Developmental Biology 429 (2017) 343–355

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

The nuclear hormone receptor gene *Nr2c1* (Tr2) is a critical regulator of early retina cell patterning

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ARTICLE INFO

Keywords:
Retina
Development
Nuclear hormone receptors
Cone cell patterning
Cell proliferation

ABSTRACT

Nuclear hormone receptors play a major role in the development of many tissues. This study uncovers a novel role for testicular receptor 2 (*Tr2*, *Nr2c1*) in defining the early phase of retinal development and regulating normal retinal cell patterning and topography. The mammalian retina undergoes an overlapping yet biphasic period of development to generate all seven retinal cell types. We discovered that *Nr2c1* expression coincides with development of the early retinal cells. Loss of *Nr2c1* causes a severe vision deficit and impacts early, but not late retina cell types. Retinal cone cell topography is disrupted with an increase in displaced amacrine cells. Additionally, genetic background significantly impacts phenotypic outcome of cone photoreceptor cells but not amacrine cells. Chromatin-IP experiments reveal NR2C1 regulates early cell transcription factors that regulate retinal progenitor cells during development, including amacrine (Satb2) and cone photoreceptor regulators thyroid and retinoic acid receptors. This study supports a role for *Nr2c1* in defining the biphasic period of retinal development and specifically influencing the early phase of retinal cell fate.

1. Introduction

Nuclear hormone receptors (NHRs) are a highly conserved group of transcriptional regulators that are involved in numerous functions including development, reproduction, metabolism, circadian cycle, and immunological responses (Bookout et al., 2006; Mangelsdorf et al., 1995; Olivares et al., 2015). NHRs are often activated in response to lipophilic ligands such as steroid hormones, thyroid hormone, vitamins and retinoids, and orphan receptors (with unidentified ligands) to repress or activate the expression of target gene networks (Bookout et al., 2006; Fuller, 1991; Egea et al., 2000; Yang et al., 2006; McKenna and O'Malley, 2002; McKenna et al., 2009) NHRs regulate gene expression by interacting with transcription factor complexes and binding to hormone response elements (HREs) (Smirnov, 2002; Chen and Evans, 1995). NHRs play a critical role in orchestrating the complex process of retinogenesis which occurs as a common pool of retinal progenitor cells (RPC) specify into more committed precursor cells that generate each of seven unique cell types (Cayouette et al., 2003; Livesey and Cepko, 2001).

Retinogenesis occurs in two overlapping phases in which the seven different types of cell are generated in a sequential and overlapping order. In the early phase ganglion, horizontal, cones and amacrine cells are produced followed by a later phase of differentiation that produce rods, bipolar, and Müller glia cells (Ashery-Padan and Gruss, 2001). As the retina develops, cells proliferate from the outer retina and migrate to the inner retina. The correct orchestration of this process is guided by transcription factors, cell-surface receptors and nuclear hormone receptors (Cepko et al., 1996). Transcriptional activators from the basic helix-loop-helix (bHLH) family; which include *Math5, Ngn2, Math3, NeuroD and Mash1*, promote neuronal fate and inhibit glial fate (Bertrand et al., 2002; Hatakeyama and Kageyama, 2004). For example, in the absence of *Math3* and *NeuroD,* amacrine cells are not generated (Inoue et al., 2002) while the lack of *Math5* contributes to the loss of ganglion cells (Wang et al., 2001). Expression of NeuroD leads to the generation of Müller cells (Morrow et al., 1999) and along with the expression of Pax6 and Six3 promotes amacrine cells (Inoue et al., 2002). Several studies show *Nr2e3* plays a critical role in retinal

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http://dx.doi.org/10.1016/j.ydbio.2017.05.021
Received 12 January 2017; Received in revised form 17 May 2017; Accepted 19 May 2017
Available online 25 May 2017

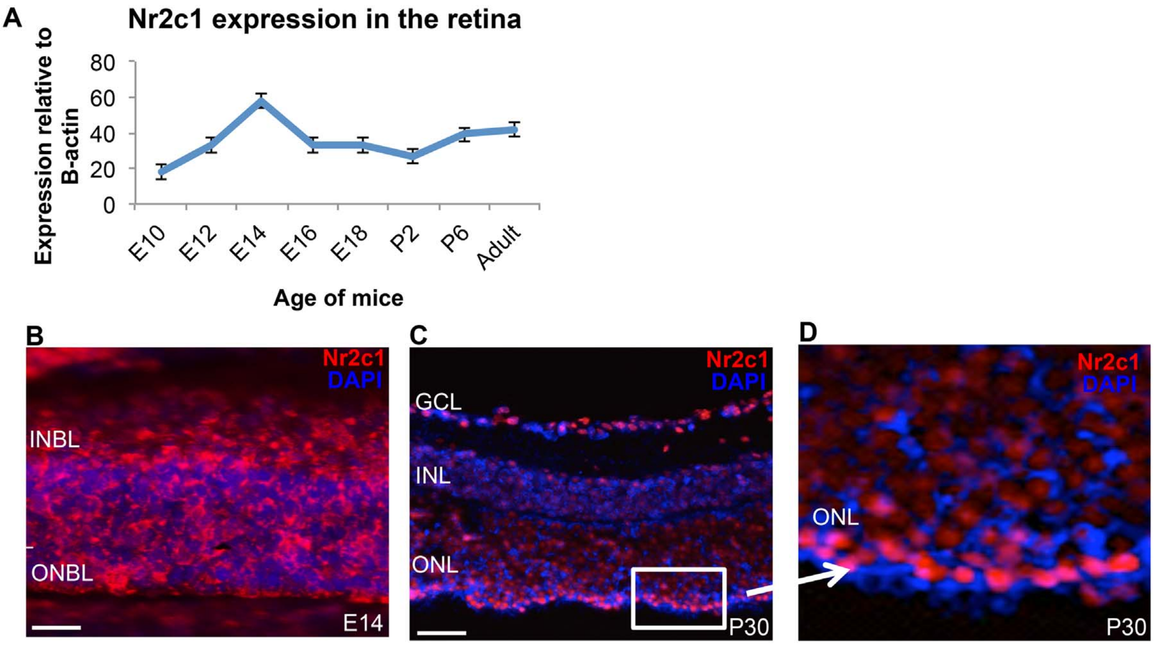

Fig. 1. Expression of *Nr2c1* in the control line C57Bl6/J. A) NR2C1 expression (red) at E14.5 in the outer neuroblastic layer (ONBL) and inner neuroblastic layer (INBL) of the developing retina. NR2C1 (red) protein is expressed in the GCL, INL, OPL and limited photoreceptor cells (likely cones) of the adult retina at P30. DAPI blue nuclear counterstain B) NR2C1 expression in the developing retina E14.5. in the INBL and ONBL. C) Expression of NR2C1 in the adult retina at P30 to GCL, INL and cone cells in the basal layer of ONL. Cone cell expression magnify and pointed by the arrow D) Inset (from box in C) to show NR2C1 label in the cone cells of the ONL. INBL: inner neuroblastic layer, ONBL: outer neuroblastic layer, IS: inner segments; OS: outer segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Bar: 50 µm. Sections were taken from fresh frozen OCT embedded medium. N = 3.

progenitors regulating cone cell production and supports *Nrl* in it's primary role of regulating rod cell development (Haider et al., 2006; Cheng et al., 2006; Mears et al., 2001; Oh et al., 2008, 2007). *Rora* in contrast supports cone cell development and *Crx* is a regulator of both rod and cone photoreceptor differentiation (Fujieda et al., 2009).

The mammalian retina is rod dominant (95–97%) (Strettoi et al., 2004; Jeon et al., 1998) with only 3–5% cone cells. Photoreceptor cells comprise approximately 70% of retinal cells in the mammalian retina (Jeon et al., 1998). Thus, it is not surprising that the visual system and retinal topography of vertebrate animals are largely influenced by photoreceptor cell patterning (Brzezinski and Reh, 2015). Further, photoreceptor patterning has uniquely evolved to each species based on behavior and adaptation to its environment (Viets et al., 2016; Mitchell and Leopold, 2015; Lamb et al., 2007; Lamb, 2009; Salvini-Plawen and Mayer, 1977). These patterns influence both visual acuity and color perception. During mouse retinal development, cone photoreceptor cells have a defined pattern to establish normal topography. Rod photoreceptors and green opsin expressing cone photoreceptors are expressed in a uniform pattern across the retina (Roberts et al., 2006). In contrast, blue opsin expressing cone photoreceptors have a dorsal to ventral gradient, with a ventral concentration. Additionally, unlike humans that express a single opsin gene in each photoreceptor cell, mouse cone photoreceptors, particularly in the ventral retina, co-express blue and green opsin (Roberts et al., 2006; Marquardt and Gruss, 2002; Zhang et al., 2006; Wang and Cepko, 2016). This patterning is established by thyroid hormone receptor β2 (*Trβ2*) that binds to thyroid hormone and inhibits S-opsin expression to create a gradient (Roberts et al., 2006). *Rora*, in contrast, is important for blue opsin expression (Alfano et al., 2010).

A key regulator of *Rora* and maintaining the correct cone cell topography is Nr2e3 (Haider et al., 2009). Mutations in human Nr2e3 are associated with enhanced S cone syndrome (ESCS), Goldman Favre syndrome, autosomal dominant retinitis pigmentosa (adRP), and clumped pigmentary retinopathy (CPRD) (Wright et al., 2004; Chavala et al., 2005; Coppieters et al., 2007; Haider et al., 2000). Mice lacking *Nr2e3* ($Nr2e3^{rd7/rd7}$, *rd7*) display pan-retinal spotting, whorls and rosettes in the photoreceptor layer, an increase in blue-opsin expressing cone cells, with progressive retinal degeneration (Haider et al., 2006; Akhmedov et al., 2000; Cheng et al., 2011). Our prior studies revealed *Nr2c1* is up-regulated in $Nr2e3^{rd7/rd7}$ mice and is co-targeted by both REV-ERB alpha (*Nr1d1*) and *Nr2e3* in the developing retina (Haider et al., 2009; Mollema et al., 2011).

