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High susceptibility to experimental myopia in a mouse model with a retinal ON pathway defect

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Abstract

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

Methods—Refractive development was measured in unmanipulated wildtype C57BL/6J (WT) and *nob* mice from 4 to 12 weeks of age 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, DOPAC, were measured using HPLC-ECD in retinas of *nob* and WT mice under light- and dark-adapted conditions.

Results—*Nob* mice had greater hyperopic refractive errors than WT mice under normal visual conditions until 12 weeks of age, when both strains had similar refractions. At 6 weeks of age, refractions became less hyperopic in *nob* mice but continued to become more hyperopic in WT mice. Following two weeks of form deprivation (6 weeks of age), *nob* mice displayed a significant myopic shift (~4 D) in refractive error relative to the opposite and control eyes, while 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 *nob* mice. However, dopamine and DOPAC levels were significantly lower in *nob* mice compared to WT.

Conclusions—Under normal laboratory visual conditions, only minor differences in refractive development were observed between *nob* and WT mice. The largest myopic shift in *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.

Introduction

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. While refractive errors are not life-threatening, 35% of the US population are affected, with over 25% having myopia [1]. In Asian countries, such as China, Taiwan and Singapore, the prevalence of myopia has reached near epidemic proportions [2–7]. 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 myopia [8–15] or hyperopia [16]; 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]. This study exploits a new model of experimental myopia, the mouse, to show an interaction between genetic background and environmental exposures in abnormal refractive development.

In this study, we examined the refractive state and dopamine levels of the *nob* mouse [18] that 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]. *Nob* mice have been shown to have loss of visual transmission in the ON pathway using the electroretinogram (ERG), behavioral tests, and immunocytochemistry [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 [25] (~10 diopters, 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 [26].

The use of mouse models provides a unique opportunity to simultaneously examine 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 wildtype (WT) C57BL/6J mice (n=5) (Jackson Laboratory, Bar Harbor Maine) and *nob* mice [18] 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 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 followed the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the local Institutional Animal Care and Use Committee.

Refractive Error Measurements

An eccentric infrared photorefractor (PowerRefractor), customized for the mouse eye was used to measure refractive errors [27]. The photorefractor consists of a CCD camera with a series of infrared LEDs positioned in front of the lens. The 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 25–40°. When positioned correctly, 10 images of the eye were collected in 0.4 seconds to determine refractive error using a custom software program [27]. 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; xylaxine 12 mg/kg) and five more refractions were taken quickly 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 *nob* mice (n=16). The mouse was lightly sedated (ketamine 60 mg/kg; xylaxine 12mg/kg) and then a series of trial lenses placed in front of the eye (−10, −5, plano, +5, +10 diopters). Relative refractive error was correlated to trial lens power to determine the linear relationship.

Data were analyzed by comparing WT and *nob* mice using repeated measures ANOVA and Holm-Sidak Multiple Comparisons (SPSS 8.0, Chicago, IL). For the refractive development data, linear regression curve fitting was performed to better define the trends in refractive error over time (Kaleidagraph, Synergy Software, Reading, PA). Based on R values, the data were best defined by two linear models that break at 6 weeks of age; the age of sexual maturity in the mouse.

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 [28]. 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 and then positioned over the eye. The goggle reduced light transmission by less than 0.13 ND. The mice were checked for compliance of goggle-wear every 2–3 hours during the 12-hour light phase of the daily light-dark cycle.

Dopamine analysis

Steady-state levels of dopamine (DA) and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), were measured in the mouse retina using high performance liquid chromatography with electrochemical detection (HPLC-ECD) as described previously [29]. Briefly, mice were sacrificed by cervical dislocation and retinas were dissected from eyes at room temperature and frozen on dry ice within ~1min. 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 [29]. For analysis, the amount of DA and DOPAC was compared between light-adapted animals in which the retinas were collected 3–4 hours into the light cycle (*nob*, n = 3; WT, n=10) versus dark-adapted animals which had been dark-adapted overnight (*nob*, n= 5; WT, n = 3).

