Diabetes Reshapes the Circadian Transcriptome Profile in Murine Retina

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Purpose. Diabetic retinopathy (DR) is a common complication of diabetes and has a high prevalence. Dysregulation of circadian rhythmicity is associated with the development of DR. This research aimed to investigate rhythmical transcriptome alterations in the retina of diabetic mice.

Methods. C57BL/6J mice were used to establish a diabetes model by intraperitoneal injection of streptozotocin (STZ). After 12 weeks, retinas were collected continuously at 4-hour intervals over 1 day. Total RNA was extracted from normal and STZ-treated retinas and RNA sequencing was performed. Meta2d algorithm, Kyoto Encyclopedia of Genes, Phase Set Enrichment Analysis, and time-series cluster analysis were used to identify, analyze and annotate the composition, phase, and molecular functions of rhythmic transcripts in retinas.

Results. The retina exhibited powerful transcriptome rhythmicity. STZ-induced diabetes markedly modified the transcriptome characteristics of the circadian transcriptome in the retina, including composition, phase, and amplitude. Moreover, the diabetic mice led to re-organized temporal and clustering enrichment pathways in space and time and affected core clock machinery.

Conclusions. Diabetes impairs the circadian rhythm of the transcriptomic profile of retinas. This study offers new perspectives on the negative effects of diabetes on the retina, which may provide important information for the development of new treatments for DR.

Keywords: diabetes, circadian rhythm, retina, transcriptome, diabetic retinopathy

Diabetic retinopathy (DR) is one of the major complications of diabetes mellitus (DM) and is the primary contributor to preventable blindness in the working-age population.[1,2] Based on the increasing number of patients with diabetes, estimations indicate that DR will affect 191 million people by 2030.[3] Unless prompt measures are undertaken, the number of people at risk of losing their vision will rise from 37.3 million to 56.3 million.[3] Hence, there is an urgent need to explore the molecular mechanisms involved in DR pathogenesis and develop new therapeutic approaches.

Circadian rhythms are intrinsic biological oscillations that organize behavior and physiology into a 24-hour program that adapts organisms to daily environmental conditions.[4,5] The mammalian retina contains an autonomous circadian clock that drives many processes within the retina, including outer segment disk shedding, electroretinogram b-wave amplitude, and visual sensitivity.[6–9] A published study has demonstrated the rhythmic expression of clock genes in neuronal cells of the inner nuclear layer and ganglion cell layer in the mouse retina.[10] Our previous study also showed that approximately 10% of genes expressed in the mouse retina had circadian rhythms and that these genes were involved in glycolysis, ATP generation, the Hif-1 pathway, and the assembly of cilia in photoreceptors.[11] Many reports have demonstrated that diabetes disrupts the regular rhythm of the retina.[12–16] Furthermore, expression of retinal clock genes was also observed to be altered in rodent models of type 1 and type 2 diabetes.[17–19] Busik et al. have reported downregulated expression of retinal clock genes (*Clock*, *Bmal1*, *Per1*, Per2, *Cry1*, *Cry2*, *Erb*, and *Rora*) in diabetic rats.[17] Additionally, Vancura et al.[18] and Lahouaoui et al.[19] have examined the expression of clock genes in the retina at multiple time points during a day and found that the circadian pattern of retinal clock genes was disrupted in diabetic mice. However, they used real-time quantitative PCR as an assay, which provided limited information. A recent study by Rajendran et al. integrated microarray datasets of nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) retinal tissue to establish common gene expression profiles of patients with NPDR and patients with PDR.[20] However, Busik et al.[17] and Rajendran et al.[20] only used data from one time point, which did not reflect the dynamic process of gene expression over a 24-hour period. More importantly, Rajendran's study focused on gene expression profiles

between different stages of DR (NPDR and PDR) rather than between normal and disease (control and DM). To date, whether diabetes affects the circadian gene expression profile in the retina over a 24-hour period remains unclear.

The current study evaluated the alterations in the transcriptome of the retina between normal and streptozotocin (STZ)-induced diabetic mice. Using a bioinformatics approach, the effect of hyperglycemic stress on the circadian rhythm of the retinal transcriptome and the underlying mechanisms were explored. It was found that diabetes significantly modified the features of the diurnal transcriptome and showed an unprecedented influence on the retinal circadian transcriptome.

Methods

Whole Research Design

The design and analysis process of this study is shown in Figure 1. Animals were injected with vehicles or STZ (see Fig 1A). Retinas were harvested at 4-hour intervals over the whole circadian cycle and subjected to RNA-Seq for circadian rhythm analysis (see Fig. 1B). The Meta2d algorithm was used for circadian rhythmicity gene identification and the phase and amplitude analysis of the circadian rhythm transcriptome. Subsequently, the molecular functions of the genes were annotated through the Kyoto Encyclopedia of Genes and Genomes (KEGG), phase set enhanced analysis (PSEA), and time series clustering analysis (see Fig. 1C).

Animals

Male 6-week-old C57BL/6 mice were purchased from Hunan Slack Jingda Experimental Animal Co., Ltd. Standard circadian rhythms were used for mouse husbandry. The animal experiments were carried out following the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Central South University Animal Care Committee (CSU-2023-0014). In this study, Zeitgeber time (ZT) was used to represent the rhythmic phase, whereby ZT0 represented the time the lights were turned on (7:00), and ZT12 represented the time the lights were turned off (19:00).

Mouse Model of Diabetes

Mice were induced with STZ to develop diabetes. Briefly, after 12 hours of fasting, STZ (Sigma Aldrich, St. Louis, MO, USA; 50 mg/Kg/d) or vehicle was injected intraperitoneally for 5 consecutive days, as described previously.[21] One week after the last STZ injection (defined as week 0), random blood glucose levels greater than 16.7 mmol/L indicated the mouse diabetes model was successfully established.

Retina Collection and RNA Extraction

After 12 weeks of diabetes onset, retinas were collected every 4 hours from mice euthanized by cervical dislocation over a 24-hour circadian cycle. Liquid nitrogen quickly froze the retina samples. In the RNA-Seq analysis, three independent biological samples at every time point were used. To avoid resetting the circadian clock, samples were collected under dim red light during the dark phase.

RNA Extraction, Library Construction, and Sequencing

The RNA isolation, library construction, and RNA sequencing were performed by Gene Denovo Biotechnology (Guangzhou, China). Fastp was used to filter reads, mapped them to the reference genome, and calculated the fragments per kilobase of mappable length and million counts (FPKM) values to quantify gene expression.

Identification of Rhythmic Gene Expression

The Meta2d is a tool comprised in the R package MetaCycle, and was used to assess the circadian rhythmicity of the transcriptome in the retina across the 24-hour cycle.[22] In a nutshell, Meta2d implements ARSER, JTK_CYCLE, and Lomb-Scargle, and integrates the results via N-version programming concepts.[23] In this study, transcripts were defined as rhythmic expressions if Meta2d BH.Q < 0.05.

Functional Annotation With KEGG and PSEA

The KEGG pathway analysis was performed using the ClusterProfiler package in R software. The significance threshold for the Q value was set to 0.05. Furthermore, PSEA

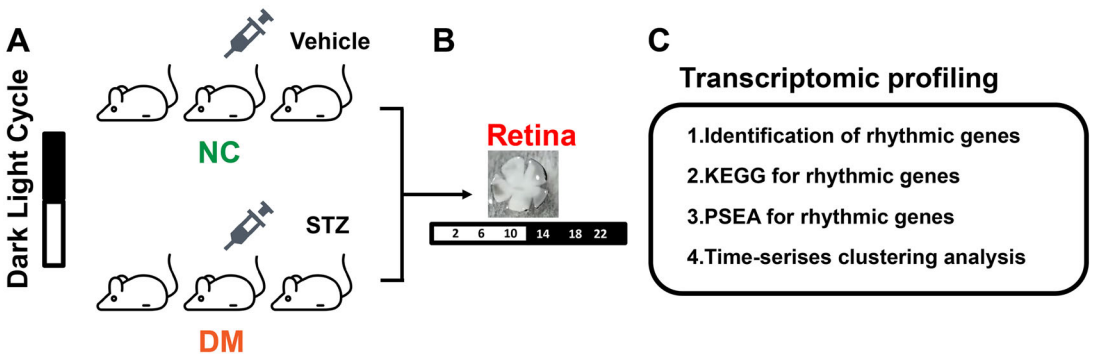

Figure 1. **Experimental design workflow**. (**A**) After 1 week of adaptive feeding under a 12 hour light/12 hour dark cycle, mice were randomly divided into the NC (vehicle injection) or DM groups (STZ injection). (**B**) Twelve weeks after establishing the diabetic model, retinas were collected every 4 hours over a 24-hour circadian cycle. (**C**) The extracted RNA from the retinas of NC and DM mice was processed for RNA-sequencing. The Meta2d algorithm, KEGG analysis, PSEA, and time series clustering analysis were used to determine the circadian transcriptional landscape of daily rhythmic genes.

