

## ORIGINAL CONTRIBUTION

# Diabetes Alters Diurnal Rhythm of Electroretinogram in db/db Mice

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Diabetic retinopathy (DR†) is the most common complications of diabetes and a leading cause of blindness in the United States. The retinal neuronal changes precede the vascular dysfunction observed in DR. The electroretinogram (ERG) determines the electrical activity of retinal neural and non-neuronal cells. The retinal ERG amplitude is reduced gradually on the progression of DR to a more severe form. Circadian rhythms play an important role in the physiological function of the body. While ERG is known to exhibit a diurnal rhythm, it is not known whether a progressive increase in the duration of diabetes affects the physiological rhythm of retinal ERG. To study this, we determined the ERG rhythm of db/db mice, an animal model of type 2 diabetes at 2, 4, and 6 months of diabetes under a regular light-dark cycle and constant dark. Our studies demonstrate that the diurnal rhythm of ERG amplitude for retinal a-wave and b-wave was altered in diabetes. The implicit time was increased in db/db mice while the oscillatory potential was reduced. Moreover, there was a progressive decline in an intrinsic rhythm of ERG upon an increase in the duration of diabetes. In conclusion, our studies provide novel insights into the pathogenic mechanism of DR by showing an altered circadian rhythm of the ERG.

## INTRODUCTION

The recent report from the Center for Disease Control and Prevention (CDC) suggests that more than 100 million Americans are living with diabetes or prediabetes [1]. Strikingly, diabetes is the 7<sup>th</sup> leading cause of death in the United States. Diabetes-related eye disease is the foremost cause of blindness among adults. The risk of developing eye disorders among diabetics is 25 times that of

the general population [2]. Diabetic retinopathy (DR) is the most common complications of diabetes with an estimated prevalence of 28.5 percent among US adults with diabetes [3]. In addition to DR, long-standing diabetes is also associated with an increased risk of diabetic macular edema (DME) with a prevalence of 3.8 percent. During the first twenty years of diabetes, nearly all patients with Type 1 diabetes (T1D) and > 60 percent of patients with Type 2 diabetes (T2D) will develop some form of DR [4].

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†Abbreviations: ERG, electroretinogram; DR, Diabetic retinopathy; DME, diabetic macular edema; T1D, Type 1 diabetes; T2D, Type 2 diabetes; SCN, suprachiasmatic nucleus; NPDR, non-proliferative DR; ZT, Zeitgeber time.

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The pathogenesis of DR is complex and multifactorial. While the pathologic impact of diabetic milieu is mainly observed on retinal vasculature, there is considerable evidence that the DR is the disease of the neurovascular unit and diabetes-induced changes in retinal neurons and glia precede the onset of clinically evident vascular injury [5-7].

Circadian rhythm plays an important role in regulating a variety of biochemical and physiological processes of the body such as the sleep-wake cycle, hormone release, and feeding behavior. The daily oscillations of circadian rhythms are governed by the suprachiasmatic nucleus (SCN). The SCN drives the rhythms of other peripheral organs including the retina. Interestingly, the retinal clock is independent of the SCN and can influence the rhythms generated by the SCN [8]. The retinal clock regulates photoreceptor survival [9], ganglion cell viability during the aging [10], and critical functions of the retina such as phagocytosis, disk shedding, and corneal thickness.

The risk of diabetes is increased due to a variety of circadian rhythm disorders such as irregular or poor quality of sleep, circadian misalignment, and melatonin dysregulation. Interestingly, experimental studies in laboratory animals where the circadian cycle was altered by changing the light exposure pose an increased risk of T2D [11] and this observation is also reciprocated in shift workers [12] constituting 15 million Americans [13]. Circadian disruption due to genetic factors also increases the risk of diabetes [14,15]. Genetic studies demonstrate that a variant in the receptor for circadian-regulated hormone melatonin (MTNR1B), which is known to be associated with increased fasting glucose and T2D is also involved with increased duration of endogenous melatonin production [16]. On the contrary, diabetes itself leads to progressive circadian dysfunction or exacerbates the existing circadian rhythm disruption. Diabetic individuals display a disrupted circadian rhythm of body temperature, heart rate, and fasting blood glucose [17]. The diabetes-associated gradual alteration in circadian rhythm might be associated with compromised retinal function. Melatonin levels, an important component of a retinal clock, are found to be decreased in diabetes irrespective of the stage of DR [18]. The retinal ganglion cells in open-angle glaucoma are known to be associated with the disrupted circadian rhythm of temperature [18].

The electroretinogram (ERG) is an objective method for evaluating a retinal function [19]. The electroretinography abnormalities can be observed at an early stage of the disease when there are no visible changes in the fundus [20]. In the early stage of DR, *i.e.* non-proliferative DR (NPDR), there is a significant reduction in scotopic ERG b-wave amplitude and a decrease in oscillatory po-

tential (OP) [21,22]. There is a further decrease in ERG amplitude upon an increase in the severity of the DR from mild NPDR to severe NPDR. Several abnormalities are observed in ERG parameters with proliferative diabetic retinopathy (PDR) such as a decrease in OP or a complete disappearance of OP along with a decrease in ERG a-wave and b-wave amplitude [20].

Notably, the retinal ERG also display diurnal rhythm both in humans [23,24] and rodents [25], however, how diabetes influences this natural rhythm of ERG is not studied yet. Moreover, how long-standing diabetes (when the retinal vascular defects are evident) influences the biological rhythm of ERG is not known. To study this, we analyzed ERG rhythm at different time intervals using a mouse model of type 2 diabetes, the db/db mice. The retinal ERG rhythm was evaluated at 2, 4, and 6 months of diabetes. Our studies unravel a progressive decline in ERG rhythm with an increase in the duration of diabetes.

## MATERIAL AND METHODS

### Animal Studies

The C57BL6/J, B6.BKS(D)-Lepr<sup>db</sup>/J (an animal model for T2D; db/db) and Lepr<sup>db/+</sup> db/m (heterozygotes; db/m) mice were purchased from The Jackson Laboratory. All animal care and experimental procedures were in accordance with The Guiding Principles in the Care and Use of Animals (NIH). The animals were maintained in a 12 hr light: 12 hr dark cycle (LD condition). The “lights on” time was considered as Zeitgeber time (ZT) 0 and the “lights off” time was considered as ZT 12. For studying the intrinsic circadian rhythm in the absence of light cues, the animals were maintained in complete darkness for 2 weeks (DD condition) before performing ERG on them. Besides the occasional movement of cages for bedding change, food & water supply, and examination by the veterinary staff (under red light), the cages were not moved from their respective locations. Prior to ERG studies under LD conditions, the animals were dark-adapted overnight. For both light conditions the ERG was recorded at ZT-3, ZT-9, ZT-15, and ZT-21. The mice were anesthetized with a mixture of Ketamine and Xylazine. The pupils were dilated using 1% tropicamide and 2.5% phenylephrine. The gold loop electrode (LKC Technologies, Inc, Gaithersburg, MD, USA) was placed over the cornea after the application of Gonak (Hypromellose 2.5% solution Akorn Inc, Lake Forest, IL), and the scotopic ERG recordings were performed using an LKC NGIT-100 recording machine (LKC Technologies, Inc, Gaithersburg, MD, USA). The values for a- and b-wave amplitudes and implicit times are obtained using an inbuilt analysis tool from LKC Technologies.

