

Comparison of refractive development and retinal dopamine in OFF pathway mutant and C57BL/6J wild-type mice

Ranjay Chakraborty,^{1,4} Han na Park,^{1,4} Moe H. Aung,^{1,2} Christopher C. Tan,¹ Curran S. Sidhu,¹ P. Michael Iuvone,^{1,2,3} Mabelle T. Pardue^{1,2,4}

(The first two authors contributed equally to this work.)

¹Department of Ophthalmology, Emory University School of Medicine, Atlanta, GA; ²Department of Neuroscience, Emory University School of Medicine, Atlanta, GA; ³Department of Pharmacology, Emory University School of Medicine, Atlanta, GA; ⁴Atlanta VA Center of Visual and Neurocognitive Rehabilitation, Decatur, GA

Purpose: Proper visual transmission depends on the retinal ON and OFF pathways. We used *Vsx1*^{-/-} mice with a retinal OFF visual pathway defect to determine the role of OFF pathway signaling in refractive development (RD) of the eye.

Methods: Refractive development was measured every 2 weeks in *Vsx1*^{-/-}, *Vsx1*^{+/+} (both on 129S1/Sv background), and commonly used C57BL/6J mice from 4 to 12 weeks of age. Form deprivation (FD) was induced monocularly from 4 weeks of age using head-mounted diffuser goggles. Refractive state, corneal curvature, and ocular biometry were obtained weekly using photorefractometry, keratometry, and 1310 nm spectral-domain optical coherence tomography. Retinal dopamine and its metabolite, 3,4-dihydroxyphenylacetate (DOPAC), were measured using high-performance liquid chromatography (HPLC).

Results: During normal development, the *Vsx1*^{-/-} and *Vsx1*^{+/+} mice showed similar myopic refractions at younger ages (4 weeks, *Vsx1*^{-/-}: -5.28 ± 0.75 diopter (D); WT: -4.73 ± 0.98 D) and became significantly hyperopic by 12 weeks of age (*Vsx1*^{-/-}: 3.28 ± 0.82 D; WT: 5.33 ± 0.81 D). However, the C57BL/6J mice were relatively hyperopic at younger ages (mean refraction at 4 weeks, 3.40 ± 0.43 D), and developed more hyperopic refractions until about 7 weeks of age (8.07 ± 0.55 D) before stabilizing. Eight weeks of FD did not induce a myopic shift in the 129S1/Sv animals (0.16 ± 0.85 D), as opposed to a significant shift of -4.29 ± 0.42 D in the C57BL/6J mice. At 4 weeks of visual development, dopamine turnover (the DOPAC/dopamine ratio) was significantly greater in the 129S1/Sv mice compared to the C57BL/6J mice. FD did not alter the levels of dopamine between the goggled and opposite eyes for any genotype or strain.

Conclusions: OFF pathway signaling may not be critically important for normal refractive development in mice. Elevated retinal dopamine turnover in early refractive development may prevent FD myopia in 129S1/Sv mice compared to C57BL/6J mice.

During normal refractive development, a perfect match between the optical power and the axial length of the eye produces a sharp, focused image on the retina, resulting in emmetropia [1,2]. Any disruption to this coordinated mechanism of ocular growth results in the development of refractive errors: eyes that are either too short (hyperopia) or too long (myopia) [2].

A large body of animal studies has suggested that the signal cascade for regulating eye growth starts at the retina [2-4]. In the retina, visual signals from photoreceptors are transmitted to ganglion cells via a heterogeneous class of retinal interneurons known as bipolar cells [5]. At least 11 distinct bipolar cell types have been characterized in the mammalian retina. These bipolar cells are classified based

on their morphology [5], immunoreactivity to specific bipolar cell markers [6], response to light as either OFF (types 1-4) or ON (types 5-9 and rod bipolar cells) [5,7,8], and their synaptic input from rod and cone photoreceptors [6,9,10]. Cone bipolar cells are essential for transmitting cone- and rod-generated visual signals in mammals since these cells make direct synapses with corresponding ganglion cells in the inner plexiform layer (IPL) for processing photopic vision [9,11]. In addition, cone bipolar cells transmit scotopic signals by connecting ON rod bipolar cells to ganglion cells through AII amacrine cell networks between rod bipolar cells and ON and OFF cone bipolar cell terminals [9]. ON and OFF circuitries of the visual system are important for processing contrast sensitivity [7,12,13], an important prerequisite for a high-acuity retinal image. Since emmetropization is driven by a sharp, in-focus image to the retina [14], abnormalities in visual transmission through ON and OFF cone pathways could potentially influence normal refractive development in mammals [15].

