REPORT

Mutations in *TRPM1* Are a Common Cause of Complete Congenital Stationary Night Blindness

Maria M. van Genderen,[1] Mieke M.C. Bijveld,[1] Yvonne B. Claassen,[2] Ralph J. Florijn,[3] Jillian N. Pearring,[7] Francoise M. Meire,[8,9] Maureen A. McCall,[6] Frans C.C. Riemslag,[1] Ronald G. Gregg,[6,7] Arthur A.B. Bergen,[3,5] and Maarten Kamermans[2,4,*]

Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous group of retinal disorders characterized by nonprogressive impaired night vision and variable decreased visual acuity. We report here that six out of eight female probands with autosomal-recessive complete CSNB (cCSNB) had mutations in *TRPM1*, a retinal transient receptor potential (TRP) cation channel gene. These data suggest that *TRMP1* mutations are a major cause of autosomal-recessive CSNB in individuals of European ancestry. We localized TRPM1 in human retina to the ON bipolar cell dendrites in the outer plexifom layer. Our results suggest that in humans, TRPM1 is the channel gated by the mGluR6 (*GRM6*) signaling cascade, which results in the light-evoked response of ON bipolar cells. Finally, we showed that detailed electroretinography is an effective way to discriminate among patients with mutations in either *TRPM1* or *GRM6*, another autosomal-recessive cCSNB disease gene. These results add to the growing importance of the diverse group of TRP channels in human disease and also provide new insights into retinal circuitry.

Congenital stationary night blindness (CSNB), caused by defective signaling from photoreceptor to bipolar cells, is characterized by a reduced or absent b-wave and a normal a-wave in the electroretinogram (ERG). Two types of CSNB can be distinguished by use of the standard flash ERG: the "complete" form (cCSNB), also known as type 1 CSNB or CSNB1 (MIM 310500),[1] is characterized by the complete absence of rod pathway function; and the "incomplete" form (icCSNB), also known as type 2 CSNB or CSNB2 (MIM 300071), is caused by impaired rod and cone pathway function. cCSNB is caused by postsynaptic defects in depolarizing or ON bipolar cell signaling, whereas the hyperpolarizing or OFF bipolar cell pathway is intact. CSNB segregates in X-linked and autosomal-recessive form. X-linked cCSNB is caused by mutations in *NYX* (MIM 300278), which encodes nyctalopin, a leucine-rich proteoglycan of unknown function.[2–4] One form of autosomal-recessive CSNB is caused by mutations in *GRM6* (MIM 604096), which encodes the metabotropic glutamate receptor 6 (mGluR6).[5,6] Nyctalopin and mGluR6 are localized on the dendrites of ON bipolar cells[7,8] and are required for signal transmission from photoreceptors to ON bipolar cells. Both are involved in modulating a nonspecific cation channel that has been identified through animal studies to be TrpM1.[9,10] Modulation of TrpM1 leads to a change in the membrane potential of ON bipolar cells, thus making TrpM1 essential for ON bipolar cell function.

If TRPM1 (MIM 603576) indeed plays a role in ON bipolar cell signaling in humans, it should also be localized postsynaptically on the ON bipolar cell dendrites in the outer plexiform layer (OPL) of the retina (Figure 1A). To examine this, we reacted transverse sections of normal human retina with antibodies to TRPM1 and Ribeye (a presynaptic marker) or peanut agglutinin (PNA) (a marker for cone terminals) (Figure 1). Images of TRPM1 and PNA staining (Figure 1B) showed dense TRPM1 puncta closely aligned with PNA-positive cone photoreceptor terminals. Weaker TRPM1 staining in the inner nuclear layer was associated with bipolar cell somata. Staining for both TRPM1 and Ribeye (Figure 1C) showed closely associated, but nonoverlapping, labeling in large cone and small rod terminals. This localization of TRPM1 strongly resembles that of nyctalopin[8] and indicated that it, too, is localized on rod ON bipolar cell dendrites.