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version 1.1 software was used to annotate the circadian pathways. "c2.cp. Kegg.v2022.1.Hs.symbols.gmt," as an annotated reference gene set, was accessed through the Molecular Signature Database. The domain circadian phase ranged from 0 to 24, and the minimum was set at 10 to 1000 with a Q value (determined by the Kuiper test) < 0.05. The PSEA parameters were set as follows: domain from 0 to 24; minimum items/ste: 5; maximum sims/test: 10000; and the significance threshold was set to 0.05 of Kuiper Q value.

Time Series Clustering Analysis

To provide an intuitive visualization of dynamic patterns of rhythmic gene expression levels in the retinas with time, a noise-proof soft clustering analysis was performed by using the fuzzy c-means clustering algorithm in the Mfuzz package. Among the parameters, the core threshold was set to 0.7, with a number of clusters of 4, whereas the remaining parameters were left at their default values.

Statistics Analysis and Software

Statistical analysis was performed using GraphPad Prism 9.0 (La Jolla, CA, USA). Heat maps were created by using the Phenomenap package in R (64-bit, version 4.2.2). Venn diagrams were produced by the OmicShare tools. The phase, period distribution, and Rayleigh vectors of the rhythm genes were analyzed by Oriana 4.01 (Kovach, Pentraeth, Wales, UK) software. Statistical analysis was performed using the student *t*-test, and $P < 0.05$ indicated a statistically significant difference.

Results

Establishment of the Diabetic Mice Model

C57BL/6 male mice were injected with STZ for 5 days to establish the diabetic mice model. Compared to the normal control (NC) group (vehicle injection), the blood glucose levels of the DM group (STZ injection) rose substantially over 12 weeks, resulting in diabetes (Fig. 2A). Over time, the body weight of both NC and DM mice increased. However, the body weight of the DM group was significantly lower than the NC group (Fig. 2B). Those results indicated that the diabetic mice model had been successfully established.

DM Increased the Proportion of Retinal Rhythmicity Genes

To map the circadian transcriptome profiles of normal and diabetic mouse during a 24-hour circadian cycle, retinas were collected every 4 hours from NC and DM mice, and RNA-seq analysis was performed. In total, 18,213 and 18,252 transcripts were obtained from the NC and DM retinas, respectively (Figs. 3A, 3B). To characterize the different transcriptome patterns, all transcriptomes were classified into three different categories as in a previous study.[24,25]: (1) rhythmic genes, showing expression changes over the 24-hour period (Meta2d BH.Q < 0.05; FPKM ≥ 0.1), (2) nonrhythmic genes, with consistent expression over the 24-hour period (Meta2d BH.Q ≥ 0.05; FPKM ≥ 0.1), and (3) low-expression genes (FPKM < 0.1). Of the 18,213 transcripts from NC, rhythmic genes accounted for 6.50% (1182; Supplementary Table S1), nonrhythmic transcripts for 84.24% (15,344), and low-expression transcripts for 9.26% (1687; see Fig 3A). The 18,252 transcripts from the DM mouse showed a comparable distribution, with 8.71% (1588) rhythmic genes (Supplementary Table S2), 81.65% (14,904) nonrhythmic genes, and 9.64% (1760) low-expression genes (see Fig 3B). In total, 18,674 non-overlapping retinal transcripts were identified over the 24-hour period, among which 17,791 (95.0%) were identified in both groups. In contrast, 422 (2.3%) were exclusively identified in the NC group and 461 (2.7%) were found only in the DM group (Fig. 3C).

To investigate the effects of diabetes on circadian gene expression, the rhythmic genes in the retina of the NC and DM groups were first subjected to Venn analysis. In total, 2340 transcripts with rhythmic expression were found in the two groups. Seven hundred fifty-two (32.14%) exhibited rhythmic expression only in NC mice, 1158 (49.49%) only in DM mice, and 430 (18.38%) in both groups (Fig. 4A). That means that in the diabetic retina, a total of 752 genes lost rhythmicity, whereas 1158 genes gained rhythmicity. The distinction in expression between the unique set of rhythmic transcripts in the retina of NC mice (Fig. 4B) and DM mice

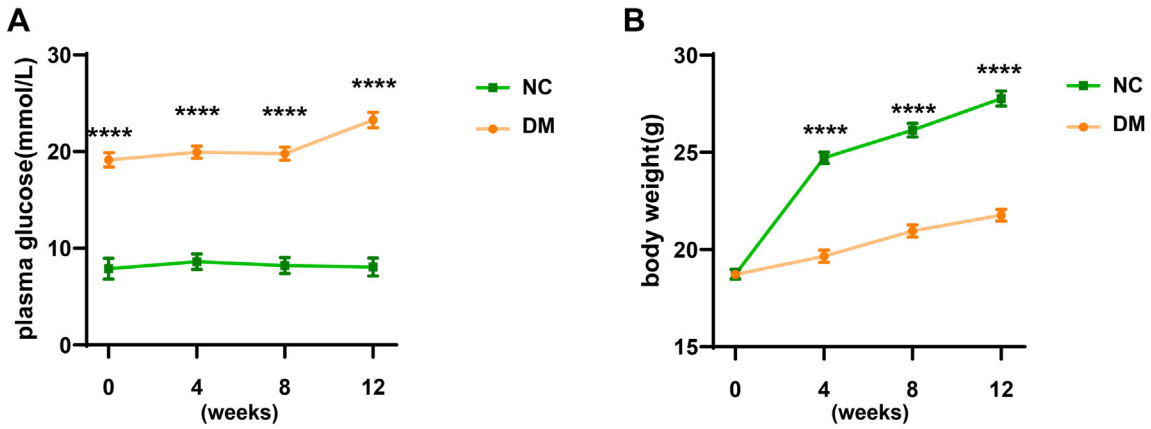

Figure 2. **Blood glucose and body weight of mice.** (**A**) Starting from 1 week after the last injection of STZ, the random blood glucose of mice was measured once every 4 weeks. (**B**) The body weight of mice was measured once every 4 weeks. Data are shown as mean ± standard deviation. Statistical analyses were conducted using the student *t*-test. ns means no statistical difference, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared with the NC group.

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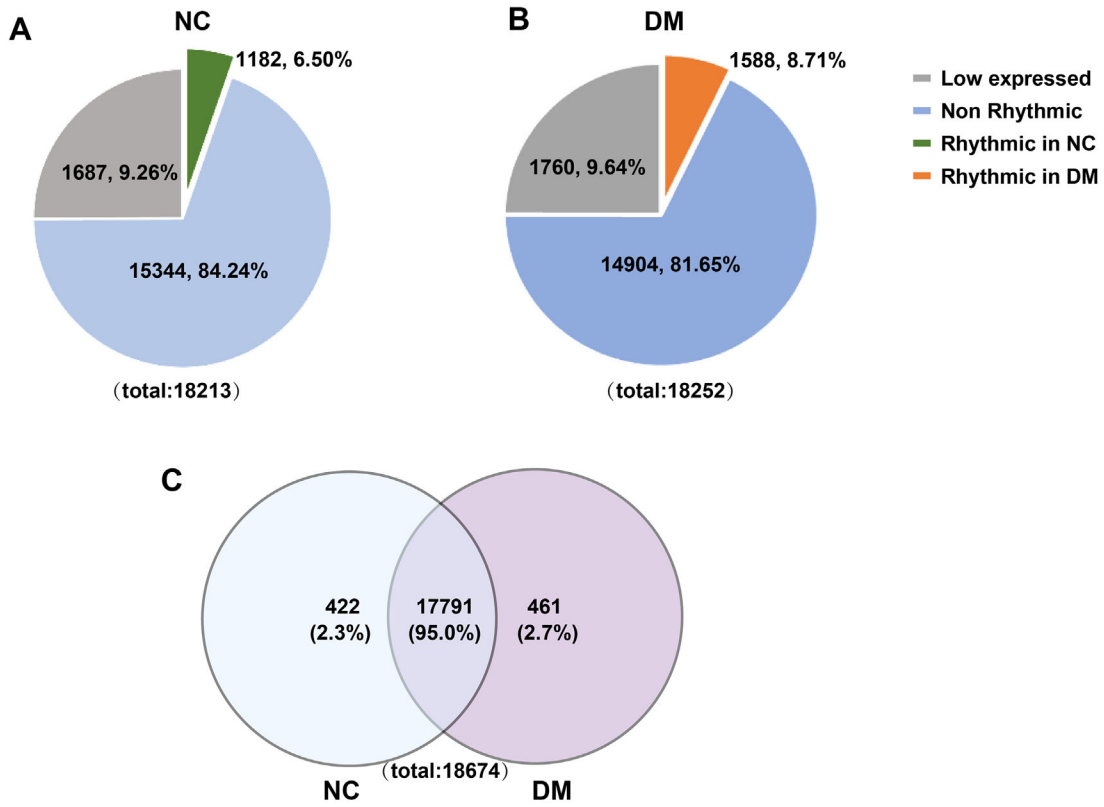

Figure 3. DM increased the proportion of retinal rhythmic genes. Pie charts of the transcriptome composition of the retinas of (**A**) the NC and (**B**) DM groups. (**C**) Venn diagram representing the overlapping sets of transcripts in the retinas of the NC and DM groups.

