

## Identification and functional characterization of gene defects underlying congenital stationary night blindness (csnb)

Marion Neuillé

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# Université Pierre et Marie Curie

Ecole doctorale Physiologie, Physiopathologie et Thérapeutique (ED394)

Institut de la Vision – Département de Génétique –Identification des défauts génétiques conduisant à des pathologies oculaires progressives ou non-progressives

# Identification and functional characterization of gene defects underlying congenital stationary night blindness (CSNB)

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Thèse de doctorat de Neurosciences

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A ma famille

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# LIST OF ABBREVIATIONS

Å: Angström AAV: adeno-associated vector ABCA4: ATP binding cassette subfamily A member 4 AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid ARVO: Association for Research in Vision and Ophthalmology ATPase: adenosine triphosphatase BCA: bicinchoninic acid BCs: bipolar cells Bgeo: beta-galactosidase BM: Bruch's membrane Ca: calcium CABP4/CABP4: calcium binding protein 4 CACN2B: calcium voltage-gated channel auxiliary subunit beta 2 CACNA1F/CACNA1F: calcium channel, voltage-dependent, alpha 1F subunit CACNA2D4: calcium channel, voltage-dependent, alpha 2/delta subunit 4 cCSNB: complete congenital stationary night blindness cd<sup>.</sup> candela cGMP: cyclic guanosine 3', 5'-monophosphate CO<sub>2</sub>: carbon dioxide CSNB: congenital stationary night blindness Da: Dalton DAPI: 4',6-diamidino-2-phenylindole DEP: Dishelved, Egl-10, Pleckstrin Dg/DG: dystroglycan DGC: dystrophin-glycoprotein complex DHEX: Disheveled, Egl-10, Pleckstrin helical extension DMEM: Dulbecco's Modified Eagle Medium DNA: deoxyribonucleic acid DOG: Genetic section of the German Association for Ophthalmology DTT: 1,4-dithiothreitol *Elfn1*/ELFN1: leucine rich repeat and fibronectin type III, extracellular 1

ELM: external limiting membrane

EM: electron microscopy

ERG: electroretinogram

ETDRS: Early Treatment Diabetic Retinopathy Study

EZ: ellipsoid zone

FAF: fundus autofluorescence

FRM: Fondation pour la Recherche Médicale

GAP: guanosine 5'-triphosphatase activating protein

GCL: ganglion cell layer

GDP: guanosine 5'-diphosphate

GFP: green fluorescent protein

GGL: G protein gamma-like domain

GluR: glutamate receptor

*Gnao1*/ $G_{o\alpha 1}$ : G protein subunit alpha o1

GNAT1/Gnat1: guanine nucleotide binding protein, alpha transducin 1

*Gnb3*/G<sub> $\beta$ 3</sub>: G protein subunit beta 3

 $Gnb5/G_{\beta5}$ : G protein subunit beta 5

GPCR: G protein-coupled receptor

GPI: glycosylphosphatidylinositol

GPR179/Gpr179/gpr179/GPR179: G protein-coupled receptor 179

GRM6/Grm6/grm6/mGluR6: glutamate receptor, metabotropic 6

GTP: guanosine 5'-triphosphate

HEK: human embryonic kidney

HRP: horseradish peroxidase

icCSNB: incomplete congenital stationary night blindness

iGluRs: ionotropic glutamate receptors

InDels: insertions/deletions

INL: inner nuclear layer

IPL: inner plexiform layer

IS: inner segments

IZ: interdigitation zone

K: potassium

KA2: glutamate receptor, ionotropic, kainate 5 (gamma 2)

kb: kilobase

KIZ: kizuna L-cone: long-wavelength light sensitive cone LF: lipofuscin LGN: lateral geniculate nucleus LRIT3/Lrit3/LRIT3: leucine rich-repeat, immunoglobulin-like and transmembrane domains 3 LRR: leucine rich-repeat LRRCT: C-terminal leucine rich-repeat LRRNT: N-terminal leucine rich-repeat m/z: mass to charge ratio M-cone: medium-wavelength light sensitive cone MEA: multi-electrode array mRNA: messenger ribonucleic acid MYO7A: myosin VIIA MZ: myoid zone NA: numerical aperture Na: sodium NaCl: sodium chloride NFL: nerve fiber layer NGS: next-generation sequencing NH<sub>4</sub>CO<sub>3</sub>: ammonium carbonate NMDA: N-methyl-D-aspartate nob: no b-wave *NYX/Nyx/nyx/*NYX: nyctalopin O<sub>2</sub>: dioxigen ONL: outer nuclear layer OPL: outer plexiform layer OS: outer segments P2: post-natal day 2 P30: post-natal day 30 PB: phosphate buffer PBS: phosphate buffer saline PDE: phosphodiesterase PDE6A: phosphodiesterase 6A *PDE6B/Pde6* $\beta$ : phosphodiesterase 6B

PDE6G: phosphodiesterase 6G PNA: peanut agglutinin ppm: parts per million PSD-95: post-synaptic density 95 PSTH: peristimulus time histogram *Puro*: puromycin R9AP: regulator of G-protein signaling 9 binding protein RGCs: retinal ganglion cells RGS: regulator of G-protein signaling Rgs11/RGS11: regulator of G-protein signaling 11 Rgs7/RGS7: regulator of G-protein signaling 7 RGS9 : regulator of G-protein signaling 9 RHO: rhodopsin RPE: retinal pigment epithelium S-cone: short-wavelength light sensitive cone SD-OCT: spectral domain optical coherence tomography SDS: sodium dodecyl sulphate SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis SGOF: Société de Génétique Ophtalmologique Francophone SLC24A1/Slc24a1/SLC24A1: solute carrier family 24, member 1 SLC24A2: solute carrier family 24, member 2 SLO: scanning laser ophthalmoscope Tris: Trishydroxymethylaminomethane TRPM1/Trpm1/TRPM1: transient receptor potential cation channel, subfamily M, member 1 UV-cone: ultraviolet light sensitive cone v/v: volume per volume V1: primary visual cortex VA: visual acuity w/v: weight per volume WES: whole exome sequencing

## **INTRODUCTION**

## I. The visual system in the retina

## A. General considerations about retinal and visual processing

1. Transmission of the visual signal from the eye to the brain

The retina is the light-sensitive part of the eye and corresponds to the inner-most layer of the posterior part of the ocular globe. Light enters the eye through the pupil; a circular muscle called the iris controls the diameter of the pupil, and it also gives the eye color. The transparent cornea covers both of these tissues anteriorly. After entering through the pupil, light passes through the crystalline lens and the vitreous chamber before finally reaching the retina. Cornea and crystalline lens accommodation is necessary for the formation of a sharp image on the retina. Moreover, transparency of the lens and vitreous humor is essential for sufficient light transmission to the retina. The retina is part of the central nervous system and is composed of several layers of neurons surrounded by glial cells and interconnected by synapses (Figure 1). The retina lays on a monolayer of epithelial cells, known as the retinal pigment epithelium (RPE).



Figure 1: Schematic representation of a human eye in cross-section with an enlargement of the retina (modified from Webvision)

Once light reaches the retina, photons (elementary particles of light) are captured by rod and cone photoreceptors which transform light into a biochemical signal. This signal is transmitted to bipolar cells (BCs) and then retinal ganglion cells (RGCs) which in turn transform the signal into action potentials. The visual signal is then carried by axons of RGCs that form the optic nerve to the lateral geniculate nucleus (LGN). Finally, the visual signal reaches the primary visual cortex (V1), a region of the brain in the posterior occipital lobe

where the information is analyzed to create images (Figure 2). A separate part of the brain, the accessory visual cortex, is responsible for image memory and higher visual processing.



Figure 2: Schematic representation of the visual input from the eye to the brain (modified from Webvision). LGN: lateral geniculate nucleus; V1: primary visual cortex

### 2. Histology of the retina

The retina is composed of 10 different layers, listed below from the inner retina (vitreous side) towards the outer retina (choroid side) (Figure 3):

- the inner limiting membrane, a basal membrane associated with Müller glial cell end feet, forming a barrier between the retina and vitreous
- ➤ the nerve fiber layer (NFL), composed of axons of RGCs that form the optic nerve
- > the ganglion cell layer (GCL), containing ganglion cell bodies
- the inner plexiform layer (IPL), formed by vertical synapses connecting bipolar and ganglion cells and horizontal synapses connecting bipolar and amacrine cells
- the inner nuclear layer (INL), containing cell bodies of amacrine, bipolar, horizontal cells and Müller cells
- the outer plexiform layer (OPL), formed by vertical synapses connecting photoreceptor and BCs and horizontal synapses connecting photoreceptors and horizontal cells
- > the outer nuclear layer (ONL), containing photoreceptor cell bodies
- the external limiting membrane, a zone of adherent junctions between Müller cells and photoreceptor inner segments, forming a barrier between the subretinal space and the neural retina
- the photoreceptor layer, which is composed of the inner and outer segments of photoreceptors
- > the retinal pigment epithelium (RPE) composed of a monolayer of epithelial cells



Figure 3: Simple representation of retinal layers (modified from Webvision)



Figure 4: Summary of retinal pigment epithelium functions (adapted from (1))

Horizontal cells play an important role in the organization of the spatially opponent receptive fields of BCs and RGCs, which modulate the photoreceptor signal for better light sensitivity and adjust the synaptic gain. Amacrine cells serve to integrate, modulate and interpose a temporal domain to the visual message presented to the ganglion cell. Müller cells are glial cells that are necessary to maintain the correct structure and function of the neural retina. Epithelial cells from the RPE form the hemato-retinal barrier and are essential for the survival of the photoreceptors. They are responsible for the phagocytic cycle of photoreceptor outer segments, the recycling of 11-*cis* retinal through the vitamin A cycle, protection against oxidative stress induced by light, the supply of nutrients to the retina, the ionic balance and the secretion of trophic factors (1) (Figure 4).

## **B.** The visual cascade in the retina

#### 1. Photoreceptors

a. Types and structure: comparison between human and mouse

Photoreceptors are the first order neurons of the retina. They are located in the outer part of the retina and transform photons (light energy) into an electrochemical signal. In mammals, two types of photoreceptors coexist: rods and cones, which are named according to the shape of their outer segments. Each photoreceptor is composed of an outer and inner segment. The phototransduction cascade takes place in the outer segment. The inner segment contains a lot of mitochondria and ribosomes and is connected to the outer segment through a connecting cilium. The cell body contains the nucleus and the synaptic terminal contacts the second order neurons of the retina (2). The outer segments of photoreceptors are filled with stacks of discs continuously extending from the connecting cilium; these stacks contain visual pigment molecules called opsins (3). Outer segments are also continuously digested by RPE cells, which serve to maintain a consistent length of the outer segments. In rods, the discs are independent from the plasma membrane, which envelops the entire outer segment, whereas in cones, the plasma membrane itself invaginates to form the stacks (Figure 5).



Figure 5: Schematic structure of rod and cone photoreceptors (adapted from (2))

Rods are responsible for vision under dim light conditions at night. They only need one photon to be activated and their function is saturable. Their outer segments contain the pigment molecule rhodopsin, encoded by *RHO*, with a peak-sensitivity around 500 nm wavelength of light. Cones mediate color vision and function in bright light. There are three types of cones in the human retina defined by the expression of three distinct opsins with

different light sensitivities (4). S-cones express a short-wavelength sensitive opsin that peaks at 437 nm, M-cones express a medium-wavelength sensitive opsin peaking at 533 nm, and L-cones express a long-wavelength sensitive opsin peaking at 564 nm. In contrast to humans, only two types of cones are present in the mouse retina, expressing two different opsins with different light sensitivities. UV-cones express an ultraviolet light sensitive opsin peaking at 370 nm and M-cones express a medium-wavelength sensitive opsin peaking at 511 nm (5).

#### b. Density and localization in the retina: comparison between human and mouse

Photoreceptors are distributed on the whole surface of the retina except above the optic disc, the nasal zone where the optic nerve fibers leave the eye. The optic disc corresponds to the "blind spot" in the visual field. Although humans are diurnal mammals and mice are nocturnal mammals, the retina of both species is dominated by rods: the human retina is composed of approximately 95% rods and 5% cone photoreceptors (6) and the mouse retina is composed of approximately 97% rods and 3% cone photoreceptors (7). The human and mouse retinas contain a specialized central area known as the macula and area centralis (8), respectively. These regions are highly enriched in cones as compared to the rest of the retina, although rods are still the predominant photoreceptor type in this region. However, the most central part of the macula in humans (but not mice) is a region called the fovea that is composed exclusively of cones (6) (Figure 6); this region is responsible for sharp central vision and maximal visual acuity. Although the murine retina lacks a fovea, the general composition and organization of photoreceptors in murine and human retinas are otherwise comparable (9).



