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Michael Bergen March 27, 2015

The Role of Dopamine in the Development of Myopia

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Abstract

The Role of Dopamine in the Development of Myopia

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Purpose

During development, the eye grows until incoming light is focused on the retina, a process called emmetropization. Dopamine (DA) has been heavily implicated as a modulator of this process. While many studies have utilized pharmacological agents and neurotoxins to elucidate the exact role of dopamine on eye growth, much remains to be known about this process. In this study, a retina-specific dopamine knockout is utilized to characterize the role of dopamine in refractive development, ocular growth, and susceptibility to experimental myopia.

Methods

Dopamine knockout (rTHKO) mice were on a C57BL/6J background and were homozygous for both the Chx10 Cre-recombinase and floxed tyrosine hydroxylase alleles. In the untreated refractive development (RD) paradigm, rTHKO mice and age-matched control (Ctrl) mice were measured every 2 weeks from post-natal day 28 (P28) to P112. Under the FD paradigm, mice received a head-mounted diffuser goggle at P28 over their right eye (OD) and were measured weekly until P77. Measurements of refractive error, corneal curvature, and ocular biometrics were obtained at each measurement session. Retinas from each group were analyzed by HPLC for dopamine and DOPAC concentrations.

Results

rTHKO mice exhibited an 85.3% loss in retinal DOPAC and an 89.5% loss in retinal DA compared to Ctrl mice. Untreated rTHKO mice became spontaneously myopic ($F_{(1,188)} = 7.602$, $p<0.001$) and had significantly steeper corneas (Main effect of genotype $F_{(1,209)} = 14.1$, $p<0.001$) compared to Ctrl mice. rTHKO mice also had thinner corneas (Main effect of genotype $F_{(1,181)} = 37.17$, $p<0.001$), thinner retinas ($F_{(6,181)} = 6.07$, $p<0.001$), and shorter axial lengths ($F_{(6,181)} = 3.78$, $p<0.01$). Form deprived rTHKO and Ctrl mice showed statistically similar myopic shifts (difference of right and left eyes).

Conclusions

Our results support the hypothesis that dopamine is a stop signal for refractive development. Loss of dopamine may affect the growth of the cornea, which would heavily impact refractive state. It is possible that the rTHKO mice show slowed axial growth due to myopic defocus imposed by the corneal steepening but were unable to fully compensate for the myopic defocus. Interestingly, the reduction in DA did not influence the response to FD.

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Introduction

In normal ocular refractive development, the mammalian eye grows until the incoming light is focused by the cornea and lens onto the photoreceptors of the eye to produce an image that is in-focus, a process called emmetropization. In a large percentage of the human population, this process occurs abnormally, leading to near-sightedness, or myopia. Myopia is characterized by excessive eye growth such that incoming light is focused in front of the retina, leading to impaired vision and the need for corrective lenses. Several studies have aimed to determine the prevalence of myopia. An estimated 41.6% are affected in the United States (Vitale et al., 2009). In Hong Kong, myopia in school children is estimated to have a prevalence of 85-88% (Edwards et al., 2004). In a study of 19 year old males in Seoul, South Korea, an unprecedented 96.5% were myopic (Jung et al., 2012). Using various animal models and genome-wide association studies, researchers have determined that both genetic (Hawthorne et al., 2013) and environmental (Norton et al., 2013) factors contribute to emmetropization.

Following the discovery that a juvenile lid-suture promoted excessive eye growth and myopia, animal models of form deprivation (FD) have been developed in order to investigate the mechanisms underlying refractive development (Wiesel et al., 1977). In the most common FD models, a diffuser goggle is placed over one eye of the animal, causing blurred visual input with reduced contrast sensitivity to the affected eye. This treatment leads to excessive eye growth and myopia development in many animal models. A similar response to FD can be induced in mice (Faulkner et al., 2007), which provides an excellent opportunity to explore various genetic factors that make the eye more or less susceptible to myopia. Several studies have helped build the conclusion that mechanisms of emmetropization act locally within the retina. One study found that severing the optic nerve of chicks produced no change in development of form deprivation myopia (Wallman et al., 2004). In addition, hemi-field lenses or hemi-diffusers, which act on only half of the visual field, produce lens-induced myopia and form deprivation myopia, respectively, in only the affected half of the eye (Diether et al.,

1997). Another study showed that exposing only the peripheral retina to hyperopic defocus, with a peripherally negative diopter lens, caused axial myopia (Alexandra Benavente-Pérez, 2014). These studies show that the peripheral retina is important in detecting refractive error, and changes in eye growth are mediated locally within the retina. To test whether the FD effect is merely a product of a reduced light level in the affected eye, a previous study placed a neutral density filter over the control eye to match the light level in the affected eye and found that the frosted goggle still produced a myopic shift compared to the eye treated with the neutral density filter (Feldkaemper et al., 1999). This confirms that the retina does, in fact, respond to changes in visual acuity, and this is responsible for the effect seen in the FD paradigm.