(Fig. 4C) is displayed in heatmaps. The 1158 rhythmic genes that were unique to DM mice were further analyzed via Venn diagram to determine if they belonged to the non-rhythmic or low-expression category in the normal retina. The results showed that these transcripts belonged to the non-rhythmic class in NC mice (Fig. 4D). Collectively, these results indicate that STZ-induced hyperglycemia modifies the constituent of the transcriptome in the retina. Specifically, diabetes increases the number of retinal rhythmic genes, which do not exhibit rhythmic expression in the normal retina.

DM Changed the Phase and Amplitude of Rhythmic Gene Expression in the Retinas

To clarify the influence of diabetes on transcripts across the retina, Oriana software was used to visualize the oscillatory phase, periods, and Rayleigh vectors for NC exclusive, DM exclusive, and shared rhythmic genes. The two groups of unique rhythmic genes showed different distributions. Specifically, the phase of 752 transcripts was mostly distributed between ZT7 and ZT11, with a mean vector (μ) of 08:59 and a mean vector length (r) of 0.386 for NC exclusive rhythm genes (Fig. 5A). However, the phases of 1158 transcripts were distributed mostly between ZT8 and ZT12, with a mean vector (μ) of 09:35 and a mean vector length (r) of 0.707 for DM-exclusive rhythm genes (Fig. 5B). In addition, the 430 shared rhythmic genes had a phase distribution between approximately ZT6.5 and ZT10.5, with an average vector (μ) of 09:26 and an average vector length (r) of 0.321 in the NC group (Fig. 5C), whereas the phase of these genes was mainly distributed from ZT8 to ZT12, with an average vector (μ) of 10:18 and an average vector length (r) of 0.347 in the DM group (Fig 5D). Remarkably, 60% (260) of the 430 shared rhythmic transcripts showed phase shifts of at least 1 hour. Among these transcripts, 9% (40) were phase-advanced and 51% (220) were phase-delayed (Fig. 5E).

A violin plot was drawn to determine the impact of diabetes on retinal rhythmic gene amplitude. The results showed that the oscillation amplitude of the 1158 rhythmic genes unique to the DM group was lower than that of the 752 rhythm genes unique to the NC group (Fig. 5F; $P < 0.05$). In contrast, the oscillation amplitude of the 430 shared rhythmic genes showed no statistically significant difference between the DM and NC groups (Fig. 5G). Collectively, these results suggest that diabetes alters the expression phases and amplitudes of rhythmic genes in the retina.

DM Reshapes KEGG and Phase-Set Enriched Pathways in Retinas

KEGG enrichment analysis was performed to further investigate the functions of rhythmic genes. In the NC group, the genes were enriched in fatty acid metabolism, fatty acid elongation, glutamatergic synapse, neurotrophin signaling pathway, long-term potentiation, and circadian entrainment (Fig. 6A). However, the DM group genes were enriched in different pathways, including the spliceosome, biosynthesis of cofactors, nucleocytoplasmic transport, alanine,

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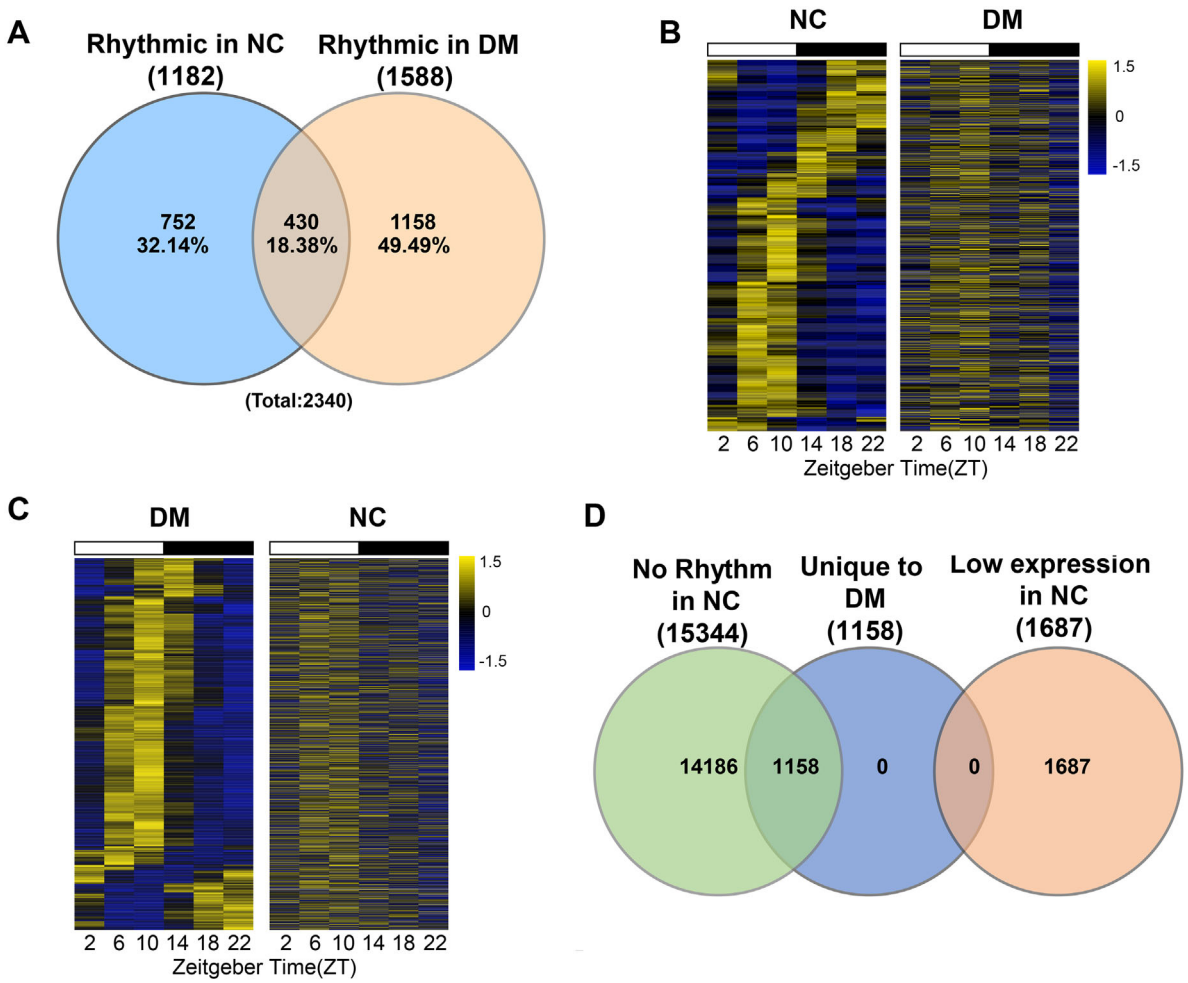

Figure 4. DM alters the characteristics of the circadian transcriptome in mouse retinas. (**A**) Venn diagram overlapping sets of rhythmic transcripts in the retinas of the NC and DM groups. (**B**) Heatmap displaying the 752 rhythmic transcripts unique to the NC group. Variations in the expression levels of these transcripts over time are shown for the NC (*left*) and DM (*right*) groups. The color bar represents the scale of transcript expression, with expression ranges normalized to ±1.5. (**C**) Heatmap illustrating the 1158 rhythmic transcripts unique to the DM group. Variations in the expression levels of these transcripts over time are shown for the DM (*left*) and NC (*right*) groups. The color bar represents the scale of transcript expression, with expression ranges normalized to ±1.5. (**D**) Venn diagram showing the number of rhythmic genes in the DM group (*blue*) overlapping with non-rhythmic genes (*green*) and low-expression genes (*orange*) in the NC group.

aspartate, and glutamate metabolism, and steroid biosynthesis (Fig. 6B). Six pathways were identified in shared rhythmic genes, including carbon metabolism, glycolysis, ribosome biogenesis in eukaryotes, biosynthesis of amino acids, central carbon metabolism in cancer, and fructose and mannose metabolism (Fig. 6C, Supplementary Table S3). These findings suggested that DM effectively reprogrammed rhythmically enriched biological pathways.

