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TITLE

Shedding light on myopia by studying complete congenital stationary night blindness

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Abstract

Myopia is the most common eye disorder, caused by heterogeneous genetic and environmental factors. Rare progressive and stationary inherited retinal disorders are often associated with high myopia. Genes implicated in myopia encode proteins involved in a variety of biological processes including eye morphogenesis, extracellular matrix organization, visual perception, circadian rhythms, and retinal signaling. Differentially expressed genes (DEGs) identified in animal models mimicking myopia are helpful in suggesting candidate genes implicated in human myopia. Complete congenital stationary night blindness (cCSNB) in humans and animal models represents an ON-bipolar cell signal transmission defect and is also associated with high myopia. Thus, it represents also an interesting model to identify myopia-related genes, as well as disease mechanisms. While the origin of night blindness is molecularly well established, further research is needed to elucidate the mechanisms of myopia development in subjects with cCSNB. Using whole transcriptome analysis on three different mouse models of cCSNB (in Gpr179\(^{-}\), Lrit3\(^{-}\) and Grm6\(^{-}\)), we identified novel actors of the retinal signaling cascade, which are also novel candidate genes for myopia. Meta-analysis of our transcriptomic data with published transcriptomic databases and genome-wide association studies from myopia cases led us to propose new biological/cellular processes/mechanisms potentially at the origin of myopia in cCSNB subjects. The results provide a foundation to guide the development of pharmacological myopia therapies.
Keywords: congenital stationary night blindness, myopia, retina, transcriptome sequencing, meta-analysis

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1. Introduction

Myopia (short-sightedness) is the most common human eye disorder worldwide, in which the image is focused in front of the retina, resulting usually from an excessive growth of the eye posterior segment (increased axial length) (reviewed by e.g. (Goss and Wickham, 1995; McBrien and Gentle, 2003), generally as a consequence of accelerated post-natal eye growth (McBrien and Gentle, 2003) but also set by the optical power of the cornea and the lens (Iribarren, 2015). It can be due to structural abnormalities of the cornea as found in keratoconus or due to changes primarily in the lens power found e.g. in older people developing cataracts (reviewed by (Flitcroft et al., 2019; Iribarren, 2015)). Recent animal studies, combined with clinical studies, have revealed that the mechanisms of optically guided eye growth are influenced by the retina (Flitcroft, 2012).

High axial myopia, defined as a refractive error of more than −6.0 diopters (D) or ocular axial length of more than 26–26.5 mm (Flitcroft et al., 2019), can lead to blinding complication such as myopic macular degeneration (MMD), retinal tears and retinal detachment, and predispose to other ocular diseases such as glaucoma and early onset cataracts (Hornbeak and Young, 2009). A subset of subjects with high myopia also demonstrate diffuse retinal changes beyond the macula, so-called “diffuse chorioretinal atrophy”, which is defined as pathologic myopia (Ohno-Matsui et al., 2016); pathologic myopia can be associated with fundus changes, regional retinal and choroidal thinning (Ohno-Matsui and Jonas, 2019; Ye et al., 2019), and increased retinal complications, such as retinal detachment (Ohno-Matsui et al., 2016). Epidemiological studies estimated that half the world’s population will be myopic by 2050, with 10% showing high myopia (Holden et al., 2016). Indeed, it was shown that the incidence of myopia is increasing, affecting now 20%-
40% of children at the age of 17-18 in **developed western countries** and up to 80% -90% in **East and Southeast Asia** (reviewed by (Morgan et al., 2018) (e.g. Ang et al., 2020; Cumberland et al., 2015; Foster and Jiang, 2014; French et al., 2013; Holden et al., 2014; Holden et al., 2016; Hysi et al., 2014; Kempen et al., 2004; Lam et al., 2004; Lin et al., 2004; Morgan et al., 2018; Morgan et al., 2012; Pan et al., 2012; Vitale et al., 2009)). However, in **less developed countries, with less developed education systems**, the prevalence of myopia in young adults is estimated to be only 5% -10% (reviewed by (Morgan et al., 2018) (e.g. Anera et al., 2009; Casson et al., 2012; Dandona et al., 2002a; Dandona et al., 2002b; Gao et al., 2012; Jimenez et al., 2004; Jimenez et al., 2012; Khandekar and Abdul-Helmi, 2004; Lewallen et al., 1995; Lindquist et al., 2011; Lithander, 1999; Pokharel et al., 2000). High myopia is a complex disorder, which is mediated by genetic and more importantly by environmental factors (Baird et al., 2010; Morgan and Rose, 2005; Morgan, 2003; Morgan et al., 2018; Wojciechowski, 2011; Young, 2009). More than 300 genes were associated with refractive error development in humans (Flitcroft et al., 2018; Hysi et al., 2020; Kiefer et al., 2013; Tedja et al., 2019; Tedja et al., 2018; Verhoeven et al., 2013). They encode proteins of vastly different functions, including **eye morphogenesis, extracellular matrix organization, visual perception, circadian rhythms, pigmentation and retinal signaling** (Flitcroft et al., 2018; Hysi et al., 2020; Tedja et al., 2018). The contribution of heritability to myopia is difficult to determine (Dirani et al., 2006; Hammond et al., 2001; Lopes et al., 2009; Lyhne et al., 2001; Peet et al., 2007; Tedja et al., 2019; Tedja et al., 2018; Teikari et al., 1988; Wojciechowski, 2011) and estimated to be rather low (Morgan and Rose, 2005), since it was shown that environmental factors play a very important role in the occurrence of myopia, especially illumination conditions. This is underpinned by the increasing prevalence of myopia taking place in East and Southeast Asia, where children spend more time inside studying as compared to less developed countries (Morgan et al., 2018).
Indeed, the intensity of ambient light was found to influence the development of myopia.

Outdoor activities have a protective effect against the development and progression of myopia (He et al., 2015; Rose et al., 2008; Wu et al., 2013; Xiong et al., 2017). Similarly for animal models raised under different lighting conditions, high illuminances have been found to slow the development of induced myopia (Smith et al., 2012). Interestingly, intermittent exposure to bright light was more efficient in suppressing myopia progression than continuous light exposure (Lan et al., 2014).

The identification of differentially expressed genes (DEG) in animals with induced myopia has been helpful in suggesting candidate genes associated with the development of human myopia (Brand et al., 2007; Frost and Norton, 2012; Gao et al., 2011; McGlinn et al., 2007; Riddell et al., 2016; Schipper et al., 2008; Shelton et al., 2008; Siegwart and Norton, 2002; Tkatchenko et al., 2016; Tkatchenko et al., 2006).

High myopia can also be syndromic. Pathogenic variants affecting proteins expressed in the retina, either in photoreceptors or in the downstream bipolar cells (BCs) can lead to progressive and non-progressive retinal monogenetic disorders, which can be also associated with other ocular disorders including myopia. One such disease is X-linked retinitis pigmentosa (RP) (MIM #300029), one of the most frequent and severe progressive form of RP, with symptoms of night blindness that occur during the first decade of life and progress to legal blindness by the third to fourth decade in most affected males (Flaxel et al., 1999). In this disease, high myopia is often observed from childhood in addition to retinal degeneration and electrophysiological abnormalities (Flaxel et al., 1999; Koenekoop et al., 2003).

Mutations in RPGR (MIM #312610) account for the majority of X-linked RP, and these patients show high myopia in addition to RP (Sanchez Tocino et al., 2019). Similarly, mutations in ARR3 (e.g. (van Mazijk et al., 2022; Xiao et al., 2016) and OPN1LW, mainly expressed in cone-photoreceptors, were also associated with high myopia (reviewed by (Neitz
and Neitz, 2021). Other syndromic and non-syndromic eye disorders associated with high myopia include Marfan syndrome (MIM #154700) (Pyeritz and McKusick, 1979), with mutations in FBN1 (MIM #134797), Knobloch (MIM #267750) (summarized by (Aldahmesh et al., 2011), with mutations in COL18A1 (MIM #120328), Wagner (MIM #143200), with mutations in VCAN (MIM #118661), Stickler syndrome (MIM #108300), with mutations in COL2A1 (MIM #120140), Ehlers-Danlos syndrome (MIM#130000), with mutations in COL5A1 (MIM# 120215) and Donnai-Barrow syndrome (MIM #222448) (Ohlsson, 1963), with mutations in LRP2 (MIM #600073) (Kantarci et al., 2007). Based on these findings, it appears that disruption of the retinal signaling pathway from photoreceptors to the inner retina can lead to myopia. Interestingly, common genetic variants within or nearby genes that are associated with syndromic myopia are enriched for variants that cause non-syndromic, common myopia (Flitcroft et al., 2018). For most of the implicated genes, expression in the retina is documented. Reinforcing these findings, degeneration of photoreceptors and/or amacrine cells (ACs) in chicken retinas, has been associated with increased axial growth of the eye, leading to myopia (Ehrlich et al., 1990; Fischer et al., 1999). E.g. this was shown using colchicine to destroy amacrine cells, which normally suppress axial growth of the eye (Fischer et al., 1999).

Studies in chicken and mice showed that light dependent ON-bipolar cell (BC) dopamine signaling is a major player in the development and protection of myopia (Boatright et al., 1994; Boelen et al., 1998; Dumitrescu et al., 2009; Iuvone et al., 1978; Schaeffel and Feldkaemper, 2015; Stone et al., 2011). Retinal dopamine content is decreased in the absence of light and during myopia development (Schaeffel and Feldkaemper, 2015). It was shown, that intravitreal or topical application of levodopa, which is widely used in the treatment of neurological disorders involving dysregulation of the dopaminergic system, inhibits the
development of experimental myopia in chickens (Thomson et al., 2019). In addition, it has been demonstrated that non-myopic children treated for attention deficit hyperactivity disorder (ADHD) with dopaminergic agents have slower developmental myopic refraction than untreated peers, providing a more direct suggestion that dopamine plays a role in human refractive development (Gurlevik et al., 2021).

Patients with **complete congenital stationary night blindness (cCSNB)**, which represents a complete ON-BC dysfunction, with unaltered OFF-BC function present as well with high myopia (median error of -7.4 D) (Bijveld et al., 2013; Zeitz et al., 2015). Multiple mutations in cCSNB-associated genes such as *NYX, GRM6, GPR179, TRPM1* and *LRIT3* lead to this condition in patients and mouse models (Audo et al., 2012; Bech-Hansen et al., 2000; Dryja et al., 2005; Gregg et al., 2003; Koike et al., 2010; Li et al., 2009; Maddox et al., 2008; Masu et al., 1995; Morgans et al., 2009; Neuille et al., 2014; Orhan et al., 2021; Orlandi et al., 2012; Pardue and Peachey, 2014; Peachey et al., 2012; Pusch et al., 2000; Qian et al., 2015; van Genderen et al., 2009; Varin, 2020; Zeitz et al., 2013; Zeitz et al., 2015; Zeitz et al., 2005).

CNSNB mouse models lacking *Nyx (nob)* and *Grm6* with ON-BC dysfunction, showed **increased susceptibility to myopia and decreased dopamine content** (Chakraborty et al., 2015; Pardue et al., 2008). While human subjects with cCSNB and high myopia have no **ON-BC transmission**, their OFF-BC transmission is largely intact (Miyake et al., 1986; Zeitz et al., 2015). By contrast, subjects with incomplete CSNB (iCSNB) show variable degrees of myopia (median refractive error of −4.8 D), associated with both reduced ON-and OFF-pathway dysfunction (Bijveld et al., 2013; Miyake et al., 1986; Zeitz et al., 2015). We are not aware that high myopia has been reported with other forms of CSNB, leading to a rod dysfunction and a Riggs-ERG (Zeitz et al., 2015). Aleman et al showed that stimulation of the ON-pathway may thicken the choroid and thus perhaps inhibit myopia in humans (Aleman et
Together, these findings suggest that **ON-pathway disruption** contributes to the development of myopia and that studies on cCSNB can be used to better understand myopia.

