Further Insights Into GPR179: Expression, Localization, and Associated Pathogenic Mechanisms Leading to Complete Congenital Stationary Night Blindness

Elise Orhan,[1–3] Laurent Prézeau,[4] Said El Shamieh,[1–3] Kinga M Bujakowska,[1–3] Christelle Michiels,[1–3] Yvrick Zagar,[1–3] Claire Vol,[4] Shomi S. Bhattacharya,[5,6] José-Alain Sahel,[1–3,5,7–9] Florian Sennlaub,[1–3] Isabelle Audo,[1–3,5,9] and Christina Zeitz[1–3]

[1]Institut National de la Santé et de la Recherche Médicale (INSERM), U968, Paris, France
[2]Centre National de la Recherche Scientifique (CNRS), UMR_7210, Paris, France
[3]Université Pierre et Marie Curie (UPMC Paris 06), Unité Mixte de Recherche (UMR)_S 968, Institut de la Vision, Paris, France
[4]Institut de Génomique Fonctionnelle CNRS UMR5203, INSERM U661, Université Montpellier 1 (UM1) & Université Montpellier 2 (UM2) Montpellier, France
[5]University College London (UCL)-Institute of Ophthalmology, London, United Kingdom
[6]Department of Cellular Therapy and Regenerative Medicine, Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Isla Cartuja, Seville, Spain
[7]Fondation Ophtalmologique Adolphe de Rothschild, Paris, France
[8]Académie des Sciences-Institut de France, Paris, France
[9]Centre Hospitalier National d'Ophtalmologie (CHNO) des Quinze-Vingts, INSERM-Direction de l'Hospitalisation et de l'Offre de Soins Centre d'Investigation Clinique (DHOS CIC) 503, Paris, France

Correspondence: Christina Zeitz, Department of Genetics, Institut de la Vision, 17, Rue Moreau, 75012 Paris, France; christina.zeitz@inserm.fr.

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Purpose. Mutations in *GPR179*, which encodes the G protein-coupled receptor 179, lead to autosomal recessive complete (c) congenital stationary night blindness (CSNB), which is characterized by an ON-bipolar retinal cell dysfunction. This study further defined the exact site of *Gpr179* expression and its protein localization in human retina and elucidated the pathogenic mechanism of the reported missense and splice site mutations.

Methods. RNA in situ hybridization was performed with mouse retinal sections. A commercially available antibody was validated with GPR179-overexpressing COS-1 cells and applied to human retinal sections. Live-cell extracellular staining along with subsequent intracellular immunolocalization and ELISA studies were performed using mammalian cells overexpressing wild-type or missense mutated *GPR179*. Wild-type and splice site–mutated mini-gene constructs were transiently transfected, and RNA was extracted. RT-PCR-amplified products were cloned, and Sanger sequenced.

Results. Mouse *Gpr179* transcript was expressed in the upper part of the inner nuclear layer, and the respective human protein localized at the dendritic tips of bipolar cells in human retina. The missense mutations p.Tyr220Cys, p.Gly455Asp, and p.His603Tyr led to severely reduced cell surface localization, whereas p.Asp126His did not. The mutated splice donor site altered *GPR179* splicing.

Conclusions. Our findings indicate that the site of expression and protein localization of human and mouse GPR179 is similar to that of other proteins implicated in cCSNB. For most of the mutations identified so far, loss of the GPR179 protein function seems to be the underlying pathogenic mechanism leading to this form of cCSNB.

Keywords: GPR179, expression and localization, cCSNB, pathogenicity, trafficking defect, mini-gene approach

Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous disorder, which is characterized by impaired night vision and is often associated with other ocular problems such as decreased visual acuity, nystagmus, high myopia, and strabismus.[1] Clinically, this disorder can be classified according to two forms distinguished by particular full-field electroretinogram (ERG) abnormalities.[2] Patients with Riggs-type ERG responses reveal a reduced a- and b-wave, whereas patients with the Schubert-Bornschein-type of ERG are characterized by an electronegative scotopic ERG response in which the a-wave is larger than the b-wave.[3,4] The latter type can be further divided into the incomplete (ic) and complete (c) forms. In the former form, the patient shows reduced scotopic b-wave and severely reduced 30-Hz flicker and single-flash photopic ERG responses; in the latter form, the patient shows severely reduced scotopic b-wave and square-shaped a-wave photopic ERG responses with relatively preserved amplitude.[5] Mutations in genes involved in the phototransduction cascade that cause autosomal dominant (ad) CSNB (*RHO*, *GNAT1*, *PDE6B*)[6–10] and one gene that causes autosomal recessive (ar) CSNB (*SLC24A1*)[11] have been reported to lead to Riggs-type CSNB. However, most cases of CSNB

reported so far have a Schubert-Bornschein-type phenotype and are associated with mutations in the genes causing icCSNB (*CACNA1F*, *CABP4*, and *CACNA2D4*)[12–15] and cCSNB (*NYX*, *GRM6*, *TRPM1*, *GPR179*, and *LRIT3*).[16–25] Genes involved in cCSNB are expressed in the upper part of the inner nuclear layer (INL) of the retina[26–28] and encode proteins localized at the dendritic tips of ON-bipolar cells.[22,24,25,29–35] All proteins are implicated in signaling from photoreceptors to bipolar cells. *GRM6* encodes the metabotropic glutamate receptor mGluR6 (also called GRM6), which is important for glutamate-induced signaling from the photoreceptors. During darkness, glutamate binding leads to the activation of Gαo, the α subunit of the G protein of mGluR6[36] and, at the end of the cascade, to the closure of a non-selective ion channel, TRPM1.[26,37,38] RGS7/Gβ5 and RGS11/Gβ5 complexes are GTPase accelerating proteins (GAP) in the same cascade and are important for the deactivation of Gαo.[39,40] In daylight, the TRPM1 channel opens, resulting in depolarization of the ON-bipolar cells and formation of the ERG b-wave, which is absent in patients with cCSNB.[41] Specific intracellular motifs present in LRIT3 and in vitro and in vivo studies of NYX and TRPM1 suggest that LRIT3 and NYX are important for the correct localization of TRPM1 at the dendritic tips of ON-bipolar cells.[25,33] *GPR179*, which encodes the orphan G protein-coupled receptor 179, has only recently been identified as mutated in patients with cCSNB.[23,24] Previous immunolabeling in mice showed that GPR179 is localized at the dendritic tips of bipolar cells[24,35] and is essential for postsynaptic targeting of the G protein-deactivating RGS-Gβ5 complex (mentioned above) to the dendritic tips of ON-bipolar cells.[35] Although we recently showed by RT-PCR experiments that *GPR179* is expressed in human retina,[23] the exact expression site and localization and the relevant pathogenic mechanism still need to be elucidated. In the current study, we aimed to define the exact expression site and protein localization of mouse and human GPR179 and to elucidate its underlying pathogenic mechanism(s) implicated in cCSNB.

