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ON pathway mutations increase susceptibility to form-deprivation myopia

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Abstract

The ON pathway mutation in *nob* mice is associated with altered refractive development, and an increased susceptibility to form-deprivation (FD) myopia. In this study, we used *mGluR6−/−* mice, another ON pathway mutant, to determine whether the *nob* phenotype was due to the *Nyx* mutation or abnormal ON pathway transmission. Refractive development under a normal visual environment for *mGluR6−/−* and age-matched wild-type (WT) mice was measured every 2 weeks from 4 to 16 weeks of age. The response to monocular FD from 4 weeks of age was measured weekly in a separate cohort of mice. Refraction and ocular biometry were obtained using a photorefractor and optical coherence tomography. Retinas were harvested at 16 weeks, and analyzed for dopamine (DA) and DOPAC using high-performance liquid chromatography. Under normal conditions, *mGluR6−/−* mice were significantly more myopic than their WT controls (refraction at 12 weeks; WT: 9.40 ± 0.16 D, *mGluR6−/−*: 6.91 ± 0.38 D). Similar to *nob* mice, two weeks of FD resulted in a significant myopic shift of −5.57 ± 0.72 D in *mGluR6−/−* mice compared to −1.66 ± 0.19 D in WT animals. No significant axial length changes were observed with either normal or FD visual conditions. At 16 weeks, *mGluR6−/−* retinas showed significantly lower DOPAC levels (111.2 ± 33.0 pg/mg) compared to their WT counterparts (197.5 ± 11.2 pg/mg). Retinal DA levels were similar between the different genotypes. Our results indicate that reduced retinal DA metabolism/turnover may be associated with increased susceptibility to myopia in mice with ON pathway defect mutations.

Keywords

refractive error; form-deprivation; ON pathway; metabotropic glutamate receptor; dopamine; myopia

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Emmetropization is an active, visually-guided process whereby the axial length and the optical power of the eye precisely match each other to eliminate neonatal refractive errors, and bring the eye into perfect focus (Smith, 1998; Wallman and Winawer, 2004). Any disruption to this mechanism of ocular growth results in the development of refractive errors; eyes being either too short (hyperopia) or too long (myopia) (Wallman and Winawer, 2004).

A large body of animal research using lens-induced (Hung et al., 1995; Irving et al., 1992; Schaeffel et al., 1988; Wildsoet and Wallman, 1995) and form-deprivation (FD) (Nickla et al., 1998; Smith and Hung, 2000; Wallman et al., 1978; Wallman et al., 1995) paradigms have shown the importance of the visual environment in the regulation of ocular growth. The ocular response to FD (Troilo et al., 1987) or lens-induced defocus (Wildsoet and Wallman, 1995) after optic nerve section in chickens indicates that the visual mechanisms involved in regulating refractive development localize primarily [if not exclusively, (Troilo et al., 1987; Wildsoet, 2003)] to the retina. Furthermore, partial diffusers only cause changes in the defocused area of the visual field in chickens (Diether and Schaeffel, 1997; Hodos and Kuenzel, 1984; Wallman et al., 1987) and primates (Smith et al., 2009), resulting in focal changes in refraction. Therefore, any defect in visual transmission through the retina could potentially influence ocular growth and lead to development of refractive errors.

Several studies have suggested a role for various retinal cell types and pathways in normal eye development. In chickens, physiological and morphological changes in photoreceptors are associated with experimentally induced myopia (Crewther, 2000). Pharmacological elimination of the OFF pathway in chicken retina using the D isomer of a gliotoxin α amino adipic acid (DαAAA) resulted in an enhanced rate of axial elongation under normal visual conditions, as well as with negative lenses (Crewther and Crewther, 1990). Conversely, inhibition of the ON pathway with the L isomer (LαAAA) caused a reduction in axial eye growth for both visually normal and lens-reared conditions (Crewther and Crewther, 1990). Furthermore, alternations in ON and OFF responses using 2-amino-4 phosphonobutyric acid (APB) or cis 2,3 piperidine-dicarboxylic acid (PDA) in chickens influence the ocular growth patterns and refractive errors (Crewther et al., 1996). More recent studies using various mutant mouse models have provided stronger evidence of retinal involvement in ocular refractive development (Chakraborty et al., 2014; Pardue et al., 2008; Park et al., 2013; Park et al., 2014). Mouse models ensure complete and selective blockage of a single pathway, and allow examination of the interaction between genetic background and visual environment in refractive development (Pardue et al., 2013).