## Statistics

The data were expressed as Mean  $\pm$  SEM. The curve fitting for raw data was performed using the GraphPad Prism 8 software (GraphPad Software, La Jolla, CA) as described [26]. The amplitude and phase values obtained from the curve fitting were further analyzed using a multifactor ANOVA. Circadian rhythmicity for ERG in C57BL6/J mice was determined using the equation (Amplitude $\cdot\cos(ZT/24\cdot(2\cdot\pi)+\text{phase})+\text{mesor}$ ) in an R program (version 3.5.1)

In addition, for studies involving diabetic mice, we evaluated the difference in circadian parameters in mice with the three factors disease/genotype (db/db vs db/m); diabetes duration (2 vs 4 vs 6 months) and light condition (LD vs DD), the animals were subgrouped accordingly. The statistical significance of circadian oscillation for each subgroup was first evaluated by a zero-amplitude test. To evaluate the circadian oscillation, a linear model that can be transferred to a single cosine function was used and a zero-amplitude test was performed using this model. For the subgroups that demonstrated circadian oscillation, we further tested whether there is any difference in circadian parameters (mesor difference; phase shift; amplitude difference) by performing a log-likelihood ratio test. If there was any significant difference in circadian parameters, we used a permutation test between any paired subgroups to test which circadian parameters were different. A p value of less than 0.05 was considered as statistically significant using an R program (version 3.5.1).

## RESULTS

### *The Diurnal Rhythm of ERG in Wild-Type Mice*

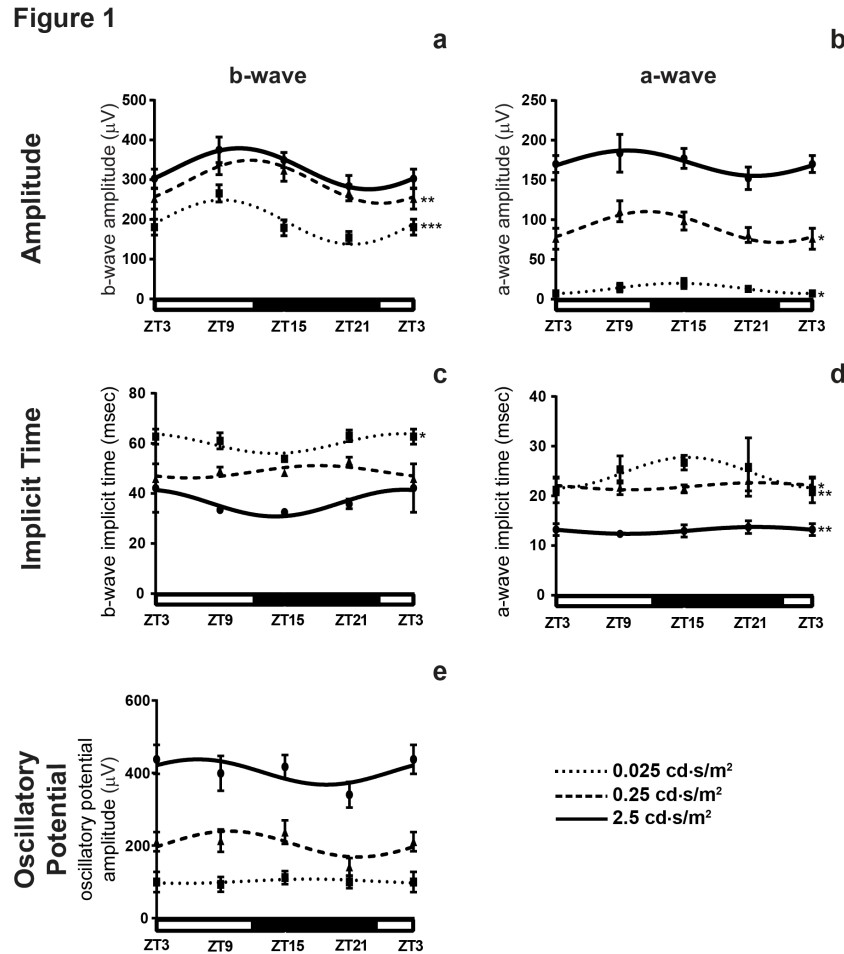
To study whether ERG exhibits a diurnal rhythm in C57BL6/J mice, we performed ERG assessments at 6 hr intervals. The amplitude of an ERG b-wave was highest in between ZT-9 and ZT-15 at all the light intensities tested. The zero amplitude test revealed that the ERG response exhibits a diurnal rhythm for ERG b-wave (Figure 1a) at 0.025 cd.s/m<sup>2</sup> and 0.25 cd.s/m<sup>2</sup>. The ERG a-wave showed a peak response between ZT-9 and ZT-15 and a circadian rhythm (Figure 1b) at the lower light intensities. The implicit time exhibited a diurnal rhythm at 0.025 cd.s/m<sup>2</sup> for b-wave, however, the other two intensities (0.25 and 2.5 cd.s/m<sup>2</sup>) did not show a diurnal rhythm, (Figure 1c, d). The implicit time for a-wave exhibited a diurnal rhythm at all the light intensities. The oscillatory potential did not show a diurnal rhythm at all the light intensities, with a peak at ZT-9 (Figure 1e).

### *Diabetes Alters ERG Rhythm in db/db Mice Under Regular Light and Dark Conditions*

We assessed the ERG rhythm of db/m (controls) and db/db mice (type 2 diabetes) at time points similar to the C57BL6/J mice under 12 hr light and 12 hr dark conditions. The ERG recordings were conducted at 2, 4, and 6 months of diabetes.

At two months' time point, the peak of ERG b-wave was observed between ZT-3 and ZT-9 while at the lowest point between ZT-15 and ZT-21 in db/m mice. For db/db mice, the peak response was at ZT-15 for 0.025 and 2.5 cd.s/m<sup>2</sup> light intensities. However, for 0.25 cd.s/m<sup>2</sup> light intensity, the peak was in between ZT-9 and ZT-15. The zero amplitude test revealed a diurnal rhythm for all light intensities for db/m mice. Overall, there was an increase in ERG b-wave amplitude for db/db mice at higher intensities, except for the lower intensity of light, 0.025 cd.s/m<sup>2</sup>. However, there was no significant diurnal rhythm of ERG b-wave for db/db mice at all the intensities. The b-wave implicit time exhibited a diurnal rhythm for db/m mice at 2.5 cd.s/m<sup>2</sup> and db/db mice for 0.025, 0.25 cd.s/m<sup>2</sup>. While the oscillatory potential exhibited a diurnal rhythm only at a higher intensity for db/m mice, none of the other groups demonstrated a diurnal rhythm for oscillatory potential. The a-wave demonstrated a peak at ZT-9 for 0.025 and 0.25 cd.s/m<sup>2</sup> intensity in db/m mice; however, the peak for 2.5 cd.s/m<sup>2</sup> was in between ZT-3 and ZT-9. In db/db mice, the peak for a-wave was at ZT-15 for 0.025 and 0.25 cd.s/m<sup>2</sup>, and at ZT-9 for 2.5 cd.s/m<sup>2</sup> light intensity. The amplitude of a-wave was higher in db/db mice when compared to db/m mice. The a-wave demonstrated a diurnal rhythm for 0.25 cd.s/m<sup>2</sup> and 2.5 cd.s/m<sup>2</sup>, however, diabetic mice did not demonstrate a circadian rhythm at all the intensities that were tested. The implicit time for a-wave showed a diurnal rhythm for db/m mice at 0.25 cd.s/m<sup>2</sup> and 2.5 cd.s/m<sup>2</sup>, however, for db/db mice lacked diurnal rhythm at all the intensities. (Figure 2).