In recent years, gene identification studies in CSNB patients led to the identification of key proteins mediating photoreceptor to ON-BC signal transmission. Because of the prevalence of myopia in CSNB patients, these ON-pathway genes are also candidate genes for myopia. Seeking new myopia genes through this purely genetic approach has its limitations since CSNB is a rare retinal condition (Zeitz et al., 2015); for example our data suggest that the prevalence of CSNB in France is ~1:400,000 (Varin, 2020), though it is likely to be under-diagnosed since correct diagnosis requires the application of a specific clinical examination (e.g. electroretinography) and/or molecular genetic testing. Given these limitations, we followed an alternative approach using whole transcriptome sequencing to seek novel CSNB-associated or CSNB-myopia related genes. This allowed us to **add novel proteins** to the retinal signaling pathway, identify new candidate genes for myopia, and **strengthen the evidence for several previously proposed myopia-associated** genes. These results provide a framework for the development of future therapeutic strategies to treat myopia.

### 2. Phenotype in human cases and animal models with complete congenital stationary night blindness (cCSNB) and associated myopia

#### 2.1. Signal transmission from photoreceptors to ON-bipolar cells

The clinical characterization of subjects with CSNB, and more specifically **cCSNB**, was recently reviewed (Zeitz et al., 2015). **Electroretinography** is crucial for a correct positive diagnosis of CSNB and distinguish its different forms, both in human subjects and animal models (Zeitz et al., 2015). A clear diagnostic electroretinogram (ERG) feature in cCSNB is
In light-adapted conditions (LA 3.0 30 Hz and LA 3.0), ERGs are altered differently (see for details (Zeitz et al., 2015)) (Figure 1). ERG findings under scotopic conditions highlight similarities between cCSNB animal models and the human phenotype. In contrast, under photopic conditions, cCSNB animal models show more variability between species (Figure 1). The photopic ERG phenotype of cCSNB mouse models is generally more severe, including the absence of the b-wave, compared to that of human cases or larger animal models (Figure 1) (e.g. (Neuille et al., 2014; Orhan et al., 2021)). In contrast, cone-driven responses are comparable between CSNB dogs, horses and human cases, i.e. mildly reduced and delayed (reviewed in (Kondo et al., 2015; Varin et al., 2021b; Witzel et al., 1978; Zeitz et al., 2015) (Figure 1). These differences may be a result of species-specific physiological differences, but could also be due to the use of different ERG protocols (Varin et al., 2021b).

ERG features associated with myopia in human subjects and animal models are less well studied (Koh et al., 2014; Westall et al., 2001). Some studies in myopic chicken suggested that the oscillatory potentials are altered (Fujikado et al., 1997). By performing multifocal ERG measurements Ho et al., showed that ERG responses originating from the human paracentral retina negative defocus are reduced compared to ERG responses originating from the central retina (Ho et al., 2012). This paracentral region of the retina is believed to predominately reflect the activity from ON- and OFF-BCs, with relatively small contributions from photoreceptors and other inner retina cells (Chu et al., 2008). Because genetic data are often unavailable for myopia subjects, it remains uncertain if myopia cases that present specific ERG-alterations represent syndromic myopia cases, as found e.g. in cCSNB. For those genes that have been implicated in myopia, they may also have roles in ON-pathway signaling. For example, a mouse model of myopia lacking Apllp2, coding for the amyloid beta precursor like protein 2, which is highly expressed in bipolar cells, revealed a reduction in the
amplitude of the ERG b-wave and oscillatory potentials (Tkatchenko et al., 2015). A genome-wide-association study (GWAS) revealed an association between APLP2 variants and myopia in humans; thus, the ERG defects in the Aplp2−/− mouse were thought to be linked to myopia (Tkatchenko et al., 2015) (Figure 2). However, the electrophysiological properties of the retina of the Aplp2−/− mouse model are also consistent with a role of APLP2 in synaptic transmission. It would be interesting to document ERGs of myopia cases linked to APLP2.

2.2. Visual acuity in cCSNB and myopia

Subjects with cCSNB have decreased visual acuity with a median of 0.30 log MAR (Minimum Angle of Resolution; about 20/40) (Zeitz et al., 2015). Relatively preserved mean best corrected visual acuity of 0.02 log MAR (about 20/20; (Ye et al., 2019)) and 0.88 decimals (better than 20/25; (Liu et al., 2010)) is observed in myopic (including high myopic) subjects without underlying retinal changes. Pathologic myopia, however, is associated with a reduced mean best corrected visual acuity of 0.56 ± 0.35 decimals (about 20/35; (Liu et al., 2010)) and 0.19 ± 0.20 log MAR (about 20/30; (Ye et al., 2019)). About 30% of cases with myopic retinal changes have visual acuity worse than 20/60. Hence, although cCSNB subjects demonstrate high axial myopia (Al-Hujaili et al., 2019) their visual acuity loss is generally more severe, due to cone ON-bipolar cell dysfunction, than in subjects with isolated myopia.

Visual acuity in mice can be measured using an optomotor test (Jellali et al., 2005). All tested mouse models for cCSNB reveal severely reduced visual acuity under scotopic conditions (Neuille et al., 2014; Orhan et al., 2021; Pinto et al., 2007). Visual acuity under photopic conditions may be also altered (Neuille et al., 2014; Pinto et al., 2007; Varin et al., 2021b). A few studies have measured visual acuity in myopia mouse models; For example, in Aplp2
knock-outs visual acuity was normal in form-deprivation myopia, but contrast vision was reduced and correlated with myopia (Tkatchenko et al., 2015). Similarly, patients with myopia exhibited reduced sensitivity to contrast in comparison to emmetropes and contrast sensitivity decreased with an increasing degree of myopia (Stoimenova, 2007).

2.3. Retinal structure in cCSNB and myopia

In contrast to progressive rod-cone dystrophy, the retinal structure is quite well preserved in human subjects with cCSNB. This is due to the stationary, functional, but not degenerative nature of disease of cCSNB. A few reports describe thinning of inner retinal layers (INLs) in cases with cCSNB as measured by spectral domain Optical Coherence Tomography (SD-OCT). However, this thinning is different from that observed in subjects with progressive rod-cone dystrophy (Al Oreany et al., 2016; Godara et al., 2012; Ivanova et al., 2019). SD-OCT measurements done in the Lrit3 mouse model of cCSNB also revealed thinning of the INL (Neuille et al., 2014). It is not clear if the INL thinning observed in human cCSNB subjects and in a few mouse models is related to the development of high myopia or if it is only caused by the mutated gene causing cCSNB. A systematic analysis of cCSNB cases compared to myopia control cohorts is needed to determine the exact cause of this thinning (Zeitz et al., 2015).

In subjects with myopia without outer retinal changes, regional changes in central retinal thickness have been described by SD-OCT; these include thinning of the INL and outer nuclear layer, and thickening of OPL and photoreceptor outer segments (Liu et al., 2015; Ye et al., 2019). In subjects with pathologic myopia, additional changes described under the term of myopic maculopathy are observed (Ohno-Matsui et al., 2016).
3. Studying myopia in mouse models

Animal models have been increasingly used to investigate mechanisms of myopia. Myopia can be induced using negative lenses or form deprivation in a variety of species as diverse as monkeys (Qiao-Grider et al., 2007; Weinstein and Grether, 1940), tree shrews (Norton and McBrien, 1992; Petry et al., 1984), cats (Berkley and Watkins, 1973; Blake et al., 1974), guinea pigs (Buttery et al., 1991; Howlett and McFadden, 2007), chickens (Irving et al., 1996; Jarvis et al., 2009; Schmid and Wildsoet, 1998), and fish (Shen and Sivak, 2007). However, most these models are not well suited for genetic research.

The mouse, has become a popular animal model for the myopia research (Barathi et al., 2014; Barathi et al., 2013; Beleggia et al., 2015; Cases et al., 2015; Hudson et al., 2015; Ma et al., 2014; Miyake et al., 2015; Pardue et al., 2008; Song et al., 2016; Storm et al., 2014; Tekin et al., 2013; Tkatchenko et al., 2012; Tkatchenko et al., 2015; Tran-Viet et al., 2013; Wu et al., 2015a; Wu et al., 2015b). The mouse offers a number of important advantages compared to other species, including well-established techniques allowing the alteration of its genome. Moreover, both genes and environment can be manipulated in the same mouse (Barathi et al., 2008; Schaeffel et al., 2004; Tejedor and de la Villa, 2003a; Tkatchenko et al., 2010).

Behavioral studies suggest that vision is critical for accurate spatial navigation in mice (Buhot et al., 2001; Cook et al., 2001; de la Cera et al., 2006; Faulstich et al., 2004). Although mice are classified as nocturnal animals they are also active during the day and have diurnal retinal features (Flores et al., 2007; Jacobs, 2013; Yang et al., 2009). Mice undergo emmetropization driven by photopic visual stimulation similar to humans (Tkatchenko et al., 2013). Basic spatial and temporal vision characteristics in mice are similar to those in humans (Histed et al., 2012; Umino et al., 2008) but with lower visual function compared to humans (Prusky and Douglas, 2004). Due to the above-mentioned differences, thresholds for rod-dominated
(scotopic) and cone-dominated (photopic) vision in mice, as well as the structure of the retinal
scotopic and photopic pathways, maybe different but still show high similarities to those in
humans (Leamey et al., 2008; Umino et al., 2008). Although mice lack the fovea found in
primates, the mouse retina has an area of increased density of photoreceptors and retinal
ganglion cells (RGC) just temporal to the optic disc, which represents an anatomical and
functional analog of the area centralis found in all non-primate mammals (Drager and Olsen,
1981; Jeon et al., 1998; Leamey et al., 2008). Induced changes in genetic background and
gene-targeted mutations affect refractive eye development and susceptibility to myopia in
mice (Schippert et al., 2007; Tkatchenko et al., 2015; Tkatchenko et al., 2019; Troilo et al.,
2019; Wisard et al., 2011; Zhou et al., 2001; Zhou et al., 2010). Experimental myopia can be
induced in mice using visual form deprivation (form deprivation myopia, FDM) or
negative spectacle lenses (lens-induced myopia, LIM), similar to other animal models
(Chakraborty et al., 2014a; Pardue et al., 2008; Pardue et al., 2013; Park et al., 2013;
Schaeffel et al., 2004; Tejedor and de la Villa, 2003b; Tkatchenko et al., 2015) (Figure 3).
Susceptibility to FDM was increased in mouse models with a defect in the ON-pathway
(Chakraborty et al., 2015; Pardue et al., 2008), highlighting that ON-pathway disruption may
be an important element in refractive error development. More specifically, cCSNB-
associated myopia as shown by myopic shifts in eyes with FDM was observed in mice
lacking Nyx (nob) (Gregg et al., 2003) and Grm6 (Masu et al., 1995) (Chakraborty et al.,
2015; Pardue et al., 2008). We hypothesize that the cCSNB models used in the present study
will similarly show a greater susceptibility to FDM- or LIM-induced myopia, although the
genetic background of the mice must be considered, as this can strongly influence both the
baseline and susceptibility to induced myopia. For example, wild-type mice in a “129
background” show high baseline myopia (~ -20D) compared to those in a C57BL/6J
background (Tkatchenko et al., 2019). Our cCSNB models lacking Gpr179 and Lrit3 are in a
C57BL/6J background; in contrast, our cCSNB mouse model lacking Grm6 is in a different genetic background (129S6.129S(Cg)) than the one used by Chakraborty et al., 2015 (Chakraborty et al., 2015). Thus, we estimate that the difference of the refractive error following FDM or LIM may not be measurable in our mice lacking Grm6. Recently we were indeed able to show that our cCSNB model lacking Gpr179 is more susceptible to LIM (Wilmet et al., 2022 under review).

All cCSNB mouse models share the same gene defects as human subjects with cCSNB (Audo et al., 2012; Bech-Hansen et al., 2000; Dryja et al., 2005; Gregg et al., 2003; Pardue et al., 1998; Peachey et al., 2012; Pusch et al., 2000; Zeitz et al., 2013; Zeitz et al., 2015; Zeitz et al., 2005). All cCNSB genetic defects result in a defective ON-BC transmission. In contrast, disruption of mainly the OFF-pathway caused by a mutation in the Vsxl gene did not significantly alter refractive eye development or the susceptibility to experimentally induced FDM (Chakraborty et al., 2014b). These findings lend further support to the selective contribution of the ON-pathway to myopia at least in animal models. Recently it was shown that Vsxl is expressed in both cone ON- and OFF-BCs, but not in rod ON-BCs (Shekhar et al., 2016). We are not aware of studies showing myopia in mice with iCSNB, which affects both ON- and OFF-BCs. However, in a recent study, patients with a variant near GJD2 had high myopia, supporting a potential role for altered signaling in cone-driven OFF pathways in myopia development. Therefore, it is possible that multiple retinal pathways contribute to myopia (Jiang et al., 2022).