Given this expression pattern and the role of TRPM1 in ON bipolar cell signaling in animals,[9,10] we hypothesized that individuals with autosomal-recessive cCSNB lacking mutations in either *GRM6* or *NYX* may have mutations in *TRPM1*. We subsequently screened eight female autosomal-recessive CSNB probands of European ancestry for mutations in *TRPM1,* after the other cCSNB genes had been excluded. We sequenced 26 exons (accession no. NM_002420_4) and adjacent splice sites and found *TRPM1* mutations in six probands. All six probands had normal retinas on the basis of funduscopy (data not shown). Their clinical characteristics, including representative dark-adaptation curves and ISCEV (International Society for Clinical Electrophysiology of Vision) standard ERG, are presented in Table S1, Figure S1, and Figure S2 (available online). Five probands carried either homozygous or compound-heterozygous mutations, and in one

[1]Bartiméus, Institute for the Visually Impaired, 3702 AD Zeist, The Netherlands; [2]Retinal Signal Processing, [3]Molecular Ophthalmogenetics, Netherlands Institute for Neuroscience, 1105 BA Amsterdam, the Netherlands; [4]Department of Neurogenetics, [5]Departments of Clinical Genetics and Ophthalmology, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands; [6]Department of Ophthalmology and Visual Sciences, [7]Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, KY 40292, USA; [8]Département of Pédiatrique Ophtalmologie, Hôpital Universitaire des Enfants Reine Fabiola, 1020 Brussels, Belgium; [9]Department of Pediatric Ophthalmology, University Hospital Ghent, 9000 Ghent, Belgium
*Correspondence: m.kamermans@nin.knaw.nl
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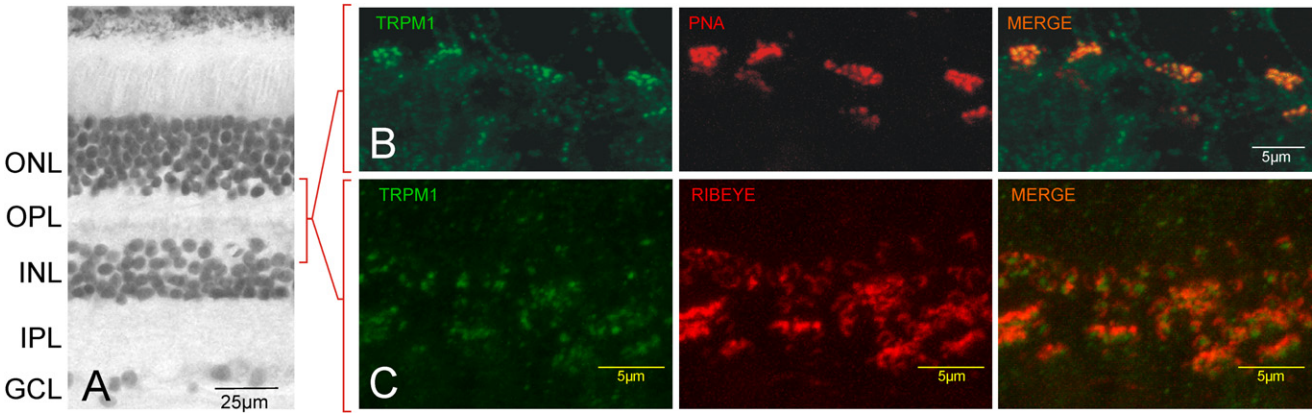