PSEA was performed to investigate the biological pathways that peaked at a specific time of day in the normal and diabetic retina. Only eight pathways were characterized as significantly enriched in the NC group (Fig. 7A). Nevertheless, 46 significantly enriched functional pathways were identified in DM retinas (Fig. 7B, Supplementary Table S4). Compared to the NC retina, the DM retina was enriched in metabolism-related pathways, such as glycerolipid metabolism, glycerophospholipid metabolism, and steroid biosynthesis, as well as immune-related pathways, such as the chemokine signaling pathway, leukocyte transendothelial migration, and Fc gamma R-mediated phagocytosis. Moreover, both the Wnt signaling pathway and the VEGF signaling pathway were significantly enriched in the DM group. Notably, although the MAPK signaling pathway, ErbB signaling pathway, and renal cell carcinoma were enriched in both groups, the peak ZT times of the pathways were different. Therefore, those findings demonstrate a temporal alteration of DM in the qualities and distributions of phase-enriched signal pathways.

DM Remodels the Cluster-Dependent Transcriptomic Maps in the Retinas

Clustering analysis is an efficient tool to uncover structures hidden in big gene expression data sets. A soft Mfuzz clustering was performed using R to identify dynamical patterns in normal and diabetic retinal transcriptome activity during the circadian cycle. According to the peak and trough distribution of the rhythmic genes over the 24-hour cycle, genes unique to the NC and DM groups were separately classified

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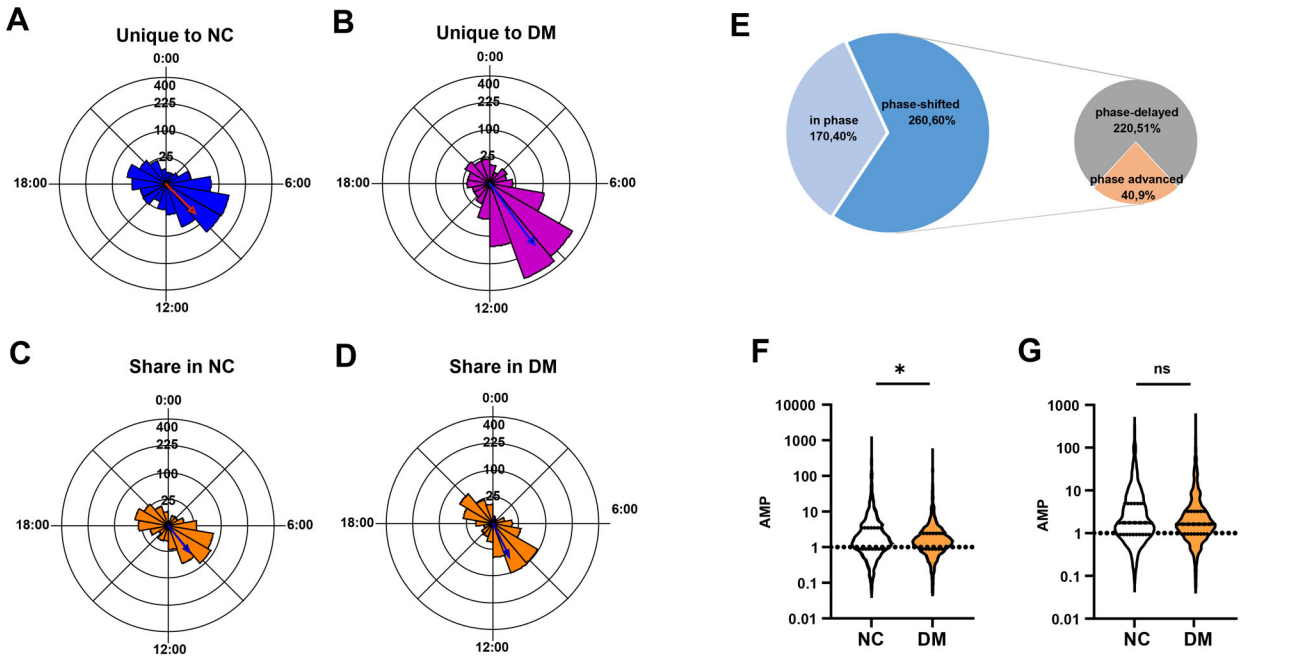

Figure 5. DM alters the phase and amplitude of the circadian transcriptome in mouse retinas. (**A–D**) Phase distribution of genes in the retina. *Grey shading* for dark cycles. (**A**) Rhythmic genes unique to the NC group, (**B**) rhythmic genes unique to the DM group, (**C**) shared rhythmic genes in the NC group, and (**D**) shared rhythmic genes in the DM group. (**E**) Pie charts showing phase analysis of shared genes. (**F**) Oscillation amplitudes of unique transcripts in the retinas of NC and DM mice. (**G**) Oscillation amplitudes of shared rhythmic gene in the retinas of the NC and DM mice. Statistical analyses were conducted using the student *t*-test, ns means no statistical significance, $^*P < 0.05$.

into 4 clusters (Figs. 8A–H, left, Supplementary Table S5). Subsequently, KEGG enrichment analysis was carried out to determine the specific biological pathways of each cluster of transcripts. The results revealed that transcripts with similar oscillatory patterns between NC and DM retinas were significantly enriched into distinct biological pathways (see Figs. 8A–H, right). Moreover, the same pathways in both groups showed opposite expression patterns, such as the butanoate metabolism and valine, leucine, and isoleucine degradation being enriched in cluster 4 in the NC group (see Fig. 8D), and in cluster 3 in the DM group (see Fig. 8G). This temporally inconsistent pathway activation may contribute to altered biological functions. Taken together, these findings suggest that STZ-induced hyperglycemia reprograms the dynamic transcriptional clustering of the biological pathways.

DM Alters Retinal Core Clock Transcription

To determine the effect of diabetes on retinal clock genes, RNA-Seq and Meta2d were used to compare the periods, phases, and amplitudes of the encoding 12 central components in normal and diabetic mouse retinas. In the NC mice, four core clock genes showed a robust rhythm (Meta BH Q < 0.05), with two (*Cry2* and *Per1*) peaking during the light cycle at ZT6 and two (*Per2* and *Npase2*) during the dark cycle (ZT14 and ZT22, respectively; Fig. 9A). However, the above genes, excluding *Per2*, showed no circadian rhythmicity in diabetic mice (Meta BH Q > 0.05). In addition, the expression of the *Rora* and *Nr1d2* was delayed by 4 hours, and the oscillation of *Nr1d1* was attenuated at its peak time (see Fig. 9A).

To characterize the phase (the time of peak expression of rhythmic genes) change of the central clock genes, the Meta2d analysis data was plotted using Oriana software. As depicted in Figure 9B, the phase distribution of the 12 clock gene transcriptions occurred at various ZT times. Seven clock genes in the NC group were distributed in the light cycle (ZT3–12), and five clock genes were distributed in the dark cycle (ZT12–22). In contrast, diabetic retinas showed a modified distribution, with five clock genes in the light cycle (ZT4–12) and seven clock genes in the dark cycle (ZT12–22). Compared to the NC group, *Bmal1* (22:00 vs. 19:30), *Per2* (13:00 vs. 12:00), *Per3* (14:30 vs. 12:30), and *Nr1d1* (10:30 vs. 8:00) were phase-advanced at least 1 hour, whereas *Rora* (18:30 vs. 22:00), *Rorc* (16:00 vs. 18:00), *Clock* (6:30 vs. 18:30), *Per1* (7:30 vs. 8:30), *Cry1* (11:30 vs. 16:00), *Cry2* (7:00 vs. 9:00), *Nr1d2* (11:30 vs. 15:00), and *Npas2* (3:00 vs. 5:00) were phase-delayed at least 1 hour. These results suggest that hyperglycemia induced by STZ re-organizes the core clock apparatus in the retinas.

