High Susceptibility to Experimental Myopia in a Mouse Model with a Retinal ON Pathway Defect

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Purpose. Nob mice share the same mutation in the Nyx gene that is found in humans with complete congenital stationary night blindness (CSNB1). Nob mutant mice were studied to determine whether this defect results in myopia, as it does in humans.

Methods. Refractive development was measured in unmanipulated wild-type C57BL/6J (WT) and nob mice from 4 to 12 weeks of age by using an infrared photorefractor. The right eye was form deprived by means of a skull-mounted goggling apparatus at 4 weeks of age. Refractive errors were recorded every 2 weeks after goggling. The content of dopamine and the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by HPLC with electrochemical detection (HPLC-ECD) in retinas of nob and WT mice under light- and dark-adapted conditions.

Results. The nob mice had greater hyperopic refractive errors than did the WT mice under normal visual conditions, until 12 weeks of age when both strains had similar refractive errors. At 6 weeks of age, refractions became less hyperopic in the nob mice but continued to become more hyperopic in the WT mice. After 2 weeks of form deprivation (6 weeks of age), the nob mice displayed a significantly myopic shift (~4 D) in refractive error relative to the opposite and control eyes, whereas WT mice required 6 weeks of goggling to elicit a similar response. As expected with loss of ON pathway transmission, light exposure did not alter DOPAC levels in the nob mice. However, dopamine and DOPAC levels were significantly lower in the nob mice compared with WT.

Conclusions. Under normal laboratory visual conditions, only minor differences in refractive development were observed between the nob and WT mice. The largest myopic shift in the nob mice resulted after form deprivation, suggesting that visual pathways dependent on nyctalopin and/or abnormally low dopaminergic activity play a role in regulating refractive development. These findings demonstrate an interaction of genetics and environment in refractive development. (Invest Ophthalmol Vis Sci. 2008;49:706–712) DOI:10.1167/iovs.07-0643

Normal refractive development results in emmetropia, a perfect match between optical power and axial length of the eye. However, the eye does not always grow to emmetropia, resulting in eyes that are too short (hyperopia) or too long (myopia) for their optical power. Although refractive errors are not life-threatening, 35% of the U.S. population are affected, with more than 25% having myopia.1 In Asian countries, such as China, Taiwan, and Singapore, the prevalence of myopia has reached near-epidemic proportions.5–7 Clinical and experimental evidence suggests that genetics and visual environment influence refractive development, yet the mechanisms coordinating the growth of the eye and optical system remain elusive. In humans, several genes have been linked to myopia8–15 or hyperopia16; however, the influence of visual environment, mainly near work, has remained inconclusive.15 In contrast, animal models have shown a clear influence of visual environment on the refractive development of the eye.17 In the present study, we explored a new model of experimental myopia, the mouse, to show an interaction between genetic background and environmental exposures in abnormal refractive development.

We examined the refractive state and dopamine levels of the nob mouse,18 which carries a null mutation in Nyx,19 leading to a loss of function of the ON pathway.18,19 Specifically, Nyx encodes the protein nyctalopin, which is located on the postsynaptic side of the photoreceptor-to-ON bipolar cell synapse.20 ERG, behavioral tests, and immunocytochemistry have shown nob mice to have loss of visual transmission in the ON pathway.18,19,21,22 Nyx mutations have been identified in patients with the complete form of X-linked congenital stationary night blindness (CSNB1).23,24 Patients with CSNB1 present with high myopia (10 D), suggesting a possible link between the genetic mutation and/or disease state and refractive development. In addition, a recent report has found mutations in Nyx in patients with high myopia without night blindness.25 The use of mouse models provides a unique opportunity for simultaneous examination of genetic and environmental influences on the refractive state of the eye.

Methods

Animals and Experimental Design

All mice were maintained as in-house breeding colonies at the Atlanta VA Medical Center. Both male and female wild-type (WT) C57BL/6J mice (n = 5; Jackson Laboratory, Bar Harbor, ME) and nob mice18 on a C57BL/6J background (n = 5) were refracted between 4 and 12 weeks of age to assess refractive development under unmanipulated visual conditions. For goggling experiments, the mice had baseline refractions at 4 weeks of age and then were goggled for 2 or 8 weeks (nob-2 weeks, n = 28; WT-8 weeks, n = 12). Refractive measurements were obtained every 2 weeks. 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 Error Measurements**

