetic and parasympathetic (vagal) influences on the modulation of HR.[10] Reduced HRV is known to be one of the earliest indicators of DCAN[11] as DCAN has been shown to involve a change in the normal balance of the autonomic nervous system (ANS).[7,11-13] Whereas the DCAN Subcommittee of the Toronto Diabetic Neuropathy Expert Group identified HRV, baroreflex sensitivity, muscle sympathetic nerve activity, plasma catecholamines, and heart sympathetic imaging as the most sensitive and specific approaches currently available to evaluate DCAN in clinical research, it also identified serious limitations of the existing methods and emphasized that efforts should be undertaken to develop new noninvasive and safe DCAN tests, with a higher sensitivity and specificity.[14]

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The limitations of the existing methods for evaluation of DCAN are clearly seen in experimental studies. For example, young streptozotocin (STZ)-diabetic rats exhibit a significant reductions in both HR and HRV, suggesting disturbance in the ANS balance.[15] Furthermore, insulin treatment of these STZ-treated diabetic rats showed no significant recovery of the autonomic nervous activity even though HR recovered to the state recorded prior to STZ administration.[16] This is in agreement with clinical studies identifying limitations of HRV as a diagnostic marker of DCAN.[17] Apparently, new quantitative methods for assessment of this diabetic complication are needed.

The literature on DCAN in mice is sparse. One intriguing mouse model of type 1 diabetes is the Akita mouse. Ins2C96Y Akita mice, which spontaneously develop insulin-dependent diabetes at about 4 weeks of age, express a mutant nonfunctional insulin isoform.[18] A further investigation provided evidence for cardiac parasympathetic dysfunction.[19] Thus, there is a strong rationale to suggest that Akita mice develop DCAN similar to those found in STZ-diabetic rodents, and can be a suitable model for studying this complication.

The goal of this work was to determine if DCAN in Akita mice can be monitored with principal dynamic mode (PDM) analysis of HRV. The HRV measurements were extracted from photoplethysmographic (PPG) pulse intervals, obtained from a pulse oximeter. The HRV parameters have been shown to be accurately obtained from pulse intervals.[20,21] The PDM algorithm has been shown to accurately separate the dynamics of the sympathetic and parasympathetic nervous systems,[22,23] which has not been possible using the estimation of low- and high-frequency spectral powers. Therefore, the onset was monitored and the progression of DCAN in Akita mice with PDM and compared its performance to the standard time-domain HRV parameters and spectral analysis techniques.

Methods

Figure 1 summarizes all in vivo and ex vivo measurements that were performed. In addition, details regarding the HRV data analyses performed on in vivo measurements are also provided in Figure 1.

Animal Population

For each experimental case, there were 10 mice in total, 5 wild-type C57BL/6 and 5 Akita mice. A different set of animals was used for each case. All animal-related experimental protocols were approved by the Institutional Animal Care and Use Committee at Stony Brook University and Worcester Polytechnic Institute. The case lettering below corresponds to the labels in Figure 1.

Case A: Tail-Cuff Pulse Oximeter Data Collection and Analysis

Given that DCAN occurs in 4-month-old Akita mice, we were interested in knowing if DCAN occurs prior to 4 months. As we were interested in examining DCAN onset in mice as early as 1.5 months, we used a tail-cuff pulse oximeter PPG instead of telemetry ECG recordings. This was necessitated because a telemetry ECG sensor is too large and heavy (~5 grams) for a 1.5-month-old Akita mouse which weighs ~16 grams, and is the age of their first restraint. Another advantage of using a pulse oximeter for HRV measurement is that surgical procedures are not needed to implant a PPG sensor.

Sex- and age-matched mice were used for this case. Mice were 1.5 months old at the start of the experiment, and the entire recording process lasted until they reached 5 months of age. Animals were conscious and restrained in a tube-shaped mouse restrainer with their tails exposed. They were trained to be acclimated in the mouse restrainer for 30 minutes per day for a period of 1 week. Starting the following week, they were again placed in the restrainer for 30-60 minutes per day, but data were collected during their awake state. The mice's temperature was maintained at 37 C. The Y-clip pulse oximeter PPG sensor was placed on the proximal (thickest) portion of the tail to produce the best signal. PPG data were recorded at a sampling rate of 600 Hz. Ten minutes of settling time was allowed. PPG recordings were collected for only 30 minutes to 1 hour each day, 5 days a week.

Pulse Oximeter Data Processing and Analysis

For each day, a 15 minute data segment was selected from pulse oximeter signals, and all analysis results were averaged (eg, HR, PSD, and PDM) based on every 1 minute interval of data. One minute of data is enough to perform HRV analysis, given the high mice's HR. Data were analyzed using Matlab R2010a (The Mathworks, Natick, MA). Automatic detection of pulse-interval series from PPG was carried out after removing erroneous spikes and premature beats. The obtained series were subjected to the cubic spline procedure to make them equally spaced. The resultant HR time series were down-sampled to 10 Hz. Prior to HRV analysis, all data were detrended, zero-meaned, and normalized to unit variance.

