Visual Image Quality Impacts Circadian Rhythm-Related Gene Expression in Retina and in Choroid: A Potential Mechanism for Ametropias

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Purpose. Stimulated by evidence implicating diurnal/circadian rhythms and light in refractive development, we studied the expression over 24 hours of selected clock and circadian rhythm–related genes in retina/retinal pigment epithelium (RPE) and choroid of experimental ametropias in chicks.

Methods. Newly hatched chicks, entrained to a 12-hour light/dark cycle for 12 to 14 days, either experienced nonrestricted vision OU (i.e., in both eyes) or received an image-blurring diffuser or a minus 10-diopter (D) or a plus 10-D defocusing lens over one eye. Starting 1 day later and at 4-hour intervals for 24 hours, the retina/RPE and choroid were separately dissected. Without pooling, total RNA was extracted, converted to cDNA, and assayed by quantitative PCR for the expression of the following genes: *Opn4m*, *Clock*, *Npas2*, *Per3*, *Cry1*, *Arntl*, and *Mtnr1a*.

Results. The expression of each gene in retina/RPE and in choroid of eyes with nonrestricted vision OU varied over 24 hours, with equal levels OU for most genes and times. Altered visual input influenced gene expression in complex patterns that varied by gene, visual input, time, and eye, affecting experimental eyes with altered vision and also contralateral eyes with nonrestricted vision.

Discussion. Altering visual input in ways known to induce ametropias alters the retinal/RPE and choroidal expression of circadian rhythm–related genes, further linking circadian biology with eye growth regulation. While further investigations are needed, studying circadian processes may help understand refractive mechanisms and the increasing myopia prevalence in contemporary societies where lighting patterns can desynchronize endogenous rhythms from the natural environmental light/dark cycle.

Keywords: circadian clock genes, melanopsin, retina/RPE, choroid, ametropia

Why refractive errors develop and why the prevalence of myopia is increasing to alarming levels in the developed world remains a puzzle. Refractive responses to visual blur and defocus in experimental animals and, to the extent observable, in children have indicated that visual input regulates eye growth. As an example, in animal models, blurring visual input with a diffuser induces ipsilateral form-deprivation myopia, and the wearing of a minus or plus spectacle lens alters eye growth to place the retina conjugate to the altered position of distant images, causing ipsilateral lens-induced myopia or lens-induced hyperopia, respectively.[1] The mechanisms governing postnatal refractive and eye growth responses to image quality are largely intrinsic to the eye, with much evidence implicating the retina as a controller.[1–3] Besides image quality, a potential role for insufficient light exposures as a cause for myopia was first proposed in the 19th century.[4–6] Most recently, clinical investigations have demonstrated a modest protective effect against myopia in children by increased outdoor exposures.[7–11] Bright light exposures in the laboratory protect against experimental myopia in animal models.[12] How light acts to limit myopia remains speculative.

Light exposure controls circadian biology, and accumulating evidence supports the notion that the dysregulation of circadian rhythms might contribute to the development of ametropias, as discussed in our recent review.[13] The eyes of animals and humans undergo diurnal oscillations in dimensions, including fluctuations in axial length

and choroidal thickness. In animals with experimentally induced myopia or hyperopia, the phase relationships of these rhythms are altered as ametropias develop,[14] but there are not yet data on whether the diurnal fluctuations in ocular dimensions are affected in children developing ametropia. From microarray and quantitative PCR (qPCR) assays that commonly compare experimental eyes to contralateral control eyes, altered expression of several circadian clock genes and a melanopsin gene has been identified in the retina/retinal pigment epithelium (RPE) in lens-induced and form-deprivation myopias of chicks.[15,16] Minus or plus lens wear affects expression of the clock gene *Arntl* in chicks,[17] and altered RPE expression of melanopsin develops in lens-induced myopia of tree shrew.[18] Experimental myopia induces only small changes in the expression of most affected genes in retina,[19] including the altered expression of these clock and melanopsin genes.[15,16]

The circadian clock is an oscillating autonomous molecular mechanism consisting of transcriptional-translational feedback loops that use a series of clock genes and their protein products; it requires about 24 hours to cycle.[20,21] The retina expresses the molecular components of the circadian clock.[20,21] Based on accumulating evidence that disordered rhythms and clock genes might contribute to the development of ametropias,[13] we here characterized the diurnal expression of selected circadian clock genes in the chick retina/RPE and separately in the chick choroid over a full 24-hour period. We also analyzed the expression of one of the two melanopsin isoforms expressed in chicks (*Opn4m*), a blue light absorbing photopigment in retinal ganglion cells and in other nonrod/noncone retinal neurons of chicks. In mammals, the melanopsin-containing neurons provide input for circadian entrainment, among other functions.[13,20–23] While the pineal is more central to circadian rhythm control in birds than in mammals,[24] melanopsin-expressing retinal neurons seem to have analogous functions in birds.[25–28] We also assayed the expression of one of the melatonin receptor subtypes (*Mtnr1a*) involved in signaling output from the clock. We included the choroid because it undergoes diurnal thickness fluctuations that are hypothesized to influence refractive development.[13,29] The expression levels of these genes undergo diurnal oscillations in the retina,[30–35] but their daily expression patterns in the choroid have not been studied.

We studied gene expression patterns in eyes of chicks with nonrestricted vision OU (i.e., in both eyes) and in eyes with three well-established methods of perturbing refractive development: diffuser wear to produce form-deprivation myopia, minus lens wear to produce lens-induced myopia, and plus lens wear to produce lens-induced hyperopia.[1] We selected clock and circadian rhythm–related genes whose expression that we previously found to be altered in chick retina at single times during the day in unilateral lens-induced myopia or form-deprivation myopia, comparing experimental eyes with altered visual input to contralateral eyes with nonrestricted vision.[15,16]

Methods

Animals and Tissue Harvesting

Newly hatched chicks (*Gallus gallus domesticus*; Cornell-K strain) were reared under a 12-hour light/12-hour dark cycle (4100K fluorescent light, ~300 lux in cage) for 12 to 14 days. At zeitgeber time (ZT) 0 (defined as lights on at ZT 0), an image-degrading diffuser, a minus 10-diopter (D) lens, or a plus 10-D lens was secured over the right eye (OD) using matching Velcro rings, with nonimpaired visual input to the contralateral left eye (OS). Other chicks experienced nonrestricted visual input OU, with no device over either eye. Starting the next day after one full 12-hour light/12-hour dark cycle of device wear, chicks were killed by decapitation without anesthesia in timed cohorts so that tissues were acquired at approximately ZT 0, 4, 8, 12, 16, or 20 hours (n = 8 chicks/time/condition). For the "night" samples, chicks were killed under dim dark yellow light from a photographic safe light (Premier Model SL1012, Doran Manufacturing, Cincinnati OH, USA; ~0.5 lux). The retina/RPE and choroid were then immediately dissected separately over ice in sterile and RNAse-free conditions from each eye, snap-frozen in liquid nitrogen, and stored without pooling at −80°C until processed. The supplementary methods detail the timing schedule for tissue sampling. The vitreous bodies from these eyes also were removed and separately assayed for the levels of 3,4-dihydroxyphenylacetic acid (DOPAC), which will be reported independently. The experiments adhered to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Use and Care Committee of New England College of Optometry.