Over the past few decades, dopamine (DA) has become more and more implicated as an important modulator of refractive eye growth. Dopamine is a retinal neuromodulator that has been shown to decrease in concentration with myopia development (Stone et al., 1989). Several studies have suggested that dopamine is a “stop” signal for eye growth (reviewed by Feldkaemper et al., 2013). Traditionally, researchers have studied this pathway in primate and chick models, utilizing pharmacological agents to affect the dopamine receptors. A previous study showed that spiperone, an antagonist for the dopamine 1 and 4 receptors, prevented the ameliorative effects of brief periods of unrestricted vision in a form deprivation model. This indicates that dopamine action in the retina plays a key role in inhibiting excess eye growth during emmetropization (Nickla et al., 2011). In addition, an inverse relationship between dopamine release and response to form deprivation was shown, indicating that lower initial dopamine concentrations correlate with higher susceptibility to FD myopia (Park et al., 2013).

Many animal models have been used in myopia research, mainly monkeys, cats, tree shrews, marmosets, chickens, and guinea pigs (Edwards, 1996). However, the mouse model provides its own set of advantages (Pardue et al., 2013). First, the mouse genome is well understood, and can be readily

manipulated to probe various biochemical pathways. With this, the mouse provides an opportunity to manipulate both genes and environment simultaneously in the same animal. In addition, the gestational period is relatively short, and the litter size is relatively large. These properties make the mouse model much easier to use than other comparable models. Some disadvantages of the mouse model include the mouse's low visual acuity, lack of a fovea, and nocturnal activity; however, the mouse eye matches the human eye in many ways and has been used to elucidate biochemical pathways of the human eye in the past (Pardue et al., 2013).

In this study, a genetic strain of mouse was utilized which contains a retina-specific dopamine knockout. By disrupting the dopamine synthesis pathway genetically, we were able to greatly reduce or eliminate dopamine in the retina. In order to achieve this, a specific part of the dopamine synthesis pathway, tyrosine hydroxylase (TH), was targeted in the retina. TH is an enzyme that catalyzes the formation of L-3,4-dihydroxyphenylalanine (L-DOPA) from the amino acid L-tyrosine. L-DOPA is then converted to dopamine by DOPA decarboxylase. Therefore, a retinal TH knockout would, in theory, eliminate all significant sources of dopamine in the retina and allow us to determine the effects of dopamine on normal refractive development and susceptibility to FD in the mouse eye. This conditional knockout must be specific to the retina, as a complete knockout would be lethal (Sotak et al., 2005). To achieve this retinal specificity, Cre-lox technology was utilized to specifically target TH excision in retinal tissue. A previous study showed that this knockout exhibits a ~90% decrease in retinal dopamine concentration and significantly dampens both contrast sensitivity and light-adapted retinal function (Jackson et al., 2012). By selectively reintroducing dopamine 1 receptor (D1R) and dopamine 4 receptor (D4R) agonists to this retina-specific dopamine knock-out model, a previous study elucidated the effects of dopaminergic activity in these two receptors on visual function. The results showed that D1 receptors, found mainly on horizontal cells and cone ON-bipolar cells (Veruki et al., 1996), are responsible for increasing visual acuity in light-adapted vision by uncoupling electrical synapses between

horizontal cells. It was also found that D4 receptors, found mainly on cone photoreceptors, are responsible for light-adapted contrast sensitivity by down regulating rod-cone coupling (Jackson et al., 2012). Thus, the retina-specific dopamine knockout model used in this study can be expected to affect these same receptor pathways, D1R and D4R, seen in previous studies.

We hypothesized that a retina-specific tyrosine hydroxylase knockout (rTHKO) would effectively knock out retinal dopamine sources, and this depletion of retinal dopamine would result in more myopic refractive errors and longer axial lengths than control mice during normal refractive development. We also hypothesized that rTHKO mice would exhibit more myopic biometric characteristics, such as longer vitreous chamber depth and steeper corneas, than control mice. Finally, this loss of retinal dopamine is expected to result in increased susceptibility to myopia in form deprivation treated mice.

Materials and Methods

Retinal Dopamine Knockout Model

In this study, C57BL/6J mice were used according to the approved IACUC protocol and the guidelines published by NIH and Association for Research in Vision and Ophthalmology (ARVO). A retina-specific dopamine knockout strain was yielded by first obtaining C57BL/6J mice homozygous for the Chx10-Cre allele, in which a gene for the Cre-recombinase protein has been spliced downstream of the WT Chx-10 promoter, which is active in all retinal progenitor cells (Liu et al., 1994). This strain was crossed with a $TH^{(lox/lox)}$ strain, in which the WT tyrosine hydroxylase (TH) gene has been flanked with two loxP sites, the recognition sequences for the Cre-recombinase protein. When the two strains are crossed and homozygotes are selected for, the result is a Chx10-Cre:$TH^{(lox/lox)}$ strain, in which the Cre-recombinase gene, expressed only in the retina, splices out the WT tyrosine hydroxylase gene in vivo,

rendering it functionless (Jackson et al., 2012). In order to control for all genetic factors other than the knocked out TH, the $TH^{(lox/lox)}$ strain was used as the wild type control (Ctrl) for each experimental paradigm. The retina-specific dopamine knockout mice, Chx10-Cre:$TH^{(lox/lox)}$, will hereafter be referred to as "rTHKO," and the wild-type controls will be referred to as "Ctrl." Mice were genotyped by Transnetyx, Inc.