An eccentric infrared photorefractor customized for the mouse eye was used to measure refractive errors. The photorefractor consists of a CCD camera with a series of infrared LEDs positioned in front of the lens. The infrared (IR) LEDs produced a reflection in the eye such that a brightness gradient was established across the pupil. The pupil of each eye was dilated with 1% tropicamide to ensure pupil sizes of >1.7 mm. The mouse was placed on a small platform positioned 60 cm from the camera at approximately 25° to 40°. When the subject was positioned correctly, a custom software program collected 10 images of the eye in 0.4 seconds, to determine refractive error. During each recording session, five refractive measurements were taken while the mouse was awake and gently restrained. The mouse was then lightly sedated (ketamine 60 mg/kg; xylazine 12 mg/kg), and five more refractive measurements were taken before the corneal surface began to dry out ("asleep refraction"). As demonstrated in Figure 2, measurements obtained under sedation (asleep) had less variability and were used for all further data analyses.

To demonstrate that the photorefractor is producing valid measurements in relation to other refractions measured the same way, we calibrated the photorefractor with trial lenses. For this experiment, refractions were obtained from the right eye of the calibrated animals that had been dark-adapted overnight (WT, n = 5; WT, n = 10) versus dark-adapted animals that had been dark-adapted overnight (nob, n = 5; nob, n = 3). Relative refractive error was correlated with asleep refractions (0.41 D) was less than half the variability obtained when the mouse was awake (1.16 D; P < 0.001, Mann-Whitney rank sum test). These results show that awake refractions provide a large range of refractive errors, which can be refined by asleep refractions, thereby increasing our accuracy. Only asleep refractions were used for further data analysis.

**Photorefraction Calibration**

To demonstrate the validity of our refractive measurements in relative terms, we calibrated the photorefractor to a series of trial lenses. Figure 1 shows that the linear relationship between refractive error and trial lens power was −0.754 (Pearson product moment correlation; Fig. 1). As expected, placing positive lenses in front of the eye reduced the measured hyperopia, whereas negative lenses increased it. The refractive errors measured in the mouse eye, however, spanned only 7 D of the potential 20 D of trial lens power. We attribute this to the relatively poor optical quality of the mouse eye, as well as the small eye artifact.

Figure 2 plots awake versus asleep refractive errors for the WT mice at ~60 days of age. The plot shows the average refractive error ± SD of both eyes of 20 mice in which awake refractive errors were taken followed by asleep refractions. The variability with asleep refractions (0.41 D) was less than half the variability obtained when the mouse was awake (1.16 D; P < 0.001, Mann-Whitney rank sum test). These results show that awake refractions provide a large range of refractive errors, which can be refined by asleep refractions, thereby increasing our accuracy. Only asleep refractions were used for further data analysis.

**Refractive Development**

When raised in normal laboratory visual environments, the nob mice had more hyperopic refractive errors than did the WT mice, until ~12 weeks of age, (Fig. 3A). At 4 weeks of age, WT mice had refractive errors of −0.28 ± 0.28 D (mean ± SEM). With increasing age, the eye became somewhat more hyperopic, plateauling at +10.45 ± 0.27 D of hyperopia by 12 weeks of age.

In comparison, the nob mice had significantly more hyperopia at young ages than did the WT mice until 12 weeks of age.

**Dopamine Analysis**

Steady state levels of dopamine (DA) and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), were measured in the mouse retina by using high-performance liquid chromatography with electrochemical detection (HPLC-ECD) as described previously. Briefly, the mice were killed by cervical dislocation and the retinas were dissected at room temperature and frozen on dry ice within ~1 minute. Frozen retinas were homogenized in 0.1 N HClO4-containing 0.01% of sodium metabisulfite and 50 ng/mL of internal standard 3,4-dihydroxybenzylamine hydrobromide and centrifuged. DOPAC and DA were measured in the supernatant fraction by HPLC with electrochemical detection. For analysis, the amount of DA and DOPAC was compared between light-adapted animals in which the retinas were collected 3 to 4 hours into the light cycle (nob, n = 5; WT, n = 10) versus dark-adapted animals that had been dark-adapted overnight (nob, n = 5; WT, n = 3).

**RESULTS**

**Form Deprivation**

Form deprivation was induced by placing a head-mounted goggling apparatus over the right eye at 4 weeks of age, as previously described. Briefly, a pedestal composed of dental cement that held a small stainless steel frame over one eye was attached to the skull. A plastic goggle painted white to create a diffuser was glued to the frame. The mouse was lightly sedated (ketamine 60 mg/kg; xylazine 12 mg/kg), and five more refractive measurements were taken before the corneal surface began to dry out ("asleep refraction"). As demonstrated in Figure 2, measurements obtained under sedation (asleep) had less variability and were used for all further data analyses.

