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Lack of cone mediated retinal function increases susceptibility to form-deprivation myopia in mice

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Abstract

Retinal photoreceptors are important in visual signaling for normal eye growth in animals. We used *Gnat2*^{cplf3/cplf3} (*Gnat2*^{-/-}) mice, a genetic mouse model of cone dysfunction to investigate the influence of cone signaling in ocular refractive development and myopia susceptibility in mice. Refractive development under normal visual conditions was measured for *Gnat2*^{-/-} and age-matched *Gnat2*^{+/+} mice, every 2 weeks from 4 to 14 weeks of age. Weekly measurements were performed on a separate cohort of mice that underwent monocular form-deprivation (FD) in the right eye from 4 weeks of age using head-mounted diffusers. Refraction, corneal curvature, and ocular biometrics were obtained using photorefractometry, keratometry and optical coherence tomography, respectively. Retinas from FD mice were harvested, and analyzed for dopamine (DA) and 3,4-dihydroxyphenylacetate (DOPAC) using high-performance liquid chromatography. Under normal visual conditions, *Gnat2*^{+/+} and *Gnat2*^{-/-} mice showed similar refractive error, axial length, and corneal radii across development ($p > 0.05$), indicating no significant effects of the *Gnat2* mutation on normal ocular refractive development in mice. Three weeks of FD produced a significantly greater myopic shift in *Gnat2*^{-/-} mice compared to *Gnat2*^{+/+} controls (-5.40 ± 1.33 D vs -2.28 ± 0.28 D, $p = 0.042$). Neither the *Gnat2* mutation nor FD altered retinal levels of DA or DOPAC. Our results indicate that cone pathways needed for high acuity vision in primates are not

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as critical for normal refractive development in mice, and that both rods and cones contribute to visual signalling pathways needed to respond to FD in mammalian eyes.

Keywords

Refractive error; cone photoreceptors; form-deprivation; Gnat2; dopamine; mouse

Emmetropization is an active, visually-driven process that brings the eye into perfect focus, or the state of emmetropia (Smith, 1998; Wallman and Winawer, 2004). Ametropia or refractive errors occur when axial eye length does not match the optical power of the eye produced by the cornea and the crystalline lens. Although mechanisms underlying refractive errors are not well understood, the retina appears to be an essential element of the signaling for ocular growth. In addition to being the first layer of photosensitive neurons that detects the visual image (Crewther, 2000), the retina secretes a number of regulatory neurotransmitters [such as DA (Iuvone et al., 1991; Stone et al., 1989), retinoic acid (McFadden et al., 2004), nitric oxide (Nickla and Wildsoet, 2004; Nickla et al., 2006), and glucagon (Feldkaemper and Schaeffel, 2002), etc.] that have been shown to alter ocular growth in chickens and/or mammals. Visual blur to the eye results in a number of cellular and biochemical changes in the retina and the retinal pigmented epithelium (RPE) (Sharpe and Stockman, 1999), which signal changes to the choroid, and eventually the sclera, leading to alterations in the overall growth and refractive state of the eye (Wallman et al., 1995). Perhaps the most important evidence of retinal signaling modulating eye growth comes from studies that show optic nerve section does not prevent myopic growth in response to form-deprivation (FD) (Troilo et al., 1987) or spectacle lenses (Wildsoet, 2003) in chickens. Furthermore, in both chickens (Wallman et al., 1987) and primates (Smith et al., 2009a), application of partial diffusers cause local changes in ocular growth, restricted to the defocused part of the visual field. Together, these studies suggest that refractive development of the eye is primarily regulated in the retina, with evidence of some modulation from the higher visual areas, at least in chicks (Troilo et al., 1987; Wildsoet, 2003).