Results

Photorefraction calibration

To demonstrate the validity of our refractive measurements in relative terms, the photorefractor was calibrated to a series of trial lenses. Figure 1 shows the linear relationship between refractive error and trial lens power was −0.754 (Pearson product moment correlation; Figure 1). As expected, placing positive lenses in front of the eye reduced the measured hyperopia, while negative lenses increased it. The refractive errors measured in the mouse eye, however, only spanned 7D of the potential 20D of trial lens power. We attribute this to the relatively poor optical quality of the mouse eye [27], as well as the small eye artifact [30].

Figure 2 plots awake versus asleep refractions for *nob* mice at ~60 days of age. The plot shows the average refraction ± SD of both eyes of 20 mice in which awake refractions 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 showed 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

Nob mice had more hyperopic refractive errors compared to WT mice until ~12 weeks of age when raised under normal laboratory visual environments (Figure 3A). At 4 weeks of age, WT mice had refractive errors of +6.38 ± 0.28 D (mean ± SEM.). With increasing age, the eye became somewhat more hyperopic, plateauing 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 WT mice until 12 weeks of age [Figure 3A; Two factor repeated measures ANOVA, F (7, 122) = 9.89, p <0.001]. Linear curve fitting of the data demonstrated that the *nob* mice had ~2 D more hyperopia between 4 and 6 weeks of age (Figure 3B). However, at 6 weeks of age, the refractive errors of *nob* mice began to shift towards less hyperopia (relative myopic shift) while those of WT mice continued to shift towards more hyperopic refractions. At 12 weeks of age there was no significant difference between *nob* and WT refractions (10.40 ±0.27 vs 10.45 ±0.27, respectively; Holm-Sidak Multiple Comparisons). Refractions of *nob* mice >12 weeks of age did not reveal any differences from WT controls (data not shown).

Form Deprivation

To test whether the *Nyx* gene defect affected environmentally-induced myopia, we compared the susceptibility to form deprivation myopia between *nob* and WT mice.

The goggled eyes of WT mice had significantly different refractive error measurements over the form deprivation period compared to opposite and control eyes (Two way repeated measures ANOVA, $F(8, 106) = 5.80$, $p<0.001$). The goggled eyes showed a trend towards 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 (Figure 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 post-goggling (Holm-Sidak Multiple Comparison, $p<0.001$).

In contrast, when diffuser goggles were applied to *nob* mice, a significant shift in refraction was detectable after only 2 weeks of deprivation (Figure 4B; Two way repeated measures ANOVA, $F(2, 101) = 54.34$, $p<0.001$). While 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 towards 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 *nob* mice compared to WT (Figure 5). In WT mice, a significant myopic shift was only found after 6 weeks of form deprivation (Holm-Sidak Multiple Comparison, $p<0.05$). The overall myopic shift after 8 weeks of goggling was −4.25 ±0.44 D (Holm-Sidak Multiple Comparison, $p<0.001$). In contrast, *nob* mice developed a significant myopic shift of −4.96 ±0.32 D after only 2 weeks (Two-way repeated measures ANOVA, $F(1,50) = 58.842$, $p<0.001$). This myopic shift was nearly identical to that produced in the WT mice after 8 weeks of goggling.

Dopamine analysis

DA and its metabolite, DOPAC, were measured in WT and *nob* mice at 12 weeks of age under two conditions, dark-adapted or light-adapted (Figure 6). In WT mice, retinas collected 4 hours into the light cycle showed significant increases in DOPAC levels compared to dark-adapted

controls (Figure 6A; Mann-Whitney Rank Sum Test, $t = 21.0$, $p<0.001$). In contrast, no differences in DOPAC levels were observed between dark-adapted and light-adapted retinas of *nob* mice (Student's t-test, $t = -0.05$, $p = 0.96$).