Figure 6: Rod and cone densities in the human retina (adapted from (10))

However, there are notable differences between murine and human retinas in the distribution of the different cone subtypes. In humans, M- and L-cones represent 90% of cones and are

present on the whole retinal surface, but they are the only types of cones in the fovea. S-cones represent only 10% of cones and are completely absent from the fovea (11). In mice, M-cones localize to the dorsal half of the retina whereas UV-cones localize mainly to the ventral half (12).

#### c. The phototransduction cascade

As previously described, photoreceptors transform light into a biochemical signal. The first steps of this transformation occur as part of the phototransduction cascade, whereby the capture of a photon leads to the hyperpolarization of the photoreceptor plasma membrane (13) (Figure 7).



Figure 7: Schematic representation of rod phototransduction (modified from Webvision). R\*: activated rhodopsin; G\*: activated transducin

Rod phototransduction is a G-protein-signaling cascade. Rhodopsin is the G protein-coupled receptor, anchored in the outer segment discs and associated with the chromophore 11-*cis* retinal. Photon absorption by 11-*cis* retinal triggers its isomerization into all-*trans* retinal and leads to the activation of rhodopsin. Activated rhodospin binds the  $\alpha$ -subunit of the heterotrimeric G protein transducin, encoded by *GNAT1*, catalyzing the exchange of guanosine 5'-triphosphate (GTP) for guanosine 5'-diphosphate (GDP). The activated  $\alpha$ -subunit of transducin interacts with the two inhibitory  $\gamma$ -subunits of the cyclic guanosine 3', 5'-monophosphate (cGMP) phosphodiesterase (PDE), encoded by *PDE6A*, *PDE6B* and *PDE6G*, respectively. The catalytic  $\alpha$ - and  $\beta$ -subunits of PDE are released and catalyze the

hydrolysis of cGMP. The consequent decrease in the cytoplasmic free concentration of cGMP leads to closure of the cGMP-gated cation channels located in the plasma membrane of rod outer segments. Consequently, the influx of cations into rod outer segments is inhibited, resulting in their hyperpolarization. During recovery from the photoresponse, transducin is deactivated by hydrolysis of bound GTP, allowing the inhibitory  $\gamma$ -subunits of PDE to rapidly re-inhibit the  $\alpha$ - and  $\beta$ -subunits. Rhodopsin is also deactivated and the all-*trans* retinal is recycled by RPE cells. In darkness, cGMP-gated cation channels are open and mediate the influx of cations (primarily Na<sup>+</sup> but also Ca<sup>2+</sup>) into the cell (the so-called "dark current") (14). Sodium ions are actively expelled by a Na/K-adenosine triphosphatase (ATPase) whereas a Na/Ca<sup>2</sup>-K exchanger, SLC24A1 in rods and SLC24A2 in cones (15), balances the Ca<sup>2+</sup> current in photoreceptor outer segments.

#### d. Glutamate release

In the synaptic terminal of photoreceptors, light stimulus is finally transformed into a biochemical signal. Photoreceptors use the neurotransmitter glutamate to transmit the visual signal to the second order neurons of the retina. Glutamate release at the photoreceptor synapse is controlled by Ca<sup>2+</sup> currents and requires a Ca<sup>2+</sup> channel (13). Photoreceptors specifically express CACNA1F, which is the  $\alpha_1$ -subunit of a voltage-dependent L-type Ca<sup>2+</sup> channel. This subunit forms the pore of a heteromultimeric protein complex, which transmits the calcium influx across the synaptic membrane. Auxiliary subunits  $\beta$ ,  $\gamma$  and  $\alpha 2\delta$ , the last one being encoded by *CACNA2D4*, modulate calcium currents and are involved in the correct localization and assembly of the complex at the synaptic membrane. Moreover, CABP4 is a Ca<sup>2+</sup> binding protein that is associated with CACNA1F for its modulation. In darkness, photoreceptors' membrane potential is around -30 mV. CACNA1F is in an open conformation and calcium influx triggers a continuous release of glutamate in the synaptic cleft. Conversely, when the phototransduction cascade is initiated by light, photoreceptors hyperpolarized and glutamate release is reduced (Figure 8).



Figure 8: Schematic drawing of the molecular pathways at the first visual synapse between photoreceptors and ON-BCs without considering LRIT3 (adapted from (16))

#### e. Photoreceptor synapses

Photoreceptors release glutamate at a specialized chemical synapse, the ribbon synapse, allowing a sustained neurotransmitter release at high rates (17). The ribbon is a large electrondense plate-like structure that is encountered by a large number of regularly aligned glutamate vesicles. One of the proteins present in the ribbon is ribeye. The number of ribbons differs between rod and cone synaptic terminals. The synaptic terminal of rod photoreceptors is small and is called the rod spherule. It usually contains only one ribbon (Figure 9). The synaptic terminal of cone photoreceptors is larger than the rod terminal and is called the cone pedicle. It may contain between 20 and 42 ribbons (18) (Figure 10).



Figure 9: Organization of the rod spherule (modified from Webvision). Electron micrograph (left) and schematic representation (right) of a rod spherule. The presynaptic ribbon is opposed to the invaginating axons of horizontal cells (HC, yellow) and dendrites of rod bipolar cells (rb, orange)



Figure 10: Organization of the cone pedicle (adapted from (18)). (A) Schematic drawing of a cone pedicle in crosssection. Four presynaptic ribbons flanked by synaptic vesicles and four triads are shown. Invaginating dendrites of horizontal cells (medium gray) form the lateral elements, and invaginating dendrites of cone ON-BCs (light gray) form the central elements of the triads. Cone OFF-BCs dendrites (dark gray) make flat contacts at the cone pedicle base. (B) Reconstruction of the horizontal view of a cone pedicle. The short black lines represent the ribbons. Invaginating cone ON-BC dendrites (light gray) and horizontal cell dendrites (darker gray) form the 40 triads associated with the ribbons. Flat contacts are not shown. (C) Electron micrograph of a vertical section through the synaptic complex of a cone pedicle base. The upper third of the micrograph shows the cone pedicle filled with vesicles. A typical triad containing a presynaptic ribbon (arrowhead), two lateral horizontal cell processes ("H"), and one invaginating bipolar cell dendrite (star) is present in the upper left part. A flat (basal) contact is indicated by an asterisk

Photoreceptor synaptic terminals end in the OPL where they synapse with BCs, the second order neurons of the retina. It is also at this level that ON- and OFF-pathways originate as the two major parallel pathways of the visual system (19). These two distinct pathways extend from the retina to the visual cortex.

#### 2. ON pathways

#### a. Different subtypes of ON-BCs and their proximal synapse

The term "ON-BCs" comes from the fact that this type of BC depolarizes at the switch from dark to light conditions. This sign-inverting synapse between photoreceptors and ON-BCs exists because of the expression of the metabotropic glutamate receptor 6 (GRM6/mGluR6), which initiates the G protein-signaling cascade, at the dendritic tips of ON-BCs (20-22). In mouse, there are six subtypes of ON-BCs including rod BCs (23) (Figure 11). ON-BCs synapse with photoreceptors in a particular orientation named a triad. One dendrite of one ON-BC invaginates the rod spherule and the cone pedicle in line with a ribbon. Two dendrites of horizontal cells invaginate the photoreceptor terminal laterally to the ON-BC dendrite (24) (Figure 9, Figure 10).



Figure 11: The different subtypes of BCs in mouse retina (adapted from (23)). RBC: rod BCs; IPL: inner plexiform layer

b. The mGluR6 signaling cascade at the ON-BC dendritic tip

As described above, photoreceptors have a resting potential of -30 mV in the dark, continuously releasing glutamate at the ribbon synapse (25). mGluR6 is a G protein-coupled receptor that binds glutamate (20-22) at the dendritic tips of ON-BCs (Figure 8). Following this binding event, mGluR6 catalyses the exchange of GTP for GDP on the splice variant 1 of the  $\alpha$ -subunit of its G protein, G<sub>0</sub>, which dissociates from  $\beta\gamma$ -subunits (26-28). G<sub>B3</sub> is proposed to be the  $\beta$ -subunit and G<sub> $\gamma$ 13</sub> is proposed to be the  $\gamma$ -subunit of this heterotrimeric G-protein (29-31). At the end of the cascade, activated G<sub>0</sub> inhibits the closure of the transient receptor potential melastatin 1 (TRPM1) cation channel (32-34). A glycosylphosphatidylinositol (GPI)-anchored protein, NYX, has been demonstrated to be essential for the correct localization of TRPM1 at the dendritic tip of ON-BCs (35). Upon light stimulus, photoreceptor glutamate release drops, the G protein-signaling cascade is deactivated, and TRPM1 opens, leading to an influx of cations and to the depolarization of ON-BCs.

It is hypothesized that the  $G_o$  protein directly acts on TRPM1 (36). According to this hypothesis, both  $\alpha$ - and  $\beta\gamma$ -subunits would bind TRPM1, with the  $\beta\gamma$ -subunits at the N-terminus and the  $\alpha$ -subunit at both the N- and C-termini. When the  $G_o$  protein is activated,  $G_{o\alpha 1}$ -GTP would dissociate from  $G_{\beta 3\gamma 13}$  at the N-terminus of TRPM1 and would bind the C-terminus of TRPM1, inducing a conformational change of TRPM1 and leading to its closure. When the  $G_o$  protein is deactivated,  $G_{o\alpha 1}$ -GDP would swing from C- to N-terminus of TRPM1 to bind  $G_{\beta 3\gamma 13}$ , inducing a conformational change of TRPM1, leading to its opening (36). The  $G_o$  protein is able to naturally deactivate, but at a rate that is incompatible with proper visual signal transmission (37). Thus the assistance of regulator of G protein signaling (RGS) proteins of the R7 group is required for the rapid hydrolysis of GTP. At the ON-BC dendritic

tips, these RGS proteins are RGS7 and RGS11 (38-40). In mouse retina, RGS7 and RGS11 are in excess compared to mGluR6, indicating that the deactivation of the G-protein cascade is not a rate-limiting step in generation of the depolarizing response (41).

These RGS proteins contain four distinct structural domains and form heterotrimeric complexes with two binding partners (42). The catalytic domain, also called guanosine 5'triphosphatase activating protein (GAP), is located at the C-terminus of the molecule and is responsible for the stimulation of GTP hydrolysis on  $G_{o\alpha 1}$ . The second RGS protein domain, known as the G protein gamma-like domain (GGL), is involved in an interaction with a specific  $\beta$ -subunit of G protein named G<sub>B5</sub> (40, 43, 44). The Disheveled, Egl-10, Pleckstrin (DEP) and the DEP helical extension (DHEX) domains are located at the N-terminus. These domains are necessary for the interaction of RGS7 and RGS11 with membrane proteins in order to keep them in close proximity to the plasma membrane of the dendritic tips of ON-BCs. Two different anchored proteins have been described at the ON-BC dendritic tips: RGS9 anchored protein (R9AP) and GPR179. R9AP is responsible for the anchoring of RGS11, forming a RGS11/G<sub>B5</sub>/R9AP complex (44, 45). GPR179 is responsible for the anchoring of RGS7, forming a RGS7/G<sub>b5</sub>/GPR179 complex (46). GPR179 is also responsible for the anchoring of R9AP and therefore, indirectly, for the anchoring of RGS11 (47). However, since GPR179 is a G protein-coupled receptor and because it interacts with mGluR6 (48), it may have another function in addition to the mGluR6 signaling cascade regulation (e.g as a co-receptor) (49).

#### c. The distal synapses of ON-BCs and ON-RGCs

The mammalian IPL is subdivided into five strata of equal thickness. Between the second and third strata is the boundary between the OFF-sublamina and the ON-sublamina (50) (Figure 11). Axons of ON-BCs end in the inferior part of the IPL, in the ON-sublamina, where they synapse with their specific third order neurons of the retina, the ON-RGCs. They also synapse with ON/OFF-RGCs. ON-RGCs have an increased spiking activity at the light onset (51). Only cone ON-BCs axons directly contact ON-RGCs. Rod BCs first synapse with a subtype of amacrine cells, AII amacrine cells, which in turn form electrical synapses onto the axon terminals of cone ON-BCs (52). Alternative routes for rod pathways have recently been demonstrated including one route through gap junctions that exist between rod spherules and cone pedicles (53, 54) (Figure 12).



