Image Defocus and Altered Retinal Gene Expression in Chick: Clues to the Pathogenesis of Ametropia

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PURPOSE. Because of the retina’s role in refractive development, this study was conducted to analyze the retinal transcriptome in chicks wearing a spectacle lens, a well-established means of inducing refractive errors, to identify gene expression alterations and to develop novel mechanistic hypotheses about refractive development.

METHODS. One-week-old white Leghorn chicks wore a unilateral spectacle lens of +15 or −15 D for 6 hours or 3 days. With total RNA from the retina/retinal pigment epithelium, RPE, chicken gene microarrays were used to compare gene expression levels between lens-wearing and contralateral control eyes (n = 6 chicks for each condition). Normalized microarray signal intensities were evaluated by analysis of variance, using a false discovery rate of <10% as the statistical criterion. Selected differentially expressed genes were validated by qPCR.

RESULTS. Very few retina/RPE transcripts were differentially expressed after plus lens wear. In contrast, approximately 1300 transcripts were differentially expressed under each of the minus lens conditions, with minimal overlap. For each condition, low fold-changes typified the altered transcriptome. Differentially regulated genes under the minus lens conditions included many potentially informative signaling molecules and genes whose protein products have roles in intrinsic retinal circadian rhythms.

CONCLUSIONS. Plus or minus lens wear induce markedly different, not opposite, alterations in retina/RPE gene expression. The initial retinal responses to defocus are quite different from those when the eye growth patterns are well established, suggesting that different mechanisms govern the initiation and persistence or progression of refractive errors. The gene lists identify promising signaling candidates and regulatory pathways for future study, including a potential role for circadian rhythms.

The molecular mechanisms governing normal refractive development or underlying the development of refractive errors are poorly understood. Genetic factors have been implicated in both myopia1 and hyperopia.2 It has been long maintained that myopia in particular arises from complex interactions of both environmental and genetic influences, but the relative roles of environment versus genes remain to be fully defined.3–5 Contemporary laboratory and clinical research indicates that the visual image modulates refractive development because image blur or defocus alters eye growth; the retina in large part governs these processes.6–9 Much laboratory research in this area has used one of two experimental approaches: (1) form-deprivation myopia, in which wearing an image-diffusing goggle or lid suturing blurs the retinal image and induces ipsilateral myopia; and (2) wearing defocusing spectacle lenses to shift the image plane in front of or behind the retina, inducing compensating changes in eye growth to reposition the retina at the image location. With spectacle lens wear, plus (i.e., convex or [+]) spectacle lenses shift the visual image forward and slow eye growth to permit the anatomic adjustments for distant images to focus in the retina; minus (i.e., concave or [−]) spectacle lenses shift the visual image farther back and accelerate eye growth, also permitting distant images to focus on the retina. These image-related adjustments in ocular growth include not only changed scleral growth but also altered choroidal thickness: choroidal thinning with goggles or (−) lens wear that stimulate eye growth and choroidal thickening after (+) lens wear.10 The early kinetics of choroidal thickness responses vary somewhat between these eye growth models in chick.11 With spectacle lens removal, eyes previously beneath a (+) spectacle lens are hyperopic; those previously beneath a (−) spectacle lens are myopic.9 Form-deprivation myopia occurs in children,12 and the eyes of young adults adjust axial dimensions to acute defocus.13 The extent to which the signaling and molecular mechanisms that underlie the responses to goggles or spectacle lens wear account for clinical ametropia is unknown; spectacle lens wear may be most useful for studying emmetropization mechanisms.9,14 Although it is unclear whether emmetropia is the physiologic end point of human refractive development.

Through such experimental approaches, numerous neurotransmitters or other signaling molecules have been implicated in the pathway(s) linking visual input and refractive development.1 However, an organized framework is not available for the retinal signaling that underlies mechanisms for either emmetropization or the development of refractive errors. Visual stimuli that alter eye growth induce changes in gene expression at the level of the transcriptome (molecular signatures) in the retina.15 We hypothesized that these molecular signatures not only can identify important retinal mediators of refractive development but also can identify signaling pathways or networks, which may provide leads for novel approaches to understand laboratory and clinical conditions.
Paralleling a protocol we had used previously to study form-deprivation myopia, we profiled gene expression in the combined retina and retinal pigment epithelium (RPE) of chicks wearing a unilateral spectacle lens of (+) or (−) power. We conducted these assays after either 6 hours or 3 days of lens wear. The shorter duration activates a retina-to-sclera signaling pathway that subsequently modulates scleral biochemistry, indicating initiation of at least some aspect(s) of the eye growth signaling pathway; the longer duration corresponds to a time when changes in refraction and eye size are manifest. Because of their links to perturbed eye growth, rapid alterations in retinal dopamine physiology and the immediate early gene EGR1 have been studied as potential individual differences in gene expression complicate statistical approaches to interbird comparisons, and our bioinformatics approach accordingly emphasized the experimental-to-contralateral control eye comparison in individual birds.

METHODS

One-day-old white Leghorn chicks (Moyer’s Chick, Inc., Quakertown, PA) were maintained under a 12-hour light–dark cycle, under incandescent lighting (General Electric, Fairfield, CT) with irradiance of approximately 1600 μW/cm² at chick eye level. They received food (Purina Chick Chow, Indianapolis, IN) and water ad libitum. When the chicks were 1 week of age and under inhalation ether anesthesia, a mixture of ketamine (20 mg/kg) and xylazine (5 mg/kg), and both eyes were enucleated and opened for comparison in individual birds.