As observed previously[29], the level of dopamine in the light-exposed WT retinas was not significantly different than that in dark-adapted retinas (Figure 6B; Student's t-test, $t = 1.14$, $p = 0.27$). DA levels were also not significantly different between light and dark conditions in *nob* mice (Figure 6B; Student's t-test, $t = 0.34$, $p = 0.74$).

The overall levels of DOPAC and DA in light-adapted *nob* retinas were significantly less than those in 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 *nob* mice compared to 187.36 ± 10.67 pg/retina in WT mice. Similarly, DA levels were 363.59 ± 54.39 pg/retina in *nob* mice versus 545.33 ± 14.50 pg/retina in WT mice.

Discussion

Refractive development is a complicated process that has both genetic and environmental components. While 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 glucagonergic [36, 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 oxide [43] and retinoic acid[44] may inhibit eye growth.

Studies in human populations have found links between myopia and near work as well as hereditary components [see [15] for review]. In addition, a number of human diseases are associated with myopia [15]. In this study, we focus on a genetic mouse model with a *Nyx* mutation. In humans, *NYX* mutations have been found in patients with CSNB1 which is characterized by an ON pathway defect and high myopia [23–25, 45] and in patients with high myopia and no night blindness [26].

Mouse models

The use of transgenic and mutant mouse models provides an opportunity to functionally test pathways and specific elements of the proposed pathways. In this way, we can begin to more clearly determine 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. Due to 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) using lid suture [48, 49], diffuser goggles [27] and spectacle lenses [48, 50]. The data shown here confirm the refractive shift reported in WT mice from other studies [27, 48, 49] 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[51]. Egr1 is the mouse orthologue of ZENK, a transcription factor found in chicken glucagon amacrine cells. While glucagon-containing amacrine cells have not been found in the mouse, Egr1 may be involved in the regulation of eye growth. Additionally, we have reported that mice with retinal defects have different unmanipulated refractive errors [52]. 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 current study is our inability to determine what eye size parameters are changing to produce altered refractive errors. Myopia has been shown to be associated with increased axial length in other myopia models [13, 17]. In chickens and primates, changes in axial length are easily measured with calipers[53, 54], cryosections [55] or ultrasound [56, 57]. In contrast, for the small mouse eye, a 1 D change in refractive error is calculated to correspond to a 5 micron change in axial length [58]. Thus, ultrasound does not have the needed sensitivity to detect changes in axial length. Similarly, in our experience video morphology and crysections produced measurement errors of 0.08 mm and 0.14 mm, respectively [59]. Based on the model eye calculations, the mouse would need to shift 16 to 28D in order to detect differences in axial length with these techniques. Coherence interferometry has been shown to have the accuracy to measure the mouse eye [47, 60]; however, the only commercial instrument with this technology is currently not FDA-approved for use in the US. Additionally, to date, no study has reported axial length changes and refractive errors that agree with the theoretical measurements based on the mouse model eye [47, 49]. 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 [25]. The ON pathway defect has been demonstrated by the absence of the ERG b-wave, which is derived from depolarizing bipolar cells [61–63], while the a-wave, generated from photoreceptor activity [64, 65], remains normal [18, 25]. In contrast, patients with the incomplete form of congenital stationary night blindness (CSNB2), who have partial ON pathway function as evidenced by a small b-wave, do not develop high myopia [25, 66].

Another human retinal disease with associated myopia and 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 [67].

The possible contributions of the ON and OFF pathways have been investigated in FDM in the chicken model by trying to selectively block the pathways with pharmacological agents [68–70] or selectively stimulate the pathways with light stimuli [71]. These approaches do not ensure complete and selective blockage 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 [18], but has a striking loss of visual function along the ON pathway as measured by ERG [18] and visual behavior [19]. Along the visual pathway, depolarizing bipolar cells appear to have abnormal projections into the inner plexiform layer [72] and ganglion cell firing and eye-specific segregation in the dorsal-lateral geniculate nucleus are abnormal [22]. Thus, our results are consistent with the possibility that 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 *Nyx*, 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 [26]. Further studies will be needed to determine if the ON pathway defect or some other aspect of the *Nyx* defect, is causing this increased susceptibility to FDM.