Photoreceptors may play an important role in emmetropization as the plane of focus in an emmetropic eye lies within the photoreceptors, and the alignment and directionality of photoreceptors facilitate blur detection at the retina (Crewther, 2000). Reduced photoreceptor cell density (Beresford et al., 1998) and elongated outer segments of rods (Liang et al., 1995) in experimentally induced myopic eyes suggest that photoreceptors may play an important role in ocular refractive development. While there is evidence of both rods and cones contributing to normal refractive growth in chicken and mammalian eyes [see reviews: (Chakraborty and Pardue, 2015; Crewther, 2000)], circumstantial and experimental evidence suggest that cone pathways may have a greater influence on visual signaling for refractive eye growth. Examples of cone signalling in emmetropization and experimental myopia include chromatic cues detected by cone opsins that influence experimental myopia (Rucker, 2013), constant light exposure in mice that stimulated cones and suppresses rods which would increase susceptibility to form deprivation (Tkatchenko et al., 2013), development of myopia in chickens reared under dim lighting (Lauber and Kinnear, 1979), and individuals with specific cone opsin mutations which have increased incidence of

myopia (Greenwald et al., 2017). However, these studies have relied on indirect evidence from the visual environment that may not specifically isolate cones or may be due to other secondary effects.

The current study used a genetic mouse model of cone dysfunction, *Gnat2*^{cplf3/cplf3} (*Gnat2*^{-/-}) mice (Chang et al., 2006) to directly test the contribution of normal cone function on refractive development and myopia susceptibility. Transgenic mouse models can ensure complete and selective blockage of a single pathway or cell type, and allow simultaneous manipulation of both gene and visual environment in the same animal (Pardue et al., 2013). Using a mouse model of non-functional rods (*Gnat1*^{-/-} mice), a previous study found functional rod photoreceptors to be important for normal refractive development and FD response in mice (Park et al., 2014). *Gnat2*^{-/-} mice have a missense mutation in the guanine nucleotide binding protein, a heterotrimeric G-protein that encodes the α -subunit of cone transducin, necessary for hyperpolarization of cones in the phototransduction cascade (Chang et al., 2006; Lerea et al., 1986). *Gnat2*^{-/-} mice exhibit abnormal cone electroretinography responses that were 25% of wild-type mice as early as 4 weeks of age (undetectable by 9 months), progressive loss of cone α -transducin, but no changes in the cone outer segment structure (Chang et al., 2006). In humans, mutations in the GNAT2 gene cause achromatopsia, characterized by poor cone electroretinography, total color blindness, low visual acuity, photophobia, nystagmus and variable refractive errors from high myopia to high hyperopia (Haegerstrom-Portnoy et al., 1996; Michaelides et al., 2003; Sloan, 1954). Thus, *Gnat2*^{-/-} mice provide a model in which cone function is lost, but cone photoreceptor structure is intact during the experimental period.

To examine refractive development in mice, an in-house mouse breeding colony was maintained at the Atlanta Department of Veterans Affairs Medical Center with *Gnat2*^{cplf3/cplf3} mice purchased from Jackson Laboratories (Stock number: 006795, Bar Harbour, ME). All mice were kept in 12:12 hour light-dark cycles (ranged from 20–200 lux depending on location in rack/room) with food and water *ad libitum*. To confirm the loss of cone function, *Gnat2*^{+/+} and *Gnat2*^{-/-} mice at P28 were dark-adapted overnight and an electroretinogram recorded using both dark-adapted and light-adapted stimuli, as previously described (Mocko et al., 2011). To assess refractive development, age-matched male and female wild-type (*Gnat2*^{+/+}) and *Gnat2*^{-/-} mice, both on C57BL/6J background, underwent one of two experimental conditions during development: either normal visual development or FD. Mice raised under normal visual conditions (*Gnat2*^{+/+}: n = 7, *Gnat2*^{-/-}: n = 9) were tested for ocular parameters every two weeks from 4 to 14 weeks of age. For FD experiments, after obtaining baseline ocular measurements on both *Gnat2*^{+/+} (goggled n = 5–7; naïve controls n = 5–6) and *Gnat2*^{-/-} (goggled n = 7–9; naïve controls n = 10–11) mice at 4 weeks of age, the monocular head-mounted diffuser goggles were attached, as described previously (Faulkner et al., 2007). Weekly ocular measurements were performed on the FD mice for 3 weeks (i.e. up to 7 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 Atlanta Veterans Affairs Institutional Animal Care and Use Committee.