Experimental Overview

In order to better understand both normal refractive development and the susceptibility to environmentally-induced myopia, two unique experiments were conducted. In the first experiment, normal refractive development, mice underwent testing to measure refractive error, corneal curvature, and ocular biometrics every 2 weeks starting at post-natal day 28 (P28) until P112 while being raised in a standard mouse cage with unrestricted visual input on a 12:12 light:dark cycle (200 lux). At P114, mice were sacrificed, and their retinas were collected for dopamine analysis. In the second experiment, form deprivation, the mice underwent a surgical procedure at P28 in which a pedestal was outfitted to the top of the skull in order to hold a form deprivation diffuser goggle over the right eye (OD) (Faulkner et al., 2007). An image of the form deprivation model can be seen in Figure 1. The goggled mice as well as untreated naïve mice subsequently underwent ocular measurement, as described above, weekly until P77. Two days following the final testing, retinas were collected for dopamine analysis, as described above.

Ocular Measurement

In order to quantify refractive development and measure ocular growth, the mice underwent testing in a "circuit," which consisted of three measuring devices. The eyes were first treated with 1%

tropicamide to dilate the pupil. Relative refractive error of each eye was measured with an automated photorefractor. The photorefractor calculates relative refractive error by shining an infrared light into the dilated eye and measuring the pupil brightness profile (F. Schaeffel, 2008). A refractive error was first obtained with the mouse awake and gently restrained 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 (Faulkner et al., 2007). Mice that showed signs of amblyopia (> 2.0 diopter (D) difference in anesthetized refractive errors between the eyes at P28) were excluded from the study. If a mouse exhibited significant tear film aberrations as a result of the anesthetization, the refraction 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 LED lights. Since the cornea reflects an image of the LED ring, an infrared sensitive camera can calculate the radius of curvature of the cornea based on the radius of the reflected image (Frank Schaeffel et al., 1987).

Finally, biometric measurements of the mouse eye were taken with a spectral domain optical coherence tomography (SD-OCT) system (Bioptigen Envisu 4300) calibrated at a refractive index of 1.433 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 border between the retina and retinal pigment epithelium (RPE), can be calculated. Following testing, the effects of xylazine were reversed using Yohimbine (2.1 mg/kg) in order to reduce the possibility of corneal lesions (Turner et al., 2005). The mice were kept warm on a heating pad until they regained sternal recumbency, and care was taken to ensure that their eyes remained moist with saline drops.

During this experiment, the OCT system was upgraded from a 1310 nm spectral domain Bioptigen OCT to a 2100 nm spectral domain Bioptigen OCT. Our method of measuring the precise

lengths of the ocular parameters involves using software to measure the coordinates of the ocular borders in each OCT image. Since the 2100nm OCT produces significantly enhanced spatial resolution, especially in the retina, we were able to more precisely determine which structure in the OCT image corresponds with the retinal pigment epithelium (RPE) border. This new information created a slight discrepancy between values obtained from the 1310 system versus the 2100 system. To determine the magnitude of this change, the distance between where we previously measured the RPE border and where we currently measure the RPE border was measured for both rTHKO and Ctrl mice across age on the 1310 nm OCT. This distance ended up matching the discrepancy in retinal thickness measurements between the 1310 nm OCT and 2100 nm OCT. There was no significant difference in this value across age nor genotype. Thus, to correct for this difference, all retinal thickness and axial length values acquired by the 1310 nm OCT were reduced by 0.0411 mm.

Dopamine Analysis

In order to determine the concentrations of retinal dopamine and DOPAC, the primary metabolite of dopamine (Witkovsky, 2004), retinas were collected from each mouse two days following the final measurement session to allow time for residual effects of anesthesia to be eliminated. The mice were sacrificed by cervical dislocation between 4-6 h after light onset to control for retinal dopamine circadian rhythms. Each eye was quickly enucleated under controlled lighting conditions, and retinal tissue was collected and kept on dry ice and stored at −80 °C. Retinal samples were subsequently processed for dopamine analysis as described previously (Nir et al., 2000). The retinas were homogenized in 0.1 N $HClO_4$ solution (0.01% sodium metabisulfite and 50 ng/ml internal standard 3, 4-dihydroxybenzylamine hydrobromide) and centrifuged. Supernatant fractions were separated with high-performance liquid chromatography (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 the internal standard method with a standard curve generated with 0.1–1 ng DA and DOPAC.