mRNA Extraction and cDNA Synthesis

Total RNA extraction was performed in batches using the Purelink RNA mini kit (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized in 96-well plates on a SimpliAmp thermal cycler (Applied Biosystems, Foster City, CA, USA) with Superscript SS IV VILO Mastermix (Life Technologies). The cDNA samples were frozen at −80°C until they were shipped on dry ice to the University of Pennsylvania. They were maintained at −80°C until assayed.

qPCR

Gene expression levels for each eye were determined individually with real-time qPCR using an Applied Biosystems 7300/7500 qPCR machine and TaqMan Assays (Thermo Fisher Scientific, Waltham, MA, USA). Then, 20 µL of the above cDNA solution was diluted to 400 µL with pure water for each of the seven genes for the qPCR assay; 9 µL of the diluted cDNA was combined with 1 µL TaqMan Gene Expression Assay and 10 µL TaqMan Universal Master Mix (#4304437) to a total 20 µL volume for each reaction. The housekeeping gene *Gapdh* served as an endogenous control, and the expression of each gene at each time was normalized to the *Gapdh* expression level measured for each sample. Samples were run in triplicate and averaged. The $\Delta\Delta$Ct value was used as the relative expression levels for each of the seven genes under study. Table 1 identifies gene symbols, gene names, and corresponding TaqMan assays.

Statistical Analysis

Because of nonnormal data distributions, a natural log transformation was applied to the gene expression levels for model fit and better model diagnostics. The mean transformed OS value at ZT 0 was subtracted from the transformed expression values for each gene and each eye at each time point, such that the mean value of the left eye across individuals is 0 at ZT 0. To account for intereye correlation, the gene expression responses for each eye over time were

Table 1. Assayed Genes

Gene Symbol	Gene Name	TaqMan Assay
Opn4m	Melanopsin	Gg03359959_m1
Clock	Circadian locomotor output cycles kaput	Gg03362343_m1
Npas2	Neuronal PAS domain protein 2	Gg03350049_m1
Per3	Period 3	ARWCWE2
Cry1	Cryptochrome 1	Gg03364195_mH
Arntl	Aryl hydrocarbon receptor nuclear translocator-like protein 1	Gg03345653_m1
Mtnr1a	Melatonin receptor subtype 1a	Gg03339711_m1
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Gg03346983_g1

modeled using the generalized estimating equation (GEE) to estimate a robust variance structure.[36] The model formula was specified such that the transformed gene expression response was modeled using the main effects of eye and time as well as the interaction between them, with time being a categorical variable with levels ranging from ZT 0 to ZT 20.

Type III tests were performed to obtain the *P* value for the overall effects of eye and time. Post hoc pairwise comparisons were analyzed for those models that reached a Bonferroni significance level for either an overall eye effect or for an eye-time interaction effect to identify time points where the left and right eyes differed. The number of genes analyzed in each condition at each time established the Bonferroni criterion for a $P \leq 0.05$ significance level. For retinal comparisons, seven genes were analyzed giving a Bonferroni significance level of $P \leq 0.0071$; for choroidal comparisons, six genes were analyzed giving a Bonferroni significance of $P \leq 0.0083$. Unless otherwise specified, data are reported as mean ± SEM of the natural log values in both the figures and supplementary tables.

Results

By qPCR, both the retina/RPE and choroid expressed melanopsin (*Opn4m*) and each of the circadian clock genes at all time points studied. The melatonin receptor gene (*Mntr1a*) was expressed in the retina/RPE but not in the choroid. Altered visual input impacted the gene expression over time in patterns that varied between visual condition, gene, eye and time. Tables 2 to 4 summarize these complex patterns, and Figures 1 to 4 show the expression for these genes in both eyes over 24 hours. Detailed data and statistical analyses appear in Supplementary Tables S1 to S8.

The study design permitted assessment of relative gene expression within each visual condition and emphasized gene expression over time, eye effects (i.e., OD vs. OS), and the interactions of eye-time. The technical features of our molecular assays did not permit unambiguous identification of absolute differences in expression levels between the four visual cohorts, but bilateral effects could be identified by the loss of gene expression variation over time in both eyes with altered visual input to OD only, a conservative criterion. This criterion for a bilateral effect was evident in each visual condition in retina/RPE and/or in choroid and most frequently involved the expression of *Opn4m*, *Clock*, and *Cry1* (Table 2). OD-OS differences occurred only for some genes under each visual condition and only at specific times during the 24 hours of the assays. It was not possible to determine unambiguously whether an increased expression in one eye or a reduced expression in the contralateral eye accounted for OD-OS differences.

Nonrestricted Vision OU

The expression of each gene varied over time in retina/RPE and in choroid ($P < 0.001$ for each gene and each tissue; Tables 2 and 3, Figure 1, Supplementary Tables S1 and S2). In the retina/RPE, there were no differences in the expression values of each gene between the right and left eyes at any time. In the choroid, the gene expression levels were generally equivalent for most genes and times comparing right and left eyes; only *Clock* at ZT 8 and *Arntl* at ZT 4 and 16 demonstrated OD-OS differences. Although there was an overall eye effect for choroidal *Opn4m* expression, no specific time was identified by post hoc testing for an OD-OS difference. In eyes with nonrestricted vision OU, the time of peak gene expression varied between gene and between tissue (Table 4, Fig. 1, Supplementary Tables S1 and S2).

Unilateral Diffuser Wear

In retina/RPE, unilateral blur from diffuser wear eliminated the changes in gene expression over time for four genes (*Opn4m*, *Clock*, *Npas2*, and *Mntr1a*), not only in the visually deprived eye but also in the contralateral eye (Tables 2 and 3, Fig. 2, Supplementary Table S3). Those genes that continued to vary over time showed highest expression levels at the same times in both eyes and at close to the same times as eyes with nonrestricted vision OU (Table 4, Fig. 2, Supplementary Table S3). Except for *Clock* at ZT 0 and *Per3* at ZT 20, the OD versus OS expression levels of all genes remained comparable at each time (Table 2, Supplementary Table S3).