Microarray Target Preparation and Hybridization

Microarray services were provided by the Penn Microarray Facility of the University of Pennsylvania School of Medicine. All protocols were conducted as described in the manufacturers’ manuals (Ovation Manual, NuGen Technologies, Inc., San Carlos, CA; GeneChip Expression Analysis Technical Manual, Affymetrix Inc., Santa Clara CA). Briefly, 100 ng of total RNA was converted to first-strand cDNA using reverse transcriptase primed by a poly(T) oligomer that incorporated a synthetic RNA sequence. Second-strand cDNA synthesis was followed by ribo-SPIA (Single Primer Isothermal Amplification, NuGEN Ovation kit) for linear amplification of each transcript, and the resulting cDNA was fragmented, assessed with the bioanalyzer, and biotinylated. cDNA yields ranged from 7.1 to 10.2 μg, and 3.75 μg was added to hybridization cocktails (Affymetrix), heated at 99°C for 2 minutes, and hybridized for 16 hours at 45°C to chicken gene microarrays (Chicken Genome GeneChips; Affymetrix) (http://www.osa.sunysb.edu/udmf/ArraySheets/chicken_datsheet.pdf). The microarrays were then washed at low (6× SSEP) and high (100 mM MES and 0.1 M NaCl) stringency and stained with streptavidin-phycocerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycocerythrin stain. A confocal scanner was used to collect fluorescence signal after excitation at 570 nm.

Bioinformatics Analyses

Hybridization signals were quantified (Command Console and Expression Console; Affymetrix) for each probe: default values provided by Affymetrix were applied to all analysis parameters. Border pixels were removed, and the average intensity of pixels within the 75th percentile was computed for each probe. Probe intensities were exported in .cel file format (Affymetrix).

The .cel files were imported into genomics software (Genomics Suite, ver. 6.4; Partek Inc., St. Louis, MO). To permit comparison between arrays, RMA (robust multiarray average) was applied to yield background-adjusted, normalized, log-transformed signal intensities. We performed a three-way, mixed-model analysis of variance (ANOVA; factors: time, [6 hours, 3 days]; lens, +, −, or [i.e., minus, no or plus lens]; chick ID, [random effect], with an interaction term [time × lens]). With the ANOVA, four pairwise comparisons were also calculated: 6 hours, + vs. 0; 6 hours, − vs. 0; 3 days, + vs. 0; and 3 days, − vs. 0. All resulting P values were corrected for multiple comparisons using the Benjamini-Hochberg step-up method to yield false discovery rates (FDRs), as implemented in the genomics software (Partek). An FDR of <10% was considered significant and was used as the primary cutoff criterion for identifying differentially expressed transcripts. For the pairwise comparisons, fold-change in gene expression was also calculated, comparing the lens-wearing to contralateral control eye.

Using Affymetrix probe set identifiers, two web-based tools were used to evaluate the differentially expressed transcripts meeting the above <10% FDR statistical criterion. VENNY (http://bioinfopgcncbscies/tools/venny/index.html) was used to generate the Venn diagram of the overlapping transcripts between the two lens conditions and two times (Fig. 1). To evaluate potential networks and pathways implicated by the differentially expressed transcripts, we used pathway analysis (IPA ver. 8.6-3003, build 9247; Ingenuity Systems, Redwood, CA; http://www.ingenuity.com/) with the following general analysis settings: chicken genome array as the reference set, direct and indirect
relationships, endogenous chemicals included, 35 molecules/network, 25 networks, all data sources and species, nervous system tissues/CNS (central nervous system) cell lines with a relaxed filter on the molecules, and their relationships. For conformity in data reporting, we used the Affymetrix notations for gene symbols throughout.

Real-Time Quantitative RT-PCR
To ascertain the reliability of the microarray results, we conducted both biological and technical validations of the expression profiling for selected known genes, using real-time quantitative reverse transcription-polymerase chain reaction (qPCR). Biological replicates are assays on animals different from those subjected to microarray analysis but reared and processed contemporaneously; here, we used the retina/RPE from the two other chicks reared contemporaneously under each of the four conditions. Technical validations are measurements of aliquots from the same biological specimen with a different measuring technique; here, for technical validations, we used residual RNA from the retina/RPE assayed by microarray. For both types of qPCR validations, 1 µg of retina/RPE RNA was converted into cDNA (Superscript III First Strand cDNA Synthesis Supermix for qPCR; Invitrogen).

For the biological validations, we selected transcripts of known genes with a fold-change of approximately ±1.4 in either the up- or downregulated direction, including some genes that were differentially expressed under more than one condition. Within that group, we included vasoactive intestinal peptide (VIP), noggin (NOG), and bone morphogenetic protein 2 (BMP2), because of their relation to findings described in the Methods (i.e., ANOVA corrected for multiple comparisons with FDR <10%). See Supplementary Tables S1 to S4 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6727/-/DCSupplemental) for complete gene lists.

For the technical validations, we selected transcripts of known genes with a fold-change of approximately ±1.4 in either the up- or downregulated direction, including some genes that were differentially expressed under more than one condition. Within that group, we included vasoactive intestinal peptide (VIP), noggin (NOG), and bone morphogenetic protein 2 (BMP2), because of their relation to findings described in the Methods (i.e., ANOVA corrected for multiple comparisons with FDR <10%). See Supplementary Tables S1 to S4 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6727/-/DCSupplemental) for complete gene lists.

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RESULTS
Refractive Responses
As described,11 spectacle lens wear altered ocular growth to shift the retina toward the image plane of distant objects; at 3 days, the optical compensation was somewhat more advanced for +15-D lens wear (+11.8 ± 1.3 D; mean ± SEM) than for −15-D lens wear (−8.9 ± 0.7 D), although eye growth had not yet completely compensated for lenses of either sign. Eyes beneath the (+) lens were shorter than contralateral eyes, because of a shorter vitreous chamber (by 0.47 ± 0.05 mm); eyes beneath the (−) lens were longer than their contralateral eyes, because of a longer vitreous chamber (by 0.35 ± 0.08 mm).