At 4 months, there was a shift in ERG peak to ZT-21 for intensities 0.025 and 0.25 cd.s/m<sup>2</sup>, however, for the light intensity of 2.5, the peak was similar to a two-month time point for db/m mice. In db/db mice, the peak response was earlier at ZT-9 in comparison to a 2-month time point at 0.025 cd.s/m<sup>2</sup> intensity. Overall, there was an increase in the amplitude for b-wave in db/db mice as compared to db/m mice. By 4 months, both groups lost a diurnal rhythm of ERG response. The implicit time for b-wave showed a diurnal rhythm at all intensities for db/db mice, however, this rhythm was only observed at 2.5 cd.s/m<sup>2</sup> for db/m mice. The oscillatory potentials demonstrated a diurnal rhythm in all groups and at all intensities except 2.5 cd.s/m<sup>2</sup> for db/m mice. There was a shift in peak response for a-wave for both db/m and db/db mice at 4 months when compared to a two-month time point. The peak response for 0.025 cd.s/m<sup>2</sup> shifted earlier to ZT-9, while for other two intensities the peak response was moved in between ZT-15 and ZT-21. The db/db mice



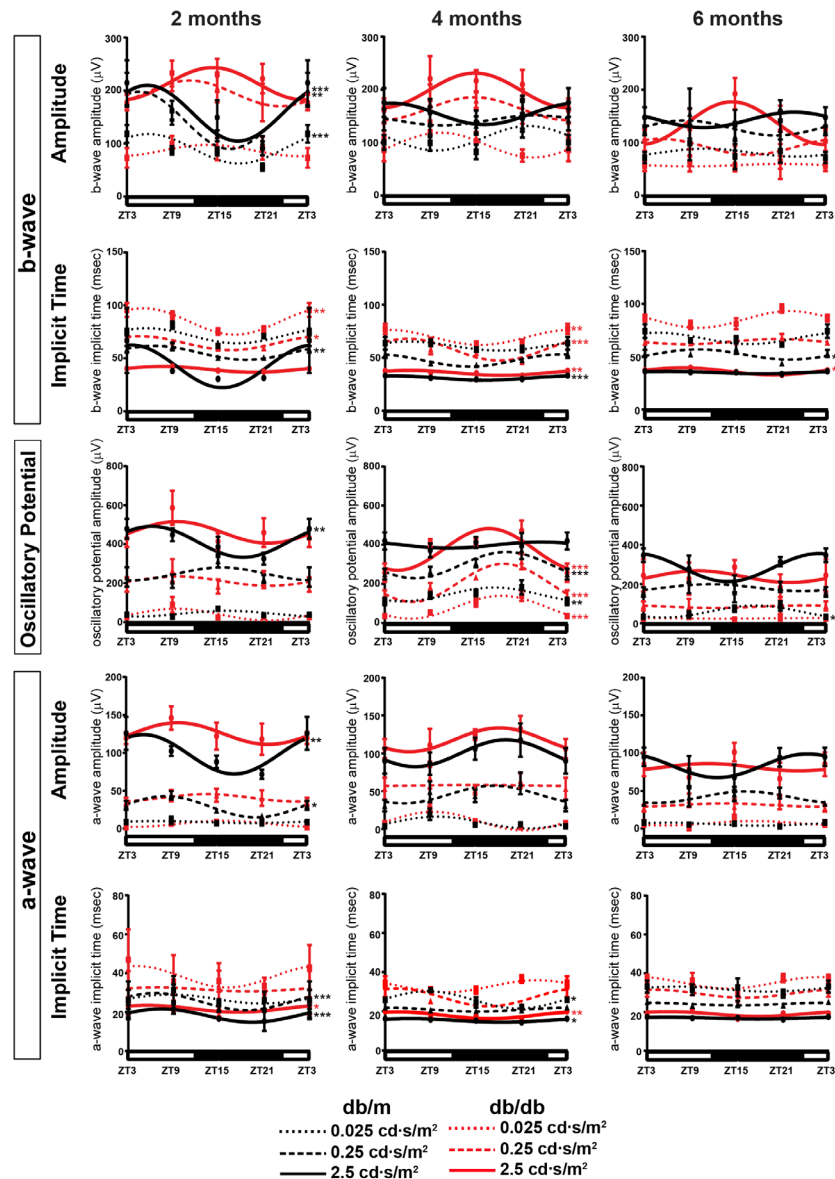
**Figure 1. Diurnal ERG rhythm in C57BL6/J mice.** The ERG recordings were performed at 6 hr time intervals. Each time point for ERG assessment is expressed as Zeitgeber (ZT) time, which corresponds to 12 hr light-dark cycles with lights being turned on at ZT-0 and off at ZT-12. A scatter plot with a curve fitting for a raw ERG data of (a) b-wave and (b) a-wave, implicit time of (c) ERG b-wave (d) a-wave and (e) an oscillatory potential for ERG,  $n=5$  \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .

followed a similar pattern. There was an overall increase in a-wave amplitude in db/db mice. None of the groups exhibited a diurnal rhythm for ERG a-wave. The a-wave implicit time did show a diurnal rhythm at higher intensities for both db/m and db/db mice and at the lowest intensity of db/m mice (Figure 2).

As diabetes progressed to 6 months and db/m mice aged, there was a progressive decline in ERG amplitude compared to 2- and 4-months' time points. The peak response of b-wave in db/m mice was at ZT-9 for 0.025, 0.25 cd.s/m<sup>2</sup> intensity, while at ZT-21 for at 2.5 cd.s/m<sup>2</sup>. The ERG b-wave peaked at ZT-3 and ZT-15 for 0.25 and 2.5 cd.s/m<sup>2</sup> light intensity respectively in db/db mice. The ERG amplitude was lower for db/db mice at lower intensities, however, was increased at 2.5 cd.s/m<sup>2</sup> when compared to db/m mice. There was no diurnal rhythm for both groups at all intensities for the b-wave. The im-

PLICIT time for b-wave exhibited a diurnal rhythm at 0.25 cd.s/m<sup>2</sup> for db/m mice and 2.5 cd.s/m<sup>2</sup> intensity for db/db mice. The oscillatory potential showed a diurnal rhythm only at 0.025 cd.s/m<sup>2</sup> for db/m mice. The a-wave peaked in between ZT-15 and ZT-21 for lower intensities in db/m mice, while at the higher intensity the peak response was observed at ZT-3. The db/db mice did not demonstrate an obvious peak, the highest response was observed between ZT-9 and ZT-15. There was an overall decrease in ERG a-wave amplitude and loss of diurnal rhythmicity. The implicit time of a-wave did not demonstrate a diurnal rhythm for both db/m and db/db mice at all the light intensities tested (Figure 2).

Figure 2



**Figure 2. ERG rhythm in db/db mice under regular light-dark conditions.** ERG recordings were performed at 2, 4 and 6 months of diabetes at different time points. Scatter plot showing ERG b-wave amplitude, implicit time, oscillatory potential, and ERG a-wave amplitude and implicit time. Each data point represents average value; the connecting line corresponds to fitted curve line for the respective group (2-months db/m n=5, db/db n=5; 4-months db/m n=5, db/db n=7; 6-months db/m n=4, db/db n=5), \*p<0.05, \*\* p<0.01, \*\*\* p<0.001.