Figure 12: Rod and cone pathways in the mammalian retina (adapted from (55)). There are five different rod pathways: 1) rods→rod BCs→AII amacrine cells→cone ON-BCs→ON-RGCs; 2) rods→rod BCs→AII amacrine cells→cone OFF-BCs→OFF-RGCs; 3) rods→cones→cone ON-BCs→ON-RGCs; 4) rods→cones→cone OFF-BCs→OFF-RGCs; 5) rods→cone cone OFF-BCs→OFF-RGCs. There are two different cone pathways: 1) cones→cone ON-BCs→ON-RGCs; 2) cones→cone OFF-BCs→OFF-RGCs. OS/IS: outer segments/inner segments

#### 3. OFF pathways

#### a. Different subtypes of OFF-BCs and their proximal synapse

The name "OFF-BCs" comes from the fact that this type of BC is inhibited by light. This sign-conserving synapse between photoreceptors and OFF-BCs exists because of the expression of ionotropic glutamate receptors (iGluRs) that form integral and non-selective cation channels at the dendritic tips of OFF-BCs (56). In mice, there are five subtypes of OFF-BCs that contact mainly cones (23) but it has been shown that type 3a, type 3b and type 4 OFF-BCs also contact rods (57, 58) (Figure 11). OFF-BCs synapse with photoreceptors by forming flat contacts (Figure 10).

#### b. Ionotropic glutamate receptors

In the dark, photoreceptors have a resting potential of -30 mV and continuously release glutamate at the level of the ribbon synapse (59). This means that glutamate is not directly released in immediate proximity of OFF-BC dendrites, but rather that it spills over ribbon synapses and diffuses towards the dendritic tips of OFF-BCs, which express iGluRs (25, 60). Two different families of iGluRs are present at the dendritic tips of OFF-BCs: AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors (GluR1-4) (61) and kainate (2-carboxy-3-carboxymethyl-4-iso-propenyle-pyrrolidine) receptors (GluR5 and KA2) (62, 63). They form heteromeric complexes and act as integral, non-selective cation channels (56). Channels open in response to glutamate binding, leading to OFF-BC depolarization. It has

functionally and anatomically been demonstrated that OFF-BCs express distinct types of dendritic iGluRs in the mouse retina (64, 65) (Figure 13). Moreover, kainate receptors have been shown to mediate transient responses whereas both AMPA and kainate receptors contribute in sustained OFF-BC subtypes (65).



Figure 13: Patterns of iGluRs expressed by the different subtypes of OFF-BCs (adapted from (64)). GluA1=GluR1; GluK1=GluR5; n.d: not determined

#### c. The distal synapse of OFF-BCs and OFF-RGCs

Axons of OFF-BCs end in the superior part of the IPL, in the OFF-sublamina, where they synapse with their specific third order neurons of the retina, the OFF-RGCs. They also synapse with ON/OFF-RGCs. OFF-RGCs have an increased spiking activity at the light offset (51). Only OFF-BC axons directly contact OFF-RGCs. Rod BCs first synapse with AII amacrine cells, which in turn form inhibitory chemical synapses onto the axon terminals of cone OFF-BCs (52). A third rod-driven OFF-pathway is through gap junctions that exist between rod spherules and cone pedicles (53, 54) (Figure 12).

#### 4. Receptive fields of RGCs

The receptive field of an RGC may be considered as the portion of the visual field in which light stimuli evoke responses in a ganglion cell (51). It corresponds to all the photoreceptors that connect (indirectly) an RGC. The receptive field of an RGC is subdivided into two concentric regions, the center and the surround. Two major types of ganglion cells are thus described: ON-center/OFF-surround and OFF-center/ON-surround RGCs. ON-center/OFF-surround RGCs increase their firing frequency when a spot of light is presented within the center of their receptive field and when the surround remains in the dark. On the contrary, a light stimulus presented in the surround region inhibits the cells' response at light offset (Figure 14). OFF-center/ON-surround RGCs have

an opposite behavior. OFF-center/ON-surround RGCs increase their firing frequency when a light stimulus is presented in the surround region of their receptive field and when the center remains in the dark. On the contrary, a spot of light presented within the center inhibits the cells' response at light onset but increases the cells' response at light offset (Figure 14) (66). The center-surround organization of the receptive fields allows the RGCs to optimally respond to contrasts rather than only to absolute luminance.

ON-center/OFF-surround



OFF-center/ON-surround



Figure 14: The receptive fields of RGCs and their behavior (modified from: http://lecerveau.mcgill.ca). ONcenter/OFF-surround RGCs increase their firing frequency when the light stimulus is presented at the center of their receptive field (left, top), whereas a light stimulus presented in the surround region inhibits the cells' activity at light onset but increases the cells' activity at light offset (left, middle and bottom). OFF-center/ON-surround RGCs increase their firing frequency when the light stimulus is presented in the surround of their receptive field (right, top), whereas a light stimulus presented in the center inhibits the cells' activity at light onset but increases the cells' activity at light offset (right, middle and bottom). The first dashed line represents light onset, and the second dashed line represents light offset

# C.Diagnostic tools for retinal diseases: comparison between human and mouse

In this part, I will develop only diagnostic tools that have been used in experiments performed during my thesis. Depending on the study, I will compare these tools in human and in mouse.

## 1. Visual acuity (VA)

Visual acuity (VA) represents the spatial resolving capacity of the visual system or, in simpler terms, the ability of the eye to see fine details. VA can be affected by different factors such as refractive errors and eye movement.

#### a. In human: Early Treatment Diabetic Retinopathy Study (ETDRS) chart



Figure 15: ETDRS chart and table showing ETDRS score with decimal conversion

The Early Treatment Diabetic Retinopathy Study (ETDRS) chart is one of the most commonly used test to evaluate the VA in humans. It is based on the recognition of optotypes, which may be letters or shapes. For this chart, the subject is placed 4 meters away from a sheet of paper covered with lines of letters with different sizes (biggest letters are on the top, smallest at the bottom) (Figure 15). The determination of VA is made based on the number of letters or shapes the subject can correctly identify. The ETDRS score is correlated to the VA expressed in the decimal form (Figure 15).

#### b. In mouse: the optomotor response

VA assessment using optotypes is not applicable for mice as they are not able to communicate. Thus, it was necessary to develop new tools to evaluate visual function in animals. It has been shown that the optomotor response allows assessment of all components of visual integration in mice in both scotopic (dark-adapted) and photopic (light-adapted) conditions (67). For this experiment, following dark or light adaptation, the mouse is placed in a rotating drum covered with vertical white and black stripes at various spatial frequencies (Figure 16). Normal mice are able to track the rotating stripes by moving their head whereas animals with altered vision cannot. The VA is determined based on the smallest spatial frequency for which the animal fails to follow the rotating drum.



Figure 16: Picture of the dispositive used for the optomotor test (adapted from (67))

#### 2. Fundus examination

a. Funduscopy

Funduscopy refers to the inspection of the structures lying at the innermost part of the globe: the retina, retinal blood vessels, optic nerve head (disc), and (to a limited degree) the subjacent choroid (68). The patient is placed in front of an ophthalmoscope that illuminates the retina, using a magnifying lens to observe the fundus, preferably after pharmacological dilatation of the pupil. The optic nerve head is a yellow-pink disc that is visually distinct from the surrounding retina, which is typically more red, brown, or orange in color and contains photoreceptors. The central retinal artery and vein emerge from the optic nerve and immediately bifurcate into superior and inferior branches, which run parallel with the retinal surface. Arteries appear smaller in diameter and lighter in color than veins. In humans, the macula is located temporally relative to the optic disc (Figure 17).



Figure 17: Normal human (left) and mouse (right) fundus (adapted from (69) and (70)). A, artery; V, vein; arrow, disc; asterisk, central retina

#### b. Fundus autofluorescence (FAF)

RPE cells play an essential role in photoreceptor survival. RPE cells phagocytose the distal part of photoreceptor outer segments, stimulating their continuous renewal. Lipofuscin (LF) is an autofluorescent pigment that normally accumulates in the lysosomes of RPE cells as a by-product of the degradation of photoreceptor outer segments (71); it can be visualized by the fundus autofluorescence (FAF) of the retina (Figure 18). LF levels increase with age (72). FAF images can be obtained through a dilated pupil with a scanning laser ophthalmoscope (SLO) with an exciting 488 nm-laser and a > 500 nm barrier filter.



Figure 18: Normal human (left) and mouse (right) FAF images (modified from Macula specialists and adapted from (73))

# 3. Structure of the retina: spectral domain optical coherence tomography (SD-OCT)

Spectral domain optical coherence tomography (SD-OCT) is a fast non-invasive method that allows the production of high-resolution cross-sectional images of the human and mouse retina. Scans of transverse sections are used to morphologically study and measure the layers of the retina and to dissect the outer retina (Figure 19). The retinal layers include: RPE/Bruch's membrane (RPE/BM) complex, the hyper reflexive band corresponding to the interdigitation zone (IZ), the photoreceptor outer segments (OS), the ellipsoid zone (EZ), the myoid zone (MZ), the external limiting membrane (ELM), ONL, OPL, INL and a complex comprising IPL, GCL and NFL (IPL+GCL+NFL). The morphology and/or thickness of some layers can be locally or globally affected in retinal diseases (71, 74), so the SD-OCT is a useful tool with which to analyze these changes over time.



Figure 19: Normal human (left) and mouse (right) SD-OCT scans (courtesy from Thomas Pugliese, MD, Centre d'Investigation Clinique des Quinze-Vingts, Paris, France and adapted from (75)). RPE/BM, retinal pigmentary ephithelium/Bruch's membrane; IZ: interdigitation zone; OS, outer segments; EZ: ellipsoid zone; MZ: myoid zone; ELM, external limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL+GCL+NFL, inner plexiform layer + ganglion cell layer + nerve fiber layer

#### 4. Function of the retina

#### a. Full-field electroretinogram (ERG)

The full-field electroretinogram (ERG) records the light-induced electrical activity of the retina in response to standardized flashes and is used to assess overall retinal function. The specific parameter that is recorded by ERG is the difference of electrical potential between the retina and the cornea on which an electrode is placed (active electrode). For humans, standardized international recommendations exist for ERG testing (76), but similar recommendations are not available for mice. In humans, after at least 20 min of dark-adapted 0.01 ERG) to elicit a rod-driven ERG trace. Then, a second and a third flash of 3.0 (dark-adapted 3.0 ERG) and 10 cd.s/m<sup>2</sup> (dark-adapted 10 ERG), respectively, are generated to obtain mixed rod-cone-driven ERG traces dominated by the rods which outnumber the cones. The subject is then light-adapted for at least 10 min with a 30 cd/m<sup>2</sup> background light to be in photopic conditions in which case the rods are saturated. A first flash of 3.0 cd.s/m<sup>2</sup> (light-adapted 3.0 ERG) elicits a cone-driven ERG trace. Finally, a 30 Hz-flicker (light-adapted 30 Hz flicker) is applied. The same types of ERG backgrounds and stimuli can be applied to mice with some adaptations (77).

The resulting ERG traces have two major components reflecting the retinal physiology: (1) the a-wave, mainly corresponding to the hyperpolarization of photoreceptors and OFF-BCs, and (2) the b-wave, mainly corresponding to the depolarization of ON-BCs in response to light onset that is counterbalanced by OFF-BCs responses (Figure 20). Further detail about the cellular origins of each ERG wave under different background and flash intensity

conditions is shown in Table 1. ERG study results include considerations of the morphology, implicit time, and amplitude of both a- and b-wave.