Alterations in Retina/RPE Gene Expression
With the chicken gene microarrays (Affymetrix), we profiled gene expression in the retina/RPE of chicks wearing a unilateral +15- or −15-D spectacle lens for 6 hours or 3 days, using the contralateral eye as the control. With a conservative 10% FDR, most identified transcripts were differentially expressed at a relatively low fold-change compared with the contralateral eyes (Table 1). In fact, all identified transcripts were differentially expressed at less than a 2.3 fold-change, with the exception of 22 of the 605 differentially expressed transcripts after 3 days of (−) lens wear (Table 1, Supplementary Tables S1–S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6727/-/DCSupplemental). In striking contrast, (−) lens wear resulted in some 1300 differentially expressed genes at each time (Table 1, Fig. 1). At 6 hours of (−) lens wear, most altered transcripts were downregulated; at 3 days, there was a more even balance in the number of downregulated and upregulated transcripts.

A surprisingly small number of differentially expressed transcripts were common to two or more different conditions (Fig. 1, Supplementary Table S4, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6727/-/DCSupplemental). The microarrays
TABLE 1.

<table>
<thead>
<tr>
<th>Transcript number</th>
<th>Total number</th>
<th>Down- vs. up-regulated</th>
<th>Fold-change</th>
<th>SEM</th>
<th>Maximum</th>
<th>Minimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-D Lens</td>
<td></td>
<td>Down-regulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td>6</td>
<td>2</td>
<td>-1.29 ± 0.12</td>
<td>-1.29</td>
<td>-1.29</td>
<td></td>
</tr>
<tr>
<td>3 d</td>
<td>2</td>
<td>3</td>
<td>+1.02 ± 0.07</td>
<td>+1.02</td>
<td>+1.84</td>
<td></td>
</tr>
<tr>
<td>+15-D Lens</td>
<td></td>
<td>Up-regulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td>6</td>
<td>6</td>
<td>-1.49 ± 0.15</td>
<td>-1.49</td>
<td>-2.00</td>
<td></td>
</tr>
<tr>
<td>3 d</td>
<td>6</td>
<td>3</td>
<td>+1.92 ± 0.14</td>
<td>+1.92</td>
<td>+2.41</td>
<td></td>
</tr>
</tbody>
</table>

The data represent those differentially expressed transcripts comparing the lens-wearing to contralateral control eyes and meeting the statistical criteria described in the Methods (i.e., ANOVA corrected for multiple comparisons with FDR).

Validations by qPCR

For the genes/conditions selected for biological validations, qPCR was performed on retina/RPE RNA from two chicks not used for microarray profiling. Of these genes, seven were confirmed by qPCR and six were not confirmed (Table 2). The technical validations used the same six RNA samples as were assayed by microarray for four genes/conditions in Table 2; all four confirmed the profiling results. For the technical validations, the mean (±SEM) fold-changes with statistical significances were: BMP2 minus lens, 6 hours: -3.25 ± 2.41, P = 0.03; BMP2 minus lens, 3 days: -3.26 ± 1.42, P = 0.05; VIP minus lens, 6 hours: -1.84 ± 0.20, P = 0.01; DUSP4 minus lens, 3 days: -2.00 ± 0.31, P = 0.03.

Analysis of Patterns of Altered Gene Expression of Individual Genes and Patterns of Genes

To organize the long lists of differentially expressed transcripts under the two (-) lens conditions, we used both pathways analysis (Ingenuity Systems) and a manual evaluation based on the authors’ familiarity with laboratory and clinical studies of refractive mechanisms. We sought both to identify novel signaling molecules and to group known genes into categories potentially informative for mechanisms governing the accelerated eye growth and myopic refraction developing under these (-) lens conditions. We did not perform such analyses for the (+) lens conditions because so few genes were differentially expressed.

We used a database of biological and functional data (IPA, Ingenuity Systems) to generate de novo networks of interactions among the differentially expressed genes at both 6 hours and 3 days of (-) lens wear (Supplementary Table S5, http://www.iovs.org/lookup/suppl/dot:10.1167/iovs.10-6727/-/DCSupplemental). At each experimental time point, the proportion of network molecules from the experimentally identified gene lists fell considerably after the first few networks; and accordingly, only the top five networks are shown for each condition (Supplementary Table S5, http://www.iovs.org/lookup/suppl/dot:10.1167/iovs.10-6727/-/DCSupplemental). The database also contains directionally informative canonical pathways based on previously established biological and functional models of relationships between genes and gene products. From this database’s assessment of the statistical significance between its database of canonical pathways and the lists of differentially expressed genes at either 6 hours or 3 days (IPA, Ingenuity Systems), Supplemen-
Table 2. Selected Differentially Expressed Genes Studied by qPCR as Biological Validations