Materials and Methods

Preparation of Mouse and Human Retinas for RNA In Situ Hybridization and Protein Localization Studies

Six-week-old C57BL/6JRj (Janvier, Genest Saint Isle, France) female mice were anesthetized with a mixture of 140 mg/kg xylazine (Bayer, Leverkusen, Germany)/14 mg/kg ketamine (Virbac, Carros, France) saline solution and were perfused transcardiacally with 4% paraformaldehyde (PFA) in 0.12 M phosphate buffer, pH 7.4. Eyes were collected and postfixed for 1 hour in 4% PFA before being dehydrated in 30% sucrose phosphate-buffered saline (PBS) solution. Thereafter, eyes were embedded in 7% gelatin/10% sucrose PBS and frozen in −40°C isopentane. Subsequently, 20-μm retinal sections were made with a cryostat (model HM560, Microm; Thermo Fisher, Walldorf, Germany). Animal handling was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the local institutional review board. Human donor eyes without known history of retinal diseases were collected through the Minnesota Lions Eye bank after due consent in accordance with the Declaration of Helsinki. The posterior segment was dissected, postfixed, dehydrated, embedded, and sectioned as described above for the mouse retinas.

RNA In Situ Hybridization Studies

A cDNA fragment encompassing exon 7 to 9 of mouse *Gpr179* was cloned into a pBluescript II SK vector using a commercial cloning service (GeneCust, Dudelange, Luxembourg). The plasmid was linearized using the restriction enzymes *Sac*I and *Kpn*I. Antisense and sense RNA in situ hybridization probes were synthesized using T7 and T3 RNA polymerase (Roche Diagnostics, Basel, Switzerland), respectively, and labeled with digoxigenin-UTP (Roche Diagnostics). Mouse retinal sections were postfixed in 4% PFA for 10 minutes, washed with PBS, and treated with proteinase K (10 μg/mL; Invitrogen, Carlsbad, Czech Republic) for 2 minutes. Following a wash with PBS, the sections were postfixed in 4% PFA, washed in PBS, and then acetylated in an acetylation buffer of 1.3% triethanolamine (Sigma-Aldrich, St. Quentin Fallavier, France), 0.25% acetic anhydride (Sigma-Aldrich), and 0.06% hydrochloric acid solution. The sections were washed in 1% Triton X-100 PBS solution and blocked for 2 hours in hybridization buffer containing 50% formamide, 5× SSC (saline sodium citrate [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), 1× blocking solution (Denhardt's solution; Sigma-Aldrich), 250 μg/mL yeast tRNA (Roche Diagnostics), and 240 μg/mL salmon sperm (Roche Diagnostics), pH 7.4. The sections were hybridized with digoxigenin-labeled probes overnight at 72°C, after which they were rinsed for 2 hours in 0.2× SSC at 72°C and blocked for 1 hour at room temperature in 0.1 M Tris, 0.15 M NaCl (B1), 10% normal goat serum (NGS; Vectorshield, Burlingame, CA), pH 7.5. After blocking, slides were incubated overnight at room temperature with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:5000 dilution; Roche Diagnostics) in B1 containing 1% NGS. After additional washes, the alkaline phosphatase activity was detected using nitro blue tetrazolium chloride (337.5 μg/ml; Roche Diagnostics) and 5-bromo-4-chloro-3-indolyl phosphate (175 μg/ml; Roche Diagnostics). Eight hours later, sections were mounted (Mowiol; Calbiochem/Merck, Carlstadt, NJ). Slides were scanned with a Nanozoomer 2.0 high throughput (HT) equipped with a 3-charge–coupled device time delay integration (TDI) camera (Hamamatsu Photonics, Hamamatsu, Japan).

Protein Immunolocalization in Human Retina

Retinal sections were incubated overnight with primary rabbit anti-GPR179 (product code, HPA017885-100UL; Sigma-Aldrich), mouse anti-Gαo (Merck-Millipore, Billerica, MA), mouse PKCα (Sigma-Aldrich), mouse calbindin (Swant, Marly, Switzerland), and mouse C-terminal-binding protein 2 (CtBP2; BD Transduction Laboratories, San Jose, CA) antibodies at dilutions of 1:400, 1:400, 1:200, 1:500, and 1:10,000. Prior to GPR179/Gαo antibody staining, retinal sections were postfixed for 5 minutes in methanol at −20°C. Thereafter, sections were washed 3 times for 5 min each in 1× PBS and then incubated with donkey anti-rabbit Alexa Fluor 488 (Jackson ImmunoResearch Laboratories, Baltimore, MD) and donkey anti-mouse Cy3 (Jackson Immunoresearch Laboratories) secondary antibodies and 4′,6-diamidino-2-phenylindole (DAPI; Euromedex, Souffelweyersheim, France) at a dilution 1:1000 each for 1 hour at room temperature. Negative controls were performed with only the use of secondary antibodies. Sections were washed 3 times for 5 min in PBS and mounted with coverslips (Mowiol preparation; Calbiochem/Merck). Confocal fluorescence microscopy images were taken (model FV1000; Olympus, Hamburg, Germany).