Retinal dopamine and DOPAC analysis by HPLC yielded concentrations for both dopamine and DOPAC, which were normalized to aggregate protein concentration (pg/mg). These raw concentrations were then normalized to dopamine and DOPAC levels from Ctrl animals in each run, to account for variation among different HPLC runs. To assess this data, dopamine and DOPAC levels were compared between groups. In addition, the ratio of DOPAC/dopamine was calculated, as it is thought to be a good indicator of dopamine turnover in the eye.

Head Pedestal Surgery

Under the FD experiment, P28 mice were measured in the myopia "circuit" and subsequently outfitted for a head-mounted pedestal, as described previously (Faulkner et al., 2007). An image of the surgically attached pedestal can be seen in Fig. 1. Mice were given an extra 0.02 ml of intraperitoneal ketamine/xylazine and 0.02 ml of subcutaneous Meloxicam (metacam; 5mg/ml). All tools were sterilized and kept on sterile gauze throughout the procedure. The mouse's head was shaved to reveal a patch of skin of about 1 cm in diameter, which was cleaned with chlorohexidine, and saline drops were applied to each eye to keep the cornea moist. A small incision was made in the scalp, and the exposed fascia and periosteum were removed. Three small holes were drilled using a bone drill bit, one in each parietal bone and one in the occipital bone, into which three small stainless steel bone screws were inserted. Super Glue and dental cement were applied to the exposed area, and a small piece of tubing was placed in the dental cement and covered. Once the cement had dried, a frosted goggle was inserted into the pedestal and secured in the metal cube, which holds a balancing bar on the left side of the head. Small

adjustments were made to the goggle using needle-nose plyers to ensure a smooth fit on the mouse's face. Mice were then treated with 0.05 ml intraperitoneal Yohimbine (2.1 mg/kg) and 0.30 ml of subcutaneous lactated Ringers and were allowed to recover on a heating pad with wet food ad libitum.

This procedure, when compared to the practice of gluing a frosted lens onto the fur surrounding the eye, has been shown to lead to much higher compliance and significantly fewer ocular health issues (Faulkner et al., 2007). In addition, this method allows for much easier removal of the goggle during routine measurement.

Statistics

In order to assess differences between the two genotype groups across age, a Two Way Repeated-Measures ANOVA was run for each set of data using SigmaStat with Holm Sidak post hoc comparisons. Results are reported as an interaction effect unless stated otherwise. The differences between genotypes for dopamine levels were analyzed using an unpaired two-tailed Student's t-test. No significant differences were found between refractive errors of untreated, RD mice and naïve FD mice. Thus, the RD data were used as control values for the FD experiment and some control data from the FD experiment were used in the analysis of the RD data. To quantify the myopic shift seen in an animal undergoing the FD myopia treatment, the difference in refractive error between the right (OD) and left (OS) eyes was calculated. This value corresponds to the myopic shift induced in the form deprived eye compared to the naïve eye. Goggled mice were compared to untreated mice to control for random variation.

Results

Refractive Development

rTHKO mice became spontaneously myopic by 3.28 ± 0.27 D from 6 to 12 weeks of age compared to Ctrl mice (Fig. 2; $F_{(1,188)} = 7.602$, $p<0.001$). 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 to Ctrl mice (Fig. 3; Main effect of genotype $F_{(1,209)} = 14.1$, $p<0.001$).

Analysis of ocular parameters showed differences in ocular growth among the two genotypes. First, rTHKO mice had significantly smaller corneal thicknesses across time with an average difference of 0.010 ± 0.0009 mm (Fig. 4A; Main effect of genotype $F_{(1,181)} = 37.17$, $p<0.001$). Additionally, rTHKO mice had significantly thinner retinas by 0.014 ± 0.0027 mm compared to Ctrl mice (Fig. 4B; $F_{(6,181)} = 6.07$, $p<0.001$). This interaction was significant at all time points except 4 and 8 weeks of age.

Finally, and most surprisingly, rTHKO mice had significantly shorter axial lengths by 0.040 ± 0.0049 mm compared to Ctrl mice across age (Fig. 4C; $F_{(6,181)} = 3.78$, $p<0.01$). This interaction was significant at all time points.

Dopamine Analysis

Figure 5 shows that retinal DOPAC was reduced by 85.3% ± 1.7% and retinal DA was reduced by 89.5% ± 2.9% in the rTHKO mice compared to Ctrl (Fig. 5A,B; Student's t-test, ***$p<0.001$). rTHKO mice exhibited a significantly higher DOPAC/DA turnover ratio compared to Ctrl mice (Fig. 5C; Student's t-test, *$p<0.05$).