Correspondence to: Mabelle T. Pardue, Research Service (151Oph), Atlanta VA Medical Center, 1670 Clairmont Road, Decatur, GA, 30033; Phone: (404) 321-6111 x17342; FAX: (404) 728-4847; email: mpardue@emory.edu

Although a few studies have reported abnormalities in visual transmission through the ON pathway [16-20] are associated with myopia in humans [21], and an increased susceptibility to form-deprivation myopia in a mutant mouse carrying a null mutation in *Nyx* [22], no previous study has examined the refractive state associated with an OFF visual pathway defect in mammals. In this experiment, we examined the refractive state and retinal dopamine (DA) levels of *Vsx1*^{-/-} mice [23], which carry a null mutation in the visual system homeobox 1 gene, *Vsx1* [24]. *Vsx1* is a paired-like homeo- and CVC-domain transcription factor [24,25] expressed in mammalian cone bipolar cells [23,26]. Previous studies with immunolabeling for specific cone bipolar cells in *Vsx1*^{-/-} retinas have shown a significant decrease in the axonal termini of various OFF [23,26,27] and a few ON [28] cone bipolar cells, suggesting that *Vsx1* is essential for late terminal differentiation and functioning of cone bipolar cells, especially OFF cone bipolar cells. *Vsx1*^{-/-} mice have been reported to have deficits in bipolar cell function measured with an electroretinogram (ERG) [26]. In humans, *VSX1* (Gene ID 30813; OMIM 605020) has been associated with missense mutations that cause inherited corneal dystrophies and abnormalities in inner retinal function [29]. We hypothesize that anomalous terminal differentiation of OFF cone bipolar cells, causing an abnormal visual transmission from the photoreceptors to the ganglion cells in *Vsx1*^{-/-} retinas, would result in altered refractive development and form deprivation in *Vsx1*^{-/-} mice. Since *Vsx1*^{-/-} and *Vsx1*^{+/+} mice are on a 129S1/Sv background, a strain of mice not previously reported for refractive development [30], the results were compared with the commonly used C57BL/6J mice.

METHODS

Animals and experimental design: We examined the refractive development of *Vsx1*^{-/-} mice with a genetically induced OFF visual pathway defect [23]. In the mouse, *Vsx1* expression is detected as early as postnatal day 5 in the bipolar cell region of the inner nuclear layer (INL) [24]. An in-house breeding colony with male and female heterozygous *Vsx1* mutants (a generous gift from Dr. Robert L. Chow, University of Victoria, Victoria, Canada) was maintained at the Atlanta Department of Veterans Affairs Medical Center. Mice were kept in 12 h:12 h light-dark cycles (lights on at 06:00 with about 17 lux during the light phase) with mouse chow and water available ad libitum.

Age-matched male and female wild-type (*Vsx1*^{+/+}) and *Vsx1*^{-/-} mice on a 129S1/Sv background were subjected to two different experimental paradigms: refractive development (RD) and form deprivation (FD). For the RD experiments

(*Vsx1*^{+/+}, n=8; *Vsx1*^{-/-}, n=7), refractive error and axial length measurements were obtained every 2 weeks from 4 to 12 weeks of age under non-manipulated visual conditions. For the FD experiments, baseline refractive measurements for the *Vsx1*^{+/+} (goggled, n=7; naïve controls, n=8) and *Vsx1*^{-/-} (goggled, n=8; naïve controls, n=9) mice were obtained at 4 weeks of age, and then the mice were subjected to monocular form deprivation in the right eye using head-mounted goggling apparatus as described previously [31]. Goggles were applied for a period of 8 weeks (i.e., up to 12 weeks of age). Refractive development in the 129S1/Sv *Vsx1*^{+/+} mice was also compared with the commonly used C57BL/6J mice under normal (n=8) and FD (goggled, n=8; naïve controls, n=8) visual paradigms. Ocular compliance with goggling was monitored daily, and animals that showed poor compliance or ocular health were excluded from further measurements. 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.

Refractive state, corneal curvature, and ocular biometry: For the RD and FD paradigms, refractive error and ocular biometry were acquired, as described previously [22,32,33]. Eyes were dilated with 1% tropicamide, and the refractive state was obtained using an automated infrared photorefractor [34] by gently restraining the mouse by the tail in a dark room. A small subset of mice (n=13 for both RD and FD) had axial length measurements acquired before anesthesia using a custom-made 845 nm time-domain partial coherence interferometer (PCI) [33,35]. Mice were stabilized in a conical tube and the head pedestal stabilized with a clip [32]. Following the awake measurements, the mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). The refractive error measurements were repeated under anesthesia to obtain a more stable measurement with standard deviations of less than 0.5 diopter (D) [22].

Subsequently, the corneal radius of curvature was measured using an automated keratometer [36,37]. Upon proper alignment of the eye, the device used infrared light-emitting diodes (LEDs) to create eight Purkinje images on the corneal surface, which was recorded with an infrared light-sensitive video camera to calculate the overall corneal radius of curvature [37].

Axial length measurements were obtained using 1310 nm spectral-domain optical coherence tomography (SD-OCT; Bioptigen, Durham, NC), as previously described [32]. Axial length was measured from the anterior cornea to the RPE border using the OCT calipers (calibrated at a refractive index of 1.433) [38]. After the measurements were completed, the

mice were given yohimbine (2.1 mg/kg) to reverse the effects of anesthesia and reduce the development of corneal lesions [39], and placed on a warming pad to recover.

Retinal dopamine quantification: To determine the levels of retinal DA and DOPAC (the primary metabolic by-product of DA) [40] during the critical period of normal refractive development in mice [30], the retinas of 4-week-old *Vsx1*^{-/-} (n=9), *Vsx1*^{+/+} (n=10), and C57BL/6J (n=10) mice were harvested between 4 and 6 h after light onset. For the FD experiments, the retinal DA and DOPAC levels were measured after the final endpoint at 8 weeks for the *Vsx1*^{+/+} (control: n=3, goggled: n=6) and *Vsx1*^{-/-} (control: n=4, goggled, n=7) mice and at 4 weeks for the C57BL/6J mice (control: n=6, goggled: n=4). Experimental mouse retinas were collected 48 h after the final measurements to eliminate any effect of anesthesia. Harvested retinas were immediately frozen on dry ice and stored at -80 °C. The frozen retinas were processed as previously described [41]. Briefly, 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 high-performance liquid chromatography (HPLC) using a mobile phase of 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. All FD cohort retinas were homogenized individually; the retinas from the right and left eyes of the RD cohort were pooled together for analysis.