Expression Constructs

The DNA coding sequence and *Bam*HI and *Not*I linkers of the wild-type and mutated human *GPR179* genes were synthesized in an optimized way and cloned in an expression vector (pcDNA3; Invitrogen, Courtaboeuf, France) by a company (GeneCust). To validate the commercially available human anti-GPR179 antibody (product code HPA017885-100UL; Sigma-

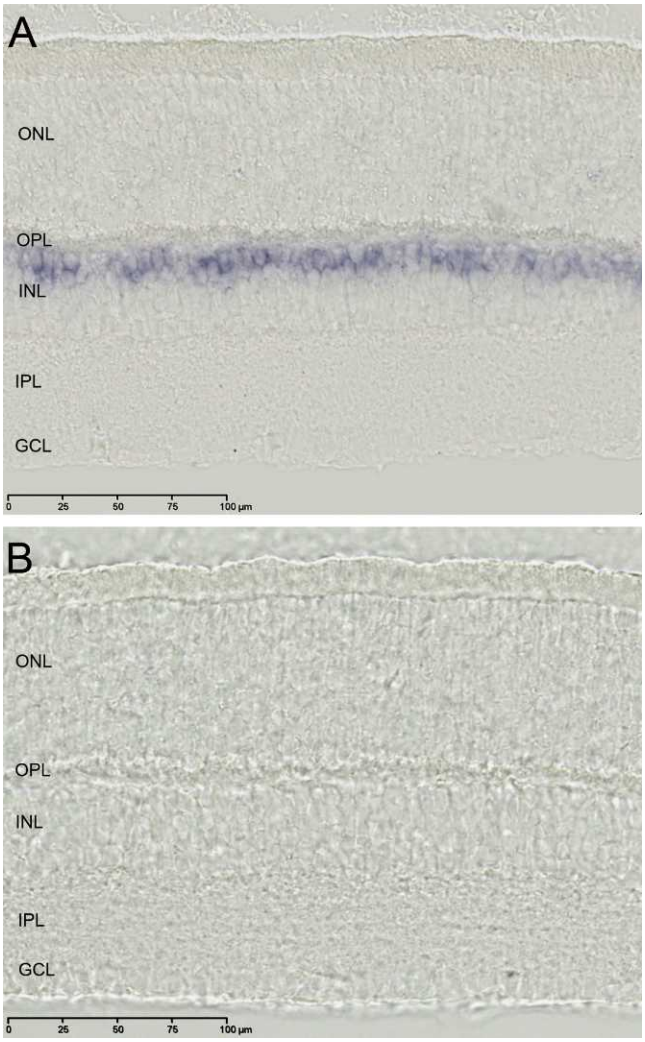

FIGURE 1. *GPR179* is expressed in the somata of the upper part of the INL in mouse retina. Hybridization was performed with antisense (**A**) and sense (**B**) Gpr179 (exons 9–11) riboprobes (signal in *purple*). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Aldrich), we inserted in frame a flag-tag between the predicted signal sequence (after amino acid 26) and the main sequence. The sequences of the respective plasmids were verified by the company and in our laboratory by Sanger sequencing using standard conditions with an automated 48-capillary sequencer (BigDye Terminator version 1.1 cycle sequencing kit, model 3730 genetic analyzer; Applied Biosystems, Courtaboeuf, France) with specific primers designed against the wild-type and optimized synthetic GPR179 sequence (see Supplementary Table S1) and vector oligonucleotides (T7, SP6, and BGH oligonucleotides).

Cell Culture, Transfection, and Immunofluorescence

Transient transfection studies were performed in COS-1 cells. In 24-well plates, 130,000 cells per well were seeded over coated coverslips and transfected after 6 hours with 10 µg of human wild-type and mutated GPR179 plasmids, applying the calcium phosphate method.[42] To validate in vitro the human anti-GPR179 antibody mentioned above, cells were permeabilized after 36 hours of transfection and stained for intracellular GPR179 protein with the human anti-GPR179 antibody and mouse anti flag-tag antibody (product code M2 F3165; Sigma-Aldrich) in the same experiment and visualized with anti-mouse Alexa Fluor 488 (Jackson Immunoresearch Laboratories) and donkey anti-rabbit Cy3 (Jackson Immunoresearch Laboratories) antibodies, respectively, at a dilution of 1:1000 each. To investigate the localization of wild-type and mutated GPR179 proteins, extracellular live cell staining and subsequent intracellular staining were performed as previously described.[43] Stained cells were analyzed with confocal fluorescence microscopy (model FV1000; Olympus). Using standard protein extraction methods, we were not able to obtain a clear signal with this GPR179 antibody by using Western blot analysis (data not shown).

Transfection of HEK293 Cells, ELISA for Quantification of Cell Surface Receptor Expression

HEK293 cells were cultured in modified Eagle medium supplemented with 10% fetal calf serum (FCS) and transfected by electroporation as previously described.[44] Ten million cells were transfected with 5 µg of plasmid DNA encoding wild-type or mutated GPR179 or wild-type GPR158 used as a specificity control. Control cells were transfected with the empty vector. Briefly, cells were cultured at 30°C for 24 hours; fixed with 4% paraformaldehyde; and when needed, incubated for 3 minutes with 0.05% Triton X-100; and then blocked with blocking buffer (1% FCS in PBS). Rabbit polyclonal anti-GPR179 antibody (product code HPA017885; Sigma-Aldrich) was applied for 30 minutes at 0.5 mg/L. After cells were washed with blocking buffer, the horseradish peroxidase-conjugated donkey anti-rabbit (product code NA934V, 1:1500 dilution; GE-Healthcare, Little Chalfont, UK) secondary antibody was applied for 30 minutes, and cells were washed with blocking buffer and then PBS. Chemiluminescence was detected using SuperSignal substrate (Pierce, Rockford, IL) and an Infinite F500 reader (Tecan, Männedorf, Switzerland). Data were collected using Tecan i-control software (Tecan).

