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Altered Refractive Development in Mice With Reduced Levels of Retinal Dopamine

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During normal ocular refractive development, the mammalian eye grows until the incoming light is focused by the cornea and lens onto the retina to produce an image that is in-focus, a process called emmetropization. In a large percentage of the human population (41.6% of US residents from 1999-20041 and 96.5% of 19-year-old males in South Korea in 20122) this process occurs abnormally, leading to near-sightedness, or myopia. Human myopia is characterized by excessive axial eye growth such that incoming light is focused in front of the photoreceptors, resulting in a blurred image of distant objects. Negative corrective lenses focus light back on the retina and provide improved vision. Even with corrective lenses, myopia is associated with long-term risk for ocular pathologies such as glaucoma, cataract, and retinal detachment.3

Over the past few decades, increasing evidence has indicated that retinal dopamine (DA) is an important modulator of refractive errors and eye growth. Dopamine concentration has been shown to decrease with myopia development4 and therefore DA has been suggested as a “stop” signal for eye growth (see review in Ref. 5). Traditionally, researchers have studied this pathway in primate and chick models, using pharmacological agents to affect DA receptors. For example, spiperone, a D2-like receptor antagonist, prevented the ameliorative effects of brief periods of unrestricted vision in chicks undergoing form deprivation (FD).6 This suggests that DA plays a key role in inhibiting excess eye growth during emmetropization. Another study showed that apomorphine, a DA agonist, inhibits axial growth and myopia development in primates during visual deprivation, again suggesting that DA prevents myopic growth.7 Overall, current findings support the idea that retinal DA is an important protective factor against myopia, yet these findings have been mostly supported by pharmacological experiments.

This study used a mouse model in which DA was selectively removed from the retina by genetically targeting the DA enzyme in this process. To achieve retinal specificity, Cre-lox
technology was used to target TH excision in retinal tissue using a Chx10 promoter. Retina-specific TH knockout (rTHKO) mice have approximately 90% reduction in retinal DA and DOPAC levels compared with wild-type (WT) controls, showing that a low level of retinal DA still remains. Previous studies in which visual input was altered, followed by measurements of DA levels, refractive error, and eye size, suggest that changes in dopaminergic amacrine cell activation may represent a “blur detector,” such that disrupted visual input decreases retinal DA release, leading to myopic refractive errors. Using rTHKO mice, we tested the effect of chronic removal of retinal DA on refractive error development under normal and FD conditions. Because DA is considered a “stop signal” for myopia, we hypothesized that the absence of DA during the critical period of refractive development would result in myopia without FD, mimicking the effect of altered visual input.

**Materials and Methods**

**Retinal DA Knockout Model**

In this study, mice were used according to the approved Institutional Animal Care and Use Committee protocol and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retina-specific TH knockout (THKO) mice were described previously and showed significantly reduced contrast sensitivity and light-adapted retinal functions. Briefly, THloxP/loxP mice, in which exon 1 of the TH gene was flanked with two loxP sites, were bred with mice expressing Cre-recombinase driven by the Chx-10 promoter, which is expressed in retinal progenitor cells. The THloxP/loxP mice were used as the WT control (“Ctrl”) for each experimental paradigm. Mice were genotyped by Transnetx, Inc. (Cordova, TN, USA).

**Experimental Overview**

In order to better understand refractive development under normal and FD visual conditions, two experimental paradigms were followed. First, mice underwent testing to measure refractive error, corneal curvature, and ocular biometrics every 2 weeks starting at postnatal day 28 (P28) until P112 (n = 12 Ctrl; n = 17 rTHKO mice) while being raised in standard mouse cages with unrestricted visual input on a 12:12 light:dark cycle (~70 [range, 20–200] lux; 4100K, 32W; Sylvania Octron®800 Ecologic fluorescent bulb; Sylvania, Wilmington, MA, USA). This lighting emits three major spectral peaks at 430, 545, and 610 nm with 70% of the spectral power greater than 530 nm. Retinas were collected from each mouse for DA analysis 2 days after light onset to control for circadian rhythms in retinal DA. HPLC. Mice were killed by cervical dislocation between 4 and 6 hours after light onset to control for circadian rhythms in retinal DA. Each eye was quickly enucleated under controlled lighting conditions (fluorescent lighting, 600 lux), and retinal tissue was collected, immediately frozen on dry ice, and stored