Data analysis: Statistical analyses were performed using commercial software (SigmaStat 3.5, Aspire Software International, Ashburn, VA). Changes in refractive error, keratometry, and axial length between the *Vsx1*^{+/+}, *Vsx1*^{-/-}, and C57BL/6J animals with age under normal non-manipulated (RD cohort) and form-deprived (FD cohort) conditions were analyzed with repeated-measures two-way ANOVA (ANOVA) and Holm-Sidak post-hoc tests for statistical significance. To minimize the influence of body size (mean bodyweight at 4 weeks; *Vsx1*^{+/+}: 15.94±0.91 g, *Vsx1*^{-/-}: 13.6±0.44 g, and C57BL/6J: 13.19±0.34 g, one-way ANOVA on Rank, H=7.07, p=0.029) on eye size [42], the axial length measurements were normalized to the respective bodyweight across age. For the RD cohort, where the right and left eyes received the same treatment, the refraction and biometric measurements from the two eyes were averaged to represent a single value from each mouse. For animals for which PCI and SD-OCT measurements were available, the axial length

values were averaged since the techniques have good agreement [32]. For the FD cohort experiments, refractive errors are presented as “myopic shift,” the difference between goggled and opposite eyes or right minus left eyes for naïve controls. To eliminate any inter-subject variability, the axial length measurements of the FD cohort were normalized to 4-week-old values (i.e., baseline values). After the values were normalized to baseline, “axial shift” was determined by calculating the difference in eye length between the right eye and the left eye. The DA and DOPAC measurements from the 4-week-old RD cohorts were analyzed using one-way ANOVA with Holm-Sidak post-hoc comparisons, or when normality failed, a one-way ANOVA on Rank with Dunn’s method post-hoc comparisons. Additionally, the DA and DOPAC values from the FD cohorts are presented as a ratio of the right eye to the left eye (the OD/OS ratio) to illustrate the changes in the retinal DA levels between the goggled and control eyes, where a ratio of 1 would indicate similar DA levels in the goggled (right) and opposite (left) eyes. DA data from the FD cohorts were analyzed using two-way ANOVA with Holm-Sidak post-hoc comparisons.

RESULTS

Refractive development altered by strain, but not the OFF pathway defect: Under a normal laboratory visual environment, the 129S1/Sv (*Vsx1*^{-/-} and *Vsx1*^{+/+}) and C57BL/6J mouse strains showed a significant increase in hyperopic refraction with increasing age from 4 to 12 weeks (Figure 1A; two-way repeated-measures ANOVA main effect of age, F(8,195)=76.88, p<0.001). However, there were significant differences in the pattern of refractive development for these strains across age (two-way repeated-measures ANOVA interaction, F(16,195)=6.69, p<0.001). At 4 weeks of age, the *Vsx1*^{-/-} and *Vsx1*^{+/+} mice were relatively myopic (mean refractive error; mean ± standard error of the mean (SEM), *Vsx1*^{+/+}: -4.73±0.98 D, *Vsx1*^{-/-}: -5.28±0.75 D) and became significantly hyperopic by the end of 12 weeks (*Vsx1*^{+/+}: 5.33±0.81 D, *Vsx1*^{-/-}: 3.28±0.82 D). Throughout the course of refractive development, refractive errors were not significantly different between the *Vsx1*^{-/-} and *Vsx1*^{+/+} mice (Holm-Sidak multiple comparisons, p<0.05), suggesting that the *Vsx1* mutation had no significant effect on normal refractive development of the eye. In comparison, the C57BL/6J mice had relatively hyperopic refractive errors at younger ages (mean refraction at 4 weeks, 3.40±0.43 D), and developed more hyperopia until about 7 weeks of age (8.07±0.55 D) before stabilizing.

From 4 to 12 weeks of age, there was significant flattening in corneal curvature for each group (mean