Mini-Gene Approach

Patient genomic DNA containing the heterozygous c.1784+1G>A mutation was amplified between intron 6 and intron 9 with GPR179 oligonucleotides used for the initial mutation screening (GPR179_EX7F and GPR179_EX9R)[23] with a DNA polymerase (HOT FIREPol; Solys Biodine, Tartu, Estonia). The amplicon was subcloned in a vector (pCRII-TOPO vector; Invitrogen). Subsequent Sanger sequencing using standard M13 oligonucleotides was performed to verify the presence of the wild-type and splice site mutation in the obtained constructs. The inserts of the sequence-validated constructs were cloned into a vector (pBudCE4.1 vector; Invitrogen) using the *Hind*III and *Xba*I restriction sites. Transient transfection studies were performed in COS-1 cells in 6-well plates, and total RNA was extracted using a kit (RNeasy mini-kit; Qiagen, Hilden, Germany). Reverse transcription was performed using a reverse transcriptase (SuperscriptII; Invitrogen). To analyze the in vitro splicing products, PCR was performed using oligonucleotides present in exons 7 and 9 of GPR179 (RT_GPR179_EX7F 5′GTGCTGCAGCTGTTTCTGTC3′ and RT_GPR179_EX9R 5′ AAGAGGAGGAGGGTCCAGTC3′). Five microliters of the RT-PCR products was investigated by electrophoresis on a 2% agarose gel; 1 µL was cloned in a vector (pCRII-TOPO; Invitrogen), and 16 clones per condition were picked and Sanger sequenced using standard M13 oligonucleotides. To normalize GPR179 RT-PCR values, a beta-actin PCR (using the primers ACTNBqPCR Ex4F CGCCAACACAGTGCTGTCTG and ACTNB_qPCR_Ex5R GGAGTACTTGCGCTCAGGAG) was performed on the obtained cDNA and was investigated as mentioned

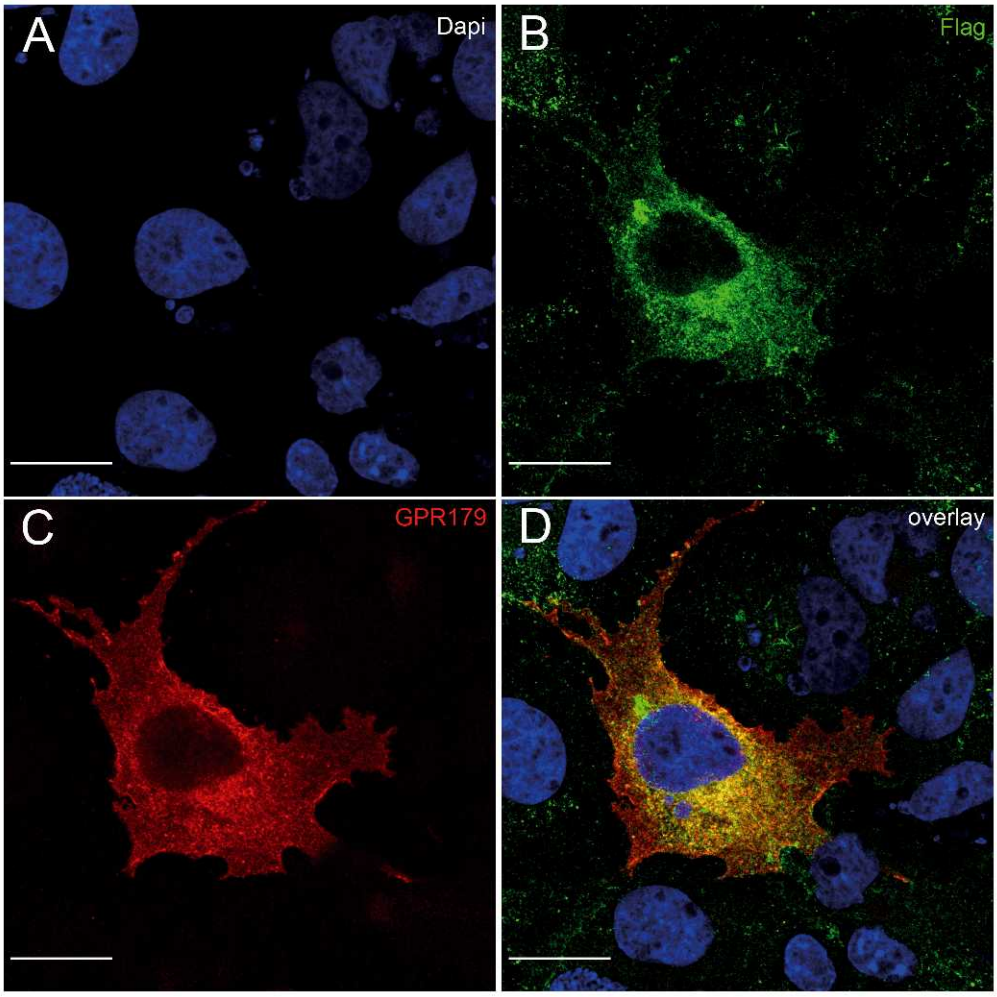

FIGURE 2. Commercial antibody raised against human GPR179 effectively detects human GPR179 in overexpressing COS-1 cells. Nuclei were stained with DAPI (*blue*) (**A**). The protein was detected by an anti-flag-tag antibody (*green*) (**B**) and an anti-GPR179 antibody (*red*) (**C**). (**D**) An overlay of the staining is shown. *Scale bars*: 20 μm.

above for the GPR179 mini-gene RT-PCR. All PCR experiments were performed 5 times. Negative controls without DNA were included. Assessments of *GPR179* mRNA and ACTB (beta-actin) mRNA levels were performed using a semiquantitative analysis (ChemiDoc XRS and Quantity One version 4.4.0 software; Bio-Rad, Hercules, CA). Domain prediction was performed by applying the Uniprot algorithm (http://www.uniprot.org).

Statistical Analyses

Statistical analyses were performed using SPSS software (version 19.0; SPSS, Inc., Chicago, IL). An assessment of normality was performed prior to applying the required statistical tests. The threshold for statistical significance was set at a P value of ≤ 0.05. To study the influence of the p.Asp126His, p.Tyr220Cys, p.Gly455Asp, and p.His603Tyr mutations on GPR179 chemiluminescence levels, mean comparisons between groups were analyzed by paired sample *t*-tests. The background noise was removed by subtracting its chemiluminescence value (plasmid pcDNA3) from the total chemiluminescence of other plasmids. Subsequently, all chemiluminescence values were proportionally transformed into percentages by fixing the permeabilized values to 100%. To study the influence of the c.1784+1G>A mutation on *GPR179* mRNA levels, mean comparisons between groups (wild-type and mutant) were analyzed by paired sample *t*-tests.