To demonstrate that the photorefractor is producing valid measurements in relation to other refractions measured the same way, we calibrated the photorefractor with trial lenses. For this experiment, refractions were obtained from the right eye of the nob mice (n = 16). The mouse was then lightly sedated (ketamine 60 mg/kg; xylazine 12 mg/kg) and five more refractive measurements were taken before the corneal surface began to dry out ("asleep refraction"). As demonstrated in Figure 2, measurements obtained under sedation (asleep) had less variability and were used for all further data analyses.

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**FIGURE 1.** Refractive errors measured with a photorefractor calibrated to the mouse eye with the addition of a series of trial lenses. The regression line showed good correlation between the measured refractive error and trial lens power. Each point represents one recording of a single eye (n = 16 eyes).
control mice (data not shown). Multiple comparisons). Refractions of the nob mice (10.40 ± 0.27 D vs. 10.45 ± 0.27 D; Holm-Sidak multiple comparison; P < 0.001). Although the control eyes and the (contralateral) fellow eyes of goggled nob mice all had very similar, hyperopic refractions, the refractions of goggled eyes quickly shifted toward less hyperopia (Holm-Sidak multiple comparison, P < 0.001).

The myopic shift (the difference between the goggled and opposite eye) occurred much more quickly in the nob mice than in the WT (Fig. 5). In the WT mice, a significant myopic shift was only found after 6 weeks of form deprivation (Holm-Sidak multiple comparison; P < 0.001). In contrast, the nob mice developed a significant myopic shift of −4.96 ± 0.32 D after only 2 weeks (two-way, repeated-measures ANOVA, F_{(2,101)} = 58.842, P < 0.001). This myopic shift was nearly identical with that produced in the WT mice after 8 weeks of goggling.

Dopamine Analysis

DA and its metabolite, DOPAC, were measured in the WT and nob mice at 12 weeks of age under two conditions, dark adapted or light adapted (Fig. 6). In the WT mice, retinas collected 4 hours into the light cycle showed significant increases in DOPAC levels compared with the dark-adapted control animals (Fig. 6A; Mann-Whitney rank sum test, t = 21.0, P < 0.001). In contrast, no differences in DOPAC levels were observed between dark- and light-adapted retinas of the nob mice (Fig. 6A; Student’s t-test, t = −0.05, P = 0.96).

As observed previously, 29 the level of DA in the light-exposed WT retinas was not significantly different from that in dark-adapted retinas (Fig. 6B; Student’s t-test, t = 1.14, P = 0.27). DA levels were also not significantly different between light and dark conditions in the nob mice (Fig. 6B; Student’s t-test, t = 0.34, P = 0.74).

The overall levels of DOPAC and DA in the light-adapted nob retinas were significantly less than those in the WT retinas (Student’s t-test, t = −6.345, P < 0.001 and t = −4.675, P < 0.001, respectively). DOPAC levels were 47.52 ± 8.56 pg retina in the nob mice compared with 187.36 ± 10.67 pg retina in the WT mice. Similarly, DA levels were 363.59 ± 46.75 pg retina in the nob mice compared with 792.0 ± 65.19 pg retina in the WT mice.

Form Deprivation

To test whether the Nyx gene defect affects environmentally induced myopia, we compared the susceptibility to form deprivation myopia of the nob versus the WT mice.

The goggled eyes of the WT mice had significantly different refractive error measurements over the form deprivation period compared with the opposite and control eyes (two-way, repeated-measures ANOVA, F_{(6,106)} = 5.80, P < 0.001). The goggled eyes showed a trend toward less hyperopic refractions starting at 2 weeks, but these differences did not reach significance compared to the opposite eye until 6 weeks of goggle wear (Fig. 4A; Holm-Sidak multiple comparison, P < 0.05). Note that the two eyes of the control mice had very little variability between them. The goggled eye continued to become significantly less hyperopic at 8 weeks after goggling (Holm-Sidak multiple comparison; P < 0.001).

In contrast, when diffuser goggles were applied to the nob mice, a significant shift in refraction was detectable after only 2 weeks of deprivation (Fig. 4B; two-way, repeated measures ANOVA, F_{(2,101)} = 54.34, P < 0.001). Although the control eyes and the (contralateral) fellow eyes of goggled nob mice all had very similar, hyperopic refractions, the refractions of goggled eyes quickly shifted toward less hyperopia (Holm-Sidak multiple comparison, P < 0.001). The myopic shift (the difference between the goggled and opposite eye) occurred much more quickly in the nob mice after 8 weeks of goggling.