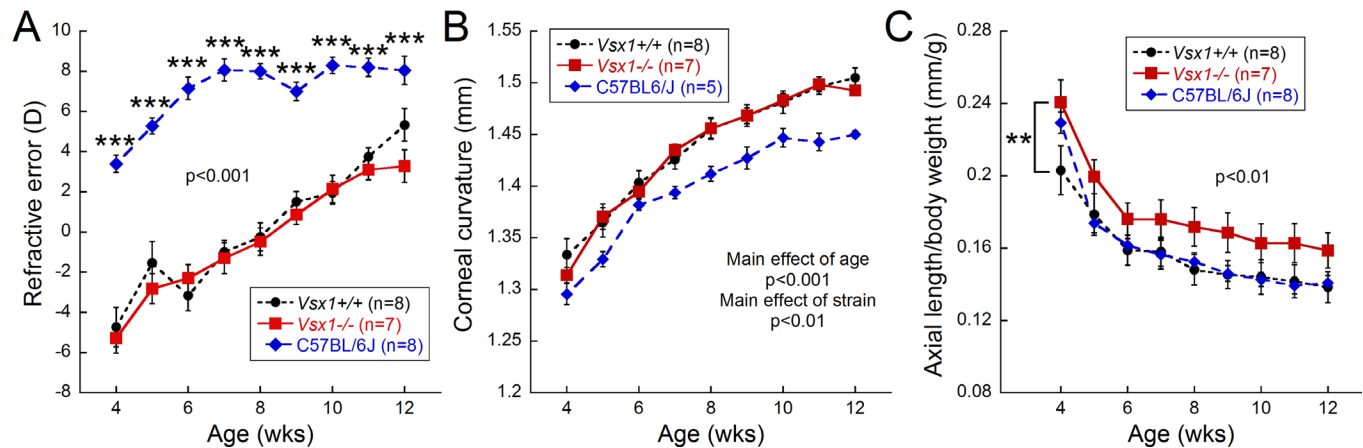


Figure 1. Refractive development of *Vsx1*^{+/+}, *Vsx1*^{-/-}, and C57BL/6J mice. **A**: All mice showed a significant increase in hyperopic refractions with increasing age from 4 to 12 weeks (two-way repeated-measures ANOVA main effect of age, $F(8,195)=76.88$, $p<0.001$). Significant differences were observed in the pattern of refractive development between the 129S1/Sv (*Vsx1*^{+/+} and *Vsx1*^{-/-}) and C57BL/6J mice (two-way repeated-measures ANOVA main effect of strain, $F(2,195)=84.41$, $p<0.001$). **B**: All mice exhibited a significant flattening of corneal curvature with increasing age (two-way repeated-measures ANOVA, $F(8,126)=197.70$, $p<0.001$). Corneal curvatures were significantly flatter for mice on the 129S1/Sv genetic background (*Vsx1*^{+/+} and *Vsx1*^{-/-}) compared to the C57BL/6J mice across development (two-way repeated-measures ANOVA main effect of strain, $F(2,171)=6.46$, $p=0.008$). **C**: Except for a small difference between the *Vsx1*^{+/+} and *Vsx1*^{-/-} mice at 4 weeks of age (two-way repeated-measures ANOVA, $F(16,199)=2.245$, $p=0.006$), the changes in axial length normalized to bodyweight were not significantly different between either the genotype or strain throughout the developmental period (two-way repeated-measures ANOVA main effect of strain, $F(2,199)=2.50$, $p=0.107$). The P values indicate the ANOVA interaction effect, unless otherwise stated. Holm-Sidak post-hoc comparisons ** $p<0.01$, *** $p<0.001$. Data are expressed as the mean \pm standard error of the mean (SEM).

flattening, 0.17 ± 0.001 , 0.18 ± 0.001 , and 0.15 ± 0.007 mm for *Vsx1*^{-/-}, *Vsx1*^{+/+}, and C57BL/6J, respectively; Figure 1B; two-way repeated-measures ANOVA main effect of age, $F(8,171)=281.95$, $p<0.001$). However, the corneal curvatures were significantly flatter for the 129S1/Sv (*Vsx1*^{-/-} and *Vsx1*^{+/+}) mice (Holm-Sidak multiple comparisons at all ages, $p>0.05$) compared to the C57BL/6J mice across development (two-way repeated-measures ANOVA main effect of strain, $F(2,171)=6.47$, $p=0.008$).

Consistent with the increase in bodyweight with age, there was a significant decrease in the axial length normalized to bodyweight across all groups (mean change in axial length between 4 and 12 weeks of age; *Vsx1*^{-/-}: -0.08 ± 0.003 , *Vsx1*^{+/+}: -0.06 ± 0.005 , C57BL/6J: -0.09 ± 0.002 mm; Figure 1C; two-way repeated-measures ANOVA main effect of age, $F(8,199)=169.83$, $p<0.001$). Except for a small difference in axial length between the *Vsx1*^{-/-} and *Vsx1*^{+/+} mice at 4 weeks of age, the changes in axial length were not significantly different between either the genotype or the strain throughout the developmental period (two-way repeated-measures ANOVA main effect of strain, $F(2,199)=2.50$, $p=0.107$). The standard deviation for raw axial length measurements (about 50 μ m) was greater than the inter-user measurement variability of the instrument (21 μ m) [32] and the predicted axial length difference based on eye modeling

(1 D of refractive change= \approx to about 5 μ m) [36]. Therefore, the SD-OCT measurements may not have been able to detect small changes in axial length between strains despite the significant differences in refractive errors (Figure 1A).