Numerous retinal signaling pathways have been shown to be involved in refractive eye development and myopia in animal models (Summers et al., 2021; Tkatchenko and Tkatchenko, 2019; Troilo et al., 2019). Dopamine signaling plays an important role in these
processes and dopamine (DA) and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) have been implicated in refractive error development in mice (Schaeffel and Feldkaemper, 2015). Stimulation of DA biosynthesis in light is due to the activation of tyrosine hydroxylase (TH) (Iuvone et al., 1978). Dopaminergic ACs receive excitatory glutamatergic input from ON-BCs (Boatright et al., 1994; Boelen et al., 1998; Dumitrescu et al., 2009; Stone et al., 2011) and transmit the signal further to RGCs in the retina. Retinal DA and DOPAC content drop during the development of FDM and remain at reduced levels (Schaeffel and Feldkaemper, 2015). Further support for the role of DA in myopia development comes from the cCSNB mouse models lacking Nyx (nob) and Grm6, as they are characterized by not only an increased susceptibility to FDM but also by lower levels of DA and DOPAC (nob mice) and DOPAC (Grm6<sup>−/−</sup>) compared to wild-type animals (Chakraborty et al., 2015; Pardue et al., 2008). Compared to measuring the refractive error in animal models with FDM or LIM, measuring DA and DOPAC by ultra-performance liquid chromatography is less time consuming, since the induction of experimental myopia is not needed. Indeed, recent findings revealed that levels of DOPAC are reduced in genetic retinal disease mouse models and are not affected by myopia induction (Chakraborty et al., 2015; Park et al., 2013). As shown in Figure 4, we detected lower levels of DA and DOPAC after light adaptation in mice lacking Gpr179, Lrit3 and Grm6 compared to wild-type animals, further demonstrating that these cCSNB mouse models provide a valid basis to study myopia.

4. Differentially expressed genes in cCSNB

4.1. RNA-Seq studies in mouse models for cCSNB

Several studies have identified novel myopia-associated candidate genes by whole transcriptome sequencing in several animal models of myopia (Riddell and Crewther, 2017;
Here, we performed whole transcriptome sequencing (RNA-Seq) in three different adult mouse models of cCSNB (Gpr179−, Lrit3− and Grm6−) and their age-matched littermates (each, n = 5) to investigate the molecular basis of myopia in cCSNB and to identify novel proteins involved in the retinal signaling cascade, including the ON-pathway. After identification and validation of differentially expressed genes (DEGs), a meta-analysis was performed by comparing our data with: i) published transcriptomic data from purified retinal cells and single cell RNA-Seq data (Macosko et al., 2015; Shekhar et al., 2016; Siegert et al., 2012; Siegert et al., 2009; Woods et al., 2018) and ii) pathway analyses and myopia databases and/or publications concerning the role of identified DEGs in normal vision. Figure 5 shows DEGs with fold changes (FC) of at least 1.2, P-values of ≤ 0.01 and an expression value of at least 5 transcripts per million reads (TPM) from RNA-Seq data of adult Gpr179−, Lrit3− and Grm6− mice compared to data from age-matched wild-type littermates (Supplementary Table S1-S4). As expected, all three mouse models exhibited significantly lower expression of their respective “knocked out” genes (Gpr179, Lrit3, Grm6) compared to the corresponding wild-type strains (Supplementary Tables S1-S4). Overall, 155, 133, and 222 genes were differentially expressed in the Gpr179−, Lrit3− and Grm6− mouse strains, respectively (Supplementary Tables S1-S4 and Figure 5a). Differences in numbers and types of DEGs between the strains may be due in part to differences in genetic background, which may also influence differential regulation of the gene network (Tkatchenko et al., 2019). While cCSNB mouse models lacking Gpr179 and Lrit3 are on a C57BL/6J background, mice lacking Grm6 are on a 129S6.129S(Cg) background. However, to minimize background related issues, for each knock-out strain, the respective littermate wild-type strain was used for comparison. DEGs, appearing in all three cCSNB mouse strains may be implicated in a common physiological
role, while DEGs appearing in only one cCSNB mouse strain may have a unique role important for the specific gene missing in the model. Here, we focused mainly on DEGs shared by at least two of the three mutant strains.

4.2. Validation of RNA-Seq studies

To identify genes playing a role in the cCSNB phenotype, DEGs with at least 1.2 FCs and P-value ≤ 0.01 were investigated, including their expression in specific retinal cell-types and their association with the term “myopia”. Overall, there were 52 DEGs, that appeared in at least two cCSNB mouse strains (Supplementary Table S5 and S6) and 7 in all three (Supplementary Table S6, bold, Figure 5A and B). The Gpr179+/− and Lrit3−/− mouse strains shared the majority of the DEGs (38), followed by Gpr179+/− and Grm6−/− (16) and Grm6−/− and Lrit3−/− mouse strains (12) (Figure 5A). The 52 DEGs present in at least two mouse strains could be sorted into 10 clusters (Figure 5B). The largest cluster consisted of genes down-regulated in the cCSNB models (cluster 1, Figure 5B). Quantitative RT-PCR (RT-qPCR) performed for 24 of those genes confirmed that all 24 were differentially expressed in the retina of knock-out mouse strains compared to the corresponding wild-type mice (Figure 6 shows some of the validated genes, Supplementary data). Of note, some genes were found to be differentially expressed only in mice lacking either Lrit3 or Gpr179 genes by RNA-Seq, but were found to be significantly differentially expressed in all three knock-outs by RT-qPCR (e.g. Prkca and Tphg, Figure 6). Although not all differences observed with RNA-Seq were validated by RT-qPCR, which may be explained by the lesser sensitivity of RT-qPCR experiments (Costa et al., 2013), the trend was the same for the majority of the 24 DEGs, thus validating the transcriptome sequencing approach.

4.3. Known genes implicated in CSNB
Mutations in NYX, GRM6, GPR179, TRPM1 and LRIT3 that cause cCSNB affect protein localization in retinal ON-BCs (Audo et al., 2012; Bech-Hansen et al., 2000; Dryja et al., 2005; Li et al., 2009; Peachey et al., 2012; Pusch et al., 2000; van Genderen et al., 2009; Varin, 2020; Zeitz et al., 2013; Zeitz et al., 2005). The proteins localize in the outer plexiform layer (OPL) and in most cases in the dendritic tips of ON-BCs (Zeitz et al., 2015). Similar to human subjects, ON-BC function is affected in cCSNB mouse models (Gregg et al., 2003; Koike et al., 2010; Masu et al., 1995; Morgans et al., 2009; Neuille et al., 2014; Peachey et al., 2012; Zeitz et al., 2015). Proteins involved in cCSNB often show an interdependency in their localization, and may or may not show correlated changes in expression levels (Neuille et al., 2015). For example, nyctalopin (encoded by Nyx) and TRPM1 interact. Localization of TRPM1 at the ON-BC dendritic tips is dependent on the presence of nyctalopin, mGluR6 and LRIT3 (Cao et al., 2011; Hasan et al., 2020; Neuille et al., 2015; Pearring et al., 2011; Zeitz et al., 2015). Using RNA-Seq, we showed that Trpm1 mRNA levels are dependent on the expression of Gpr179, Lrit3, and Grm6, and that expression levels of Grm6 are dependent on the expression of Lrit3 (Figure 7). However, although Trpm1 expression levels seems to be dependent on Gpr179 expression, the localization of TRPM1 at the dendritic tips of BCs does not appear to be affected in Gpr179−/− mice (Orhan et al., 2021; Orlandi et al., 2012). Aside from the targeted gene in each knock-out strain, most of the other known genes underlying cCSNB, are not strongly differentially expressed (Supplementary Tables S1 and S6), suggesting that the 52 DEGs in the cCSNB mouse models are not necessarily candidate genes for cCSNB.

4.4. Transcriptome analyses of DEGs in cCSNB
All 52 DEGs that we identified are expressed in the human retina (Supplementary Table S6, Figure 8) (Farkas et al., 2013). Most of them were already reported in retina transcriptome databases (Blond and Leveillard, 2019; Farkas et al., 2013; Kalathur et al., 2008; Macosko et al., 2015; Shekhar et al., 2016; Siegert et al., 2012; Siegert et al., 2009). Here, we mainly focus either on genes expressed in ON-BCs or in downstream ACs and RGCs. Twenty genes (530(5730419F03Rik, Cxcr7, Ampd3, Arc, Bdnf, Cacna1s, Car8, Lect1, Edn2, Eomes, Gabrr1, Ldb2, Lrp2bp, Olfm3, Plekhf1, Rgs16, Slco5a1, Syn2, Tgbf2 and Tpbg) showed an age-dependent relative increase in expression in the rd1-transcriptome database, as a result of the age-dependent photoreceptor degeneration (Blond and Leveillard, 2019; Kalathur et al., 2008). These genes are expressed in the INL or ganglion cell layer (GCL) and they seem to be important for BC, AC and RGC function and maintenance (Supplementary Table S6). In addition, a number of novel candidate genes absent in the current transcriptome databases were also identified. Several of these genes were found in rod (Col5a1 and Vgll4), cone (Cxcr7, Car8, Casp7, Gabrr1, Ptprr, Spred3, Tac2 and Tpbg), rod-cone (Lect1, Gng4, Tgbf2 and Trpc1), BC (Cxcr7, Bdnf, Cacna1s, Car8, Casp7, Cenjl, Cdc25c, Lect1, Col5a1, Dusp4, Gabrr1, Gng4, Grm6, Ldb2, Lrp2bp, Plekhf1, Prkca, Ptprr, Rgs16, Slco5a1, Spred3, St6galnac5, Tac2, Tpbg, Tgbf2), AC (Arc, Cxcr7, Car8, Lect1, Fbxo32, Gabrr1, Gng4, Ldb2, Olfm3, Plekhf1, Ptprr, Tac2, Tgbf2, Tpbg and Vgll4) and RGC (Cxcr7, Car8, Lect1, Eomes, Gabrr1, Gng4, Plekhf1, Ptprr, Tgbf2, Trpc1, Tpbg and Vgll4) databases (Figure 8).

Interestingly, 530(5730419F03Rik, Ampd3, Car8, Casp7, Cenjl, Lect1, Dusp4, Gabrr1, Gm20754, Lrp2bp, Prkca, Ptprr, Rgs16, Slco5a1 and Tpbg were also shown to be differentially expressed in mice lacking the BC-specific basic helix-loop-helix (bHLH) transcription factor, Bhlhe23, alias Bhlhb4 (Bramblett et al., 2004; Woods et al., 2018). This transcription factor was found to be expressed in rod bipolar cells (RB). ERGs in the adult Bhlhb4 knockout mice, in a C57Bl6 strain, showed that the loss of BHLBH4 resulted in
disrupted rod signaling and profound retinal dysfunction resembling CSNB, characterized by
the loss of the scotopic ERG b-wave. A depletion of INL cells in the adult Bhlhb4 knockout
has been ascribed to the abolishment of the RB cell population during postnatal development.
Other retinal cell populations including photoreceptors were unaltered. In the absence of
information from these databases, our data was completed using expression data available
from the literature and single cell RNA-Seq analyses (https://www.proteinatlas.org)
(Supplementary Table S6, Figure 8).

Strikingly, Lect1 (Cnmd) was up-regulated in Gpr179−/− and Lrit3−/− mice and slightly down-
regulated in Grm6−/− mice compared to wild-type mice. These findings were validated by RT-
qPCR (Figure 5B, cluster 2, Figure 6). In contrast, Etv4 was down-regulated in Gpr179−/− and
Lrit3−/− mice and up-regulated in Grm6−/− mice compared to wild-type animals (Figure 5B,
cluster 9).