Dopamine

DA is synthesized and released by a subset of amacrine/interplexiform cells[73, 74]. In the rate-limiting step of DA synthesis, L-tyrosine is hydroxylated to form L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase. L-DOPA is subsequently decarboxylated to DA. Dopamine is released by the neuron and metabolized to dihydroxyphenylacetic acid (DOPAC)[29]. In the rodent retina, DOPAC is the main DA metabolite[29].

DA release is stimulated by light exposure via the ON pathway [75–77]. 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 [29]. In this study, we hypothesized that the level of DOPAC would not change upon light exposure in *nob* mice due to the ON pathway defect. As predicted, Figure 6 shows that DOPAC and DA levels do not change in *nob* mice between dark and light conditions. In addition, the data show that *nob* mice have significantly lower levels of DOPAC and DA compared to WT mice, perhaps due to the loss of ON pathway stimulation.

In FDM, DA has been implicated as a possible stop signal for eye growth. DA levels are decreased after FDM in chicks and non-human primates [33, 34, 78, 79]. In addition, the DA receptor agonist, apomorphine, has been shown to block the expected axial elongation in a dose-dependent fashion in chickens and macaques [31, 32], and recent studies have shown DA levels linked to eye growth using dopamine agonist and antagonists [80] . In contrast, low doses of 6-hydroxy dopamine (6-OHDA), a neurotoxin of catecholaminergic cells which inhibits dopaminergic pathways, have been shown to suppress FDM [81]. 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. While the low level of DA in the *nob* mice may have produced a slight myopic shift from 6–12 weeks of age, it was only after form deprivation that a significant myopic shift was measured. Further studies will need to be done to determine whether DA is decreased in FDM in the mouse model.