Nr2c1 is a nuclear hormone receptor gene expressed during tissue differentiation and is regulated by retinoic acid (Shyr et al., 2009). *Nr2c1* was isolated from testes as well as the prostate and therefore, originally named Testicular Receptor (*Tr2*) (Chang and Kokontis, 1988). While *Nr2c1* has not been studied in the retina, it directly targets thyroid hormone receptor and retinoic acid receptor by regulating the cellular retinoic acid binding protein I and II involved in retinoic acid metabolism (Chang and Pan, 1998; Wei et al., 2000), both of which are critical for establishing proper patterning of cone cells (Fujieda et al., 2009; Ng et al., 2001, 2009; Lu et al., 2009; Khanna et al., 2006). Further studies in ES cells concluded that *Nr2c1* also plays a role in early embryogenesis and regulates the pluripotentiality of stem cells (Shyr et al., 2009; Hu et al., 2002).

Our prior studies revealed that *Nr2c1* is the in the *Nr2e3* gene network. However, the role of *Nr2c1* in the retina remains unknown. In this study, we generated a knockout model of *Nr2c1* to examine its role in the developing and mature retina. Mice lacking *Nr2c1* exhibit severe vision loss associated with defects in early retinal proliferation, pattering, and topography. Specifically, *Nr2c1* appears to regulate cone photoreceptor topography. Lack of *Nr2c1* generates an aberrant gradient of green opsin expressing cone cells. Additionally, our data suggests *Nr2c1* may suppress amacrine cell proliferation and migration. Mice lacking *Nr2c1* have an increase in displaced amacrine cells in the ganglion cell layer. Interestingly, cells of the later phase of retinal development are not impacted by loss of *Nr2c1*. Our studies reveal a novel role for the *Nr2c1* in the retina in the generation and organization of early retinal cell types. This study reveals that *Nr2c1* helps define the biphasic development of the early retina.

2. Results

2.1. Nr2c1 is expressed in the developing retina and loss of Nr2c1 impacts ganglion cell morphology

To determine the expression profile of *Nr2c1*, we collected ocular tissue from the control strain, C57Bl6/J (B6), at embryonic time points E10, E12, E14, E16, E18 and postnatal time points P2, P6 and P30. *Nr2c1* expression corresponds to the period of early retina cell generation with a peak at E14.5 [35], a second peak of expression at postnatal day 6 and continued expression in the adult retina (Fig. 1A). Immunohistochemistry on B6 retinas and brain revealed that NR2C1 localizes to the inner and outer neuroblastic layer (INBL, ONBL respectively) of the developing retina (Fig. 1B) at E14. The ONBL is the most basal layer where retinal progenitors reside. The INBL, in contrast, is where newly postmitotic cells migrate once leaving the ONBL and exiting the cell cycle. Newly differentiated ganglion cells are located in the most apical region of the INBL (Carter-Dawson and LaVail, 1979). In the adult retina, NR2C1 was detected in ganglion cells (GCL), the inner nuclear layer (INL), outer plexiform layer (OPL), and cone photoreceptor cells in the retina (Fig. 1C,D).

To determine if the loss of *Nr2c1* has an impact on the retina, we generated a knockout mouse model of *Nr2c1* using gene trap technology from the Sanger International Gene Trap Resources. The gene trap (XS0212) was designed using a 9 kb beta galactosidase insert located at the 3′ region of intron 2 of the *Nr2c1* gene. The gene trap results in a truncated message for both the full-length *Nr2c1* transcript, and the two alternative transcripts that lack exon 1 (Fig. 2A). Direct sequencing revealed that the gene trap insertion lacks the first 128 bp of the beta galactosidase intron one. The site of the gene trap insertion is 403bp from the end of intron 2 of the *Nr2c1* gene. Chimeric mice were generated on a C57BL6/J (B6) and 129sv/J genetic background. As *Nr2c1* was first identified as testicular receptor 2, we monitored breeders to determine the effect of the loss of *Nr2c1* on fertility. The colony was maintained as $Nr2c1^{+/-}$ × $Nr2c1^{+/-}$. A total of 436 animals were generated from 29 different breeder pairs with an approximately equal ratio of females (207) to males (219). No reduction in the number of litters or litter size (6–8 pups/litter) was observed when compared to strain control colonies B6 (average litter size 6–8) and 129/SvJ (average litter size 4–6). Additionally, while $Nr2c1^{+/-}$ × $Nr2c1^{-/-}$ or $Nr2c1^{-/-}$ × $Nr2c1^{-/-}$ breeders also did not exhibit reduction in litter size it was noted that the $Nr2c1^{-/-}$ × $Nr2c1^{-/-}$ set up took in average 2–3 months longer to have the first litter.

Animals from each genotype were characterized clinically, histologically and functionally to determine the effects of the lost of *Nr2c1*. Examination of the fundus at postnatal day (P) 30 showed no difference in the retina for any of the genotypes (Fig. 3A). Localization of *Nr2c1* was determined thru X-gal staining for beta galactosidase in N1 animals at P30 (Fig. 3B). X-gal staining was detected in cells of each nuclear layer in the retina with the highest expression in the ganglion cell layer (GCL). A lighter staining was observed in the inner nuclear layer (INL), outer plexiform layer (OPL), inner segments and cone nuclei. As expected, X-gal staining was not detected in the wild-type retina. No gross morphological abnormalities were observed in the retina, however an increase in cells of the ganglion cell layer is apparent (Fig. 3B) which typically, has a single cell layer of cells as opposed to multiple layers observed in the GCL of $Nr2c1^{-/-}$ retinas.

2.2. Loss of Nr2c1 results in abnormal retinal function

Electroretinogram (ERG) studies of $Nr2c1^{+/+}$, $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ animals at P30 were performed. Evaluation of retinal function in light- (photopic) and dark-(scotopic) adapted conditions shows heterozygous and knockout mice exhibit reduction in both A-wave responses (from photoreceptor cells) and B-wave response (from second order neurons) (Fig. 3C) with 50% reduction in $Nr2c1^{+/-}$ and complete loss in $Nr2c1^{-/-}$ retinas.

2.3. Nr2c1 is important to establish normal cone photoreceptor cell patterning

Immunohistochemistry labeling of mature cone photoreceptor cells (P30) was performed to determine if retinal patterning and topography are affected by loss of *Nr2c1*. As stated earlier, the ratio and distribution of the blue and green opsins varies along the entire retina. Green opsin is normally distributed throughout the retina while blue opsin is found predominantly in the ventral region (Applebury et al., 2000). Immunolabeling revealed that $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retinas show loss of the uniform expression of green opsin. $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retinas have an aberrant dorsal-ventral gradient of green opsin expression with a reduction in the green opsin positive cones in the ventral and central retina compared to the wild type (Fig. 4A). In contrast, $Nr2c1^{-/-}$ blue opsin expressing cone cells show no loss or altered patterning and are similar to $Nr2c1^{+/+}$. (Fig. 4B). Double labeling with peanut agglutinin (PNA),

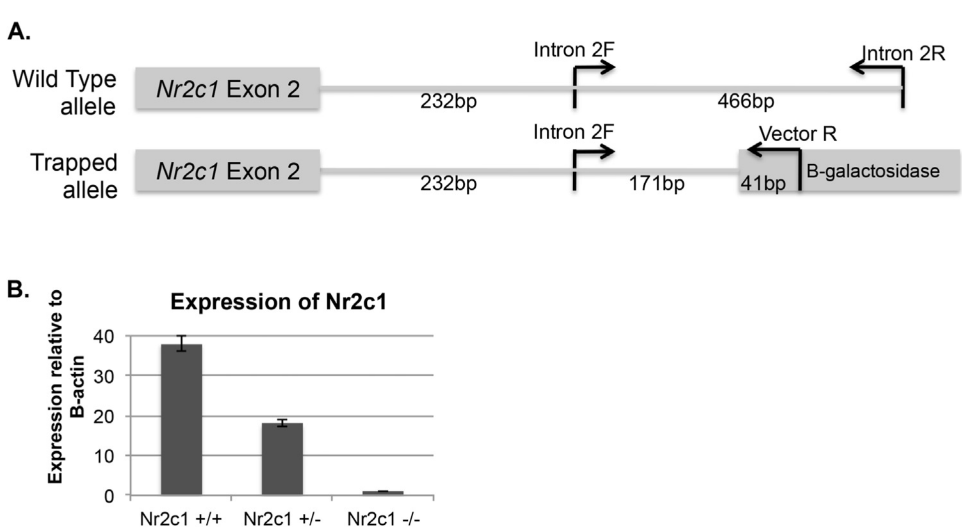

Fig. 2. Expression of *Nr2c1* is ablated in knockout mice. A) Gene trap insertion was located within exon two of the *Nr2c1* gene. Direct sequencing identified that the gene trap insertion lacks the first 128 bp of the beta galactosidase intron one, and that the site of the gene trap insertion is 403 bp from the end of intron 2 of the *Nr2c1* gene. **B)** Quantitative Real Time PCR shows reduced expression in heterozygotes and complete loss of *Nr2c1* expression in homozygous animals. Expression relative to β-actin. Mean ± standard error.