**Head Pedestal Surgery**

Under the FD experimental paradigm, P28 mice had ocular measurements taken and were subsequently outfitted with a head-mounted pedestal and a monocular diffuser goggle, as described previously. Briefly, the scalp and peristium of the anesthetized mouse were removed, and three stainless steel screws were placed in the skull. A mix of cyanosacrylate glue (Krazy Glue, Westerville, OH, USA) and dental cement was used to create a pedestal that held in place a diffuser goggle over the right eye. Mice were checked daily to ensure proper goggle compliance. Goggles were repositioned when needed. Temporary loss of goggles (<4–6 hours) did not appear to alter the myopia shift, and no mice were removed from the study for lack of goggle compliance.

**DA Analysis**

In order to determine the levels of retinal DA and DOPAC (the primary metabolite of DA) retinal samples were analyzed by HPLC. Mice were killed by cervical dislocation between 4 and 6 hours after light onset to control for circadian rhythms in retinal DA. Each eye was quickly enucleated under controlled lighting conditions (fluorescent lighting, 600 lux), and retinal tissue was collected, immediately frozen on dry ice, and stored
Effects of Reduced Retinal Dopamine on Myopia

FIGURE 1. Relative refractive error is shown across age for the two genotypes, rTHKO and Ctrl. The eyes of rTHKO mice had significantly less hyperopic refractive errors than Ctrl mice, corresponding with relative myopia (two-way repeated ANOVA interaction effect: F(1,188) = 7.602, P < 0.001; post hoc analysis: **P < 0.01; ***P < 0.001). Symbols represent average ± SEM.

FIGURE 2. Corneal radius of curvature is shown across age for the two genotypes. rTHKO and Ctrl. Retina-specific THKO mice had significantly smaller corneal radii of curvature, corresponding with steeper corneas and therefore, presumably shorter focal lengths (two-way repeated ANOVA main effect of genotype F(1,180) = 5.1, P < 0.05). Symbols represent average ± SEM.

RESULTS

Loss of Retinal DA Leads to Myopia During Normal Refractive Development

Under normal visual conditions, rTHKO mice had significant myopic refractions compared with Ctrl mice from 6 to 14 weeks (average difference in refractive error, 3.28 ± 0.27 D, F(1,188) = 7.602, P < 0.001; Fig. 1). Retrieval errors of rTHKO and Ctrl mice were similar at 4 weeks of age, but both genotypes became more hyperopic by 6 weeks, with Ctrl mice reaching 6.06 ± 0.72 D and rTHKO only 3.16 ± 0.58 D (Holm-Sidak post hoc comparison, P < 0.001). Within each genotype the refractive errors were not statistically different from 6 to 12 weeks of age, with refractions becoming less hyperopic at 14 and 16 weeks.

In addition, rTHKO mice had significantly steeper corneas (smaller corneal radius of curvature) by 0.023 ± 0.003 mm from 4 to 16 weeks of age compared with Ctrl mice (Fig. 2; main effect of genotype F(1,180) = 5.1, P < 0.05). Unlike refractive errors that were similar at 4 weeks of age between the genotypes, the corneas of rTHKO mice were steeper at 4 and 6 weeks of age.

Analysis of ocular parameters showed differences in ocular growth between the two genotypes. First, rTHKO mice had significantly smaller CIs at all age, with an average difference of 0.010 ± 0.001 mm (Fig. 3A; main effect of genotype F(1,181) = 37.17, P < 0.001). Additionally, as shown in Figure 3B, rTHKO mice had significantly thinner retinas compared with Ctrl mice (F(6,181) = 6.07, P < 0.001). Control and rTHKO RTs began at 0.170 ± 0.003 and 0.169 ± 0.002 mm, respectively at P28, but Ctrl mice showed a thickening trend, reaching 0.186 ± 0.003 mm at 12 weeks, while rTHKO mice showed a slight thinning trend, reaching 0.163 ± 0.003 mm at 12 weeks.