Figure 20: Origin of the a- and b-waves in the dark-adapted ERG. A schematic representation of the retina (left, from Webvision) and a representative ERG trace from the mixed rod-cone response (right) are shown. In scotopic conditions, the a-wave originates mainly from the hyperpolarization of rods and the b-wave originates mainly from the depolarization of rod BCs in response to light. Implicit times are measured from the time of the flash to the trough or the peak of the corresponding wave. The amplitude of the a-wave is measured from the baseline to the trough of the wave whereas amplitude of the b-wave is measured from the trough of the b-wave to the peak of the b-wave

ERG test	Main physiological generator(s)
Dark-adapted 0.01 ERG	- no a-wave
	- b-wave: rod BCs
Dark-adapted 3.0 ERG	- a-wave: (mainly rods) with a minor contribution of cone OFF-BCs
	- b-wave: ON-BCs (mainly rod-driven) slightly counterbalanced by cone OFF-BCs
Dark-adapted 10 ERG	- a-wave: (mainly rods) with a minor contribution of cone OFF-BCs
	- b-wave: ON-BCs (mainly rod-driven) slightly counterbalanced by cone OFF-BCs
Light-adapted 3.0 ERG	- a-wave: cones + cone OFF-BCs
	- b-wave: cone ON-BCs counterbalanced by cone OFF-BCs
Light-adapted 30 Hz flicker	- cone-driven ON- and OFF-pathways

Table 1: Physiological cellular origins of ERG waves under different testing conditions (adapted from (76))

In humans, it is also possible to separately assess the ON- and OFF-pathways of the cone visual system. In photopic conditions, when a brief flash is used, the b-wave illustrates both the depolarization of cone ON-BCs counterbalanced by cone OFF-BCs. By using a long-duration flash (> 100 ms), a positive d-wave is generated, reflecting the retina's response to light cessation and the depolarization of cone OFF-BCs at the light offset (78) (Figure 21). Several studies describe attempts to separately assess the ON- and OFF-pathways of the cone visual system in mice but none of these protocols is currently used in routine (79-83).



Figure 21: Normal human photopic ERG duration series for 10- to 300-ms flashes to isolate the d-wave (courtesy from Graham Holder, PhD, UCL Institute of Ophthalmology, UK Moorfields Eye Hospital, London, UK)

#### b. Multi-electrode array (MEA)

In mice, ON- and OFF-pathways of the cone visual system may be evaluated by multielectrode array (MEA), which records light-evoked spiking activity from RGCs from *ex vivo* isolated flat-mounted retina (84) (Figure 22). A flat-mounted retina is pressed against a MEA composed of 256 electrodes and is continuously perfused with a warmed oxygenated medium to keep it alive throughout the experiment. Light-evoked spiking activity from RGCs is elicited by applying full-field light stimuli of different intensities and wavelengths. Interpretation and analysis of the MEA results includes consideration of the morphology of the responses, their maximum firing frequency, and the time at which the firing frequency is maximal.



Figure 22: Peristimulus time histogram representing the average response to a full-field flash (adapted from (84)). The first dashed line represents light onset, and the second dashed line represents light offset. ON- and ON/OFF RGCs spike at the light onset whereas OFF- and ON/OFF RGCs spike at the light offset

## II. Congenital stationary night blindness (CSNB)

The first steps in vision occur when rod and cone photoreceptors transform light into a biochemical signal, which is subsequently processed by the retina. The initial steps occur in photoreceptors (the phototransduction cascade), and are well understood, whereas the process of signal transmission from the photoreceptors to their postsynaptic neurons (e.g. bipolar cells) remains to be further characterized. Our understanding about the phototransduction cascade was obtained by studying the molecular pathophysiology of retinal diseases such as retinitis pigmentosa and Leber congenital amaurosis, two disorders resulting from mutations in specific components of the phototransduction pathway. On the other hand, congenital stationary night blindness (CSNB) is a useful model to characterize the signal transmission cascade from the photoreceptors to postsynaptic neurons. CSNB is a clinically and genetically heterogeneous group of retinal disorders caused by mutations in genes implicated in the phototransduction cascade or in signaling from photoreceptors to adjacent bipolar cells (13, 16). These disorders are non-progressive and non-degenerative, and unlike many other retinal disorders, they are present at the time of birth. The common clinical sign in CSNB patients is either night or dim-light vision disturbance or delayed dark adaptation (Figure 23). The true prevalence of CSNB is unknown (and probably underestimated) because the night vision disturbance may be overlooked in a well-lit urban environment. In addition, CSNB is a clinical diagnosis and requires a clinical examination with specialized equipment that is only available in some hospitals, so many affected individuals may not be identified.



Figure 23: Normal vision (left and right) compared with what an individual suffering from CSNB sees in dim-light conditions (center) (from Retina Suisse)
## **A. Different forms of CSNB**

CSNB can be subdivided into different types according to the pattern of clinical signs present in affected patients, but, in all cases, the full-field ERG is critical for precise diagnosis.

## 1. The Riggs-type of CSNB

The Riggs-type of CSNB [MIM 610445, MIM 610444, MIM 616389, MIM 163500, MIM 613830] is the less common type of CSNB; only a few cases have been reported. Individuals affected with this type of CSNB display specific ERG abnormalities, as first reported by Riggs (85). These patients exhibit generalized rod photoreceptor dysfunction, with dark-adapted responses being dominated by cone responses, which are normal. Therefore, the a-wave amplitude is reduced in response to a bright flash under dark adaptation, with a possible additional reduction of the b/a ratio and an electronegative waveform due to the photopic hill phenomenon that is characteristic of the cone pathway function (86). Photopic ERG responses are preserved due to the normal cone system function (Figure 24). These CSNB patients exhibit a relatively mild phenotype as compared to CSNB patients affected with the Schubert-Bornschein-type of CSNB. They report night blindness but have normal visual acuity, normal color vision, normal fundus appearance, and no sign of high myopia, nystagmus or strabismus. They have elevated final thresholds on dark-adaptometry, which is compatible with a prolonged dark adaptation phase with normal daylight vision (16).



Figure 24: Representative ERG traces from a patient suffering from the Riggs-type of CSNB (left) compared to a non-affected individual (right) (adapted from (87))

## 2. The Schubert-Bornschein-type of CSNB

Most CSNB patients are affected by the Schubert-Bornschein subtype, and they exhibit characteristic ERG abnormalities as first described by Schubert and Bornschein. In this CSNB subtype, the b-wave amplitude is smaller than the a-wave (which is globally normal) in the dark-adapted bright flash condition (88). This electronegative waveform can be subdivided in two subtypes, complete (c)CSNB [MIM 310500, MIM 257270, MIM 613216, MIM 614565, MIM 615058] and incomplete (ic)CSNB [MIM 300071], characterized by ON- or both ON- and OFF-pathway dysfunction (89, 90), respectively. Patients suffering from the Schubert-Bornschein-type of CSNB display a more severe phenotype as compared to patients affected with the Riggs-type, and they often exhibit additional ocular abnormalities such as reduced visual acuity, myopia, nystagmus, photophobia and strabismus (13, 16). The Schubert-Bornschein-type of CSNB results from a post-phototransduction defect and affecting signal transmission between photoreceptors and BCs.

#### a. Incomplete CSNB (icCSNB)

icCSNB results from both ON- and OFF-pathway dysfunction. The ERG b-wave is present in response to the dark-adapted 0.01 ERG, but it is of subnormal amplitude, giving the term "incomplete CSNB". In response to a scotopic brighter flash, the a-wave is normal, confirming normal rod phototransduction, but the b-wave is severely reduced, giving an electronegative waveform (89). Photopic responses are also affected. The light-adapted single flash reveals a reduced b/a ratio. The light-adapted 30 Hz-flicker is markedly subnormal with reduced amplitude and implicit time shift, with most having a distinctive bifid peak. Finally, the photopic long-duration flash confirms both ON- and OFF-pathway defects (Figure 25). Patients suffering from icCSNB may present with little or no night vision disturbances, as only 54% of icCSNB patients report night blindness that actually does impact their daily life (91, 92). Patients with icCSNB have variable degrees of refractive error, from myopia to hyperopia, with a median refractive error of -4.8 D and various degrees of nystagmus and strabismus. Fifty three percent of icCSNB patients report photophobia. In addition, icCSNB patients generally have low visual acuity (median of 20/60) and variable color vision defects despite having normal visual fields; these daylight symptoms are consistent with the involvement of both cone-driven ON- and OFF-pathways in this condition. The fundus examination of icCSNB patients is usually normal apart from myopic changes. However, changes in retinal structure and thinning of the retina have been reported in icCSNB patients as compared to patients with isolated myopia (93, 94).



Figure 25: Representative ERG traces of individuals with cCSNB (top) and icCSNB (middle) as compared to a normal subject (bottom) (adapted from (16)). DA 0.01, dark-adapted 0.01 ERG; DA 10.0, dark-adapted 10 ERG; LA 3.0 30Hz, light-adapted 30 Hz-flicker ERG; LA 3.0, light-adapted 3.0 ERG; ON-OFF, photopic long-duration flash

b. Complete CSNB (cCSNB)

cCSNB results from ON-pathway dysfunction. In this form of CSNB, the ERG b-wave is not detectable in response to the dark-adapted 0.01 ERG, giving the term "complete CSNB". In response to a scotopic brighter flash, the a-wave is normal, confirming normal rod phototransduction, but the b-wave is severely reduced, producing an electronegative waveform (89). Under photopic conditions, the single flash response frequently has a normal a-wave amplitude, but with a square shape; the waveform has a sharply rising b-wave and a mildly reduced b/a ratio. The light-adapted 30 Hz-flicker ERG response is of normal amplitude but may have a flattened trough and mild implicit time shift. Finally, the photopic long-duration flash confirms the isolated ON-pathway defect with a normal OFF-response (Figure 25). Patients suffering from cCSNB always present with night vision disturbances (91, 92), and they have variable degrees of myopia (median refractive error of -7.4 D) and nystagmus. Strabismus is also frequently reported. Visual acuity in cCSNB patients (median of 20/40) is generally superior to that of icCSNB patients, and they usually have normal color vision and visual fields. The fundus examination is usually normal apart from myopic changes. However, retinal thinning outside of the foveal region with preservation of the outer

retina has been reported in three cCSNB patients, although the authors do not comment on whether or not the same findings were evaluated in a myopic population (95).

## **B. CSNB genes and mutations**

## 1. Gene identification strategies

Disease causing variants in many genes associated with CSNB have been identified through classical linkage approaches (96-101) or through candidate gene approaches by comparing the human phenotype to similar phenotypes observed in knock-out or naturally occurring animal models (102-110). One limitation of the linkage approach is the requirement to examine large families. Techniques using massively parallel sequencing of human exons implicated in inherited retinal disorders (targeted sequencing) and all human exons (whole exome sequencing, WES), have successfully identified disease causing variants in known and novel genes underlying many heterogeneous diseases (111, 112), including CSNB (111, 113). WES is an unbiased method to identify the gene defect in families with only few family members available, but data from unaffected family members and proper filtering procedures are crucial to the rapid identification of "the" disease-causing variant in each CSNB family. A first filtering step is performed based on the mode of inheritance and the frequency of the identified genetic variants in the general population. Priorities are then given to frameshift, nonsense, missense and splice site variants as well as in-frame insertions or deletions. Identified amino acid substitutions are evaluated with respect to the evolutionary conservation at that position, as well as pathogenicity predictions and *in-silico* splice-site predictions. The expression of candidate genes of interest in the eye and retina is evaluated by use of publically available transcriptomic databases. In addition, literature searches are performed for each candidate gene to find further evidence of putative pathogenicity and/or retinal function based on animal models, metabolic pathways, or other supporting experimental of clinical data, including 3-D modeling. The most likely disease causing variants are confirmed by Sanger sequencing and co-segregation analyses. Further patients are screened for mutations in the same gene.

## 2. Riggs-type of CSNB

The Riggs-type of CSNB has been associated with disease causing variants in *RHO* [MIM 180380] (110), *GNATI* [MIM 139330] (114), *PDE6B* [MIM 180072] (96, 115) and *SLC24A1* [MIM 603617](101), in both autosomal dominant (*RHO*, *GNAT1* and *PDE6B*) and autosomal

recessive (*GNAT1*, *SLC24A1*) modes of inheritance (Figure 26). Only four families with four different mutations in *RHO* (110, 116-118), three families with three different mutations in *GNAT1* (114, 119, 120), two families with two different mutations in *PDE6B* (87, 96) and one family with one mutation in *SLC24A1* (101) have been described to date, leading to a total of 10 different mutations in 4 genes. All of these mutations are only missense mutations (change of amino acid) or frameshift mutations, leading to a premature stop codon. The proteins encoded by these genes are localized in rod photoreceptors and play an important role in the phototransduction cascade (16).