Discussion

Diabetes can lead to progressive circadian rhythm disturbances, causing the loss of circadian fluctuations in blood pressure and sleep-wake patterns in db/db mice.[26,27] Diurnal oscillations in murine extraorbital lacrimal glands were also found to be remodeled in STZ-induced mice.[28] To our knowledge, this is the first research demonstrating that STZ-induced diabetes distinctively impacts the circadian transcriptome in the mouse retina. This conclusion is predominantly supported by the current observational findings that (1) diabetes alters the rhythmicity of pre-existing rhythmic genes while inducing new rhythmic expression;

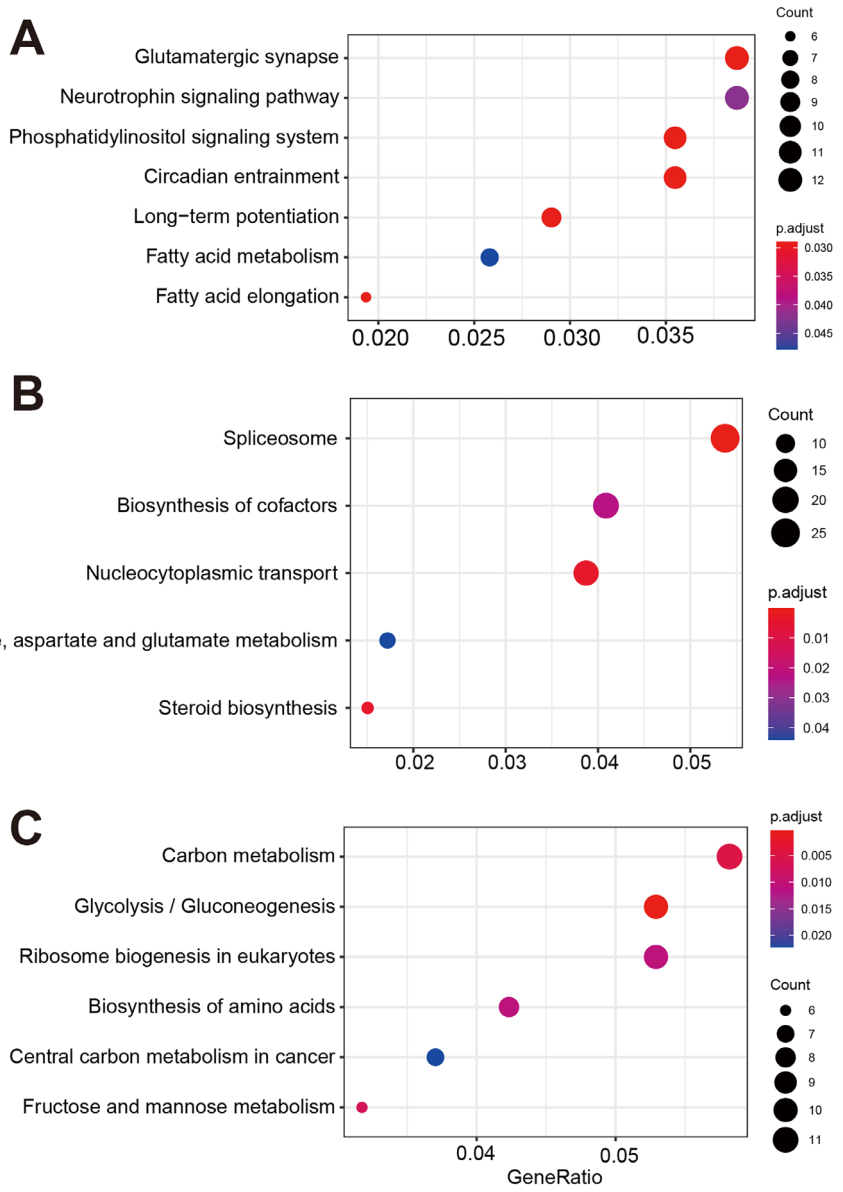

Figure 6. DM alters the KEGG pathways of the circadian transcriptome in mouse retinas. The gene annotation of enriched KEGG pathways of the rhythmic genes (**A**) unique to the NC group, (**B**) unique to the DM group, and (**C**) shared by both groups. The figure displays the pathways with adjusted P <0.05. The size of the dots represents the number of genes in the corresponding pathway, whereas the color of the dots represents the adjusted P values.

(2) STZ-induced diabetes significantly modifies the components, phase, and amplitude of rhythmic gene expression in the retina; (3) STZ-induced diabetes reshapes the KEGG biological pathway, the PSEA pathway, and the cluster-dependent transcriptome maps of enriched rhythmic genes in the retina; and (4) core clock transcription was altered in STZ-induced retinas. Interestingly, Ryan et al. (2023, Preprint) used the same approach to investigate circadian rhythm changes in the retina of another diabetic mouse, the Ins2Akita mice. They found that diabetics gained more rhythmic genes compared to controls. In addition, the authors performed a differential rhythmicity gene analysis and found that diabetes led to an advanced phase of the transcriptome. More importantly, they propose the hypothesis that in the early stages of diabetes, the retina experiences phase advancement driven by hypoxia and metabolic responses, which may generate internal time lag, leading to disruption of coordination between metabolic and circadian rhythms within the retina. These findings are confirmed by the present study and further define the negative impact of diabetes on the circadian physiology of the retina.

DR is a common complication of diabetes that leads to extensive retinal damage.[29] However, the detailed molecular mechanisms are not fully understood. High-throughput RNA-Seq and bioinformatics are formidable tools for analyzing the molecular events underlying phenotypic alterations.[30,31] Using RNA-seq techniques, the transcriptomes of the retinas of normal and diabetic mice were analyzed over a 24-hour period. The data revealed that STZ-induced hyperglycemia caused more genes to gain rhythmicity in the retina compared to controls. Further analysis revealed that the "novel" rhythmic genes in the diabetic retina were derived

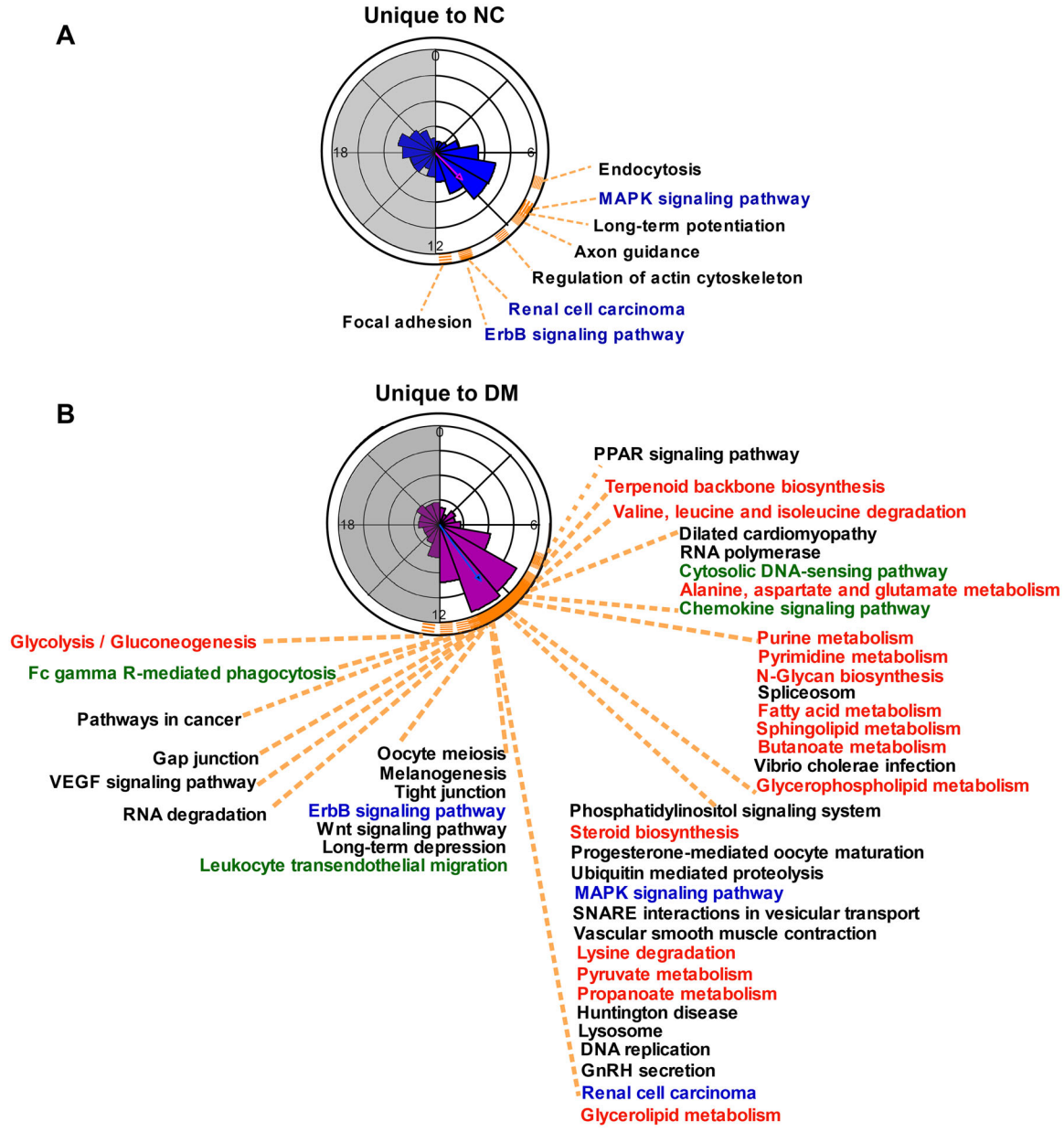

Figure 7. DM alters the phase-set enriched pathways of the circadian transcriptome in mouse retinas. (**A**) Significant phase-specific (Kuiper Q-value < 0.05) pathways of the rhythmic genes unique to the NC group, (**B**) unique to the DM group. The *inner circle* represents the phase distribution of the rhythmic genes, whereas the area of the wedge represents the gene counts in that phase. The *orange line* on the *outside circle* represents the KEGG pathways enriched at different ZTs based on the phase distribution of the *inner circle* (Q < 0.05 and >5 genes/pathways). *Gray shading* represents the dark phase. The pathways marked in *blue* are enriched in both groups. The *green* ones are immune-related pathways and the *red* ones are metabolism-related pathways.

from non-rhythmic genes in the normal retina. This finding was consistent with previous observations of increased numbers of rhythmical transcripts in mouse lacrimal gland tissues following short-term fructose or STZ-induced treatment.[25,28] The induction of such genetic oscillations is likely to be a compensatory mechanism of the retina's natural response to hyperglycemic stress.