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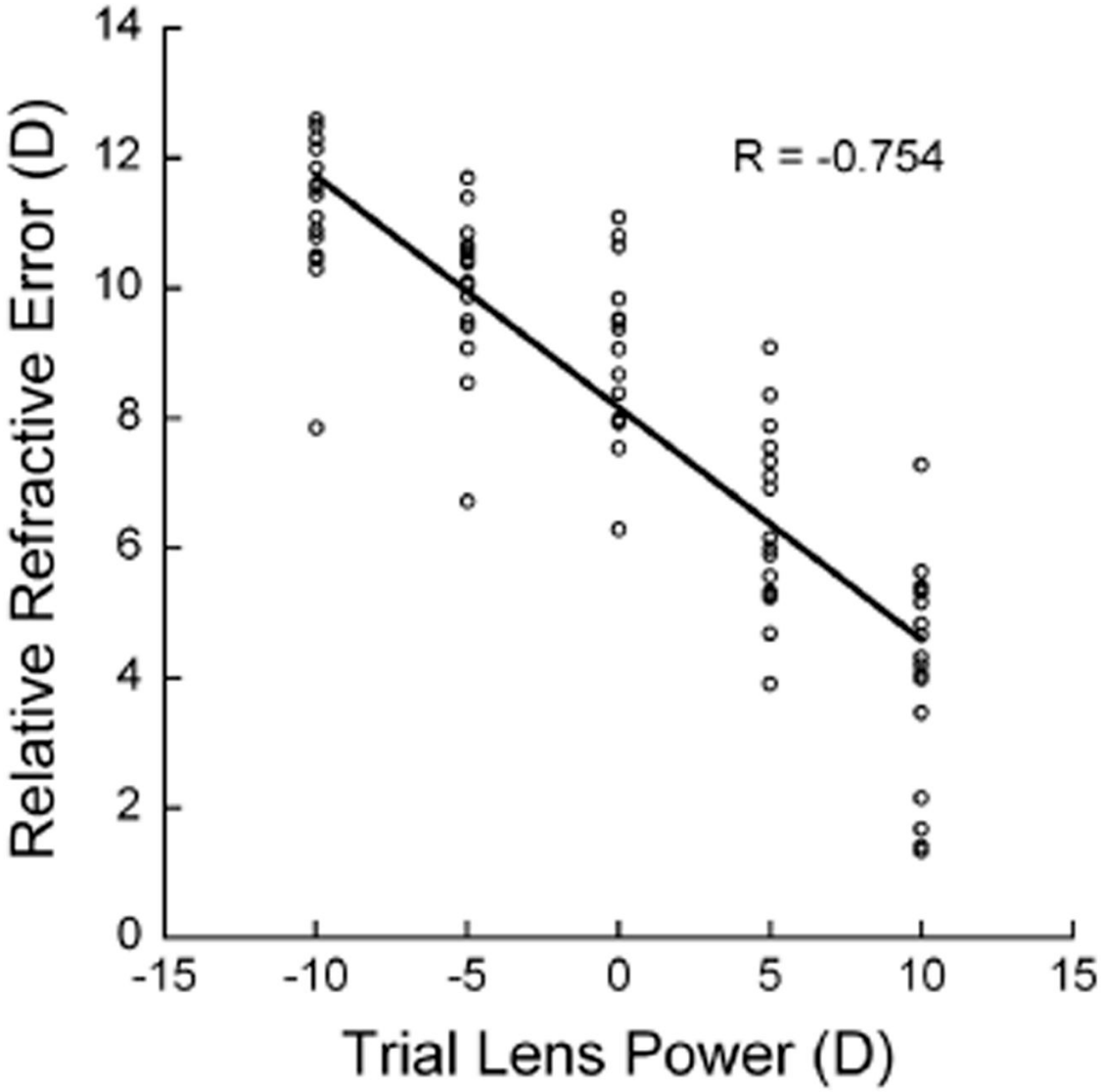

Figure 1.
Refractive errors measured with a photorefractor calibrated to the mouse eye with the addition of a series of trial lenses. The regression line showed good correlation between the measured refractive error and trial lens power. Each point represents one recording from a single eye (n=16 eyes).

