Excess cone cell proliferation due to lack of a functional NR2E3 causes retinal dysplasia and degeneration in rd7/rd7 mice

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The *rd7* mouse is a model for hereditary retinal degeneration characterized clinically by retinal spot-phenotype results from a splicing error created by a genomic deletion of an intron and part of an exon. Hematoxylin/eosin staining of *rd7* tissue shows that the whorls in the outer nuclear layer of the retina do not appear during embryonic development but manifest by postnatal day 12.5 (P12.5). Furthermore, *in situ* hybridization data indicates that the *Nr2e3* message is first present at barely discernable levels at embryonic day 18.5, becomes abundant by P2.5, and reaches maximal adult levels by P10.5. Results from these experiments indicate that *Nr2e3* message is expressed prior to the development of S-cones. This data coincides with studies in humans showing that mutations in *Nr2e3* result in a unique type of retinal degeneration known as enhanced S-cone syndrome, where patients have a 30-fold increase in S-cone sensitivity compared to normal. Immunohistochemical staining of cone cells demonstrates that *rd7* retinas have an increased number of cone cells compared to wild-type retinas. Thus, *Nr2e3* may function by regulating genes involved in cone cell proliferation, and mutations in this gene lead to retinal dysplasia and degeneration by disrupting normal photoreceptor cell topography as well as cell–cell interactions.

INTRODUCTION

The *rd7* mutation causes an autosomal recessive form of hereditary retinal degeneration. Clinical examination of *rd7/rd7* mice at 1 month of age by indirect ophthalmoscopy shows the presence of evenly spaced white spots across the entire retina (1). Histological examination of these eyes by light microscopy reveals retinal dysplasia with whorls and rosettes in the outer nuclear layer (onl), which attenuate by 5 months and disappear by 16 months (2). These whorls are thought to correspond to the white spots observed clinically as they disappear parallel to onl flattening. Electroretinographs (ERGs) show progressive reduction of cone and rod signals by 16 months such that the amplitudes are reduced to 50% of normal. These recordings correlate to the progressive loss of cones and rods seen histologically (2).

A previous study reported that the *rd7* mouse results from a deletion of exons 4 and 5 in the message of the photoreceptor cell-specific nuclear receptor gene, *Nr2e3* (2). This mutation appears to encompass part of the ligand binding region of *Nr2e3*. Although the function of *Nr2e3* is currently unknown, analysis of the protein sequence reveals significant similarity to the steroid receptor superfamily of ligand-dependent transcription factors. These genes include cellular receptors for steroid hormones and nuclear receptors for hormonal and non-hormonal molecules (3–5). They share signature structural motifs such as DNA and ligand binding and dimerization domains. In addition, the DNA binding region contains a P-box, involved in discriminatory binding of the transcription factor to a response element in the gene it is regulating, and a D-box, thought to be involved in protein–protein interactions (6).

*Nr2e3* was first identified through a search for genes related to *Nr2e1* (formerly *TLX* or *tailless*), an orphan receptor involved in the development of the eye and forebrain (7). Studies in *Drosophila* and *Xenopus* demonstrate the importance of *Nr2e1* in driving pluripotent cells to a particular cell fate (7) and in evagination of the eye vesicle (8). Furthermore, *in vitro* experiments demonstrate that *Nr2e3* is capable of binding to a subset of response element sequences also recognized by *Nr2e1* (9). Thus, *Nr2e3* may have a similar role to *Nr2e1* in driving pluripotent cells to a particular fate.

Recently, the *NR2E3* gene was mapped to human chromosome 15q24 and mutations within this gene have been associated with a unique retinal dystrophy known as enhanced S-cone syndrome (ESCS) (10). In 29 families studied, mutations were found exclusively within the highly conserved ligand or DNA binding regions of the gene. ESCS is an autosomal-recessive retinal disorder that manifests as an increased sensitivity of the S-cones (the least populous cone type) and reduced rod and L/M cone function (11–13). The visual sensitivity mediated by S-cones is significantly higher in patients compared to controls. Most hereditary retinal degenerative disorders affect the mature photoreceptor topography.