Figure 26: Genes implicated in CSNB according to ERG and mode of inheritance at the beginning of my thesis (adapted from (113)). XL, X-linked; ar, autosomal recessive; ad, autosomal dominant

## 3. Schubert-Bornschein-type of CSNB

a. icCSNB

icCSNB has been associated with disease causing variants in *CACNA1F* [MIM 300110] (97, 98), *CABP4* [MIM 608965] (105) and *CACNA2D4* [MIM 608171] (121). *CACNA1F* mutations are responsible for the X-linked form of icCSNB whereas *CABP4* and *CACNA2D4* mutations are inherited as an autosomal recessive trait (Figure 26). One hundred and thirty-three different mutations have been reported for icCSNB, with the majority identified in *CACNA1F*, followed by *CABP4* and then *CACNA2D4*. Many different types of mutations were identified in these three genes, missense mutations, nonsense mutations, in-frame insertions-deletions (Indels), mutations putatively affecting splicing, and frameshift mutations

leading to a premature stop codon or to an elongated protein. The proteins encoded by these three genes localize presynaptically and are important for the continuous release of glutamate at the photoreceptor synapse as previously described (13, 16).

### b. cCSNB

cCSNB has been associated with disease causing variants in *NYX* [MIM 300278] (99, 100), *GRM6* [MIM 604096] (107, 108), *TRPM1* [MIM 603576] (102-104) and *GPR179* [MIM 614515] (109, 113). *NYX* mutations are responsible for the X-linked form of cCSNB whereas *GRM6*, *TRPM1* and *GPR179* mutations are inherited as an autosomal recessive trait (Figure 26). One hundred and fifty-six different mutations have been reported for cCSNB, with the majority identified in *NYX* followed by *TRPM1*, *GRM6*, and finally *GPR179* (16, 122). The identified genetic variants includes missense mutations, nonsense mutations, in-frame Indels, mutations putatively affecting splicing, frameshift mutations leading to a premature stop codon and the putative loss of the initiation codon. The proteins encoded by these genes localize at the dendritic tips of ON-bipolar cells and are part of the mGluR6 signaling cascade as previously described (13, 16).

## **C.Animal models for cCSNB**

Several animal models have been described for the Riggs-type of CSNB and for icCSNB (16), but these models will not be discussed in detail for the purpose of this thesis. The primary focus of this work is the study of animal models of cCSNB and the pathogenic mechanisms associated with mutations in genes underlying cCSNB. Animal models of CSNB have played a critical role in our understanding of the molecular physiopathology of this disease, and well-characterized animal models are also crucial for the development of pharmaceutical or genetic treatments. The advantage of animal models, in addition to the possibility of *in vivo* functional and structural assessments similar to those performed on human patients (e.g. ERG recording, retinal imaging including FAF and SD-OCT, etc), is to allow *post mortem* studies for a more precise analysis of retinal structure and function. In addition, protein localization and interaction studies may be performed in samples from affected animals and compared to unaffected controls.

Twelve animal models for the Schubert-Bornschein-type of CSNB (primarily cCSNB) have been created or spontaneously occurred, including eight mouse models, three zebrafish models and one horse model. These animal models of cCSNB all exhibit molecular defects that impair signal transmission from photoreceptors to BCs. More specifically, they all exhibit defects in the mGluR6 signaling cascade at the dendritic tips of ON-BCs. The phenotype in these animal models is non progressive, stationary and characterized by night blindness as in human patients. All animals exhibit absent scotopic ERG b-waves, a phenotype now commonly known as "*nob*" for "*no b-wave*" phenotype. There are no obvious morphological changes in these animals. The ERGs in most models differ from those of humans under photopic conditions. In almost all mice, the photopic b-wave amplitude is undetectable, whereas in cCSNB patients, photopic b-waves are clearly present although of abnormal waveform. These differences in ERG pattern may reflect inter-species differences in cone populations and properties (123).

## 1. Animal models for Nyx gene defect

#### a. Mouse model for Nyx gene defect

For the Nyx gene defect, one naturally occurring mouse model carrying a deletion that is predicted to lead to a truncated protein has been described (nob) (123-125). This mouse model displays a stationary nob phenotype with an absent scotopic ERG b-wave, whereas the a-wave is normal. The photopic b-wave is also not detectable (123). For the photopic ERG, a long-latency negative wave is followed by a positive-going intrusion, illustrating the contribution of the cone-driven OFF-pathways (80). The rod-driven OFF-pathways are also functional (126). For the photopic flicker ERG, responses are smaller than in control mice at low temporal frequencies but with a different waveform. However, they become similar when the stimulus frequency increases, indicating that the derived ON-BC response is maximal at low frequencies (127). In a recent paper, Tanimoto et al. showed that rod- and cone-driven ON-responses are abolished in nob mice, whereas cone-driven OFF-responses are normal (82). ON-BCs fail to respond to glutamate or mGluR6 antagonist puffs (35, 128). The ONdefect is also highlighted in RGCs, the LGN, and in the cortex (123, 129). RGCs display an abnormally elevated spontaneous rhythmic activity after eye opening, as well as small and delayed ON-responses with decreased OFF-responses. Furthermore, RGCs projections are desegregated in the LGN. Finally, visual evoked potentials reveal no response to light onset and a slow response at the light offset. The nob mice also show decreased sensitivity to light (125). No gross morphological abnormalities are reported in the retina and even in the rod BC dendrites of these animals (123, 130).

#### b. Zebrafish model for *nyx* gene defect

One zebrafish model for the nyx gene defect was generated by injection of antisense morpholino nucleotides directed against the translation site (109, 131). At 4 days post fertilization, injected zebrafish exhibit abolition of the ERG b-wave as well as reduced contrast sensitivity. No evidence of morphological changes was reported.

## 2. Animal models for *Grm6* gene defect

#### a. Mouse models for *Grm6* gene defect

Four mouse models have been described for Grm6: one laboratory-generated knock-out  $(Grm6^{tm1Nak/tm1Nak})$  (22), one chemically-induced mouse model harboring a missense mutation (nob4, p.Ser185Pro) (132) and two naturally occurring mouse models carrying mutations that are predicted to lead to a truncated protein (nob3 and nob7) (133, 134). All of these mouse models display a stationary *nob* phenotype with an absent scotopic ERG b-wave and a normal a-wave (22, 132-134). The photopic ERG is electronegative with an increased a-wave and a severely reduced b-wave, with a small and slow positive component remaining, showing the contribution of the OFF-pathways (135). Intact functioning of the OFF-pathway is confirmed by the photopic long-duration flash ERG with the presence of a d-wave (135, 136). Tanimoto *et al.* showed an abolition of rod- and cone-driven ON-responses in these mice, whereas cone-driven OFF-responses are normal (82, 83). In addition, rod BCs fail to respond to light and are hyperpolarized (137).

These mouse models also exhibit behavioral abnormalities.  $Grm6^{tm1Nak/tm1Nak}$  mice show reduced sensitivity of papillary responses to light stimulus and a severely impaired ability to drive optokinetic nystagmus in response to visual contrasts (138). They also fail to suppress activity upon light onset despite a normal circadian system (139). *nob3* and *nob4* mice also display reduced scotopic and photopic contrast sensitivity (132, 133). At the level of RGCs and their projections to the cortex,  $Grm6^{tm1Nak/tm1Nak}$  mice show visual evoked potentials with no ON-responses and normal OFF-responses (22). Neurons of the visual cortex and RGCs in this mouse model have long-latency ON-responses of small amplitude and normal OFFresponses (136). Moreover, recording light evoked fields potentials from the superior colliculus shows a drastically reduced and delayed ON-response that further decreases as light intensity increases (140). For *nob3* and *nob4* mice, RGCs give drastically reduced ONresponses with a long latency whereas OFF-responses are relatively normal (132, 133). In addition, these mouse models display reduced orientation selectivity and contrast sensitivity in cortical cells (141).

The gross retinal morphology remains normal in the *Grm6* mouse models (22). They have a normal fundus appearance, normal retinal laminar structure (132, 133), no alteration in the cellular organization of BCs, amacrine cells and RGCs, and no defects in the stratifications of both ON- and OFF-BCs in developing and mature RGCs (142). Finally, there are no changes in the RGC fiber projections (22). However, despite normal retinal gross morphology, some ultrustructural defects have been reported in the *Grm6* mouse models. In *Grm6*<sup>tm1Nak/tm1Nak</sup> mice, rod spherules were completely normal, but cone pedicles showed a reduced number of invaginating ON-BC dendrites. This decrease was correlated with a reduced number of ribbons per cone pedicle (143). In *nob4* mice, both rod spherules and cone pedicles showed a reduced number of mouse of invaginating ON-BC dendrites (44, 144).

## b. Zebrafish model for grm6 gene defect

One zebrafish model for the grm6 gene defect was developed by injection of antisense morpholino nucleotides directed against the grm6b paralog (145). In this model, a concentration-dependent reduction of the ERG b-wave was shown at 5 days post-fertilization.

### 3. Animal models for *Trpm1* gene defect

### a. Mouse models for *Trpm1* gene defect

Two mouse models have been described for the *Trpm1* gene defect: one laboratory-generated knock-out (*Trpm1*<sup>tm1Lex/Tm1Lex</sup>) (32-34) and one naturally occurring mouse model harboring a missense mutation (*Trpm1*<sup>tvrm27/tvrm27</sup>, p.Leu134Pro) (146). Both display a *nob* phenotype with an absent scotopic b-wave, a preserved a-wave, and a photopic electronegative ERG (32-34, 146, 147). Vision in these mice is impaired with reduced contrast sensitivity and spatial frequency thresholds (32). Rod BCs are unresponsive and cone ON-BC responses are dramatically altered whereas cone OFF-BCs remain responsive (32, 34, 35). Structurally, these mouse models show normal cellular and synaptic layers. Moreover, rod and cone photoreceptor terminals have normal ribbon synapses (146).

## b. Horse model for *Trpm1* gene defect

One horse model with a 1378 base pair-insertion in the intron 1 of *Trpm1* has been described (148). This insertion corresponds to a long terminal repeat of an endogenous retrovirus that

leads to a premature poly-adenylation and disruption of the *Trpm1* transcript. This horse model displays an ERG that is similar to patients with cCSNB; there is no scotopic b-wave, a reduced photopic b-wave, and an a-wave with both a larger trough and a longer implicit time (149, 150). The photopic flicker is normal but the scotopic one is abnormal; the waveform is altered with reduced amplitude and increased implicit time (150). These horses do not suffer from myopia but show visual deficits in reduced ambient illumination (149, 150). Horses with *Trpm1* gene defects exhibit normal gross retinal morphology, including normal rod and cone inner and outer segments and normal rod and cone synapses (149).

## 4. Animal models for Gpr179 gene defect

### a. Mouse model for *Gpr179* gene defect

One mouse model for the Gpr179 gene defect has been described (*nob5*) (109). This naturally occurring mouse model harbors the insertion of an endogenous retroviral element in intron 1 of Gpr179. The dark-adapted ERG initially shows a normal a-wave and an absent b-wave (109) but a small b-wave-like response is present with low light luminance flashes (151). A slow b-wave is also present in response to long-duration scotopic flashes (151). The light-adapted ERG is electronegative (109). Rod BCs generate small but detectable responses after application of an mGluR6 antagonist (151). Structurally, the Gpr179 mice show normal cellular and synaptic layers as well as normal rod and cone ribbon synapses (109).

#### b. Zebrafish model for gpr179 gene defect

One zebrafish model for the *gpr179* gene defect was generated by injection of antisense morpholino nucleotides directed against the translation site (109). At 4-6 days post-fertilization, this zebrafish model displays significantly reduced ERG b-wave amplitudes (109).

## D. Pathogenic mechanism(s) of gene defects underlying cCSNB

The known molecular components of the mGluR6 signaling cascade (described in I.B.2.b. The mGluR6 signaling cascade at the ON-BC dendritic tip) include transmembrane proteins, GPI-anchored proteins, and cytoplasmic proteins, all of which are localized at the dendritic tips of ON-BCs. Some of proteins components, when mutated, are responsible for cCSNB. *In vitro* and *ex vivo* studies of mutations identified in patients as well as in animal models have enabled the elucidation of the pathogenic mechanism(s) underlying this disorder.