In the choroid, the effects of diffuser wear were somewhat less pronounced. The time-variable expression level of only *Clock* was negated by form deprivation. Except for *Npas2* whose maximum expression time was the same as in chicks with nonrestricted vision OU, the time of highest expression level for the time-varying genes shifted by 4 or 8 hours in the visually deprived and/or contralateral eye. The OD versus OS expression levels of *Cry1* were not equivalent at ZT 16 and ZT 20, but comparable expression levels occurred OU in the other assayed genes (Tables 2–4, Fig. 2, Supplementary Table S4).

Unilateral Minus 10-D Lens Wear

In retina/RPE, unilateral defocus from wearing a minus 10-D lens eliminated the changes in gene expression over time for two genes, *Opn4m* and *Cry1* (Tables 2 and 3, Fig. 3, Supplementary Table S5). Most time-varying genes showed highest expression levels at the same time in both eyes. Compared to the maximum expression in eyes with nonrestricted vision OU, the actual times of highest expression were the same or were shifted by up to 8 hours (Table 4). The expression

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Table 2. Gene Expression Over 24 Hours: Summary of Statistically Significant Effects

	Condition															
	Nonrestricted Vision OU			Diffuser OD				Minus 10-D Lens OD				Plus 10-D Lens OD				
Gene	Eye	Time	Eye-Time	Eye	Time	Eye-Time	Bilateral Effect	Eye	Time	Eye-Time	Bilateral Effect	Eye	Time	Eye-Time	Bilateral Effect	
Retina/RPE																
Opn4m	—	X	—	—	—	—	XX	—	—	—	XX	X	—	—	XX	
Clock	—	X	—	X	—	—	XX	X	X	—	—	—	—	—	XX	
Npas2	—	X	—	—	—	—	XX	—	X	—	—	—	X	—	—	
Per3	—	X	—	—	X	X	—	—	X	—	—	—	X	—	—	
Cry1	—	X	—	—	X	—	—	—	—	—	XX	—	—	—	XX	
Arntl	—	X	—	—	X	—	—	—	X	—	—	—	X	—	—	
Mntr1a	—	X	—	—	—	—	XX	—	X	—	—	—	X	X	—	
Choroid																
Opn4m	X	X	—	—	X	—		X	X	—	—	X	—	—	XX	
Clock	—	X	X	—	—	—	XX	X	—	—	XX	—	—	—	XX	
Npas2	—	X	—	—	X	—	—	X	X	—	—	—	X	—	—	
Per3	—	X	—	—	X	—	—	X	X	—	—	X	X	X	—	
Cry1	—	X	—	X	X	—	—	X	X	X	—	—	—	—	XX	
Arntl	—	X	X	—	X	X	—	X	X	X	—	—	X	—	—	

Mntr1a was detectable in retina but not in choroid by qPCR.
X indicates significant effect over time, OD versus OS, and/or for the interaction of eye-time based on the Bonferroni criteria of $P \leq 0.0071$ for retina, $P \leq 0.0083$ for choroid;
XX, bilateral effect based on loss of variation OU over time with visual alteration only in OD;
—, no significant effect.

Table 3. Gene Expression Over Time: OD Versus OS

	Condition							
	Nonrestricted Vision OU		Diffuser OD		Minus 10-D Lens OD		Plus 10-D Lens OD	
Gene	OD vs. OS	ZT Times When OD ≠ OS	OD vs. OS	ZT Times When OD ≠ OS	OD vs. OS	ZT Times When OD ≠ OS	OD vs. OS	ZT Times When OD ≠ OS
Retina/RPE								
Opn4m	OD = OS		OD = OS		OD = OS		**OD < OS**	**4, 8, 12**
Clock	OD = OS		**OD < OS**	**0**	**OD < OS**	**20**	OD = OS	
Npas2	OD = OS		OD = OS		OD = OS		OD = OS	
Per3	OD = OS		**OD < OS**	**20**	OD = OS		OD = OS	
Cry1	OD = OS		OD = OS		OD = OS		OD = OS	
Arntl	OD = OS		OD = OS		OD = OS		OD = OS	
Mntr1a	OD = OS		OD = OS		OD = OS		**OD < OS: 4, 8** **OD > OS: 16**	**4, 8, 16**
Choroid								
Opn4m	OD = OS		OD = OS		**OD < OS**	**16, 20**	**OD < OS**	**0**
Clock	**OD < OS**	**8**	OD = OS		**OD < OS**	**0, 8**	OD = OS	
Npas2	OD = OS		OD = OS		**OD < OS**	**0, 4, 8**	OD = OS	
Per3	OD = OS		OD = OS		**OD < OS**	**8**	**OD < OS**	**8, 12, 16**
Cry1	OD = OS		**OD < OS**	**16, 20**	**OD < OS**	**0**	OD = OS	
Arntl	**OD < OS: 4** **OD > OS: 16**	**4, 16**	OD = OS		**OD < OS**	**0, 4, 8**	OD = OS	

Bold font highlights the genes and the corresponding times when the expression levels differ between OD and OS. See Supplementary Tables S1-S8. ZT 0 = light phase onset.

Table 4. Times of Highest Gene Expression

	Nonrestricted Vision OU		Diffuser OD		Minus 10-D Lens OD		Plus 10-D Lens OD	
Gene	OD	OS	OD	OS	OD	OS	OD	OS
Retina/RPE								
Opn4m	8	8	—	—	—	—	—	—
Clock	8	8	—	—	16	16	—	—
Npas2	12	12	—	—	12	12	12	12
Per3	0	0	0	0	20	0	20	20
Cry1	12	12	8	8	—	—	—	—
Arntl	8	8	8	8	12	12	12	12
Mntr1a	20	20	—	—	20	20	20	20
Choroid								
Opn4m	8	8	8	4	0	0	—	—
Clock	12	12	—	—	—	—	—	—
Npas2	12	12	12	12	12	12	12	12
Per3	0	0	4	4	0	20	0	20
Cry1	12	12	4	4	12	12	—	—
Arntl	12	12	12	4	12	12	16	12

Time of highest numerical value for gene expression with a time effect, from Supplementary Tables S1 to S8. ZT 0 = light phase onset. —, no time effect based on the Bonferroni criteria of $P \leq 0.0071$ for retina, $P \leq 0.0083$ for choroid.

levels were comparable between the two eyes of chicks in the minus lens cohort, except for *Clock* at ZT 20 that was lower in the retina/RPE of the eye with altered visual input relative to its contralateral eye (Tables 2 and 3, Fig. 3, Supplementary Table S5).