Figure 1. TRPM1 Is Localized to the OPL in the Human Retina
Human eyes, obtained from the Kentucky Lions Eye Bank, were prepared as described in Gregg et al.[8] Antibodies and their dilutions were as follows: anti-ctbp2/ribeye (BD Biosciences PharMingen, San Diego, CA, USA; 1:1000), rhodamine-labeled PNA (Vector Labs, Burlingame, CA, USA; 1 μg/ml), and anti-TRPM1 (Sigma, Saint Louis, MO, USA; 1:300).
(A) Transverse section through a "peri-foveal" area of a normal human retina. Abbreviations are as follows: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
(B) Immunolocalization shows close apposition of TRPM1 puncta with PNA-positive cone photoreceptor terminals in the OPL.
(C) Immunolocalization shows close apposition of TRPM1 with Ribeye, a rod and cone photoreceptor synaptic ribbon marker.
Scale bar represents 25 μm in (A) and 5 μm in (B) and (C).

proband we found only a single heterozygous mutation (Table S1). None of the described mutations were present in 210 control chromosomes.

Proband 1 was homozygous for a 36,445 bp deletion that includes exons 2–7 (Figure 2Ai). The deleted chromosome was present in both parents and was absent from the proband's unaffected sister. This deletion removes exons used in four additional isoforms recently described by Oancea et al.[11] and should produce a null allele for all isoforms of *TRPM1*. Proband 2 (Figure 2Aii) was compound heterozygous with a single-bp deletion in exon 3 (c.83delA), resulting in a frameshift (p.Asn28Metf62) on one allele. The other allele carried a c.1600G>A transition in exon 14, which generates a p.Gly534Arg missense mutation. The proband's unaffected parents were carriers (mother for c.83delA; father for c.1600G>A), whereas the unaffected sister had no mutations. The parents were not aware of any possible consanguinity. Alamut analysis, which predicts mutation impact on function, classifies p.Gly534Arg as weak, although p.Gly534 is conserved in TRPM1 channels from human to frog (Figure 2B), suggesting functional importance. Proband 3 had a c.296T>C transversion in exon 4, which results in a p.Lys99Pro substitution on one allele, and a c.1832C>T transversion in exon 16, which results in p.Pro611His missense mutations (Figure 2Aiii) on the other allele. DNA samples from the parents were not available. However, the c.296T>C mutation was found in the unaffected sister of proband 3, and the c.1832C>T mutation was found in the unaffected brother. Alamut analysis predicts that both are likely to interfere with the function of the protein, and each contains highly conserved residues. Proband 4 carries a c.220C>T transition in exon 4, which results in a p.Arg74Cys substitution on one allele, and a c.1091T>G transversion in exon 9, which results in a p.Leu364Arg substitution (Figure 2Aiv) on the other allele. Only the c.220C>T mutation was found in proband 3's mother, and DNA was not available for her father. Alamut analysis predicts that both are highly conserved residues likely to interfere with the function of the protein, and each contains highly conserved residues (Figure 2B). We identified two mutations (c.3061+1G>A and c.3142G>A) in proband 5; the c.3142G>A mutation is located in the ion transport domain and, according to Alamut analysis, interferes with the function of the protein. The c.3061+1G>A mutation most likely affects the splicing pattern of the gene (deletion of exon 22 expected). Finally, we identified a single heterozygous splice-site mutation (c.2250+1G>A) in proband 6. The mutations that were predicted to alter splicing (probands 5 and 6) require further functional confirmation.

Scotopic (rod), photopic (cone), mixed cone-rod, and 30 Hz photopic ERG responses were measured in control subjects (Figure S2A) and in cCSNB probands with *GRM6*, *NYX*, and *TRPM1* mutations (Figures S2B–S2D), with the use of ISCEV standard conditions.[12,13] In control subjects, the mixed cone-rod ERG has a negative-going a-wave, due to photoreceptor activation, preceding a positive-going b-wave, caused by ON bipolar cell depolarization (mixed; Figure S2A). Consistent with their diagnosis, all cCSNB probands showed mixed ERG responses of normal a-wave and no b-wave phenotype (mixed; Figure S2B–S2D). No scotopic ERG response could be identified. Their photopic, 30 Hz flicker ERG responses were normal, and oscillatory potential (OP) responses were normal to subnormal. So far, all of our probands had a phenotype similar to those of mouse models with mutations in *Grm6*, *TrpM1*,[10,14] and *Nyx*,[15] in which the absence of ON bipolar cell activity has been directly confirmed.[8]