Form Deprivation

Ctrl mice underwent a myopic shift of 3.41 ± 0.80 D after roughly 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. 6A; $F_{(3,90)} = 5.54$, $p<0.01$). rTHKO mice showed a myopic shift of 2.62 ± 1.56 D after 2 to 4 weeks of FD, but there was markedly more variation in response to FD (Fig. 6B; Main effect of treatment $F_{(1,97)} = 7.96$, $p<0.01$). The two genotypes' responses to form deprivation were not statistically different from one another.

No significant trend was seen in corneal curvature as a result of the FD treatment for either genotype. Dopamine and DOPAC analysis by HPLC showed no statistical changes in either dopamine or DOPAC as a result of the FD treatment. Analysis of ocular parameters recorded in the FD experiment yielded no statistical trends for either genotype when comparing goggled mice to untreated control mice. Finally, dopamine and DOPAC data from each mouse was compared to myopic shift, corneal curvature change, and ocular parameter change as a result of FD treatment in an attempt to correlate variation in retinal dopamine with varying response to form deprivation, yet no significant trends were found.

Discussion

Retinal Dopamine Knockout Effectiveness

We can first conclude that this model is an effective retinal-dopamine knockout model based on the dopamine and DOPAC concentrations yielded from the HPLC analysis. While not complete, this model was successful in substantially reducing both retinal dopamine and retinal DOPAC to below 15% of Ctrl concentrations. As recorded previously, this model is especially useful for retinal studies because

concentrations of dopamine, DOPAC, and other catecholamines in the brain are completely unaltered (Jackson et al., 2012). Thus, the results gathered from this model can be completely attributed to retinal dopamine pathways, rather than higher level neural pathways or other systemic effects.

While this model greatly reduced retinal dopamine and DOPAC levels, there were still residual concentrations in the retina. This can be attributed to either incomplete action of the Chx-10 promoter during development or alternative synthesis pathways of dopamine. 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 (Liu et al., 1994), yet it has been shown to be variably active in adult retinal tissue (Lefebvre et al., 2008). This does not exclude the possibility that some retinal neurons remain unaffected and evade TH excision by Cre recombinase. Another possible explanation for the incomplete knockout is that other dopamine synthesis pathways become upregulated to compensate for the TH knockout. A previous study found 2 to 22% dopamine concentration in TH-null mice compared to WT controls, but found undetectable amounts of dopamine in TH-null mice that also had the enzyme tyrosinase knocked out (Rios et al., 1999). This opens the possibility that tyrosinase is synthesizing dopamine in the absence of tyrosine hydroxylase, which could account for the trace dopamine levels seen in this model.

Corneal Steepening Underlies Myopic Refraction in rTHKO

Because the cornea is the first refractive surface in a light ray's path to the retina and there is a large difference in refractive index between air and corneal tissue, it is responsible for most of the refractive power of the eye. Thus, changes in the radius of curvature and thickness of the mouse's cornea are very powerful in changing the refractive power of the eye. It is possible that the myopic effect seen in this model is due to the change in corneal curvature and thickness. Typically, steeper

corneas have been associated with myopic refractive errors, which is the same trend seen in this study. However, myopic development is typically associated with axial lengthening, which was not seen in this study. It is possible that the cornea of an rTHKO mouse is producing such a heavy myopic defocus that axial lengthening is being slowed. Previous studies have shown that positive lens defocus, which brings the focal point of incident light in front of the retina, slows eye growth and axial lengthening. This effect has been well documented in several animal models, including tree shrews (Metlapally et al., 2008), guinea pigs (Howlett et al., 2009), chicks, marmosets, and rhesus macaques (Zhu et al., 2013). It is possible that the rTHKO mice exhibited slowed axial lengthening in response to the myopic defocus produced by the corneal shape and were not able to fully compensate, leaving them with both shorter axial lengths and relative myopia. Since many studies have characterized the inverse relationship between retinal dopamine and ability to properly emmetropize when treated with lens defocus, it makes sense that these animals would be unable to fully compensate for this defocus, given their low retinal dopamine concentrations.

Further studies are necessary to evaluate the relationship between dopamine and other parameters that contribute to optical power, such as lens curvature, posterior corneal curvature, and relative refractive indices of ocular structures.

It was observed that the rTHKO animals tended to weigh less on average than Ctrl counterparts. This trend was heavily investigated, but no statistically significant difference in body weight was recorded between the two groups. While normalization to body weight is sometimes used to control for eye size, the fact that the difference in body weight between the two groups was not statistically different precluded us from further using this to normalize other parameters. However, it remains possible that some degree of the biometric differences between the two genotypes observed in this study might be due to differences in body weight.

Dopamine's Effect on Myopia and Form Deprivation

Dopamine is considered a stop signal for myopic eye growth, and many studies have focused on the dopamine receptors in the retina as the potential signaling mechanisms (reviewed by Feldkaemper et al., 1999). However, our data suggest that dopamine may also affect corneal curvature and thickness. It has been previously shown that dopaminergic receptor activity was found in the cornea of rabbits (Cavallotti et al., 1999) as well as bovine corneas (Grub et al., 2012). Dopamine's effect on development of the cornea is not currently very well understood, but our results suggest that dopamine is important for the development of both the curvature and thickness of the cornea.