Traditional HRV Analysis

The mean HR and all HRV parameters were calculated from the pulse-intervals series. The time domain parameters of standard deviation of the normal-to-normal (SDNN) beats (eg, standard deviation of the beat-by-beat variations) and root mean square of successive difference (RMSSD) values were computed. SDNN reflects overall autonomic nervous activities

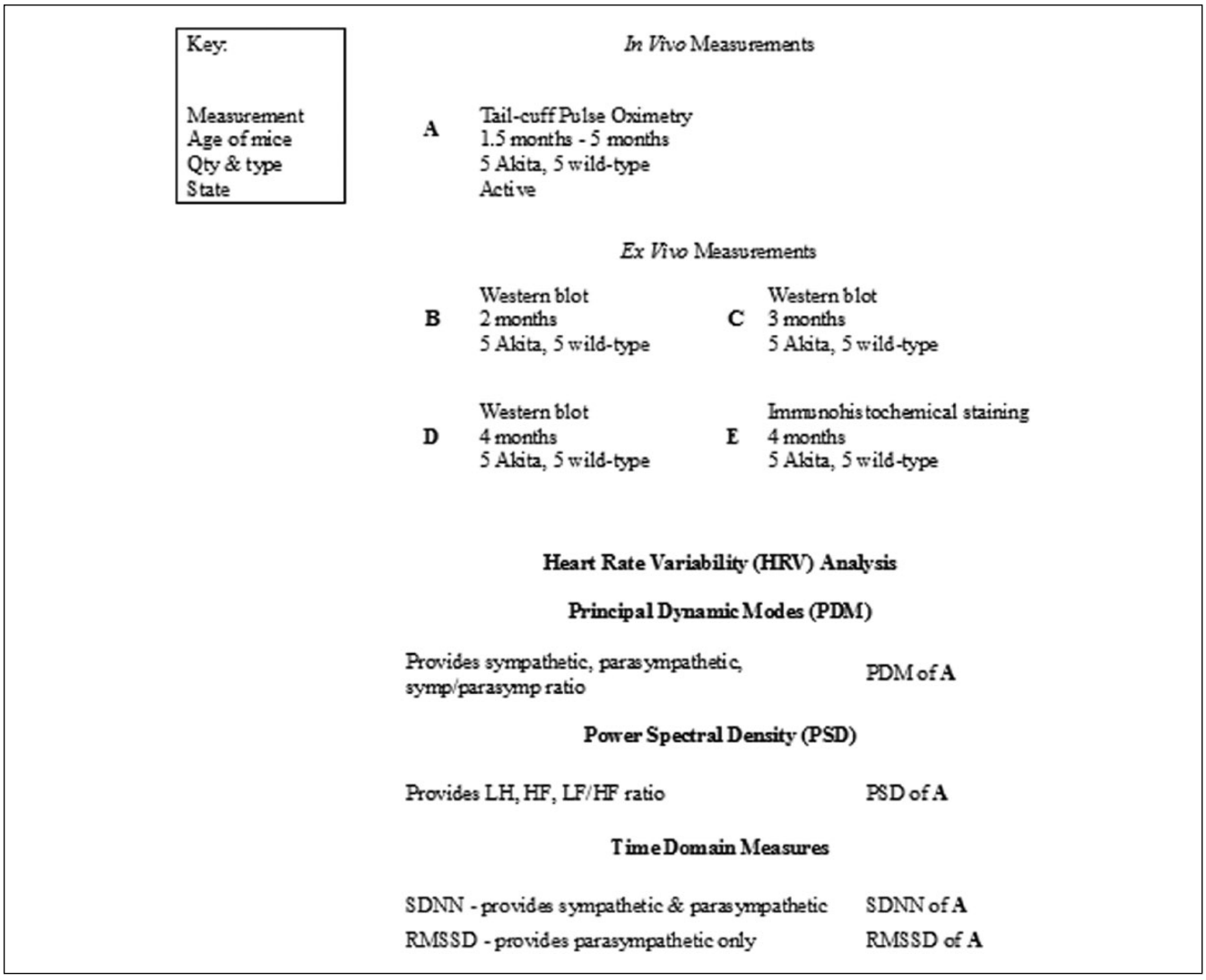

Figure 1. Experimental protocol and data analyses of heart rate variability using principal dynamic mode (PDM), power spectral density (PSD) of the low frequency (LF) and high frequency (HF), root mean square of successive difference (RMSSD), and standard deviation of normal-to-normal (SDNN) beats.

and RMSSD reflects the parasympathetic dynamics.[24] For frequency domain parameters, Welch periodogram (a 256-point FFT with a Hanning window and 50% overlapping segments) was computed, and spectral powers in both the low frequency (LF) (0.4-1 Hz) band and high frequency (HF) (1-4 Hz) band were calculated as these frequency bands have been determined for mice in previous studies.[6] It is well established that the LF band represents both sympathetic and parasympathetic nervous activities while the HF band represents parasympathetic nervous activity.[24,25] We also computed LF/HF ratio to assess the autonomic balance of the C57BL/6 and Akita mice.

Principal Dynamic Mode Analysis of HRV

The PDM was compared to the traditional HRV measures. The advantages of PDM have been well documented and its most salient feature is that it enables accurate separation of the sympathetic and parasympathetic dynamics.[22] Basically, the PDM takes into account the second-order nonlinearities of HR dynamics and among many frequencies in the HRV signal, and extracts the principal components using an eigen decomposition approach. The feasibility of separation of the ANS dynamics via the PDM has been validated using ANS pharmacological blockades in our previous study.[22] We have modified the PDM technique for use with even a single output signal of HRV data, whereas the original PDM required both input and output data.[23] A comparison to the PSD has shown that the PDM is more accurate.[22,26] While the PDM is a time-domain representation, we convert it to the frequency domain via the fast Fourier transform (FFT). Therefore, hereafter we will describe the PDM's dynamic characteristics in the

frequency domain. For this study, 8 Laguerre functions were used with a memory length of 60. The calculation of PDMs as well as determining Laguerre functions and memory lengths have been previously described.[23] The derived PDM's 2 main dynamics will be referred to here as the sympathetic and parasympathetic.