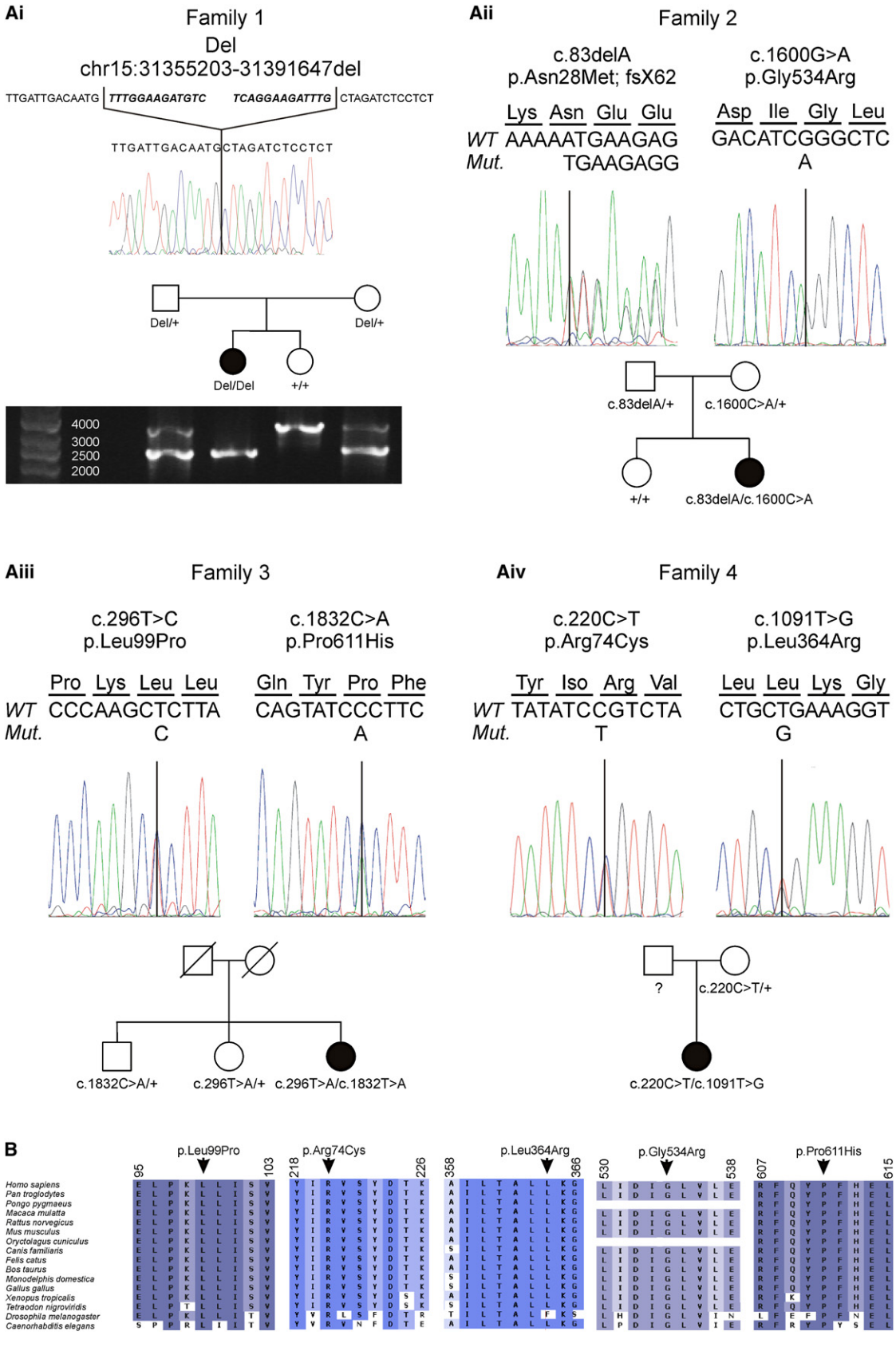

Figure 2. ***TRPM1*** **Mutations Are Present in Six Probands with Autosomal-Recessive cCSNB**
(Ai-Aiv) Four families in which two deleterious and/or highly conserved mutations in the TRPM1 channel were identified. Each of the subsections, Ai–Aiv, shows the mutation, the predicted impact on the amino acid sequence, and a small portion of the chromatogram showing the mutant sequence. (Ai) Proband 1 has a 36,455 bp deletion in *TRPM1* (chromosome 15). To confirm that the deletion was

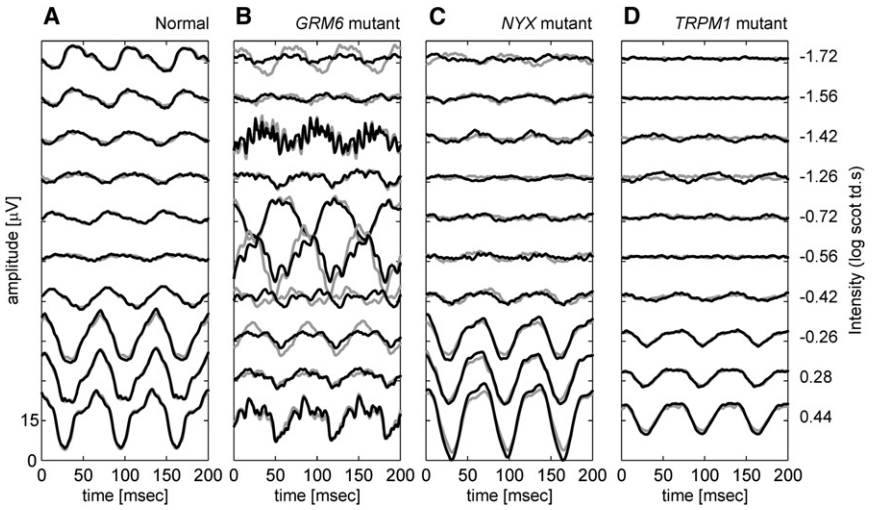

Figure 3. ERG Responses from Control Subjects and Probands with *GRM6*, *NYX*, and *TRPM1* Mutations

The 15 Hz flicker ERG responses from representative healthy control subjects and probands with *GRM6*, *NYX*, and *TRPM1* mutations differ (B–D, respectively). ERG responses were recorded with the use of DTL electrodes (right eye: gray; left eye: black). Shown are scotopic 15 Hz flicker ERG responses to a series of ten stimuli of increasing intensities starting at −1.72 log scot td×s (top trace) up to 0.44 log scot td×s (bottom trace). Between −1.72 and −0.56 log scot td×s rod signals in the normal retina use the primary (rod → ON bipolar cell → AII amacrine cell) pathway, and at mesopic levels from −0.56 log scot td×s upwards, they use the secondary (rod → cone → bipolar cell) pathway. At approximately −0.56 log scot td×s, the signals transferred through the two rod pathways are equal in amplitude but lag one another by half a phase and interfere destructively[6,16,17] (see control subject in A: "Normal"). In probands with *NYX* (C) or *TRPM1* (D) mutations, ERG responses from the primary rod pathway could not be identified, whereas responses from the secondary rod pathway were within normal limits. In the proband with *GRM6* mutations, ERG responses could be measured at all intensities, but the responses had abnormal phase behavior.