RESULTS

Expression and Immunolocalization of Mouse and Human GPR179 in the Retina

RNA in situ hybridization studies performed on mouse retina with a riboprobe against mouse *Gpr179* revealed expression in the somata of the upper part of the INL (Fig. 1A). No staining was observed using the respective sense riboprobe (Fig. 1B). To confirm that the antibody raised against the human GPR179 effectively detected it, we overexpressed a wild-type flag-tagged GPR179 plasmid in COS-1 cells and detected the protein with the anti-flag as well as with the anti-GPR179 antibody, indicating that this antibody indeed recognizes the human GPR179 protein in immunolocalization studies (Fig. 2). This antibody was then applied to a human retinal section, which revealed a clear staining in the outer plexiform layer (OPL) (Fig. 3A), more specifically in the dendritic tips of ON-bipolar cells costained for the specific markers of ON-bipolar cells: Gαo (Fig. 3B) and PKCα (Fig. 3C).[45,46] Specific labeling of the presynaptic compartments of the ribbon synapses with CtBP2 excluded presynaptic localization of GPR179 (Fig. 3D).

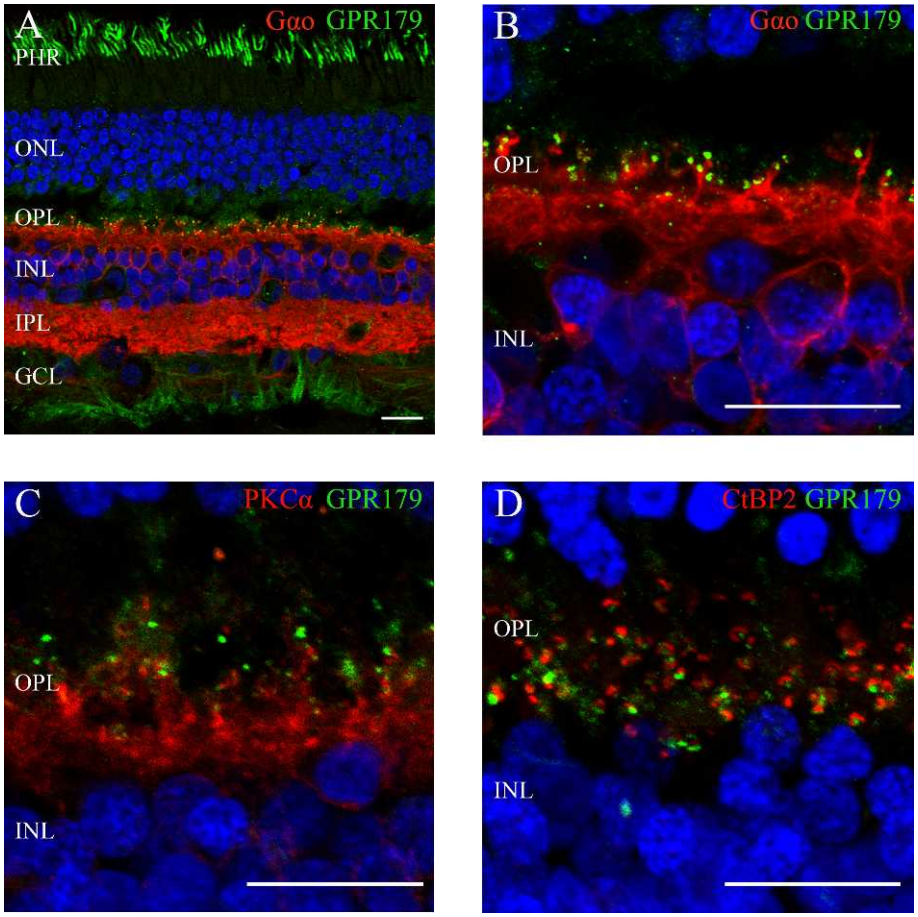

FIGURE 3. GPR179 is localized in the dendritic tips of ON-bipolar cells in whole human retina (**A**) and at ×4 magnifications (**B**–**D**). Retinal sections were double-labeled with GPR179 (*green*) and markers of distinct synapse compartments (*red*): (**A**, **B**) with Gαo (ON-bipolar cells), (**C**) with PKCα (ON-bipolar cells), and (**D**) with CtBP2 (presynaptic compartment of ribbon synapse). Nuclei were stained with DAPI (*blue*). *Scale bars*: 20 μm. PHR, photoreceptor layer.

Because of the specific expression of *Gpr179* in ON-bipolar cells (shown by our RNA in situ hybridization studies) and a background signal detected using only secondary antibody in the rod photoreceptors (negative controls in the Supplementary Data), we considered that the additional antibody staining found in the photoreceptor cell layer and Müller cells (Fig. 3A) represented non-specific staining. Similarly, in a study by Klooster et al.[47] published during the review process of our manuscript, a specific punctuate staining pattern in the OPL using the same GPR179 antibody and some unspecific labeling in other retina layers was detected, thus confirming our results.

Extra- and Intracellular Localization of Wild-Type and Mutated GPR179 Variants

To further investigate the impact of missense mutations leading to cCSNB, the extra- and intracellular immunolocalization of GPR179 in COS-1 cells overexpressing the wild-type and four mutated variants (p.Asp126His, p.Tyr220Cys, p.Gly455Asp, and p.His603Tyr) were investigated. Using live-cell staining, we showed that the GPR179 protein localizes at the surface of the cell and in intracellular compartments, presumably in the endoplasmic reticulum and Golgi apparatus as expected for G protein-coupled receptors (Fig. 4A). This was also true for the p.Asp126His variant. However, the p.Tyr220Cys, p.Gly455Asp, and p.His603Tyr mutations abolished GPR179 surface staining, and these mutant proteins were seen only in intracellular compartments (Fig. 4A). To validate this outcome with an independent method, we performed ELISA using the anti-GPR179 antibody to detect either the wild-type or the mutated GPR179 receptor transiently expressed in HEK293 cells. Again, while the p.Asp126His mutation was present at the cell surface at levels similar to those of the wild-type GPR179, levels of the other variants (p.Tyr220Cys, p.Gly455Asp, and p.His603Tyr) were severely reduced on the cell surface ($P = 0.002$ for p.Tyr220Cys and p.His603Tyr mutation and 0.0007 for the p.Gly455Asp mutation) (Fig. 4B).