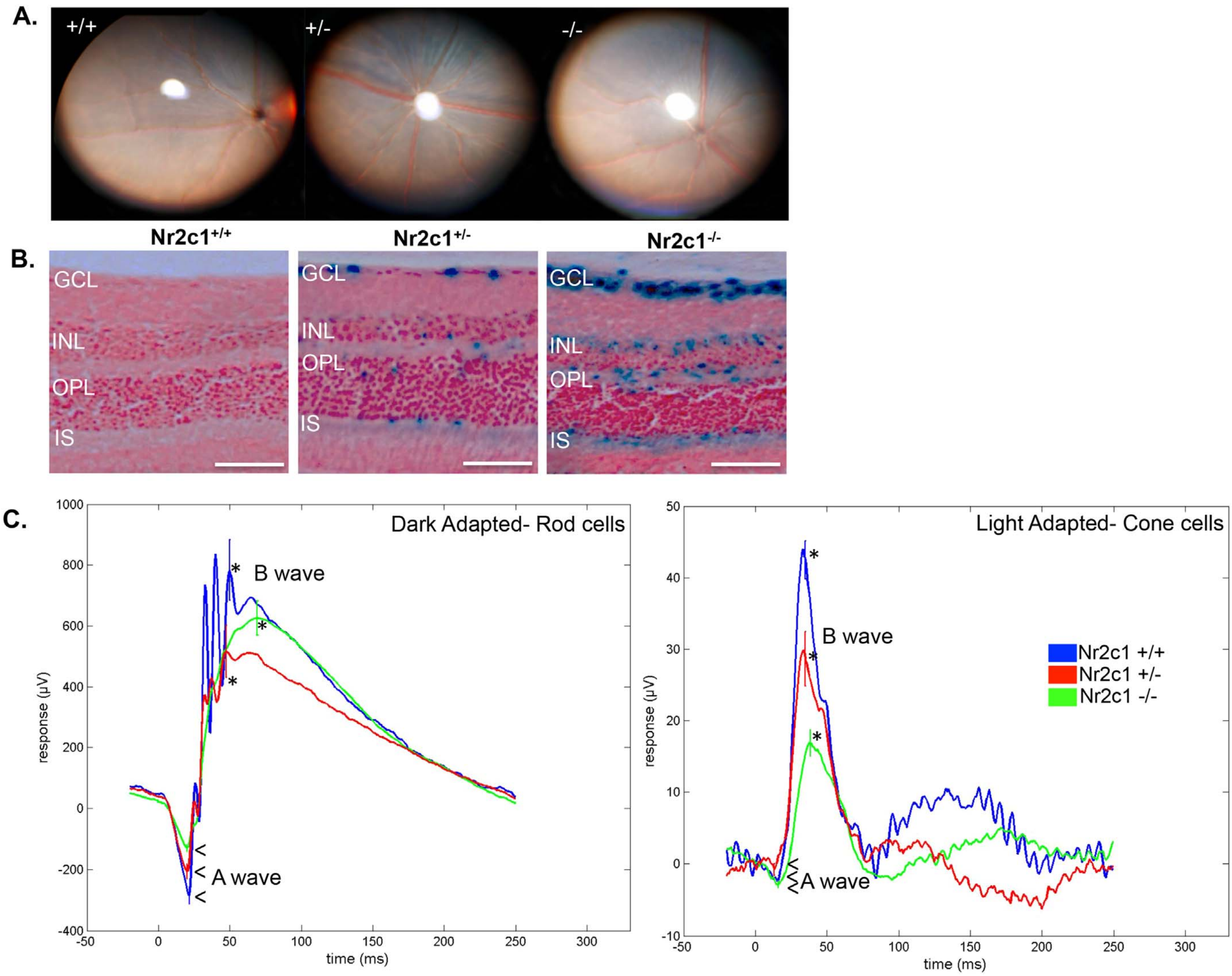

Fig. 3. Clinical and functional characterization of mutant mice shows the loss of *Nr2c1* results in reduce cone and rod function. A) Clinical characterization showed no apparent abnormalities in the fundus of knock out mice compare to wildtype animals. **B)** X-gal staining (blue) expression in $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retinas was highest in ganglion cells with lighter staining in the inner nuclear, outer plexiform layer, and cone photoreceptors. Increase number of cells in the ganglion layer of $Nr2c1^{-/-}$ retinas is seen. Bar: 50 µm. n = 10 **C)** Dark- and light-adapted electroretinogram recordings show sequential reduction of a and b wave response in $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ compared to wildtype normal retinas. Graphs represent average of 30 responses obtained in replicated experiments for a n = 10/genotype of P30 animals.

which labels the inner and outer segments (IS,OS) of all cones, show that $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ have a normal number of cone cells suggesting the gradient does not result in reduction in total cone photoreceptor cell number (Fig. 4B). Further while there is loss of uniform green opsin patterning in the $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retinas, there is no loss of total cone cells (as indicated by PNA labeling) and the ventral cone cells only express blue opsin instead of co-expressing both blue and green opsins (Fig. 4B) (Haider et al., 2006, 2009; Applebury et al., 2000). Finally, a cell count for green and blue opsin positive cells was performed that confirmed the immunohistochemistry data (Fig. 4C). Retinal flat mounts labeled with green also illustrate the loss of green opsin expression in $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retinas (Fig. 5A). Whole mount retinas labeled with blue opsin, PNA, and DAPI confirm no change in expression or patterning is observed in $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retinas compared to wildtype (Fig. 5B). Interestingly, our initial observations revealed significant variation in the green opsin gradient phenotype. We observed reduced penetrance of the green opsin gradient as the genetic background shifted from 129sv/J to B6 (Fig. 6). Approximately 80% of $Nr2c1^{-/-}$ at N1 exhibited a gradient green opsin phenotype compared to < 20% at N3, suggesting a strong modifier effect from the B6 allele can suppress the green opsin phenotype (Fig. 6). There are several examples of genetic modifier genes in the retina and other tissues that influence time of disease onset and progression (Houlston and Tomlinson, 1998; Cruz et al., 2014). To be consistent, these studies were performed using only $Nr2c1^{-/-}$ animals that exhibit the green opsin gradient phenotype.

2.4. Nr2c1 promotes cell proliferation and topography of early retinal cell types

To determine if other early retinal cells are also impacted by loss of *Nr2c1*, immunohistochemistry was performed on P30 animals of each genotype. Histological analysis showed an increase of cells in the ganglion cell layer, which is typically a single layer comprised of ganglion cells and a small population of displaced amacrine cells. There are over 30 different types of amacrine cells with distinct morphological characteristics and functions (Cherry et al., 2009). While there are not sufficient unique molecular markers to identify each subtype of amacrine cells, antibodies such as the calcium binding

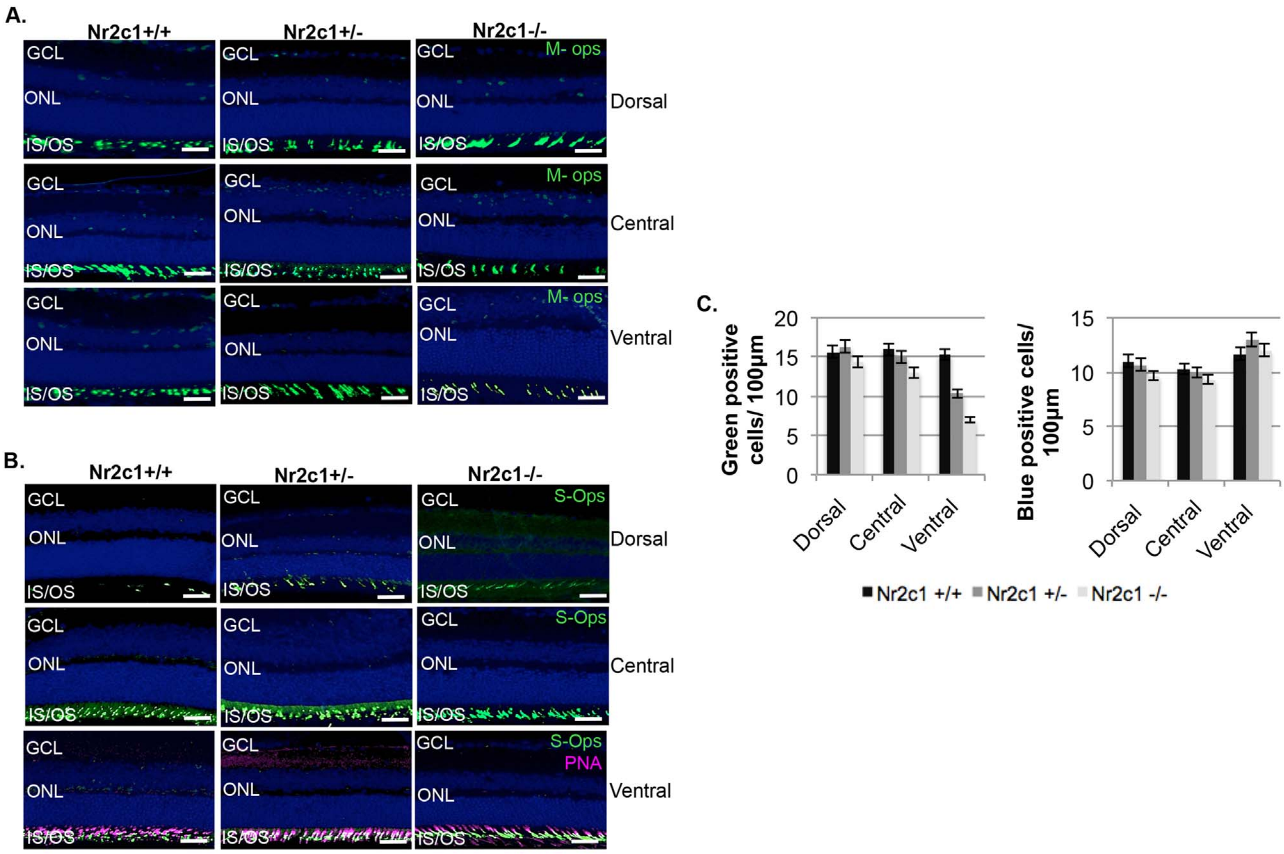

Fig. 4. Green Opsin (M-opsin) Expressing Cone Cells Affected In Central and Ventral Retina of *Nr2c1* Mutant Retinas. A) Green opsin (M-opsin) expressing cone cells reduced in ventral and central, regions of the $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retina but not in the dorsal region. **B)** Blue opsin (S-opsin) double labeled with peanut agglutinin (PNA) for all cones in the ventral retina show no difference between normal and *Nr2c1* mutant retinas. IS: inner segments; OS: outer segments; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Bar: 50 µm. **C)** Cell count of green and blue opsin cells confirm IHC section and whole mount data showing loss of green opsin expressing cells in the ventral retina of $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$. scale bar: 50 µm. Paraffin embedded sections of P30 animals. N = 20 for IHC of each genotype (A, B); N = 5 for C.

protein parvalbumin and choline acetyl transferase (CHAT) label all amacrine cells (Cherry et al., 2009; Massey and Mills, 1999). Labeling of amacrine cells with parvalbumin and CHAT antibodies showed an increase in the number displaced amacrine cells in the ganglion cell layer (Fig. 7A,B). In contrast, when labeling ganglion cells with Beta Tubulin III we observed no difference between normal and *Nr2c1* mutant retinas (Fig. 7C). Blinded serial section cell counts. confirmed an increase in Parvalbumin and Chat positive amacrine cells. No changes were observed in the number of Beta Tubulin III positive ganglion cells between normal and *Nr2c1* mutant retina. No significant differences were observed in $Nr2c^{-/-}$ or $Nr2c1^{+/-}$ horizontal cells labeled with calbindin compared to $Nr2c1^{+/+}$ (Fig. 7D). Importantly, the increase in amacrine cells phenotype, unlike the green opsin gradient phenotype, is fully penetrant and observed in all $Nr2c1^{-/-}$ mutants of each generation.