ON and OFF pathways are important for efficiently transferring information about changes in light stimuli to the higher visual centers, and processing contrast sensitivity information (Schiller, 1992; Schiller et al., 1986). These pathways have been implicated in refractive development of mutant mice with selective ON or OFF pathway defects (Chakraborty et al., 2014; Pardue et al., 2008). A non-functional ON pathway (Gregg et al., 2003; Pardue et al., 1998) in *nob* mice due to a mutation in *Nyx* (Gregg et al., 2003) causes low retinal dopamine (DA) levels and has previously been associated with a small myopic shift under normal visual conditions, and increased susceptibility to myopia in response to visual FD (Pardue et al., 2008). Conversely, non-functional OFF pathways in *Vsx1*−/− mice (Chow et al., 2001;

Chow et al., 2004) had no significant effect on either normal or visually deprived refractive development (Chakraborty et al., 2014). These findings suggest that abnormal visual transmission through the ON pathway causes a greater refractive effect than disruption of the OFF pathway, perhaps due to changes in retinal dopaminergic activity, and may be more critical for normal ocular development in mammals.

In this study, we examined the refractive development and dopamine levels of *mGluR6−/−* mice (Masu et al., 1995; Sugihara et al., 1997; Tagawa et al., 1999; Takao et al., 2000), with a null mutation in the metabotropic glutamate receptor (*mGluR6*), which is located on the postsynaptic membrane of ON bipolar cells in both rod and cone systems (Nakajima et al., 1993; Vardi and Morigiwa, 1997). As a result of defective synaptic transmission through the ON-bipolar cells, *mGluR6−/−* mice have normal electroretinogram (ERG) a-waves, but non-recordable b-waves without significant change in responses from the OFF pathway (Masu et al., 1995). Additionally, *mGluR6* mutants show unmeasurable ON-responses from the superior colliculus (Masu et al., 1995). Furthermore, the loss of *mGluR6* produces these functional abnormalities without morphological changes in the retina (Tagawa et al., 1999; See reviews of mouse b-wave mutants McCall and Gregg, 2008; Pardue and Peachey, 2014). In humans, *mGluR6* mutations are associated with complete autosomal recessive congenital stationary night blindness (CSNB) (Dryja et al., 2005; Zeitz et al., 2005), abnormal cone ERG ON responses (Dryja et al., 2005) and high myopia (Xu et al., 2009) suggesting a potential link between the genetic mutation and refractive error development. In this study, we measured refractive changes in *mGluR6−/−* mice to determine whether altered refractive development in this mutant was similar to *nob* mice, which might implicate abnormal ON pathway transmission (and related changes in retinal DA levels) in refractive development versus some other aspect of the mutations.

An in-house breeding colony with both male and female homozygous *mGluR6* mutants (Jackson Laboratory, Bar Harbor, ME) on C57BL/6J background was maintained at the Atlanta Department of Veterans Affairs Medical Center. Mice were kept in 12:12 hour light cycles of ~17 lux with mouse chow and water *ad libitum*.

Age-matched male and female *mGluR6−/−* and C57BL/6J wild-type (WT) mice were subjected to one of two different experimental conditions: a normal visual environment or form- deprivation (FD). For mice raised in a normal visual environment (WT n=10; *mGluR6−/−* n=10), refractive measurements were obtained every 2 weeks from 4 to 16 weeks of age. For FD experiments, baseline refractive error measurements for WT (goggled n= 6; naïve controls n=6) and *mGluR6−/−* (goggled n= 5; naïve controls n=5) mice were obtained at 4 weeks of age and then the mice were subjected to monocular visual deprivation in the right eye using head-mounted diffuser goggles, as described previously (Faulkner et al, 2007). Weekly measurements were performed on the FD cohort for a period of 2 weeks (i.e. to 6 weeks of age). 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.