LECT1 (MIM# 605147), coding for the leukocyte cell-derived chemotaxin 1 protein (also
called chondromodulin 1) is an angiogenesis inhibitor first isolated from fetal bovine
cartilage. Although confined to cartilage and the eye at later stages of development, fetal
whole-mount in situ hybridization revealed expression of Lect1 mRNA in somites, heart,
bronchial arches, roof plate, retina and limb buds. LECT1 expression pattern suggested a role
in morphogenesis during embryonic development (Shukunami et al., 1999). In adult retina,
LECT1 was found in the GCL and INL as revealed by RNA in situ hybridization and
immunohistochemistry studies (Funaki et al., 2001). A knock-out mouse model showed no
overt abnormality during embryogenesis (Brandau et al., 2002; Nakamichi et al., 2003).
Analysis of expression databases revealed that LECT1 is expressed in the inner retina,
photoreceptors, BCs, ACs and RGCs and that its expression appears to be reduced in 
*Bhlhe23*/*−−* mice (Woods et al., 2018).

### 4.4.1. Single cell analyses of DEGs in subpopulation of bipolar cells

To investigate in which BCs (rod BCs, cone ON- or OFF-BCs) the more abundant DEGs 
were expressed, our data was compared to a single-cell BC-specific transcriptome database 
(Shekhar et al., 2016). We found that most genes were enriched in rod BCs and cone ON-
BCs, but some were also expressed in OFF-BCs (BC1-4 = OFF-BCs, BC5-BC7 = ON-BCs 
(Figure 9). These data are further explored below for genes where additional functional 
information has been described previously.

### 4.5. Pathway analyses of DEGs involved in CSNB and correlation with 
myopia

In rare cases, subjects with mutations in *NYX* (Yip et al., 2013; Zhang et al., 2007; Zhou et al., 
2015), *TRPM1* (Zhou et al., 2016) and *GRM6* (Jin et al., 2017; Xu et al., 2009) present with 
early-onset high myopia despite the absence of documented cCSNB diagnosis. However, it is 
believed that most mutations in *NYX, LRIT3, TRPM1, GRM6* and *GPR179* are not directly 
responsible for high myopia. Instead, it is hypothesized that myopia in cCSNB is caused by a 
disruption of the ON-pathway (Chakraborty et al., 2015). Our analysis revealed that mitogen-
activated protein kinase (MAPK) pathways, synaptic signaling, G protein-coupled 
receptor ligand binding pathways, and proteins implicated in eye, endoderm and 
connective tissue development were affected in cCSNB (Figure 10A). Some of the genes 
appeared in multiple pathways (Figure 10B). In the following paragraphs we detail these 
genes and pathways and describe correlations between cCSNB and associated ocular 
alterations, focusing on myopia. When genes appeared in multiple pathways, they are 
described only once. In addition, we focus on genes with additional data available in the 
literature. These genes are highlighted in bold. A detailed description of all DEGs, their
expression pattern in different cell types of the retina, their roles and putative involvement in
myopia development can be found in the supplementary data and supplementary Tables S6
and S7).

4.5.1. MAPK pathway

To our knowledge, the MAPK signaling pathway has not previously been implicated in
cCSNB. In the normal retina, phosphorylated MAPKs are barely detectable. However,
subjects and mice lacking functional retinoschisin (RS1) exhibit decreased ERG b-wave
amplitudes and increased MAPK activation (Gehrig et al., 2007; Plossl et al., 2017). In
addition, under certain pathological conditions, such as induced retinal ischemia, MAPKs are
activated by phosphorylation in several retinal layers, including in the INL with a concomitant
reduction of the b-wave amplitude (Zeitz et al., 2015). Interestingly, inhibitors of MAPK
phosphorylation significantly normalized the b-wave amplitude after ischemia (Roth et al.,
2003). Although MAPK signaling in the retina has been investigated, its role in cCSNB and
accompanying ocular manifestations are poorly understood. Fifteen DEGs associated with the
MAPK pathway were identified: Ackr3/Cxcr7, Bdnf, Cacna1s, Casp7, Ccnj1, Cdc25c,
Dusp4, Ldb2, Prkca, Ptprr, Spred3, Synj2, Tgfb2, Tpbg and Vgl14 (Figures 10 and 11).

LRIT3 (MIM# 615004) encoding leucine-rich repeat, immunoglobulin-like, and
transmembrane domain-containing protein 3 (LRIT3) is mutated in human subjects with
cCSNB (MIM# 615058) and mice (Neuille et al., 2014). Interestingly, LRIT3 is itself a
modulator of the fibroblast growth factor receptor 1 (FGFR1) (Kim et al., 2012), which also
plays an important role in the MAPK signaling pathway, though not identified as such in
pathway analyses. Phosphorylated FGFR1 regulates different signaling pathways including
MAPK.
**TGFB2** (MIM# 190220) encodes the transforming *growth factor* beta 2 (Supplementary Table S6 and S7, Figures 8-11). Mice lacking **Tgfb2** exhibit perinatal mortality and a wide range of developmental defects, including congenital heart defects, skeletal defects and *eye* defects (Sanford et al., 1997), which suggests that TGFB2 also plays a role in eye development, albeit with a different outcome dependent on the species tested. Studies in tree shrews revealed that expression of **Tgfb2** was reduced in animals with experimentally induced *myopia* (Jobling et al., 2004) but increased in the experimentally-induced myopia guinea pig model (Chen et al., 2013). Studies of *human aqueous humor* also found that levels of TGFB2 positively correlated with axial length in cases with *myopia* (Jia et al., 2014). In addition, a genetic variant in **TGFB2** (Lin et al., 2009) and the myopia Harmonize GAD database (Rouillard et al., 2016) linked **TGFB2** to myopia (Supplementary Tables S6 and S7). To our knowledge, a connection between down-regulation of **TGFB2** in inner retinal cells, including rod BCs and cone ON-BCs, and myopia has not been previously established.

**BDNF** (MIM# 113505) encoding brain-derived neurotrophic factor (B)DNF (Supplementary Table S6 and S7, Figures 8-11) serves as a survival, mitogenic, and differentiation factor in both the developing and adult central and peripheral nervous systems (reviewed in (Klocker et al., 2000)). The cellular effects of neurotrophins are mediated by the activation of Trk tyrosine kinase receptors and the p75 receptor. Upon binding to these receptors, neurotrophins are known to activate various intracellular signal transduction pathways, including the MAPK pathway (Figure 11), which affect cell survival and cell death mechanisms. BDNF is expressed in the inner retina (Herzog and von Bartheld, 1998) including all bipolar cells (Supplementary Table S6, Figures 8 and 9). Its neuroprotective and healing properties in these
cells are well known (Afarid et al., 2016). Several polymorphisms in BDNF have been associated with myopia (Musolf et al., 2017). In addition, BDNF appeared in the myopia Harmonize GAD database (Rouillard et al., 2016) (Supplementary Table S6 and S7, Figure 8).

ACKR3 (MIM# 610376), also known as CXCR7, and located on chromosome 2q37.3, encodes the atypical chemokine receptor 3. It is expressed in several regions of the CNS, in endothelial and various immune cells (Salazar et al., 2014), as well as in retina (Supplementary Table S6, Figures 8-11). ACKR3 is able to heterodimerize with CXCR4, which increases ligand stimulated membrane recruitment of β-arrestin, thus causing sustained activation of the ERK1/2 and p38 MAPK signaling pathways (Quinn et al., 2018) (Figure 11). Interestingly, ACKR3/CXCR7 was linked to myopia in a mapped region on chromosome 2q37 (Figure 8) (Schache et al., 2009). A few mutations in ACKR3 have been shown to cause oculomotor synkinesis, characterized by involuntary eye movements in mouse and humans (Whitman et al., 2019).

CACNA1S (MIM# 114208), coding for the L-type voltage-dependent calcium channel, alpha-1S subunit, is required for normal skeletal and cardiac muscle function (Elbaz et al., 1995). CACNA1S is homologous to other calcium channel alpha-1 subunits, such as CACNA1F (Cav1.4), which is mutated in subjects with incomplete CSNB (Bech-Hansen et al., 1998; Strom et al., 1998). In the retina, Cacna1s was found to be expressed in BCs and more recently in cones and rods (Supplementary Table S6, Figures 8-11). Initially, it was thought to localize in dendritic tips of ON-BCs, similar to other genes implicated in cCSNB (Specht et al., 2009; Tummala et al., 2014) but its exact protein localization in the retina remains
unclear, since commercially available CACNA1S antibodies have been shown to cross-react with GPR179 (Hasan et al., 2016), which is localized at the dendritic tips of ON-BCs. Calcium channels and their subunits in general have been linked to MAPK signaling (Ikeda, 2001) (Figure 11). L-type voltage-gated calcium channels in both retinal photoreceptors and bipolar neurons are under circadian control (Hull et al., 2006; Ko et al., 2009; Ko et al., 2007).

**PRKCA** (MIM# 176960) encodes protein kinase C, alpha (PKAlpha, PRKCA), which is a calcium-activated, phospholipid- and diacylglycerol (DAG)-dependent serine/threonine-protein kinase (Supplementary Table S6, Figures 8-11). It is involved in regulation of cell proliferation, apoptosis, differentiation, migration and adhesion, tumorigenesis, cardiac hypertrophy, angiogenesis, platelet function and inflammation, by directly phosphorylating targets or activating signaling cascades involving ERK1/2 (Figure 11). In the retina, PRKCA present at glutamatergic rod-BC synapses could act as a molecular switch to trigger intracellular signaling cascades in response to the activation of G-protein-coupled receptors (Woods et al., 2018). Its role in activating MAPK pathways or in cCSNB have not been studied. We found reduced expression of Prkca in cCSNB models by RNA-Seq and validated it by RT-qPCR (Figure 6). In addition, we showed a trend for reduced PRKCA protein levels in *Gpr179*<sup>−/−</sup> and *Lrit3*<sup>−/−</sup> mice (Figure 12). PRKCA expression at both transcript and protein levels in *Grm6*<sup>−/−</sup> mice was more variable (compare Figure 6 and Figures 12). A reduced PRKCA immunofluorescent labeling was also seen in *Gpr179*<sup>−/−</sup> and *Lrit3*<sup>−/−</sup> mice (Figure 13).

**PTPRR** (MIM: 602853) encoding protein-tyrosine phosphatase receptor type R, (PTPRR) was found to be expressed in many different retinal cells including in rod BCs and cone ON-
BCs (Supplementary Table S6 and S7, Figures 8-11). PTPRR has been implicated in MAPK deactivation in the brain (for review (Chirivi et al., 2007)). Mice lacking Ptprr showed increased phosphorylation of ERK1/2 in Purkinje cells and impaired motor coordination (Chirivi et al., 2007). In the retina, PTPRR was suggested to play an important role in neuronal development and maintenance of the mature retina (Horvat-Brocker et al., 2008).

Mutations in PTPRR could be involved in the development of myopia by a change in the enzyme’s ability to bind to, or dephosphorylate MAPK, leading to increased MAPK growth signals. Increased expression of PTPRR in rapidly growing fetal retina and choroid may be the result of normal developmental or growth regulation, suggesting that PTPRR plays a role in controlling ocular growth. Disruption of this protein’s function may account for the rapid eye growth seen in highly myopic individuals during early childhood, which slows in adulthood. As growth decreases, the need for this protein would be reduced (Hawthorne et al., 2013). In addition, PTPRR was identified by GWAS as linked to myopia (Supplementary Table S6 and S7).

TPBG (MIM# 190920), encoding trophoblast glycoprotein (Supplementary Table S6 and S7, Figures 8-11), was found to activate MAPK signaling in smooth muscle cells and adventitial pericyte-like cells (Spencer et al., 2019). TPBG is directly connected to ACKR3 (CXCR7; see above). Indeed, TPBG silencing downregulated phosphorylated ERK and ACKR3. In the retina, TPBG is highly expressed by rod-BCs, where it has been shown to exhibit PRKCA-dependent phosphorylation (Wakeham et al., 2020; Wakeham et al., 2019). TPBG phosphorylation was decreased in Prkca<sup>−/−</sup> compared to wild-type mice. Our studies revealed lower expression of Tpbg at the mRNA level, and a trend of lower protein levels in all cCSNB mouse strains (Figures 7 and 12). In addition, TPBG immunofluorescence was severely
reduced in the OPL and inner part of the IPL representing the dendritic tips and synaptic terminals of rod-BCs, in mice lacking Lrit3 and Gpr179 (Figure 13).