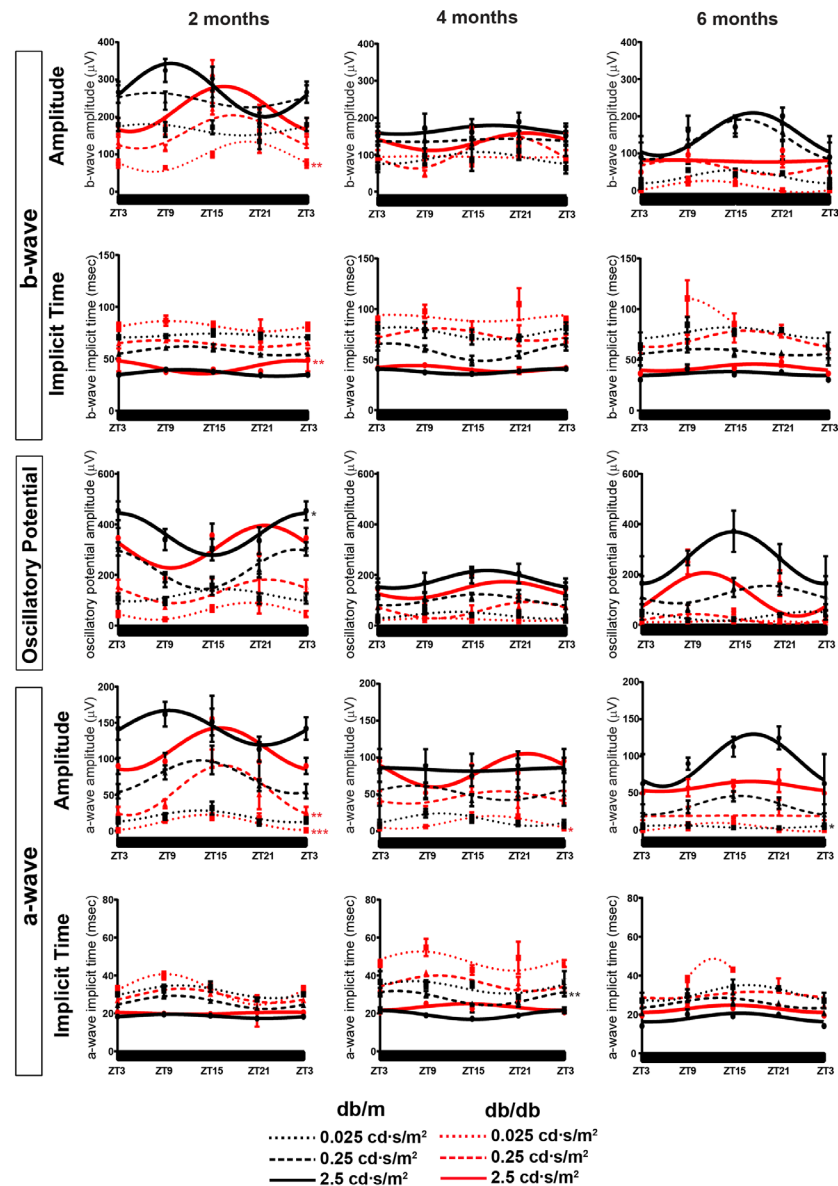
### *Intrinsic Circadian Rhythm of ERG is Decreased in Diabetes*

Next, we tested the intrinsic rhythm of retinal ERG in mice with different duration of diabetes. The animals were maintained in constant dark for two weeks to nullify the effect of external light stimuli on the biological clock.

At 2 months' time point, the b-wave exhibited a peak response at ZT-9 in db/m mice, however, with diabetes, this peak shifted in between ZT-15 and ZT-21. The zero

amplitude test demonstrated a circadian rhythm only at 0.025 cd.s/m<sup>2</sup> intensity for db/m mice, none of the other groups demonstrated a diurnal rhythm. There was an overall decrease in amplitude of ERG b-wave in db/db mice when compared to db/m mice. The implicit time of ERG b-wave exhibited a circadian rhythm at 2.5 cd.s/m<sup>2</sup>, for db/db mice, however, none of the other groups demonstrated a circadian rhythm. The oscillatory potentials showed a circadian rhythm only at 2.5 cd.s/m<sup>2</sup> for

Figure 3

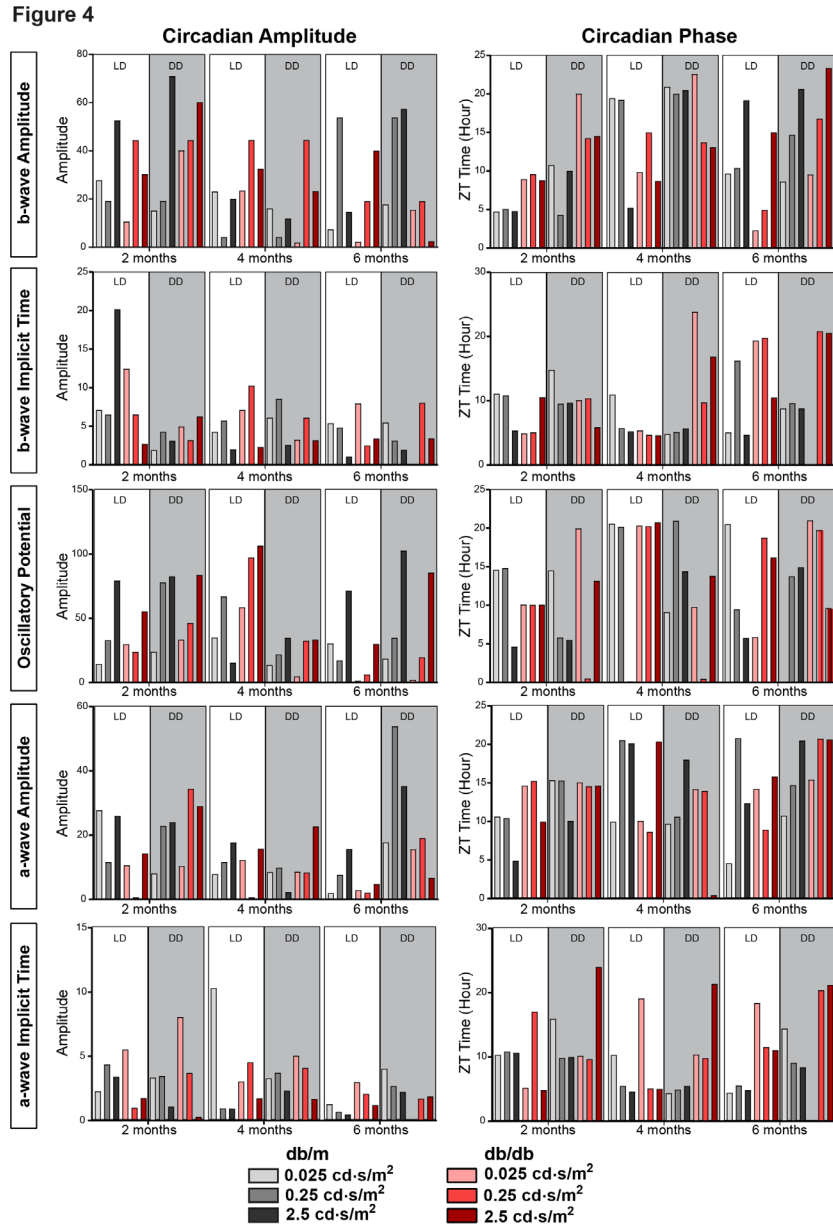


**Figure 3. An intrinsic rhythm of ERG is decreased in db/db mice.** The animals were maintained in the constant dark to nullify the effect of extrinsic light stimuli and the ERG recordings were performed at different time intervals at 2, 4, and 6 months of duration of diabetes. Scatter plot showing ERG amplitude and implicit time for b-wave and a-wave, and an oscillatory potential for ERG. Each data point showing an average value, the connecting line corresponds to fitted curve, (2-months db/m n=9, db/db n=9; 4-months db/m n=5, db/db n=7; 6-months db/m n=5, db/db n=5), \*p<0.05, \*\*p<0.01, \*\*\* p<0.001.

db/m mice, and was lower for db/db mice and did not exhibit any rhythm. The peak of a-wave amplitude was observed between ZT-15 and ZT-21 for db/db mice. There was an overall decrease in the amplitude of a-wave in db/db mice when compared to db/m animals. The a-wave implicit time did not show a circadian rhythm in both db/m and db/db animals (Figure 3).

