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Altered Refractive Development in Mice With Reduced Levels of Retinal Dopamine

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PURPOSE. The neuromodulator dopamine (DA) has been implicated in the prevention of excessive ocular elongation and myopia in various animal models. This study used retina-specific DA knockout mice to investigate the role of retinal DA in refractive development and susceptibility to experimental myopia.

METHODS. Measurements of refractive error, corneal curvature, and ocular biometrics were obtained as a function of age for both untreated and form-deprived (FD) groups of retina-specific tyrosine hydroxylase knockout (rTHKO) and control (Ctrl) mice. Retinas from each group were analyzed by HPLC for levels of DA and its primary metabolite (DOPAC).

RESULTS. Under normal visual conditions, rTHKO mice showed significantly myopic refractive shifts compared with Ctrl mice. Retina-specific THKO mice also had thinner corneas (main effect of genotype F(1,180) = 37.17, P < 0.001), thinner retinas (F(6,181) = 6.07, P < 0.001), and shorter axial lengths (F(6,181) = 3.78, P < 0.01) than Ctrl mice. Retina-specific THKO retinas contained less than 15% of DA and DOPAC compared with Ctrl retinas, and the remaining DA had a significantly higher turnover, as indicated by DOPAC/DA ratios (Student’s t-test, P < 0.05). Retina-specific THKO mice showed similar, yet more variable, responses to 6 weeks of FD compared with Ctrl mice.

CONCLUSIONS. Diminished retinal DA induced spontaneous myopia in mice raised under laboratory conditions without form deprivation. The relative myopic shift in rTHKO mice may be explained by steeper corneas, an unexpected finding. The chronic loss of DA did not significantly alter the FD myopia response in rTHKO mice.

Keywords: dopamine, myopia, refractive error

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 synthesis pathway through tyrosine hydroxylase (TH). This conditional knockout is specific to the retina, as a complete knockout would be lethal.8 Tyrosine hydroxylase catalyzes the formation of L-3,4-dihydroxyphenylalanine (L-DOPA) from the amino acid L-tyrosine. L-DOPA is then converted to DA by DOPA decarboxylase. Tyrosine hydroxylase is the rate-limiting 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.9

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.5 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 (rTHKO) mice were described previously and showed significantly reduced contrast sensitivity and light-adapted retinal functions.9 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.10 The ThloxP/loxP mice were used as the WT control (“Ctrl”) for each experimental paradigm. Mice were genotyped by Transnetyx, 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.11 Retinas were collected from each mouse for DA analysis 2 days following the final measurement session to allow time for residual effects of anesthesia to be eliminated. This group is referred to as normal refractive development (NRD). In the second experimental paradigm (FD), the mice underwent a surgical procedure at P28 in which a pedestal was fitted to the top of the skull in order to hold a diffuser goggle over the right eye.12 Goggled (n = 11 Ctrl; n = 6 rTHKO mice) and untreated naïve littermates (n = 20 Ctrl; n = 19 rTHKO mice) subsequently underwent weekly ocular measurements, as described below, until P77. Two days following the final testing, retinas were collected for DA analysis, as described below.

Ocular Measurements

In order to quantify refractive development and ocular growth, we performed ocularometry and measurements of refractive error and corneal curvature. Eyes were first dilated with 1% tropicamide. Refractive error of each eye was measured with an automated photorefractor.13 Refractive errors were first obtained with the mouse awake and allowed to move freely to get a baseline recording with a natural head position. After the mouse was anesthetized with ketamine and xylazine (ketamine 80 mg/kg; xylazine 16 mg/kg), a second set of refractive measurements was taken.12 Mice that showed a difference of greater than 2.0 diopter (D) in refractive error between the two eyes at P28 were excluded from the study. If a mouse exhibited significant tear film aberrations after anesthetization, the refractive values from the awake measurements were used instead. Next, a photokeratometer was used to measure the radius of curvature of the cornea using a ring of infrared light-emitting diodes (LED).13,14