2.5. Phototransduction is disrupted in Nr2c1 mutant retinas

As observed earlier in ERG analysis, *Nr2c1* mutants ($Nr2c1^{+/-}$ and $Nr2c1^{-/-}$) show severe vision loss (Fig. 3C). In order to determine if components of the phototransduction signaling cascade may be impacted by loss of *Nr2c1*, we evaluated the expression and localization of cone and rod specific phototransduction genes of $Nr2c1^{+/+}$, $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ in P30 retina sections. There is no significant difference in expression of visual Arrestin 1 (Arr1), that labels both rods and cones (Song et al., 2011), (Fig. 8A) cone transducin, GNAT2, (Fig. 8B) or rod transducin, GNAT1 (Fig. 8C) between normal and mutant retinas.

2.6. Late phase retinal cells are not affected by Nr2c1

We performed IHC analysis of P30 mutant and normal *Nr2c1* animals to determine if cells generated in the late phase of retinal development (rods, bipolar, and müller glia cells) are impacted by the loss of *Nr2c1*. Rods labeled with rhodopsin show no changes in normal versus *Nr2c1* mutant retinas (Fig. 9A). Similarly, there appears to be no difference between normal and *Nr2c1* mutant retinas of rod bipolar cells labeled with PKCα (Fig. 9B). PKCα is mainly expressed in rod bipolar cells (Fyk-Kolodziej et al., 2002; Fukuda et al., 1994; Haverkamp and Wassle, 2000) and can be found in the cell body as well as in the dendrites and synaptic terminals (Wassle, 1991; Vaquero et al., 1997). The Müller glial cells, which differentiates in the second phase of retinogenesis (Reichenbach et al., 1995), labeled with vimentin and glial fibrillary associated protein (GFAP) also showed no clear differences in normal versus *Nr2c1* mutant retinas (Fig. 9C, D).

The results of the ERG suggest that abnormalities in the synaptic connections between photoreceptors and higher order neurons may impact vertical transmission. To elucidate synaptic contribution to ERG loss in *Nr2c1* mutant retinas, we evaluated expression of pre- and post-synaptic markers. No significant changes in expression or localization were observed for the post-synaptic marker PSD-95, found in the axon terminals of photoreceptors (Fig. 10A) (Koulen et al., 1998) In contrast, Synaptophysin, expressed within the synaptic vesicles of both OPL and less in IPL of normal retina, is mislocalized with significant increased expression in the IPL of $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ mice (Fig. 10B). Similar to PSD-95, the synaptic ribbon marker C-terminal

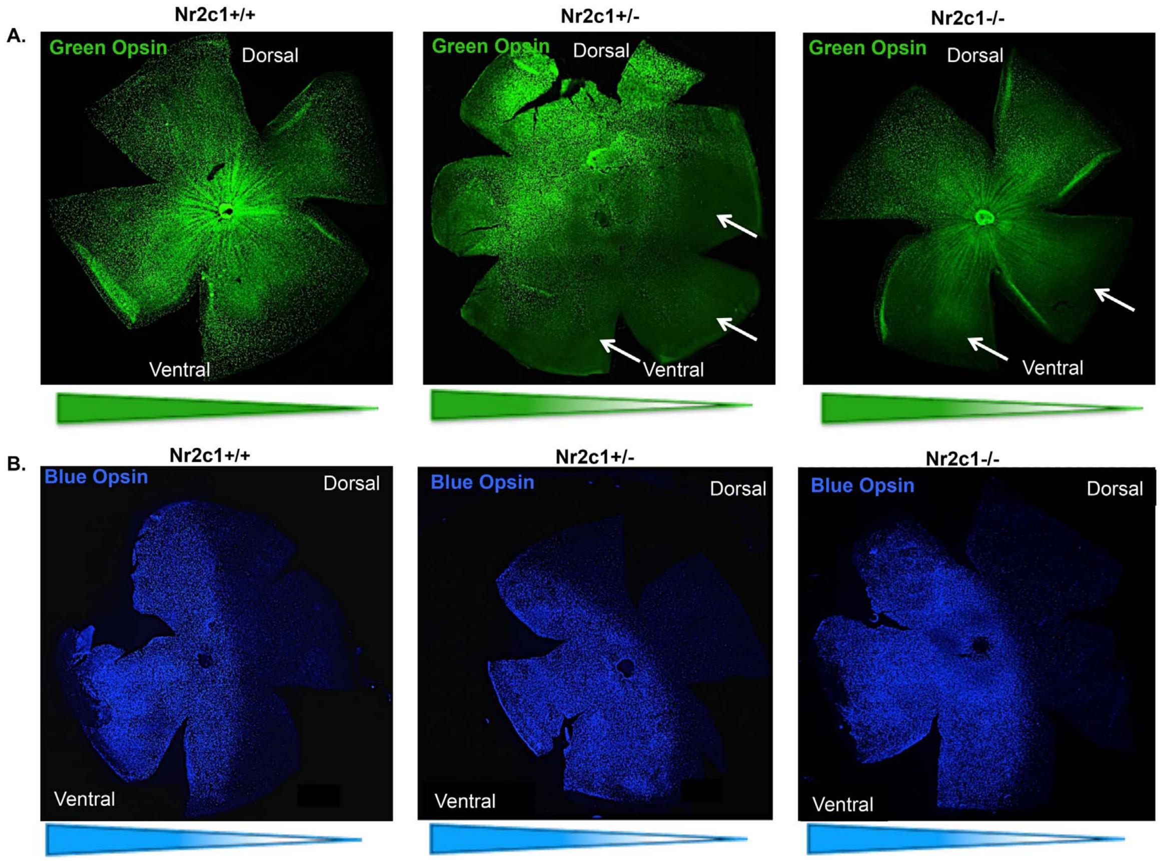

Fig. 5. Cell pattering is disturbed in the absence of *Nr2c1*. Retinal flat mounts for green opsin show a reduction in expression for the mutant mice in the ventral region compare to the uniformal expression in *Nr2c1*$^{+/+}$retinas. Arrows indicate the area where there is diminished green opsin expression. In contrast flat mounts for blue opsin shows no change in ventral concentration of blue opsin expressing cones in mutant retinas. N = 3/genotype.

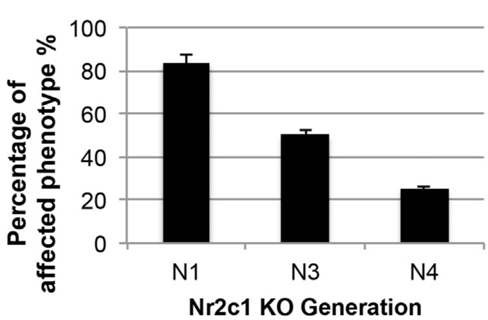

Fig. 6. Reduced penetrance of green opsin gradient indicates strong modifier gene effect from B6 background. Animals were bred from 129sv/J to B6 at each back-cross (N) generation. Percentage of animals in the incipient congenic lines found to have a *Nr2c1*$^{-/-}$ genotype and gradient expression of green opsin.

binding protein 2 (CTBP2) shows no significant difference between normal and mutant *Nr2c1* retinas (Fig. 10C).

2.7. Direct binding of NR2C1 and regulatory elements in early cone and amacrine and phototransduction genes

Our studies reveal two main cells affected by loss of *Nr2c1*: cone and amacrine cells. To identify genes regulated by NR2C1 that may impact the development and or function of cone or amacrine cells, we performed chromatin IP (ChIP) and quantitative real time PCR analysis (Supplementary Table 1). Retinal progenitor and phototransduction genes were scanned for variations of putative NR2C1 and general nuclear hormone response elements (RE) sequence AGGTCA n AGGTCA of NR2C1 (Olivares et al., 2015). A maximum of 100 kilobases (kb) upstream region of each gene to the end of intron 1 was scanned to identify RE sites. Genes containing putative NR2C1 binding sites were further evaluated by ChIP and qPCR at E14, the peak for *Nr2c1* gene expression and the peak of the early phase of retinal cell proliferation, and P30. Chromatin IP experiments revealed NR2C1 binds to 28 development genes that impact retinal progenitors and differentiating cone and amacrine cells at E14 (Fig. 11A) and 4 of the 28 genes are specific to cone and amacrine function in the mature retina at P30 (Fig. 11B). These results are congruent with the IHC observations made for the each retinal cell type.