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by reducing the number of cells through apoptotic mechanisms. ESCS is the only known retinal dystrophy to date that appears to manifest as a gain in function of a photoreceptor subtype.

Mouse photoreceptor cells include rods (97%) and cones (3%). Unlike humans, who have three types of cone cells, mice have only two types: S-cones (blue cones, sensitive at 350 nm) and M-cones (green cones, sensitive at 510 nm) (14,15). These cone cells develop between embryonic day 10.5 (E10.5) and E16.5, whereas rods develop over a longer period from E12.5 to postnatal day 0.5 (P0.5) (16,17). While many reports indicate two distinct cone populations, a recent report suggests a single cone cell type which co-expresses S (blue) opsin and M (green) opsin, as well as some cone cells expressing only one type of opsin to create a dorsal–ventral gradient in the level of expression of a particular opsin (18). The purpose of this study was to further delineate the role of Nr2e3 in the development and function of the retina. The phenotypes of the rd7 mice and ESCS patients, and the similarity of Nr2e3 to other nuclear receptors, in particular Nr2e1, indicate a possible role for Nr2e3 in photoreceptor cell development. This study reports the causative genomic mutation in the rd7 mouse and examines the temporal changes in retinal morphology of rd7 mice in relation to the gene expression profile of Nr2e3, as well as immunohistochemical data indicating an abnormal abundance of S-cones and total cone cell number in rd7 mice. Together, these data indicate a role for Nr2e3 in repressing the proliferation of photoreceptor cone cells.

RESULTS

Identification of the rd7 mutation

The human NR2E3 genomic sequence (accession no. AJ276674) was used to predict the intron/exon boundaries of mouse Nr2e3. PCR products spanning exons 4 and 5, reported to be deleted in the Nr2e3 message (2), were sequenced in B6Cg-rd7/rd7 and B6 mice. Sequence analysis revealed a deletion of the last 36 nucleotides from exon 4 as well as intron 4 (Fig. 1A and B). In addition, single nucleotide changes within exon 5, including a nonsense codon, were identified. These changes cause a splicing error in which the altered exon4/5, normally part of the ligand-binding region of Nr2e3, are skipped (Fig. 1C).

Fundus examination

Fundus examination was performed on P16.5 F1 mice generated from an F1 intercross of a B6Cg-rd7/rd7 female mouse...
mated with a B6 male. Previous reports indicated retinal spotting at P28.5, the earliest time point examined (1). Examination of P16.5 mice revealed spots across the entire retina, indicating that the phenotype is detectable soon after eye opening (P14.5).

**Phenotype profile of rd7 mice**

Prior to histological examination, rd7/rd7 and B6 (+/+) mice were distinguished by using tail DNA to amplify the region spanning the rd7 deletion. No retinal histological differences were observed by light microscopy between rd7/rd7 and B6 mice at any of the embryonic stages examined. Typical retinal whorls and rosettes are not seen in rd7/rd7 mice at P10.5, begin to appear at P12.5 before the eyes open and persist into adulthood (Fig. 2).