Effect of Splice Site GPR179 Mutation

To investigate the effect of the *GPR179* splice site mutation c.1784+1G>A, we performed a mini-gene approach with wild-type (mini-wt) and mutant (mini-mut) exon 7 to exon 9 regions of *GPR179* amplified from the patient and wild-type genomic DNA, cloned in an expression vector, and tested their transcripts in COS-1 cells (Fig. 5A). RT-PCR analysis of the mini-wt transcript showed two bands: one of 286 base pairs (bp) and one of the expected size of 426 bp. In contrast, RT-PCR analysis of the mini-mut revealed one band of 286 bp but also another of 488 bp with the 426-bp band missing. Sequencing confirmed that the 426-bp band contained correctly spliced exons 7, 8, and 9 of *GPR179*. The band at 286 bp corresponds to a hitherto unknown alternatively spliced *GPR179* transcript lacking exon 8, whereas the 488-bp product contains exon 7 and 8 and part of intron 8 and

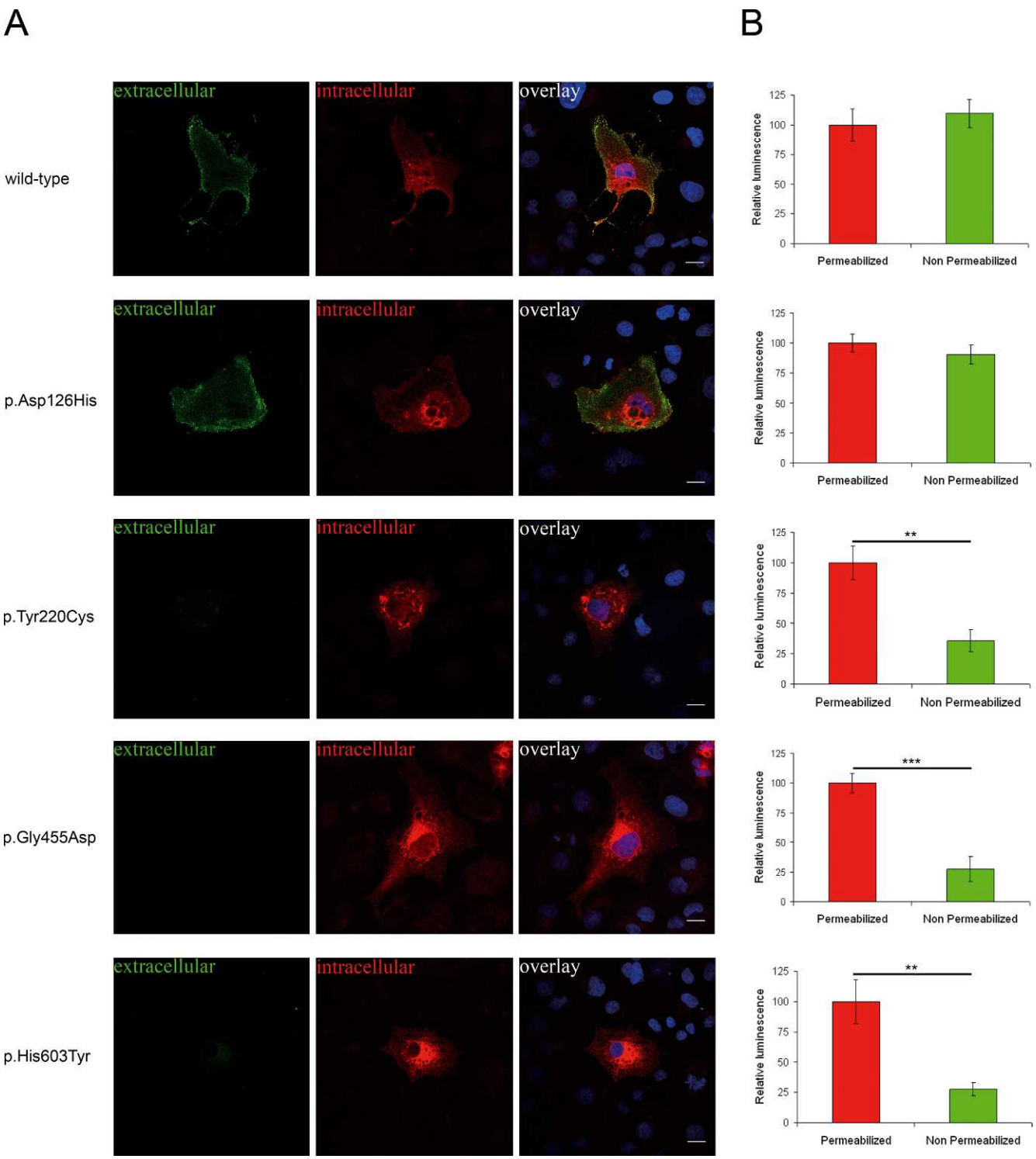

FIGURE 4. p.Tyr220Cys, p.Gly455Asp, and p.His603Tyr mutations affect cellular localization of GPR179. (**A**) Immunolocalization assay results are shown. Extracellular (*green*, *column 1*) and intracellular (*red*, *column 2*) staining were performed with COS-1 cells expressing wild-type GPR179 (*row 1*) and p.Asp126His (*row 2*), p.Tyr220Cys (*row 3*), p.Gly455Asp (*row 4*), and p.His603Tyr (*row 5*) mutated GPR179. An overlay of the these stains and DAPI-stained nuclei are presented in *column 3. Scale bar*: 20 μm. (**B**) ELISA results are shown. The wild-type GPR179 (*row 1*) and p.Asp126His (*row 2*), p.Tyr220Cys (*row 3*), p.Gly455Asp (*row 4*), and p.His603Tyr (*row5*) mutated GPR179 receptors were transiently expressed in HEK293 cells. Their presence at the cell surface was detected by ELISA (*green columns*), the total expression being detected after permeabilization of the cell with Triton X-100 (*red columns*) ($n = 3$; $^{**}P = 0.002$, $^{***}P = 0.0007$, respectively).