Cases B-D: Western Blot Analysis

As shown in Figure 1, 30 male 2-, 3-, and 4-month-old wild-type C57BL/6 (n = 5 for each age) and Akita (n = 5 for each age) mice were studied to quantify the development of nerve degeneration by Western blotting. The atrial section of the heart was used for Western blotting of heart tissue since autonomic nerves are more prevalent in the sinoatrial and atrial ventricular nodes.[27] The atrial tissues were homogenized, the proteins fractionated according to size by SDS-PAGE and then transferred onto a membrane for probing with specific antibodies. In this study, we focused on 3 autonomic nerve protein markers: tyrosine hydroxylase (TH), a marker for sympathetic nerves (anti-TH, ab112, Abcam Inc; 1:200 dilution); choline acetyltransferase (ChAT), a marker for parasympathetic nerves (anti-Choline acetyltransferase, AB144P, Millipore Inc; 1:200 dilution); and synaptophysin (SYN), a nonselective marker for nerves (anti-SYN, sc-9116, Santa Cruz Biotechnology, Inc; 1:500 dilution). Actin (anti-actin, ab50412, Abcam, Inc; 1:5,000 dilution) was used as a loading volume control. The secondary antibody consisted of anti-rabbit horseradish peroxidase antibody from goat, A6154, Sigma-Aldrich (1:10,000 dilution).

To quantitatively compare protein density in C57BL/6 and Akita mice, the integrated density of each protein expression bar was recorded and compared after normalization to actin density in each column.

Case E: Immunohistochemistry Analysis

For immunohistochemistry, the atrial section of the heart was fixed in paraformaldehyde after dissection. The slides were prepared by sectioning the tissue into 6 micrometers thick. The sections were fixed in paraformaldehyde and then stained with SYN as well as with an HCN4 antibody,[28] which is a channel protein only found in the SA node; therefore it is used as a location control for the SA node. The stained cardiac sections were examined on a Zeiss confocal microscope, and the SA and AV nodes were assessed to quantify sympathetic and parasympathetic innervation. It is well known that the SA and AV nodes are densely innervated by the autonomic nerves.[27] All samples for each mouse were from the same block, and the images were subjected to overall contrast enhancement.

Statistical Analysis of HRV Methods and Western Blot Results

For each week's HRV analysis, Student's *t* test was applied to test the difference between Akita and wild-type mice, followed by the Jarque-Bera normality test. One-way analysis of variance (ANOVA) with repeated measurement was used to test any significant difference between all weeks. Data from two other independent data segments were also tested using one-way ANOVA. The null hypothesis was rejected when the *P* value was less than .05. All values are reported as the mean ± standard deviation. All statistics were performed using SigmaStat 3.0 (SPSS Inc, Chicago, IL) and Matlab (The Mathworks, Natick, MA).

For each Western blot comparison, the image was inverted and the integrated density of each relevant protein band was recorded (using Adobe Photoshop CS4, Adobe). This density value was divided by the integrated density value of actin band in the same lane to obtain a normalized value. Student's *t* test was also applied to test the normalized integrated density value between Akita and wild-type mice, followed by the Jarque-Bera normality test. The null hypothesis was rejected when the *P* value was less than .05.

Results

Time and Frequency Parameters From HRV Analysis of Case A

The standard time- and frequency-domain parameters were investigated on a weekly basis. Results were recorded from week 6 through week 19; thus, 14 weeks of data comparison are presented. Each data point in Figures 2 to 4 is the average of each week, which consists of 15 minutes per day for 5 days per week. We found a consistently and significantly decreased mean HR in Akita mice starting at week 9, as shown in the top panel of Figure 2. SDNN values decreased significantly in Akita mice in some weeks (12th, 16th, and 19th weeks) but not in others, and increased in week 14. The RMSSD, on the other hand, showed a significant decrease in week 12, no difference in week 16, and a significant increase in week 19.

As shown in the top panel of Figure 3, no significant change in the normalized LF power was observed between Akita and C57/BL6 mice throughout the experiment (except for week 11). The HF power did not change initially in Akita when compared to C57/BL6 but a significant reduction in weeks 10 to 14 and also week 16 is noted; on week 18, HF power was increased in Akita when compared to C57/BL6. Consequently, the LF/HF ratio showed similar patterns as those of LF and HF powers in that some weeks there was significant decrease (week 11 and 16), whereas in other weeks there was either no difference or significant increase. Similar to RMSSD and SDNN values, the frequency domain parameters between the two strains were not consistent across time throughout the experiment.