<table>
<thead>
<tr>
<th>Probeset ID*</th>
<th>Gene Title (Gene Symbol)</th>
<th>Lens Sign</th>
<th>Wearing</th>
<th>Fold-Change by Microarray†</th>
<th>Fold-Change by qPCR‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gga.395.1.S1_at</td>
<td>Dual specificity phosphatase 4 (DUSP4)</td>
<td>+</td>
<td>6 h</td>
<td>-1.64</td>
<td>-1.29 (-1.23, -1.35)</td>
</tr>
<tr>
<td>Gga.395.1.S1_at</td>
<td>Bone morphogenetic protein 2 (BMP2)</td>
<td>-</td>
<td>3 d</td>
<td>-1.78</td>
<td>-2.82 (-2.38, -3.26)</td>
</tr>
<tr>
<td>Gga.395.1.S1_at</td>
<td>Bone morphogenetic protein 2 (BMP2)</td>
<td>-</td>
<td>3 d</td>
<td>-2.25</td>
<td>-2.42 (-2.71, -3.13)</td>
</tr>
<tr>
<td>Gga.666.1.S1_at</td>
<td>Vasactive intestinal peptide (VIP)</td>
<td>+</td>
<td>6 h</td>
<td>-1.68</td>
<td>-2.39 (-1.28, -3.50)</td>
</tr>
<tr>
<td>Gga.9482.1.S1_at</td>
<td>Urotensin 2 domain containing (UTS2D)</td>
<td>-</td>
<td>3 d</td>
<td>-1.46</td>
<td>-2.15 (-1.90, -2.40)</td>
</tr>
<tr>
<td>Gga.449.1.S1_at</td>
<td>Noggin (NOG)</td>
<td>+</td>
<td>6 h</td>
<td>+1.64</td>
<td>+1.48 (+1.45, +1.51)</td>
</tr>
<tr>
<td>Gga.449.1.S1_at</td>
<td>Noggin (NOG)</td>
<td>+</td>
<td>6 h</td>
<td>+1.38</td>
<td>+1.35 (+1.14, +1.55)</td>
</tr>
<tr>
<td>Gga.9482.1.S1_at</td>
<td>Urotensin 2 domain containing (UTS2D)</td>
<td>+</td>
<td>6 h</td>
<td>-1.44</td>
<td>Not confirmed (-1.00, -1.59)</td>
</tr>
<tr>
<td>GgaAffx.511.1.S1_at</td>
<td>Myosin, heavy chain 13, skeletal muscle (MYH13)</td>
<td>+</td>
<td>3 d</td>
<td>+2.78</td>
<td>Not confirmed (-1.03, +1.00)</td>
</tr>
<tr>
<td>GgaAffx.22824.1.S1_s_at</td>
<td>Oxysterol binding protein-like 6 (OSBPL6)</td>
<td>-</td>
<td>6 h</td>
<td>-1.34</td>
<td>Not confirmed (+1.01, +1.05)</td>
</tr>
<tr>
<td>GgaAffx.22824.1.S1_s_at</td>
<td>Oxysterol binding protein-like 6 (OSBPL6)</td>
<td>-</td>
<td>3 d</td>
<td>-1.42</td>
<td>Not confirmed (+1.03, +1.60)</td>
</tr>
<tr>
<td>Gga.14256.1.S1_s_at</td>
<td>Phosphodiesterase 3A, cGMP-inhibited (PDE3A)</td>
<td>-</td>
<td>6 h</td>
<td>-1.47</td>
<td>Not confirmed (-1.42, +1.07)</td>
</tr>
</tbody>
</table>

For all fold-change representations, negative numbers represent downregulation of a transcript in the retina/RPE of lens-wearing eyes compared with its expression in contralateral control eyes; positive numbers, upregulation of a transcript relative to contralateral control eyes.

‡ Mean fold-change of confirmed genes from the qPCR (quantitative RT-PCR) of two independent biological replicates; individual qPCR values in parentheses; lens-wearing vs. contralateral control eye.

The tables S6A and S6B (http://www.iovs.orglookup/suppl/doi:10.1167/iovs.10-6727/DCSupplemental) provide those canonical pathways for which P < 0.05.

We also manually generated a list of potentially informative differentially expressed genes (Table 3) based on gene products related to the extensive published pharmacology or prior reports on retinal gene expression in models of refractive development, areas that have been reviewed recently.7,15 These include genes related to amino acid neurotransmitters, acetylcholine, neuropeptides, proteins, and clock/circadian rhythms. We also manually generated a list of differentially expressed genes common to both the current investigation and prior reports on retinal gene expression in models of refractive development,26–29 as generally seen in chick eyes beneath either a 6-hour or 3-day spectacle lens. Spectacle lens wear is a well-established technique for modifying eye growth and refractive development in young chicks and mammals, and it is widely used to study developmental mechanisms for emmetropia and refractive errors. Given the current lack of a cohesive biological understanding of the regulatory mechanism(s) for either emmetropization or refractive errors, we approached these profiles with a view toward generating novel, mechanistic, conceptual hypotheses about refractive development as well as exploring individual genes and potential definable regulatory networks.

Low Fold-Changes in Altered Genes

Similar to prior reports using chicken genome-wide microarrays in whole retina with binocular (+) lens wear24 or in retina/RPE with form-deprivation myopia,16 low fold-changes characterize most differentially expressed transcripts induced by either (+) or (−) lens wear for either duration (Table 1). As before,16 we included a large number of chicks for microarray profiling without pooling of tissues, as a strategy to increase the statistical likelihood of identifying differentially expressed genes with fold-changes ratios while minimizing the risk of confounding by outliers. Evidently, the robust developmental response of chick eyes to image defocus develops without major alteration in the expression level of retinal genes, at least those genes included in the microarray platform. To enrich the list of dysregulated genes and both identify affected gene networks and generate novel hypotheses, we identified differentially expressed genes on statistical significance and not arbitrary fold-change levels.

qPCR Validations

We assayed a large number of chicks (n = 6/cohort), analyzing each eye by a separate microarray to bolster the statistical validity of the core profiling results. How to best validate microarray data is an open question.25 Here, we conducted biological validations with qPCR on the retina/RPE from two chicks in each cohort reared contemporaneously with the chicks whose retinas were evaluated by microarrays. The correlation between microarray platforms and qPCR is 0.70 to 0.89, suggesting reasonably good (though not necessarily excellent) agreement between the two methods; the agreement between microarrays and qPCR deteriorates with low fold-changes in differential gene expression,26–29 as generally seen here. We found that 7 (54%) of 13 of the qPCR assessments validated the fold-change direction of the microarrays. Although the utility of validating microarrays with technical replicates has been questioned,25 we also validated the microarrays with technical replicates for four genes/conditions for thoroughness. Considering our 10% FDR and the low fold-changes in differentially expressed genes, this proportion of altered genes validated by qPCR on biologically independent birds and the confirming technical replicates preclude a major systematic error in the entire microarray procedure. Together, these validations indicate that the profiling can be interpreted in terms of its primary statistical analysis.
The results of the microarray analyses form the basis for the considerations below. Any future study of individual genes and/or biological pathways will require independent assessments because of the low fold-changes and the inherent statistical nature of microarray profiling analysis.