Wearing a minus 10-D lens abolished the time variable expression in the choroid of *Clock* but not that of other genes (Tables 2 and 3, Fig. 3, Supplementary Table S6). The times of peak gene expression levels shifted by 8 hours for *Opn4m* OU and by 4 hours for *Per3* in the contralateral eye (Table 4), relative to eyes with nonrestricted vision OU. Compared to their contralateral eyes, the expression levels of all genes in the minus lens group were lower in the choroid at one or more times (Tables 2 and 3, Fig. 3, Supplementary Table S6).

Unilateral Plus 10-D Lens Wear

In retina/RPE, the expression of three genes (*Opn4m*, *Clock*, and *Cry1*) showed no variability over time (Tables 2 and 3, Fig. 4, Supplementary Table S7). The times of highest expression of time-varying genes were the same for both eyes, which were either identical to or shifted by 4 hours from the retinas of eyes with nonrestricted vision OU (Table 4, Fig. 4). The expression level of *Opn4m* was lower in retinas of chicks in the plus lens group at ZT 4, ZT 8, and ZT 12. For Mtnr1a, there was an OD-OS difference in expression at ZT 4, ZT 8, and ZT 16, but the direction of the difference depended on time (Tables 2 and 3, Fig. 4, Supplementary Table S7).

As in retina/RPE under a plus 10-D lens, the choroidal levels of the same three genes (*Opn4m*, *Clock*, and *Cry1*)

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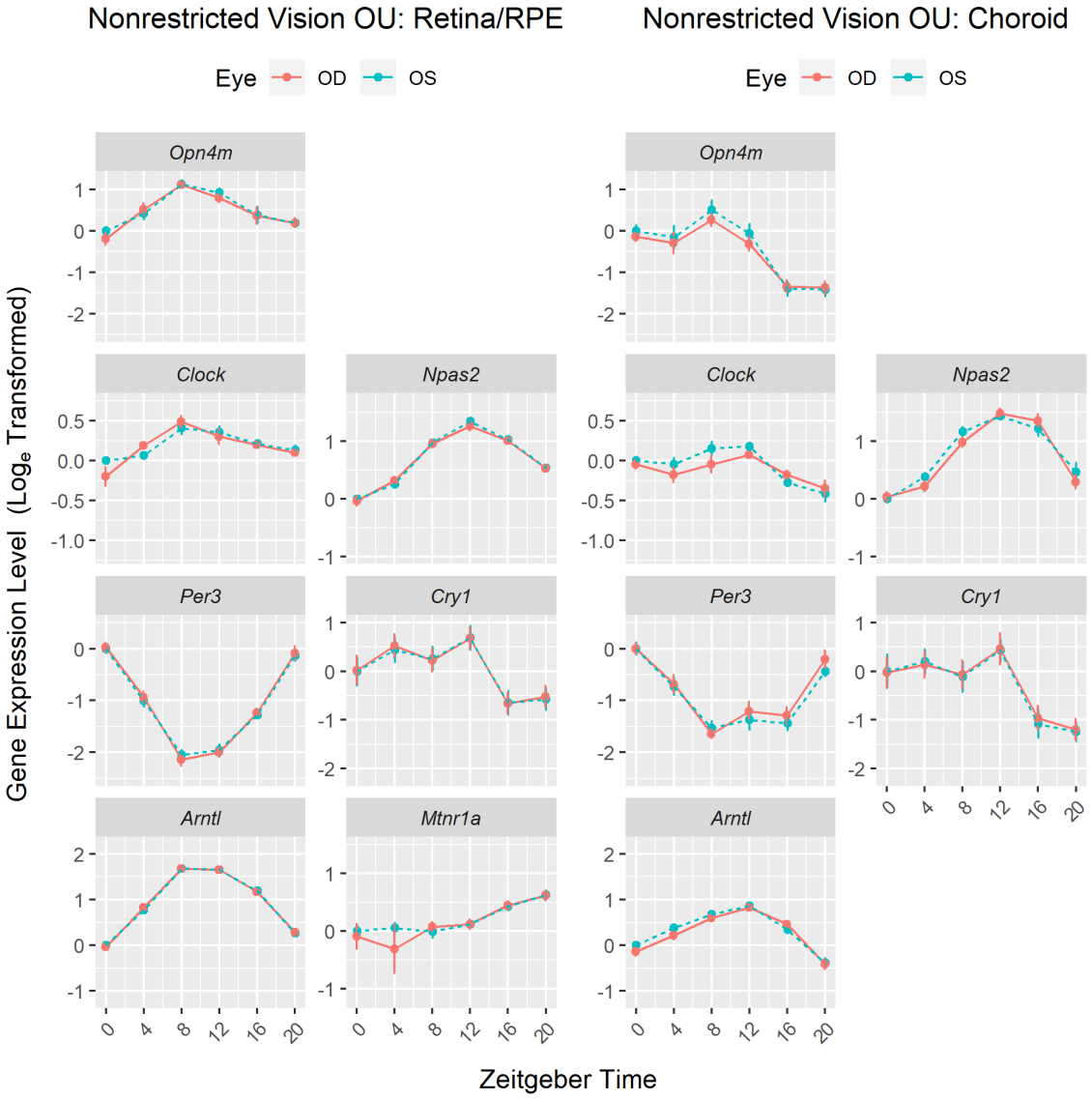

Figure 1. Nonrestricted vision OU. Expression of clock and circadian rhythm–related genes over 24 hours in the retina and choroid of chicks with nonrestricted visual input OU. Because of nonnormal data distribution, the data were log$_e$ transformed. The mean transformed OS value at ZT 0 was subtracted from the transformed expression values for each gene and each eye at each time point, such that the mean value of the left eye across individuals is 0 at ZT 0. To aid visualization, the y-axis is scaled differently between genes, but individual genes are represented at the same scale between tissues and between the visual conditions. See Supplementary Tables S1 and S2 for data and statistical analyses. *Red symbols*, OD; *blue symbols*, OS.

did not vary over time (Tables 2 and 3, Fig. 4, Supplementary Table S8). Of those that varied, their peak choroidal expression levels occurred at the same time or with a 4-hour shift compared to those of eyes with nonrestricted vision OU (Table 4, Fig. 4, Supplementary Table S8). The choroidal expression level of *Opn4m* was lower at ZT 0, and that of *Per3* was lower at ZT 8, ZT 12, and ZT 16 compared to the contralateral eyes (Tables 1 and 2, Fig. 4, Supplementary Table S8). No other OD-OS differences were identified.