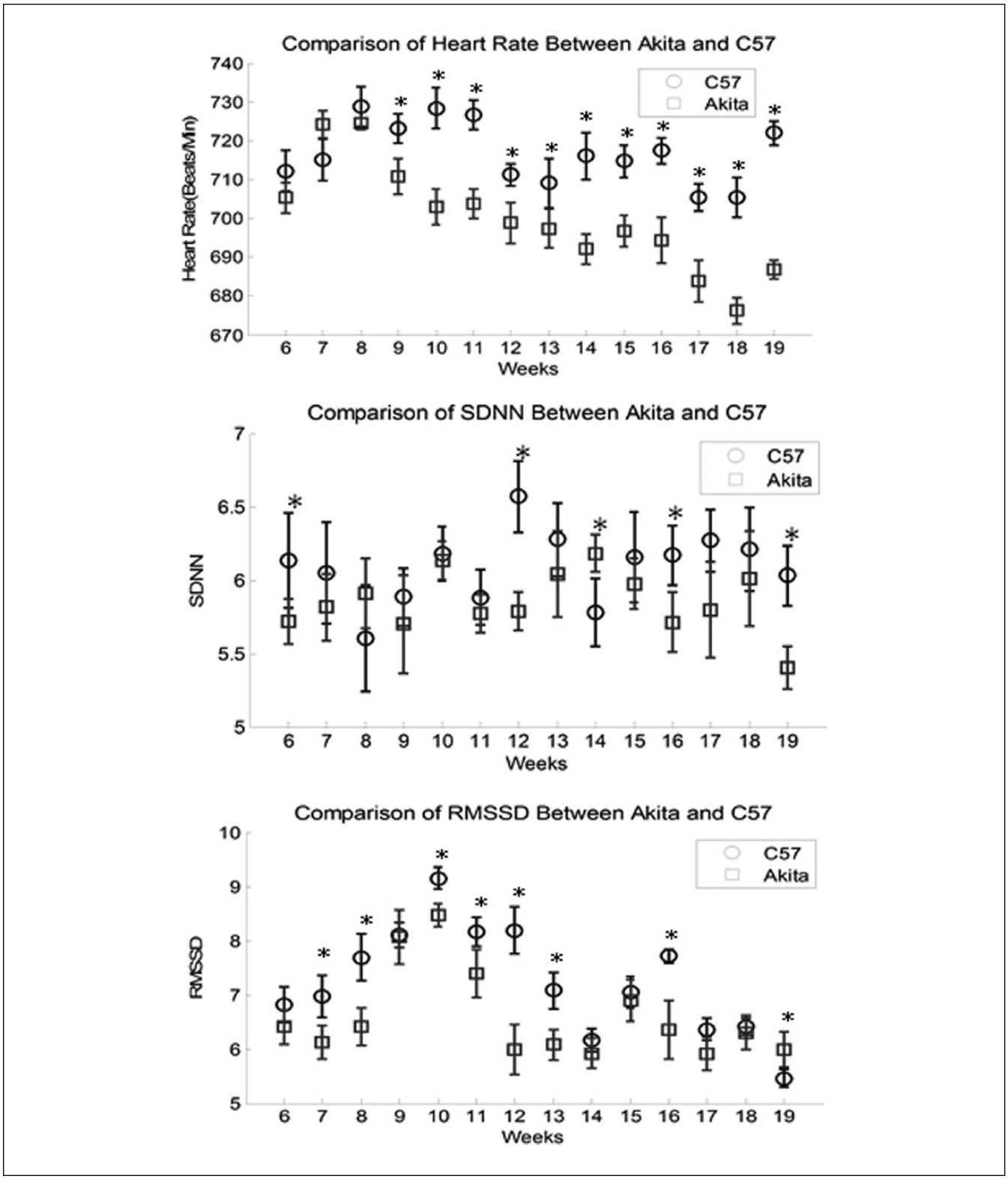

Figure 2. Comparison of heart rate (top panel), SDNN (middle panel), and RMSSD (bottom panel) between Akita and wild-type mice. Data were recorded from a pulse oximeter. *Denotes statistically significant difference between Akita and wild-type mice. There was a statistically significant downward trend in HR of Akita between all of the data in the first 13 weeks to weeks 17-19 as shown in the top panel.