**Myopic versus Hyperopic Defocus**

Comparing (+) lens wear to (−) lens wear, marked differences developed in the altered retinal transcriptome. Myopic defocus from (+) lens wear caused differential expression of very few transcripts at either time. Two other available microarray studies assessed the response of whole retina to myopic defocus. One assayed retina after bilateral (+) lens wear for 24 hours, and the other assayed retina/RPE/choroid after 1 or 4 days of recovery from myopia induced by a unilateral goggle. Each, particularly the former, identified more differentially expressed genes than the current investigation; but differences in experimental design and analytical methods limit direct comparison between these studies. For instance, bilateral lens wear, different assay times and use of interbird, not intrabird, controls in the other available study of retinal gene expression after (+) lens wear are major experimental differences that could account for the differences from the present investigation.

In contrast to the few retina/RPE genes we identified after myopic defocus, the expression levels of many transcripts were affected by hyperopic defocus from (−) lens wear (Table 1, Fig. 1). Despite their opposite effects on refraction and eye growth, (+) or (−) lens wear exerted nonreciprocal effects on the retinal transcriptome. Choroidal compensation per se does not provide a simple explanation for these differences in gene expression because choroidal thickness changes rapidly in each condition, although in different directions with different time courses. Refractive compensation to either lens is incomplete by 3 days, so that defocus persists with lens wear of either sign. Further, the limited number of genes common to (+) or (−) lens wear were each downregulated under these conditions and not altered in opposite directions. In other direct comparisons of the effects of (+) and (−) lens wear on the retinal transcriptome, a limited number of common differentially expressed genes and nonreciprocal effects similarly were seen after 24 hours in the whole retina with binocular treatments and in the amacrine cell layer with monocular treatments. These results suggest that distinctive and not opposite mechanisms may underlie eye growth acceleration from (−) lens wear and eye growth arrest from (+) lens wear.

**Onset versus Persistence and/or Progression of Refractive Changes**

Pronounced differences with limited overlap occurred in the altered retinal/RPE transcriptome comparing 6 hours to 3 days of image defocus, particularly for (−) lens wear (Table 1, Fig. 1). Similarly, marked differences with limited overlap in the retinal/RPE transcriptome developed in form-deprivation myopia at these same two experimental time points. The 6-hour time point should reflect the retinal gene changes occurring at the onset of the visually driven growth change. Transcriptome changes after 3 days of lens wear reflect a time when the altered growth patterns are well established but before lens compensation is completed. In another study, none of the altered retinal genes identified by microarray after 24 hours of (+) lens wear were affected at 4 hours when assayed by qPCR. These findings suggest a hypothesis that mechanisms initiating a change in eye growth may differ from those underlying the persistence and/or progression of altered eye growth. A few, initial clinical observations support this hypothesis: an increase in axial growth develops before the onset of myopia; outdoor activity may relate to myopia onset, not progression, while visual nearwork may relate to myopia progression rather than its onset (Mutti DO, et al. IOVS 2010;51:ARVO E-Abstract 2968). Children with ametropia are typically older developmentally than the young laboratory animals investigated in most mechanistic studies. Firmly establishing whether the mechanisms responsible for myopia onset differ from those responsible for its persistence and/or progression in children would seem fundamental to understanding myopia’s pathogenesis and to developing future therapies.

**Hyperopic Defocus versus Form-Deprivation Myopia**

The wearing of (−) spectacle lenses or image-diffusing goggles each cause ocular elongation and a myopic refraction, but they are known to differ in a variety of other parameters, such as the time course of the biochemical response in sclera and in the electroretinogram. The methods in the present study were selected to closely parallel those in our prior investigation of the altered retinal transcriptome after unilateral goggle wear, and comparing the profiling results substantiates differences between lens-induced and form-deprivation myopia. While also revealing a transcriptome difference between the onset and progression of form-deprivation myopia, the 15 altered retina/RPE genes at 6 hours and 280 altered genes at 3 days of goggle wear differ dramatically from the more extensive response to (−) spectacle lens wear (Table 1, Fig. 1). The somewhat higher FDR of 13% in the goggle study is less restrictive than the 10% rate in the present study and presumably would bias toward longer, not shorter, gene lists. Potentially contributing to shorter gene lists, though, we did use a ≥1.2 fold-change filter in the bioinformatics analysis of the goggle study. Nevertheless, after (−) lens wear, 1015 genes after 6 hours and 608 genes after 3 days were differentially expressed at a ≥1.2 fold-change (Supplementary Tables S2, S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6727/-/DCSupplemental). Further, very few differentially regulated genes were common to the two conditions (Supplementary Table S7, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6727/-/DCSupplemental). Whether related to the differences in the time course of the axial or choroidal compensations, to the nature of the altered visual input or to some other yet to be defined parameter, the retina/RPE transcriptome response to (−) lens wear differs considerably from the response to goggle wear despite the growth and refractive similarities between the two conditions.

**Networks and Pathways after Minus Lens Wear**

For the (−) lens conditions, the networks and canonical pathways generated (IPA, Ingenuity Systems; Supplementary Tables S5, S6, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6727/-/DCSupplemental) each differed between the two experimental time points.

After 6 hours of (−) lens wear, the top network functions generated by pathway analysis related to nervous system development/function, cell signaling, small molecule biochemistry, the cell cycle, and gene expression. After 3 days of (−) lens wear, the top functions included gene expression, cellular growth/proliferation, cell death, cell morphology, cellular assembly/organization, cell-to-cell signaling/interaction, cellular movement, and metabolism of amino acids and carbohydrates (Supplementary Table 5, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6727/-/DCSupplemental). As one hypothesis from these general functions, the differentially expressed genes at 6 hours more prominently include signaling and developmental phenomena, and the differentially expressed genes at 3 days may also incorporate mechanisms by which the
retina adjusts its area anatomicall to the increased expansion of the vitreous chamber.