At 4 months' time point there was a gradual decrease in ERG b-wave amplitude as compared to two months'

time point. The peak time of amplitude was shifted in between ZT-15 and ZT-21 for db/m animals. The db/db mice showed an overall decrease in ERG amplitude. None of the groups demonstrated circadian rhythmicity. The b-wave implicit time showed an overall increase in db/db mice in comparison to the db/m animals without the presence of circadian rhythm. The oscillatory potential did not show a circadian rhythm at all the light intensities and was lower for db/db animals. The a-wave amplitude



**Figure 4. Circadian amplitude and phase of ERG parameters.** Bar chart showing circadian amplitude and phase for b-wave, a-wave amplitude, oscillatory potential and implicit times. (LD condition: 2-months db/m n=5, db/db n=5; 4-months db/m n=5, db/db n=7; 6-months db/m n=4, db/db n=5; DD conditions: 2-months db/m n=9, db/db n=9; 4-months db/m n=5, db/db n=7; 6-months db/m n=5, db/db n=5).

showed a peak response at ZT-9 in db/m mice for 0.025 and 0.25 cd.s/m<sup>2</sup>. In db/db mice the peak of ERG a-wave was observed in between ZT15 and ZT-21. There was no circadian rhythm for ERG a-wave. The a-wave implicit time demonstrated an increase in db/db mice with a circadian rhythm at 0.25 cd.s/m<sup>2</sup> (Figure 3).

As diabetes progressed to 6 months a further decrease in ERG b-wave amplitude was observed as compared to 2 and 4 months' time points. The peak of ERG b-wave was observed in between ZT-15 and ZT-21 for db/m mice. In

db/db mice, a decrease in ERG amplitude with a loss of circadian rhythmicity was observed at all intensities. The b-wave implicit time and oscillatory potentials of both db/m and db/db mice lacked circadian rhythmicity. The a-wave exhibited a peak response in between ZT-9 and ZT-15 for db/m mice at lower light intensities, which was shifted to in between ZT-15 and ZT-21 as the light intensity is increased to 2.5 cd.s/m<sup>2</sup>. The a-wave at 0.025 cd.s/m<sup>2</sup> showed a circadian rhythm. The peak increase in ERG amplitude for db/db mice was observed between ZT-15

and ZT-21. The implicit time for ERG a-wave did not show a circadian rhythm at any of the light intensities in db/m and db/db mice. (Figure 3).

In order to further determine an effect of genotype (db/m vs db/db), light conditions (LD vs DD) and duration of diabetes, we determined amplitude and phase for each of the parameters tested from the curve fitting data obtained from GraphPad Prism. For the b-wave the circadian phase for the duration of diabetes and light conditions differed significantly; there was also a difference between diabetes duration and genotype as well as diabetes duration and light conditions. The phase of b-wave implicit time also differed significantly at the above parameters as well as all three conditions (Appendix A Table 1). There was no statistical difference for circadian amplitude for b-wave. Overall, there was a trend towards an increase in circadian phase at 2, 6 months, however not for 4 months' time point (Figure 4). While the a-wave circadian amplitude did not differ significantly across the different comparisons, the circadian phase differed significantly for the duration of diabetes as well as in different comparisons between the groups. The a-wave implicit time for circadian phase differed significantly across all the statistical comparisons (Appendix A Table 1). There was a general trend towards a decrease in circadian amplitude for a-wave, however the data was inconclusive as some of the frequencies (0.025 cd.s/m<sup>2</sup> and 0.25 cd.s/m<sup>2</sup> at 2 months and 0.25 cd.s/m<sup>2</sup> at 4 months) did show some increase in circadian amplitude for a-wave. There was an increase in circadian phase at 4 months under DD condition and at 6 months for both LD and DD conditions for db/db mice (Figure 4). The oscillatory potential for circadian amplitude for the duration of diabetes across lighting conditions did differ significantly as well as there was a significant difference in circadian phase in all the statistical comparisons (Appendix A Table 1). By 6 months of diabetes, there was a decrease in circadian amplitude of oscillatory potential for db/db mice under both LD and DD conditions, the changes in a circadian phase were inconclusive (Figure 4).

To further understand the changes in a circadian rhythm across the genotype, duration of diabetes, lighting conditions and individual intensities, we developed a robust mathematical model in R and performed a zero-amplitude test followed by a log-likelihood test for an individual subgroup that passed the zero-amplitude test. The significance of the difference in circadian parameters (mesor; amplitude; phase) was evaluated by 500 times model residuals resampling and the p-value was obtained from the empirical distribution. Out of all the comparisons, only the a-wave implicit time differed significantly for 2.5 cd.s/m<sup>2</sup> between 2 and 4 months, (Appendix A Table 2).

## DISCUSSION

Our study demonstrates that C57BL6/J mice exhibit a diurnal rhythm of scotopic ERG, for both a- and b-wave and the implicit time. Overall, the pattern of ERG wave was altered in db/db mice, there was a complete loss of diurnal rhythm from 2 months of diabetes for both ERG b- and a-wave in db/db mice. Increase in acellular capillaries is a pathologic hallmark of DR. In db/db mice, the acellular capillaries are evident after 4 months of diabetes [22,27]. The ERG abnormalities are also prevalent between 4 and 6 months of diabetes [28]. Therefore, we predict that in our studies the changes in ERG parameters, such as ERG amplitude and OPs, will correlate with the numbers of acellular capillary numbers. However, it was not studied in this manuscript.

One of the key findings of our study using C57BL6/J mice was defining the time points for a peak and trough response. Overall, the greatest response of ERG amplitude was observed between ZT-9 and ZT-15, and at the lowest level at ZT-21. While the previous studies performed in the mouse retina, demonstrated that the ERG b-wave amplitude is highest during the middle of the day (*i.e.* resting phase for nocturnal animals) and at the lowest at midnight (activity phase) [25], our study for the first time performed ERG assessments at four different time intervals to define the time-point for a peak ERG response. This observation warrants that timing of ERG assessment is critical for interpretation of the data and maintaining consistency of timing is critical for ERG studies. ERG studies on humans exhibit a similar response to some extent, where there is an evident increase (20 to 40 percent) in ERG during night time as compared to the daytime [23,29], and an increase in ERG implicit time earlier in the morning [30]. However, some studies observed conflicting findings where ERG amplitude was at the highest at 12 PM (activity phase) and lowest at 6 AM (resting phase) in humans [24]. Previous studies demonstrate that only photopic ERG is under circadian control, whereas a circadian clock does not regulate the scotopic ERG [31]. Moreover, deficiency of melatonin in C57BL6/J mice [32] might be attributed to lack of clock regulation of scotopic ERG [33]. While the above aspects were beyond the scope of this study, our study highlighted the presence of diurnal rhythm of ERG amplitude in C57BL6/J mice. It is noteworthy that retinal rhythms are predominantly mediated by local oscillations, and SCN clock cannot compensate for the loss of local retinal oscillators. Storch *et al.* further reinforced the concept of the local retinal clock by demonstrating that conditional deletion of a clock gene *Bmal1* in SCN does not affect ERG rhythm [8].