## 1. GRM6/Grm6 mutations

mGluR6 is a GPCR containing seven transmembrane domains and a bi-lobed, extracellular Nterminal domain containing the glutamate binding site. An extracellular cystein-rich region is placed between the glutamate binding site and the first transmembrane domain. In humans, 22 mutations have been identified in *GRM6* (16) (Figure 27A). Approximately half of them are nonsense or frameshift mutations potentially leading to a truncated protein. For these mutations, the pathogenic mechanism is most likely a loss of function of the receptor due to nonsense mediated mRNA decay or as a consequence of the receptor missing one or several critical functional domains. For six of the missense mutations, *in vitro* cellular localization of the mutated proteins has been evaluated in stable cell lines (152). None of these mutant proteins were demonstrated at the cell surface, suggesting an intracellular transport defect of the receptor. Although the mutations affect different domains of mGluR6, all probably result in misfolding of the protein. In the four mouse models with a *Grm6* gene defect, the encoded proteins do not show the typical ON-BC dendritic tip localization, indicating loss-of-function mutations (22, 40, 132-135, 137, 142, 153). Many other proteins of the mGluR6 signaling cascade, including TRPM1, are also mislocalized in these mouse models (Table 2).



Figure 27: Schematic drawing of proteins implicated in cCSNB at the beginning of my thesis with identified mutations (adapted from (16)). Nonsense and frameshift mutations are depicted in red, missense mutations are depicted in green, and in frame deletions and insertions are depicted in yellow. The presumed splice site mutations as well as the two most recent mutations identified in *NYX* (122) are not depicted. LRR, leucine-rich repeat; LRRNT, N-terminal LRR; LRRCT, C-terminal LRR; TM, transmembrane domain; GPI, glycosylphosphatidylinositol; ml, mislocalization; nf, no functional defect identified

## 2. NYX/Nyx mutations

Nyctalopin is a GPI-anchored protein containing 11 leucine-rich repeat (LRR) flanked by an N-terminal LRR (LRRNT) and a C-terminal LRR (LRRCT). To date, 71 mutations in this gene have been identified in cCSNB patients of which 75% are missense mutations (16, 122) (Figure 27D). Similar to the case for *GRM6* mutations, nonsense or frameshift mutations are most likely leading to a loss of function of the protein due to nonsense mediated mRNA decay or to a non-functional truncated protein. The subcellular localization of three mutant proteins has been studied, but they do not show a trafficking defect (154). Trafficking defects are thus probably not the pathogenic mechanism associated with *NYX* mutations. However, it has been shown that nyctalopin interacts with both TRPM1 and mGluR6 (35, 155) and that it is necessary for the correct localization of TRPM1 at the dendritic tips of ON-BCs (35) (Table 2). Consistent with this, rod BCs of *nob* mice lack functional TRPM1 channels as demonstrated by patch clamp recordings (35). In addition, restoration of nyctalopin

expression in *nob* mice restores proper TRPM1 cellular localization, thereby restoring a normal phenotype (128, 156). Thus, the pathogenic mechanism associated with *NYX* mutations is most likely related to the mislocalization of TRPM1 and the incapacity of ON-BCs to depolarize upon light stimulus.

## 3. TRPM1/Trpm1 mutations

TRPM1 is a non-selective cation channel with six transmembrane domains. To date, 51 *TRPM1* mutations have been identified in cCSNB patients (16) (Figure 27C). Again, nonsense or frameshift mutations leading to potentially truncated proteins are most likely leading to a loss of function of the protein due to nonsense mediated mRNA decay or to a non-functional protein. Two splice-site mutations are predicted to abrogate normal splicing and lead to abnormal protein production, suggesting that they are also loss-of-function alleles (147). In addition, two missense mutations located in the N- and C-terminal ends of the sixth transmembrane domain (one mutation in each end) have been studied and exhibit no ON-BC dendritic tip staining when electroporated in the mouse retina, indicating that mislocalization of TRPM1 may lead to cCSNB (147). In the *Trpm1* knock-out mouse model, TRPM1 is absent (Table 2) and there is no ON-response in ON-BCs after application of a TRPM1 agonist (34, 35, 151). Interestingly, a different mouse model carrying a missense mutation in *Trpm1* (*Trpm1*<sup>tvrm27/tvrm27</sup>) shows correct localization of TRPM1 (Table 2) at the dendritic tips of ON-BCs, indicating that the *nob* phenotype is the result of a loss of channel function (146).

## 4. GPR179/Gpr179 mutations

To date, 14 different mutations have been identified in *GPR179* leading to cCSNB (16) (Figure 27B). Again, nonsense or frameshift mutations are most likely leading to a loss of function of the protein. One predicted splice site mutation was confirmed to result in a missiplicing event, probably leading again to the loss of function of the protein (49). In another report, of the four missense mutations studied, three resulted in proteins that are not properly localized to the plasma membrane, indicating a trafficking defect (49). In the case of the fourth missense mutation (which had proper localization to the plasma membrane), the mutated residue is located at the predicted extracellular N-terminal region of the protein and is probably involved in ligand binding. Thus, even if the ligand of GPR179 is still unknown, this missense mutation could lead to a ligand binding defect. In the spontaneous occurring mouse model with a *Gpr179* gene defect, the corresponding mRNA level is dramatically decreased

and nonsense-mediated mRNA decay leading to a null allele is the predicted pathogenic mechanism (109).

Gene defect	Mouse model	mGluR6	GPR179	Nyctalopin	TRPM1	RGS7	RGS11	Gß5	R9AP
Grm6	$Grm6^{tm1Nak/tm1Nak}$ (22)	no DTB	DTB	n.d.	reduced DTB (137)	no DTB	no DTB	reduced	no DTB
		(22, 135, 137, 142, 153)	(151, 153)			(153)	(137, 153)	DTB (137)	(153)
	nob3 (133)	no DTB (133)	no DTB (48)	n.d.	no DTB (48, 155)	n.d.	n.d.	n.d.	n.d.
	nob4 (132)	no DTB (40, 132)	n.d.	n.d.	no DTB (155)	reduced	no DTB	no DTB	no DTB
						DTB (44)	(40, 44)	(44)	(44)
	nob7 (134)	no DTB (134)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gpr179	nob5 (109)	DTB (47, 151, 153)	no DTB	DTB	DTB (46, 47, 151, 153)	no DTB	no DTB	no DTB	no DTB
			(46, 47, 109, 151, 153, 157)	(151, 153)		(46, 153)	(47, 153)	(47, 158)	(47, 153)
Nyx	nob (123, 125)	DTB (130, 153)	DTB	n.d.	no DTB (35, 153)	DTB	DTB	n.d.	DTB
			(151, 153)			(153)	(153)		(153)
Trpm1	Trpm1 <sup>tm1Lex/tm1Lex</sup> (32-34)	DTB	DTB	DTB	no DTB	DTB	DTB	n.d.	DTB
		(34, 35, 48, 144, 146, 153)	(48, 151, 153)	(35, 153)	(32, 34, 35, 144, 151, 153)	(153)	(153)		(153)
	$Trpm1^{tvrm27/tvrm27}$ (146)	DTB (146)	n.d.	n.d.	DTB (146)	n.d.	n.d.	n.d.	n.d.

Table 2: Localization of many known components of the mGluR6 signaling cascade at the dendritic tips of ON-BCs in cCSNB mouse models without considering LRIT3 (adapted from (16)). DTB, dendritic tip staining

## **III. Objectives of the study**

Despite comprehensive genotyping studies of our own CSNB cohort, some cases remain with no identified disease-causing variants in known CSNB genes. This finding strongly indicates that additional disease-causing variants in other genes remain to be discovered, or that defects in the regulatory elements or introns of known CSNB genes may be involved. Therefore, the objectives of this thesis are:

- 1) Identification of novel gene defects underlying CSNB
- 2) Expression studies of genes underlying CSNB and immunolocalization studies of the encoded proteins in the human retina
- Genetic and phenotypic characterization of a new mouse model with a new gene defect underlying CSNB
- 4) Localization analysis of photoreceptor synaptic proteins in this mouse model
- 5) Study of the ultrastructure of the photoreceptor ribbon synapses in this mouse model
- 6) Evaluation of the ON- and OFF-pathways function in this mouse model
- Identification of binding partners of the protein of interest in the human and/or mouse retina.

These objectives will result in the functional characterization of the corresponding protein and to the elucidation of pathophysiological mechanism(s) by which mutations in this protein lead to CSNB.

## **MATERIALS AND METHODS**

This section includes only the materials and methods which have not yet been published as part of this doctoral work. All previously published materials and methods are not described here.

## I. Ethics statements

All animal procedures were performed according to the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes, as well as the National Institute of Health guidelines. The procedures were granted formal approval by the Institutional Animal Care and Use Committee of the Scripps Research Institute.

## II. Animal care

The generation and characterization of the *Lrit3* knock-out mouse model (*Lrit3<sup>nob6/nob6</sup>*) was previously described (75) (http://www.taconic.com/knockout-mouse/lrit3/tf2034). The *Grm6* knock-out mouse model (*Grm6<sup>-/-</sup>*) [129S6.129S(Cg)-Grm6<sup>tm1Nak</sup>] mouse model was obtained commercially (Riken, Ibaraki, Japan). Nine *Lrit3<sup>nob6/nob6</sup>* and 4 *Grm6<sup>-/-</sup>* homozygous mutant adult mice of both sexes and 15 control animals (10 for *Lrit3*, 5 for *Grm6*) were used in this study. Mice were housed in groups in a temperature-controlled room with a 12-h light/12-h dark cycle. Fresh water and rodent diet were available *ad libitum*.

## **III. DNA constructs and antibodies**

The murine and human *Lrit3/LRIT3* coding sequences without the stop codon were subcloned into a pBudCE4.1 expression vector at the *SalI* and *XbaI* or *BamHI* restriction sites, respectively, by GeneCust Europe (Dudelange, Luxembourg). These plasmids encode mouse and human LRIT3-Myc fusion proteins. The Myc tag is located at the C-terminal end.

Rabbit anti-mouse LRIT3 antibody was obtained from Eurogentec (Seraing, Belgium) (159). Rabbit anti-GluR1 antibody (AB1540, Merck, Darmstadt, Germany), goat anti-GluR5 antibody (sc-7616, Santa-Cruz Biotechnology, Dallas, TX, USA), rabbit anti-Pikachurin antibody (011-22631, Wako, Osaka, Japan), mouse anti-PSD-95 antibody (MABN68, Merck), mouse anti-Myc antibody (11667149001, Roche Diagnostics, Meylan, France) and rabbit polyclonal anti-human LRIT3 antibody (HPA013454, Sigma-Aldrich, Saint-Louis, MO, USA) were purchased.

## **IV. Electron microscopy**

Electron microscopy (EM) studies were performed in collaboration with Kirill Martemyanov (Department of Neuroscience, The Scripps Research Institute, Jupiter, FL, USA). Eyes were enucleated, cleaned of extra-ocular tissue, and pre-fixed for 15 min in cacodylate-buffered half-Karnovsky's fixative containing 2 mM calcium chloride. The eyecups were then hemisected along the vertical meridian and fixed overnight in the same fixative. The specimens were rinsed with cacodylate buffer and postfixed in 2% osmium tetroxide in buffer for 1 hour, then gradually dehydrated in a series of solutions with increasing ethanol and acetone concentrations (30–100%), and embedded in Durcupan ACM resin (Electron Microscopy Sciences, Hatfield, PA, USA). Blocks were cut to 70-nm-thickness sections, and were stained with 3% lead citrate. Sections were examined in a Tecnai G2 spirit BioTwin (FEI, Hillsboro, OR, USA) transmission electron microscope at 80 or 100 kV accelerating voltage. Images were captured with a Veleta CCD camera (Olympus, Tokyo, Japan) operated with TIA software (FEI).

## V. Immunohistochemistry

## **A. Preparation of retinal sections**

Mice were euthanized by  $CO_2$  administration and cervical dislocation. Eyes were enucleated and prepared as previously described (160) with some modifications. The anterior segment and lens were removed and the eyecup was fixed in 2% (w/v) paraformaldehyde in 0.12 M phosphate buffer, pH 7.2 (PB) for 10 min at room temperature. The eyecup was washed three times in PB and cryoprotected with increasing concentrations of ice-cold sucrose in PB (10%, 20% for 1 h each and 30% overnight). Subsequently, the eyecup was embedded in 7.5% gelatin-10% sucrose and frozen in a dry ice-cooled bath of isopentane. Sections were cut at a thickness of 16 µm on a cryostat and mounted onto glass slides (Super-Frost, Thermo Fisher Scientific, Waltham, MA, USA). The slides were air dried and stored at -80°C.