Discussion

Stimulated by the experimental evidence implicating circadian rhythms in the mechanisms of refractive development, we assayed the expression of clock and circadian rhythm–related genes in chicks over a full 24-hour period following unilateral alteration of visual input by methods known to induce ametropias. In our prior findings and in the experimental refraction literature, study designs commonly compare results in eyes with unilateral visual impairment to those of contralateral control eyes. Accordingly, we optimized the technical aspects of our assays to emphasize OD-OS differences in gene expression, as well as the established daily cycling of these genes in retina and other tissues. The assayed genes included melanopsin (*Opn4m*), transcription activators (*Clock*, *Npas2*, and *Arntl*) and transcription repressors (*Per3* and *Cry1*) of the circadian clock,[20,21] and one of the melatonin receptor subtypes (*Mntr1a*). While some evidence implicates a potential role for conventional photoreceptors in refractive control mechanisms,[37–39] we addressed here the photopigment melanopsin specifically because of its role in regulating circadian rhythms.[13]

For this investigation, we selected clock and circadian rhythm–related genes whose expression we previously found altered in lens-induced myopia using microarrays[16] and confirmed in form-deprivation myopia.[15] These

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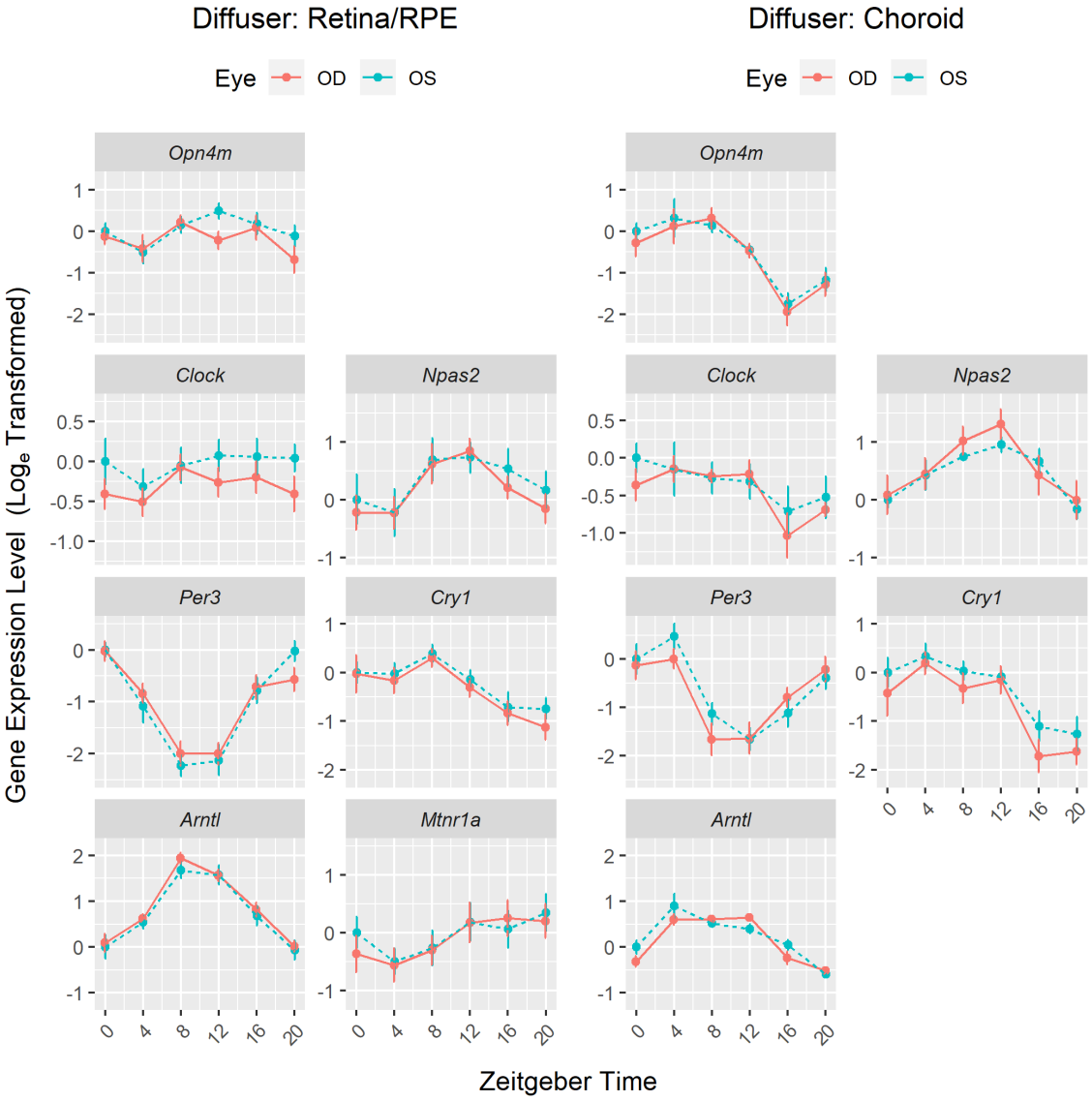

Figure 2. Diffuser wear OD. Expression of clock and circadian rhythm–related genes over 24 hours in the retina and choroid of chicks wearing a diffuser OD. Because of nonnormal data distribution, the data were log$_e$ transformed. The mean transformed OS value at ZT 0 was subtracted from the transformed expression values for each gene and each eye at each time point, such that the mean value of the left eye across individuals is 0 at ZT 0. To aid visualization, the y-axis is scaled differently between genes, but individual genes are represented at the same scale between tissues and between the visual conditions. See Supplementary Tables S3 and S4 for data and statistical analyses. *Red symbols*, diffuser-wearing eye (OD); *blue symbols*, contralateral eye with intact vision (OS).

genes represent only a subset of known clock genes in the primary transcription-translation feedback loop of the circadian clock; as examples from known avian genes, we assayed one of two period genes, one of two cryptochrome genes, none of the genes in the secondary transcription-translation feedback loop, one of two melanopsin genes, and one of two melatonin receptor genes.[24,25,35] As discussed below, we cannot exclude influences of visual input on the expression of nonstudied circadian genes or their potential to interact with the genes assayed here.