We demonstrate that ERG b-wave was altered in diabetic retinas. While the diurnal rhythm of ERG was



maintained in non-diabetic mice, there were no diurnal rhythms for ERG in diabetic mice. Our data are consistent with previous studies, which report similar changes in ERG response for diabetes [34]. Furthermore, our studies, for the first time, traced the circadian rhythm of ERG at different time intervals, over the course of 6 months of diabetes. These findings demonstrate a progressive decline in the circadian rhythm of ERG and a substantial loss of the circadian rhythm by 6 months of diabetes in db/db mice. The scotopic retinal ERG b-wave is mainly attributed to rod bipolar cells [35], while the late radial currents around the bipolar cells are contributed by the Müller cells, therefore our studies suggest that the bipolar and Müller cell function is highest during the middle of the day as opposed to other times. The a-wave of the ERG indicates the response of photoreceptors [36], in our studies, the amplitude of a-wave was higher earlier in diabetes, which became dysrhythmic and declined in diabetes suggesting a progressive decline in photoreceptor function in diabetes. The OPs are four to six wavelets on the rising phase of the ERG b-wave. While the OPs arise from the inner plexiform layer, it is unclear which cells of the retina are exactly responsible for the OPs, some studies suggest that amacrine cells are responsible for OPs [37]. The decrease in OPs at lower frequencies is an indication of a decrease in amacrine cell function, however, further studies are necessary to support this assertion.

A Zeitgeber implies to the prominent signal impacting SCN and the light is the most prominent zeitgeber. While light does not generate the periodicity of circadian rhythms, it plays an important role in setting the timing of circadian rhythms related to gene expression, activity, and behaviors. While the current study design for DD conditions did not allow us to precisely define the exact time of ERG response, the amplitude of the ERG wave was consistently downregulated progressively with an increase in the duration of diabetes, and the intrinsic circadian rhythm of ERG was dramatically reduced after 6 months of diabetes. The pathologic hallmark of DR, an increase in acellular capillaries are evident after 4 months, however, neuronal and glial dysfunction precedes an increase in acellular capillaries [5-7]. While the present study design did not allow us to study the underlying mechanisms for alteration in an ERG response for diabetic mice, our studies provide novel insights in understanding the pathogenesis of DR by demonstrating that intrinsic circadian rhythms of the retinal electrical function are lost in diabetes. Future studies using genetically knock out mice for clock genes with hyperglycemia would further help in understanding the cause and effect relations due to diabetes and circadian rhythms.

In conclusion, our study unravels that T2D alters the ERG rhythm. The decrease in intrinsic ERG rhythm is progressive and long-term diabetes substantially reduces

circadian rhythm of an ERG.

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Appendix A Table 1

**b-wave amplitude**

Source of Variation	Circadian Amplitude				Circadian Phase							
	0.025 cd.s/m <sup>2</sup>		0.25 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>		0.025 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>			
	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value		
Diabetes duration	F (2, 103) = 0.8919	P=0.4130	F (2, 130) = 0.3856	P=0.6808	F (2, 115) = 2.781	P=0.0662	F (2, 103) = 48.28	P<0.0001	F (2, 105) = 53.02	P<0.0001	F (2, 115) = 27.83	P<0.0001
Light condition	F (1, 103) = 0.06591	P=0.7979	F (1, 130) = 0.000	P>0.9999	F (1, 115) = 0.2314	P=0.6314	F (1, 103) = 51.82	P<0.0001	F (1, 105) = 18.95	P<0.0001	F (1, 115) = 36.73	P<0.0001
Genotype	F (1, 103) = 0.07411	P=0.7860	F (1, 130) = 0.9293	P=0.3368	F (1, 115) = 0.2689	P=0.6051	F (1, 103) = 0.026	P=0.8712	F (1, 105) = 0.020	P=0.8863	F (1, 115) = 0.229	P=0.6332
Diabetes duration x Light condition	F (2, 103) = 1.070	P=0.3467	F (2, 130) = 0.000	P>0.9999	F (2, 115) = 0.7016	P=0.4979	F (2, 103) = 3.189	P=0.0453	F (2, 105) = 9.395	P=0.0002	F (2, 115) = 1.940	P=0.1484
Diabetes duration x Genotype	F (2, 103) = 0.2046	P=0.8153	F (2, 130) = 3.846	P=0.0238	F (2, 115) = 0.5671	P=0.5687	F (2, 103) = 19.29	P<0.0001	F (2, 105) = 30.09	P<0.0001	F (2, 115) = 3.410	P=0.0364
Light condition x Genotype	F (1, 103) = 0.4115	P=0.5226	F (1, 130) = 0.000	P>0.9999	F (1, 115) = 0.8793	P=0.3504	F (1, 103) = 22.23	P<0.0001	F (1, 105) = 5.844	P=0.0174	F (1, 115) = 0.277	P=0.5995
Diabetes duration x Light condition x Genotype	F (2, 103) = 1.387	P=0.2545	F (2, 130) = 0.000	P>0.9999	F (2, 115) = 1.223	P=0.2983	F (2, 103) = 1.324	P=0.2706	F (2, 105) = 3.608	P=0.0305	F (2, 115) = 5.018	P=0.0081

**b-wave implicit time**

Source of Variation	Circadian Amplitude				Circadian Phase							
	0.025 cd.s/m <sup>2</sup>		0.25 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>		0.025 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>			
	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value		
Diabetes duration	F (2, 106) = 1.405	P=0.2499	F (2, 122) = 1.840	P=0.1632	F (2, 110) = 0.6375	P=0.5306	F (2, 106) = 22.32	P<0.0001	F (2, 122) = 0.489	P=0.6143	F (2, 110) = 22.87	P<0.0001
Light condition	F (1, 106) = 0.1018	P=0.7503	F (1, 122) = 10.35	P=0.0017	F (1, 110) = 0.3331	P=0.5650	F (1, 106) = 39.75	P<0.0001	F (1, 122) = 0.140	P=0.7088	F (1, 110) = 0.017	P=0.8958
Genotype	F (1, 106) = 0.4155	P=0.5206	F (1, 122) = 2.164	P=0.1438	F (1, 110) = 0.3871	P=0.5351	F (1, 106) = 76.32	P<0.0001	F (1, 122) = 0.545	P=0.4616	F (1, 110) = 0.534	P=0.4662
Diabetes duration x Light condition	F (2, 106) = 5.505	P=0.0053	F (2, 122) = 2.070	P=0.1306	F (2, 110) = 0.3463	P=0.7081	F (2, 106) = 1.220	P=0.2994	F (2, 122) = 0.531	P=0.5889	F (2, 110) = 35.87	P<0.0001
Diabetes duration x Genotype	F (2, 106) = 1.168	P=0.3150	F (2, 122) = 0.9081	P=0.4060	F (2, 110) = 1.143	P=0.3225	F (2, 106) = 13.29	P<0.0001	F (2, 122) = 0.846	P=0.4316	F (2, 110) = 20.75	P<0.0001
Light condition x Genotype	F (1, 106) = 0.4823	P=0.4889	F (1, 122) = 0.006925	P=0.9338	F (1, 110) = 0.2316	P=0.6313	F (1, 106) = 3.468	P=0.0653	F (1, 122) = 3.924	P=0.0498	F (1, 110) = 14.83	P=0.0002
Diabetes duration x Light condition x Genotype	F (2, 106) = 2.045	P=0.1344	F (2, 122) = 1.478	P=0.2321	F (2, 110) = 0.6245	P=0.5374	F (2, 106) = 12.47	P<0.0001	F (2, 122) = 2.885	P=0.0597	F (2, 110) = 8.709	P=0.0003