**Validation of Nr2e3 probes for expression studies**

Before performing *in situ* hybridization experiments to determine the gene expression profile for Nr2e3, we determined the validity of the probe used. *In situ* hybridization and northern blot analysis of human retina shows that NR2E3 is expressed exclusively in the retina and specifically within the onl (10). Previous experiments in mouse (2,9) also detect the expression of Nr2e3 in the onl of the retina. This pattern of expression differs from that observed in a recent report, which indicated that Nr2e3 was expressed solely in the retinal pigment epithelium (RPE) and Müller glial cells (19). To investigate this discrepancy, we studied albino mice [used by Chen *et al.* (19)] as well as pigmented mice and generated probes from three regions spanning the Nr2e3 gene. All three probes hybridized to the same distinct transcript, of ~2.5 kb, corresponding to the Nr2e3 mRNA (data not shown). Probe 3 is identical to that used by Chen *et al.* (19). These probes were then used for *in situ* hybridization experiments on eye sections from albino and B6 mice. Our results agree with previous reports (9,10) and indicate that the Nr2e3 message is expressed in the onl and is not detectable in the RPE or Müller glial cells with the present methods. Probe 1 (nucleotides 1–711) was found to be the most robust and was thus used for the gene expression studies described below.

**Gene expression profile of Nr2e3**

Once the discrepancy between the various probes for Nr2e3 and their expression patterns were clarified, a temporal gene expression profile for Nr2e3 was carried out. *In situ* hybridization experiments were performed using probe 1. No message was detected at the embryonic time points prior to E18.5; however, low levels of Nr2e3 expression were observed at E18.5 and P0.5 with moderate expression at P2.5, and maximal adult level expression by P10.5 (Fig. 3). The Nr2e3 message is equally distributed throughout the retina. These results indicate that the Nr2e3 gene is expressed at the transcriptional level prior to S-cone differentiation. Interestingly, the Nr2e3 message was also detected in rd7 tissue by *in situ* hybridization.
Immunohistochemical analysis of retinas from rd7 and control mice

Immunohistochemical analysis using peanut lectin (PNA) and blue opsin antibodies revealed a significant increase in the total number of cone cells in rd7/rd7 retinas compared to wild-type (Fig. 4). Cone cells were not only observed in regions surrounding the whorls but also appeared to completely fill the whorls. Blue opsin expressing cone cells also increased 2–3-fold in rd7 compared to wild-type retinas (Fig. 5). In the dorsal retina, where whorls were rarely observed, not only was there a significant increase in the percentage of cone cells that express blue opsin but spatially they appeared to cluster together. In the wild-type dorsal retina, blue opsin was expressed in ~20% of the total cone cells, whereas in rd7 retinas blue opsin was expressed in 30–50% of the total cone cells in the region. In the ventral region, where whorls were abundant, the most striking feature was observed within each whorl, where as many as 25–65 cone cells were present. This number varies according to the size of each whorl and accounts for the larger standard deviations seen for the cone cell counts from the ventral region. This significant increase in the actual number of cone cells found in rd7 mice may account for the retinal dysplasia observed where the space available for photoreceptor cells is not sufficient to accommodate the increase in total cone cell number.

Figure 3. Temporal expression of Nr2e3 in control mice. In situ hybridization using probe 1. Brightfield image is shown on left, and corresponding antisense image on right. No signal was detected with the sense probe at any of the time points (data not shown). Nr2e3 is expressed in the onl at barely discernable levels at E18.5, is highly expressed by P2.5 and persists through P42.5.
DISCUSSION

Characterization of the rd7 genomic mutation

A previous study by Akhmedov et al. (2) demonstrated that the Nr2e3 mRNA of rd7 mice lacks exons 4 and 5. The genomic mutation underlying the rd7 phenotype is identified here as a deletion of the last part of exon 4 and all of intron 4 as well as the presence of several nucleotide changes in exon 5, including a nonsense codon. The remaining exon 4/5 sequences lack an open reading frame and are skipped during splicing (2). The presence of the nonsense codon suggests that a mechanism such as nuclear scanning may be causing the exons to be skipped (20).

The rd7 mutation lies within the ligand binding region of the Nr2e3 gene. Recent reports suggest that motifs within the ligand binding region of nuclear receptors are required for the association of co-activators and co-repressors (21). Thus, although an Nr2e3-derived mRNA is created in rd7 mice, the protein is likely to be functionally inactive as it would be unable to provide a recognizable ligand binding site and/or site for the association of potential transcription co-activators or co-repressors.