Like the networks, the archived canonical pathways to which the pathway analysis assigned differentially expressed genes were largely different between the two times (Supplementary Tables S6A, S6B, http://www iovs org/lookup/suppl/ doi:10.1167/ iovs.10-6727/-/DCSupplemental). The most statistically significant association occurred for the glutamate receptor signaling pathway, discussed further below. Many of the assigned canonical pathways related generally to receptor signaling, as might occur in neurons. Understanding how, or whether, these canonical pathways relate to either image defocus or the resultant accelerated eye growth response requires future study.

Selected Differentially Expressed Genes

**Amino Acid Signaling Genes.** A marked disparity in differentially regulated genes related to amino acid signaling developed between the two (-- lens wear times (Table 3). Transcriptome alterations related to the excitatory amino acid glutamate occurred chiefly at the 6-hour time point. In fact, glutamate receptor signaling comprised the most significant canonical pathway identified at 6 hours of (-- lens wear (Supplementary Table S6A, http://www iovs org/lookup/suppl/doi:10.1167/ iovs.10-6727/-/DCSupplemental). Supporting a role for glutamate signaling in refraction, local administration of excitatory neurotoxins and drugs interacting with excitatory amino acid receptors influence ocular growth and refractive development in the chick.54–58 A major excitatory neurotransmitter for vertical transmission through the retina, glutamate signaling also impacts the ON and OFF pathways previously implicated in eye growth of the chick.59–61

In contrast to the altered expression of glutamate-related genes mostly at 6 hours, altered expression of genes related to the inhibitory amino acids GABA and glycine occurred only at the 3-day time point (Table 5). Daily administration of drugs for 4 or 4.5 days that interact with GABA receptors alters the growth of chick eyes with either intact or altered visual input.62–65 These dosage schedules extend beyond the 3-day profiling time, when GABA genes were measured, and no data are available for shorter drug dosing times. Whether from adaptation to altered contrast or another mechanism, this shift in amino acid signaling genes dramatically underscores the 6-hour versus 3-day differences in retinal/RPE transcriptome alterations.

The changes in amino acid signaling genes may be particularly relevant to the role of retinal dopamine in regulating eye growth.66 Dopaminergic amacrine cells receive excitatory glutamatergic input from ON bipolar cells,67–69 and mice with an ON bipolar cell defect have low levels of dopamine and increased sensitivity to form-deprivation myopia.70 Conversely, dopamine amacrine cells are tonically inhibited by GABA.69–71

**Immediate-Early Gene: EGR1.** The retinal expression of the immediate-early gene EGR1 (or ZENK) by RT-PCR or its gene product by immunohistochemistry diminished in conditions stimulating eye growth (e.g., (-- lens or goggle wear) and increased in conditions inhibiting eye growth (e.g., [+ lens wear or myopia recovery). Accordingly, it has been suggested that the early activity of EGR1 may mediate the direction of eye growth.18,20,21,52 Among chick retinal neurons immunoreactive for this transcription factor are amacrine cells expressing glucagon20 (discussed below). Available data on EGR1 expression within the first few hours of altered unilaterar visual input differ on whether the altered retinal gene expression occurs bilaterally or ipsilaterally.53 We did not detect differential retinal/RPE expression of EGR1 after 6 hours of minus lens wear (Table 3). Our analysis compared gene expression to the contralateral eye, however, and parallel EGR1 effects in both eyes after 6 hours of lens wear would have masked changes in expression as a result of our experimental design. With longer periods of (-- lens wear, available data suggest that EGR1 expression differences between defocused and contralateral eyes may increase,53 and we did find EGR1 expression to be downregulated in experimental eyes relative to contralateral eyes at 3 days in the (-- lens-wearing cohort (Table 3). Although more work is required to resolve detailed differences between specific studies, much evidence implicates EGR1 in the mechanism signaling the direction of eye growth.10,20,21,52,53 Including our profiling results after 3 days of lens wear.

**Acetylcholine-Related Genes.** Acetylcholine has long been implicated in refractive development, but much is uncertain about the underlying mechanisms. Evidence of a role for acetylcholine has been the antimyopia activity of atropine and other muscarinic acetylcholine receptor antagonists in children54,55 and experimental animals.56,57 Including chick.58 Whether these drugs inhibit myopia by acting at muscarinic acetylcholine receptors, though, has been questioned.52,59 Besides antimuscarinic drugs, nicotinic acetylcholine receptors may also have a role in refractive development; but only a few potentially pertinent laboratory and clinical epidemiologic studies are available.60–62 Organophosphate insecticides inhibit acetylcholinesterase (ACHE) and could elevate synaptic acetylcholine levels; environmental exposure has been associated with increased myopia prevalence.63 In contrast, ACHE inhibitors suppress form-deprivation myopia in chick.64,65

Despite the evidence implicating the retina in refractive control,66–69 it is uncertain whether acetylcholine influences refractive development by action at the retina or through action elsewhere.10,18,52,64,66,67 Our profiling results suggest potential involvement of cholinergic signaling in the retina (Table 3): the chick cholinergic receptor CHR2 transcript was downregulated at 6 hours, and two transcripts representing the gene for ACHE were upregulated at 3 days. Despite extensive interest in acetylcholine signaling, understanding the questions and contradictory results about the role of acetylcholine in refractive development requires future research.

**Biologically Active Peptides, Proteins, and Other Genes.** We identified altered expression of genes for biologically active peptides, proteins, and other signaling molecules, some but not all of which have been identified in refractive research (Table 3). Two—vasoactive intestinal peptide (VIP) and glucagon—have generated interest as potential mediators of refractive development.7 The gene for VIP, downregulated only after 6 hours of (-- lens wear, is also downregulated in retina/RPE after 3 days of goggle wear.16 The retinal level of VIP is elevated in monkeys but not in chicks with form-deprivation myopia.7,68 Form-deprivation myopia in chicks, however, is fully inhibited by VIP antagonists but partially blocked by VIP.69 While consistent with a role for retinal VIP in eye growth control, research is needed to clarify its role in refractive development.