**VGLL4** (MIM# 6186962) encodes vestigial-like family member 4 (Supplementary Table S6 and Figures 8-11). Studies in zebrafish (Xue et al., 2018), xenopus (Barrionuevo et al., 2014; Faucheux et al., 2010) and mice suggested that VGLL4 takes part in early embryonic development, including eye development. Interestingly, this gene is a part of the Hippo signaling pathway, which has also been implicated in myopia (Zhu et al., 2018).

**DUSP4** (MIM# 602747 alias *MKP-2*), located on chromosome 8p12, codes for dual-specificity phosphatase 4 (Guan and Butch, 1995). It belongs to the DUSP protein family that inactivates their target kinases, namely MAPKs, by dephosphorylating both phosphoserine/threonine and phosphotyrosine residues. Expression of DUSP proteins is induced by various extracellular stimuli, and has diverse tissue distribution and subcellular localization (Dickinson and Keyse, 2006; Patterson et al., 2009). In melanocytes, **DUSP4** expression may be regulated by the microphthalmia-associated transcription factor (MITF), which is a melanocytic lineage-specific transcription factor with a role in the development of malignant melanoma. Similarly, MITF regulates **TRPM1** expression, which is implicated in both cCSNB and melanoma-associated retinopathy (MAR) (Hoek et al., 2008; Li et al., 2009; van Genderen et al., 2009; Varin et al., 2020; Xiong et al., 2013). Targeting the MAPK signaling pathway has transformed the treatment of metastatic melanoma. It has been recently shown that the genetic inactivation of DUSP4 reduced the proliferation of melanoma cells, thus making DUSP4 an interesting therapeutic target (Christodoulou et al., 2021). **DUSP4** is expressed in different tissues, including the eye, and more importantly in retinal BCs.
(Supplementary Table S6, Figures 8-11). In tissues other than the retina, DUSP4 was mainly localized to the nucleus and found to be induced by multiple agents, including serum, growth factors, UV-light, and oxidative stress. Depending on the tissue, it dephosphorylates MAPKs, such as ERK, JNK or p38 (Figure 11). In addition, DUSP4 was shown to play a role in innate and adaptive immunity, in cancer, and in brain function (Lawan et al., 2012; Seternes et al., 2019). Supporting our RNA-Seq findings, a significant downregulation of Dusp4 expression was found in Lrit3−/− and Gpr179−/− mice by RT-qPCR and a trend was noted in Grm6−/− mice (Supplementary Figure 1). In Trpm1−/− mice, significantly reduced expression of Dusp4 was also observed (Supplementary Figure 1). These findings were confirmed at the protein level, but with more variability (Figure 12). Western blot analyses of retinal extracts from wild-type mice revealed a band at 43 kDa, the calculated molecular weight of DUSP4. This band was absent in Dusp4−/− mice (data not shown). The role of DUSP4 in the retina is largely unknown. Transcriptional profiling of endogenous germ layer precursor cells identified Dusp4 as an essential gene in zebrafish endoderm specification (Brown et al., 2008). Although most of the vertebrate eye is derived from the ectoderm (Bassnett and Sikic, 2017), zebrafish Dusp4 morphants had small necrotic heads, suggesting an essential role of DUSP4 in anterior development (Brown et al., 2008). However, the role of DUSP4 in eye development was not further described. Interestingly, in well-established models of refractive error induction, Dusp4 appeared to be down-regulated in the eye upon inductions of both FDM and LIM in chicks and mice (McGlinn et al., 2007; Stone et al., 2011; Tkatchenko et al., 2016). Subjects with an 8p deletion, which includes the 8p12 DUSP4, often present with eye defects including nystagmus, strabismus and myopia, among other clinical syndromes (https://www.rarechromo.org/media/information/Chromosome%20%208/8p%20interstitial%20deletions%20including%208p12%20FTNP.pdf) (e.g. (Beighle et al., 1977; Chilcote et al., 1987; Devriendt et al., 1999; Klopocki et al., 2006)). Such defects are also found in cCSNB
cases. Together, these findings suggest that DUPS4 may be one of the genes that underlies cCSNB-related high myopia (Supplementary Table S6, Figure 8).

4.5.2. Synapse

Several signaling pathways involved in synaptic transmission were strongly associated with cCSNB (Figure 10A), including glutamatergic, serotonergic, cholinergic and GABAergic (gamma-aminobutyric acid) signaling. GABA is one of the major inhibitory neurotransmitters of the retina and central nervous system (Cheng et al., 2011). Twelve DEGs were associated with GABAergic signaling: Ackr3/Cxcr7, Cacna1s, Car8, Edn2, Gabrr1, Gng4, Grm6, Prkca, Ptprr, Rgs16, Tac2 and Trpc1 (Figure 10A). A detailed description of their expression in the different cell types of the retina, focusing on the inner retina, as well as their function and possible involvement in myopia can be found in the supplementary data (Supplementary Tables S6 and S7) and Figure 8).

GABRR1 (MIM# 137161), coding for the gamma-aminobutyric acid receptor, rho-1 (GABRR1) (alternatively named GABA-C Receptor, rho-1 subunit), is a member of a family of ligand-gated chloride channels that are the major inhibitory neurotransmitter receptors in the central nervous system (Cutting et al., 1991). It represents one of the subunits of the GABA-C receptor (rho1-rho3) (e.g. (Enz, 2001)) expressed in the inner retina in cone photoreceptors, bipolar cells and horizontal cells (Supplementary Table S6, Figures 8 and 9). In addition, GABRR1 was found to be differentially expressed in bipolar cell-deficient Bhlhe23−/− mice (Woods et al., 2018). Mice deficient for the Gabrr1 subunit lacked GABA-C receptor-mediated GABA-evoked responses, and signaling from rod BCs to third order cells was altered (McCall et al., 2002). The elimination of GABRR1 was demonstrated to result in
the absence of GABA-C receptors in the retina. While global retinal morphology was not
affected and the scotopic electroretinogram revealed a normal shape of the a-wave (rod-
function) and b-wave (mainly rod-BC function), the implicit time of the b-wave was shorter
and more oscillatory potentials (OPs) were found in mice lacking Gabrr1 (McCall et al.,
2002). It has been suggested that some of the OPs are generated by a negative feedback loop
in the IPL between the GABAergic ACs and the axon terminals of rod BCs (Kolb and Nelson,
1981), which may indicate that the altered ERG is due to a transmission defect between ACs
and rod BCs. Together, these findings confirm that GABRR1 has an important function in
normal visual processing. Several studies identified an association between GABRR1 and
myopia (Supplementary Table S6 and Figure 8). A genome-wide association study identified
GABRR1 as one of the genes associated with refractive error in Asian populations (Fan et al.,
2016; Lin et al., 2020). TPMPA ((1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid)), a
GABA-C antagonist has been shown to inhibit synaptic transmission in rat spinal cord (Rozzo
et al., 1999), modulate sleep-waking behavior in rats (Arnaud et al., 2001), enhance memory
in chicks (Gibbs and Johnston, 2005) and most importantly, inhibit myopia development in
chicks and guinea pigs (Chebib et al., 2009; Cheng et al., 2014; Stone et al., 2003), indicating
possible roles for GABA-C receptors in memory, circadian rhythms, and myopia (Cheng et
al., 2011). Although GABA-C receptors are pentameric structures formed from three possible
subunits, the rho1 subunit, encoded by Gabrr1, was found to be the primary target of GABA-
C antagonists (cis- and trans-(3-aminocyclopentanyl)butylphosphinic acid (cis- and trans-3-
ACPBPA) inhibiting myopia (Chebib et al., 2009). In addition to its expression in the retina,
Gabrr1 is also expressed in the fibrous and cartilaginous layers of the chick sclera, where it is
localized to the sclera fibroblasts and chondrocytes, which have been previously connected to
myopia (Cheng et al., 2011). Together, these findings are consistent with reduced levels of
**Gabrr1** expression in cCSNB (Figure 6), as a possible cause for the development of high myopia in cCSNB.

**CA8** (MIM# 114815) and its mouse orthologue Car8, encoding carbonic anhydrase VIII (CA8), has been found to be important for GABAergic synaptic transmission. Car8 is mainly expressed in the inner retina, with some expression in cone photoreceptors (Supplementary Table S6, Figures 8 and 9). In addition, it was found to be differentially expressed in *Bhlhe23<sup>−/−</sup>* mice (Woods et al., 2018). Car8 has been mostly studied in the brain, where it is highly expressed in cerebellar Purkinje neurons (Jiao et al., 2005; Kato, 1990) and other parts of the brain (Lakkis et al., 1997). Mice lacking functional Car8 (Jiao et al., 2005) show gait ataxia and dystonia with some ultrastructural differences in synapses between Purkinje cell dendritic spines, indicating a role for carbonic anhydrase VIII in synapse formation and/or maintenance. Similarly, subjects with mutations in CA8 exhibit ataxia and mild mental retardation (Turkmen et al., 2009). Recent studies in mouse retina showed CAR8 immunolabelling in rod BCs co-labelled for PRKCA, with particular bright staining in the axon terminal regions and weaker staining in the cytoplasmic compartments (Puthussery et al., 2011). In addition, some labelling was observed in a subset of cone BCs and GABAergic ACs (Puthussery et al., 2011). Mice lacking Car8 do not have a CSNB phenotype, as measured by electroretinography, but light-evoked current responses from AII-ACs were significantly larger and more prolonged than in control mice, suggesting that CAR8 is important for modulating retinal neurotransmission (Puthussery et al., 2011). Interestingly, genome-wide association studies found a potential association between CA8 and myopia (Kiefer et al., 2013) (Supplementary Table S6 and Figure 8). The reduced expression of Car8 in *Gpr179<sup>−/−</sup>* and *Lrit3<sup>−/−</sup>* mice, as identified by RNA-Seq studies, was confirmed by RT-qPCR and at the protein level (Figures 6 and 12).
**GRM6** (MIM# 604096) encodes metabotropic glutamate receptor 6 (GRM6 or mGluR6), which has been well described as causing cCSNB when mutated (Dryja et al., 2005; Zeitz et al., 2005) and which we used here as a mouse model (Masu et al., 1995) to identify DEGs in cCSNB. *Grm6* seems to be the only “cCSNB gene” with a significantly reduced expression in other cCSNB models (here *Lrit3*), indicating that the expression of *Lrit3* may influence *Grm6* transcript levels. *Grm6* is uniquely expressed in ON-BCs, where the ON-visual pathway originates (Supplementary Table S6) (Zeitz et al., 2015). As mentioned above, several studies have described mutations in *GRM6* (Jin et al., 2017; Xu et al., 2009) that have been associated with early-onset high myopia despite undocumented cCSNB (Supplementary Table S7, Figure 8).

**RGS16** (MIM# 602514) encodes regulator of G-protein signaling 16, which is expressed in the inner retina (Dhingra et al., 2008) (Supplementary Table S6 and Figures 8 and 9) and differentially expressed in *Bhlhe23*−/− mice (Woods et al., 2018). While other regulatory proteins such as GNB5 (GTP-binding protein beta 5), RGS9 and RGS11 are known to be implicated in ON-BC signal transduction, the role of RGS16 in this signaling cascade remains to be elucidated. Further, we are not aware of a link between *RGS16* and retinal disorders, including myopia. Genome-wide association studies revealed a variant in this gene associated with circadian rhythms in individuals claiming to be morning persons (Hu et al., 2016). Interestingly, *Rgs16*−/− mice were shown to have a longer circadian period (Doi et al., 2011).