Finally, biometric measurements of the mouse eye were taken with a 1310 nm spectral domain optical coherence tomography (SD-OCT) system (intrasubject variability: 10 ± 10 µm15; Bioptigen, Durham, NC, USA) calibrated with a refractive index of 1.43316 to obtain the following biometric lengths: corneal thickness (CT), anterior chamber depth (ACD), lens thickness (LT), vitreous chamber depth (VCD), and retinal thickness (RT). With these values, axial length (AL), defined as the distance from the anterior surface of the cornea to the anterior surface of the RPE, was calculated. For these measurements, we assumed that the refractive index of each structure was constant, and thus applied a single refractive index to allow for comparisons between groups (see Refs. 17, 18 for a discussion of refractive index measurements in mice). Following testing, the effects of xylazine were reversed using yohimbine (2.1 mg/kg) in order to reduce the possibility of corneal lesions.19 The mice were kept warm on a heating pad during recovery from anesthesia, and care was taken to ensure that their eyes remained moist at all times with saline drops.

During these experiments, the OCT system was upgraded to an Envisu R4300 SD-OCT (Bioptigen). Because the Envisu OCT produces significantly enhanced spatial resolution (intrasubject variability 4.1 ± 2.3 µm), especially in the retina, we were able to more accurately determine which structure in the OCT image corresponds with the RPE border. To correct for the difference between the instruments, a careful comparison of the images produced with the two devices was made. Based on this analysis, all RT and AL values acquired by the 1310 nm OCT were reduced by 0.0411 mm.

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.12 Briefly, the scalp and periostium of the anesthetized mouse were removed, and three stainless steel screws were placed in the skull. A mix of cyanoacrylate 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 DA20) 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.
at -80°C. Retinal samples were subsequently processed for DA analysis as described previously.21 The 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. Supernatant fractions were separated with HPLC using a 0.1 M sodium phosphate, 0.1 mM EDTA, 0.35 mM sodium octyl-sulfate, and 6% acetonitrile (pH 2.7) mobile phase to quantify the DA and DOPAC levels with coulometric detection. The DA and DOPAC levels were calculated using a standard curve generated with 0.1 to 1 ng DA and DOPAC and normalized to aggregate protein concentration (ng/mg). Dopamine and DOPAC levels were compared between groups, as well as the ratio of DOPAC/DA as an indicator of DA turnover in the eye.

Statistics
Two-way repeated-measures ANOVA with Holm Sidak post hoc comparisons (SigmaStat, San Jose, CA, USA) was performed to examine the differences between the two genotypes across age. Results are reported as an interaction effect unless otherwise stated. Normal distributions and equal variances were verified for each test. The differences between genotypes for DA levels were analyzed using an unpaired two-tailed Student’s t-test. Because no significant differences were found between refractive errors of untreated NRD mice and naive FD mice, the refractive error data were combined. To determine the effect of FD treatment, the difference in refractive error for each mouse across time was calculated as a shift.

RESULTS
Loss of Retinal DA Leads to Myopia During Normal Refractive Development
Under normal visual conditions, rTHKO mice had significant myopic refractive errors 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). Refractive 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 CTs 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 mm 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.$^9$ 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$^{10}$; however, it has been shown to be only variably active in adult retinal tissue,$^{23}$ leaving the possibility that some retinal neurons remain unaffected and evade Th excision by Cre recombinase.$^{24}$ Consistent with this interpretation, Jackson et al.$^9$ 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
The myopic shift (OD minus OS) induced by FD treatment is shown for the two genotypes, Ctrl (A) and rTHKO (B). The dashed lines show data for the FD treated mice, while the solid lines represent data from the naïve, untreated mice. (A) Control mice undergoing the FD treatment showed a significant myopic shift after 2 weeks of treatment (two-way repeated measures ANOVA interaction effect: F(3,90) = 5.54, P < 0.01; post hoc analysis: *P < 0.05, **P < 0.01, ***P < 0.001). All goggled time-points have greater than three Ctrl mice except at 10 weeks. (B) Retina-specific THKO mice undergoing the FD treatment showed a myopic shift after 2 weeks of treatment (two-way repeated measures ANOVA main effect of treatment: F(1,93) = 11.1, P < 0.01). Symbols represent average ± SEM.

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. 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 antagonists or models in which retinal DA stores are reduced or abolished 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 defects or photoreceptor degeneration, or producing no response to FD in a model with nonfunctional rod photoreceptors. 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.

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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**References**