Expression of Nr2e3 in the retina

As previous reports were contradictory with regard to the localization of Nr2e3 within the retina cell layers (9,10,19),

Figure 4. Total and S-opsin expressing cone cells in the dorsal and ventral retinas of rd7/rd7 mice compared to controls. Immunohistochemistry of rd7 and wild-type retinal sections using PNA and blue opsin antibody. Rd7 mice have an increase in S-cones as well as an increase in the total number of cone cells.
several probes were generated from the Nr2e3 sequence in order to clarify the location of Nr2e3 gene expression. Our study consistently observed Nr2e3 mRNA within the onl regardless of the probe used. The Nr2e3 message is observed minimally at E18.5, becomes abundant by P2.5 and persists throughout adulthood. This time course is critical in understanding the putative role that this transcription factor plays in retinal development and function. It appears that Nr2e3 mRNA is expressed minimally immediately following the completion of cone cell development and maximally after the completion of rod cell development. The expression pattern suggests that it may serve as a repressor for cone cell proliferation.

A role for rd7 in cone cell differentiation

The gene defective in rd7 mice, Nr2e3, is a member of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors. It is most similar to the orphan receptor tailless (tll, Nr2e1), which has been implicated in anterior brain differentiation, in particular, the determination of the eye and nose (22). In Drosophila, expression of Nr2e1 drives cells toward optic lobe fate, and the loss of Nr2e1 function results in an increase in the number of Bolwig’s organ cells (7). Similarly, it has been suggested that loss of Nr2e3 function may lead to a default S-cone pathway (10) in which the number of cones expressing S-opsin is increased with a concomitant reduction in L and M cones (23). Previous studies demonstrate that ligands of steroid/thyroid receptors direct progenitors to a cone photoreceptor specific fate (24). More recently, the loss of thyroid hormone receptor β-2 function was shown to lead to selective loss of cones expressing green opsin and an increase in the number of cones expressing blue opsin (25).

Cone cell counts support the hypothesis that Nr2e3 may act as a repressor of S-cone proliferation by clearly showing that rd7 mice, which are Nr2e3 deficient, have more blue opsin expressing cone cells than wild-type mice. This is especially apparent within the whorls where as many as 25–65 cone cells may be found, and within the dorsal region where a significant increase in the percentage of blue opsin expressing cells is observed, suggesting that when altered, cone photoreceptors default to blue cones. This is consistent with reports of S-opsin expression preceding M-opsin expression (14,17); when genes regulating M-opsin expression are non-functional, all cones revert to blue cones (25). Our data also suggest, however, that Nr2e3 may play a larger role in regulating the differentiation of cone cells, as rd7 retinas contained 30–60% more total cone cells than normal (Fig. 5). It has been shown in primate development that cellular interactions between cones lead to proper spatial arrangement, opsin phenotype and proper ratios of mature photoreceptor cells (26–28). Light microscopy data indicate the presence of rd7 whorls at P12. Perhaps at P12 a threshold is reached where the number of cone cells overwhelm the normal area provided for them, thus causing the retinal dysplasia which manifests as whorls.

The rd7 mouse serves as the first model where a gain of photoreceptor cell types results in retinal dysplasia and degeneration. The increase in total cone cells of rd7 mice disrupts not only the normal spatial arrangement of photoreceptor cells but also the proper ratio of total and blue opsin expressing cone cells. The presence of Nr2e3 message in adults, as well as the delay of retinal degeneration in rd7 mice, further suggests a more complex role including the regulation of genes involved in photoreceptor function and maintenance. Identifying genes regulated by Nr2e3 as well as co-factors that contribute to its function will provide further insight into what is required for the maintenance of proper retinal morphology and function.