For both a normal visual environment and FD conditions, refractive error and axial length measurements (measured from the anterior cornea to the retinal pigment epithelium) were

acquired using an automated infrared photorefractor (Schaeffel et al., 2004) and a 1310 nm spectral-domain optical coherence tomography system (SD-OCT; Bioptigen Inc., Durham, NC), as described previously (Chakraborty et al., 2014; Pardue et al., 2008; Park et al., 2012; Park et al., 2013). Statistical analyses were performed using commercial software (SigmaStat 3.5, Aspire Software International, Ashburn, VA). Changes in refractive error and axial length between the *mGluR6*−/− and C57BL/6J WT animals across age under normal and FD visual conditions were analyzed by repeated-measures two-way analysis of variance (ANOVA), and Holm-Sidak post-hoc tests for statistical significance.

Under a normal visual environment, both *mGluR6*−/− and WT mice showed significantly increased hyperopic refractions (values averaged between the two eyes for each mouse) from 4 to 16 weeks of age (Figure 1A; two-way repeated-measures ANOVA main effect of age, $F(6,135)=58.69$, $p<0.001$). However, *mGluR6*−/− mice were significantly more myopic (mean refraction at 12 weeks ± standard error of the mean (SEM); WT: 9.40 ± 0.16 D, *mGluR6*−/−: 6.91 ± 0.38 D) than their age-matched WT controls throughout the developmental period (two-way repeated-measures ANOVA main effect of genotype, $F(1,135)=75.04$, $p<0.001$), suggesting that the *mGluR6* mutation had a significant effect on normal refractive development of the eye.

From 4 to 16 weeks of age, both genotypes exhibited a significant increase in axial length (mean change in axial length between 4 to 16 weeks of age; WT: 0.37 ± 0.01, *mGluR6*−/−: 0.37 ± 0.007 mm; two-way repeated-measures ANOVA main effect of genotype, $F(6,135)=1214.2$, $p<0.001$). However, no significant differences were observed between the two genotypes at any measured time point (two-way repeated-measures ANOVA main effect of genotype, $F(1,135)=0.25$, $p=0.623$). In order to elucidate the refractive changes in *mGluR6* mutants, we further examined the changes in corneal curvature using automated keratometry. However, no significant differences in corneal curvatures were observed between the *mGluR6*−/− and WT mice under either normal visual or FD conditions (data not shown). Given the inadequacy of the axial length and corneal curvature changes in explaining the myopic refractive error in *mGluR6*−/− mice, we hypothesize that it could be due to differences in other ocular optical parameters, such as changes in thickness, curvature or refractive index of the crystalline lens.

To examine the interaction between visual environment and genetic background, mice were form-deprived from 4 to 6 weeks of age, and the effect of goggling on refraction were compared between the two genotypes (Figure 1B). For the FD cohort, refractive errors are presented as “myopic shift”, the difference between right, goggled and left, opposite eyes or right minus left eyes for non-goggled naïve controls. For both genotypes, naïve untreated controls showed no significant differences in refraction between the left and right eyes (myopic shift at 6 weeks, WT: −0.32 ± 0.65 D, *mGluR6*−/−: −0.37 ± 0.33 D; two-way repeated-measures ANOVA main effect of genotype, $F(1,30)=0.2$, $p=0.664$). Although both goggled *mGluR6*−/− (−5.57 ± 0.72 D) and WT (−1.66 ± 0.19 D) mice exhibited a significant myopic shift after 2 weeks of goggling (two-way repeated-measures ANOVA interaction effect, $F(2,29)=16.31$, $p<0.001$), the magnitude of refractive shift at 6 weeks was significantly greater in *mGluR6*−/− compared to WT mice (Holm-Sidak multiple comparisons, $p<0.001$, Figure 1B).