In the present study, KEGG enrichment analysis showed that rhythmic genes of the NC group were enriched to lipid metabolism pathways, suggesting that under normal conditions, lipid metabolism in the retina follows a circadian rhythm, which is consistent with those reported in other organs.[32,33] In various peripheral organs, clock proteins rhythmically activate and repress genes involved in lipid biosynthesis and fatty acid oxidation, coupling the molecular clock to the transcriptional network involved in lipid metabolism.[34] This circadian oscillation has an important role in retinal function, as illustrated by Debarshi et al. They found that circadian cycling of key elements of the phosphatidylinositol lipid signaling process is an important driver of the RPE phagocytosis process in the outer segments of photoreceptors.[35] In this study, STZ induction resulted in a loss of rhythm in the key genes of fatty acid metabolism, including *Elovl4*, *Elovl6*, and *Hadha* (see Supplementary

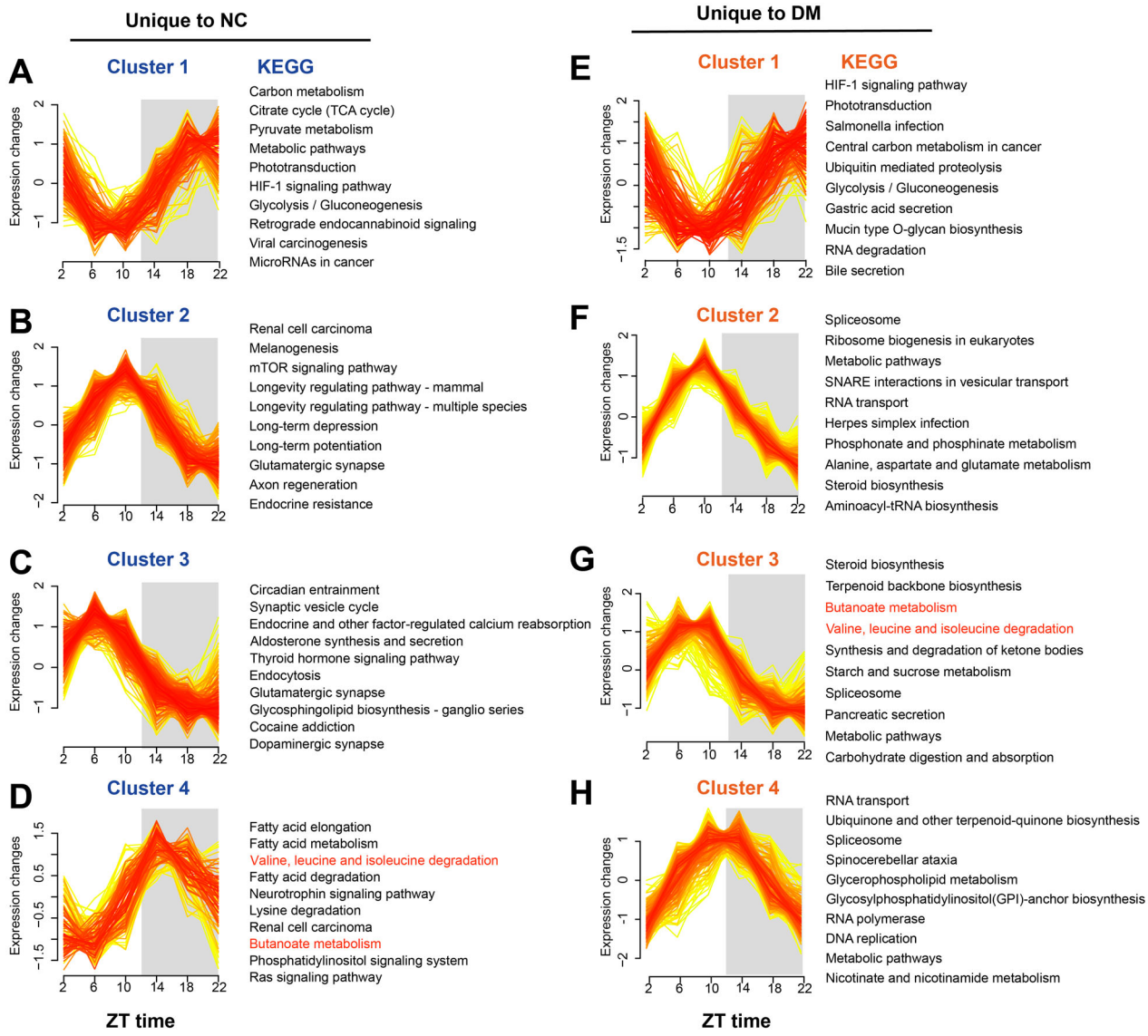

Figure 8. DM alters the cluster-dependent transcriptomic map. (**A–D**) Temporal pattern of Z-cores of four enriched clusters for rhythmic genes unique to the NC group (*left*). The top 10 KEGG pathways for each cluster were significantly enriched ($P < 0.05$) (*right*). (**E–H**) Temporal pattern of Z-cores of four enriched clusters for rhythmic genes unique to the DM group (*left*). The top 10 KEGG pathways for each cluster were significantly enriched ($P < 0.05$) (*right*). The expression values were standardized to have a mean of zero and a standard deviation of one. *Gray shading* represents the dark phase. The paths marked in *red* are enriched in both groups but distributed in different clusters.

Table S3). This result is consistent with the previous study by Wang et al.[16] The retina is rich in a variety of polyunsaturated fatty acids (PUFAs).[36] Animal and cellular experiments demonstrated that n3 PUFA has significant anti-inflammatory and anti-apoptotic effects in the retina and retinal cells,[37,38] whereas n6 PUFA exerts pro-inflammatory effects by increasing ICAM-1 expression.[39] In addition, the retina contains very long-chain polyunsaturated fatty acids (VLC-PUFAs), which have protective effects on the retina.[40] In the vertebrate retina, VLC-PUFAs are synthesized in vivo only from specific precursors (n3 and n6 PUFAs) under the effects of Elovl4 protease for carbon chain elongation.[40] Recent studies have found that a decrease in Elovl4 leads to a decrease in VLC-PUFA in the retina and an increase in the n3/n6 PUFA ratio, ultimately resulting in an inflammatory state and leading to the development of DR.[41] However, further research is required to investigate whether the rhythmic changes in the key genes of fatty acid metabolism observed in this study cause a mismatch between lipid metabolic processes and retinal metabolic demands, and the role of these changes in the development of DR.

Noteworthy, PSEA analyses revealed far more activated biological pathways in the retina of diabetic mice compared to normal mice and multiple metabolic and immune pathways associated with DR progression were activated. Interestingly, several signaling pathways were activated in both sets but peaked at different times. However, the biological significance of the temporal cues assigned to these pathways requires further investigation.

Time-series clustering approach is an effective way to assess temporal features associated with big data sets.[42,43] Four different types of rhythmic clustering oscillations are observed in the normal retina, each of them showing distinct pathway characteristics. However, significant variations in