Form deprivation myopia response absence in Vsx1^{-/-} and *Vsx1*^{+/+} mice: To examine the effects of the altered visual environment on ocular development, mice were form deprived from 4 to 8 weeks of age, and the magnitude of the myopic shift in response to goggling was compared between the genotypes (Figure 2). The *Vsx1*^{+/+} (Figure 2A) and *Vsx1*^{-/-} (Figure 2B) mice showed no significant myopic shift associated with goggling at any measured time point compared to their age-matched non-goggled naïve controls (*Vsx1*^{+/+}: myopic shift at 8 weeks; goggled animals: 0.48 ± 1.25 D, controls: 0.02 ± 0.74 D, two-way repeated-measures ANOVA treatment by time interaction, $F(4,65)=0.131$, $p=0.97$; *Vsx1*^{-/-}: mean myopic shift at 8 weeks; goggled animals: -0.32 ± 0.57 D, controls: -1.81 ± 1.2 D, two-way repeated-measures ANOVA interaction effect, $F(4,65)=0.609$, $p=0.658$), even after an extended follow-up for up to 12 weeks (data not shown). In comparison, the C57BL/6J mice exhibited a significant myopic shift in the goggled animals (-4.29 ± 0.42 D) compared to the naïve controls (0.09 ± 0.69 D) at 4 weeks after the FD treatment (two-way repeated-measures ANOVA, $F(4,72)=3.085$, $p=0.024$, Figure 2C). FD did not cause significant changes in the axial

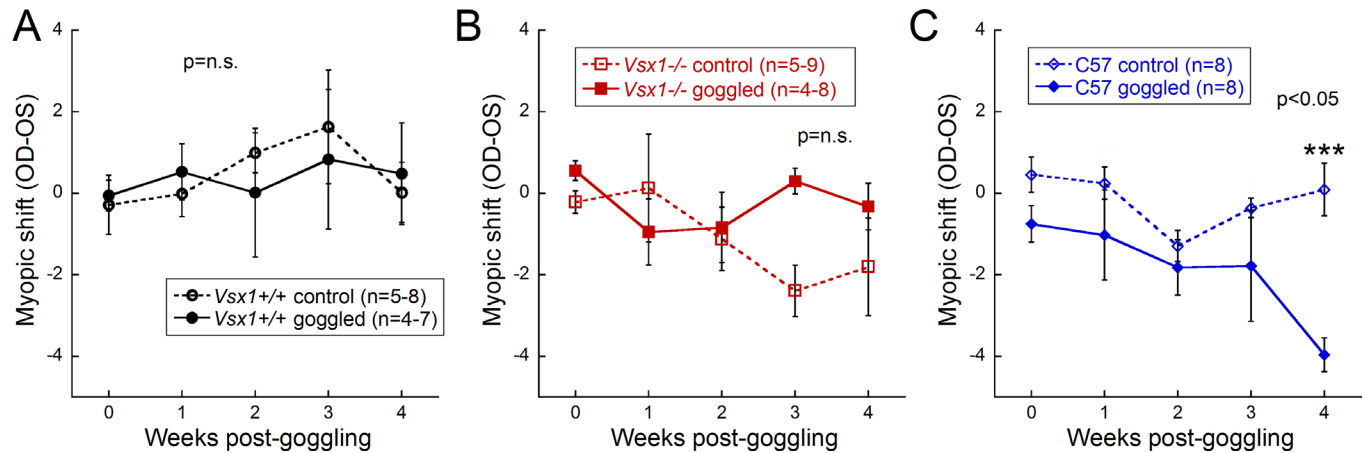


Figure 2. *Vsxl*^{+/+} and *Vsxl*^{-/-} mice do not respond to FD. **A:** *Vsxl*^{+/+} animals were unresponsive to goggling and showed a similar refractive myopic shift (goggled minus opposite eye) as their age-matched non-goggled controls across 4 weeks of form deprivation (FD) (two-way repeated-measures ANOVA treatment-age interaction, $F(4,65)=0.131$, $p=0.97$). **B:** *Vsxl*^{-/-} mice did not respond to FD and showed no change in refractive error across the goggling period (two-way repeated-measures ANOVA, $F(4,65)=0.609$, $p=0.658$). **C:** C57BL/6J mice showed a significant myopic shift in the goggled animals compared to the naïve controls at 4 weeks after FD treatment (two-way repeated-measures ANOVA, $F(4,72)=3.085$, $p=0.024$). Holm-Sidak post-hoc comparisons *** $p<0.001$. Data are expressed as the mean \pm standard error of the mean (SEM).

length or corneal curvature of the goggled mice compared to the untreated naïve controls for any genotype or strain (two-way repeated-measures ANOVA, $p>0.05$; data not shown).

We observed a considerable degree of tear film instability in the 129S1/Sv mice, which may have induced some variability in ocular response to FD between animals. Therefore, to ensure minimal effects of tear film on measurements, repeatability of measurements was closely observed, and saline eye drops were frequently applied to retain corneal hydration during the OCT measurements.

Retinal dopamine turnover significantly elevated in 129S1/Sv mice compared to C57BL/6J mice: DA and its metabolite, DOPAC, were measured in the 129S1/Sv and C57BL/6J retinas during the critical period of visual development (Figure 3) and after FD (Figure 4) to investigate the role of DA in refractive development. When the mice were 4 weeks old, the retinal DOPAC levels in the *Vsxl*^{-/-} mice (564 ± 67 pg/mg) were significantly greater than in the *Vsxl*^{+/+} (364 ± 23 pg/mg) mice (one-way ANOVA on Rank, $H=16.78$, $p<0.001$, Dunn's method multiple comparisons, $p<0.05$; Figure 3A). When we compared the strains, we found no significant differences in retinal DOPAC levels between the *Vsxl*^{+/+} and C57BL/6J (284 ± 21 pg/mg) mice. Although the DA levels were similar between the two genotypes on the 129S1/Sv background (mean DA levels; *Vsxl*^{+/+}: 1673 ± 169 pg/mg; *Vsxl*^{-/-}: 1955 ± 95 pg/mg), the levels were significantly elevated in the C57BL/6J mice (2238 ± 159 pg/mg) compared with the *Vsxl*^{+/+} strain (one-way ANOVA, $F(2,28)=3.76$,

