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Visually-Driven Ocular Growth in Mice Requires Functional Rod Photoreceptors

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PURPOSE. Proper refractive eye growth depends on several features of the visual image and requisite retinal pathways. In this study, we determined the contribution of rod pathways to normal refractive development and form deprivation (FD) myopia by testing Gnat1+/− mice, which lack functional rods due to a mutation in rod transducin-α.

METHODS. Refractive development was measured in Gnat1−/− (n = 30–36) and wild-type (WT) mice (n = 5–9) from 4 to 12 weeks of age. FD was induced monocularly from 4 weeks of age using head-mounted diffuser goggles (Gnat1−/−, n = 9–10; WT, n = 7–8). Refractive state and ocular biometry were obtained weekly using a photorefactor, 1310 nm optical coherence tomography, and partial coherence interferometry. We measured retinal dopamine and its metabolite, DOPAC, using HPLC.

RESULTS. During normal development, the refractions of WT mice started at 5.36 ± 0.68 dipters (D) and became more hyperopic before plateauing at 7.78 ± 0.64 D. In contrast, refractions in Gnat1−/− mice were stable at 7.39 ± 1.22 D across all ages. Three weeks of FD induced a 2.54 ± 0.77 D myopic shift in WT mice, while Gnat1−/− mice did not respond to FD at any age. Axial lengths of Gnat1−/− and WT mice increased with age, but differences between genotypes or with goggling did not reach statistical significance and fell within the precision of the instruments. The DOPAC levels were significantly lower in Gnat1−/− mice from 2 to 12 weeks of age with DOPAC/dopamine ratio peaking earlier in Gnat1−/− compared to WT mice. No differences in dopamine were seen in response to FD or between genotypes.

CONCLUSIONS. Functional rod photoreceptors are critical to normal refractive development and the response to FD in mice. Dopamine levels may not directly modulate the refractive state of the mouse eye, but tonic levels of dopamine during development may determine susceptibility to myopia.

Keywords: refractive development, myopia, rod photoreceptors, dopamine

Visually-driven eye growth is responsible for matching the power of the eye with ocular length to acquire in-focus images; a process called emmetropization. Local signaling by the retina mediates refractive development, as shown by the selective effect of partial occluders altering growth in only the corresponding region of the globe in chickens1–3 and primates,4 preservation of the response to form deprivation (FD)5 or lens-induced defocus6 after optic nerve section in chickens, or pharmacologically blocking retinal ganglion cell transmission in tree shrews7 (see review8). However, it should be noted that the response to lens defocus is altered after blocking input from higher visual processing areas in chickens.9–11

Since an in-focus image is the ultimate goal of refractive development, it may be presumed that the visual image should be of high acuity and temporal resolution,12 qualities attributed to cone-mediated visual processing. Thus, there is an assumption that cone pathways likely underlie the signaling needed for proper eye growth control. However, results from a few studies suggest that cone pathways may not dominate the signaling of mammalian eye growth: (1) Laser ablation of the cone-rich fovea region in monkeys did not prevent the development of FD myopia13,14 and (2) imposing FD on the rod-dominated peripheral regions of the monkey eye produced similar magnitudes of FD myopia as when the entire visual field was affected.15

The limitation of these studies is that rods and cones are present in the periphery of the retina and, thus, a small population of cones still could be contributing signals for eye growth. In addition, these experiments were performed under photopic conditions in which cones would be functionally predominant. Nonetheless, they do suggest that the rod-rich peripheral regions of the retina may be important for visually-guided eye growth and a few other studies have suggested a role for photoreceptors in refractive development.16 In fact, spatial frequency thresholds of mice without functional cones (cyclic nucleotide-gated cation channel subunit A3 knock-out, CNGA3−/−, mice), are the same as those of wild-type (WT) mice, suggesting that rod and/or rod pathways are capable of providing visual signals under photopic conditions for the
optokinetic response. In contrast, mice with nonfunctional rods (CNGB1−/− mice) have much poorer spatial resolution.17