Figure 3. Refractive development in the nob and WT mice from 4 to 12 weeks as measured with an automated photorefractor. (A) Nob mice had significantly more hyperopic refractions between 4 and 10 weeks of age than WT mice (repeated-measures ANOVA, F_{(2,122)} = 8.989, P < 0.001 and Holm-Sidak multiple comparisons). (B) Linear curve fitting of the combined mean from both eyes demonstrated that the nob mice reached the highest hyperopic refraction at 6 weeks of age and then shifted toward less hyperopia. The linear equation and regression fit are given for each line. Data are expressed as the mean ± SEM.
54.39 pg/retina in the nob mice versus 545.33 ± 14.50 pg/retina in the WT mice.

**DISCUSSION**

Refractive development is a complicated process that has both genetic and environmental components. Although the exact signaling pathway is not known, visual blur appears to be detected by the retina, which begins a signaling cascade that is transmitted through the RPE and eventually alters scleral growth. Potential candidates in this signaling pathway come from extensive work in animal models, which have implicated dopaminergic,31–34 muscarinic,35 and glucagonergic36,37 systems. It has been proposed that a signaling cascade triggers “stop” and “go” signals for eye growth.38 “Stop” signal candidates include dopamine, 31,33 glucagon,36,37 and fibroblast growth factor.38 Potential “go” signals include acetylcholine,35 transforming growth factor β,38 nitric oxide,39 and retinoic acid.40–42 However, it should be noted that studies have also shown evidence that nitric oxide43 and retinoic acid44 may inhibit eye growth.

Studies in human populations have found links between myopia and near work as well as hereditary components (for a review, see Ref. 15). In addition, several human diseases have been associated with myopia.15 In this study, we focus on a genetic mouse model with an Nyx mutation. In humans, NYX mutations have been found in patients with CSNB1, which is characterized by an ON pathway defect and high myopia23–25,45 and in patients with high myopia and no night blindness.46

**Mouse Models**

The use of transgenic and mutant mouse models provides an opportunity to test functionally the pathways and specific elements of the proposed pathways. In this way, we can begin to determine more clearly the signals controlling refractive development.

Other mammalian and avian species undergo emmetropization during early development which starts with hyperopia and decreases to near-zero refractive error.17,46 As shown in this study and others,27,47 refractive development for the mouse begins with hyperopic refractions but then continues to progress to more hyperopic refractive errors. Because of the small-eye artifact, all refractive measurements in the mouse appear hyperopic, presumably due to the retinoscopic reflection coming from the inner limiting membrane instead of the outer limiting membrane.30

Mice, like other mammalian and avian models, are susceptible to form-deprivation myopia (FDM) induced by lid suture (Beuerman RW, et al. IOVS 2003;44:ARVO E-Abstract 4338),48 diffuser goggles,27 and spectacle lenses (Beuerman RW, et al. IOVS 2003;44:ARVO E-Abstract 4338; Barathi VA, et al. IOVS 2007;48:ARVO E-Abstract 4418). The data shown herein confirm the refractive shift reported in the WT mice from other studies (Beuerman RW, et al. IOVS 2003;44:ARVO E-Abstract 4338)27,48 and demonstrate an increased susceptibility to myopia in a mouse model of a human disease also associated with high myopia. Another mutant mouse model with reported refractive abnormalities is the Egr1 KO mouse, which also exhibits relative myopia.49 Egr1 is the mouse orthologue of ZENK, a transcription factor found in chicken glucagon amacrine cells. Although glucagon-containing amacrine cells have not been found in the mouse, Egr1 may be involved in the regulation of eye growth. In addition, we have reported that mice with retinal defects have different unmanipulated refractive errors (Faulkner AE, et al. IOVS 2007;48:ARVO E-Abstract 4419). These studies demonstrate the power of mouse models
in which specific genetic mutations, disease states, and environmental conditions can be studied simultaneously.