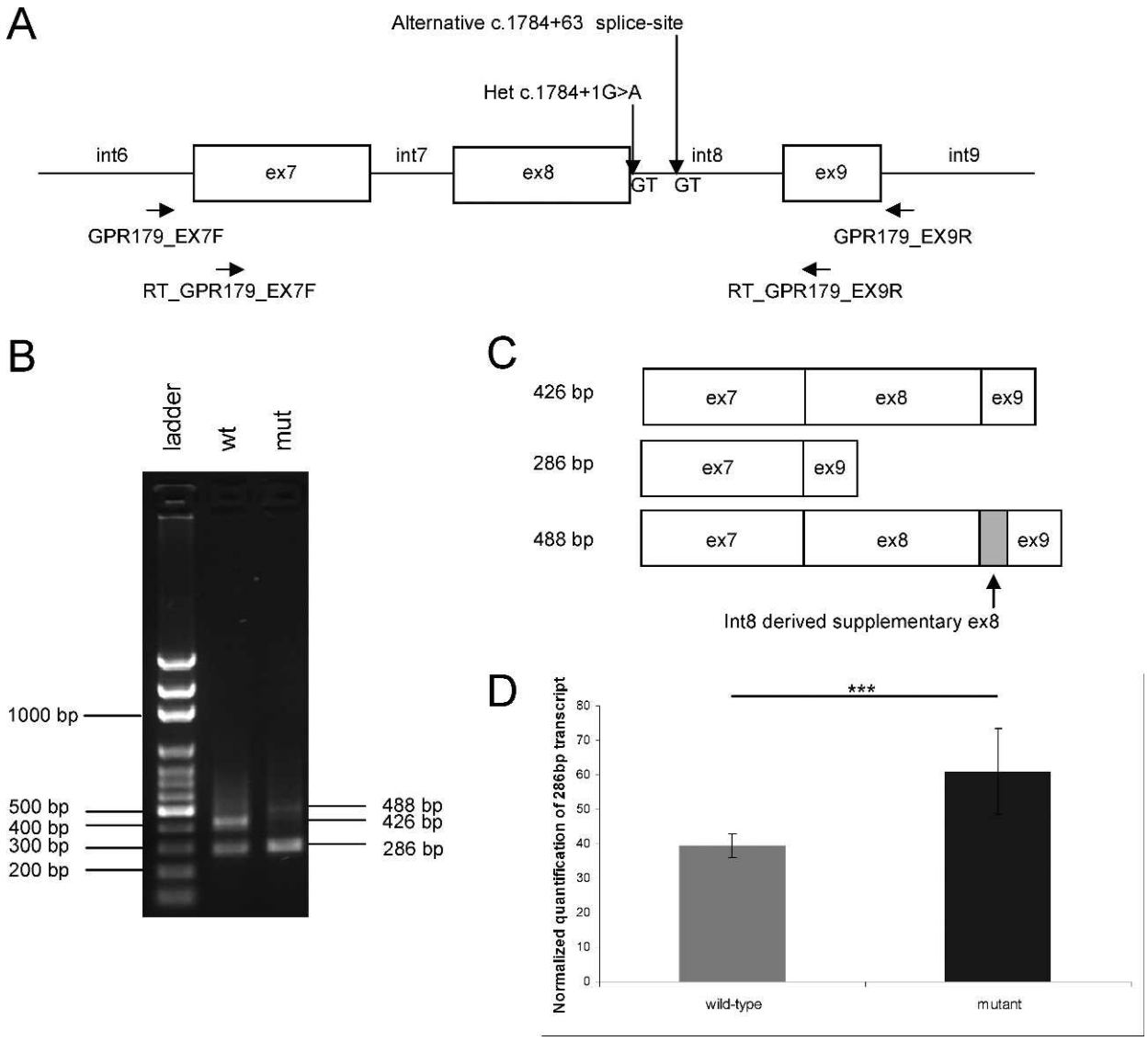

Figure 5. The *GPR179* carrying the c.1784+1G>A mutation interferes with splicing. (**A**) Schematic shows mini-genes used to analyze *GPR179* (NM_001004334.2) splicing. We compared splicing of *GPR179* control (mini-wt) and mutated (mini-mut) alleles with amplicons spanning genomic (g) regions of intron 6 to intron 9. The *horizontal arrows* show binding sites of GPR179_EX7F and GPR179_EX9R oligonucleotides used for patient gDNA PCR and the RT_GPR179_EX7F and RT_GPR179_EX9R primers used for RT-PCR analysis of mini-gene transcripts. The mutation c.1784+1G>A and the alternative c.1784+63 splice site are marked by *vertical arrows*. (**B**) Representative RT-PCR analyses of transfected COS-1 cells revealed two major transcripts (286 bp and 426 bp) for wild-type (wt) and mutated (mut) constructs (286 bp and 488 bp), respectively. (**C**) Schematic shows different splice transcripts identified by sequencing. The mini-wt 426-bp transcript includes complete exons 7, 8, and 9, whereas the mini-wt 286-bp isoform skips exon 8. The mini-mut 286-bp transcript is the same as the mini-wt 286-bp isoform, and the mini-mut 488-bp isoform includes exons 7 and 8 and a part of intron 8 and exon 9. (**D**) Semiquantitative RT-PCR showed a significant increase in skipped exon 8 PCR product in the mini-mut compared to those in mini-wt ($n = 5$; $^{***}P = 0.005$).

exon 9. This supplementary part of intron 8 is presumably due to a cryptic splice donor site localized at c.1784+63 (Figs. 5B, 5C). The difference of a 286-bp transcript between cells transfected with mini-wt and mini-mut has been observed in 5 independent PCR experiments. Five independent actin PCRs were performed with the obtained cDNA to normalize the GPR179 mini-gene values. The 286-bp product was 1.5-fold more highly expressed in mini-mut than in mini-wt cells ($P = 0.005$) (Fig. 5D).

Discussion

The present study reports the expression and localization pattern of GPR179 in mouse and human retina, using RNA in situ hybridization and immunohistochemistry. Furthermore, it describes the impact of the previously reported missense mutations (p.Tyr220Cys, p.Gly455Asp, and p.His603Tyr) by immunocytochemistry and ELISA and of a known splice site mutation (c.1784+1G>A), using a mini-gene approach.