Abnormal refractive development also is associated with retinal diseases involving rod photoreceptors and/or pathways. For instance, patients with the complete form of congenital stationary night blindness (CSNB) have disrupted visual transmission between rods and ON bipolar cells due to a mutation in Nrx gene and also present with high myopia.18 In addition, patients with cone–rod dystrophy19,20 or retinitis pigmentosa with a mutation in the Pde6b gene21 have increased incidence of myopia. Similarly, we found that mice with the Nrx mutation22 and mouse models of retinitis pigmentosa with a mutation in the Pde6b gene (Pde6b<sup>rd10</sup>/rd10 [rd1] or Pde6b<sup>rd10</sup>/rd10 [rd10])23 are more susceptible to FD myopia. Finally, in the retinopathy of prematurity (ROP) rat model, myopia is present (although paradoxically with shorter than normal axial length)24 and abnormal rod photoreceptor function has been implicated.25

Dopamine (DA), a key neuromodulator in the retina that regulates circadian rhythms and mediates adaptation to different lighting conditions, has been proposed as a stop signal for visually-driven eye growth.26 In the retina, DA is synthesized from L-3,4-dihydroxyphenylalanine (L-DOPA) and metabolized into 3,4-dihydroxyphenylacetate (DOPAC). Synthesis and release of DA are stimulated by light via the ON pathway.27–30 Initial light exposure increases retinal DA synthesis, release, and metabolism; however, the system then reaches equilibrium, such that the steady state level of DA does not change appreciably and only DOPAC levels vary during the light phase.31 Thus, an increase in the amount of DOPAC is an indicator of DA turnover (often reported as DOPAC/DA ratio) and use. Light regulation of dopamine levels is mainly through rods, cones, and possibly melanopsin cells.32–34 Dopamine increases in a log-linear relationship with illumination,35–37 although these studies did not examine rod-isolating illumination levels. Moreover, rod pathways and the dopaminergic system interact structurally and functionally; for instance, DA neurons synapse onto All and A17 amacrine cells in the rod pathway; rod-driven ON pathways stimulate DA release, which in turn decrease rod function as the retina adapts to daylight function, and loss of rods results in decreased DA levels in the retina.32–38

The mouse recently has been adopted as an experimental model for myopia, offering the ability to manipulate genes and environment (see review).39 The mouse eye responds with myopic shifts when exposed to FD;40–45 or negative lens defocus.43,46 In addition, a number of studies using mice have confirmed signaling pathways implicated in previous chicken studies as influencing refractive development, such as the early growth response protein-1,47–49 muscarinic receptors,50 adenosine receptors,51 retinoic acid,52 and dopamine.25,53 (Zhou X, et al. IOVS 2014;55:ARVO E-abstract 3038).

To more fully explore the contributions of rod photoreceptors to emmetropization and myopia development, we tested mice with nonfunctional rod photoreceptors, carrying alleles for the gene of the rhodopsin-associated G protein, transducin α1 (Gnat1) under normal and form deprived visual conditions. We then evaluated DA and DOPAC levels in the retina across postnatal development and following FD.

**METHODS**

**Animals and Experimental Design**

The Gnat1−/− mice were a generous gift from Janis Lem, PhD (Tufts-New England Medical Center, Boston, MA, USA). Importantly, loss of Gnat1 renders the rods nonfunctional, but does not induce rod degeneration until 13 weeks of age.54 Mice were maintained at the Atlanta Veterans Affairs Medical Center, on 12:12-hour light cycles (∼17 lux; lights on at 6 AM) and housed in typical shoe box cages with mouse chow and water available ad libitum.

Refractive development (RD) was characterized with weekly measurements of refractive error and axial length from 4 to 12 weeks of age in Gnat1−/− and age-matched WT control mice without any visual manipulation (see Table for animal numbers used). The response to FD was characterized by subjecting separate cohorts of WT and Gnat1−/− mice to monocular diffuser goggles at 4 weeks of age. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the local Institutional Animal Care and Use Committee.