The other ocular parameter that differed between the two genotypes in this study was retinal thickness. Figure 4B shows that the retinal thickness of the Ctrl animals increased slowly over time from 0.17 mm to roughly 0.18 mm, while the rTHKO retinas showed a slight decrease over time. This could be explained by a small amount of retinal degeneration in the retinas of the rTHKO mice. It has been shown that dopamine loss precedes photoreceptor loss in both RCS (Royal College of Surgeons) and rds (retinal degeneration slow) mice, both of which are predisposed to retinal degeneration (Djamgoz et al., 1997). In addition, retinal degeneration is commonly associated with retinal thinning. Thus, it is possible that the relatively low levels of dopamine in this model play a role in the relative decline in retinal thickness observed in this study.

Previously, many studies have aimed to understand the effect of dopamine on susceptibility to Form Deprivation myopia. Several studies have shown that utilizing either dopamine antagonists or models in which retinal dopamine stores are reduced show either no effect on FD or a reduced response to FD (reviewed by Feldkaemper et al., 2013). In this study, it was found that rTHKO mice had no significant difference in response to FD treatment when compared to Ctrl mice; however, the rTHKO response to FD was much more variable and less statistically significant. It is possible that the

incomplete loss of dopamine in this model accounts for the highly variable response to the FD treatment; however, all attempts to correlate retinal dopamine or DOPAC in both the goggled and naïve eyes with myopic shift were met with no statistical significance. It is also possible that the fluctuations in dopamine following form deprivation that some previous studies have found have simply been secondary to the mechanism behind form deprivation. The results of this study suggest that having low levels of dopamine does not substantially alter an individual's response to form deprivation, indicating that dopamine may play less of a role in susceptibility to environmental myopia than previously thought.

Finally, it was discovered that the rTHKO mice showed a higher rate of dopamine turnover than Ctrl mice in normal refractive development. Since DOPAC concentrations have been shown to be highly variable between individuals, the ratio of DOPAC/DA has been accepted as a more robust indicator of dopamine use in the eye. While some studies have gathered conflicting data, one study shows that chicks treated with positive lens defocus exhibited shorter axial lengths and an increase in retinal DOPAC when compared to untreated chicks (Guo et al., 1995). While causality cannot be determined from this finding, it remains possible that the increase in dopamine turnover seen in the rTHKO mice is correlated with the shortened axial length. It is also possible that the retina has a mechanism to upregulate dopamine metabolism when dopamine stores are low. With regards to the Form Deprivation results seen in this experiment, it is possible that the excessive dopamine turnover ratio seen in the rTHKO mice is what is preventing them from growing excessively myopic during FD. While one would expect their low levels of dopamine to make them very susceptible to FD, this extra turnover could provide a protective effect.

Future Directions

While many studies have aimed to elucidate the exact mechanisms behind spontaneous myopia and susceptibility to form deprivation myopia, the exact mechanism is proving to be very elusive. In this study, we showed that a Cre-mediated, retina-specific dopamine knockout was a successful model in depleting a substantial amount of retinal dopamine and inducing spontaneous myopia. We were somewhat successful in elucidating the ways in which retinal dopamine shapes the eye during refractive development. It is clear that corneal growth is mediated in some way by dopaminergic pathways, and failure of these pathways leaves a myopic cornea and the inability to properly compensate for this myopic defocus.

While the overall effect of dopaminergic signaling on refractive development and ocular growth are becoming more well-documented, it will become important for future studies to gain a better understanding of how specific dopamine receptors and signaling pathways contribute to myopia. Studies are currently being conducted to characterize the refractive development, ocular growth, and myopia susceptibility of mice with dopaminergic pathway defects, such as dopamine 4 receptor knockout (D4RKO) and dopamine 1 receptor knockout (D1RKO). It would also be useful to selectively reintroduce dopamine receptor agonists to this rTHKO model to determine the specific rescue effects of each receptor. Having a better understanding of the precise role of dopamine in refractive development will allow us to determine the best targets for pharmacological intervention. As myopia incidence continues to rise around the world, research in myopia and vision science are becoming key to address this epidemic.

Figures

Figure 1: A picture of the form deprivation goggle used to induce myopia in this study. Three screws are placed in the skull, two in each parietal bone and one in the occipital bone, and dental cement is used to hold a piece of stainless steel tubing in place. This pedestal holds the goggle and fastening cube into place over the mouse's right eye. A balance bar on the opposite side of the head prevents movement of the goggle.