In contrast to the standard flash ERG, a 15Hz flicker ERG paradigm can subdivide cCSNB patients with and without *GRM6* mutations and provide insight into dysfunction in the various rod pathways[6] (Figure 3). At low scotopic intensities, the response is dominated by the primary rod pathway (Figure 4Ai; rod → rod ON-BC → AII amacrine cell, whereas the response is dominated at high scotopic intensities by the secondary rod pathway (Figure 4Aii; rod →cone [via gap junctions between rod and cone pedicles] → BC).[16,17] In control subjects (Figure 3A), the ERG amplitude decreases and then reaches a null at about −0.56 log scot td×s before increasing again at higher intensities. This occurs because of destructive interference, caused by out-of-phase signals from the primary and secondary rod pathways. None of the 15 Hz ERG responses of our *TRPM1* probands showed a pattern similar to either the control subject (Figure 3A) or our autosomal-recessive cCSNB proband with *GRM6* mutations (Figure 3B). Rather, the ERG responses of all *TRPM1* probands (Figure 3D) were identical to those of cCSNB patients with *NYX* mutations (Figure 3C). Specifically, responses at low intensities were completely absent, whereas they were similar to normal at higher intensities, revealing the complete absence of the primary rod pathway and mildly reduced activity of the secondary rod pathway. As reported previously for autosomal-recessive cCSNB patients with *GRM6* mutations, our *GRM6* proband had responses at all intensities that were markedly dissimilar in phase as compared to normal responses.[6] It has been suggested that this is due to the interaction between the secondary and tertiary rod pathways (Figure 4Aiii; rod → OFF-BCs).[6]

Distinct CSNB phenotypes also occur in retinal ganglion cell activity in *Nyx* and *Grm6* mutant mice.[18] *Nyx* mutant ganglion cells show spontaneous rhythmic bursting activity with a fundamental frequency of about 4 Hz; whereas the spontaneous activity of *Grm6* mutant ganglion cells is the same as that of the wild-type.[19] Furthermore, the spatial organization of all *Nyx* mutant ganglion cells is significantly altered, whereas *Grm6* OFF ganglion cells have normal receptive field center/surround organization.[18] Because the genes implicated in cCSNB are all located in the ON bipolar cell dendrites, our data imply that, in both humans and mice, the state of the ON bipolar

present in family members, we performed a PCR, using forward primers 5′ UTR (for the mutant allele) and TRPM1 exon 6F (for the WT allele) in combination with a common reverse primer, TRPM1 exon 7R. This PCR generated the predicted 3411 bp fragment from the WT allele and a 2293 bp fragment from the mutant allele. The ethidium-bromide-stained gel shows that both parents were carriers of this mutation and that it is absent in the proband's unaffected sister. (Aii) The pedigree for proband 2 showed that she inherited one mutant *TRPM1* allele from each parent (c.83delA, c.1600C>A), whereas her unaffected sister inherited two WT *TRPM1* alleles. (Aiii) The pedigree of proband 3 also showed consistent segregation of the mutant alleles with the disease phenotype. The proband had two mutant TRPM1 alleles (c.296T>C; c.1832C>A), whereas each of her two unaffected siblings had only one. Both parents died, but it can be deduced from the segregation pattern that each parent carried only one of the mutations found in the proband. (Aiv) Proband 4 inherited one of the mutant *TRPM1* alleles (c.220C>T) from her mother. DNA from her father was not available. The second mutant allele (c.1091T>G) could have been inherited from her father or could be a de novo mutation.

(B) Conservation of the five missense mutations indentified in probands 2–4. In every species for which data were available, all five mutations were located in highly conserved regions. The darkness of the blue color indicates the amount of conservation, with dark blue indicating the most conserved. All sequence changes were evaluated for their pathogenicity with the Alamut software. The study followed the tenets of the Declaration of Helsinki and was approved by the ethics committee of the Academic Medical Centre, Amsterdam. All participants provided signed informed consent for participation in the study.