MATERIALS AND METHODS

Animals

The mice used in this study were bred and maintained under standard conditions at The Jackson Laboratory, C57BL/6J,77-2C2a-rd7/rd7 (B6.Cg-rc7/rd7) female mice were mated to C57BL/6J (B6) males to produce rd7 heterozygous offspring.
These F₂ offspring were then intercrossed to produce mice that were \( N_r2e3^{rd}/N_r2e3^{rd}, N_r2e3^{rd}+/+ \) or +/+ . This breeding scheme was chosen to ensure that both the \( rd7/rd7 \) and wild-type offspring were of the same genetic background. Tissues from F₂ mice were harvested at the following embryonic and postnatal time points: E10.5, E11.5, E16.5, E18.5, P0.5, P2.5, P6.5, P8.5, P10.5, P12.5, P14.5, P16.5, P18.5 and adult (P42.5). A minimum of three \( rd7/rd7 \) and three wild-type mice were analyzed for each time point. Tissues from albino (BALB/cJ) mice were harvested at 8 weeks.

Genotyping

Tail DNA was extracted using standard procedures (29). Homozygous \( rd7 \) animals were distinguished from heterozygous and wild-type controls by PCR analysis of tail DNA using primers designed from the \( Nr2e3 \) gene. A forward primer in exon 4 (5′-GAGCTTGGAAAAACACAGGC-3′) and a reverse primer in exon 5 (5′-CAGGTTGGAAAACACAGGC-AAG-3′) amplified a 339 bp fragment in wild-type animals and a 239 bp fragment in \( rd7 \) mutants and heterozygotes harboring the deletion. The fragments were amplified using 20 ng of tail DNA in a 10.0 µl PCR reaction containing 1× PCR buffer (10 mM Tris–HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.001% w/v gelatin), 250 µM each dATP, dCTP, dGTP, dTTP, 20 pmol of each forward and reverse primer and 0.25 U Taq polymerase. PCR reactions were subjected to the following cycling conditions: 94°C for 3 min followed by 10 cycles of 94°C for 10 s, 60°C for 30 s and 68°C for 60 s, followed by 20 cycles of 94°C for 10 s, 55°C for 30 s and 68°C for 60 s and a final extension of 68°C for 7 min. PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining. The PCR products were then cloned into the Topo-XL vector (Invitrogen) and sequenced to ensure accuracy of the probes. All three probes prepared for in situ hybridization were also used as probes on a northern blot containing poly(A)⁺ selected whole-eye RNA to ensure that each probe hybridized to the same gene (\( Nr2e3 \)). These probes were labeled with \([35S]-dCTP \) (Amersham Pharmacia, Buckinghamshire, UK) using a random prime DNA-labeling kit (Amersham Pharmacia) and hybridized at 65°C. After hybridization, the blot was washed with 0.2× SSC/0.1% SDS at 60°C and exposed overnight to X-ray film at room temperature. RNA riboprobes were synthesized with T7 RNA polymerase in the presence of [35S]-UTP. In situ hybridization was performed on 4% PFA-fixed, paraffin-embedded sections at 65°C as described by Ikeda et al. (30).

**Clinical examination**

F₂ mice were clinically evaluated by indirect ophthalmoscopy at P16.5–P42.5. Pupils were dilated with 1% atropine and a 60-diopter lens was used to examine the fundus.

**Histology**

Tissue samples were collected from F₂ littermates at E10.5 to P6 and immediately immersed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.5) for 24 h. Eyes enucleated from mice that were older than P10 were immersed in 4% PFA. Samples were then transferred into 70% ethanol and processed for paraffin embedding. Once embedded, 6.0 µm sections of tissues were prepared for hematoxylin and eosin staining as well as for in situ hybridization.

**Sequencing**

PCR amplified products from B6Cg-\( rd7/rd7 \) and B6 tail DNA were cloned into the Topo-XL PCR cloning vector (Invitrogen, Carlsbad, CA) and sequenced using Big Dye Terminator (version 2. ABI, Foster City, CA) to identify the genomic mutation in \( rd7 \) mice. Cycling conditions were performed as specified by the manufacturer. DNA fragments were analyzed on an Applied Biosystems 3700 Sequencer. Sequence data was analyzed using the Sequencer 3.1 software (Gene Codes, Ann Arbor, MI).