**a-wave amplitude**

Source of Variation	Circadian Amplitude				Circadian Phase							
	0.025 cd.s/m <sup>2</sup>		0.25 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>		0.025 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>			
	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value		
Diabetes duration	F (2, 106) = 1.405	P=0.2499	F (2, 122) = 1.840	P=0.1632	F (2, 110) = 0.6375	P=0.5306	F (2, 106) = 22.32	P<0.0001	F (2, 122) = 0.489	P=0.6143	F (2, 110) = 22.87	P<0.0001
Light condition	F (1, 106) = 0.1018	P=0.7503	F (1, 122) = 10.35	P=0.0017	F (1, 110) = 0.3331	P=0.5650	F (1, 106) = 39.75	P<0.0001	F (1, 122) = 0.140	P=0.7088	F (1, 110) = 0.017	P=0.8958
Genotype	F (1, 106) = 0.4155	P=0.5206	F (1, 122) = 2.164	P=0.1438	F (1, 110) = 0.3871	P=0.5351	F (1, 106) = 76.32	P<0.0001	F (1, 122) = 0.545	P=0.4616	F (1, 110) = 0.534	P=0.4662
Diabetes duration x Light condition	F (2, 106) = 5.505	P=0.0053	F (2, 122) = 2.070	P=0.1306	F (2, 110) = 0.3463	P=0.7081	F (2, 106) = 1.220	P=0.2994	F (2, 122) = 0.531	P=0.5889	F (2, 110) = 35.87	P<0.0001
Diabetes duration x Genotype	F (2, 106) = 1.168	P=0.3150	F (2, 122) = 0.9081	P=0.4060	F (2, 110) = 1.143	P=0.3225	F (2, 106) = 13.29	P<0.0001	F (2, 122) = 0.846	P=0.4316	F (2, 110) = 20.75	P<0.0001
Light condition x Genotype	F (1, 106) = 0.4823	P=0.4889	F (1, 122) = 0.006925	P=0.9338	F (1, 110) = 0.2316	P=0.6313	F (1, 106) = 3.468	P=0.0653	F (1, 122) = 3.924	P=0.0498	F (1, 110) = 14.83	P=0.0002
Diabetes duration x Light condition x Genotype	F (2, 106) = 2.045	P=0.1344	F (2, 122) = 1.478	P=0.2321	F (2, 110) = 0.6245	P=0.5374	F (2, 106) = 12.47	P<0.0001	F (2, 122) = 2.885	P=0.0597	F (2, 110) = 8.709	P=0.0003

**a-wave implicit time**

Source of Variation	Circadian Amplitude				Circadian Phase							
	0.025 cd.s/m <sup>2</sup>		0.25 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>		0.025 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>			
	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value		
Diabetes duration	F (2, 116) = 1.291	P=0.2790	F (2, 112) = 0.0865	P=0.9171	F (2, 112) = 0.0865	P=0.9171	F (2, 108) = 81.56	P<0.0001	F (2, 114) = 9.529	P=0.0001	F (2, 114) = 9.529	P=0.0001
Light condition	F (1, 116) = 1.413	P=0.2370	F (1, 112) = 0.00021	P=0.9884	F (1, 112) = 0.00021	P=0.9884	F (1, 108) = 9.970	P=0.0021	F (1, 114) = 155.0	P<0.0001	F (1, 114) = 155.0	P<0.0001
Genotype	F (1, 116) = 0.07126	P=0.7900	F (1, 112) = 0.5778	P=0.4488	F (1, 112) = 0.5778	P=0.4488	F (1, 108) = 114.8	P<0.0001	F (1, 114) = 120.7	P<0.0001	F (1, 114) = 120.7	P<0.0001
Diabetes duration x Light condition	F (2, 116) = 0.01592	P=0.9842	F (2, 112) = 5.938	P=0.0035	F (2, 112) = 5.938	P=0.0035	F (2, 108) = 46.21	P<0.0001	F (2, 114) = 1.068	P=0.3472	F (2, 114) = 1.068	P=0.3472
Diabetes duration x Genotype	F (2, 116) = 1.843	P=0.1630	F (2, 112) = 1.367	P=0.2590	F (2, 112) = 1.367	P=0.2590	F (2, 108) = 18.79	P<0.0001	F (2, 114) = 6.632	P=0.0019	F (2, 114) = 6.632	P=0.0019
Light condition x Genotype	F (1, 116) = 0.1636	P=0.6866	F (1, 112) = 0.4475	P=0.5049	F (1, 112) = 0.4475	P=0.5049	F (1, 108) = 2.550	P=0.1132	F (1, 114) = 111.2	P<0.0001	F (1, 114) = 111.2	P<0.0001
Diabetes duration x Light condition x Genotype	F (2, 116) = 2.054	P=0.1329	F (2, 112) = 0.8775	P=0.4186	F (2, 112) = 0.8775	P=0.4186	F (2, 108) = 22.39	P<0.0001	F (2, 114) = 8.187	P=0.0005	F (2, 114) = 8.187	P=0.0005

**Oscillatory potential**

Source of Variation	Circadian Amplitude				Circadian Phase							
	0.025 cd.s/m <sup>2</sup>		0.25 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>		0.025 cd.s/m <sup>2</sup>		2.5 cd.s/m <sup>2</sup>			
	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value		
Diabetes duration	F (2, 105) = 1.277	P=0.2831	F (2, 115) = 2.042	P=0.1344	F (2, 110) = 0.7743	P=0.4635	F (2, 107) = 8.333	P=0.0004	F (2, 122) = 28.85	P<0.0001	F (2, 129) = 40.74	P<0.0001
Light condition	F (1, 105) = 2.675	P=0.1049	F (1, 115) = 0.01964	P=0.8888	F (1, 110) = 0.2753	P=0.6009	F (1, 107) = 20.40	P<0.0001	F (1, 122) = 27.66	P<0.0001	F (1, 129) = 28.39	P<0.0001
Genotype	F (1, 105) = 0.01592	P=0.8997	F (1, 115) = 0.09851	P=0.7542	F (1, 110) = 0.00444	P=0.9470	F (1, 107) = 3.976	P=0.0487	F (1, 122) = 6.119	P=0.0148	F (1, 129) = 214.3	P<0.0001
Diabetes duration x Light condition	F (2, 105) = 3.468	P=0.0348	F (2, 115) = 4.155	P=0.0181	F (2, 110) = 0.9225	P=0.4006	F (2, 107) = 59.42	P<0.0001	F (2, 122) = 12.63	P<0.0001	F (2, 129) = 2.351	P=0.0993
Diabetes duration x Genotype	F (2, 105) = 1.909	P=0.1534	F (2, 115) = 0.8899	P=0.4135	F (2, 110) = 1.143	P=0.3226	F (2, 107) = 1.863	P=0.1602	F (2, 122) = 22.96	P<0.0001	F (2, 129) = 20.28	P<0.0001
Light condition x Genotype	F (1, 105) = 0.3200	P=0.5728	F (1, 115) = 0.3084	P=0.5797	F (1, 110) = 0.1214	P=0.7282	F (1, 107) = 142.7	P<0.0001	F (1, 122) = 15.82	P<0.0001	F (1, 129) = 177.0	P<0.0001
Diabetes duration x Light condition x Genotype	F (2, 105) = 0.6871	P=0.5053	F (2, 115) = 0.03918	P=0.9616	F (2, 110) = 0.9309	P=0.3973	F (2, 107) = 54.96	P<0.0001	F (2, 122) = 11.20	P<0.0001	F (2, 129) = 86.40	P<0.0001