**EDN2** (MIM# 131241) encodes endothelin2 (EDN2). *Edn2* is one of the few genes expressed at higher levels in cCSNB mouse strains compared to the wild-type mice (Supplementary Table...
S6, Figures 5 and 10A). It was found to be a potent vasoconstrictor that binds to two G protein-coupled receptors, i.e., EDNRA (which resides on bipolar dendrites) and EDNRB (which is present on Mueller and horizontal cells). Both receptors are also present on choroidal vessels (Prasanna et al., 2003). Interestingly, it has been shown that Edn2 was also up-regulated in the retina of several models of photoreceptor degeneration (Chen et al., 2004; Cottet et al., 2006; Rattner and Nathans, 2005; Van Schil et al., 2016), as well as following retinal damage, such as retinal detachment (Rattner and Nathans, 2005; Zacks et al., 2006). These observations suggest that increased expression of Edn2 may be a general response to retinal insult (reviewed in (Bramall et al., 2013)). EDN2 was linked to myopia by a recent genome-wide association study (Tedja et al., 2018), suggesting its increased expression as a potential cause of cCSNB-related myopia (Supplementary Table S6 and Figure 8).

**LRP2BP** (MIM# 619020) encodes low density lipoprotein receptor-related protein 2b (LRP2B). We are including it here among BC synaptic proteins because of its apparent retinal localization (Woods et al., 2018) (Supplementary Table S6 and Figure 8). LRP2BP is a scaffold protein, known to bind and recruit proteins to the megalin receptor (LRP2), which participates in endocytosis and signal transduction (Petersen et al., 2003). Interestingly, mutations in LRP2 cause the Donnai-Barrow/Facio-Oculo-Acoustico-Renal syndrome, which is partially characterized by high-grade myopia (Kantarci et al., 2007; Stora et al., 2009). Histological investigations of ocular mouse tissues also identified a severe myopia phenotype in megalin-deficient mice (Storm et al., 2014) and suggested that LRP2 is required for normal eye growth (Cases et al., 2015). Similarly, a zebrafish model for Lrp2 developed myopia (Veth et al., 2011). Even more importantly, **LRP2BP** is located on chromosome 4q35.1, a region deleted in cases with high myopia, among other disorders (Manolakos et al., 2013; Rossi et al., 2009) (Figure 8).
SLCO5A1 (MIM# 613543) encoding solute carrier organic anion transport family, member 5A1 (SLCO5A1), was also included in this section, because of a recent finding suggesting that this protein is an important component of retinal synapses and appears to be localized in the OPL (Woods et al., 2018). It is not clear if it is also located elsewhere in the retina (Supplementary Tables S6 and Figure 8). Subjects with the mesomelia-synostoses syndrome have many extraocular disorders but may also present with myopia. This syndrome is due to a non-recurrent microdeletion on chromosome 8q13 encompassing SULF1 and SLCO5A1 (Isidor et al., 2010) (Figure 8).

4.5.3. GPCR ligand binding

Signaling related to G protein-coupled receptor ligand binding was also overrepresented in our database. Twelve different genes were associated with G protein-coupled receptor ligand binding, i.e., Ackr3/Cxcr7, Bdnf, Edn2, Gng4, Grm6, Plekhf1, Prkca, Tac2, Tgfb2, Tpbg, Trpc1 and Umodl1 (Figure 10). A description of their expression patterns in different cell types of the retina, focusing on the inner retina, and their putative role in myopia can be found in the supplementary data (Supplementary Tables S6 and S7 and Figure 8).

One of these genes, UMODL1 (MIM# 613859) encodes an extracellular matrix adhesion and cellular migration uromodulin-like 1 protein (UMODL1) (Di Schiavi et al., 2005; Shibuya et al., 2004). It is known to be expressed in olfactory and vomeronasal sensory neurons (Di Schiavi et al., 2005). It is also present in retina gene expression databases, but the specific cell types expressing Umodl1 remain unknown. UMODL1 was found to be associated with
myopia in a genome-wide association study (Nishizaki et al., 2009) (Supplementary Tables S6, S7 and Figure 8).

4.5.4 Eye, endoderm, and connective tissue development

The vertebrate eye is a complex organ. Its development is guided by highly organized processes that take place during embryonic development, and mutations in key genes involved in these processes lead to severe congenital disorders as well as myopia (Graw, 2010). Several DEGs identified in cCSNB mouse models were associated with eye, endoderm and connective tissue development, including Arc, Bdnf, Cacna1s, Cnmd/Lect1, Col5a1, Eomes, Etv4, Dusp4, Grm6, Olfm3, Prkca, Spred3, Tgfb2, Unmodl1 and Vgll4 (Supplementary Tables S6 and S7, Figures 7 and Figure 10).

ARC (MIM# 612461) encodes the activity-regulated cytoskeleton-associated protein (ARC) (Supplementary Table S6 and Figure 8). ARC mRNA is rapidly produced and delivered into dendrites following synaptic activity in neurons (Dynes and Steward, 2007). Interestingly, its expression in rodent brains is robustly induced by prolonged wakefulness (Cirelli and Tononi, 2000). Converging evidence has led to the hypothesis that wakefulness potentiates, and sleep reduces, synaptic strength. Recently, ARC was demonstrated to be important for the induction of multiple behavioral and molecular responses associated with sleep homeostasis (Suzuki et al., 2020).

COL5A1 (MIM# 120215) encodes the type V collagen alpha-1 (COL5A1) protein, which belongs to group V and XI collagens. These collagens control fibrillogenesis, probably by...
forming a core within the fibrils. Another characteristic of these collagens is the partial retention of their N-propeptide extensions (Fichard et al., 1995). Collagen V was found to be expressed in the human placenta and skin, but later studies showed that it is also present in other organs. Our transcriptome meta-analyses revealed that Col5a1 is expressed in rods and in BCs in the retina (Supplementary Table S6 and Figure 8). Mutations in COL5A1 were found in subjects with the classic type 1 Ehlers-Danlos syndrome (MIM#130000). Ehlers-Danlos syndromes (EDS) are a group of heritable connective tissue disorders that share the common features of skin hyperextensibility, articular hypermobility, and tissue fragility. The main features of classic Ehlers-Danlos syndrome are loose-jointedness and fragile, bruisable skin that heals with peculiar 'cigarette-paper' scars (Beighton, 1970). Ophthalmic clinical features have also been described, including retinal detachment (Pemberton et al., 1966) and high pathologic myopia (Gharbiya et al., 2012; Perez-Roustit et al., 2019).

EOMES (MIM# 604615) encodes eomesodermin (EOMES), also called Tbr2. It is directly regulated by Brn3b and is important for RGC development (Mao et al., 2008). In agreement with these observations, our gene expression data revealed that Eomes is mainly expressed in the inner retina and more specifically, in RGCs (Supplementary Table S6 and Figure 8). Homozygous silencing of EOMES leads to microcephaly with polymicrogyria and corpus callosum agenesis (Baala et al., 2007). We are not aware of a retinal phenotype in subjects with this gene defect; however, mice lacking Eomes (alias Trpb2) have reduced retinal projections to non-image-forming brain nuclei and an attenuated pupillary light reflex (Sweeney et al., 2014). Interestingly, subjects with CSNB have a reduced redilation and smaller baseline pupil diameters, suggesting a disinhibition of intrinsically photosensitive RGCs due to affected post-photoreceptor transduction via BCs (Schatz et al., 2019).
OLFM3 (MIM# 607567), encoding olfactomedin 3, is a downstream target of PAX6, which plays a critical role in eye development (Grinchuk et al., 2005). Although the function of OLFM3 is not yet understood, it may be involved in cell-cell adhesion and cell attachment to the extracellular matrix, and affect cell migration and axon growth (Grinchuk et al., 2005). Our gene expression data revealed that Olfm3 is expressed in ACs in the retina (Supplementary Table S6 and Figure 9). OLFM3 was also implicated in glaucoma and myopia (Supplementary Table S6 and Figure 8) (Podkolodnaya et al., 2011).

4.5.5. Genes not associated with any pathway, unknown function and limited amount of expression in the retina

Several not confirmed but annotated mouse genes (5730419F03Rik, Dbhos (dopamine beta hydroxylase, opposite strand, long coding RNA), Gm20754, Gm40477, Gm49760 and Gm5478) of unknown function and only limited information about their retinal expression patterns were differentially expressed in several cCSNB mouse lines (Supplementary Table S6). Two genes (5730419F03Rik and Gm20754) appeared to be also differentially expressed in mice lacking Bhlhe23. In addition, 5730419F03Rik is most likely expressed in the inner retina, suggesting that it may have a role in inner retinal signaling. Interestingly, Dbhos was also differentially expressed in mice exposed to blue light (Ouyang et al., 2020). Future studies aimed at investigating the exact cellular expression patterns of these genes and their roles in the retina, as well as identification of the human orthologs will shed light on their contribution to retinal pathophysiology.

Although some information is available about the expression in the retina of the following DEGs, AFAP1L1 (MIMI# 614410), Pdzph1, PLEKHF1 (MIM# 615200), ST6GALNAC5

39
(MIM# 610134) and MAST2 (MIM# 612257) (Supplementary Table S6, supporting material), their roles in retinal physiology and possible pathologies remains to be elucidated.

4.5.6 Other genes from myopia databases that are differentially regulated in at least one mouse model with cCSNB

Several other genes identified in myopia databases appear to be significantly differentially expressed as determined by RNA-Seq analyses in at least one mouse model of cCSNB (Supplementary Tables S6 and S7 and Figure 8).

5. Cause of reduced expression of many DEGs in cCSNB

Reduced expression of DEGs and their respective proteins in cCSNB may be due to reduced expression levels or a lower number of cells expressing the DEGs. Genes implicated in cCSNB share a common phenotype, namely an ON-BC signaling defect. Although the morphology of the retina in human subjects and animal models with cCSNB is believed to be non-degenerative and stable, subtle cellular changes have been described in mice lacking Lrit3 and Trpm1 (Neuille et al., 2014; Takeuchi et al., 2018). SD-OCT revealed thinning of the INL, IPL, GCL, and nerve fiber layer in Lrit3−/− mice (Neuille et al., 2014). This was not found in Gpr179−/− mice (Orhan et al., 2021). Interestingly, rod BC terminals were significantly smaller in Trpm1−/− retinas than in Grm6−/− retinas, suggesting that deficiency of TRPM1, but not GRM6 affects rod BC synaptic terminal maturation and alter signaling between rod BCs and ACs (Kozuka et al., 2017).
Notably, we found that *Prkca* transcripts were reduced in cCSNB retinas, and that, similar to TPBG, the level of PRKCA protein, which is specifically localized to rod BCs, was greatly reduced in *Gpr179<sup>−/−</sup>* and *Lrit3<sup>−/−</sup>* mice, but not in *Grm6<sup>−/−</sup>* mice (Figure 13), while the numbers of nuclei present in the INL were unaffected (Figure 14). Visualizing synapses with a protein-independent marker needs to be performed to evaluate if the lower amounts of PRKCA and TPBG are due to morphological changes in rod BCs, particularly their synaptic terminals.

Similarly, we found that levels of DA and DOPAC were also reduced in cCSNB retinas. Since the number of nuclei in the INL (Figure 14), remained unchanged in the cCSNB mouse models, and reduction of TH-positive dopaminergic amacrine cells was not significantly different in cCSNB compared to wild-type mice (tested for *Gpr179<sup>−/−</sup>* versus *Gpr179<sup>+/+</sup>*) (Figure 15), we conclude that the reduced expression of some genes in cCSNB, as well as the reduction in DA and DOPAC levels are not due to degeneration of retinal cells, but may be linked to altered synapse morphology and/or lower RNA expression and/or reduced protein levels. We speculate that the absence of cCSNB genes such as *Gpr179* and *Lrit3*, leads to the classical ERG-defect but that other differentially regulated genes may contribute to myopia in those subjects with cCSNB. Similarly, exposure to reduced light levels in genetically unaffected individuals may also lead to lower expression of these genes and thus to myopia.