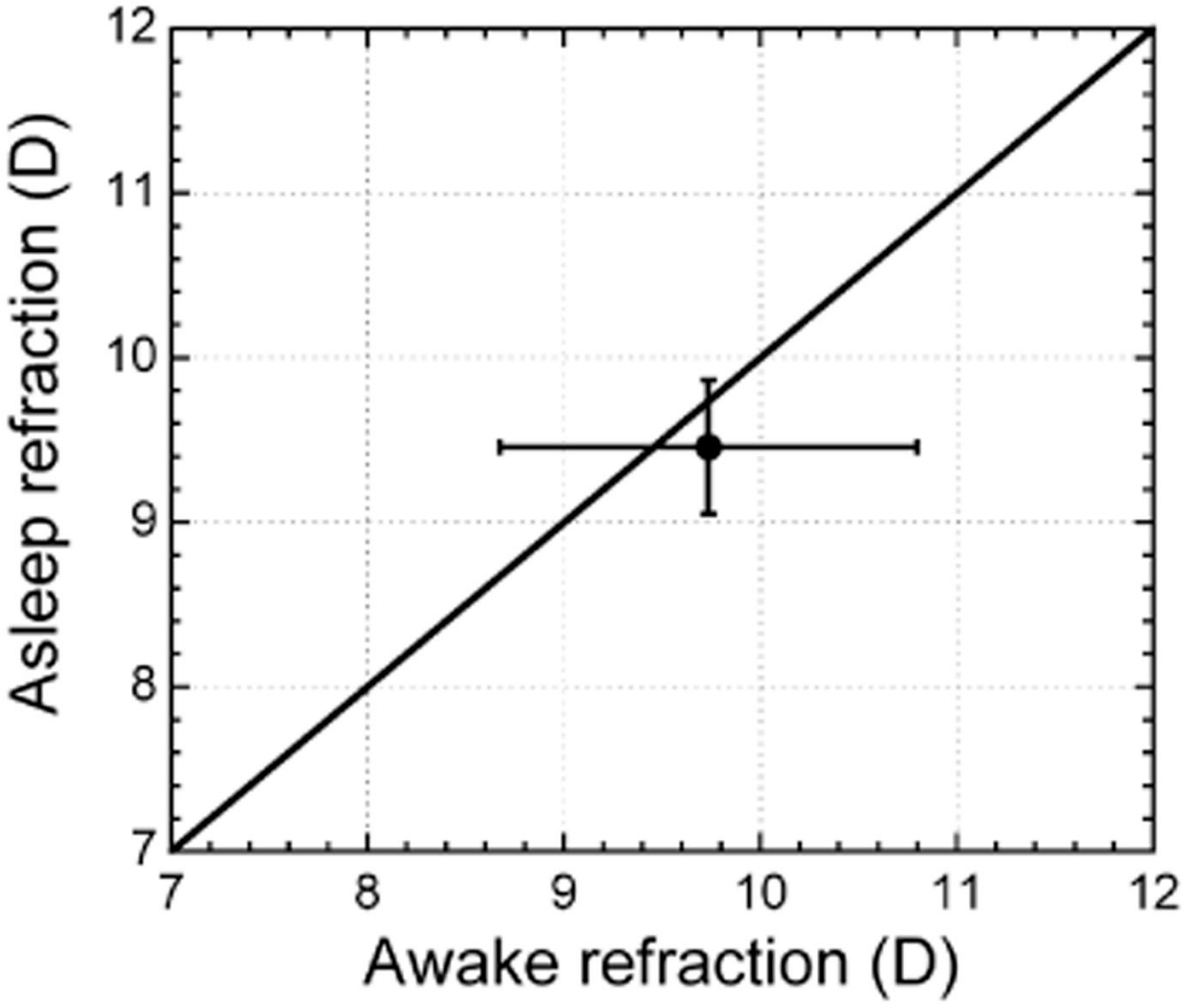

Figure 2.
Plot of average asleep vs. awake refractions for the same *nob* mice (n=20). The error bars represent standard deviation. Note less variability was obtained when the mouse was asleep.