Appendix A Table 2

<b>b-wave amplitude</b>	
	P value
db/m_0.025 cd-s/m <sup>2</sup> _2months_LD	0.00038
db/m_0.25 cd-s/m <sup>2</sup> _2months_LD	0.0004
db/m_2.5 cd-s/m <sup>2</sup> _2months_LD	0.00373
db/db_0.025 cd-s/m <sup>2</sup> _2months_DD	0.00588

Comparison groups	Amplitude		Phase		Group mean	
	P value	difference	P value	difference	P value	difference
db/m_0.025 cd-s/m <sup>2</sup> _2months_LD vs. db/db_0.025 cd-s/m <sup>2</sup> _2months_DD	0.168	-15.38205	0.136	3.4967	0.48927	-6.64564

<b>b-wave implicit time</b>	
	P value
db/m_0.25 cd-s/m <sup>2</sup> _2months_LD	0.00816
db/m_2.5 cd-s/m <sup>2</sup> _4months_LD	0.00092
db/m_0.25 cd-s/m <sup>2</sup> _6months_LD	0.0284
db/db_0.025 cd-s/m <sup>2</sup> _2months_LD	0.00125
db/db_0.25 cd-s/m <sup>2</sup> _2months_LD	0.0145
db/db_0.025 cd-s/m <sup>2</sup> _4months_LD	0.00767
db/db_0.25 cd-s/m <sup>2</sup> _4months_LD	0
db/db_2.5 cd-s/m <sup>2</sup> _4months_LD	0.00487
db/db_2.5 cd-s/m <sup>2</sup> _6months_LD	0.00377
db/db_2.5 cd-s/m <sup>2</sup> _6months_DD	0.04389

Comparison groups	Amplitude		Phase		Group mean	
	P value	difference	P value	difference	P value	difference
db/m_2.5 cd-s/m <sup>2</sup> _4months_LD vs. db/db_2.5 cd-s/m <sup>2</sup> _4months_LD	0.762	0.31143	0.272	-0.64412	0	4.83522
db/db_0.025 cd-s/m <sup>2</sup> _2months_LD vs. db/db_0.25 cd-s/m <sup>2</sup> _4months_LD	0.11	-5.64191	0.308	0.44967	0	-15.77774
db/db_0.25 cd-s/m <sup>2</sup> _2months_LD vs. db/db_0.25 cd-s/m <sup>2</sup> _4months_LD	0.33	3.13999	0.432	-0.39617	0.00337	-7.0884
db/db_2.5 cd-s/m <sup>2</sup> _4months_LD vs. db/db_2.5 cd-s/m <sup>2</sup> _6months_DD	0.28	1.26434	0.48	-0.35188	0.74649	0.4595

<b>a-wave amplitude</b>	
	P value
db/m_0.25 cd-s/m <sup>2</sup> _2months_LD	0.0101
db/m_2.5 cd-s/m <sup>2</sup> _2months_LD	0.00168
db/m_0.025 cd-s/m <sup>2</sup> _6months_DD	0.01716
db/db_0.025 cd-s/m <sup>2</sup> _2months_DD	0.0004
db/db_0.25 cd-s/m <sup>2</sup> _2months_DD	0.00171
db/db_0.025 cd-s/m <sup>2</sup> _4months_DD	0.03098

Comparison groups	Amplitude		Phase		Group mean	
	P value	difference	P value	difference	P value	difference
db/db_0.025 cd-s/m <sup>2</sup> _2months_DD vs. db/db_0.025 cd-s/m <sup>2</sup> _4months_DD	0.736	-1.31091	0.108	-0.855	0.52431	-0.13927

**a-wave implicit time**

	P value
db/m_0.25 cd·s/m <sup>2</sup> _2months_LD	0.00028
db/m_2.5 cd·s/m <sup>2</sup> _2months_LD	0.00053
db/m_0.025 cd·s/m <sup>2</sup> _4months_LD	0.03127
db/m_2.5 cd·s/m <sup>2</sup> _4months_LD	0.03055
db/m_0.25 cd·s/m <sup>2</sup> _4months_DD	0.00469
db/db_2.5 cd·s/m <sup>2</sup> _2months_LD	0.04209
db/db_2.5 cd·s/m <sup>2</sup> _4months_LD	0.00785

Comparison groups	Amplitude		Phase		Group mean	
	P value	difference	P value	difference	P value	difference
db/m_2.5 cd·s/m <sup>2</sup> _2months_LD vs. db/m_2.5 cd·s/m <sup>2</sup> _4months_LD	<b>0.008</b>	-2.58087	0.762	0.12825	0.00001	-2.97329
db/m_2.5 cd·s/m <sup>2</sup> _2months_LD vs. db/db_2.5 cd·s/m <sup>2</sup> _2months_LD	0.102	-1.685	0.224	0.47793	0.00001	3.49772
db/m_2.5 cd·s/m <sup>2</sup> _4months_LD vs. db/db_2.5 cd·s/m <sup>2</sup> _4months_LD	0.476	0.64089	0.54	0.48204	0.0001	2.54497
db/db_2.5 cd·s/m <sup>2</sup> _2months_LD vs. db/db_2.5 cd·s/m <sup>2</sup> _4months_LD	0.824	-0.25499	0.846	0.14235	0	-3.92604

**oscillatory potential**

	P value
db/m_2.5 cd·s/m <sup>2</sup> _2months_DD	0.03483
db/m_2.5 cd·s/m <sup>2</sup> _2months_LD	0.00738
db/m_0.025 cd·s/m <sup>2</sup> _4months_LD	0.03845
db/m_0.25 cd·s/m <sup>2</sup> _4months_LD	0.00026
db/m_0.025 cd·s/m <sup>2</sup> _6months_LD	0.02016
db/db_0.025 cd·s/m <sup>2</sup> _4months_LD	0.00029
db/db_0.25 cd·s/m <sup>2</sup> _4months_LD	0.0001
db/db_2.5 cd·s/m <sup>2</sup> _4months_LD	0.00076

Comparison groups	Amplitude		Phase		Group mean	
	P value	difference	P value	difference	P value	difference
db/m_2.5 cd·s/m <sup>2</sup> _2months_DD vs. db/m_2.5 cd·s/m <sup>2</sup> _2months_LD	0.962	-2.71738	0.186	-0.92438	0.10716	50.2001
db/m_0.025 cd·s/m <sup>2</sup> _4months_LD vs. db/m_2.5 cd·s/m <sup>2</sup> _2months_LD	0.288	24.20915	0.674	-0.22768	0.00023	-65.304
db/m_0.25 cd·s/m <sup>2</sup> _4months_LD vs. db/db_0.25 cd·s/m <sup>2</sup> _4months_LD	0.37	27.55133	0.792	0.1032	0.00016	-89.08891
db/m_0.025 cd·s/m <sup>2</sup> _4months_LD vs. db/m_0.025 cd·s/m <sup>2</sup> _6months_LD	0.904	-2.60838	0.922	-0.06239	0	-87.07567