It is important to note that we found the rd8 gene55 in the Gnat1−/− mice, as well as in some of the WT animals (31% as homogenous mutant of rd8, 26% as heterozygotes). It is unlikely that the slow rod photoreceptor degeneration caused by the rd8 mutation had any significant contributions to our results as the mice reported here were younger than 6 months, when electroretinograms still are normal in rd8 mice.56 and no statistically significant differences were found between the different rd8 genotypes (WT mice 2-way repeated ANOVA on myopic shift, main effect for rd8 genotype F<sub>2,48</sub> = 0.125, P = 0.88).

**Refractive State and Axial Length Measurements**

Each mouse underwent the following experimental measurements of refractive error and ocular biometry, as described previously.57 First, eyes were dilated with 1% tropicamide and measurements of refractive state and axial length were obtained using 1310 nm spectral-domain optical coherence tomography (SD-OCT; Bioptigen, Inc., Durham, NC, USA), as described previously.58 Axial length measurements of a subset of mice (see Table for animal numbers) were acquired using a custom-built 845-nm time-domain partial coherence interferometer (PCI) after being placed in an open-ended conical tube with the mouse’s head pedestal stabilized by a clip.58,59

Mice then were anesthetized (ketamine 80 mg/kg and xylazine 16 mg/kg) and refractions repeated to obtain a more stabilized measurement with standard deviations of less than 0.5 diopters (D).57 While still anesthetized, axial length measurements were obtained using 1310 nm spectral-domain optical coherence tomography (SD-OCT; Bioptigen, Inc., Durham, NC, USA), as described previously.58 After measurements, mice were given yohimbine (2.1 mg/kg) to reverse the effects of anesthesia and reduce the development of corneal lesions.60 Mice recovered on a warming pad with saline drops applied to the eyes. The entire measurement routine lasted approximately 30 minutes.

**Retinal Dopamine Quantification**

Separate cohorts of 8-week-old WT and Gnat1−/− mice were sacrificed at Zeitgeber time 3 (ZT3) and ZT22 (or 3 hours after and 2 hours before light onset, respectively) to confirm abnormal DOPAC/DA ratio phenotype in Gnat1−/− mice (see Table for animal numbers). To determine the levels of retinal DA during development, retinas from both genotypes were collected at 1, 2, 4, 6, 8, 10, and 12 weeks of age between 10 and 12 AM (see Table). In addition, retinal DA and DOPAC levels also were measured from mice after the final endpoint of the FD experiments (see Table). Retinas were collected 48 hours after ocular parameters assessment to provide a recovery period from any effects of anesthesia. In brief, DA and DOPAC levels were quantified using HPLC with coulometric detection.
as described previously. Retinas were homogenized in 0.1 N HClO₄ solution (0.01% sodium metabisulfite and 50 ng/mL internal standard 3,4 dihydroxybenzylamine hydrobromide) and centrifuged. The HPLC conditions included an Ultrasphere ODS 5 μm 250 × 4.6 mm column (HiChrom, Berkshire, England, or Beckman Coulter, Fullerton, CA, USA) for separation with a mobile phase containing 0.1 M phosphoric acid, 0.1 mM EDTA, 0.3 to 0.35 mM sodium octylsulfate, 6% acetonitrile, adjusted to pH 2.7 with NaOH. The retinal DA and DOPAC levels were quantified using standard curves generated with 0.1 to 1 ng DA and DOPAC. All FD cohort retinas were homogenized individually, while the right and left retinas were pooled together in the RD cohort for DA and DOPAC quantification.

Data Analysis

All statistical analyses were performed using commercial software (SigmaStat 3.5; Aspire Software International, Ashburn, VA, USA). Data plotted in Figures are presented as mean ± SEM, underwent repeated-measures 2-way ANOVA, and Holm-Sidak post hoc tests for statistical significance. For RD refraction and axial length data both eyes received the same treatment and the values from the two eyes were averaged to represent a single value from each individual mouse. In a selection of animals that underwent PCI and SD-OCT measurements, the axial length values were averaged together, since measurements by the two techniques were in good agreement (interclass correlation coefficient = 0.94). For FD results, refractive errors are presented as “myopic shift” (difference between right and left eyes), since the refractive errors of untreated opposite eyes were not statistically different from those of naive control eyes. Axial lengths of FD cohort were normalized to 4-week-old values (baseline) to eliminate individual variability in eye size due to differences in body size. “Axial shift” represents the difference in length between the right and left eye after values had been normalized to baseline. The DA and DOPAC levels across age were normalized to the Gnat1⁻/⁻ and WT values obtained at ZT3. The DA and DOPAC values from FD cohorts were analyzed by taking the difference between the two eyes. When normality failed for DA analysis, Student’s t-test was used with P value corrected with the robust false discovery rate method (calculated as P[^#tests + 1]/[2 * #tests]).