Ocular parameters included refractive error acquired using an automated infrared photorefractor (Schaeffel et al., 2004), corneal radius of curvature measured with a

photokeratometer (Schaeffel, 2008; Schmucker and Schaeffel, 2004), and axial length measured from the anterior cornea to the retinal pigment epithelium (RPE) using a 1310 nm spectral-domain optical coherence tomography system (SD-OCT; Bioptigen Inc., Durham, NC). These methods have been described previously (Chakraborty et al., 2014; Pardue et al., 2008; Park et al., 2012).

As mutations in cone photoreceptors could influence retinal DA release (and therefore myopia susceptibility) through cone ON bipolar pathways (Ghosh et al., 2004; Hartveit, 1997), retinal levels of DA and DOPAC (3,4-dihydroxyphenylacetate, primary metabolite of DA) were measured. To determine the changes in DA levels associated with FD, retinas from both goggled (right eye) and non-goggled eyes (left eye) were collected after the final end point at 7 weeks of age (*Gnat2*^{+/+}: n = 6, *Gnat2*^{-/-}: n = 7). To avoid any effects of anesthesia and circadian rhythms on DA measurements, all mouse retinas were harvested 48h after the final ocular measurement and between 4 to 6h after light onset. Harvested retinas were immediately frozen on dry ice and stored at -80°C, and were quantified using high-performance liquid chromatography (HPLC), as previously described (Nir et al., 2000; Pozdeyev et al., 2008). Changes in ocular measurements between the *Gnat2*^{-/-} and *Gnat2*^{+/+} mice across age under normal and FD conditions were analyzed by two-way repeated-measures analysis of variance (RM-ANOVA), and Holm-Sidak post-hoc tests for multiple comparisons using commercial software (SigmaStat 3.5, Aspire Software International, Ashburn, VA). In FD animals, differences in DA and DOPAC levels (ng/mg of retinal protein) between the goggled and non-goggled control eyes across both genotypes were analyzed using a two-way ANOVA.

As previously reported (Chang et al., 2006), we found that *Gnat2*^{-/-} mice at P28 had normal dark-adapted ERGs, indicating normal rod function. However, light-adapted ERGs were unrecordable until the brightest flash stimuli (90.6 cd s/m²) was presented and then were only ~15% of the wild-type b-wave amplitude (Supplemental Figure 1). Mice were housed in lighting conditions that were 2.5 log units dimmer than the dimmest ERG flash stimuli. Thus, *Gnat2*^{-/-} mice have non-functional cone photoreceptors during the experimental period.

Under normal visual conditions, both *Gnat2*^{+/+} and *Gnat2*^{-/-} mice showed a significant increase in hyperopic refractive errors from 4 through 6 weeks of age (values averaged for the two eyes from each mouse), at which it stabilized and remained relatively steady thereafter (two-way RM-ANOVA main effect of age, F(5, 82)=23.821, p<0.001). However, the pattern of refractive development was not different between the two genotypes across age (mean refraction at 10 weeks ± standard error of the mean (SEM); *Gnat2*^{+/+}: +6.57 ± 0.78 D, *Gnat2*^{-/-}: +5.74 ± 0.56 D) (two-way RM-ANOVA main effect of genotype, F(1, 82)=1.178, p=0.296, Figure 1A). Both genotypes also exhibited similar changes in axial length (F(1, 59)=1.014, p=0.343) and corneal radius of curvature (F(1, 82)=0.774, p=0.394) at all time points across development (data not shown). Together, these results suggest that the loss of cone function due to the *Gnat2* mutation had no significant effect on normal refractive development in mice.