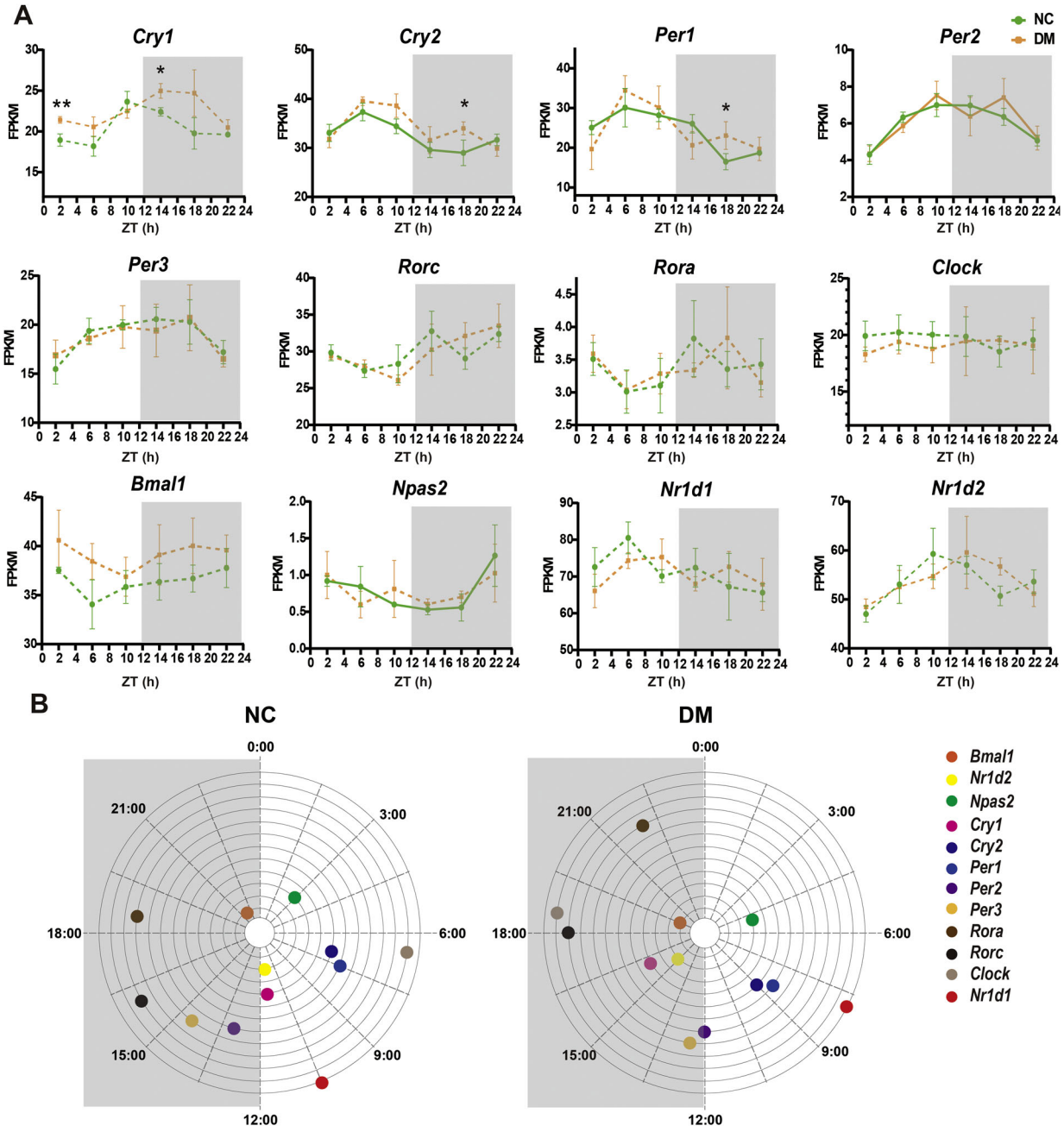

Figure 9. Effect of DM on circadian transcription of core clock genes in the retinas. (**A**) The temporal abundance of transcripts for the 12 core clock genes. The x-axis indicates the time points sampled. Y-axis indicates the expression of each gene at a specific ZT time point. The *gray shading* indicates dark cycles. The *solid lines* for clock genes with circadian rhythms (Meta BH Q < 0.05), and *dotted lines* for clock genes with non-circadian rhythms (Meta BH Q>0.05). *$P < 0.05$, **$P < 0.01$, between the NC and DM groups at each time point. (**B**) The peak phase distribution in ZT for core clock genes. The *gray shading* indicates dark cycles.

the circadian transcription landscape were observed after the onset of diabetes. This reconfiguration may involve metabolic cues within the diabetic process, although the mechanism of the dysregulation needs deeper analysis.

Notably, butanoate metabolism and valine, leucine, and isoleucine degradation pathways showed opposite expression patterns in the NC group and DM group. Disturbances in the circadian rhythms of those pathways may affect glutamate levels. On the one hand, the first step of butyrate metabolism involves the decarboxylation of glutamate to form gamma-aminobutyric acid (GABA).[44] On the other hand, branched-chain amino acids (BCAAs) including valine, leucine, and isoleucine in the retina, are nitrogen donors and promote glutamate synthesis by transamination of α-ketoglutarate in glial cells.[45,46] The glutamate excitotoxic effects play an important role in neuronal death in DR by increasing free radical production and inducing an apoptotic cascade response.[47,48] Therefore, this non-temporally coordinated pathway activation may also contribute to the development of DR.

All mammalian cells contain a range of core clock genes, which make up a self-regulated transcription-translation

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feedback loop to control downstream clock control genes.[49–52] Modification in the transcription of core clock genes, such as phase and amplitude of oscillations, may dramatically affect physiological functions. Similar to previous studies,[17,19] our research also showed significant changes in the transcription of the core clock genes in the retina under diabetic conditions. These changes include the advanced or delayed expression peaks of 12 core clock genes, the loss of rhythmicity in *Cry2*, *Per1*, and *Npase2*, and the weakening of oscillation of *Nr1d1* at its peak time. However, we are not certain of the effects of diabetes on clock genes in specific types of cells in the retina. The retina is a heterogeneous tissue composed of different types of cells, and several biological clocks with independent oscillatory cycles are distributed among the retinal layers.[53] In this study, we took the whole retina for mRNA sequencing and obtained the average transcriptional profile of all retinal cells. Wang et al. have shown that rod cells are the major contributors to the rhythmic signature of the retina, with estimates of the abundance of rod photoreceptors in the whole retinal tissue being above 60%.[11] This implies that in our results, changes in clock gene expression in some specific cell types with lower proportions in the retina may have been masked. Altogether, we observed dysregulation of retinal core clock gene expression in diabetic mice, which may be involved in the pathogenesis of DR. Further research on the underlying mechanisms may help develop a therapeutic approach to minimize the negative impact of diabetes on the retina by focusing on core clock gene expression and function.

Compared with previous studies, the present research offers some improvement. For example, we used intensive sampling time and advanced sequencing techniques to represent the dynamic retinal transcriptome more precisely. In addition, in-depth analyses of the function and gene expression patterns associated with rhythmic genes in the normal and diabetic groups were performed to reveal the effects of diabetes on physiological processes during the circadian cycle in the retina. Nevertheless, the limitations of the present study should be acknowledged. First, C57BL/6 mice are nocturnal and have a different sleep-wake cycle to that of humans. Second, this study highlights the bioinformatical interpretations of the effects of diabetes on the transcriptomic rhythmicity of retinas; future studies will focus more on the cellular and molecular mechanisms. Third, due to the complexity of the different cell types in the retinas and their oscillatory cycles, we are not certain of the effects of diabetes on clock genes in specific types of cells in the retina. Single-cell RNA-seq sequencing technology might be applied in the future. Finally, the present study collected retinal samples over a 24-hour period from mice with a 12-week course of diabetes, with results that may only reflect alterations of the transcriptome in the early stages of DR. In future studies, repeating the 24-hour experiment and extending the duration of DM would be beneficial.

Conclusions

In summary, the transcriptomic and extensive bioinformatic analyses showed that STZ-induced diabetes drastically alters the circadian oscillations of the transcriptomic profile across the mouse retina. Our findings suggest that alterations in the circadian rhythms of transcriptomes and signaling pathways in the retina contribute to retinal impairment in diabetes. A deeper exploration of this intricate molecular network might reveal potential targets for DR treatment.