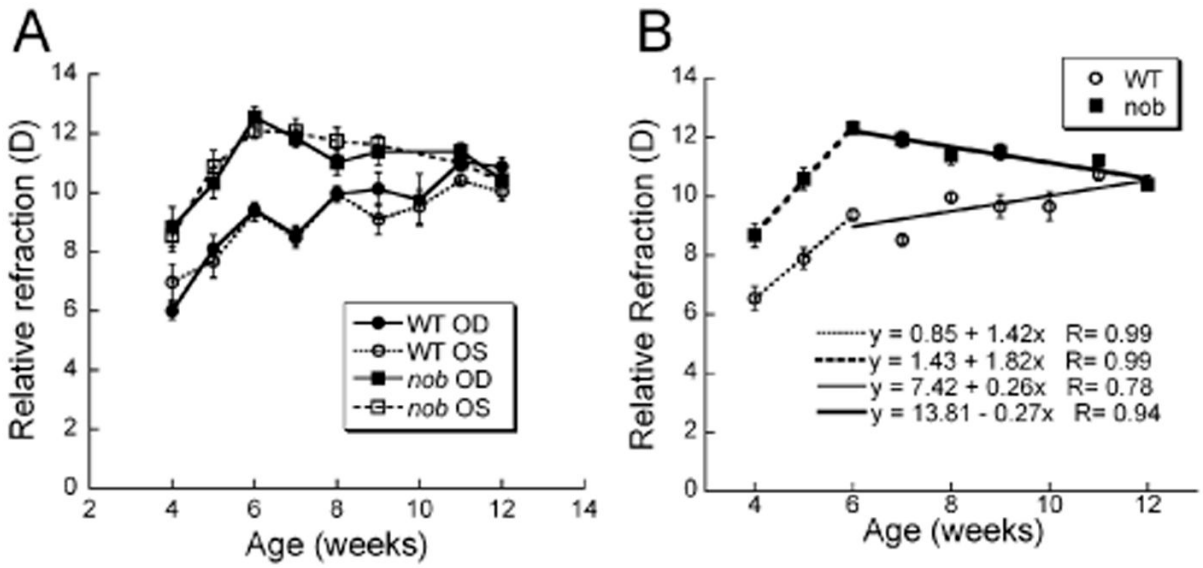

Figure 3.

Refractive development in *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 compared to WT mice (Repeated measures ANOVA, F (7, 122) = 9.89, $p < 0.001$ and Holm Sidak Multiple Comparisons). B) Linear curve fitting of the combined mean from both eyes demonstrated that *nob* mice reached the highest hyperopic refraction at 6 weeks of age and then shifted towards less hyperopia. The linear equation and regression fit are given for each line. Symbols and error bars represent mean and standard error of the mean, respectively.

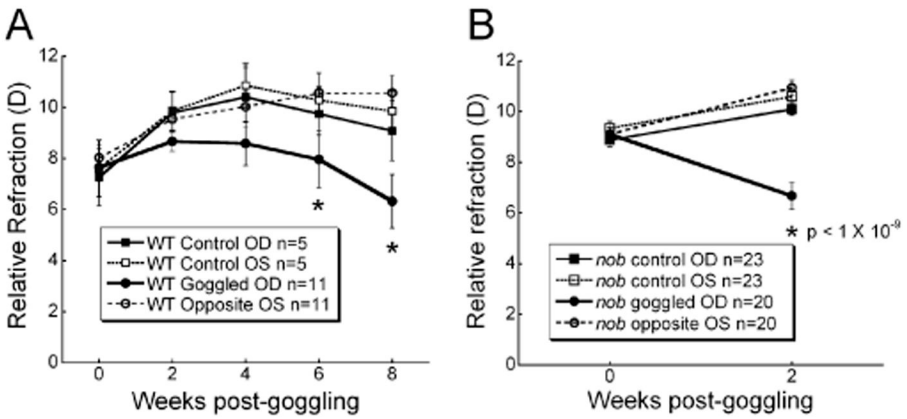

Figure 4.

Refractive errors from WT (A) and *nob* (B) eyes with form deprivation induced by diffuser goggles compared to the ungoggled opposite eye and naïve control mice. A) Refractions recorded every two weeks in WT mice over the goggling period showed myopic shifts in eyes with form deprivation at 6 and 8 weeks after goggling. Asterisk indicates $p<0.001$ with Holm-Sidak Multiple Comparison. B) Refractions in the *nob* mice at baseline and 2 weeks after goggling. Symbols represent mean ±SEM.