$p<0.05$, Holm-Sidak multiple comparisons, $p<0.05$; Figure 3B). The DOPAC/DA ratios were similar between the 129S1/Sv *Vsxl*^{+/+} (0.23 ± 0.02) and *Vsxl*^{-/-} (0.30 ± 0.05) genotypes (Figure 3C). However, the ratios were significantly lower for the C57BL/6J mice (0.13 ± 0.01) compared to the *Vsxl*^{+/+} mice (one-way ANOVA on Ranks, $H=16.66$, $p<0.001$, Dunn's method multiple comparisons, $p<0.05$), suggesting considerable strain differences in DA turnover during the critical phase of refractive development.

Under FD conditions, the ratio of the right eye to the left eye (the OD/OS ratio) for retinal DOPAC was not significantly different between the goggled and control animals for any strain or genotype (Figure 4A), indicating goggling had no significant effects on DA metabolism between the different genotypes. Similar results were found for retinal DA and the DOPAC/DA ratio (Figure 4B,C).

DISCUSSION

In the mammalian retina, cone bipolar cells are essential for transmitting cone- and rod-generated visual signals [9]. Given the importance of clear, high-quality retinal images in normal ocular development [14], abnormalities in the morphology, development, or function of ON/OFF bipolar cells could potentially influence normal refractive development of mammalian eyes. In the current study, we found that the *Vsxl*^{-/-} mutation, which causes selective impairment of the retinal OFF visual pathway, does not significantly alter normal refractive development. Additionally,

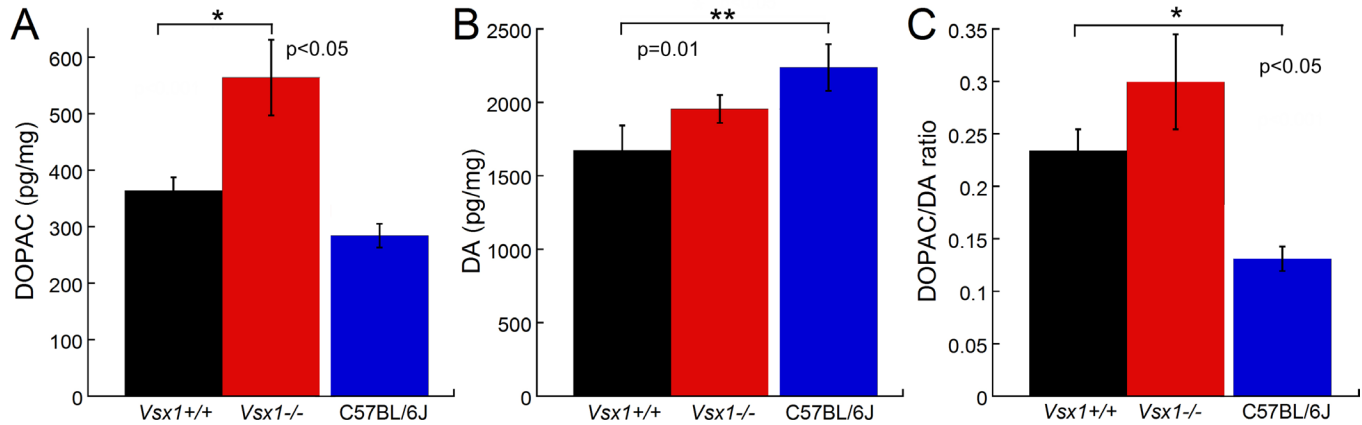


Figure 3. Higher retinal dopamine turnover in 129S1/Sv mice compared to C57BL/6J. **A:** At 4 weeks of age, the 3,4-dihydroxyphenylacetate (DOPAC) levels in the *Vsx1*^{-/-} mice were significantly higher than those in the *Vsx1*^{+/+} mice (one-way ANOVA on Rank, $H=16.78$, $p<0.001$, Dunn's method multiple comparisons, $p<0.05$). No significant differences were found in the retinal DOPAC levels between the *Vsx1*^{+/+} and C57BL/6J strains. **B:** Although the dopamine (DA) levels were similar between the 129S1/Sv *Vsx1*^{+/+} and *Vsx1*^{-/-} genotypes, the levels were significantly elevated in C57BL/6J compared with the *Vsx1*^{+/+} strain (one-way ANOVA, $F(2,28)=3.76$, $p<0.05$, Holm-Sidak multiple comparisons, $p<0.05$). **C:** The DOPAC/DA ratios were similar between the two genotypes on the 129S1/Sv background. However, the ratios were significantly lower for the C57BL/6J mice compared to the *Vsx1*^{+/+} mice (one-way ANOVA on Ranks, $H=16.66$, $p<0.001$, Dunn's method multiple comparisons, $p<0.05$).

the *Vsx1*^{-/-} mice were also unresponsive to imposed form deprivation, as were the *Vsx1*^{+/+} controls on the same genetic background. Significantly increased dopamine turnover at the age of FD induction in the 129S1/Sv mice compared to C57BL/6J mice may underlie the difference in myopia susceptibility.