One limitation of the present study was our inability to determine what eye size parameters were changing to produce altered refractive errors. Myopia has been shown to be associated with increased axial length in other myopia models. In chickens and primates, changes in axial length are easily measured with calipers, cryosections, or ultrasound. In contrast, in the small mouse eye, a 1-D change in refractive error is calculated to correspond to a 5-μm change in axial length. Thus, ultrasound does not have the needed sensitivity to detect changes in axial length. Similarly, in our experience video morphology and cryosections produced measurement errors of 0.08 and 0.14 mm, respectively (Pardue MT, et al. 2004; 45: ARVO E-Abstract 4281). Based on the model eye calculations, the mouse would need to shift 16 to 28 D to calculate differences in axial length. In contrast, the small mouse eye, a 1-D change in refractive error is calculated to correspond to a 5-μm change in axial length. Thus, ultrasound does not have the needed sensitivity to measure the mouse eye; however, the only commercial instrument with this technology is currently not FDA-approved for use in the United States. In addition, to date, no study has reported axial length changes and refractive errors that agree with the theoretical measurements based on the mouse model eye. Resolution of these discrepancies in measurements and further characterization of the changes in eye dimensions in the mouse will occur as more sensitive imaging technologies are applied to the mouse eye.

ON-Pathways in FDM

CSNB1 is characterized by a selective defect in the ON pathway and high myopia. The ON pathway defect has been demonstrated by the absence of the ERG b-wave, which is derived from depolarizing bipolar cells, whereas the a-wave, generated from photoreceptor activity, remains normal. In contrast, patients with the incomplete form of congenital stationary night blindness (CSNB2), who have partial ON pathway defects is retinopathy of prematurity (ROP). Lu et al. have shown that the ON response, as recorded with the multifocal ERG, decreased as the amount of myopia increased in patients with ROP.

The possible contributions of the ON and OFF pathways have been investigated in FDM in the chicken model by trying to block the pathways selectively with pharmacologic agents or to stimulate the pathways selectively with light stimuli. These approaches do not ensure complete and selective blockade of a single pathway, thus, making specific statements about the role of ON and OFF pathways in FDM difficult. However, it appears that disruption of ON and OFF pathway transmission alters eye growth.

The use of mutant models with specific visual pathway defects provides a new approach to investigate the role of the ON and OFF pathways in FDM. In this study, the nob mice were shown to have greater susceptibility to FDM. The retina of nob mice has normal laminar structure, but it has a striking loss of visual function along the ON pathway as measured by ERG and visual behavior. The ON pathway transmission influences refractive development. Furthermore, since the largest differences in the refractive error were induced after alterations in the visual environment, these experiments may suggest that visual disruptions, and not an ON transmission defect alone, are needed to influence refractive development.

Alternatively, nyctalopin, the protein encoded by NXY, may have a separate role in detecting visual blur than that related to the ON pathway. Recent studies in humans have identified novel mutations in NYX associated with myopia, but not night blindness. Further studies are needed to determine whether the ON pathway defect or some other aspect of the NYX defect causes this increased susceptibility to FDM.

Dopamine

DA is synthesized and released by a subset of amacrine-interplexiform cells. In the rate-limiting step of DA synthesis, L-tyrosine is hydroxylated to form L-3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. DOPA is subsequently decarboxylated to DA. DA is released by the neuron and metabolized to DOPAC. In the rodent retina, DOPAC is the main DA metabolite.

DA release is stimulated by light exposure via the ON pathway. DOPA and DA synthesis increase to compensate for increased DA release and metabolism with light exposure; consequently, the steady state level of DA does not change. In this study, we hypothesized that the level of DOPAC would not change on light exposure in the nob mice, due to the ON pathway defect. As predicted, Figure 6 shows that DOPAC and DA levels do not change in the nob mice between dark and light conditions. In addition, the data show that DOPAC and DA levels were significantly decreased in the nob compared with the WT mice. Data are expressed as the mean ± SE (*P < 0.001).
agonist, apomorphine, has been shown to block the expected axial elongation in a dose-dependent fashion in chickens and macaques, and recent studies have shown DA levels linked to eye growth using dopamine agonists and antagonists. In contrast, low doses of 6-hydroxy dopamine (6-OHDA), a neurotoxin of catecholaminergic cells that inhibits dopaminergic pathways, have been shown to suppress FDM. Thus, the role of DA and visual pathways in FDM is complex. The current studies provide further support that decreased levels of DA are associated with increased FDM. Although the low level of DA in the nob mice may have produced a slight myopic shift from 6 to 12 weeks of age, it was only after form deprivation that a significant myopic shift was measured. Further studies are needed to determine whether DA is decreased in FDM in the mouse model.

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References
38. Rohrer B, Stell WK. Basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-beta) act as stop and go signals to modulate postnatal ocular growth in the chick. Exp Eye Res. 1994;58:553–561.