Finally, eyes of rTHKO mice had significantly shorter ALs compared with Ctrl mice as a function of age (Fig. 3C; F(6,181) = 5.78, P < 0.01). Axial length was shorter by an average of 0.040 ± 0.005 mm in rTHKO compared with Ctrl across all ages. Measurements of ACD, LT, and VCD did not show any significant differences between the genotypes (Supplementary Table S1).

Retinal DA and DOPAC Significantly Reduced in rTHKO Mice

Figure 4 shows that retinal DA was reduced by 93.5 ± 3.1% and retinal DOPAC was reduced by 93.4 ± 0.8% in rTHKO mice compared with Ctrl mice in the NRD group (Fig. 4A, 4B; Student’s t-test, ***P < 0.001). Retina-specific THKO mice exhibited higher DOPAC/DA turnover ratios compared with Ctrl mice (Fig. 4C; Student’s t-test, **P < 0.001).

Loss of Retinal DA Did Not Alter the Response to FD

Control mice underwent a significant myopic shift (OD-OS) of 3.54 ± 0.51 D after 2 weeks of treatment. This myopic shift showed a statistically significant difference from untreated Ctrl mice, and the post hoc analysis was significant for all time points after 4 weeks (Fig. 5A; F(3,90) = 5.54, P < 0.01). Retina-
specific THKO mice showed a significant myopic shift of 4.07 ± 1.5 D after 6 weeks of FD, but there was markedly more variation in the degree of response to FD (Fig. 5B; main effect of treatment \( F(1,93) = 11.1, P < 0.01 \)). There was no statistical difference in the response to FD between the two genotypes \( F(1,52) = 0.239, P = 0.63 \).

Corneal curvature did not change as a result of the FD treatment for either genotype (Supplementary Table S1). Analysis of ocular parameters from the FD experiments yielded no statistically significant differences for either genotype when comparing goggled mice with untreated control mice or between genotypes (Supplementary Table S1). Dopamine and DOPAC analysis by HPLC also showed no statistically significant changes in either DA, DOPAC, or DOPAC/DA ratio as a result of the FD treatment.

**DISCUSSION**

Our findings show that rTHKO mice raised under normal, unaltered visual conditions have relative myopia compared with Ctrl mice. In this mouse model, the shift toward myopia appeared to be due to increased corneal steepening, and not increased AL, indicating potential interactions between dopaminergic signaling in the retina and development of the cornea. The significant reduction of DA had no effect on the response to FD in rTHKO mice. Potential explanations for the normal response to FD include that residual retinal DA turnover preserved the signaling for FD myopia, or that DA signaling is not involved in the response to FD in mice.

**Effectiveness of rTHKO in Eliminating Retinal DA**

Retina-specific THKO mice have substantially reduced retinal DA and DOPAC levels. As previously reported, retinal DA and DOPAC levels were below 10% of Ctrl while concentrations of DA, DOPAC, and other catecholamines in the brain were completely unaltered. Thus, the results gathered from this model can be attributed to changes in retinal DA pathways, rather than higher level neural pathways or other systemic effects. The residual levels of DA and DOPAC may be attributed to either incomplete action of the Chx-10 promoter during development or alternative synthesis pathways of DA. The Chx-10 promoter serves as a good tool for studying the retina because it has been shown to be actively transcribed in all neuroblasts in the developing optic cup; however, it has been shown to be only variably active in adult retinal tissue, leaving the possibility that some retinal neurons remain unaffected and evade Th excision by Cre recombinase. Consistent with this interpretation, Jackson et al. found that some TH-immunoreactive amacrine cells persist in the retinas of rTHKO mice, accounting for approximately 10% of the number of cells in control retinas. Alternatively, other DA

**Figure 3.** Ocular parameters of both rTHKO and Ctrl mice measured at different ages in the refractive development experiment. (A) Retina-specific THKO mice had significantly thinner corneas compared with Ctrl mice (two-way repeated ANOVA main effect of genotype \( F(1,181) = 37.17, P < 0.001 \)). (B) Retina-specific THKO mice had significantly thinner retinas compared to Ctrl mice (two-way repeated ANOVA interaction effect: \( F(6,181) = 6.07, P < 0.001 \)). (C) Retina-specific THKO mice had significantly shorter ALs across time compared with Ctrl mice (two-way repeated ANOVA interaction effect: \( F(6,181) = 3.78, P < 0.01 \)). Post hoc analysis: * \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \). All symbols represent average ± SEM. Note that some errors bars are obscured by the symbols.