Quantitative real time PCR (q-RTPCR) was performed to determine if genes putatively targeted by NR2C1 at P30 are misexpressed in *Nr2c1*$^{+/-}$ or *Nr2c1*$^{-/-}$ retinas (Fig. 12). At present, it is not possible to evaluate the E14 putative NR2C1 target genes for misexpression as there is no distinguishable phenotype in mutants at that age. Interestingly, *Satb2,* a key amacrine cell fate factor, expression was significantly decreased in both *Nr2c1*$^{+/-}$ and *Nr2c1*$^{-/-}$ animals. *Satb2* specifically regulates cell fate change in the progenitor cells to generate glycinergic amacrine cells (Balasubramanian and Gan, 2014; Kay et al., 2011). Similar to immunohistochemical observations, blue opsin showed no significant difference in expression between normal and mutant *Nr2c1* retinas. Parvalbumin did not show significant difference in expression in *Nr2c1*$^{-/-}$ but seems to be modestly increased in *Nr2c1*$^{+/}$ retinas. *Satb2* expression is reduced in both *Nr2c1*$^{+/-}$ and *Nr2c1*$^{-/-}$ retinas compared to normal. Interestingly, there was increased expression of green opsin transcript in the *Nr2c1*$^{-/-}$ animals. This contrasts with protein expression showing reduction of cells

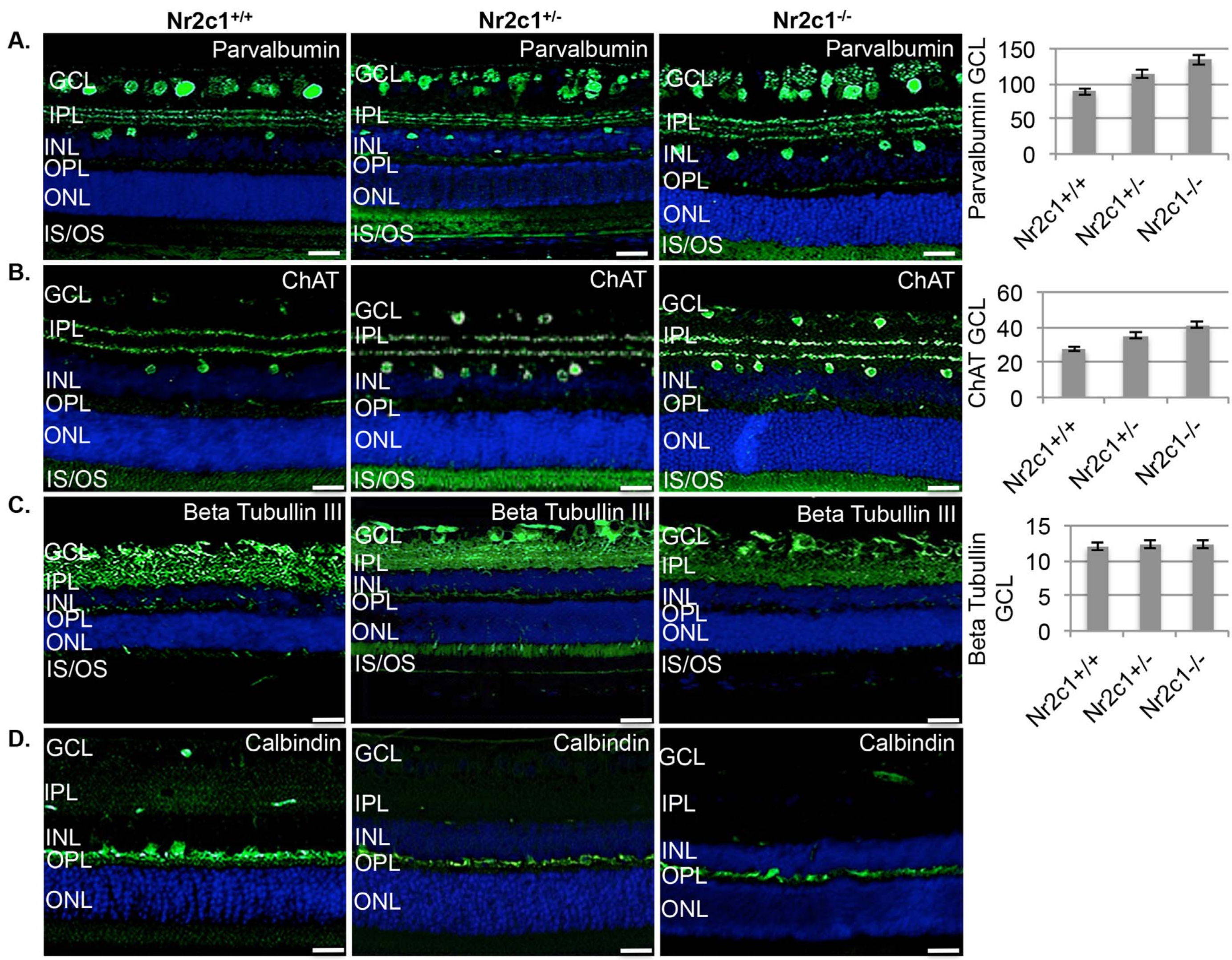

Fig. 7. Loss of *Nr2c1* affects amacrine and cone cells. A) Increase of parvalbumin positive amacrine cells displaced in GCL of *Nr2c1*$^{+/-}$ and *Nr2c1*$^{-/-}$ mutant retinas. **B)** ChAT positive amacrine cells also show modest increase of cells in the ganglion layer. **C)** No major differences observed in ganglion cells labeled with Beta Tubullin III. **D)** No difference observed in Calbindin expression in horizontal cells of *Nr2c1*$^{+/-}$ and *Nr2c1*$^{-/-}$. IS: inner segments; OS: outer segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. N = 3 for the cell count and N = 20 for IHC. scale bar: 50 µm. Paraffin embedded sections of P30 animals.

expressing green opsin. It is possible that the retina is producing more green opsin message to compensate for the loss in the number of cone cells expressing green opsin.

3. Discussion

In this study, we identified a novel and important role for the nuclear hormone receptor gene *Nr2c1* in the retina. Development is a dynamic complex process requiring concerted effort of many biological pathways converging to generate specific tissues. Nuclear hormone receptors are well known to play a major role in the development of many tissues including the retina. The mammalian retina undergoes an overlapping and biphasic period of development to generate all seven retinal cell types. Early cells such as the ganglion, cone, amacrine, and horizontal cells are generated during E10.5-E18.5 with a peak around E14.5 while late cells begin proliferation at approximately E12.5 and continue to develop to P5 in the central retina and P11 in the peripheral retina (Marquardt and Gruss, 2002; Zhang et al., 2006; Wang and Cepko, 2016). Our studies reveal *Nr2c1* expression profile coincides with peak time of progenitor cell proliferation of early retina cells. Consistent with this expression profile, ChIP-qRTPCR revealed that NR2C1 targets genes regulating early retinal types during development. Importantly, no changes were observed in late phase retinal cell types.

Development involves proliferation, differentiation and cell patterning to establish proper topography. These complex processes require the careful orchestration of multiple genes and networks. Cone photoreceptor cell patterning is regulated by the interaction and many transcription factors and their ligands including *Nr2c1*, *Trβ*, thyroid hormone, and retinoic acid (Fujieda et al., 2009; Haider et al., 2009; Gan and Flamarique, 2010). In vitro studies show *Nr2c1* regulates genes in the retinoic acid signaling pathway. This pathway is known to regulate the patterning of S opsin expression (Lin et al., 1995). It has been demonstrated that both RORα (RORA) and RORβ have a common binding site in the promoter region of the *Opn1sw* gene (Fujieda et al., 2009). Green opsin, however, is regulated by Trβ2. which plays a role in opsin expression by inhibiting S-opsin and promoting M-opsin expression in cone cells (Yanagi et al., 2002). Loss of *Trβ* thus results in all cones expressing only blue opsin (Roberts et al., 2006; Ng et al., 2001). Our study on NR2C1 further expands knowledge of cone cell patterning. Loss of *Nr2c1* results in gradient distribution of the normally evenly distributed green opsin.