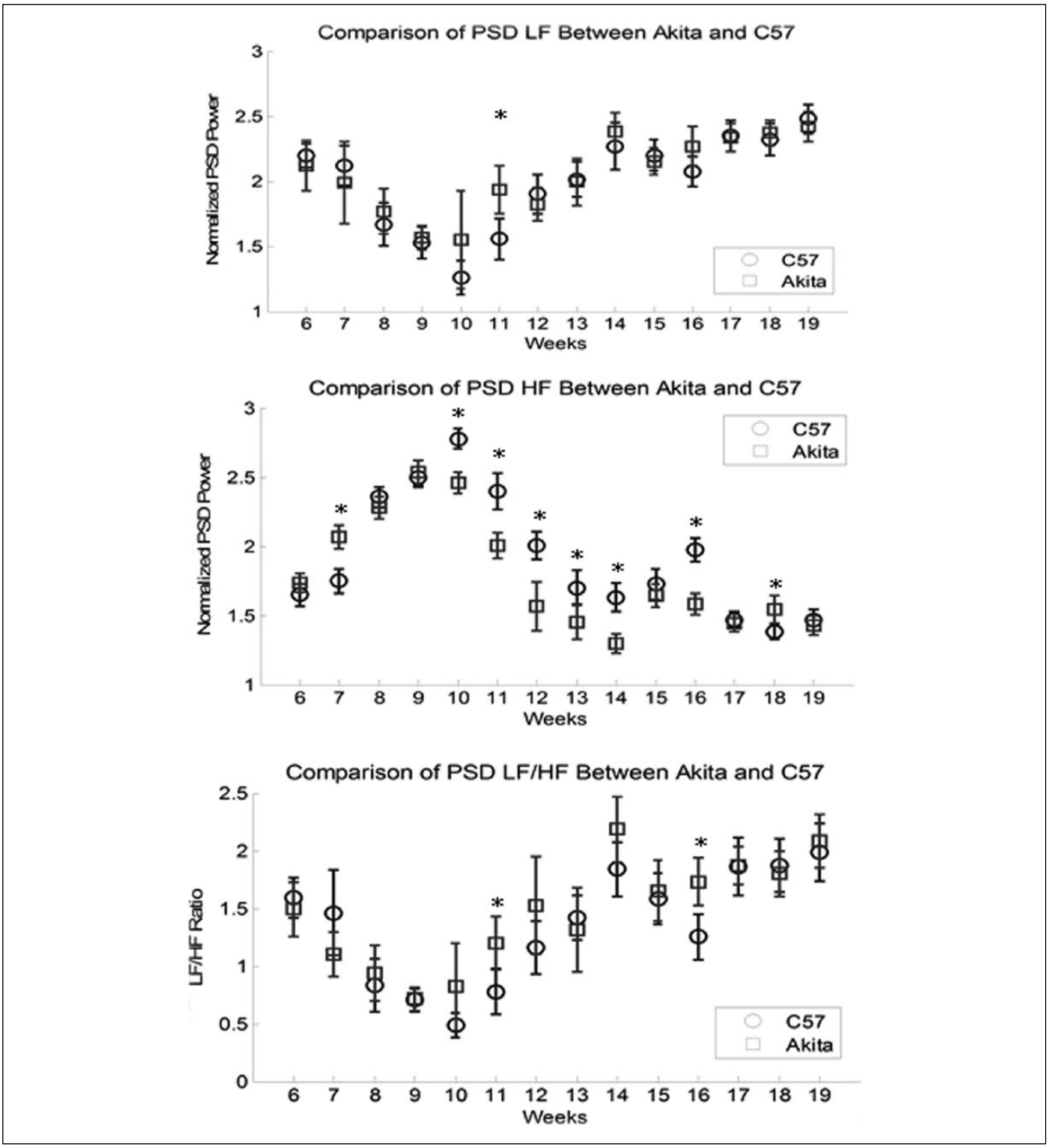

Figure 3. Comparison of low frequency (LF; top panel), high frequency (HF; middle panel), and LF/HF (bottom panel) between Akita and wild-type mice. Data were recorded from a pulse oximeter. *Denotes statistically significant difference between Akita and wild-type mice.

PDM Analysis of HRV

As shown in the bottom panel of Figure 4, the PDM-derived sympathetic power remains unchanged between the 2 strains of mice initially but then is significantly lower from week 11 to week 19 in Akita mice as compared to C57/BL6. This result suggests that depressed or impaired sympathetic activities are present as early as 3