**Figure 4.** Retinal DA levels in rTHKO and Ctrl mice at P70 when housed under normal laboratory conditions. In rTHKO mice DA (A) and DOPAC (B) concentrations were significantly reduced compared with those in Ctrl mice (Student’s t-test, \( P < 0.001 \)). (C) Retina-specific THKO mice exhibited a significantly higher DOPAC/DA ratio than Ctrl mice (Student’s t-test, \( P < 0.001 \)). Bars represent average ± SEM.
synthesis pathways may become upregulated to compensate for the absence of TH. A previous study found 2% to 22% DA concentration in the brains of TH-null mice compared with WT controls, but found undetectable amounts of DA in TH-null mice that also had the enzyme tyrosinase knocked out.25 Thus, tyrosinase may be synthesizing DA in the absence of TH, which could account for the trace DA levels seen in this model.

**Ocular Parameter Changes in rTHKO Mice Raised Under Unaltered Visual Conditions**

Retina-specific THKO mice had significantly less hyperopic refractive errors and steeper corneas compared with Ctrl mice. The large difference in refractive index at the air and corneal interface as well as the asphericity of the anterior corneal surface makes the cornea the most important refracting surface of the eye. We hypothesize that the relative myopia seen in the rTHKO mice is due to steeper corneal curvature, instead of the typical axial elongation observed with myopia. A previous study found corneal curvature to be significantly correlated with refractive error in mice.26 It is possible that the corneal steepening in rTHKO mice is producing such a large myopic defocus that axial growth is slowed. Previous studies in several animal models, including tree shrews,27 guinea pigs,28 chicks,29 marmosets,30 and rhesus macaques31 have shown that positive lens defocus, which brings the focal point of incident light in front of the photoreceptors, slows eye growth and axial lengthening. Based on these observations, we predict that the short ALs in rTHKO mice may be due to slowed axial lengthening during development in response to myopic defocus produced by the decreased corneal radius of curvature.

The corneal curvature changes in the rTHKO mice may indicate that DA directly acts on the cornea, as some dopaminergic receptor activity is located in the corneas of rabbits32 and bovines.33 Alternatively, corneal changes may be due to DA-regulated growth factors released from the retina that act on the cornea, or due to DA-influenced retinal functions that alter parasympathetic output to the anterior segment. Future experiments on optical models of the mouse eye and the potential role of DA in corneal development may help elucidate the mechanisms driving corneal curvature and AL in mice.

The reduction in retinal DA may induce developmental changes that result in decreased RT in the rTHKO mice compared with Ctrl mice (Fig. 3B). The RT of the rTHKO mice was relatively stable from 4 to 16 weeks of age (0.169 ± 0.001 to 0.165 ± 0.002, respectively), indicating the absence of a progressive retinal degeneration phenotype. Because DA is an essential neuromodulator in the retina, the loss of DA likely influences retinal signaling and may lead to reduced survival of specific neurons. Future studies are needed to more fully characterize the retinal morphology of the rTHKO mice.