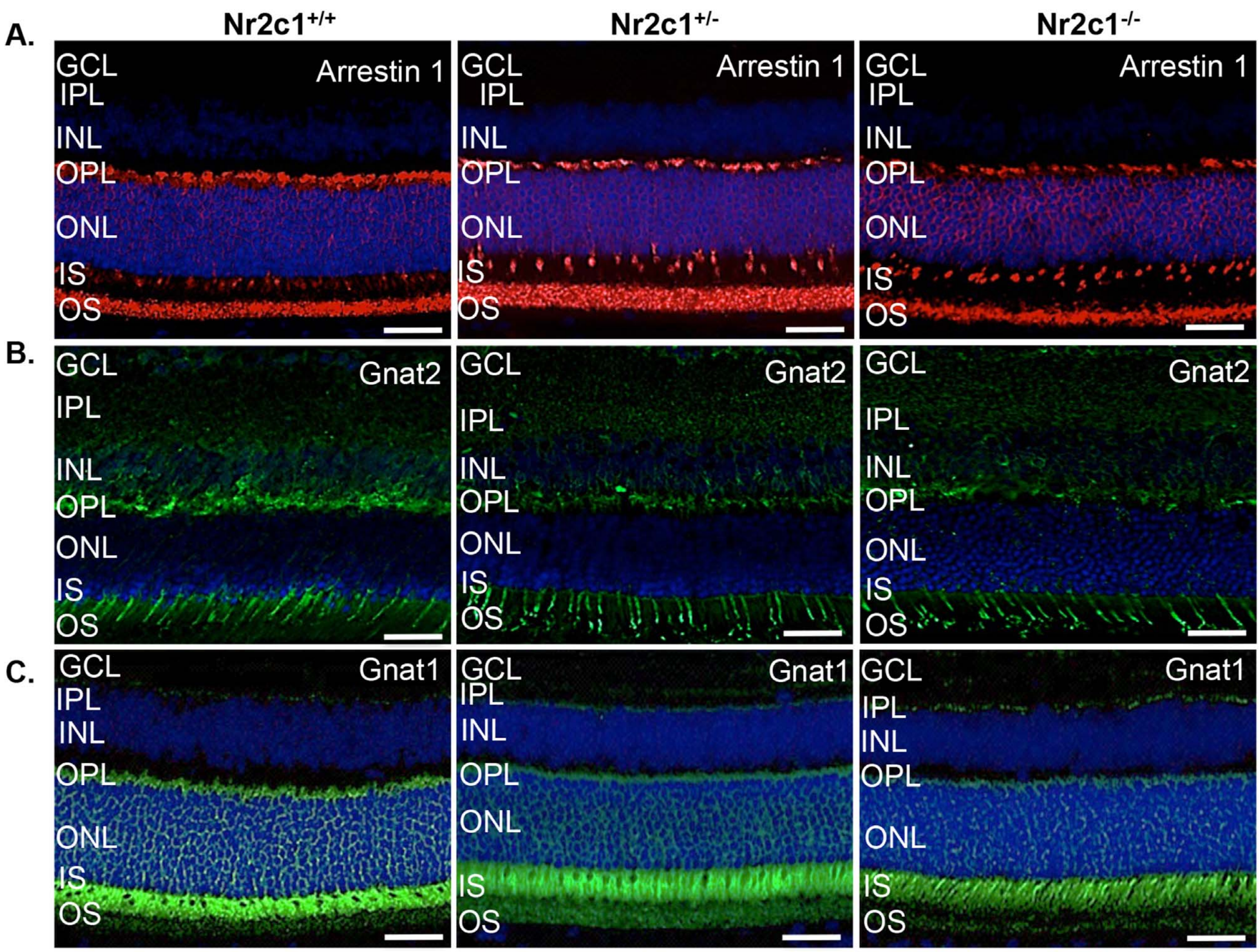

Fig. 8. Phototransduction genes are not misexpressed in *Nr2c1* Mutant Retina. No observable difference in **A**: Visual Arrestin 1 expression in rod and cone cells. **B:** Cone cells expressing Gnat2. Or **C**: Rod cells expressing Gnat1 of *Nr2c1* mutant vs normal retina. IS: inner segments; OS: outer segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. N = 20 scale bar: 50 µm. Paraffin embedded sections of P30 animals.

Additionally, ChIP results suggest NR2C1 regulates both *Trβ* and RORA expression in the developing retina. That loss of *Nr2c1* does not lead to complete loss of green opsin expression or change in blue opsin patterning or expression, suggests that other factors work with *Nr2c1*, *Trβ* and RORA in concert and are able to mitigate total cell loss.

Interestingly, we observed that expression of one of the key factors of amacrine cell fate, *Satb2*, was reduced in both $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ animals. Further, we noticed an increase in displaced amacrine cells in the GCL of both $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retinas. These results indicate that *Nr2c1* also has a key role in early retinal cell proliferation. *Satb2* promotes *NeuroD6* expression*;* previously shown to interact with *Nr2c1*, to direct the cells towards a glycinergic amacrine fate (Balasubramanian and Gan, 2014; Xiang, 2013). In addition to the developmental abnormalities, loss of *Nr2c1* resulted in cellular disorganization within the mature retina and had a severe impact on retinal function. Further, a shift of the synaptic ribbon marker, Synaptophysin, into the IPL of *Nr2c1* mutant retinas was observed. This shift may contribute to halted vertical transmission of electrical stimuli as observed in the ERG responses of $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ mice. Our ChIP studies also reveal a role for NR2C1 in the mature retina to modulate phototransduction genes and transcription factors that are key to normal retinal function.

Interestingly, shifting the *Nr2c1* mutation onto a B6 genetic background resulted in reduced penetrance of only the green opsin phenotype, suggesting there is a potent genetic modifier gene from B6 that modulates cone cell patterning. Reduction in penetrance was observed immediately after one generation of backcrossing and was reduced by 80% in N3. Genetic modifiers are allele variants that are "normal" not mutations (disease associated) and are capable of influencing the disease onset, progression, and severity (Cruz et al., 2014; Haider et al., 2002; Nadeau, 2001). As there are over 100 inbred strains of mice, many genetic modifier genes have been discovered by shifting genetic backgrounds in mice that harbor mutations, including those that modulate retinal degeneration (Cruz et al., 2014; Rozmahel, 1996; Dietrich et al., 1993; Bourdeau et al., 2001; McCright et al., 2002). Our prior studies mapped and identified genetic modifier genes for $Nr2e3^{rd7/rd7}$ (*rd7)*. The $Nr2e3^{rd7/rd7}$ model has been extensively evaluated to study the heterogeneity observed in retinal degeneration diseases associated to the *Nr2e3* gene, underscoring the importance of genetic modifier genes in understanding disease pathology, etiology, and in developing strong therapies (Wright et al., 2004; Haider et al., 2000; Cheng et al., 2011; Bernal et al., 2008; Escher et al., 2009; Bandah et al., 2009).

The present study revealed a novel and crucial role for *Nr2c1* in regulating cell-fate specification and cell patterning in the retina and is critical for the proper development of early cell types such as ganglion cells. Interestingly, *Nr2c1* is also expressed in the developing and mature brain. Future studies include understanding the role of *Nr2c1* in the brain and further deciphering the complex interaction of *Nr2c1*, thyroid hormone, and retinoic acid in regulating neuronal cell fate. Recent studies implicate *Nr2c1* in cancer (Hu et al., 2002). We will also evaluate the potency of *Nr2c1* for stem cell and cancer related

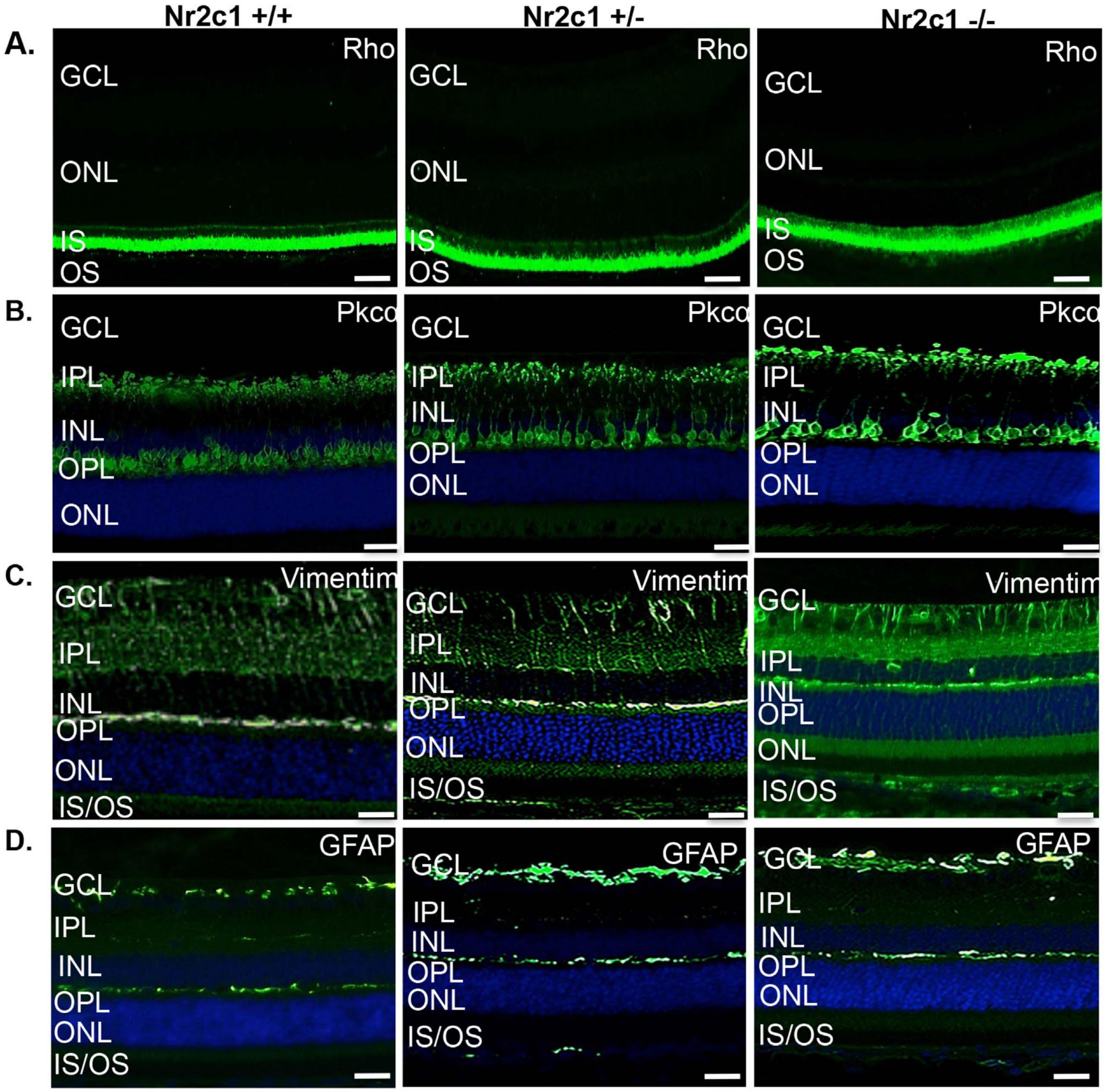

Fig. 9. Loss of *Nr2c1* shows no loss of bipolar or Müller glial cells and no evidence of reactive gliosis. Late retina cells show no observable phenotype in *Nr2c1*$^{+/-}$ and *Nr2c1*$^{-/-}$ compared to wildtype **A)** rods labeled with rhodopsin. **B)** rod bipolar cells labeled with PKC alpha. **C)** Müller glia cells labeled with vimentin **D)** Müller glia cells labeled with GFAP show no evidence of reactive gliosis in *Nr2c1*$^{+/-}$ and *Nr2c1*$^{-/-}$ retinas. IS: inner segments; OS: outer segments; ONL: outer nuclear layer; INL: inner nuclear layer; OPL: outer plexiform layer; GCL: ganglion cell layer. N = 20 scale bar: 50 µm Paraffin embedded sections of P30 animals.

therapies. *Nr2c1* is clearly an important transcription factor in the central nervous system and likely also plays an important role in stem cell and cancer biology and could serve as a novel factor to be considered for targeted therapeutics.