Opn4m Expression in the Choroid

Other than our meeting report of the early results of this study (Stone RA et al., *IOVS* 2018;59:ARVO E-Abstract 5054), *Opn4m* had not been identified directly in choroid earlier, and its origin and function are currently unknown. Melanopsin has been identified in human lens epithelial cells, where it seemingly regulates melatonin synthesis.[40] The soma of trigeminal neurons express melanopsin,[41] and these neurons might innervate the choroid, which has peripheral sensory nerves.[42,43] Whether light activates melanopsin-expressing trigeminal neurons is equivocal.[41,44] Other potential sources are choroidal blood vessels or melanocytes as melanopsin has been identified in mouse aorta and in cultured chicken melanocytes.[45,46] Regarding potential function, the normal light-induced increase in choroidal thickness in mice is not observed with systemic knockout of melanopsin,[47] suggesting that melanopsin might regulate diurnal or defocus-induced choroidal thickness alterations.[13,29] Thus, the roles of melanopsin in the choroid are yet to be established, including the intriguing possibility that the choroid might be photosensitive.

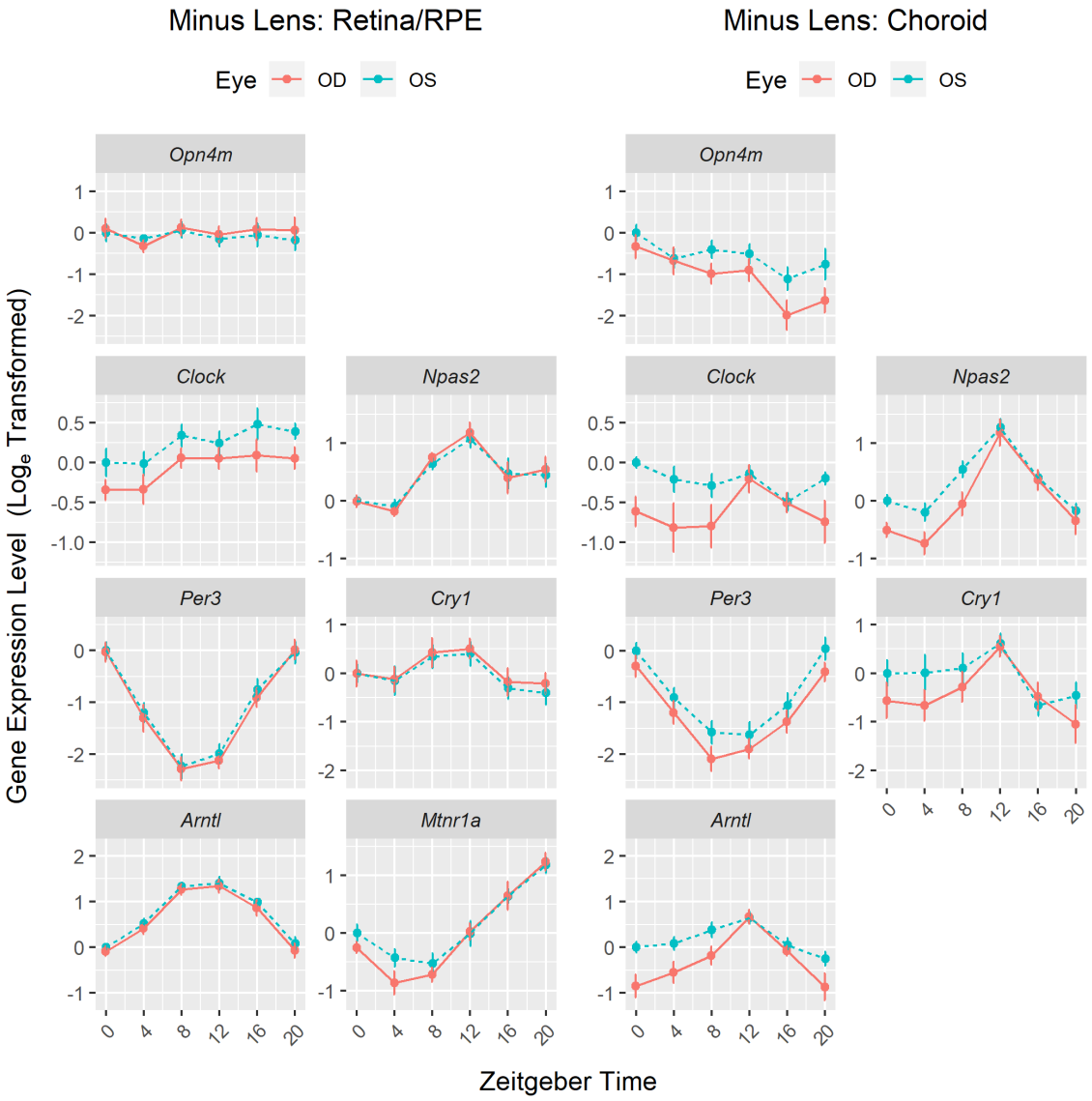

Figure 3. Minus 10-D lens wear OD. Expression of clock and circadian rhythm–related genes over 24 hours in the retina and choroid of chicks wearing a minus 10-D spectacle lens OD. Because of nonnormal data distribution, the data were log$_e$ transformed. The mean transformed OS value at ZT 0 was subtracted from the transformed expression values for each gene and each eye at each time point, such that the mean value of the left eye across individuals is 0 at ZT 0. To aid visualization, the y-axis is scaled differently between genes, but individual genes are represented at the same scale between tissues and between the visual conditions. See Supplementary Tables S5 and S6 for data and statistical analyses. *Red symbols*, lens-wearing eye (OD); *blue symbols*, contralateral eye with intact vision (OS).

Interpreting Gene Expression Data

In the retina/RPE and choroid of chicks with nonrestricted visual input OU, the expression levels of each of these genes varied over time during the 24-hour day (Fig. 1, Table 1). While *Mtnr1a* was detectable only in retina/RPE, the other genes showed variable expression patterns over 24 hours in both retina/RPE and in choroid of eyes with nonrestricted vision OU in patterns that generally were similar between the two tissues (Fig. 1). Altering visual input modifies the expression of the assayed genes in complex patterns that vary by gene, method of image alteration (diffuser, minus lens, or plus lens), tissue (retina/RPE versus choroid), and time. The gene expression changes affected not only experimental eyes with altered vision but also, unexpectedly, contralateral "control" eyes with nonrestricted vision. The breadth of the gene effects links circadian clock function to established visual parameters that govern eye growth.

Retinal/RPE and choroidal tissues were isolated 24 to 48 hours after the initiation of visual alteration, and the altered gene expressions identified here thus reflect changes occurring at the onset of visually induced ametropias. Because the molecular alterations identified at myopia onset or during myopia progression are not identical,[15–17,19,48,49] longer duration experiments will be needed to learn whether similar effects on clock and circadian rhythm–related genes occur as ametropias progress at later times.