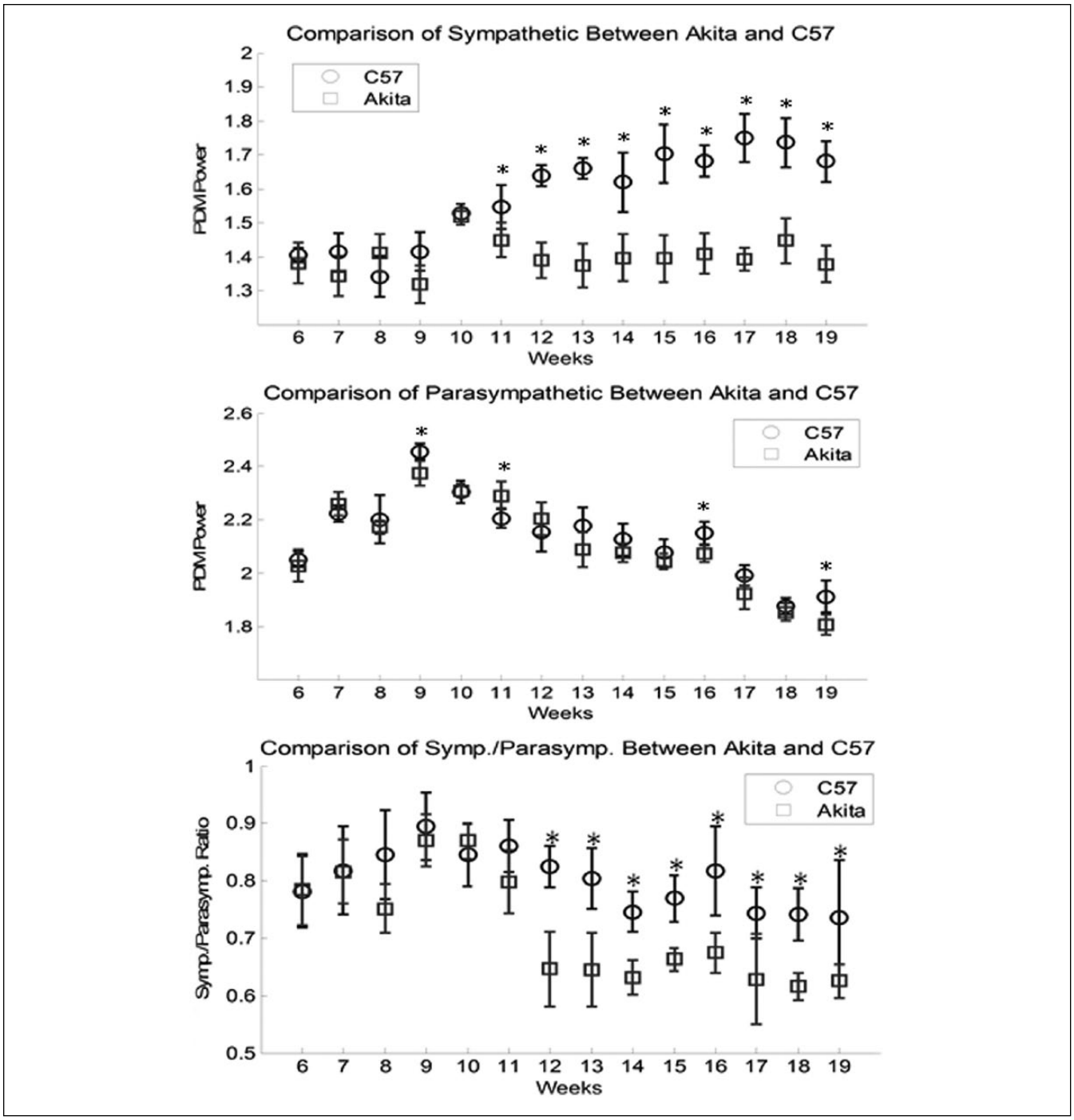

Figure 4. Comparison of sympathetic (top panel), parasympathetic (middle panel), and symp/parasymp ratio (bottom panel) between Akita and wild-type mice. Data were recorded from a pulse oximeter and analysis was by PDM. *Denotes statistically significant difference between Akita and wild-type mice.

months in Akita mice and become more severe as they age. On the contrary, the parasympathetic power via PDM shows no significant change for most time points between the two strains. Note that data were recorded in an active state where parasympathetic nervous dynamics are depressed.[24] Thus differences between the parasympathetic activities of Akita and C57 were not likely to be detectable. The sympathetic/parasympathetic ratio was

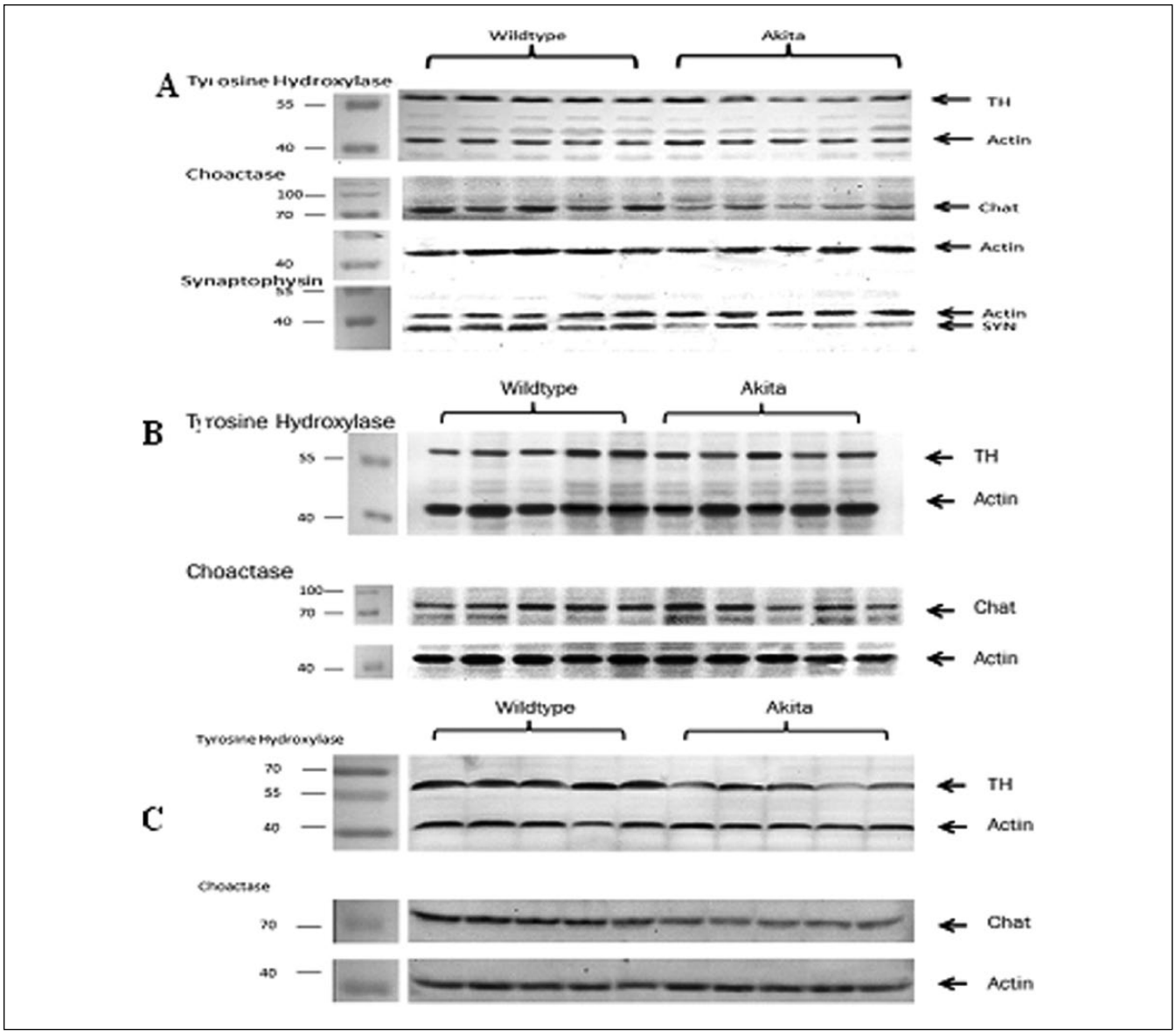

Figure 5. Western blot analysis of heart tissue extracts from Akita (n = 5) versus wild-type (n = 5) mice at (A) 4 months, (B) 3 months, and (C) 2 months of age. SYN is a general nerve protein marker, Chat is a marker of the parasympathetic nerves, and TH is a marker of the sympathetic nerves. Protein molecular weight markers (kDa) are shown at the left of each blot.