DOPAC and DA levels were similar for *Gnat2^{+/+}* and *Gnat2^{-/-}* mice (DOPAC: *Gnat2^{+/+}* 0.289 ± 0.020 ng/mg; *Gnat2^{-/-}* 0.332 ± 0.018 ng/mg, two-way RM-ANOVA main effect of genotype, $F(1, 23)=2.237$, $p=0.150$; DA: *Gnat2^{+/+}* 2.201 ± 0.122 ng/mg; *Gnat2^{-/-}* 2.358 ± 0.062 ng/mg, two-way RM-ANOVA main effect of genotype, $F(1, 24)=1.372$, $p=0.225$; DOPAC/DA ratio: *Gnat2^{+/+}* 0.131 ± 0.004 ng/mg; *Gnat2^{-/-}* 0.140 ± 0.006 ng/mg, two-way RM-ANOVA main effect of genotype, $F(1, 23)=1.677$, $p=0.210$; Figure 2), indicating that loss of cone function did not affect retinal dopamine content or metabolism.

To study the effects of altered visual environment with the absence of cone function, monocular FD was induced in mice from 4 to 7 weeks of age, and the effect of goggling was compared between the two genotypes. For the FD cohort, measurements are presented as “myopic shift”, calculated as the difference in ocular measurements between the goggled (right eye) and non-goggled (left) eyes. Untreated naïve controls from either genotype showed no significant difference in refraction between the right and left eyes (myopic shift at 7 weeks, *Gnat2^{+/+}*: -0.36 ± 0.27 D, *Gnat2^{-/-}*: -0.03 ± 0.45 D; two-way RM-ANOVA main effect of genotype, $F(1,64)=0.065$, $p=0.802$). Although goggled animals from both genotypes developed significant myopia after 3 weeks of goggling (*Gnat2^{+/+}*: -2.28 ± 0.28 D; *Gnat2^{-/-}*: -5.40 ± 1.33 D), the magnitude of the refractive shift was two times greater in *Gnat2^{-/-}* compared to *Gnat2^{+/+}* mice (two-way RM-ANOVA main effect of genotype, $F(1,50)=4.599$, $p=0.042$, Figure 1B). To account for this genotypic difference in FD response, differences in corneal curvature (corneal shift) and axial length (axial shift) between the two eyes were also compared. However, no significant differences were observed in axial length or corneal radii between goggled and naïve animals for either genotype (two-way RM-ANOVA, $p>0.05$; data not shown). This could be due to limited resolution of the OCT to detect the RPE surface (Park et al., 2012) or may be indicative of other optical parameters, such as the crystalline lens, playing a greater role in mediating optical changes in the mouse eye associated with FD.

Under FD conditions, the FD eyes of *Gnat2^{-/-}* mice did not show any significant differences compared to their untreated left eyes or either eyes of FD *Gnat2^{+/+}* mice for DOPAC (*Gnat2^{+/+}* goggled: 0.296 ± 0.028 ng/mg, control: 0.284 ± 0.031 ng/mg; *Gnat2^{-/-}* goggled: 0.335 ± 0.021 ng/mg, control: 0.329 ± 0.030 ng/mg), DA (*Gnat2^{+/+}* goggled: 2.198 ± 0.162 , control: 2.205 ± 0.195 ng/mg; *Gnat2^{-/-}* goggled: 2.293 ± 0.053 , control: 2.422 ± 0.110 ng/mg), or DOPAC/DA ratio (a measure of DA turnover) (*Gnat2^{+/+}* goggled: 0.134 ± 0.007 , control: 0.128 ± 0.004 ng/mg; *Gnat2^{-/-}* goggled: 0.146 ± 0.009 , control: 0.134 ± 0.006 ng/mg) (two-way ANOVA, $p>0.05$, Figure 2). These results suggest that increased myopia susceptibility in eyes with cone dysfunction may be independent of changes in retinal DA.