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References

1. Semeraro F, Morescalchi F, Cancarini A, Russo A, Rezzola S, Costagliola C. Diabetic retinopathy, a vascular and inflammatory disease: therapeutic implications. *Diabetes Metab.* 2019;45(6):517–527.
2. Wong TY, Cheung CM, Larsen M, Sharma S, Simo R. Diabetic retinopathy. *Nat Rev Dis Primers.* 2016;2:16012.
3. Zheng Y, He M, Congdon N. The worldwide epidemic of diabetic retinopathy. *Indian J Ophthalmol.* 2012;60(5):428–431.
4. Lowrey PL, Takahashi JS. Genetics of circadian rhythms in Mammalian model organisms. *Adv Genet.* 2011;74:175–230.
5. Hamnett R, Crosby P, Chesham JE, Hastings MH. Vasoactive intestinal peptide controls the suprachiasmatic circadian clock network via ERK1/2 and DUSP4 signalling. *Nat Commun.* 2019;10(1):542.
6. Teirstein PS, Goldman AI, O'Brien PJ. Evidence for both local and central regulation of rat rod outer segment disc shedding. *Invest Ophthalmol Vis Sci.* 1980;19(11):1268–1273.
7. Jackson CR, Ruan GX, Aseem F, et al. Retinal dopamine mediates multiple dimensions of light-adapted vision. *J Neurosci.* 2012;32(27):9359–9368.
8. Sengupta A, Baba K, Mazzoni F, et al. Localization of melatonin receptor 1 in mouse retina and its role in the circadian regulation of the electroretinogram and dopamine levels. *PLoS One.* 2011;6(9):e24483.
9. Storch KF, Paz C, Signorovitch J, et al. Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information. *Cell.* 2007;130(4):730–741.
10. Ruan GX, Zhang DQ, Zhou T, Yamazaki S, McMahon DG. Circadian organization of the mammalian retina. *Proc Natl Acad Sci USA.* 2006;103(25):9703–9708.
11. Wang Z, Ji S, Huang Y, et al. The daily gene transcription cycle in mouse retina. *Exp Eye Res.* 2021;207:108565.
12. Qi X, Mitter SK, Yan Y, Busik JV, Grant MB, Boulton ME. Diurnal rhythmicity of autophagy is impaired in the diabetic retina. *Cells.* 2020;9(4).
13. Alex A, Luo Q, Mathew D, Di R, Bhatwadekar AD. Metformin corrects abnormal circadian rhythm and Kir4.1 channels in diabetes. *Invest Ophthalmol Vis Sci.* 2020;61(6):46.
14. Bhatwadekar AD, Rameswara V. Circadian rhythms in diabetic retinopathy: an overview of pathogenesis and investigational drugs. *Expert Opin Investig Drugs.* 2020;29(12):1431–1442.
15. Di R, Luo Q, Mathew D, Bhatwadekar AD. Diabetes alters diurnal rhythm of electroretinogram in db/db mice. *Yale J Biol Med.* 2019;92(2):155–167.
16. Wang Q, Tikhonenko M, Bozack SN, et al. Changes in the daily rhythm of lipid metabolism in the diabetic retina. *PLoS One.* 2014;9(4):e95028.

17. Busik JV, Tikhonenko M, Bhatwadekar A, et al. Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. *J Exp Med.* 2009;206(13):2897–2906.
18. Vancura P, Oebel L, Spohn S, et al. Evidence for a dysfunction and disease-promoting role of the circadian clock in the diabetic retina. *Exp Eye Res.* 2021;211:108751.
19. Lahouaoui H, Coutanson C, Cooper HM, Bennis M, Dkhissi-Benyahya O. Diabetic retinopathy alters light-induced clock gene expression and dopamine levels in the mouse retina. *Molec Vis.* 2016;22:959–969.
20. Rajendran S, Seetharaman S, Vetrivel U, Kuppan K. Integrative study of gene expression datasets in retinal samples of diabetic retinopathy. *Exp Eye Res.* 2022;223:109194.
21. Shimokawa C, Kato T, Takeuchi T, et al. CD8(+) regulatory T cells are critical in prevention of autoimmune-mediated diabetes. *Nat Commun.* 2020;11(1):1922.
22. Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB. MetaCycle: an integrated R package to evaluate periodicity in large scale data. *Bioinformatics (Oxford, England).* 2016;32(21):3351–3353.
23. Benegiamo G, Mure LS, Erikson G, et al. The RNA-binding protein NONO coordinates hepatic adaptation to feeding. *Cell Metab.* 2018;27(2):404–418.e407.
24. He J, Jiao X, Sun X, et al. Short-term high fructose intake impairs diurnal oscillations in the murine cornea. *Invest Ophthalmol Vis Sci.* 2021;62(10):22.
25. Lu D, Lin C, Jiao X, et al. Short-term high fructose intake reprograms the transcriptional clock rhythm of the murine extraorbital lacrimal gland. *Invest Ophthalmol Vis Sci.* 2019;60(6):2038–2048.
26. Hou T, Su W, Guo Z, Gong MC. A novel diabetic mouse model for real-time monitoring of clock gene oscillation and blood pressure circadian rhythm. *J Biol Rhythms.* 2019;34(1):51–68.
27. Laposky AD, Bradley MA, Williams DL, Bass J, Turek FW. Sleep-wake regulation is altered in leptin-resistant (db/db) genetically obese and diabetic mice. *Am J Physiol Regul Integr Comp Physiol.* 2008;295(6):R2059–2066.
28. Jiao X, Lu D, Pei X, et al. Type 1 diabetes mellitus impairs diurnal oscillations in murine extraorbital lacrimal glands. *Ocul Surf.* 2020;18(3):438–452.
29. Yang H, Gan S, Jiang Z, et al. Protective effects of essential oil from Fructus Alpiniae zerumbet on retinal Müller gliosis via the PPAR-γ-p-CREB signaling pathway. *Chinese Med.* 2020;15:4.
30. Louer EMM, Yi G, Carmone C, et al. Genes involved in energy metabolism are differentially expressed during the day-night cycle in murine retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 2020;61(5):49.
31. DeVera C, Tosini G. Circadian analysis of the mouse retinal pigment epithelium transcriptome. *Exp Eye Res.* 2020;193:107988.
32. McCarthy JJ, Andrews JL, McDearmon EL, et al. Identification of the circadian transcriptome in adult mouse skeletal muscle. *Physiol Genomics.* 2007;31(1):86–95.
33. Rey G, Cesbron F, Rougemont J, Reinke H, Brunner M, Naef F. Genome-wide and phase-specific DNA-binding rhythms of BMAL1 control circadian output functions in mouse liver. *PLoS Biol.* 2011;9(2):e1000595.
34. Gooley JJ. Circadian regulation of lipid metabolism. *Proc Nutrit Soc.* 2016;75(4):440–450.
35. Mustafi D, Kevany BM, Genoud C, Bai X, Palczewski K. Photoreceptor phagocytosis is mediated by phosphoinositide signaling. *FASEB J.* 2013;27(11):4585–4595.
36. Wang J, Li M, Geng Z, et al. Role of oxidative stress in retinal disease and the early intervention strategies: a review. *Oxid Med Cell Longev.* 2022;2022:7836828.
37. Opreanu M, Tikhonenko M, Bozack S, et al. The unconventional role of acid sphingomyelinase in regulation of retinal microangiopathy in diabetic human and animal models. *Diabetes.* 2011;60(9):2370–2378.
38. Chen W, Esselman WJ, Jump DB, Busik JV. Anti-inflammatory effect of docosahexaenoic acid on cytokine-induced adhesion molecule expression in human retinal vascular endothelial cells. *Invest Ophthalmol Vis Sci.* 2005;46(11):4342–4347.
39. Chen W, Jump DB, Grant MB, Esselman WJ, Busik JV. Dyslipidemia, but not hyperglycemia, induces inflammatory adhesion molecules in human retinal vascular endothelial cells. *Invest Ophthalmol Vis Sci.* 2003;44(11):5016–5022.
40. Gorusupudi A, Rallabandi R, Li B, et al. Retinal bioavailability and functional effects of a synthetic very-long-chain polyunsaturated fatty acid in mice. *Proc Natl Acad Sci USA.* 2021;118(6).
41. Tikhonenko M, Lydic TA, Wang Y, et al. Remodeling of retinal fatty acids in an animal model of diabetes: a decrease in long-chain polyunsaturated fatty acids is associated with a decrease in fatty acid elongases Elovl2 and Elovl4. *Diabetes.* 2010;59(1):219–227.
42. Huang S, Si H, Liu J, et al. Sleep loss causes dysfunction in murine extraorbital lacrimal glands. *Invest Ophthalmol Vis Sci.* 2022;63(6):19.
43. Zou S, Liu J, Si H, et al. High-fat intake reshapes the circadian transcriptome profile and metabolism in murine meibomian glands. *Front Nutr.* 2023;10:1146916.
44. Tyagi N, Lominadze D, Gillespie W, et al. Differential expression of gamma-aminobutyric acid receptor A (GABA(A)) and effects of homocysteine. *Clin Chem Lab Med.* 2007;45(12):1777–1784.
45. Gowda K, Zinnanti WJ, LaNoue KF. The influence of diabetes on glutamate metabolism in retinas. *J Neurochem.* 2011;117(2):309–320.
46. Ola MS, Hosoya K, LaNoue KF. Regulation of glutamate metabolism by hydrocortisone and branched chain keto acids in cultured rat retinal Müller cells (TR-MUL). *Neurochem Inl.* 2011;59(5):656–663.
47. Lipton SA, Rosenberg PA. Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med.* 1994;330(9):613–622.
48. Niizuma K, Endo H, Chan PH. Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. *J Neurochem.* 2009;109(Suppl 1):133–138.
49. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. *Nature.* 2002;418(6901):935–941.
50. Levi F, Schibler U. Circadian rhythms: mechanisms and therapeutic implications. *Annu Rev Pharmacol Toxicol.* 2007;47:593–628.
51. Hardin PE, Panda S. Circadian timekeeping and output mechanisms in animals. *Curr Opin Neurobiol.* 2013;23(5):724–731.
52. Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. *Trends Cell Biol.* 2014;24(2):90–99.
53. Ko GY. Circadian regulation in the retina: from molecules to network. *Eur J Neurosci.* 2020;51(1):194–216.

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