found to be significantly lower with Akita than wild-type mice starting at week 12 and continuing to the end of the experiment, which is indicative of the autonomic imbalance in diabetic mice.

Cases B-D: Western Blot Assessment of Autonomic Nerve Density

Only the PDM approach indicated consistently significantly reduced autonomic activity in Akita mice when compared to C57/Bl6 mice. Western blotting was performed to determine whether the autonomic nervous function data obtained by the PDM method correlated with nerve rarefaction in the SA and AV nodes in both strains of mice. As shown in Figure 5A, the autonomic nerve protein markers SYN, TH, and ChAT were all significantly less abundant in the 4-month-old Akita than the age-matched wild-type mice (see Table 1).

We also investigated the possibility of autonomic nerve rarefaction in younger (3 months old) Akita and age-matched wild-type mice. In Figure 5B, we show the Western blot of TH, SYN, and ChAT antibodies in 3-month-old mice (Akita n = 5 and wild-type n = 5). After normalizing the integrated density to actin, a significant reduction was found in Akita mice. The same experiments were performed on 2-month-old

Table 1. Quantification of Western Blot Analysis.

	TH	Chat	SYN
4 months			
Wild-type	1.52 ± 0.18	0.99 ± 0.27	1.14 ± 0.43
Akita	1.18 ± 0.20*	0.66 ± 0.13*	0.50 ± 0.15*
3 months			
Wild-type	1.11 ± 0.12	1.06 ± 0.07	
Akita	0.87 ± 0.15*	0.84 ± 0.02*	
2 months			
Wild-type	0.48 ± 0.08	0.65 ± 0.12	
Akita	0.49 ± 0.05	0.62 ± 0.07	

Wild-type: n = 5 and Akita: n = 5 for each age group. These values are normalized.
*Denotes statistical significance with $P < 0.05$.

mice (Figure 5C; Akita n = 5 and wild-type n = 5). There was no significant reduction in abundance of any of the 3 proteins at 2 months. These results are summarized in Table 1.

Case E: Immunohistochemistry of Cardiac Autonomic Nerves

Immunohistochemical analysis was performed on 4-month-old mice to examine if indeed SYN expression is reduced in Akita mice. Figure 6 shows a stack of deconvolved optical sections of the immunostaining of SA nodes for HCN4 (red) and SYN (green). The negative control was obtained by omitting both primary antibodies and the image was not deconvolved. The tissue sections were stained concurrently and imaged with the same settings. As shown, the SA node in this Akita mouse is clearly less densely innervated than that of the C57BL/6 control mouse.

Discussion

We illustrated an alternative to the power spectral density approach, termed PDM analysis, to estimate the dynamics of the ANS from HR fluctuation data. Unlike the PSD, our PDM method provides separation of the dynamics associated with the parasympathetic and sympathetic nervous systems.[22,23] The advantages of using the PDM method over the traditional HRV measures have already been demonstrated in our previous study involving healthy subjects with the aid of pharmacological blockade.[22] In this study, we found significant depression of the parasympathetic dynamics with application of atropine without affecting the sympathetic dynamics obtained via the PDM. With application of propranolol, the reverse effect was noted.[22]

The results obtained from the PSD and the traditional time-domain HRV measures derived from the PPG (pulse oximeter) recordings did not correlate with the reduction in nerve marker proteins observed in Akita mouse heart tissue by Western blot analysis. In particular, the Western blot results indicated that nerve rarefaction in Akita mice started at 3 months and persisted as they aged, but the PSD and time-domain measures did not show such differences in ANS dynamics between the wild-type and Akita mice for all time durations. The PDM, consistent with the Western blot analysis, showed depressed sympathetic dynamics and autonomic imbalance starting at 3 months. Thus, using PPG recordings we were able to discern the onset of DCAN in Akita mice starting at 3 months, which was corroborated by the Western blot analysis. The parasympathetic dynamics derived from the PDM, however, did not show consistent nerve rarefaction in Akita mice when compared to wild-type at either 3 or 4 months. We believe this is due to the fact that PPG recordings were made during an active state that is dominated by the sympathetic and depressed parasympathetic nervous dynamics.[24] Hence, the parasympathetic difference between Akita mice and wild-type mice is likely to be minimal during the active state.[24]

Reduced HRV is known to be one of the earliest indicators of DCAN[11] in human as it involves an imbalance of the ANS in humans.[6,7] Sustained hyperinsulinemic hypoglycemia in type 1 diabetics and their nondiabetic counterparts has been shown to result in reduced cardiac vagal outflow in all patients.[7] One of the consequences of hyperinsulinemic hypoglycemia is decreased autonomic function present in the early development of diabetes, but moreover prolonged diabetes leads to a progressive decline in autonomic function.[13] These human studies clearly indicate autonomic imbalance with only either parasympathetic or sympathetic nervous tone reduced. This is similar to our current study with the PSD method: consistent depression of the 2 branches of the ANS is not often found.