Previous observations suggesting that high-resolution vision is an important prerequisite for emmetropization have led to speculation that cone pathways dominate the visual signaling for normal refractive development (Carmichael Martins and Vohnsen, 2018; Gawne et al., 2017; Gisbert and Schaeffel, 2018; Nevin et al., 1998; Rucker and Wallman, 2008). Contrary to this hypothesis, we found *Gnat2^{-/-}* mice with non-functional cone photoreceptors develop normal refractive error, axial length and corneal radii when exposed to normal laboratory conditions, suggesting that functional cone photoreceptors may not be critical for emmetropization in mice. Interestingly, the absence of rod signaling in the *Gnat2^{-/-}* mice

results in abnormal refractive development (Park et al., 2014). Thus, we hypothesize that normal refractive development in *Gnat2*^{-/-} mice may be due to normal processing of visual information through functional rod photoreceptors in the retina. Alternatively, the absence of refractive abnormalities with cone dysfunction in mice may be related to their rod-dominated retina and poor cone vision, as reported previously (Carter-Dawson and LaVail, 1979; Schmucker et al., 2005). However, more recent evidence suggest that the mouse can effectively use both rods and cones for more sensitive vision, and can switch between the two photoreceptors at different luminance levels (Naarendorp et al., 2010; Umino et al., 2008). In fact, mice appear to use rod pathways for visual acuity as *CNGA3*^{-/-} mice without functional cones (mutation in cyclic nucleotide-gated cation channel subunit A3) show similar spatial frequency thresholds as wild-type controls, whereas mice without functional rods (*CNGB1*^{-/-} mice) exhibit poorer spatial acuity (Schmucker et al., 2005). Finally, rod pathways have been shown to function under both dim and bright light environments in mice (Tikidji-Hamburyan et al., 2017). Together, these data suggest that rod pathways are important for visual function and normal refractive development across a wide range of light levels in mice, while cone pathways may not be as critical.

FD is commonly used in mice to induce experimental myopia, and the magnitude of myopia observed in *Gnat2*^{+/+} mice was in close agreement with previous reports on C57BL/6J wild-type mice (Barathi et al., 2008; Chakraborty et al., 2015b; Pardue et al., 2008; Park et al., 2014; Schaeffel et al., 2004). After 3 weeks of FD, *Gnat2*^{-/-} mice developed significant myopia (~ 5.5 D, two times greater than *Gnat2*^{+/+} mice), indicating that the absence of cone function in conjunction with FD visual environment increases the myopic shifts in murine eyes. These data suggest that normal cone activity decreases the sensitivity to applied FD. However, rod pathway contributions also seem to be important for the response to FD as evidenced by 1) imposing peripheral FD (Smith et al., 2005) or lens induced defocus (Smith et al., 2009b) on the rod-dominated peripheral retina of the monkey eye produce similar magnitudes of myopia as when imposed on the entire visual field, 2) laser ablation to the cone-rich fovea has no effect on emmetropization or FD myopia in monkey eyes (Smith et al., 2007), and 3) absence of rod signaling suppresses the FD response in mice (Park et al., 2014). Together these studies suggest that retinal signaling of refractive development is more complicated than just cone-mediated signaling pathways, and there appears to be a significant contribution from rods (or even intrinsically photosensitive retinal ganglion cells (Chakraborty et al., 2015a)) in this process.

DA is released by activity in rod pathways through ON bipolar cell stimulation (Daw et al., 1990; Newkirk et al., 2013; Witkovsky, 2004). Thus, the absence of DA or DOPAC changes in *Gnat2*^{-/-} mice with normal visual input was not surprising since the rod pathways are still intact and could stimulate DA release. In the FD studies, no significant differences were found in the levels of retinal DA or DOPAC with 3 weeks of goggling in either *Gnat2*^{+/+} or *Gnat2*^{-/-} mice. Previous studies have reported no significant changes in retinal DA levels of C57BL/6J wild-type mice (Wu et al., 2015) or wild-type mice of other backgrounds (Chakraborty et al., 2014; Park et al., 2013; Park et al., 2014) with FD, suggesting that retinal DA may not directly modulate the refractive state of the mouse eye (Zhou et al., 2017).