RESULTS

Abnormal Refractive Development in Gnat1⁻/⁻ Mice

Nonfunctional rod photoreceptors had the most profound effect on normal refractive development at young ages. At 4 weeks of age, WT animals had refractive errors of 5.02 ± 0.52 D (mean ± SEM, n = 12) and became more hyperopic with age, reaching a refractive error of 7.78 ± 0.64 D at 12 weeks old (n = 12, Fig. 1A). In contrast, the eyes of Gnat1⁻/⁻ mice did not have a period of growth toward relative hyperopia, but had stable refractive errors in the range from 6.85 to 7.88 D during the entire study period (n = 31–38/timepoint). This produced significant differences between Gnat1⁻/⁻ and WT mice at 4 and 5 weeks of age (Fig. 1A; 2-way repeated ANOVA, F(7,336) = 9.33, P < 0.001).

While axial length significantly increased with age for WT and Gnat1⁻/⁻ mice, there were no differences between Gnat1⁻/⁻ and WT mice (Fig. 1B; 2-way repeated ANOVA, main effect of age F(8,154) = 2.17, P = 0.034; n = 5–8/timepoint for WT and 9–15 mice/timepoint for Gnat1⁻/⁻).

Gnat1⁻/⁻ Mice Unresponsive to FD

First, regardless of genotype, eyes in naive control mice (not goggled) had similar refractive errors (Fig. 2; myopic shift [difference between right and left eyes]) in WT mice, 0.04 ± 0.45 D, n = 9; Gnat1⁻/⁻ mice, 0.11 ± 0.18 D, n = 12. Conversely, the response to FD differed depending on the
genotype. The WT mice exhibited a relative myopic shift (−2.54 ± 0.77 D, n = 10) compared to the contralateral eye after 3 weeks of goggle wear (Fig. 2A; 2-way repeated ANOVA, \( F_{(3,77)} = 3.1, P = 0.055 \)). In contrast, Gnat1−/− mice did not respond to FD. After 3 weeks of goggle wear, the refractive errors of Gnat1−/− form-deprived eyes did not shift and remained similar to the contralateral eye (−0.04 ± 0.10 D, n = 10; Fig. 2B; 2-way repeated ANOVA, \( F_{(3,86)} = 0.9, P = 0.447 \)). Therefore, results from form-deprived Gnat1−/− mice were not significantly different from the naive nongoggled Gnat1−/− animals (Fig. 2B). At 8 weeks after goggling, Gnat1−/− animals still had no significant myopic shift (−0.12 ± 0.14 D, n = 9) compared to WT mice (−2.15 ± 1.27 D, n = 7). A direct comparison of WT and Gnat1−/− mice at 3 weeks after goggling showed significant differences in response to FD between the genotypes (data not shown; 2-way ANOVA, \( F_{(1,42)} = 7.36, P = 0.01 \)).

Axial length differences were not detected after form deprivation with the instruments used. In WT mice, goggled and opposite eyes had longer axial lengths after 3 weeks of FD compared to naive controls; however, this was not statistically significant (n = 8, Fig. 3A). In Gnat1−/− mice, similar axial lengths were measured between goggled and opposite eyes, with naive control eyes showing a trend for longer axial lengths (n = 9, Fig. 3B). No statistically significant differences were found between the body weights of goggled and control mice (data not shown). Schematic models of the mouse eye photoreceptor 5 to 6 μm change in axial length is needed for 1 D change in optical power.43 Thus, it is possible that the interuser measurement variability of the SD-OCT instruments (21 μm) used here46 is not sufficient to detect differences in axial length, as the measured axial shifts of goggled mice were less than the resolution limit.