For melanopsin (*Opn4m*) in the retina/RPE, the variation in gene expression over time was lost in eyes wearing a diffuser and for both lens conditions, not only in the experimental eyes but also in contralateral eyes. In the choroid, the visual effects on melanopsin expression were

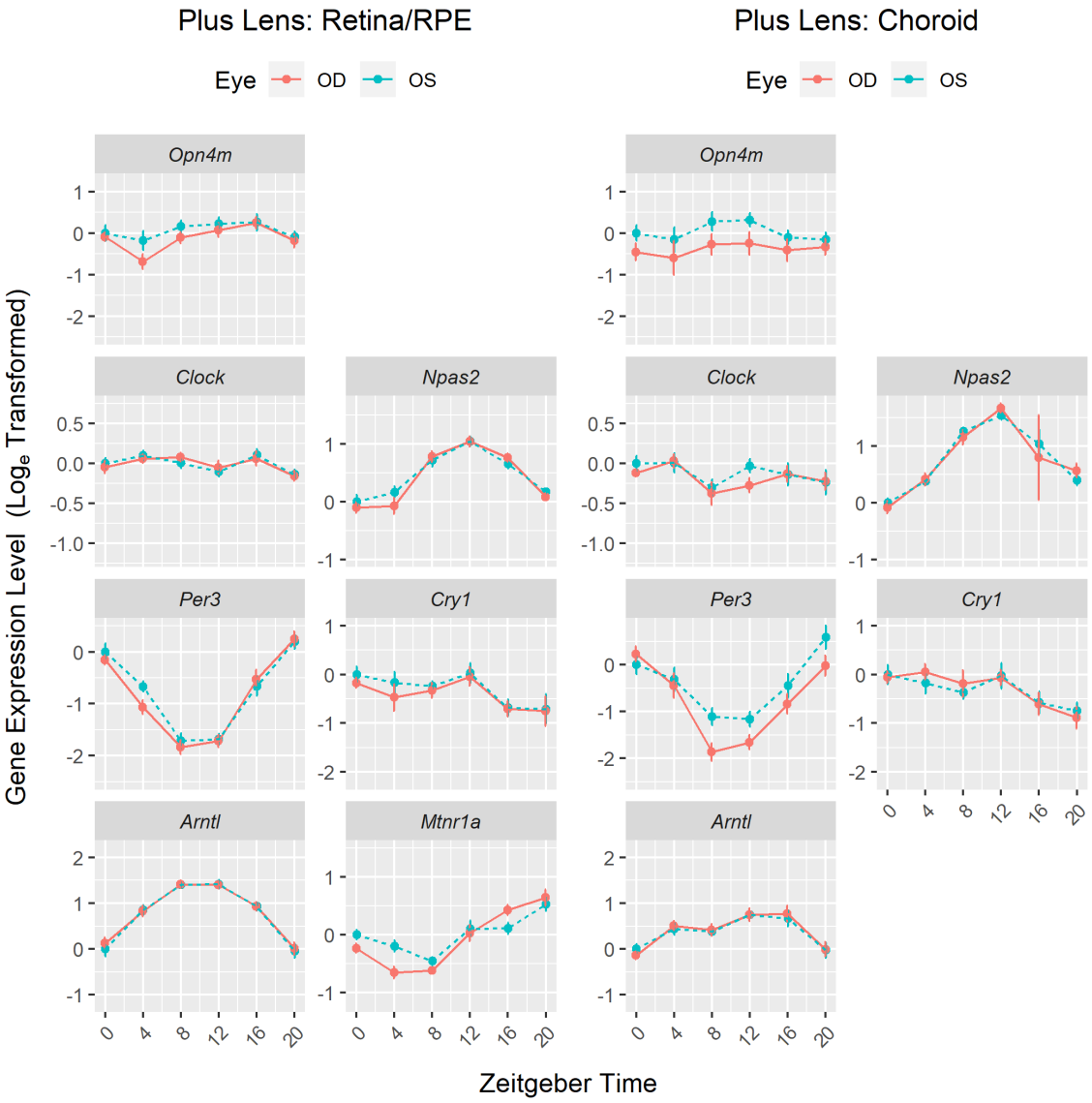

Figure 4. Plus 10-D lens wear OD. Expression of clock and circadian rhythm–related genes over 24 hours in the retina and choroid of chicks wearing a plus 10-D spectacle lens OD. Because of nonnormal data distribution, the data were log$_e$ transformed. The mean transformed OS value at ZT 0 was subtracted from the transformed expression values for each gene and each eye at each time point, such that the mean value of the left eye across individuals is 0 at ZT 0. To aid visualization, the y-axis is scaled differently between genes, but individual genes are represented at the same scale between tissues and between the visual conditions. See Supplementary Tables S7 and S8 for data and statistical analyses. *Red symbols*, lens-wearing eye (OD); *blue symbols*, contralateral eye with intact vision (OS).

different. The choroidal melanopsin expression continued to vary over time in eyes of chicks wearing a diffuser or minus lens but was stable over time in eyes of the plus lens group. In mice, knockout of melanopsin gene expression induces an exaggerated myopia response to form deprivation (Chakraborty R, et al., *IOVS* 2015;56:ARVO E-Abstract 5843). The current gene expression results justify seeking a direct influence of melanopsin on refractive development, including potential influences on the ocular rhythms that modulate eye growth.[13,50,51]

Of the circadian clock genes, only *Per3* and *Arntl* continued to vary by time both in retina/RPE and in choroid under all three altered visual conditions. The expression of the other clock genes (*Clock*, *Npas2*, and *Cry 1*) no longer consistently varied over time in either tissue or under each altered visual condition. The visual image, as distinct from light per se, impacts the daily expression of specific circadian clock genes.

The expression of *Mntr1a* no longer varied with time in the retina/RPE of chicks wearing a diffuser but continued to vary with time in chicks wearing either minus or plus lenses. Exogenously administered melatonin exerts some effects on the growth of normal and form-deprived chick eyes,[35] and human myopic subjects have elevated serum melatonin levels.[52] However, retinal melatonin and retinal dopamine interact reciprocally during the diurnal cycle, and retinal dopamine has been implicated repeatedly in the mechanism of myopia.[13,53,54] Retinal dopaminergic cells express clock genes, interact with melanopsin containing neurons, and act to entrain endogenous retinal rhythms to the light/dark cycle.[13] While needing direct study, the effects of altered vision on melatonin receptor expression may be secondary

to effects on retinal dopamine and/or the expression of melanopsin or retinal clock genes.