**Absence of Retinal DA Does Not Significantly Alter Response to FD Myopia in rTHKO Mice**

Retina-specific THKO mice showed no significant differences in mean magnitude of response to FD treatment compared with Ctrl mice. Previous studies have shown that retinal DA levels decrease after FD or lens defocus in animal models of experimental myopia (see review in ref. 5). However, in the mouse model of myopia, reductions in retinal DA levels with FD have not been reported.34–38 Furthermore, the consequences of chronically reduced DA levels on the response to FD have been variable. Several chicken studies have shown that using either nonselective DA antagonists39 or models in which retinal DA stores are reduced40–43 or abolished44 has either no effect on FD or a slight reduction in response to FD. In mice with retinal gene mutations that result in chronic reductions in DA signaling, the response to FD has had opposite effects: enhancing myopic shifts in models with ON pathway defects22 or photoreceptor degeneration,38 or producing no response to FD in a model with nonfunctional rod photoreceptors.37 The results of this study suggest that low levels of retinal DA do not substantially alter the response to FD in mice. Perhaps due to the residual levels of retinal DA in rTHKO mice, DA turnover was present and in fact, significantly greater, when expressed as the DOPAC/DA ratio, than in Ctrl mice, and may have provided sufficient signaling for a normal response to FD. Compensatory increases in DA turnover following partial DA depletion may be a common property of DA neurons. For example, compensatory increases in DA synthesis and turnover have been observed in brain DA neurons following partial lesions with 6-hydroxydopamine.45 It should be noted that the rTHKO mice responded to FD with a trend for smaller myopic shifts with greater SDs (~2.67
driven eye growth. Finally, specific mutations may amplify signaling to certain ocular structures (for instance the change in corneal curvature in the rTHKO mice). While this may not produce the same phenotype as seen in most cases of human myopia, it may reveal new information about the importance or influence of particular pathways on refractive development in isolation.

CONCLUSIONS
Retina-specific THKO mice with low retinal DA developed spontaneous myopia and retained a myopic response to FD, albeit with greater variability. The spontaneous myopia in rTHKO mice was associated with steeper corneas rather than increased ALs. Additional studies are needed to further explore the role of DA in myopia development in mice, including using inducible knock-outs to maintain normal gene expression during early development and using pharmacological agents in combination with genetic mutations to further elucidate mechanisms. This knowledge from mouse models, combined with that from other animal models of experimental myopia, is important for elucidating the role of DA in human myopia in the future.

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Relevance of rTHKO Mice to Human Myopia
Axial length is the primary ocular component associated with myopia in human eyes, and changes in AL have been previously associated with DA level changes (see review in ref. 5). Thus, whether the results from rTHKO mice that show relative myopia and shorter ALs are relevant to human myopia has yet to be determined.

Retinopathy of prematurity (ROP) in human children and oxygen-induced retinopathy (OIR) in rats result in myopic eyes with shorter than normal ALs. The myopic refractive errors in ROP and OIR are due primarily to changes in optical power of the cornea. In mice, the role of DA is critical to the development of FD myopia, as previously reported. Future studies in which all retinal DA is removed are needed to determine the role of retinal DA in susceptibility to environmental myopia.

A puzzling aspect of the FD data is the absence of changes in ocular parameters to explain the measured refractive shift. One possible explanation is that the sensitivity of our instruments is not great enough to detect the changes in mouse eyes. Due to the small size of the mouse eye, small changes in AL have large effects on refractive power, such that an approximately 5 μm change in AL has been calculated to produce a 1 D myopic shift. The resolution of the newest SD-OCT used here is near this limit. A second possibility is that the mouse does not respond consistently with axial myopia as observed in other animal models. The absence of axial elongation in mice after FD-induced myopic shifts, as found in this study and others, is contrasted with studies reporting a correlation between AL and refractive error with FD or lens defocus. Finally, because we did not measure all possible ocular parameters, it is possible that there are changes in one or more of these parameters that could explain the myopic refractive errors in the rTHKO mice. For instance, LT changes have been reported for other experimental myopia models and during emmetropization in humans. We have previously reported that the crystalline lens refractive index increases with FD in a mouse model with an ON pathway defect, suggesting another potential factor influencing ocular parameter measurements. The development of new and improved instruments to image the eye and perform ocular biometry will improve our ability to determine which changes in ocular parameters produce the refractive change in the mouse eye.


driven eye growth. Finally, specific mutations may amplify signaling to certain ocular structures (for instance the change in corneal curvature in the rTHKO mice). While this may not produce the same phenotype as seen in most cases of human myopia, it may reveal new information about the importance or influence of particular pathways on refractive development in isolation.

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