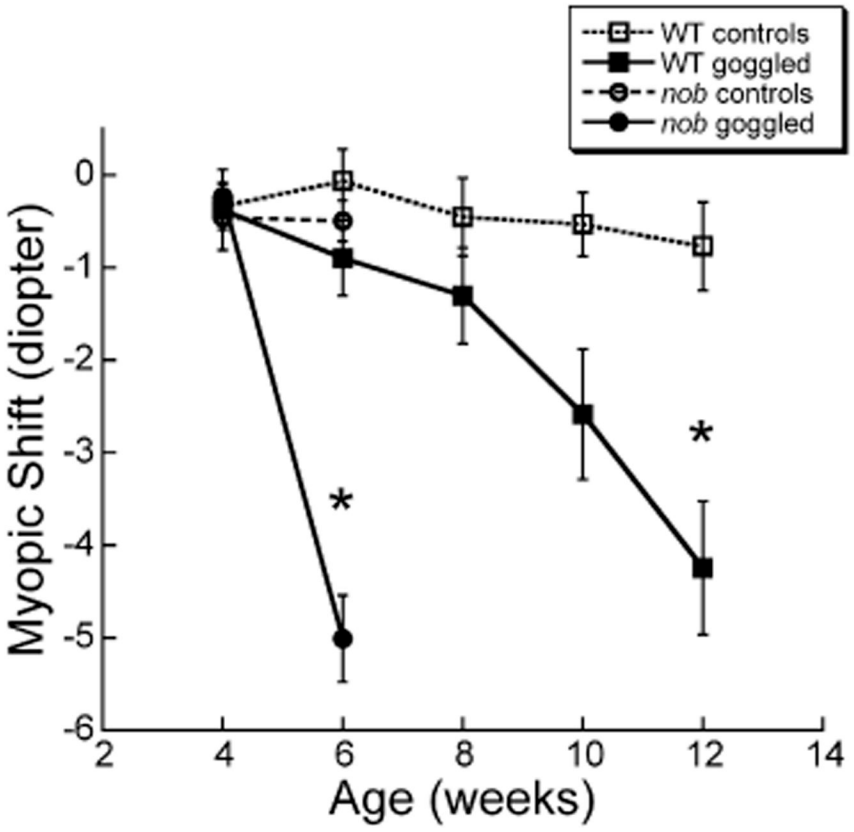

Figure 5.
The myopic shift (goggled eye – unmanipulated opposite eye) in response to form deprivation in WT and *nob* mice. Square symbols (mean± SEM) represent the WT mice (n = 11) while the round symbols represent the *nob* mice (n = 20). Asterisks indicate significant *p* values less than 0.001 with Holm-Sidak Multiple Comparison tests.

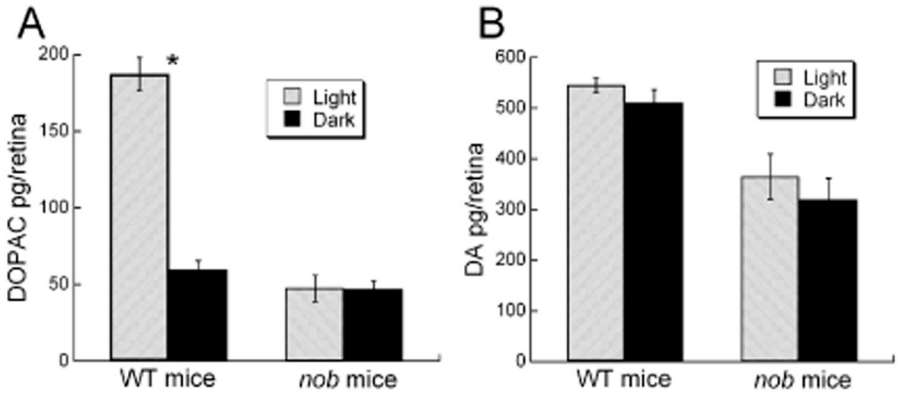

Figure 6.

Average DOPAC and DA levels from *nob* and WT mice at 12 weeks of age. Retinas were collected 4 hours into the light cycle (light) or under dark-adapted (dark) conditions. A) While the levels of DOPAC were significantly greater after light exposure in WT mice, no differences were found between light- and dark-adapted retinas in *nob* mice. B) No significant differences in dopamine (DA) levels were seen between light and dark conditions for either strain. Note that DOPAC and DA levels were significantly decreased in *nob* compared to WT mice. Symbols and error bars represent mean and standard error, respectively. Asterisk indicates $p<0.001$.