Because we assayed only a subset of circadian clock, melanopsin, and melatonin receptor genes, we cannot exclude an impact from nonassayed but related genes. Altered vision produces complex effects on circadian gene expression that include variable outcomes between genes, tissue, and type of visual alteration; irregular and not clearly cyclical patterns of some of the gene expression patterns over the day; persistent variability over 24 hours of only some genes; and modified expression only at specific times for particular genes. Because our study represents the first day of the responses to visual modification, these "nonregular" alterations could arise from transitioning during this interval of circadian signaling from that occurring during nonrestricted vision to that occurring with persistent perturbed visual input. Alternatively, these "nonregular" findings could arise from complex, perhaps complementary or partial interactions between the different forms of particular circadian genes, only one of which was measured. Clearly, more research is needed to understand the effects of visual input on the clock and circadian rhythms in the eye.

Despite these considerations, visual perturbations known to induce experimental myopia or hyperopia promptly affect circadian gene expression in retina/RPE and in choroid, tissues believed to govern refractive development. A transcription activator with numerous pleiotropic effects, *Bmal1* (brain and muscle arnt-like protein 1; also termed *Arntl*) is a nonredundant component of the mammalian circadian clock. Knockout in retina of *Bmal1* induces bilateral myopia in mice even with nonrestricted visual input and supports a potential role of circadian and related mechanisms in regulating refraction.[55] To our knowledge, this study in mice is the only prospective investigation of refractive development in mammals with a known circadian signaling abnormality.[55]

Bilateral Effects

The growth and refraction responses to unilateral diffuser or lens wear affect primarily the experimental eyes,[1] with only minor refractive effects on contralateral eyes with nonrestricted visual input.[17,56] Surprisingly, unilateral diffuser or lens wear affected the expression of some clock and circadian rhythm–related genes bilaterally by the criterion of bilateral loss of variation over time following unilateral visual alteration (Table 2, Figs. 1-4). Our experimental design did not permit unambiguous identification of quantitative differences in gene expression between the contralateral eyes of chicks with unilateral visual alteration and the eyes of chicks with nonrestricted vision OU. In contrast to the symmetrical gene expression levels in the chicks with nonrestricted vision, OD-OS differences in gene expression developed most frequently in the choroid after minus lens wear, but depending on the gene, these occurred at one to three of the sampling times in each tissue and visual impairment (Table 3, Fig. 3).

Given the interocular differences in growth and refraction from diffuser or lens wear,[1] an explanation for bilateral effects on clock and circadian rhythm–related gene expression is not presently apparent. Perhaps, pathways directly responsive to visual input may modify a putative general effect of clock-dependent signaling to normalize growth in eyes with nonrestricted vision contralateral to eyes wearing a diffuser or lens. Alternatively, both clock-dependent and clock-independent signaling pathways may exist and only partly interact to regulate refractive development. Given the apparent roles of diurnal rhythms of ocular dimensions in governing eye growth,[13] further study clearly is needed to learn the implications of the bilateral effects on ocular clock and circadian rhythm–related genes in eyes with unilateral experimental ametropias and to learn whether bilateral effects on these genes contribute to the apparent binocular similarity of refraction and refractive errors in human eyes.

Light and Refraction

Clinically, the long-standing notion that inadequate lighting or insufficient exposure to the outdoors might comprise an etiology of myopia and that outdoor light exposures are protective against myopia[4–6,57] is now generating much interest.[13,58,59] Whether the antimyopia properties of outdoor exposures relate to light intensity, as often suggested, to circadian entrainment by light or to some other property of being outdoors remains to be proven.[7,9,15,60,61]

The impact of light exposure on experimental refractive development is frequently studied in chicks. As examples, rearing under constant light elongates the chick eye while flattening the cornea; hyperopia results because marked corneal flattening so reduces corneal power that images focus behind the retina despite the enlongated eye.[62–65] A limited period of daily darkness inhibits this response, an early suggestion of a circadian effect.[66] Besides photoperiod length, varying light intensity impacts eye development, studied initially in chicks but also in other species.[12,64,67–72] Long-term rearing of chicks in low-intensity light even induces myopia.[71]

These prior reports, though, largely do not provide a biologic mechanism for how ambient lighting might influence refractive development. Recent clinical reports on the antimyopia effects of outdoor exposures hypothesize increased retinal dopamine release, but an explanation involving dopamine was not identified in the only available study that actually measured retinal dopamine outdoors in an experimental myopia model.[15]

Nonetheless, genes identified in a human genome-wide association meta-analysis suggest light-related retinal signaling as a mechanism underlying ametropias.[73] Further, another recent meta-analysis of human genome-wide association studies of refractive errors, applying Gene Ontology, identified "Circadian Rhythm" and "Circadian Regulation of Gene Expression" as two of the enriched gene sets.[74] Besides clinical genetics, the results here and our prior findings on altered clock and circadian rhythm–related genes in myopia,[15,16,48] support the possibility that the circadian clock and melanopsin may provide the mechanistic link between environmental light exposures and refractive development.[13]

A Potential Unification of Refractive Mechanisms?

That altered vision by diffuser or lens wear induces refractive errors and also alters the expression of circadian rhythm–related genes in both retina/RPE and choroid provides further evidence that visual mechanisms regulating eye growth interact with ocular circadian biology. The results here, the first detailed investigation of gene expression patterns over 24 hours at the onset of ametropia, buttress

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the notion that circadian biology may be essential to understand refractive development of the juvenile eye.

Inducing ametropia in animals by altering visual input impacts a perplexing range of signaling molecules and pathways using conventional pharmacologic methods or genome-wide screens by microarray, RNA-sequencing, or other techniques.[17,48,49,59,75,76] Importantly, the circadian clock is now known to influence many and varied biological processes since the diurnal expression of 10% to 40% or more of protein coding genes overall is under diurnal control in organ-dependent patterns.[77,78] If circadian clock disruption alters the diurnal expression of a substantial number of retinal/RPE and/or choroidal genes, studying mediators downstream of clock genes could provide a framework for understanding the breadth of the signaling pathways influencing refraction, beyond just cataloging them.

Artificial lighting distorts natural light exposures at day and/or at night and desynchronizes endogenous circadian rhythms from the environmental light/dark cycle. Such circadian desynchronizations in contemporary societies now seem to contribute to many disorders, including certain cancers, neurologic diseases, obesity, diabetes, and disorders of sleep and mood.[79–83] The increasing prevalence of myopia, particularly in developed and developing countries,[84] is both worrying and unexplained. As we have suggested and reviewed,[13,55,59,85] the patterns of ambient lighting in contemporary societies may contribute to the increasing prevalence of myopia through a mechanism involving desynchronization of endogenous ocular circadian rhythms from the external light/dark cycle. If so, direct study of circadian biology during childhood could ultimately lead to much-needed mechanistic understanding of ametropias and to clinically acceptable, behavioral therapies based on modifying circadian dysregulations that may contribute to ametropias.

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