For axial length data, “axial shift” was determined by calculating the difference in eye length between the right (FD) and the left (control) eye after normalizing the data to 4 week-old baseline values. FD did not cause significant change in axial lengths of goggled mice and no significant differences were observed in axial shift of untreated control animals for any genotype (two-way repeated measures ANOVA, $p>0.05$; data not shown). Please note that in the current study, the standard deviation for raw axial length measurements was ~ 35 μm, mainly due to limited resolution of the instrument to detect posterior retinal borders. This was greater than the inter-user measurement variability of our SD-OCT (21 μm) (Park et al., 2012), and predicted axial length difference based on mouse eye modeling (1 D of refractive change = ~ 5 μm change in eye length) (Schmucker and Schaeffel, 2004). Therefore, our instrument may not have been able to detect small changes in axial length, despite obvious differences in refractive errors as shown in Figure 1A and 1B.

To determine the changes in retinal dopamine activity associated with the *mGluR6* mutation, levels of retinal DA and DOPAC (3,4-dihydroxyphenylacetate, the primary metabolite of DA) (Witkovsky, 2004) were quantified for both normal refractive development and FD experiments. For normal refractive development, retinas of *mGluR6*−/− (n=7) and WT (n=7) mice were harvested at the end of the experiment (i.e. 16 weeks of age) between 4 and 6 h after light onset. For the FD experiments, retinas of *mGluR6*−/− (control: n=5, goggled: n=5) were harvested after the final end point at 6 weeks of age. Given the evidence that C57BL/6 WT mice (Wu et al., 2015) or WT mice of different backgrounds (Chakraborty et al., 2014; Park et al., 2013; Park et al., 2014) do not show significant changes in retinal DA levels with imposed FD, changes in retinal dopamine activity for goggled WT animals were not measured in this study. To avoid the effects of anesthesia on measurements, all experimental mouse retinas were collected 48 hours after the final measurements. Harvested retinas were immediately frozen on dry ice and stored at −80°C. The frozen retinas were processed using high-performance liquid chromatography (HPLC), as previously described (Nir et al., 2000; Pozdeyev et al., 2008). Right and left eyes of mice with a normal visual environment were pooled together for analysis, while eyes of FD mice were analyzed individually. For normal visual environment cohorts, all HPLC measurements were compared between *mGluR6*−/− and WT mice using independent two-tailed t-test. For the FD experiments, DA and DOPAC values were individually analyzed for the goggled (right eye), opposite (left untreated eye) and naïve control (average of untreated right and left) eyes using one-way ANOVA with Holm-Sidak post-hoc comparisons. An estimate of retinal dopamine turnover was presented as the ratio of DOPAC to DA (DOPAC/DA ratio) for both normal visual environment and FD experiments.

For 16 week old normal visual environment cohorts, retinas from *mGluR6*−/− mice yielded significantly lower DOPAC levels (111.2 ± 33.0 pg/mg) compared to their WT counterparts (197.5 ± 11.2 pg/mg, Student’s t-test, $t=2.761$, $p=0.019$, Figure 2A). However, retinal DA levels were found to be similar between the two genotypes (*mGluR6*−/−: 1609 ± 53.5 pg/mg, WT: 1491 ± 69.1 pg/mg, Student’s t-test, $t=-1.352$, $p=0.201$, Figure 2B). The DOPAC/DA ratios in WT mice (0.14 ± 0.002) were approximately two fold greater than those in *mGluR6*−/− mice (0.07 ± 0.02) (Student’s t-test, $t=3.001$, $p=0.012$, Figure 2C), indicating a significantly lower dopamine turnover in *mGluR6*−/− retinas.

For the FD experiments, goggled eyes of *mGluR6−/−* mice did not yield significant differences in any measured dopamine variable compared to their untreated left eyes or eyes of naïve control animals ($p>0.05$), suggesting that goggling had no significant effect on retinal dopaminergic activity of *mGluR6−/−* animals.

Under normal unmanipulated visual conditions, we found *mGluR6−/−* mice to be significantly more myopic than their WT controls throughout the developmental period measured from 4 to 16 weeks of age (Figure 1A). Using *nob* mice, another genetic model for the ON pathway defect (Pardue et al., 1998), Pardue et al., reported a relative myopic shift (decrease in hyperopic refraction) in *nob* mice during adolescence (from 6 to 12 weeks of age) compared to WT mice (Pardue et al., 2008). Together, these findings suggest that defects in the ON visual pathway significantly disrupt normal refractive development, and may predispose the eye to myopia in rodents.