## **B.** Immunostaining of retinal sections

Immunohistochemistry on retinal sections was performed as previously described (160) with some modifications. Sections were incubated with primary antibodies in 3% (v/v) normal donkey serum, 1% bovine serum albumin, and 0.5% Triton X-100 in PB overnight at room temperature. Primary antibodies were: rabbit anti-GluR1 (1:100), goat anti-GluR5 (1:100), rabbit anti-Pikachurin (1:1000), and mouse anti-PSD-95 (1:500). After washing in PB, the sections were incubated with secondary antibodies coupled to Alexa Fluor 488 or Cy3 (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:1000 in PB for 1.5 h at room temperature. The slides were stained with DAPI and a cover slip was applied with mounting medium (Mowiol, Merck Millipore, Billerica, MA, USA). None of the secondary antibodies used gave recordable signal when used as a negative control without primary antibodies (data not shown).

## C. Image acquisition

Fluorescent staining signals were captured with a confocal microscope (FV1000, Olympus, Hamburg, Germany) equipped with 405, 488, and 559 nm lasers. Confocal images were acquired with a 40x objective compatible with oil (lens NA: 1.3) imaging pixels of 310 nm and 77 nm in width and height for zoom 1 and 4, respectively, and using a 0.52  $\mu$ m step size. Each image corresponds to the projection of three optical sections. For figures, brightness and contrast were optimized (ImageJ, v.1.50; National Institutes of Health, Bethesda, MD, USA).

## VI. ON/OFF ERG

ON/OFF ERG studies were performed in collaboration with Stuart Coupland (Ottawa Hospital Research Institute, University of Ottawa, Ottawa ON, Canada). We tried to adapt the protocol established by Khan *et al.* in primate for mice in order to separately record ERG responses originating from cone driven ON- and OFF-bipolar cells (BCs) (161). After overnight dark adaptation, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Eye drops were used to dilate the pupils (0.5 % mydriaticum, 5 % neosynephrine) and anesthetize the cornea (0.4 % oxybuprocaine chlorhydrate). Body temperature was maintained at 37°C through the use of a heating pad. Gold wire loop electrodes for mice (Diagnosys LLC, Lowell, MA, USA) were placed on the corneal surface. A needle electrode placed in the

back served as a ground electrode. Recordings were made from both eyes simultaneously. Stimulus presentation and data acquisition were provided by the Espion  $E^2$  system (Diagnosys LLC). Mice were first light-adapted for 10 min by exposure to a white 150 cd/m<sup>2</sup> saturating background. Ramping rapid-ON/OFF-flicker white stimuli were then applied at a frequency of 8 Hz to selectively elicit ON- and OFF-responses. The stimuli had a miminum light intensity of 0 cd/m<sup>2</sup> and a maximum light intensity of 150 cd/m<sup>2</sup>. Twenty consecutive sweeps were recorded.

## VII. MEA

MEA studies were performed in collaboration with Serge Picaud (Institut de la Vision, Paris, France). MEA recordings were obtained from ex vivo isolated flat-mounted retina as previously described (84). Mice were euthanized by  $CO_2$  inhalation followed by cervical dislocation and eyes were rapidly enucleated and dissected under dim red light at room temperature in Ames medium (Sigma-Aldrich, St-Louis, MO, USA), oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (Air Liquide, Paris, France). Retinas were placed on a Spectra/Por membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA) and gently pressed against a MEA (MEA256 100/30 iR-ITO, Multi Channel systems MCS, Reutlingen, Germany) using a micromanipulator, with RGCs facing the electrodes. Retinas were continuously superfused with bubbled Ames medium at 34°C at a rate of 1–2 mL/min, and let to rest for 1 hour before the recording session. Ten repeated full-field light stimuli at a 480 nm-wavelength were applied to the samples at 10<sup>14</sup> photons/cm<sup>2</sup>/s for 2 sec with 10 sec intervals by using a Polychrome V monochromator (Olympus) driven by a STG2008 stimulus generator (MCS). Raw RGC activity recorded by the MEA was amplified (gain 1000-1200) and sampled at 20 kHz using MCRack software (MCS). Resulting data were stored and filtered with a 200 Hzhigh-pass filter. Raster plots were obtained using threshold detection (using the mean amplitude of the spontaneous activity + 3 standard deviations (SD) as a threshold) and peristimulus time histograms (PSTH) were plotted with a bin size of 50 ms using a custom made script in MATLAB v.R2014b (MathWorks, Natick, MA, USA). Only RGCs with a mean spontaneous firing frequency superior to 1 Hz were considered. We subsequently determined for each sorted RGC the maximum firing frequency in an interval of 2 s after the light onset (for ON-responses) and in an interval of 2 s after the light offset (for OFFresponses). These values were normalized to the mean spontaneous firing frequency of the corresponding RGC. Considering that significant responses have a maximum firing frequency that is superior to the mean spontaneous firing frequency + 5SD, we determined the time at which these significant frequencies were reached after the light onset for ON-responses and after the light offset for OFF-responses. The histograms were traced using Prism v6 software (Graphpad, La Jolla, CA, USA).

# VIII. Analysis of mouse and human LRIT3 in overexpressing cells though mass spectrometry

These studies were performed in collaboration with Manuela Argentini (Institut de la Vision, Paris, France) and the structural and functional proteomic/mass spectrometry platform (Institut Jacques Monod, Paris, France).

## A. Cell culture and transfection

GripTite<sup>TM</sup> 293 MSR cells, a subtype of human embryonic kidney (HEK) cells, were purchased (Thermo Fisher Scientific, Waltham, MA, USA) and maintained in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 0.01 mg/mL of gentamycin (Life Technologies) and 0.6 mg/mL of geneticin (Life Technologies) to maintain the selection pressure. One day before transfection, GripTite<sup>TM</sup> 293 MSR cells were seeded at a concentration of 300,000 cells/mL in a 10-cm petri dish. The following day, cells reached approximately 80% confluence and were transfected with either the human *LRIT3*myc or the mouse *Lrit3*-myc construct using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Cell pellets were collected and frozen 48 h after transfection.

## **B.** Immunoprecipitation and Western blotting

Transfected GripTite<sup>TM</sup> 293 MSR cells from one 10-cm plate were lysed using 200  $\mu$ L of 50 mM Tris, pH 7.5 supplemented with 150 mM NaCl, 1% Triton-X100 (Sigma-Aldrich) and protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The homogenate was incubated for 30 min on ice with vortexing every 10 min. It was subsequently sonicated three times for 10 s with at least 30 s between each sonication step, then centrifuged at 13,000 g for 10 min at 4°C. The total protein concentration of each sample was determined with a bicinchoninic acid assay (BCA Protein Assay Kit, Thermo Fisher Scientific). 10  $\mu$ g of mouse anti-Myc or rabbit anti-human LRIT3 antibody or 2.5  $\mu$ g of rabbit anti-mouse LRIT3 antibody were incubated

with 100 or 25  $\mu$ L of magnetic beads covalently coupled to G protein (Dynabeads, Life Technologies), respectively, on a rocker at room temperature for 1 h. Supernatants were then incubated with antibody-bead complexes on a rocker at 4°C overnight. After five washes with lysis buffer, the proteins were eluted from the beads with 20  $\mu$ L of a sodium dodecyl sulphate (SDS) sample buffer (240 mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS) with 10 nM of 1,4-Dithiothreitol (DTT) at room temperature for 30 min with vortexing every 5 min.

Two microliters of each eluate were mixed with a loading buffer (SDS sample buffer with 0.04% bromophenol blue) with 20 nM of DTT in final concentration and were then subjected to 4-15% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were transferred onto nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked for 1 h at room temperature in 5% dry milk diluted in phosphate buffer saline (PBS) with 0.1% Tween 20 and subjected to primary antibodies in 1% dry milk diluted in PBS with 0.1% Tween 20 overnight at 4°C. After washing in PBS, 0.1% Tween 20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) in 1% dry milk diluted in PBS, 0.1% Tween 20, and bands were subsequently detected by using a chemiluminescent substrate (ECL2, Thermo Fisher Scientific).

## **C.Silver staining**

The remaining eluates from the immunoprecipitation experiment were subjected to 4-15% SDS-PAGE (Bio-Rad Laboratories). The gel was fixed for 30 min in 30% ethanol, 5% acetic acid. After washes with water, the gel was incubated with sensitizing solution (0.02% sodium thiosulfate) for 1 min. The staining was performed for 20 min in a solution containing 0.2% (w/v) of silver nitrate and 0.01% of formaldehyde before development with 1% (w/v) sodium carbonate, 0.00125% sodium thiosulfate and 0.01% formaldehyde. Once the bands had reached the desired intensity, development was stopped by adding a solution containing Tris 4% (w/v) and 2% acetic acid. Finally, individual bands were cut and stored in 50 $\mu$ L of acetonitrile at -20°C until submission for mass spectrometry analysis.

## **D. Mass spectrometry analyses: LC-MS/MS acquisition**

Plugs were reduced with 10mM DTT, alkylated with 55mM iodoacetamide and incubated with 20  $\mu$ L of 25 mM NH<sub>4</sub>HCO<sub>3</sub> containing sequencing-grade trypsin (12.5  $\mu$ g/mL; Promega,

Madison, WI, USA) overnight at 37°C. The resulting peptides were sequentially extracted with 70% acetonitrile, 0.1% formic acid. All digests of protein extracts were analyzed using a Q Exactive Plus equipped with an EASY-Spray nanoelectrospray ion source and coupled to an Easy nano-LC Proxeon 1000 system (all devices are from Thermo Fisher Scientific). Chromatographic separation of peptides was performed with the following parameters: Acclaim PepMap100 C18 pre-column (2 cm, 75 µm i.d., 3 µm, 100 Å), Pepmap-RSLC Proxeon C18 column (50 cm, 75 µm i.d., 2 µm, 100 Å), 300 nL/min flow, gradient rising from 5 % solvent B (100 % acetonitrile, 0.1% formic acid) to 35% solvent B (solvent A is water, 0.1% formic acid) in 120 min followed by column regeneration for 50 min. Peptides were analyzed in the orbitrap in full ion scan mode at a resolution of 70000 (at mass to charge ratio (m/z) 400) and with a mass range of m/z 350-1500. Fragments were obtained with a Higher Energy Collision dissociation activation with a collisional energy of 27%, an isolation width of 1.4 m/z. MS/MS data were acquired in the orbitrap in a data-dependent mode in which the 20 most intense precursor ions were fragmented, with a dynamic exclusion of 30 s. The maximum ion accumulation times were set to 50 ms for MS acquisition and 45 ms for MS/MS acquisition. MS/MS data were processed with Proteome Discoverer 2.1 software (Thermo Fisher Scientific) coupled to an in house Mascot search server (version 2.5.1; Matrix Science, Boston, MA, USA). The mass tolerance was set to 6 parts per million (ppm) for precursor ions and 0.02 Da for fragments. The following modifications were used in variable modifications: oxidation (Methionine), phosphorylation (Serine, Threonine, and Tyrosine), acetylation (N-terminal). The maximum number of missed cleavages by trypsin was limited to two. MS/MS data were searched against Homo Sapiens and Mus musculus databases retrieved from the NCBInr database.

## **RESULTS**

# I. Identification of novel mutations in *SLC24A1* in the Riggs-type of CSNB

This work led to one publication and one poster presentation at an international meeting.

## Publication:

1) **Neuille, M.**, Malaichamy, S., Vadala, M., Michiels, C., Condroyer, C., Sachidanandam, R., Srilekha, S., Arokiasamy, T., Letexier, M., Demontant, V., Sahel, J.A., Sen, P., Audo, I., Soumittra, N. & Zeitz, C. (2016) Next-generation sequencing confirms the implication of SLC24A1 in autosomal-recessive congenital stationary night blindness (CSNB). *Clin Genet*. Epub ahead of print.

## Poster presentation:

1) **Neuille, M.**, Malaichamy, S., Vadala, M., Sachidanandam, R., Arokiasamy, T., Sahel, J.A., Sen, P., Audo, I.S., Soumittra, N. & Zeitz, C. - Novel mutations in *SLC24A1* leading to congenital stationary night blindness (CSNB) - Annual meeting of the Association for Research in Vision and Ophthalmology (ARVO) (May 1-5 2016) – Seattle (WA, USA).

# Next-generation sequencing confirms the implication of *SLC24A1* in autosomal-recessive congenital stationary night-bindness

The aim of this study was to identify the gene defect(s) in a previously diagnosed icCSNB family lacking and in two other families with unclassified CSNB. Disease causing variants in the known genes *CACNA1F*, *CABP4*, *CACNA2D4* and in the candidate gene *CACN2B* had already been excluded in the icCSNB family by Sanger sequencing. In contrast, the two families with unclassified CSNB had not previously been investigated. This work was performed in collaboration with an Indian group.