Normal refractive development in Vsx1-/- mice: In this study, we observed no significant differences in the ocular refractive development curve between the *Vsx1*^{-/-} mice and their age-matched *Vsx1*^{+/+} controls, which suggests the *Vsx1* mutation does not have a significant effect on the normal refractive development of mammalian eyes. The *Vsx1*^{-/-} and *Vsx1*^{+/+} mice were substantially myopic at younger

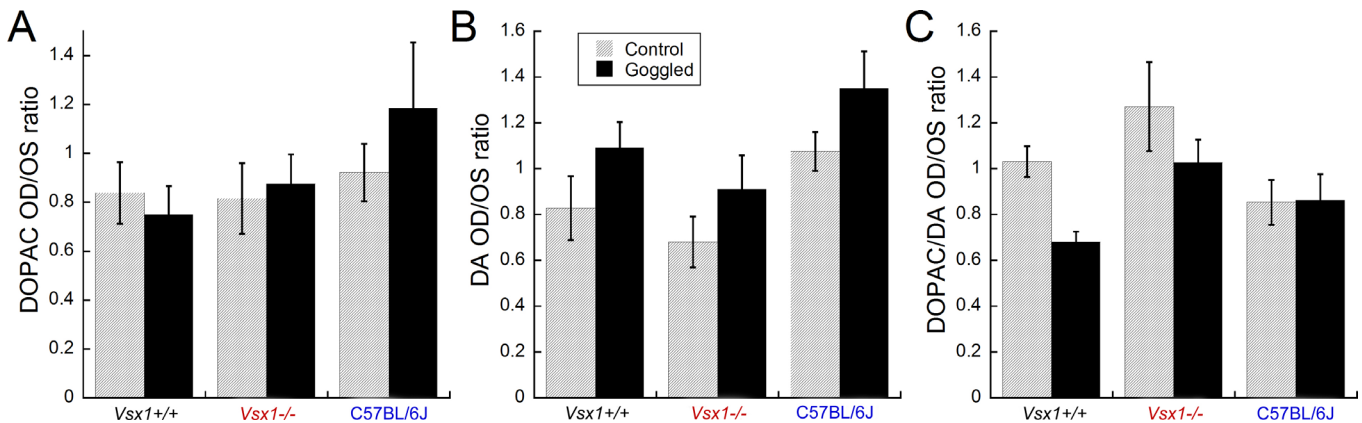


Figure 4. No effects of goggling on DA metabolism in *Vsx1*^{+/+} and *Vsx1*^{-/-} mice. **A:** Following 4 (C57BL/6J mice) or 8 (*Vsx1*^{+/+} and *Vsx1*^{-/-} mice) weeks of form deprivation (FD), the ratio of the right eye to the left eye (the OD/OS ratio) for retinal 3,4-dihydroxyphenylacetate (DOPAC) was not significantly different between the goggled and control animals for any strain or genotype (two-way ANOVA interaction effect, $F(2,29)=0.545$, $p=0.587$). **B:** The OD/OS ratio for retinal dopamine (DA) was also similar between the treatment groups for all mouse groups (two-way ANOVA interaction effect, $F(2,29)=0.015$, $p=0.985$). **C:** FD did not cause significant changes in the DOPAC/DA ratios of the goggled animals compared to the untreated naïve controls for any genotype or strain (two-way repeated-measures ANOVA, $F(2,29)=0.781$, $p=0.469$). Data are expressed as the mean \pm standard error of the mean (SEM). Animal numbers used for analysis: *Vsx1*^{+/+} (control, $n=6$; goggled $n=3$), *Vsx1*^{-/-} (control, $n=7$; goggled $n=4$), and C57BL/6J mice (control, $n=6$; goggled $n=4$).

ages, and became significantly hyperopic with increasing age (Figure 1A). In this study, similar to previous reports [22,36,43-45], the juvenile C57BL/6J mice were considerably hyperopic (mean refractive error at 4 weeks, 3.4 ± 0.43 D) as opposed to the *Vsx1*^{+/+} mice that were significantly myopic (-4.73 ± 2.78 D). Given the insufficiency of the axial length (same as the C57BL/6J mice) and corneal curvature (flatter than in the C57BL/6J mice) changes in explaining the myopic refractive errors in the *Vsx1*^{+/+} mice, we hypothesize that it could be due to differences in other ocular optical parameters (such as the crystalline lens). Although this is the first report of the refractive state in 129S1/Sv mice, similar variations in refractive error [34] and ocular parameters [42] between different mice strains have been reported.

Previously, immunolabeling of the axonal termini with cone bipolar cell markers in *Vsx1*^{-/-} mice has shown that the expression of *Vsx1* is restricted to only a small subset of ON (type 7) [28] and OFF (types 2, 3, and 4) [23,24,26,27,46] cone bipolar cells, suggesting that *Vsx1* may not be essential for late terminal differentiation of all cone bipolar cell subtypes in the mammalian retina. Similarly, Chow et al. [23] found the overall number of cone bipolar cells was largely similar between *Vsx1*^{-/-}, *Vsx1*^{+/-}, and *Vsx1*^{+/+} mice, and concluded that the majority of ON and OFF cone bipolar cells do not require *Vsx1* to retain their gross morphology and viability. Given the normal morphology and function of *Vsx1* independent bipolar pathways and other retinal cell types in *Vsx1*^{-/-} mice, we hypothesize that normal refractive development in *Vsx1*^{-/-} mice may be due to normal processing of visual information through other functional non-*Vsx1* containing cone bipolar interneurons in the retina.