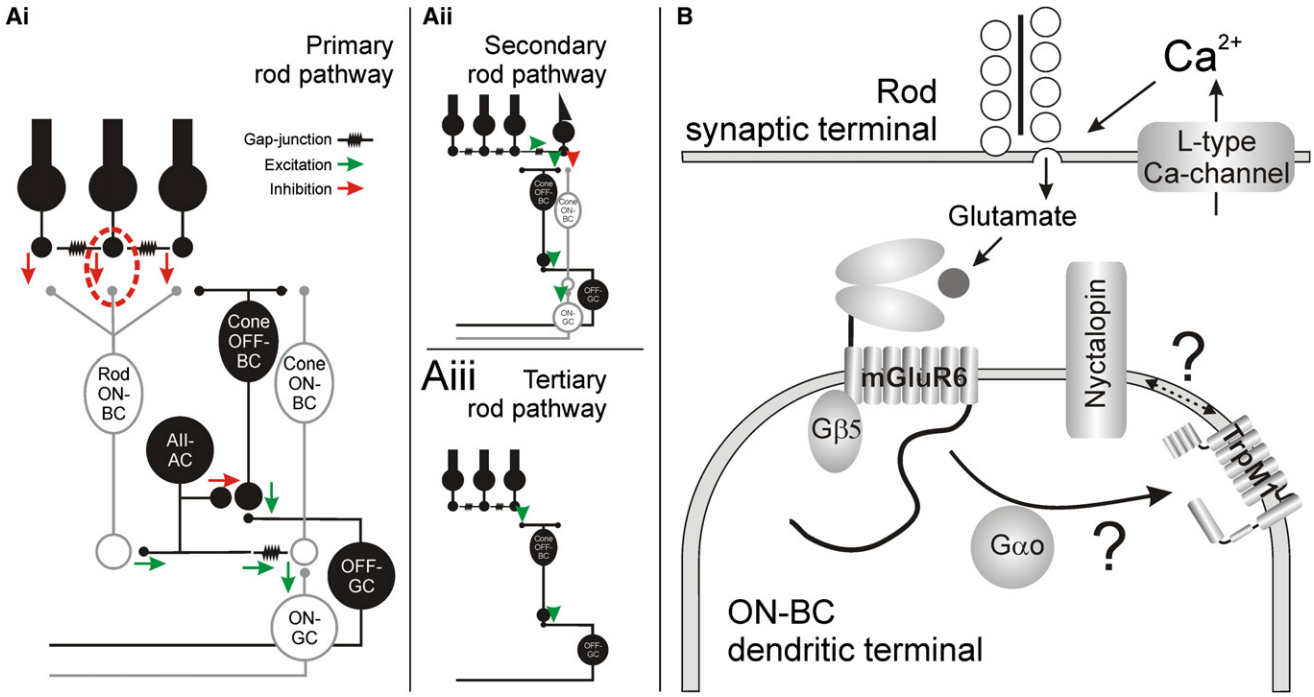

Figure 4. Rod Signaling in the Mammalian Retina Travels via Three Routes
(Ai) The primary rod pathway (rod → ON bipolar cell→ AII amacrine cell). Rods release glutamate onto rod ON bipolar cells, which provide an excitatory input to AII amacrine cells, which are electrically coupled to cone ON bipolar cells and inhibit cone OFF bipolar cells. Signaling in this pathway completely depends on the mGluR6 signaling cascade (B). This pathway is affected in cCNSB.
(Aii) The secondary rod pathway (rod → cone → bipolar cell). The electrical coupling between rods and cones allows the flow of rod signals into the cones and glutamate release into the cone ON and OFF bipolar cells.
(Aiii) The tertiary rod pathway (rod →OFF bipolar cell). A direct glutamatergic input from rods to cone OFF bipolar cell. Note that the latter two pathways are in principle independent of the rod ON bipolar cell signaling pathway (Ai). Red arrows indicate sign-inverting synapses, and green arrows indicate sign-preserving synapses. Abbreviations are as follows: BC, bipolar cell; AC, amacrine cell; GC, ganglion cell.
(B) Schematic representation of signaling between photoreceptors and bipolar cells (marked by dashed red circle in panel Ai) and the mGluR6 signaling cascade. Glutamate release from rods depends on the intracellular Ca^{2+} concentration, which is regulated by an L-type voltage-gated Ca^{2+} channel (*CACNA1F*) in the rod synaptic terminal. Glutamate binds to metabotropic glutamate receptors mGluR6 (*GRM6*) and activates an intracellular cascade involving GαO and Gβ5, leading to the closure of the TRPM1 channel. NYX also plays a role, but its function is currently unknown. GRM6, NYX, and TRPM1 have now been implicated in human cCSNB.[2,6] It is less likely that human patients with mutations in either GαO or Gβ5 will be found, given that the GαO[22] mutation is lethal in mice and Gβ5 has a primary photoreceptor-degeneration phenotype.[23]

cells differs between *TRPM1* and *NYX* mutants and *GRM6* mutants.

Figure 4B illustrates the currently identified proteins that function in ON bipolar cell signaling and cause a cCSNB phenotype in humans and/or mice. mGluR6 is the receptor sensing glutamate release by photoreceptors,[20,21] GαO and Gβ5 are second messengers in this G protein signaling cascade.[22,23] TRPM1 is most likely the cation channel that eventually modulates the light-evoked ON bipolar cell depolarization.[9,10] Nyctalopin, whose function has not yet been conclusively established, may traffic and/or anchor TRPM1 to the membrane.