**Dopamine Metabolism Altered in Gnat1−/− Mice**

The retinal dopaminergic systems in WT and Gnat1−/− mice responded differently to light and dark cycles. The DOPAC levels were lower in Gnat1−/− mice compared to WT at night (ZT22; Student’s t-test, \( t = 4.11, P = 0.004 \); Fig. 4A). Comparing the response between light (ZT5) and dark (ZT22) cycles within each genotype, Gnat1−/− mice had a diminished response to light (658 ± 153 to 331 ± 36, respectively, 98% difference) compared to WT mice (2699 ± 1062 to 1123 ± 130, respectively, 140% difference). The DOPAC/DA ratio also was significantly decreased in Gnat1−/− mice compared to WT in the dark cycle, as reported previously (Fig. 4C; Student’s t-test, \( t = 5.24, P = 0.01 \)). However, the DA levels in WT and Gnat1−/− mice were similar between the genotypes and different light phases (Fig. 4B).

Next, we examined DOPAC and DA across postnatal development. We found that DOPAC levels in WT mice (Fig. 5A) significantly increased from 1 to 4 weeks, then decreased until week 8 before rising again (2-way ANOVA, \( F_{(6,123)} = 5.499, P < 0.001 \)). In contrast, DOPAC levels remained consistent in Gnat1−/− mice, with no statistically significant differences across age. The pattern of DA in the retina was fairly similar between the two genotypes, with low levels at 1 and 2 weeks of age that increased and then became stable until 12 weeks of age (Fig. 5B; 2-way ANOVA, \( F_{(6,123)} = 4.56, P < 0.001 \)). These differences between genotypes in the pattern of DOPAC and DA indicated significant differences in dopamine metabolism, as illustrated by the DOPAC/DA ratio. The DOPAC/DA ratio was significantly higher in Gnat1−/− mice at 1 week of age, due to very low levels of DA at this age, and then decreased rapidly at 2 weeks and beyond (Fig. 5C; 2-way ANOVA, \( F_{(6,123)} = 20.17, P < 0.001 \)). In contrast, the DOPAC/DA ratio in WT retinas increased from 1 to 2 weeks of age, decreased at 4 to 6 weeks, and then further diminished at 8 to 12 weeks of age.
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DISCUSSION

Functional Rod Photoreceptors Needed for Visually-Guided Refractive Development

In most animals, refractive development follows a predictable pattern. In mammals, this typically starts with hyperopic refractions and then shifts to emmetropia during early development to adolescence. In WT mice used here and reported previously, the normal refractive development curve starts with hyperopic refractions, then shifts to greater levels of hyperopia (Fig. 1). Surprisingly, the Gnat1−/− mice did not have this same refractive development curve, but instead maintained the same range of refractions across the entire experime...
synthesis. The DA receptor expression continues to increase after birth, with dopamine D4 receptor expression reaching a peak at postnatal day 12.73 The retinal dopaminergic neurons do not reach maturation with typical ring-like axonal processes until the third postnatal week.76 Finally, L-DOPA content increases through development until the fourth postnatal week and then sharply declines.77 These observations correspond well with our data (Fig. 5) that show DOPAC levels increased until 4 weeks of age in WT mice and then decreased as the animals aged. This pattern may be indicative of a reduction in the amount of diffusible or nonvesicular dopamine with age concomitant with an increase in vesicular DA within the cell, which would increase the overall steady-state DA content (see continued increased in Fig. 5B).