Although the genetic defect in the OFF visual pathway caused by the *Vsx1* mutation does not appear to influence normal ocular refractive development, a mouse model of a non-functional ON pathway [17,18] with a null mutation in *Nyx* [18] has been shown to cause a small, but significant, myopic shift during adolescence [22]. This may be due to differences in designated rod and cone signaling through the ON and OFF pathways or differences in retinal DA levels in phenotypes expressing ON and OFF pathway defects (discussed below). A defect in the ON pathway would affect the primary rod signaling through ON rod bipolar cells to retinal ganglion cells via AII amacrine cells (although gap-junctions would retain some alternative rod signaling) [47], as well as cone signaling through ON cone bipolar cells. However, the OFF pathway defect would involve only hyperpolarizing OFF cone bipolar cells [23] with no significant effect on ON and OFF rod signaling or on cone signaling through the ON pathway. Additionally, the *Vsx1* mutation affects only a small subtype

of OFF cone bipolar cells [23,24,48] leaving a subset of *Vsx1* independent OFF bipolar cells functional for normal visual transmission. However, the *nob* [22] and *Vsx1*^{-/-} mice were on different background strains (i.e., C57BL/6J and 129S1/Sv, respectively), which could influence the refractive development and retinal DA profile observed between the two mouse strains. Future studies with mouse mutants that express complete loss of ON and OFF pathways on the same background strain are required to closely compare the effects of ON and OFF pathway deficits on normal refractive development of the eye.

Vsx1^{+/+} and *Vsx1*^{-/-} mice are unresponsive to form deprivation: To investigate the influence of the *Vsx1* mutation on refractive development with an altered visual environment, we compared the susceptibility to form deprivation myopia between *Vsx1*^{+/+} and *Vsx1*^{-/-} mice. For the *Vsx1*^{+/+} and *Vsx1*^{-/-} mice (on the 129S1/Sv background), application of the diffuser goggle even for a protracted period of 8 weeks did not produce a significant myopic shift compared to the control animals. Interestingly, in agreement with previous reports [22,34,38,49,50], the C57BL/6J mice showed a significant myopic shift of about 4 D in response to 4 weeks of FD treatment.

These variations in susceptibility to FD myopia could be due to differences in endogenous retinal DA levels and/or metabolism between strains. We found the retinal DOPAC and DA levels at 4 weeks in the C57BL/6J mice were lower or higher, respectively, than in the *Vsx1*^{+/+} mice. Importantly, the DOPAC/DA ratios, a reflection of dopamine turnover, were also significantly higher in the *Vsx1*^{+/+} mice compared to the C57BL/6J mice. The absence of response to FD could be due to a higher DA metabolism, storage, and/or availability in the 129S1/Sv retinas, making the eye resistant to imposed optical manipulation. Inhibitory effects of higher retinal dopamine levels on form-deprivation myopia have been shown in chickens [51,52], guinea pigs [53,54], and primates [55]. In addition, previous studies have indicated strain differences in response to form deprivation in mice [34], perhaps due to differences in ocular components or retinal signaling.

Following 8 weeks of form deprivation, the ratio of the retinal DA and DOPAC levels in the goggled eye (right eye) to the contralateral eye (left eye) were not significantly different between the *Vsx1*^{+/+} and *Vsx1*^{-/-} mice (which was consistent with the absence of a form deprivation response in both mouse strains). However, we also found no difference in the retinal DA and DOPAC levels in the C57BL/6J mice with a significant myopic shift. These findings suggest that baseline retinal DA metabolism and/or dopamine turnover during the critical period of visual development (Figure 3) are more

essential for determining the predisposition of the mouse eye to form deprivation myopia than changes in DA with FD. In view of recent evidence suggesting DA acts on different dopamine receptors to influence eye growth in mice [56] and synthesis of DA from other retinal sources (such as the RPE) [57], it appears that the role of DA in ocular response to visual deprivation is not simple; instead, DA participates in a complex pathway that connects the visual experience to postnatal refractive development of the eye.

*Normal corneal curvature in *Vsx1*^{-/-} mice:* Missense mutations in *Vsx1* have been associated with corneal dystrophies such as posterior polymorphous dystrophy and keratoconus in human eyes [29]. Since both corneal disorders exhibit abnormally steep corneas [58,59], we sought to examine the differences in corneal curvature between the *Vsx1*^{+/+} and *Vsx1*^{-/-} animals. In this study, no significant differences in corneal curvatures were observed between the two genotypes during their developmental period. In agreement with this observation, Chow et al. [23] also found no corneal abnormalities in *Vsx1*^{-/-} mice on gross and histological examinations.

In conclusion, our results suggest that selective impairment of retinal OFF visual pathway signaling due to the *Vsx1* mutation does not significantly alter the normal refractive development of the mouse eye, potentially due to normal visual transmission through other *Vsx1* independent ON and OFF retinal bipolar cells. Elevated retinal DA turnover in the 129S1/Sv background strain early in refractive development may prevent FD myopia in *Vsx1*^{+/+} and *Vsx1*^{-/-} mice. Finally, our study indicates that the severity of refractive deficits caused by the ON pathway defect [22] is more severe than those caused by the OFF pathway, perhaps due to altered retinal DA levels in these phenotypes.

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