The use of FD has become a standard method to induce experimental myopia in murine eyes (see review, Pardue et al., 2013). The small magnitude of myopic shift observed in WT mice after 2 weeks of goggling was in close agreement with previous studies (see review, Pardue et al., 2013). Importantly, we found *mGluR6−/−* mice to be highly susceptible to FD myopia, developing about 5.5 D of myopia in response to 2 weeks of FD (Figure 1B). Previously, a similar period of FD has been shown to induce comparable magnitude of myopia (~ 5 D) in *nob* mice (Pardue et al., 2008). In both studies, the largest differences in refractive error were induced after imposing FD, suggesting that changes in visual environment in conjunction with the ON pathway defect (and not the ON transmission defect alone) produces the most profound visual deficits in murine eyes.

The refractive deficits observed in *mGluR6−/−* mice could be due to lower DA metabolism and/or turnover in *mGluR6−/−* retinas. A defect in the ON pathway may lead to decreased DA levels in the retina as DA release is stimulated by light exposure via the ON pathway (Boatright et al., 1994; Boelen et al., 1998; Dumitrescu et al., 2009; Newkirk et al., 2013; Voigt and Wassle, 1987). At 16 weeks, we found retinal DOPAC (Figure 2A) and DOPAC/DA (Figure 2C) ratios in *mGluR6−/−* mice to be significantly lower than the WT animals. Consistent with our findings, Pardue et al. (Pardue et al., 2008) also reported retinal DOPAC levels to be significantly lower in *nob* mice compared to WT animals. Previous studies on chickens have also reported relatively greater changes in retinal DOPAC levels compared to changes in retinal DA levels associated with visual FD (Ohngemach et al., 1997). Furthermore, lower DOPAC and DA levels have been associated with FD myopia in chickens (Stone et al., 1989) and primates (Iuvone et al., 1989). However, contrary to lower DA levels in the *nob* mouse (Pardue et al., 2008), the current study found no significant differences in the endogenous retinal DA levels between the two genotypes (Figure 2B). Subtle differences in DA levels between the two studies could be due to differences in the end point of the two experiments (12 weeks vs 16 weeks in our study), as age-related dynamic changes in retinal DA levels of mice have recently been reported (Park et al., 2014).

Following 2 weeks of FD in *mGluR6−/−* mice, no significant differences in retinal DA or DOPAC levels were observed, despite a significant myopic shift. These findings suggest

that endogenous retinal DA metabolism and/or turnover are perhaps more important for determining the susceptibility of the mouse eye to experimental myopia than acute and/or induced changes in DA with FD (Park et al., 2013; Chakraborty and Pardue, 2015). Furthermore, these findings agree with the report that C57BL6J mice have unaltered dopamine with form-deprivation (Park et al., 2013; Wu et al., 2015). Reports of DA acting on different receptors to influence eye growth in rodents (Huang et al., 2014) suggest complex roles for DA in regulating visually guided ocular growth.

Our results are generally consistent with the previous study on refractive development in *nob* mice (Pardue et al., 2008), and further emphasize that ON pathway transmission is important for normal refractive development of the eye. However, both *Nyx* (Zhang et al., 2007) and *mGluR6* (Xu et al., 2009) mutations have been associated with high myopia independent of night blindness, suggesting that these mutations may play a separate role in detecting visual blur than that related to the ON pathway. If the refractive changes observed in *mGluR6*−/− and *nob* mice were occurring solely as a result of the mutation (not associated with the ON pathway defect), then both mutants would be expected to show distinct patterns of refractive development under normal visual conditions. However, given the similarities in refractive phenotypes of *mGluR6*−/− and *nob* mice under both normal and visually-deprived conditions, with significant reduction in retinal DOPAC levels, it is reasonable to conjecture that refractive defects observed in these mouse mutants are caused by the ON pathway defect (and related changes in retinal DOPAC levels), and not the mutation itself. In order to confirm this hypothesis, future studies are needed to examine whether systemic treatment with L-DOPA (L-3,4-dihydroxyphenylalanine, the precursor of DA) (Witkovsky, 2004) could rescue refractive deficits caused by the retinal DA deficiency in *nob* and *mGluR6*−/− mice.