Conclusions

In conclusion, our computational results based on the PDM approach indicate a significant cardiac autonomic impairment in diabetic Akita mice, a model of insulin-dependent type 1 diabetes. Both immunohistochemical and Western blot analyses show a progressive reduction in autonomic nerve density in Akita mice as compared to the control mice starting at 3 months of age, thus corroborating our PDM data analysis of HRV records. Utilizing a simple measurement based on tail-cuff pulse oximeter recordings, we were able to determine that the onset of DCAN in Akita mice occurs at 3 months and that the neuropathy persisted with over time.

To use our method for the assessment of DCAN in humans, studies in healthy and DCAN-bearing subjects are necessary to define age-related normal and pathologic values. One disadvantage of using PPG instead of ECG is that the former can be affected by other diabetic confounders like arterial stiffness, microvascular dysfunction, and poor peripheral circulation.

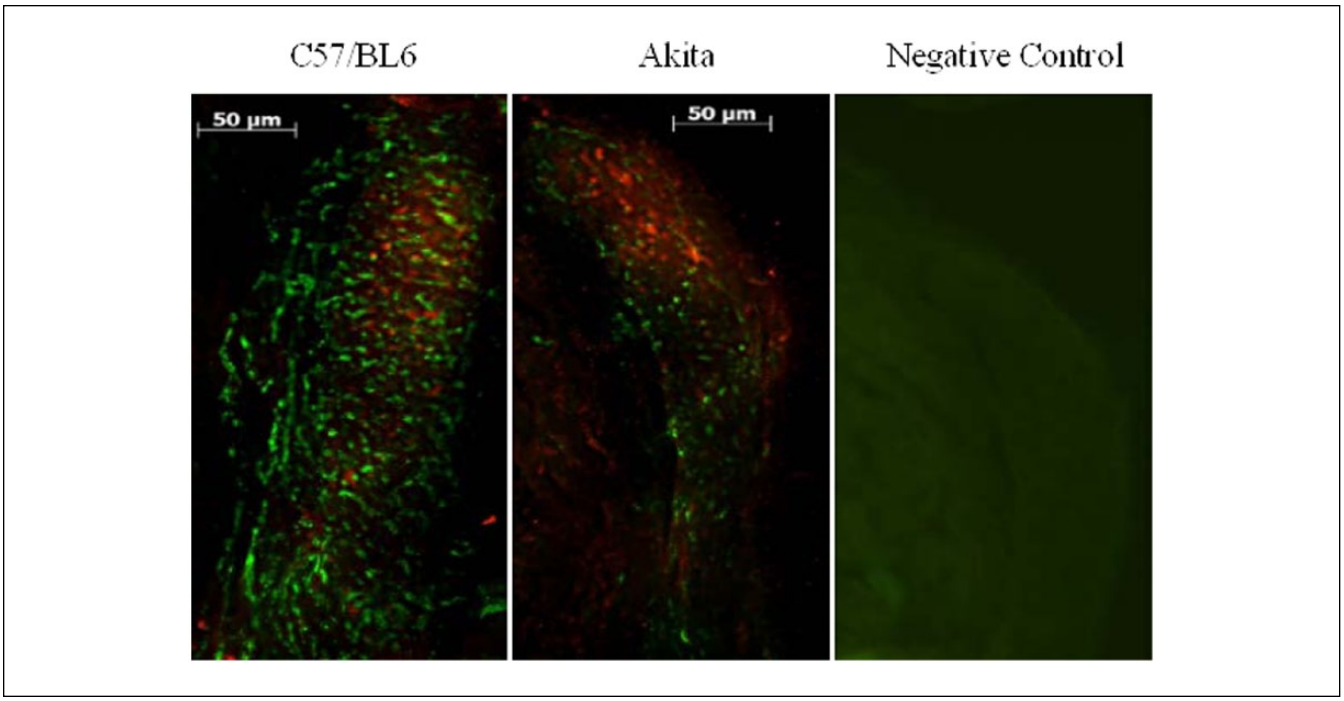

Figure 6. Representative images of immunostained heart tissue sections from wild-type and Akita mice. HCN4 immunostaining (red fluorescence) indicates that these sections are from the SA node region of the mouse heart, and SYN (green fluorescence) is a marker for nerves.

Abbreviations

ANOVA, analysis of variance; ANS, autonomic nervous system; AV, atrioventricular; ChAT, choline acetyltransferase; DCAN, diabetic cardiovascular autonomic neuropathy; FFT, fast Fourier transform; HF, high frequency; HR, heart rate; HRV, heart rate variability; LF, low frequency; PBS, phosphate buffered saline; PDM, principal dynamic mode; PPG, photoplethysmographic; PSD, power spectral density; RMSSD, root mean square of successive difference; SA, sinoatrial; SDNN, standard deviation of normal-to-normal; STZ, streptozotocin; SYN, synaptophysin; TH, tyrosine hydroxylase.

Acknowledgments

The authors thank Sharon Shaw and Drs Ray Page and Chris Malcuit for their assistance with immunohistochemistry and Western blot experiments.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Juvenile Diabetes Research Foundation (JDRF) Innovative Grant Program.

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