In the current study, we observed *Gpr179* transcript expression in the somata of the upper part of the INL in mouse retina, which resembles the expression of other genes implicated in cCSNB such as *GRM6*, *NYX*, and *TRPM1* in rat,[27] chicken,[48] zebrafish,[49] mouse,[26,32] or human.[34] Although mouse GPR179 protein localization was confined to the dendritic tips of the bipolar cells,[24] the exact localization of GPR179 protein in human retina has never been investigated. This question was addressed by using a commercially available

anti-human GPR179 antibody, which was tested for its specificity by immunolocalization in COS-1 cells overexpressing tagged human GPR179. In human retina, this antibody shows staining in the OPL and, more specifically, at the dendritic tips of bipolar cells, consistent with GPR179 location in mouse retina[24,35] and other proteins implicated in cCSNB, such as GRM6,[29] NYX,[50] TRPM1,[22,34] and LRIT3[25] and which was confirmed just very recently by an independent study.[47] The sites of expression and localization of GPR179 are in accordance with previous data for the function of GPR179 regulating G protein signaling by controlling localization and activity of the RGS7 complexes,[35] which are important for the termination of G protein-coupled receptor (GPCR) signaling pathway.[51] The authors demonstrated that GPR179 colocalizes with RGS7 and RGS11 at the dendritic tips of the ON-bipolar cells in mouse retina and forms specific complexes with RGS7. This specific immunolocalization of RGS7 and RGS11 is absent in *Gpr179*$^{nob5/nob5}$ mice, lacking functional GPR179.[35] The precise function of GPR179 needs to be further elucidated, particularly whether it has only a regulatory role or also an additional function as a coreceptor of GRM6 or is an independent receptor with its own ligand.

To elucidate pathogenic mechanisms associated with the *GPR179* gene defect, the previously reported missense mutations p.Asp126His, p.Tyr220Cys, p.Gly455Asp, and p.His603Tyr were studied in vitro.[23,24] We demonstrated both by immunostaining and ELISA that the p.Tyr220Cys, p.Gly455Asp, and p.His603Tyr mutations were associated with cell surface mislocalization, whereas there were no differences between the non-mutated and the p.Asp126His variant. The Asp126 residue is localized in the predicted extracellular N-terminal region of the protein.[23] Although, the three-dimensional structure of the amino acid residues <300 of GPR179 is currently uncertain, we know from other receptors that the N-terminus of such proteins is important for ligand binding, and we could therefore hypothesize that the p.Asp126His mutation is also associated with the loss of GPR179 ligand binding[23] and not with a trafficking defect. However, to verify this hypothesis, the relevant ligand first needs to be identified. Although Tyr220 localizes in the extracellular N-terminal region of GPR179, the p.Tyr220Cys exchange is associated with a trafficking defect. This agrees with previous predictions that this mutation replaces a highly conserved Tyr with a new cysteine, which may have an impact on the structure of the protein.[24] The Gly455 residue is localized within the third transmembrane helix, predicted to be part of the GPR179 binding pocket. Because the p.Gly455Asp mutation introduces a long negatively charged side chain, we assumed this mutation could hamper the normal functioning of the receptor.[23] Here we demonstrate that GPR179 localization at the plasma membrane is severely reduced, which implies that the charge change induced by this mutation can disrupt the correct trafficking of the protein. The His603 residue is localized in the external loop, bridging the sixth and seventh transmembrane domain,[23] and is also associated with a trafficking problem. Similarly, missense mutations in *GRM6* associated with arcCSNB in three different domains also abolish proper trafficking.[19,52] Finally, the effect of the c.1784+1G>A mutation on splicing of GPR179 was tested by using a mini-gene approach in COS-1 cells overexpressing exons 7, 8, and 9 of the wild-type and c.1784+1G>A-mutated *GPR179*. The wild-type mini-gene expression revealed two transcripts, one containing all exons and one lacking exon 8. The splice donor site mutant also showed a transcript lacking exon 8, expressed at a 1.5-fold higher level than in the wild-type construct and another transcript containing exons 7 and 8, one part of intron 8 with a cryptic donor site, and exon 9. The wild-type transcript with normal exons 7, 8, and 9 was missing. The as-yet undescribed alternative splice product found with mini-wt and mini-mut lacking exon 8 leads to a shift in the open reading frame that may induce the synthesis of a truncated protein (p.Ala549Glyfs*31), which is predicted to delete GPR179 from the fifth transmembrane domain or may lead to nonsense-mediated mRNA decay. The cryptic donor site of the 488-bp product found only in the mutant form is predicted to lead to a nonsense mutation immediately after the last amino acid encoded by the exon 8 (p.F599*), which may delete GPR179 from the fourth and last extracellular domain and thus lead to a non-functional protein or to nonsense-mediated mRNA decay. The effect of the alternative splicing that leads to the transcript lacking exon 8 present in the wild-type and mutated variants needs to be further investigated to better understand whether the shorter GPR179 protein has a function through a possible dimerization with the full-length GPR179 as seen for other GPCRs.[53] If indeed nonsense-mediated mRNA decay occurs, eliminating both alternative transcripts, which then leads to loss of protein synthesis, the pathogenic mechanism of the c.1784+1G>A splice site mutation could also be explained by a loss of function.

Our results indicate that GPR179 is expressed in the INL and localized at the dendritic tips of ON-bipolar cells in the retina and that the cCNSB phenotype is the result of mislocalization of GPR179. This protein is important for the signaling cascade that occurs postsynaptically to the photoreceptors in ON-bipolar cells. The crystal structure of the protein is not available, so the exact role of each domain of GPR179 and the effect of the mutations are not known, but, based on homology models and on our new in vitro studies, we document for most of the missense and splice site mutations that the pathogenic mechanism is caused by a loss of function as it is also predicted for the frame shift and nonsense mutations previously identified in GPR179.[23,24] Further studies are needed to better understand the exact role of GPR179 in ON-bipolar cell signal transduction cascade, including the identification of a putative ligand which may unravel other pathogenic mechanisms leading to cCSNB.

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