[24] Activation of mGluR6 leads to the closure of TRPM1 channels. Therefore, mutations in mGluR6 are likely to cause the TRPM1 channel to be open and conduct an inward current. In contrast, mutations in TRPM1 are likely to eliminate both the TRPM1 channel and the inward current. The simplest model predicts that ON bipolar cells in our probands with *TRPM1* mutations will be hyperpolarized, whereas those with *GRM6* mutations will be constitutively depolarized. This should differentially affect the sustained activity of AII amacrine cells, a vital component of information flow in the rod pathways. These changes could shift the balance between the remaining secondary and tertiary rod pathways and result in the differences that we observe in the 15 Hz ERG flicker response.

Overall, we investigated 11 autosomal-recessive cCSNB patients from nine families. Three patients from one family had mutations in *GRM6*. Six probands from six families had *TRPM1* mutations. The remaining two patients may represent the opportunity to identify additional genes that regulate either TRPM1 channel activity or the mGluR6 signaling cascade. That said, our results indicate that *TRPM1* mutations will account for more than 50% of autosomal-recessive cCSNB cases in our patient cohort of European ancestry. TRP channels are a diverse family of about 30 members that are now known to be expressed and participate in central and peripheral

nervous system function. To date, mutations in these channels have rarely been associated with diseases of the nervous system. However, our results suggest that they may represent an unexplored target for these diseases.

Supplemental Data

Supplemental data include two figures and two tables and can be found with this article online at http://www.cell.com/AJHG.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Exon Locator and eXtractor for Resequencing database (ELXRdb), http://elxr.swmed.edu/elxrdb_query.html
SNPCheck, http://ngrl.man.ac.uk/SNPCheck/SNPCheck.html
Primer3, http://frodo.wi.mit.edu/primer3/
BLAT, http://www.genome.ucsc.edu/cgi-bin/hgBlat
Human Genome Variation Society, http://www.hgvs.org
Alamut, http://www.interactive-biosoftware.com/alamut.html
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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Note Added in Proof

The involvement of TrpM1 in ON bipolar cell function in mouse retina has been confirmed by Morgans et al.: TRPM1 is required for the depolarizing light response in retinal ON-bipolar cells. PNAS. Published online October 27, 2009. doi: 10.1073/pnas.0908711106.