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Highlights

- Functional mGluR6 receptor is important for normal refractive development in mice.
- mGluR6 mutation leads to myopic eye growth in mice.
- mGluR6 mutation increases the susceptibility to form-deprivation myopia in mice.
- Reduced retinal dopamine may cause increased myopia susceptibility in mGluR6 mutant.
- Both *nob* and mGluR6 ON pathway mutants exhibit similar refractive phenotypes.

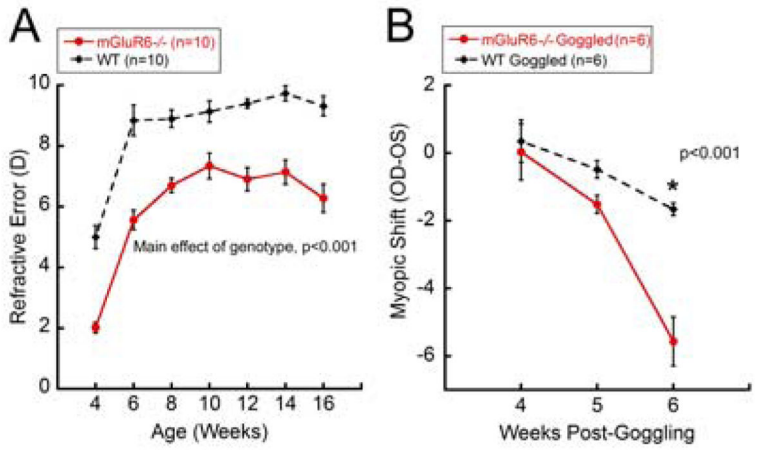

Figure 1.

Refractive development in *mGluR6−/−* mice raised under normal visual environment (A) or form-deprived (B) conditions. **A:** Both *mGluR6−/−* and WT mice showed significant increases in hyperopic refraction from 4 to 16 weeks of age (two-way repeated-measures ANOVA main effect of age, $F(6,135)=58.69$, $p<0.001$). Across normal refractive development, *mGluR6−/−* mice were significantly more myopic than WT controls (two-way repeated-measures ANOVA main effect of genotype, $F(1,135)=75.04$, $p<0.001$). **B:** After two weeks of FD, *mGluR6−/−* mice showed a significantly greater magnitude of myopic shift compared to the WT mice (two-way repeated-measures ANOVA interaction effect, $F(2,29)=16.311$, $p<0.001$, *Holm-Sidak multiple comparisons, $p<0.001$).

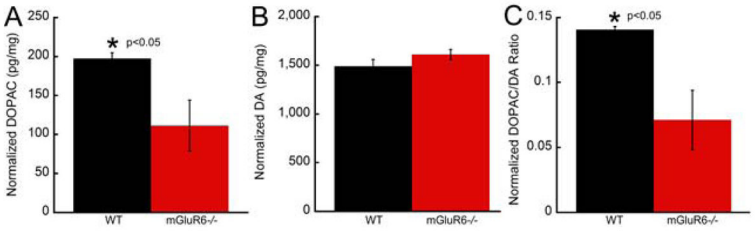

Figure 2.

Lower retinal dopamine metabolism and turnover in *mGluR6−/−* mice compared to WT mice at 16 weeks of age. **A:** Retinas harvested from *mGluR6−/−* mice exhibited significantly lower DOPAC levels compared to their WT counterparts (t-test, t=2.761, p=0.019). **B:** Retinal DA levels were similar between the two genotypes (t-test, t=−1.352, p=0.201). **C:** DOPAC/DA ratios in WT mice were approximately two fold greater than those in the *mGluR6−/−* mice (t-test, t=3.001, p=0.012).