The reduced glucagon gene expression found here after 3 days of (-- lens wear (Table 3) conforms to that notion, assuming that the reduced mRNA results in diminished retinal glucagon release and lessening of a growth inhibitory signal.
### Table 3. Differential Expression of Selected Genes by Microarray Profiling after Minus Lens Wear for 6 Hours or 3 day

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>6 h</th>
<th>3 d</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acid Signaling Genes</strong></td>
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</tr>
<tr>
<td>GLS</td>
<td>Glutamase</td>
<td></td>
<td></td>
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<tr>
<td>GRIN5</td>
<td>Glutamate receptor, metabotropic 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIN2A</td>
<td>Glutamate receptor, ionotropic, N-methyl-D-aspartate 2A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIA4</td>
<td>Glutamate receptor, ionotropic, AMPA 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIK2</td>
<td>Glutamate receptor, ionotropic, kainate 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRID2</td>
<td>Glutamate receptor, ionotropic, delta 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRID1</td>
<td>Glutamate receptor, ionotropic, delta 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIA3</td>
<td>Glutamate receptor, ionotropic, AMPA 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIP1</td>
<td>Glutamate receptor interacting protein 1</td>
<td></td>
<td></td>
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<tr>
<td>NARG1L</td>
<td>NMNAT receptor regulated 1-like</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIN1L1</td>
<td>Glutamate receptor, ionotropic, N-methyl-D-aspartate-like 1B</td>
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<td></td>
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<tr>
<td><strong>GABA-related genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GABRG3</td>
<td>Gamma-aminobutyric acid (GABA) receptor, rho 3</td>
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<td></td>
</tr>
<tr>
<td>SLC6A1</td>
<td>Solute carrier family 6 (neurotransmitter transporter, GABA), member 1</td>
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</tr>
<tr>
<td>ABAT</td>
<td>4-aminobutyrate aminotransferase</td>
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<td></td>
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<tr>
<td>LOC428967</td>
<td>Similar to gamma-aminobutyric acid (GABA) A receptor, pi</td>
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<tr>
<td>GABRG2</td>
<td>Gamma-aminobutyric acid (GABA) A receptor, gamma 2</td>
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<tr>
<td>GAD2</td>
<td>Glutamate decarboxylase 2 (pancreatic islets and brain, 65kda)</td>
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<tr>
<td><strong>Glycine-related gene</strong></td>
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<tr>
<td>GLRA2</td>
<td>Glycine receptor, alpha 2</td>
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<td></td>
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<td><strong>Acetylcholine-related genes</strong></td>
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</tr>
<tr>
<td>CHRM2</td>
<td>Cholinergic receptor, muscarinic 2</td>
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</tr>
<tr>
<td>ACH</td>
<td>Acetylcholinesterase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Miscellaneous Peptide, Protein and Other Genes</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP2</td>
<td>Bone morphogenetic protein 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noggin</td>
<td>Noggin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FST</td>
<td>Follistatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADMP</td>
<td>Anti-dorsalizing morphogenetic protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCG</td>
<td>Glucagon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
<td></td>
<td></td>
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<tr>
<td>FGFBP2</td>
<td>Fibroblast growth factor binding protein 2</td>
<td></td>
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</tr>
<tr>
<td>NGFB</td>
<td>Nerve growth factor, beta polypeptide</td>
<td></td>
<td></td>
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<tr>
<td>NPY7R</td>
<td>Neuropeptide Y7 receptor</td>
<td></td>
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<tr>
<td>NRG3</td>
<td>Neuregulin 3</td>
<td></td>
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<tr>
<td>NK3</td>
<td>Neutrophil 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PREP</td>
<td>Prolyl endopeptidase</td>
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<tr>
<td>NAAALD1</td>
<td>N-acetylated alpha-linked acidic dipeptidase-like 1</td>
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<td></td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase 2, inducible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMOX2</td>
<td>Heme oxygenase (decycling) 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNR1</td>
<td>Cannabinoid receptor 1 (brain)</td>
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<td></td>
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<tr>
<td>TRIM30A</td>
<td>Thyroid hormone receptor beta 2</td>
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<td></td>
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<tr>
<td>DIO2</td>
<td>Deiodinase, iodothyronine, type II</td>
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<tr>
<td>TRPM4</td>
<td>Thyroid hormone receptor interacting 4</td>
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<tr>
<td>INSR1</td>
<td>Insulin 1.4,5-triphosphate receptor, type 1</td>
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<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
<td></td>
<td></td>
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<tr>
<td><strong>Clock and Circadian Genes</strong></td>
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<tr>
<td>OPN4</td>
<td>Opsin 4 (melanopsin)</td>
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<td>LOC395334</td>
<td>Photopigment melanopsin-like</td>
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<tr>
<td>LOC424283</td>
<td>Similar to peripheral clock protein 2</td>
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</tr>
<tr>
<td>PER3</td>
<td>Period homolog 3 (Drosophila)</td>
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<tr>
<td>MTHFR1</td>
<td>Melatonin receptor 1A</td>
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<td></td>
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<tr>
<td>CLOCK</td>
<td>Clock homolog (mouse)</td>
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<tr>
<td>NPAR2</td>
<td>Neuronal PAS domain protein 2</td>
<td></td>
<td></td>
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<tr>
<td>CRY1</td>
<td>Cryptochrome 1 (photolyase-like)</td>
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</tr>
</tbody>
</table>

* Fold-change of gene expression by microarray for lens-wearing vs. contralateral control eye for selected genes meeting the statistical criteria described in the Methods (i.e., ANOVA corrected for multiple comparisons with FDR <10%). Negative numbers represent downregulation of a transcript in the retina/RPE of lens-wearing eyes compared with its expression in contralateral control eyes; positive numbers, upregulation of a transcript relative to contralateral control eyes.