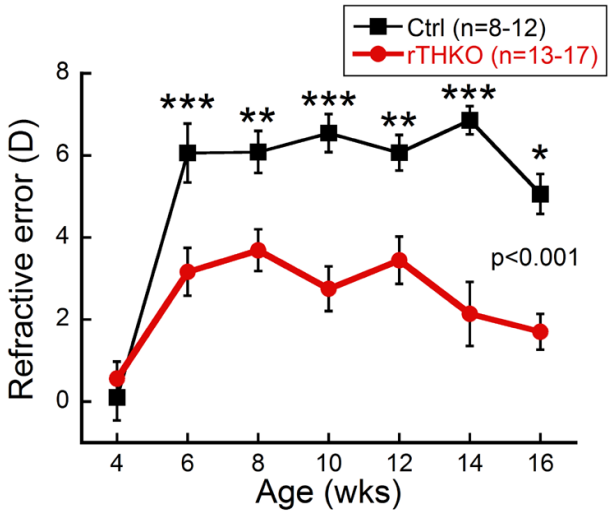

Figure 2: The relative refractive error is shown across time for the two strains, rTHKO and Ctrl. Points are plotted as average ± SEM. rTHKO mice had significantly lower relative refractive errors, corresponding with relative myopia ($F_{(1,188)} = 7.602$, $p<0.001$; post hoc analysis: *$p<0.05$; **$p<0.01$; ***$p<0.001$).

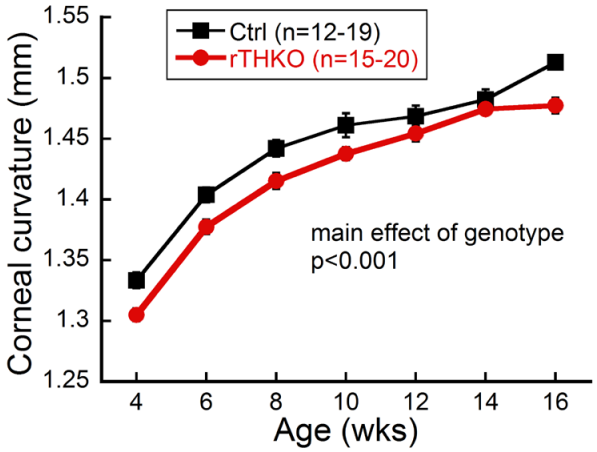

Figure 3: The corneal radius of curvature is shown across time for the two strains, rTHKO and Ctrl. Points are plotted as average ± SEM. Note that many of the error bars are obscured by the symbols. rTHKO mice had significantly smaller corneal radii of curvature, corresponding with steeper corneas (Main effect of genotype $F_{(1,209)} = 14.1$, $p<0.001$).

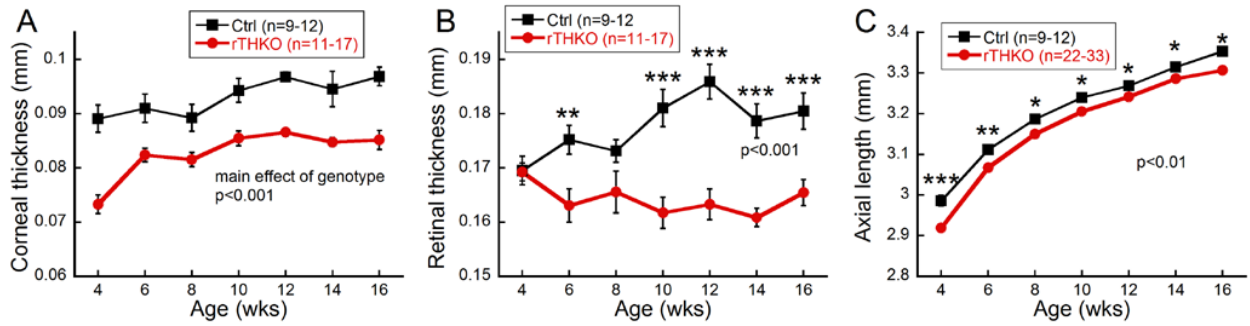

Figure 4: Ocular parameters of both rTHKO and Ctrl mice measured across age in the refractive development experiment. All points are plotted as average ± SEM. Note that some errors bars are obscured by the symbols. A) rTHKO mice had significantly thinner corneas across time compared to Ctrl mice (Main effect of genotype $F_{(1,181)} = 37.17$, $p<0.001$). B) rTHKO mice had significantly thinner retinas across time compared to Ctrl mice ($F_{(6,181)} = 6.07$, $p<0.001$). C) rTHKO mice had significantly shorter axial lengths across time compared to Ctrl mice ($F_{(6,181)} = 3.78$, $p<0.01$). Post hoc analysis: *p<0.05; **p<0.01; ***p<0.001.