**In situ hybridization**

Three mouse probes were prepared from different segments of the \( Nr2e3 \) gene for in situ hybridization and northern blot analysis: (i) probe 1 (forward primer 5′-GGGACCATCATATACTCCGATGAG-3′, reverse primer 5′-GATCCCTTGTTGC- CGGAAAGGC-3′, 700 bp PCR product); (ii) probe 2 (forward primer 5′-GCTGGAAGGCGATGAAATGAG-3′, reverse primer 5′-GACATGTCGACCTCAGAATC-3′, 480 bp PCR product); (iii) probe 3 (forward primer 5′-AAGGATGGTC- GACGACGTGGAG-3′, reverse primer 5′-TAGGAGTGGCAATGAAATGTGCC-3′, 380 bp PCR product). These primers were used to PCR amplify a retinal cDNA library under the following cycling conditions: 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s and a final extension of 72°C for 7 min. PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining. The PCR products were then cloned into the Topo-XL vector (Invitrogen) and sequenced to ensure accuracy of the probes. All three probes prepared for in situ hybridization were also used as probes on a northern blot containing poly(A)⁺ selected whole-eye RNA to ensure that each probe hybridized to the same gene (\( Nr2e3 \)). These probes were labeled with \([35S]-dCTP \) (Amersham Pharmacia, Buckinghamshire, UK) using a random prime DNA-labeling kit (Amersham Pharmacia) and hybridized at 65°C. After hybridization, the blot was washed with 0.2× SSC/0.1% SDS at 60°C and exposed overnight to X-ray film at room temperature. RNA riboprobes were synthesized with T7 RNA polymerase in the presence of [35S]-UTP. In situ hybridization was performed on 4% PFA-fixed, paraffin-embedded sections at 65°C as described by Ikeda et al. (30).

**Immunohistochemistry**

Immunohistochemical analysis was performed using blue opsin rabbit polyclonal antibody (a gift from Jeremy Nathans, JH455) and biotin-PNA (Sigma) on 6 µm serial sections of eyes (seven wild-type, seven \( rd7/rd7 \) ). Eyes were oriented dorsal/ventral and serial sections were collected over 1000 µm covering the central region of the eye from the beginning to the end of the optic nerve. Both whole retinas and sections were double labeled with blue opsin and PNA. After blocking with 2% normal goat serum in PBS, sections were incubated with blue opsin antibody (1:7000 dilution) and biotin-PNA (50 µg/ml) at 4°C overnight. Binding was detected using Cy-3 anti-rabbit IgG (1:200, Jackson Immunoresearch, West Grove, PA) for blue opsin and FITC–Avidin D (1:200, Vector, Burlingame, CA) for PNA. Images from sections were collected on a Leica DMRXE fluorescent microscope equipped with a SPO™CCD camera using an appropriate bandpass filter for the fluorochrome.

**Cone cell counts**

Cone cell counts were performed on serial sections from seven wild-type (B6) and seven \( rd7 \) retinas. Images were taken at 20× and the average number of cone cells per 100 µm retinal length was determined using Metamorph (Universal Imaging). Cone cell counts were performed at dorsal and ventral regions of the retina, ~100–200 µm from the ora serrata. Statistical analysis was performed using ANOVA (Statview 4.5, Abacus Concepts, Cary, NC). Counts were taken independently by two individuals.
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29. Buffone, G.J. (1985) Isolation of DNA from biological specimens without prior knowledge of genotypes. Differences in cell counts recorded by the two individuals were not statistically significant (ANOVA, P < 0.5–0.9). Figure 5 was generated using data collected by one individual.

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