6. **Future perspectives**

6.1. **Identification of differentially expressed genes in cCSNB models using a whole transcriptomic approach may yield novel proteins important for retinal signaling, as well as novel myopia-related genes**

Mutations in *NYX, GRM6, GPR179, TRPM1* and *LRT3* likely lead to cCSNB because the encoded proteins are necessary for ON-BC signaling (Audo et al., 2012; Bech-Hansen et al.,
The retina of cCSNB human subjects is generally well preserved and the disease course is non-progressive. The proteins are localized in the outer plexiform layer and, in most cases, in the dendritic tips of rod BCs and cone ON-BCs (Zeitz et al., 2015). Mouse models of cCSNB show a similar ON-BC transmission defect as human cases. The diagnosis is confirmed by an electronegative waveform at the full-field electroretinogram with severely reduced or absent b-wave under scotopic and photopic conditions, reflecting defective ON-pathway signaling (Zeitz et al., 2015). Candidate genes causing this disorder have been identified by linkage analyses in humans, using animal models, and more recently, by next-generation sequencing approaches (e.g. Audo et al., 2012; Pusch et al., 2000; Varin, 2020; Zeitz et al., 2013; Zeitz et al., 2005), with the help of transcriptomic data-bases to reinforce candidate genes (Zeitz et al., 2015). Recently, Woods et al. used comparative transcriptomic analyses in a mouse model lacking the rod BC transcription factor Bhlhe23 (also known as BHLHB4 or basic helix-loop-helix family member, b4), to identify potential genes implicated in CSNB (Woods et al., 2018). The phenotype of Bhlhe23−/− mice is slightly different than that of cCSNB. Although both phenotypes are stationary, rod BCs are almost completely absent in mice lacking Bhlhe23, resulting in a thinner INL but the cone pathway is not affected; thus Bhlhe23−/− mice have an isolated rod BC defect, while both, rod BC and cone ON-BC functions are affected in cCSNB. This is also reflected by the b-wave amplitude only being affected under scotopic, but not photopic conditions in Bhlhe23−/− mice (Bramblett et al., 2004). While the phenotype of Bhlhe23−/− mice is different from cCSNB, night vision may indeed be affected. To date, however subjects with mutations in BHLHE23 and a similar phenotype have not been described (Woods et al., 2018). This might be due to the fact that only scotopic vision is affected, and ERG recordings are required to detect such a phenotype in affected subjects.
Because of the absence of rod BCs, one might expect that some of the genes differentially expressed in *Bhlhe23*/*−*− mice may be important for night vision. However, expression of only a few of the “cCSNB-genes” was significantly reduced in *Bhlhe23*/*−*− mice compared to wild-type mice. Furthermore, mice deficient for genes expressed at lower levels, such as mice lacking *Car8*, did not exhibit a typical cCSNB phenotype (Puthussery et al., 2011). This is consistent with our findings, indicating that while these approaches may identify novel candidates for cCSNB this is not necessarily the case.

It is important to note that cCSNB is characterized not only by night blindness, but also by high myopia, nystagmus and often strabismus. Strikingly, since cCSNB is present from birth and young subjects are often not aware of their vision defects in dim light conditions, they are often first diagnosed due to their high myopia and/or infantile nystagmus. Thus, a main goal of this work was to identify other genes implicated in the same pathway as proteins underlying cCSNB and in that way identify additional candidates for the other associated ocular abnormalities associated with cCSNB. Since myopia represents one of the most common eye diseases worldwide, with increasing numbers of affected persons expected in coming years, we focused our study on DEGs implicated in cCSNB and associated with myopia.

### 6.2. Retinal expression of DEGs, pathway analyses and correlation to eye phenotypes

To understand in which retinal cell types the 52 DEGs are expressed, we queried several retina expression databases, including single cell transcriptome databases, as well as PubMed
and search tools such as “Google” (https://www.google.com/) (Macosko et al., 2015; Shekhar et al., 2016; Siegert et al., 2012; Siegert et al., 2009; Woods et al., 2018). These data showed that almost all 52 DEGs are expressed in the human retina, including in photoreceptors, BCs, ACs and RGCs, but for only a few of them was their retinal function described in the scientific literature (Supplementary Table S6 and Figure 8). Several DEGs, expressed in various retinal cell types were found to be associated with retinal disorders. For example, genes expressed in rods photoreceptors have been implicated in rod-cone dystrophies. Another example are genes expressed in ON-BCs, for which the encoded proteins are localized at the dendritic tips, which have been implicated in cCSNB (Zeitz et al., 2015). Variants in FRMD7 (MIM # 300628), coding for Ferm domain-containing protein 7, which is expressed in ACs (Yonehara et al., 2016), are associated with idiopathic nystagmus in mice and human subjects (MIM #310700) (Tarpey et al., 2006). Thus, other genes expressed in ACs may also be linked to nystagmus. The contribution of genes expressed in ACs to nystagmus was further substantiated in a study with cCSNB humans and mice lacking functional nyctalopin (Winkelman et al., 2019). Thus, DEGs described herein and expressed in ACs may contribute to nystagmus observed in subjects with cCSNB (Figure 8). Recently, Bui Quoc and Milleret suggested that an abnormal routing of the RGC axons at the level of the optic chiasm might cause an imbalance between contra-lateral and ipsi-lateral projections and thus lead to strabismus (Bui Quoc and Milleret, 2014). This might occur due to an abnormal expression of genes controlling RGCs axonal projections, and which may be differentially expressed in cCSNB. Further experiments and database analyses will be needed to determine if DEGs expressed in ACs and RGCs (Figure 9) contribute to nystagmus and strabismus observed in human subjects with cCSNB. In addition, these genes may also represent candidate genes in cases of isolated nystagmus and strabismus.
Our meta-analysis examined the expression of DEGs and their correlation with myopia using published literature and database analyses. About 50% of the 52 DEGs expressed in the retina, have been already linked to myopia (Figure 8), reinforcing our hypothesis that genes differentially expressed in cCSNB may cause the myopia phenotype observed in cCSNB. Indeed, GWAS studies showed that several genes expressed in various retinal cell types contribute to the development of myopia (Verhoeven et al., 2013). Some of the genes implicated in myopia are functionally involved in processes that facilitate communication between cells in the retina or control the ability of photoreceptors to respond to light. Some others are involved in pre-natal eye growth and development (Verhoeven et al., 2013). There is some overlap between genes previously described as being involved in myopia and those described here. We assume that some of the others may represent novel myopia-related genes (Hysi et al., 2020; Tedja et al., 2018; Verhoeven et al., 2013). Interestingly, many DEGs described here, when deleted or part of deleted chromosomal regions, lead to severe phenotypes that often include myopia. Our hypothesis is that the expression levels of these genes are associated with high myopia observed in cCSNB which makes cCSNB a more complex disorder than previously recognized. In addition, DEGs may also produce the cCSNB-associated ocular alterations nystagmus and strabismus. Our results combined with GWAS findings highlight the light-dependent retina-to-sclera signaling cascade that can cause myopia if disrupted and outline potential pathobiological molecular drivers (Tedja et al., 2018). It is unlikely that signals from the retina control scleral growth directly. Instead, it is suggested that retinal signals are relayed by the RPE, choroid and ciliary body, which in turn release signaling molecules to directly affect scleral growth (Wallman and Winawer, 2004). Our findings using cCSNB models suggest that due to an altered ON-pathway the expression of several genes becomes insufficient, which may result in myopia. We hypothesize, that
exposure to insufficient light levels, as found in children with not enough outdoor activities similarly leads to alterations of the ON pathway and myopia.

Not surprisingly, the cCSNB DEGs included genes that are important for synapse formation and signaling. In addition, MAPK pathways and developmental genes were identified by our study, and to our knowledge, their association with cCSNB is novel. These same genes, however, are well known to be involved in the development of myopia (Li and Zhang, 2017), further reinforcing our predictions that genes differentially expressed in cCSNB are indeed good candidates for cCSNB-associated high myopia, as well as serving as candidates for non-syndromic myopia. Other DEGs identified here were not associated with specific myopia-related search terms or pathways, but, because of their expression in the retina and described physiological function, may be nevertheless be associated with myopia. Interestingly, a few DEGs have been implicated in circadian rhythms. Circadian clocks are cellular oscillators that generate daily rhythms even in the absence of external timing cues (Dunlap, 1999; Pittendrigh, 1993) and have also been associated with eye growth and refractive error development (Chakraborty et al., 2018).

6.3 Novel candidate genes important for vision

The following DEGs represent genes not previously associated with myopia and may represent novel candidate genes underlying high myopia in cCSNB or other retinal disorders:

Adgra3/Gpr125, Afap1l1, Ampd3, Arc, Cacna1s, Ccnj1, Cdc25c, Dbhos, Eomes, Etv4, Fbox32, Frem3, Gm20754, Gm40477, Gm49760, Gm5478/Krt6c, Gng4, Insyn2b, Ldb2, Mast2, Mir670hg, Myh7b, Olfm3, Pdzph1, Plekhf1, Prkca, Rgs16, Shroom4, Spred3, St6galnac5, Synj2, Tac2, Tpg, Trpc1 and Umodl1. More analyses are needed to validate their putative functional roles in vision. For a few of them, highlighted in bold, animal models
or more functional analyses are available and may thus represent the best candidates to be analyzed in the future.

6.4 Treatment of cCSNB and myopia

Although cCSNB is believed to be a relatively mild non-degenerative stationary retinal disease, this study shows that gene defects causing cCSNB impact the expression of other genes important for the correct development of the retina and eye. Treatment efficacy may also be impacted. Indeed, at least in mice, cCSNB treatment was more successful in immature retina, and only under scotopic conditions (Hasan et al., 2019; Scalabrino et al., 2015; Varin et al., 2021a; Varin et al., 2021b), suggesting that the development of the retinal structure, e.g., synapse formation, is irreversibly impaired in cCSNB. These subtle changes of the retina may only be visible with sensitive methods such as immunohistochemistry or electron microscopy. Such developmental defects may be regulated by the genes differentially expressed in cCSNB identified in this study. Replacing the missing gene implicated in cCSNB, such as GRM6 or LRIT3, by an AAV-approach may restore the correct localization of the corresponding protein and its function, but it is not clear if this will also restore the normal expression of the cCSNB-associated DEGs. Defects in developmental processes, such as synapse formation, may lead to the observed failure to rescue photopic vision in adult mice. Furthermore, whether an AAV-based therapeutic approach can cure myopia remains unclear. Although refractive errors associated with myopia can be corrected using optical correction, myopic subjects may still develop a number of other vision-threatening complications such as myopic macular degeneration, retinal tears, retinal detachment and glaucoma (Hornbeak and Young, 2009). Therefore, identifying therapeutic approaches to prevent the development of myopia would be beneficial. Pharmacological treatments may become important means to
prevent and/or slow the progression of myopia, but they are unlikely to be able to revert or
cure established myopia.

6.4.1 Pharmacological myopia treatment

A signaling cascade, which begins in the retina, via photoreceptors, BCs and dopaminergic,
GABAergic, and glycinergic ACs, and propagate across all ocular tissues to the sclera is
likely to contribute to human myopia development and progression (reviewed by (Tkatchenko
and Tkatchenko, 2019)). This signaling cascade is triggered by optical blur, which plays a
critical role in this process. Here, we suggest that cCSNB is an additional valuable model to
study myopia, because the disruption of inner retinal signaling in cCSNB has a significant
impact on refractive eye development, potentially similar to a low light environment. As
discussed above, the neurotransmitter DA and its metabolite DOPAC have been proposed to
regulate refractive eye development and their retinal concentrations are reduced in myopia
models (Chakraborty et al., 2015; Pardue et al., 2008; Stone et al., 1989; Zhou et al., 2017).
Here, we have shown that DA and DOPAC levels are also reduced in the retina of cCSNB
mice. Intravitreal injections of DA in rabbits (Gao et al., 2006) and intraperitoneal
injections of DA in guinea pigs (Mao et al., 2010) and mice (Landis et al., 2020) inhibit FDM
myopia induction. Enhancing DA signaling by daily intraperitoneal injections of non-selective
DA receptor agonists prevented FDM in chicken, guinea pig, monkey and mice (reviewed by
(Zhou et al., 2017). More studies are needed, however, to define the cellular mechanisms of
DA action, a requirement for the development of an effective myopia treatment. Bright light
has also been shown to protect against the development of myopia and to increase DA release
in a number of studies with animal models and in children (reviewed by (Zhou et al., 2017).
It has been suggested that retinal DA may induce choroidal thickening and inhibit ocular
**growth** by triggering the release of other transmitters such as **nitric oxide** (NO) from the retina or choroid (reviewed by (Zhou et al., 2017). **Clinical trials** testing the use of **dopaminergic agents** to treat amblyopia, myopia and attention deficit disorders revealed **negative side effects of systemic activation of DA** signaling (reviewed by (Zhou et al., 2017)). In contrast, local delivery of DA by APO eye drops given to infant rhesus monkeys reduced development of FDM in the absence of systemic toxicity and other negative side effects (Iuvone et al., 1991). Despite these promising findings, we are not aware of further studies in non-human primates or clinical trials using dopaminergic compounds to inhibit myopia progression.