Contrary to *Gnat2*^{-/-} mice with non-functional cones, Park et al. found *Gnat1*^{-/-} mice with non-functional rods had abnormal refractive development and were unresponsive to imposed FD (Park et al., 2014). These refractive changes were hypothesized to be associated with decreased tonic levels of DA metabolism in the *Gnat1*^{-/-} retinas during ocular development since DA and DOPAC levels did not change with FD. Furthermore, mice with reduced tonic levels of DA due to ON pathway defects (Chakraborty et al., 2015b; Pardue et al., 2008) or photoreceptor degeneration (Park et al., 2013) also have increased susceptibility to form deprivation myopia. Taken together, these results suggest that DA levels may not directly modulate the refractive state of the mouse eye, but tonic levels of DA during development may determine susceptibility to myopia. Evidence of DA acting on different DA receptors to influence ocular growth in rodents shows that the possible action of DA on refractive development and FD myopia is complex (Huang et al., 2014; Zhou et al., 2017). Future studies are required to investigate how different photoreceptors might mediate normal refractive development under different ambient lighting conditions or visual stimuli (such as lens defocus).

A limitation of the current study is the use of *Gnat2*^{cplf3/cplf3} mice which may not have total loss of cone function at P28. As reported in (Chang et al., 2006), we also found that *Gnat2*^{-/-} mice had normal dark-adapted ERGs and non-recordable light-adapted ERGs at P28. However, it is possible *Gnat2*^{-/-} mice have some remaining cone function that provide a minimally required threshold of normal cone input that enables the *Gnat2*^{-/-} eyes to still retain normal refractive development under laboratory visual conditions, while resulting in more susceptibility to FD. Supporting this idea, Allen et al. showed that mice with both *Gnat1* and *Gnat2*^{cplf3/cplf3} mutations responded to bright stimuli (>2.0 log cd/m²), likely through cones, however, they also report evidence that rods may express *Gnat2* (Allen et al., 2010).

Finally, mutations in the GNAT2 gene have been identified in human patients with achromatopsia (Kohl et al., 2002). Along with other profound visual symptoms, patients with achromatopsia demonstrate a wide distribution of refractive errors, ranging from high myopia to high hyperopia (Haegerstrom-Portnoy et al., 1996; Michaelides et al., 2003). These findings further emphasize the importance of cone-mediated visual signaling in emmetropization. Based on our results, we hypothesize that refractive error (at least high myopia) in patients with GNAT2 mutations may be a result of visual disruptions during ocular development, and not the mutation alone.

Supplementary Material

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Figure 1:

Refractive development in *Gnat2*^{-/-} mice raised under normal visual environment (A) or form-deprived (B) conditions. A: Both *Gnat2*^{+/+} and *Gnat2*^{-/-} mice showed a significant increase in hyperopic refractive error from 4 through 14 weeks of age (two-way RM-ANOVA main effect of age, $F(5, 82)=23.821$, $p<0.001$); however, the pattern of refractive development was not different between the two genotypes at any measured time point across age (two-way RM-ANOVA main effect of genotype, $F(1, 82)=1.178$, $p=0.296$). B: After three weeks of FD, *Gnat2*^{-/-} mice showed a significantly greater myopic shift compared to *Gnat2*^{+/+} mice (two-way RM-ANOVA main effect of genotype, $F(1, 50)=4.599$, $p=0.042$).

Figure 2:

Retinal DOPAC (A), DA (B) and DOPAC/DA ratio (C) in *Gnat2*^{+/+} and *Gnat2*^{-/-} mice with FD. Levels of DOPAC and DA were not different between genotypes. FD treatment in *Gnat2*^{-/-} mice did not lead to significant changes in the levels of retinal DOPAC, DA, or DOPAC/DA ratio between the goggled (right) and control (left) eyes, or either eyes of goggled *Gnat2*^{+/+} mice (two-way ANOVA, $p > 0.05$).