† When a gene was identified as differentially expressed by more than one transcript, all fold-change values are listed.
The gene for bone morphogenetic protein 2 in retina/RPE has been found to be downregulated after both 6 hours and 3 days in form-deprivation myopia, although this is controversial. In the chick retina, melanopsin exists in two forms and is expressed by horizontal and bipolar cells in addition to ganglion cells. The diurnal physiologic activity of dopaminergic amacrine cells is believed to modulate refractive development in both experimental birds and mammals, and the effect of hyperopic defocus to alter the expression of clock genes and the genes for a melatonin receptor and melanopsin may provide mechanistic links between image clarity, dopaminergic amacrine cell activity, and refractive development.

Ambient Lighting and Refractive Development

One means of assessing circadian influences on eye development could be to examine the refractive effects of external parameters already known to modify circadian rhythms in other systems. The altered light exposure patterns that influence refractive development experimentally or associate with refractive errors clinically often conform to the types of light exposure patterns used in entrainment models to study circadian rhythms.

As extensive study has shown in the chick, disrupting the daily light–dark cycle perturbs the ordered growth of the eye, especially with rearing under constant light or constant dark. Clinically, some feature of lighting has long been hypothesized to influence human refractive development. Lighting intensity, other photoperiod properties, and the age at which lighting may act have generated most recent clinical interest. An antimony effect of outdoor activity is repeatedly noted and may relate to time spent outdoors rather than outdoor physical activity per se. Higher intensity of outdoor than indoor lighting is one hypothesized mechanism for this effect. A role for specific indoor activities (e.g., nearwork) remains uncertain.

A role for other photoperiod properties is more controversial. For instance, some but not all cross-sectional surveys associate later ametropia with disrupting the dark period in early childhood by ambient lighting at night. The only study to include ultrasound, while not reporting an overall effect on refraction, did find more high myopia and longer axial lengths with nighttime lighting exposure. Several reports have associated refraction with birthdate, suggesting...
among other hypotheses that perinatal daytime length is a myopia risk factor.\textsuperscript{123,124}

Although it may seem contradictory that both more light (e.g., from a shorter or disrupted dark period) and indoor activities (e.g., resulting in less light during the day) each associate with myopia, circadian biology may link these diverse laboratory and clinical observations. Altering the light–dark photoperiod is well known to influence circadian rhythms.\textsuperscript{1,2}\textsuperscript{6} Dim lighting affects circadian rhythms in complex ways;\textsuperscript{1,27} indoor lighting may be of inadequate intensity for proper clock entrainment.\textsuperscript{1,28} Based on our finding that hyperopic defocus alters intrinsic retinal clock and circadian gene expression while inducing myopia, addressing a role for circadian rhythms in refractive development may provide a unifying framework for understanding these disparate findings about rhythms, lighting, and refraction.

Comparison to Other Gene Expression Studies

Comparing gene expression between studies of refractive development is complicated by differences in species, experimental models, study duration, methods for detecting gene alterations, and the tissue assayed.\textsuperscript{1,5} On tissue in particular, broad sampling of a complex tissue such as the retina/RPE risks masking small effects in a limited number of discrete cells. Altered gene levels in a subtype or limited subtypes of retinal neurons may be obscured by stable or reciprocal changes or by high variability in other neurons. In this regard, higher levels of altered gene expression developed after 24 hours of the wearing of spectacle lenses wear of $+7$ or $-7$ D in the amacrine cell layer isolated by laser capture microdissection.\textsuperscript{31} Using gene screening criteria of $P \leq 0.05$ and $\geq 1.5$ fold-change, (+) lens treatment altered the expression of 58 genes (30 upregulated, 28 downregulated, with a range of $+7.7$ to $-5.7$ fold-change); and (−) lens treatment altered the expression of 128 genes (65 upregulated, 65 downregulated, with a range of $+8.0$ to $-27.4$ fold-change).\textsuperscript{31} Restricting sampling to the amacrine cell layer, as well as differences in lens power, lens wear time or analytical approach, could have accounted for the differences from the present study.

Consistent with these qualifications, most overlap in genes between the present study and prior publications tends to be limited in number, with no universally identified genes (Supplementary Table S7, http://www iovs arvojournals org lookup suppl doi 10.1167 iovs.1006727/DSupplemental). From the perspective of intercellular signaling, some of the recurring genes are those encoding bone morphogenetic protein 2, glucagon, inducible nitric oxide synthase and vasoactive intestinal peptide. For understanding the mechanisms of refractive development, microarray platforms or other approaches to gene expression are designed to identify changes in RNA abundance, not protein products or posttranscriptional protein modifications. While posttranslational regulatory mechanisms most likely affect refractive development, RNA assays as performed in this study can provide important leads and generate potentially useful hypotheses for future study.

OVERALL CONCLUSION

In the present study, we identified novel genes and sorted differentially regulated genes into pathways and signaling networks, only some of which are explicitly discussed above. Perhaps because of the experimental design and informatics approaches, the present study and our prior report on form-deprivation myopia\textsuperscript{16} conform to some mechanistic hypotheses that could lead to a general, broad biological framework to understand the mechanisms that regulate emmetropization and cause refractive errors. These include the ideas that distinctive, not opposite, processes govern the upregulation and downregulation of eye growth and that the mechanisms precipitating changes in eye growth may differ from those responsible for persistence and/or progression of ametropias. The altered retinal/RPE clock and circadian genes after hyperopic defocus substantiates a potential role for endogenous retinal rhythms in refractive development. As a testable hypothesis, disrupted endogenous ocular clock or circadian rhythms may provide a framework for understanding the extensive clinical and laboratory literature on the potential role of lighting on refractive development. Establishing any of these concepts, though, will require future laboratory and clinical investigation. Significantly, many of these hypotheses can be addressed now in children while parallel investigations progress independently in the laboratory.

References