4. Materials and methods

4.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal use and procedures were approved by the University of Nebraska Medical Center Animal Care and Use Committee and the Schepens Eye Research Institute Animal Care and Use Committee (Permit Number: S309-0714) in compliance with the Animal Welfare Act Regulations. All efforts were made to minimize animal suffering.

4.2. Generation of knockout mice

A gene trap with a random integration of beta galactosidase (9 kb) into intron two of *Nr2c1* was obtained from the Sanger International Gene Trap Resource (gene trap embryonic stem cell line XS0212). Blastocyst injection and generation of chimeras were performed at the University of Nebraska Medical Center, Mouse Genome Engineering Core Facility. Chimeras were crossed to C57BL/6 (B6) mice and agouti pups genotyped for transmission of the gene trap with primers specific for beta galactosidase (F: CAACAGTTGCGCAGCCTGA, R: GTAATGGGATAGGTCACGTTG). The insert location was identified through direct sequencing across intron two of the full-length *Nr2c1*

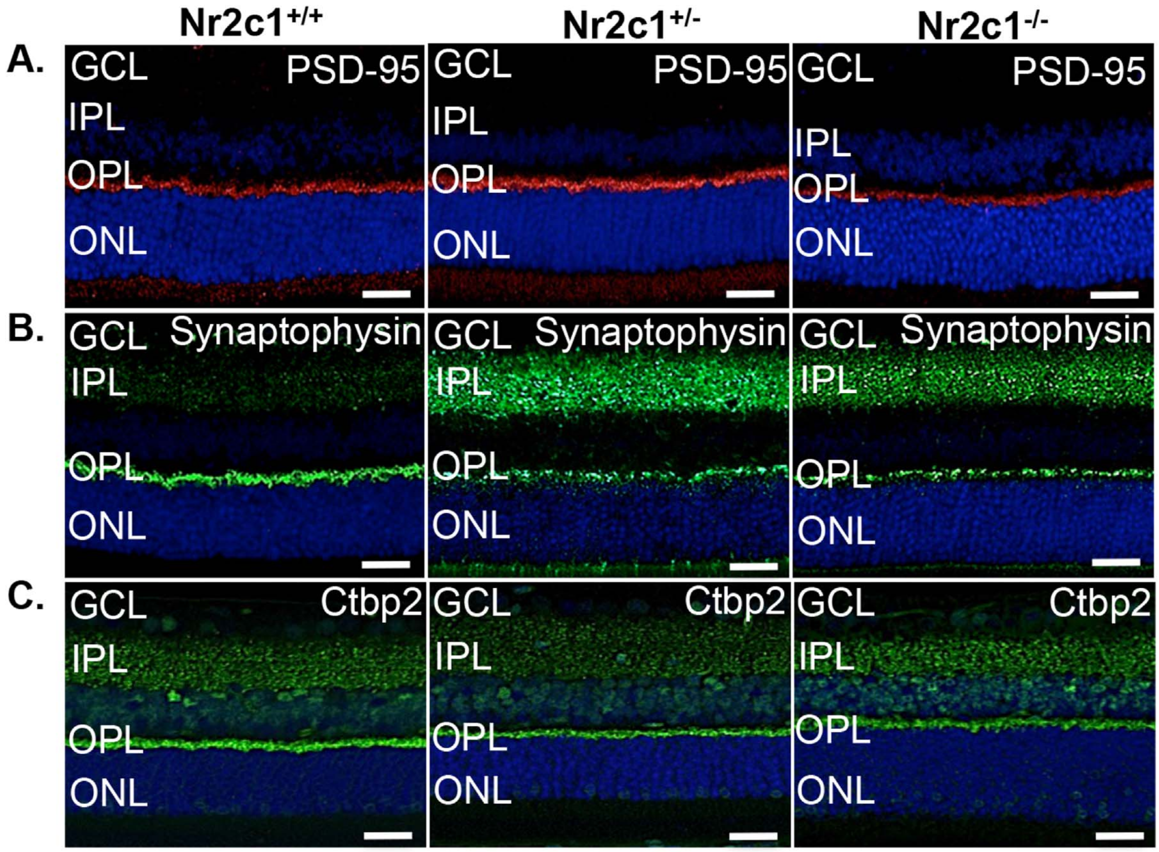

Fig. 10. Loss of *Nr2c1* results in mislocalization of synaptic ribbon marker Synaptophysin. Synaptic markers No observable difference between $Nr2c1^{+/+}$ and $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retinas in **A)** PSD-95 (post synaptic) expression or **B)** Ctbp2 (synaptic ribbons). **C)** Synaptophysin which labels synaptic vesicles however, shows a slight reduction in expression in the OPL and remarkable increase in IPL mislocalized expression. In both $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ retinas compared to normal. ONL: outer nuclear layer; OPL: outer plexiform layer; IPL: inner plexiform layer; GCL: ganglion cell layer. n = 20. scale bar: 50 µm. Paraffin embedded sections of P30 animals.

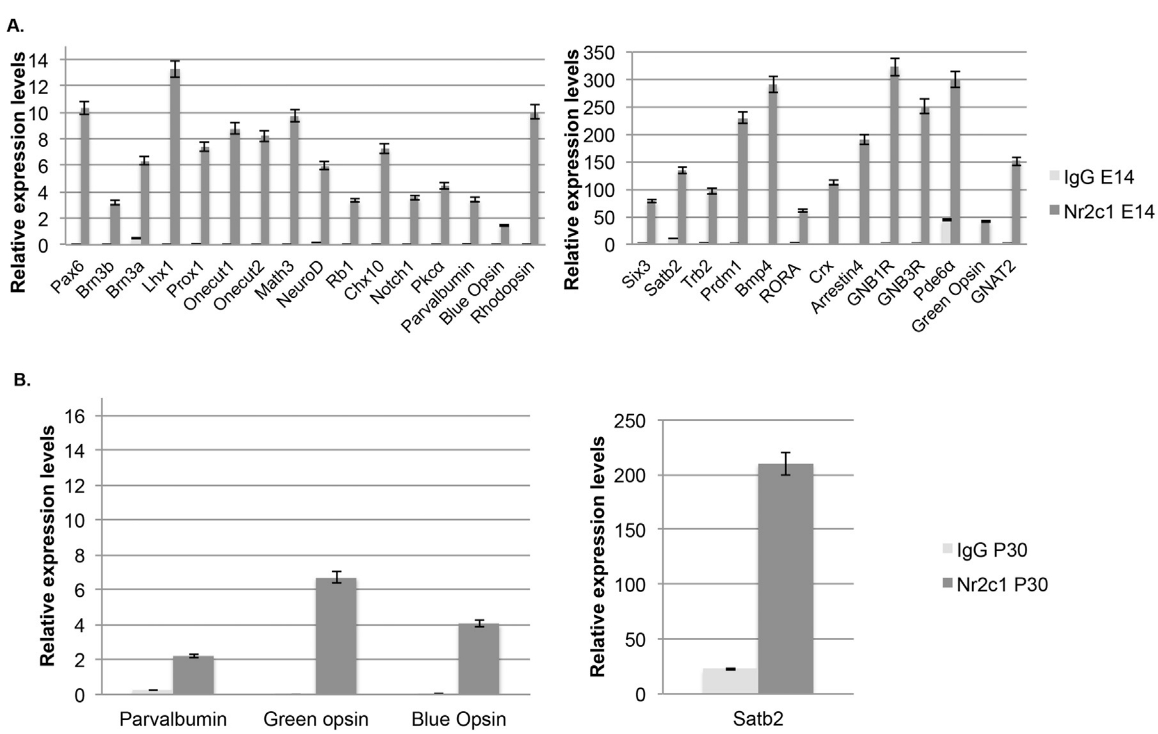

Fig. 11. *Nr2c1* regulates retinal progenitor and early amacrine and cone genes. ChIP-qPCR analysis of putative NR2C1 targets E14.5 and P30.

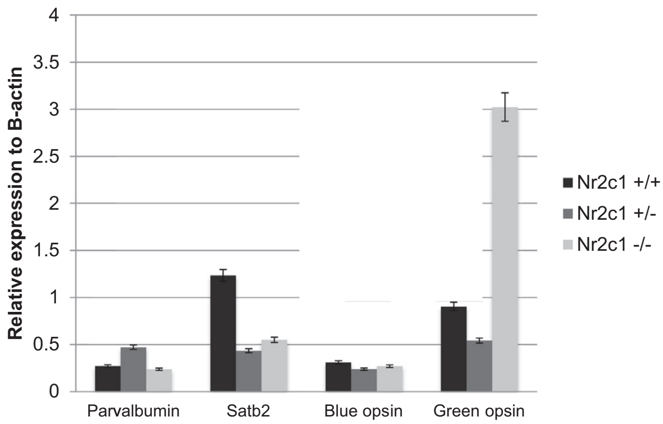

Fig. 12. P30 expression of *Nr2c1* putative cone and amacrine target genes. Differential gene expression of cone and amacrine targets of NR2C1 identified in Fig. 11 by ChIP. Expression normalized to β-actin.

transcript. F2 N3 Mice on a B6 and 129X1/SvJ background were characterized for clinical, functional and histological abnormalities.