A few other pharmacological agents targeting neurotransmitter receptors have been shown to suppress the development of experimental myopia in animals, such as the **muscarinic acetylcholine receptor (mACHR) antagonists** pirenzepine and atropine, which are expected to act on muscarinic receptors, expressed in the sclera and in the inner retina. Further long-term clinical studies with large samples sizes are needed to validate the efficacy of this potential anti-myopia therapy (reviewed (Tkatchenko and Tkatchenko, 2019; Wang et al., 2021)). Today, however, **atropine**, a **nonselective muscarinic receptor antagonist**, is the most widely used anti-myopia drug, although the exact mechanism of atropine’s inhibitory effect on myopia is not well understood. Several clinical trials using atropine eye drops in combination with optical devices such as multifocal lenses that impose a peripheral positive defocus, showed positive outcomes, albeit with low efficacy (reviewed (Tkatchenko and Tkatchenko, 2019)). It would be interesting to investigate if atropine, potentially together with optical devices would also inhibit myopia development in CSNB patients and animal models.

The recent identification of **signaling pathways**, identified by us here and others (Tkatchenko and Tkatchenko, 2019)), that control the response of the eye to optical defocus and susceptibility to myopia may be helpful for the development of new myopia therapies.
Interestingly, many of those pathways play important roles in multiple retinal cell types and in multiple ocular tissues. For example, the MAPK pathway and GABA receptor signaling influence myopia development via multiple retinal cell types as well as the RPE/choroid (reviewed (Tkatchenko and Tkatchenko, 2019)). Our study found that genes implicated in the MAPK pathway are differentially regulated in cCSNB, suggesting they may contribute to myopia in these subjects. Inhibitors of MAPK phosphorylation have been shown to inhibit myopia (She et al., 2021), but we are not aware of clinical trials testing this target.

Inhibition of GABA activity, such as with GABA receptor antagonists, may prevent excessive myopia (reviewed (Wang et al., 2021)). Schmid et al. suggested that the GABAergic pathway may interact with the dopaminergic pathway and that the balance between excitatory and inhibitory signaling is important for normal eye growth (Schmid et al., 2013). This is in line with our findings. Interestingly, we found that Gabrr1, coding for the rho-1 GABA receptor subunit, and expressed in rod BC synaptic terminals (McCall et al., 2002) is significantly downregulated in mice lacking Gpr179 and Lrit3. It is possible that this decrease in GABA receptors is a consequence of an increased release of GABA from amacrine cells, leading in turn to myopia in cCSNB. Moreover, abnormal visual processing was observed in mice lacking Gabrr1 (McCall et al., 2002) and several studies linked the human orthologue with myopia (Supplementary Table S6 and Figure 9) (Fan et al., 2016; Lin et al., 2020). We are not aware of clinical trials testing GABA receptor antagonists for myopia prevention, most likely because such drugs would be expected to have dangerous side effects.

Further understanding of additional signaling pathways and ocular tissues implicated in myopia and their interactions with various environmental factors will lead to the development of anti-myopia therapies (Tkatchenko and Tkatchenko, 2019)). Considering the complexities of the signaling cascades underlying myopia development, an ideal anti-myopia drug (or a drug combination) would target multiple pathways. Although cCSNB appears to be a good
myopia model, refractive errors are only measured following experimental myopia inductions. Alternatively, treatment efficacy could be evaluated by measuring the restoration of neurotransmitter and other metabolite levels or by experimentally assessing the normalization of gene expression within the gene regulatory networks involved in myopia development. Together, we and others showed that myopia is a complex disorder influenced by many genetic and environmental factors. Future studies are needed to develop therapies targeting these signaling cascades and determine the efficacy of potential treatment approaches (Brennan et al., 2021).

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**Figures legends:**

**Figure 1:** Schematic representation of ERG recordings of five different species known to display a cCSNB phenotype. This figure was taken from the PhD thesis of Juliette Varin (Varin, 2020), which in turn was adapted from Audo et al., 2009 (for human CSNB, subject with mutations in TRPM1) from Sandmeyer et al., 2007 (for the TRPM1−/− Appaloosa horse ERG), from Kondo et al., 2015 (for the LRIT3−/− Beagle dog ERG), from Pardue et al., 1998 (for the Nyx−/− mouse ERG) and from Peachey et al., 2012 (for the MO-gpr179 zebrafish ERG).

**Figure 2:** Example of scotopic electroretinograms in mice lacking Aplp2, a gene associated with myopia in humans. Lack of Aplp2 causes a dose-dependent decrease in the b-wave amplitude (A). The b-wave implicit time was increased in mice lacking Aplp2. Lack of Aplp2 did not have a significant impact on either a-wave amplitude or a-wave implicit time. Lack of Aplp2 caused a decrease in amplitude and an increase of the implicit time of the oscillatory potentials (B). Figure was taken from Tkatchenko et al., 2015 (Tkatchenko et al., 2015) with permission.
**Figure 3:** Lens-induced myopia in wild-type mice measured after three weeks of goggling. Myopia can be induced in mice using in-house-made goggles with negative spectacle lenses and induced refractive error can be measured using an automated photorefractor (Schaeffel et al., 2004). Wild-type mice (n=12) were goggled at P21 for three weeks. The goggles were fitted with -25.00 D lenses for the right eyes (RE) and no lens for the left eyes (LE), which were used as controls. The interocular differences between treated and control eyes was measured at P21 before goggle application and after goggle application at P42. Statistical significance was obtained with p-values, where $p \leq 0.05 = \ast$, $p \leq 0.01 = \ast\ast$ and $p \leq 0.001 = \ast\ast\ast$.

**Figure 4:** Reduced retinal DOPAC and DA in light adapted cCSNB mouse models lacking **Gpr179**, **Lrit3** and **Grm6** statistically analyzed using the Mann-Withney test. Values are reported as standard deviations. Statistical significance was obtained with p-values, where $p \leq 0.05 = \ast$, $p \leq 0.01 = \ast\ast$ and $p \leq 0.001 = \ast\ast\ast$.

**Figure 5:** A, Venn diagram of differentially expressed genes (DEGs) in the retina of three cCSNB mouse lines identified by RNA-Seq analysis. This revealed 155 (green), 133 (red), and 222 (blue) DEGs for **Gpr179**, **Lrit3** and **Grm6** mice, respectively. Of those, 7 DEGs were common to all three cCSNB mice, and 38 DEGs were shared by the **Gpr179** and **Lrit3** mice. B, Hierarchical clustering analysis of the 52 DEGs present in at least two mouse lines identified 10 clusters (different colors left column). The z-score was derived from the average of the replicates for each experimental group. Blue: low expression; orange: high expression. The largest cluster consists of genes less expressed in the cCSNB models (cluster 1).
Figure 6: Examples of genes found to be differentially expressed in three (red) and two (green) cCSNB mouse lines by RNA-Seq studies and validated by RT-qPCR experiments. Green boxes indicate the genes differentially expressed in two cCSNB mouse lines. Some genes appeared to be significantly differentially expressed only in two cCSNB mouse lines by RNA-Seq investigations, but were significantly differentially expressed in all three mouse lines by RT-qPCR studies (e.g. Prkca and Tpbg). Other genes appeared to be significantly differentially expressed in three cCSNB mouse lines by RNA-Seq investigations, but were statistically significantly differentially expressed only in two mouse lines by RT-qPCR studies (e.g. Frem3 and Ptprr). Data were tested for normality and analyzed using the nonparametric Mann-Whitney test to compare expression. Values are reported as standard deviations. Statistical significance was obtained with p-values, where p ≤ 0.05=* and p ≤ 0.01 = **. NRQ = Normalized relative quantity.

Figure 7: Interdependence of expression between genes implicated in cCSNB at the transcriptional level. There was a trend that Trpm1 expression is dependent on the expression of Gpr179, Lrit3 and Grm6, and that Grm6 expression is dependent on the expression of Lrit3.

Figure 8: Schematic drawing (created with BioRender.com) of differentially expressed genes (DEGs), localization of their expression in the retina and their association with myopia. Genes in the white box, also appeared in myopia databases and were found to be differentially expressed in at least one cCSNB mouse line. They show expression in the retina although in which cell type remains uncharacterized.
Figure 9: Schematic representation of the DEG expression in the different types of BCs. BC1-4 = OFF-BCs, BC5-BC7 = ON-BCs (generated using the data from Shekhar et al., 2016 (Shekhar et al., 2016).

Figure 10: A, DEGs were enriched in different pathways, including the MAPK signaling pathway, synapse, GPCR ligand binding, eye development, endoderm and connective tissue pathways. B, Circle presentation showed that most of the DEGs appeared in multiple pathways.

Figure 11: Schematic drawing of DEGs (shown in brown) enriched in the MAPK signaling pathway (created with BioRender.com).

Figure 12: Quantification of western blot analyses for proteins for which RNA-Seq studies showed differential expression by RNA-Seq and RT-qPCR studies in Gpr179<sup>−/−</sup>, Lrit3<sup>−/−</sup> and Grm6<sup>−/−</sup> cCSNB mouse models (n ≥ 4). The protein amounts were normalized against GAPDH, visualized with Image J and statistically analyzed using the Mann-Whitney test. Values are reported as standard deviations. Statistical significance was obtained with p-values, where p ≤ 0.05 = * and p ≤ 0.01 = **.

Figure 13: Immunostaining in Gpr179<sup>−/−</sup>, Lrit3<sup>−/−</sup> and Grm6<sup>−/−</sup> cCSNB mouse models showed a reduction of TPBG and PRKCA immunofluorescence in mice lacking Gpr179 and Lrit3 but
not in mice lacking *Grm6*. This was most obvious in the OPL and IPL (bar = 20 µm). Phr = photoreceptor layer; ONL = outer nuclear layer; OPL = outer plexiform layer; inner nuclear layer; IPL = inner plexiform layer; GCL = ganglion cell layer.

**Figure 14:** Quantification of all cell nuclei stained with DAPI revealed an equal number of cell nuclei in the inner nuclear layer (INL) in the mutant versus wild-type cCSNB mouse models (n = 3-8).

**Figure 15:** Quantification of all TH-positive cells revealed no significant differences between *Gpr179*+/+ and *Gpr179*−/− (n=4 mice).
Figure 1

Figure 2
Figure 3

Lens-induced myopia (n=12)

![Graph showing refractive state (D) for RE (-25D lens) and LE (no lens)]

<table>
<thead>
<tr>
<th>Mean interocular shift (D)</th>
<th>Standard Deviation</th>
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<tr>
<td>-5.393620263333</td>
<td>2.4257357925638</td>
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Figure 4
Figure 5
Figure 6
Figure 7

Figure 8

cCSNB genes
Figure 9
Figure 10
Figure 11

[Diagram of a signaling pathway involving proteins like MAPK, CDC25C, RAS, RAF, MEK1, MEK2, ERK, SPRED, PRKCA, DAG, Ca+, and cellular compartments like Cytoplasm and Nucleus.]

LDB2

??
Figure 12

![Graphs showing the relative expression of PRKCA and TPBG, DUSP4 and CAR8 genes.](image-url)
Figure 13

[Images of immunofluorescence microscopy showing layers of retina, including TPBG, PRKCA, and their overlays for different genotypes (Gpr179<sup>-/-</sup>, Lris3<sup>-/-</sup>, Grm6<sup>−/−</sup>).]
Figure 14

Inner nuclear layer

Figure 15

Gpr179<sup>+/+</sup>  Gpr179<sup>−/−</sup>  Lrt3<sup>+/+</sup>  Lrt3<sup>−/−</sup>  Grm6<sup>+/+</sup>  Grm6<sup>−/−</sup>

DAPI+ nuclei/mm²

Gpr179<sup>+/+</sup>  Gpr179<sup>−/−</sup>

ns

Number Th+ cell/mm²