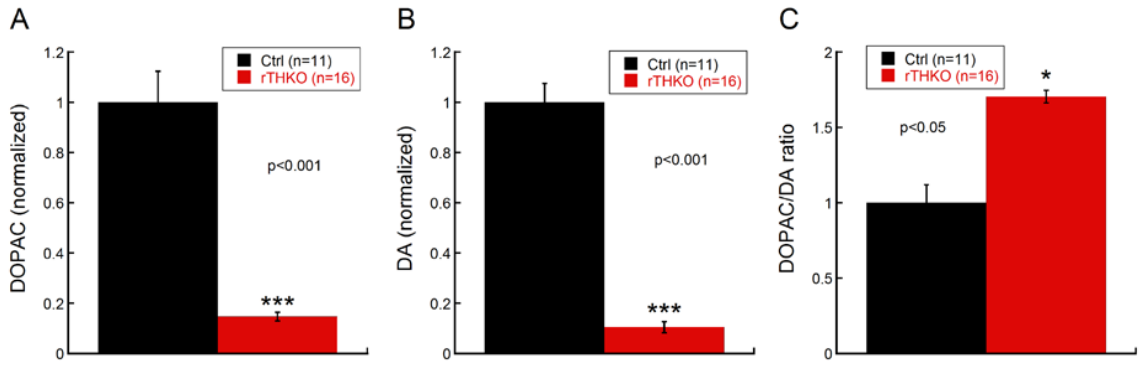

Figure 5: Retinal dopamine levels at P114. All bars show average ± SEM. A) rTHKO DOPAC concentrations were reduced by 85.3% on average compared to Ctrl mice ($p<0.001$). B) rTHKO dopamine concentrations were reduced by 89.5% on average compared to Ctrl mice ($p<0.001$). C) rTHKO mice exhibited a significantly higher dopamine turnover ratio than Ctrl mice ($p<0.05$).

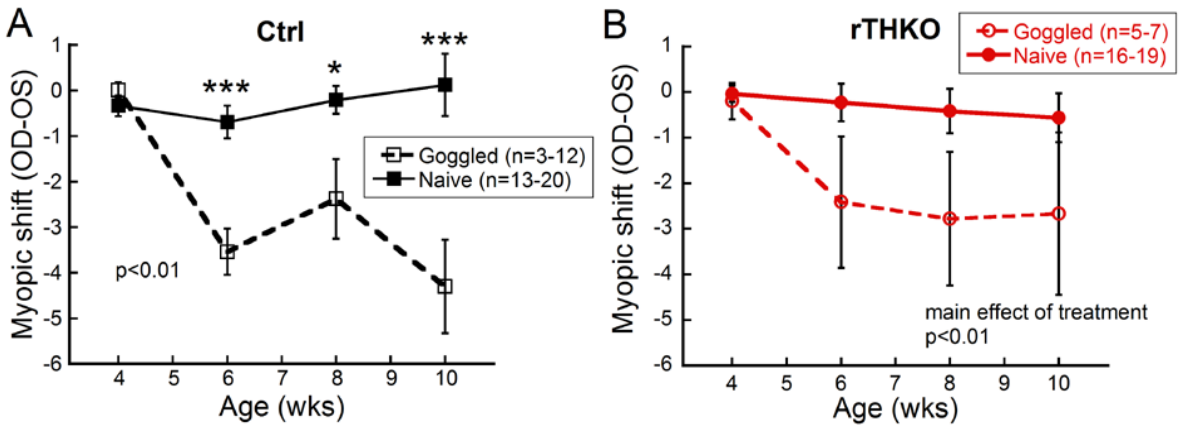

Figure 6: The myopic shift (OD minus OS) induced by the Form Deprivation treatment is shown for the two strains, Ctrl (A) and rTHKO (B). The dashed lines show the FD treated mice, while the solid lines represent the naïve, untreated mice. All plotted points show average ± SEM. A) Ctrl mice undergoing the FD treatment showed a significant myopic shift after 2 weeks of treatment ($F_{(3,90)} = 5.54$, $p<0.01$; post hoc analysis: *$p<0.05$; **$p<0.01$; ***$p<0.001$). B) rTHKO mice undergoing the FD treatment showed a myopic shift after 2 weeks of treatment (Main effect of treatment $F_{(1,97)} = 7.96$, $p<0.01$).

Appendix

Abbreviations

ACD:	Anterior Chamber Depth
AL:	Axial Length
CT:	Corneal Thickness
Ctrl:	Control
D:	Diopter
DA:	Dopamine
DOPAC:	Dihydroxyphenylacetic acid – the primary metabolite of dopamine
FD:	Form Deprivation
HPLC:	High-performance Liquid Chromatography
LT:	Lens Thickness
OCT:	Optical Coherence Tomography
OD:	Right eye
OS:	Left eye
P28:	Post-natal day 28
PCD/VCD:	Posterior/Vitreous Chamber Depth
RD:	Refractive Development
RPE:	Retinal Pigment Epithelium
RT:	Retinal Thickness
rTHKO:	Retinal Tyrosine Hydroxylase Knockout
TH:	Tyrosine Hydroxylase
WT:	Wild Type

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