4.3. PCR Amplification for Nr2c1 Genotyping

The 9 kb beta galactosidase insertion is located at the 3′ region of intron two in the *Nr2c1* gene. To differentiate between heterozygous and knockout mice, primers were designed to amplify the wild type and trapped *Nr2c1* alleles in a multiplexed PCR system The primer sequences were as follow: *Nr2c1*genoint2F: GCCTTAGGATA-GGAGGGGCTC, *Nr2c1*vectorgeoR: CTCAAACTCTCCTCCTTCCCTC, *Nr2c1*genoint2R: CCTGACGTGC CCAGTGTCTTG. A 10-μL PCR reaction volume containing 10x concentrated buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl ,15 mM $MgCl_2$), 40 mM dNTP, 10 μM each of forward and reverse primer,0.5U Taq polymerase. PCR reactions were performed using the following cycling conditions: 95 °C for 3 min, followed by 34 cycles at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 45 s. A final elongation step consisted of a 5 min extension period at 72 °C. PCR products were electrophoresed on a 2% agarose gel to decipher two bands at 171 bp for trapped alleles, and 466 bp for wild type alleles. B6 and ES cell DNA (from generation of chimeric mice) were used as controls.

4.4. Quantitative real time reverse transcriptase PCR

To confirm loss of *Nr2c1* expression in mutant retinas, retinas from mice (*$Nr2c1^{+/+}$, $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$),* were dissected. To determine the expression profile of *Nr2c1* in the retina, eyes were pooled for the embryonic time points (n = 10) and for the post natal time points each animal was done separated from E10.5, E12.5, E14.5, E16.5, E18.5, P0.5, P2.5, P6.5, P14.5, and P30.5 B6 mice.

Total RNA was isolated using Trizol as previously described (Haider et al., 2009). Two micrograms of total RNA were reverse transcribed using Retroscript (Ambion). DNase treatment was performed with DNA-*free*™ kit from Applied Biosystems AM1906. PCR amplification of cDNA was performed using the PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA) with 1 μL of 1:100 diluted cDNA, and performed using primers for *Nr2c1*: F: GCCAAGGCCATATAC-CTGTTTC, R: CTGCTTCCGGGACATGACA, and *beta actin* (control): F: ATGCCTCCCCTACCAATCTTC, R: GGATAACGTCCAGGGAACCA at 55 °C. PCR products were electrophoresed on a 2% agarose gel in triplicate.

4.5. X-gal staining

Eyes from $Nr2c1^{+/+}$, $Nr2c1^{+/-}$ and $Nr2c1^{-/-}$ mice were collected from mice age 1–3 months and fixed in 0.2% glutaraldehyde for 30 min. Samples were rinsed 3 times in wash buffer and incubated with X-gal staining solution overnight at 37 °C with mild rotation as described previously (Sanes et al., 1986). Samples were rinsed and fixed for 2 h in 4% paraformaldehyde and processed for paraffin embedding. 20 μm sections were deparafinated and counter-stained with nuclear Fast Red. Images were collected on a Zeiss Axioplan II microscope. n = 10 biological replicates/genotype.

4.6. Fundus photography

Pupils were dilated with 1% atropine sulfate and mice were examined before functional and histological analysis was performed. Images were captured on a Kowa Genesis digital fundus camera (Kowa Company Ltd., Japan). A 90-mm diopter lens (Volk, Mentor, OH) was held in place under the camera lens so that it filled the field of view (Hawes et al., 1999). Wild-type, heterozygous, and homozygous mutant mice were imaged at P30.5 and P60.5 for clinical abnormalities of the retinal fundus; n = 10 biological replicates/genotype.

4.7. Electroretinography (ERG)

ERG recordings for both the right and the left eye were performed on wild-type, heterozygous, and homozygous mutant mice at P30. The recordings were performed using the UTAS E4000 system (LKC Technologies Inc., Gaithersburg, MD). Mice were dark-adapted overnight and anesthetized with an intraperitoneal injection of normal saline solution containing ketamine (120 mg/kg i.p) and xylazine (20 mg/kg i.p). Animal body temperature was maintained at 37 °C using an electric heated platform. Pupils were dilated with 1% tropicamide ophthalmic solution and 2.5% phenylephrine hydrochloride ophthalmic solution applied on the corneal surface. One drop of 0.5% proparacaine ophthalmic solution was administered to each eye then wicked away; next gold loop electrodes were placed lightly on the cornea with a drop of Genteal severe eye gel placed in each loop to keep the cornea hydrated. A needle, inserted subcutaneously in the forehead, served as the reference electrode, while a needle inserted subcutaneously near the tail served as the ground electrode. A series of flash intensities were produced by a Ganzfeld color dome controlled by the Diagnosys Espion3 to test both scotopic and photopic responses. Rod responses are shown for measurements obtained after a light stimulus (white-6500 K; 4 ms pulse period; 6 sweeps; 55,100 ms intersweep delay) with a light intensity of 24.1 (P) cd.s/m2. Cone responses are shown for measurements obtained after 7 min of light adaptation (white-6500K, 30 cd/m^2) with a light intensity of 102.4 (P) cd s/m^2 (25 sweeps, 4 ms pulse period, 1 Hz frequency) produced by a xenon light source with a background white-6500K light of 30 cd/m^2.

The a-wave amplitude was measured from the baseline to the trough of the first negative wave; the b-wave amplitude was measured from the trough of the a-wave to the peak of the first positive wave or, if the a-wave was absent, from baseline to the peak of the first positive wave. Signal processing was performed using EM for Windows v7.1.2. Signals were sampled every 0.8 ms over a response window of 200 ms. For each stimulus condition, responses were computer averaged, with up to 50 records for the weakest signals. A signal rejection window could be adjusted during data collection to eliminate electrical artifacts. n = 10 biological replicates/genotype.

4.8. Histology

Eyes were collected from wild-type, heterozygous, and homozygous mutant mice at P30. Tissues were marked with a cautery prior to enucleation to designate dorsal ventral orientation and fixed in 3:1 methanol: acetic acid solution overnight, embedded in paraffin, sectioned at 5 μm and proceeded to Hematoxylin and eosin staining for a total of 10 animals per each genotype.

4.9. Immunohistochemistry

Immunohistochemistry analysis was performed on 10 µm OCT (E14 eyes and P30 eyes, Fig. 1B), or 5 µm paraffin embedded serial sections from *Nr2c1*$^{+/+}$, *Nr2c1*$^{+/-}$, *Nr2c1*$^{-/-}$ and B6 mice. Eyes for paraffin embedding were enaculated and oriented dorsal to ventral with a cautery and fixed in paraformaldehyde 4% or in methanol/acetic acid (3:1) overnight at 4 °C. Tissue was cut in sections of 5 µm covering an area of 100 µm. Sections were blocked with 2% horse serum (Vector, CA) in PBS and incubated with the following primary antibodies at 1:200 dilution unless otherwise indicated: *Nr2c1* (1:50, rabbit polyclonal, Novus Biological, NBP1-71803), Blue opsin (rabbit polyclonal, Millipore, AB5407), Green opsin (rabbit polyclonal, Millipore, AB5405), Rhodopsin (mouse monoclonal, Millipore, MAB5356), Biotinylated Peanut Agglutinin (Vector Laboratories, B-1075), Parvalbumin (rabbit polyclonal, Abcam, AB11427), Calbindin (mouse monoclonal, Swant, #300), PKC alpha (mouse monoclonal, Santa Cruz, SC8393), Visual Arrestin (Rabbit, Abcam, AB3435), $G_{\alpha t1}$ (Gnat1 1:200, rabbit polyclonal, Santa Cruz, SC389), PSD95 (1:200, rabbit polyclonal, Abcam, AB18258), Synaptophysin (1:200, rabbit monoclonal, Abcam, AB52636), ChAT (1:200, goat polyclonal, Millipore, AB144P), Beta Tubulin III (1:200, rabbit polyclonal, Sigma, T2200), Vimentim (1:200, rabbit polyclonal, Abcam, AB7783). N = 10 biological replicates/genotype. The next day, samples were rinsed and incubated with the corresponding secondary antibody (1:400 Alexa fluor Invitrogen) for 1 h. Images from sections were collected on a Leica DMI6000 B inverted microscope equipped with the appropriate bandpass filters for each flurochrome. Cell counts for IHC were performed using the software ImageJ (NIH, Bethesda, Maryland) in which a section of 100 µm was selected a positive stain cells were count in a double blind form for a total of 3 animals per genotype.

4.10. Chromatin immunoprecipitation and RT-PCR

A total of 8 adult retinas (P30.5) and 18 embryo retinas (E14.5) were dissected and placed in a solution of PBS and PMSF. Crosslink was done using 16% formaldehyde for an hour. Samples were then place in lysis buffer to be sonicated in 30 cycles at 50% amplitude. Immunoprecipitation was done overnight with Nr2e3, RORA and immunoglobulin (Ig) antibody. For the RT-PCR primers were pick using the following parameters: Phototransduction genes as wells as previously reported to be involve in retinogenesis were search for the nuclear receptor response element sequence (RE) for *Nr2c1* (AGGTCA n AGGTCA), as well as REs similar to Nr2e3 (AAGTCA n AAGTCA) and RORA (T/A A/T T/A C A/T A/GGGTCA) (Olivares et al., 2015). RE was selected at a maximum distance of 100kB upstream as well as into the intro 1. Quantitative RT-PCR was performed using 1ul of each sample as wells as the input. For the analysis the samples were normalize to the input.

Competing interests

The authors declare no competing financial interests.

Acknowledgements

This work was supported by the following funding agencies: NIH-NCRR P20-RRO18788-03 (NBH), RO1EY017653 (NBH), NIH-NEI RO1EY017653-01A2S1 (NBH), an unrestricted grant from Research to Prevent Blindness, Inc., New York, NY to Department of Ophthalmology, Harvard Medical School (NBH), Hope for Vision (NBH), Massachusetts Lions Eye Research Fund (NBH), American Macular Degeneration Foundation (NBH), Webster Foundation (NBH), Edward N. and Della L. Thome Memorial Foundation (NBH, MMD), NIH/NEI EY014800 (MMD), an unrestricted grant from Research to Prevent Blindness, Inc., New York, NY to the Department of Ophthalmology & Visual Sciences, University of Utah (MMD), The Skaggs Foundation for Research (MMD), The Carl Marshall Reeves & Mildred Almen Reeves Foundation, Inc. (MMD), and the Macular Degeneration Foundation, Inc. (MMD).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2017.05.021.

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