Relating DA development to refractive state, it is not clear from these data that DA is directly signaling visually-driven eye growth in the mouse. If DA directly modulates eye growth, it would be expected that refractive values would follow closely the changes in DOPAC or DA levels. However, the rapid hyperopic shift from 4 to 6 weeks in WT mice is not mirrored in the DA data, although steady DOPAC levels in Gnat1−/− mice are similar to the consistent refractive values across age. Furthermore, DOPAC and DA levels were not significantly different between strains after FD, although they had drastically different refractive responses to FD. It is important to point out that HPLC analysis of DA and DOPAC levels has limitations, as it only measures total retinal levels and does not provide information about whether DA is intra- or extracellular. Previous studies in form deprived chickens have shown that dopamine release by amacrine cells and diffusion through the retina, and not the total dopamine retinal content, is most relevant to the myopic response.78 Nevertheless, these data suggested that DA may not have a direct effect on refractive development in mice; instead, dopamine metabolism may predispose the retina to certain levels of myopia susceptibility. For instance, the loss of functional rods across development may alter dopamine release and the expression of several other associated molecules, leading to chronic changes in extracellular dopamine levels. Dopamine and DOPAC have been found to decrease with form deprivation in many species (see review26) and, thus, it would be expected that chronically low levels of retinal dopamine may increase susceptibility to FD myopia. We have found previously that low levels of DOPAC correlated with increased susceptibility to FD myopia in the rd1 and rd10 mouse models of retinitis pigmentosa.25 However, the role of DA in refractive development is likely very complex as this hypothesis did not seem to apply to Gnat1−/− mice, which are resistant to FD even though they had lower retinal DOPAC levels (Fig. 5). Furthermore, recent reports have suggested that dopamine may differentially act on dopamine receptor subtypes to influence eye growth in mice53 (Zhou X, et al. IOVS 2014;55:ARVO E-Abstract 3038), adding further complexity to the mechanisms involved.

Thus, more research is needed to elucidate how DA influences refractive development and susceptibility to FD, or myopia in general, so that it may serve as a potential therapeutic target for myopia in the future.

Effects of Gnat1 Deletion on Retinal Dopaminergic System

These studies showed that nonfunctionality of rod photoreceptors has relatively small effects on steady-state levels of retinal dopamine, as indicated by the nearly normal levels of DA in the Gnat1−/− retina in the light/dark phases and across age (Figs. 4, 5). This may indicate that DA is synthesized in Gnat1−/− retinas, but not properly metabolized. However, loss of rod function appears to disrupt DA metabolism, resulting in diminished DOPAC levels between 1 and 4 weeks postnatally and during the light phase in Gnat1−/− mice. This could be due to a variety of reasons, such as diminished neuronal activity to stimulate DA release, insufficient reuptake of DA to the presynaptic terminal, or defective metabolism of DA to DOPAC.25 Further research is needed to determine if these effects are due primarily to the loss of rod function or if the absence of rod signaling unMASKS contributions from other sources, such as the RPE or choroidal innervation.

How Could Rods Contribute to Refractive Development?

Cone-mediated visual processing has been assumed to regulate refractive development based on the fact that emmetropia produces a focused image on the retina and FD myopia occurs under photopic illumination, with little emphasis placed on a potential role of rod-mediated processing. Using the Gnat1−/− model provided a unique opportunity to isolate the contributions of functional rod photoreceptors in refractive development. Our data showed that functional rods are important for normal refractive development and the response to FD. However, the exact mechanism of how rod signaling could contribute to refractive development and the detection of FD is not known.

While rod and cone photoreceptor sensitivity traditionally has been thought to occur in a binary fashion under scotopic and photopic conditions, there is increasing evidence that retinal circuitry is much more complicated. For instance, rods in the mouse retina in vivo are capable of providing detectable signals in the presence of steady lights that are 2 log units higher than the Weber line where rod saturation is behaviorally shown to occur; although the sensitivity is decreased compared to cones.79 Furthermore, rod photoreceptors can drive circadian photoentrainment under high light intensities80 and mediate vision under photopic conditions.81 Since the ocular growth response to defocus occurs over minutes,82 compared to the millisecond response needed to detect photons for functional vision, the role of rod-mediated signaling in refractive development may involve retinal pathways with reduced sensitivity and/or roles in circadian rhythms.

Thus, our results suggested that functional rods may be a critical component of retinal signaling for refractive development. Further experiments are needed to examine the contribution of cone photoreceptors in isolation as well as testing the effects of eliminating other elements of visual pathways for their potential role in refractive development.

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Visually-Driven Ocular Growth in Mice