Whole-exome sequencing (WES) in the previously diagnosed icCSNB patient identified a homozygous nonsense variant (c.2401G>T, p.(Glu801\*)) in *SLC24A1*. Further subsequent clinical investigation and review of the family's medical records revealed the true diagnosis to be the Riggs-form of CSNB in this patient. Targeted next-generation sequencing (NGS) identified compound heterozygous deletions (c.1691\_1693delTCT, p.(Phe564del) and c.3291\_3294delATCT, p.(Val1099Glufs\*31)) and a homozygous missense variant (c.2968A>C, p.(Ser990Arg)) in *SLC24A1* in the two other patients with unclassified CSNB. The electroretinographic abnormalities varied in these three patients, but all of them had normal visual acuity without myopia or nystagmus, unlike the patients with the Schubert-Bornschein-type of CSNB. These findings confirm that *SLC24A1* defects lead to CSNB, and outline phenotype/genotype correlations in CSNB subtypes. They also demonstrate the utility of NGS techniques for diagnostic clarification in cases where the clinical characteristics are unclear.

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## **Original Article**



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## Next-generation sequencing confirms the implication of *SLC24A1* in autosomal-recessive congenital stationary night blindness

Neuillé M., Malaichamy S., Vadalà M., Michiels C., Condroyer C., Sachidanandam R., Srilekha S., Arokiasamy T., Letexier M., Démontant V., Sahel J.-A., Sen P., Audo I., Soumittra N., Zeitz C. Next-generation sequencing confirms the implication of *SLC24A1* in autosomal-recessive congenital stationary night blindness.

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Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous retinal disorder which represents rod photoreceptor dysfunction or signal transmission defect from photoreceptors to adjacent bipolar cells. Patients displaying photoreceptor dysfunction show a Riggs-electroretinogram (ERG) while patients with a signal transmission defect show a Schubert-Bornschein ERG. The latter group is subdivided into complete or incomplete (ic) CSNB. Only few CSNB cases with Riggs-ERG and only one family with a disease-causing variant in SLC24A1 have been reported. Whole-exome sequencing (WES) in a previously diagnosed icCSNB patient identified a homozygous nonsense variant in SLC24A1. Indeed, re-investigation of the clinical data corrected the diagnosis to Riggs-form of CSNB. Targeted next-generation sequencing (NGS) identified compound heterozygous deletions and a homozygous missense variant in *SLC24A1* in two other patients, respectively. ERG abnormalities varied in these three cases but all patients had normal visual acuity, no myopia or nystagmus, unlike in Schubert-Bornschein-type of CSNB. This confirms that SLC24A1 defects lead to CSNB and outlines phenotype/genotype correlations in CSNB subtypes. In case of unclear clinical characteristics, NGS techniques are helpful to clarify the diagnosis.

#### **Conflict of interest**

The funders had no role in study design, data collection, analysis and interpretation, decision to publish, or preparation of the manuscript. The authors declare no competing financial interests.

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#### Introduction

Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous group of retinal disorders caused by variants in genes implicated in the phototransduction cascade or in retinal signaling from photoreceptors to adjacent bipolar cells (1, 2).

Most individuals affected with CSNB show characteristic electroretinogram (ERG) abnormalities, named after Schubert and Bornschein who initially reported them, in which the b-wave amplitude is smaller than that of the a-wave, which is globally normal, in the dark-adapted bright flash condition (3). This electronegative waveform can be divided in two subtypes, complete (c)CSNB [MIM 310500, MIM 257270, MIM 613216, MIM 614565, MIM 615058] and incomplete (ic)CSNB [MIM 300071] (4). Patients suffering from the Schubert-Bornschein-type of CSNB often show ocular abnormalities in addition to night blindness, including reduced visual acuity, photophobia, myopia, nystagmus and strabismus (1, 2). cCSNB is characterized by a drastically reduced rod b-wave response due to ON-bipolar cell dysfunction, and specific cone ERG waveforms (5). cCSNB has been associated with disease-causing variants in NYX [MIM 300278] (6, 7), GRM6 [MIM 604096] (8, 9), TRPM1 [MIM 603576] (10-12), GPR179 [MIM 614515] (13, 14) and LRIT3 [MIM 615004] (15), whose products localize at the dendritic tips of ON-bipolar cells (2). icCSNB is characterized by a reduced rod b-wave and substantially reduced cone responses, indicative of both ON- and OFF-bipolar cell dysfunction. icCSNB has been associated with disease-causing variants in CACNA1F [MIM 300110] (16, 17), CABP4 [MIM 608965] (18) and CACNA2D4 [MIM 608171] (19), genes coding for proteins localized presynaptically and important for the continuous glutamate release at the photoreceptor synapse (1, 2).

Less commonly, individuals affected with CSNB display another type of ERG abnormalities, reported by Riggs (20) [MIM 610445, MIM 610444, MIM 616389, MIM 163500, MIM 613830]: in these cases there is a generalized rod photoreceptor dysfunction, dark-adapted responses being dominated by cone responses which are normal. Therefore, the a-wave amplitude is reduced in response to a bright flash under dark adaptation with a possible additional reduction of the b/a ratio and an electronegative waveform due to the photopic hill phenomenon characteristic of the cone pathway function (21). Photopic ERG responses are preserved due to the normal cone system function. These CSNB patients manifest distinct clinical characteristics from the Schubert-Bornschein patients, including a normal visual acuity and no clinical signs of myopia and/or nystagmus (1, 2). Riggs-type of CSNB has been associated with disease-causing variants in RHO [MIM 180380] (22), GNAT1 [MIM 139330] (23), PDE6B [MIM 180072] (24, 25) and SLC24A1 [MIM 603617] (26), underlying autosomal dominant (RHO, GNAT1 and PDE6B) or autosomal recessive (GNAT1, SLC24A1) CSNB. Only four families with four different disease-causing variants in RHO (22, 27-29), three families with three different

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disease-causing variants in *GNAT1* (23, 30, 31), two families with two different disease-causing variants in *PDE6B* (25, 32) and one family with one disease-causing variant in *SLC24A1* (26) were described. The corresponding proteins localize in rods and have a role in their phototransduction cascade (2).

To date, more than 360 different disease-causing variants have been identified in genes underlying CSNB (2). However, despite comprehensive genotyping studies on our own CSNB cohort, some cases carried no disease-causing variants in known genes underlying CSNB. This is a strong indication that disease-causing variants in other genes remain to be discovered or that defects in regulatory elements or introns might be involved. In addition, the phenotype may not be well characterized or one phenotype may be associated with different gene defects.

Disease-causing variants in many genes associated with CSNB have been identified through classical linkage approaches (6, 7, 16, 17, 25, 26) or through a candidate gene approach comparing the human phenotype with similar phenotypes observed in knock-out or naturally occurring animal models (8-12, 14, 18, 22, 33). One limitation of the linkage approach is the requirement to examine large families. Techniques using massively parallel sequencing of known human exons implicated in inherited retinal disorders and all human exons, also called whole-exome sequencing (WES) have recently been successful in identifying disease-causing variants in known and novel genes underlying heterogeneous diseases (34, 35), including rod-cone dystrophy (34, 36, 37), cone or cone-rod dystrophy (38) and CSNB (13, 15, 34). Especially, WES is an unbiased method to identify the gene defect in families with unclear phenotypes and with only few family members available.

The aim of this study was to identify the gene defect in a previously diagnosed icCSNB family lacking disease-causing variants in the known genes *CACNA1F*, *CABP4*, *CACNA2D4* and in a candidate gene *CACN2B* [MIM 600003], and in two other families with unclassified CSNB, never investigated before.

#### Materials and methods

Methods for WES, targeted next-generation sequencing (NGS) and investigation of annotated sequencing data are provided as supplementary data.

#### Ethics statements

Research procedures were conducted in accordance with institutional guidelines and the Declaration of Helsinki. Prior to genetic testing, informed consent was obtained from all CSNB-affected individuals and their family members.

#### Clinical investigation

Ophthalmic examination included best corrected Snellen visual acuity measurement, fundoscopy, D15 Farnworth

color vision test, full-field ERG incorporating the International Society for Clinical Electrophysiology of Vision (ISCEV) standards (39), fundus autofluorescence (FAF), and spectral-domain optical coherence tomography (SD-OCT) (the extent of investigation depended on the referring center).

#### Previous molecular genetic analysis

The DNA of the index patient of Family 1 was directly Sanger sequenced for the coding exonic and flanking intronic regions of the known genes underlying icCSNB and a candidate gene (*CACNA1F*, NM\_005183.3; *CABP4*, NM\_145200.3; *CACNA2D4*, NM\_172364.4; *CACNB2*, NM\_000724.3).

#### Disease-causing variants

Novel disease-causing variants identified in *SLC24A1* in this study have been deposited in the 'Leiden Open Variation Database' (http://www.lovd.nl/3.0/home) prior to publication.

#### Results

The two male patients of Family 1 were previously reported to us as affected with icCSNB (Fig. 1a). However, mutation analysis by Sanger sequencing in these cases in known genes underlying icCSNB and a candidate gene (*CACNA1F, CACNA2D4, CABP4* and *CACN2B*) did not reveal any disease-causing variant (data not shown). Subsequently, WES was performed. The index cases of Family 2 and 3 were screened for variants in all genes previously associated with CSNB by targeted NGS.

Applying our stringent criteria and filters to the sequencing data of the WES for Family 1, we reduced the number of putative variants from 1059 insertions/deletions (InDels) and 18,058 single-nucleotide variants (SNVs) to four hemizygous variants in four genes, six compound heterozygous variants in three genes and one homozygous variant in one gene (Tables S1 and S2, Supporting Information). Due to the filtering and the pathogenic prediction, the most likely disease-causing variant was the homozygous nonsense variant, c.2401G > T (p.(Glu801\*)) in SLC24A1 (Figs 1a and 2b and Table S2). The overall sequencing coverage of the captured regions in the family was 95% and 87.75% for a 10× and 25× depth of coverage, respectively, resulting in a mean sequencing depth of 77.75× per base (Table S3). Both unaffected parents were found to be heterozygous for this variant. The nucleotide T was read 104 and 74 times in the mother and father, respectively, whereas the G was found 97 and 101 times, respectively. The two affected boys showed 170 and 212 times the nucleotide T. Sanger sequencing confirmed these results (Fig. 1b). The detailed clinical data of the index case of Family 1 were only available after SLC24A1 defect identification. For this 6 year-old patient, night blindness was reported since early childhood. Visual acuity was 20/20 for both eyes, with no signs of myopia or nystagmus. Fundus examination revealed no retinal or disc abnormalities. Color sensitivity was normal. Scotopic ERG responses were severely reduced for both a- and b-waves for both eyes, whereas photopic responses were normal.

Applying our stringent filtering criteria to the sequencing data of the targeted NGS from two unclassified CSNB families never genetically investigated before and considering the pathogenic variant predictions, we found that the index patient of Family 2 carried compound heterozygous delec.1691 1693delTCT (p.(Phe564del)) tions, and c.3291 3294delATCT (p.(Val1099Glufs\*31))(Figs 1a and 2b and Table S2) and that the index patient of the consanguineous Family 3 harbored a homozygous missense variant, c.2968A > C (p.(Ser990Arg)) affecting the well conserved amino acid residue Ser at position 990 in SLC24A1 (Figs 1a and 2 and Table S2). For Family 2, the overall sequencing coverage of the captured regions was 100% for both  $10 \times$  and  $25 \times$  depth of coverage, resulting in a mean sequencing depth of 200× per base. For Family 3, the overall sequencing coverage of the captured regions was 100% for both  $10 \times$  and  $25 \times$  depth of coverage, resulting in a mean sequencing depth of  $204 \times$  per base (Table S4). All these variants co-segregated with the phenotype (Fig. 1) and were absent in 100 healthy ethnically matched control individuals. For the index patients of Family 2 and 3, night blindness was reported since early childhood. At the time of clinical examination, they were 32 and 36 years old, respectively. There were no signs of reduced visual acuity, myopia or nystagmus. Color vision was normal. Fundus examination revealed no retinal or disc abnormalities (Fig. 3a,b). No retinal degeneration was detected by SD-OCT (Fig. 3c,d). Scotopic ERG amplitudes were severely reduced in both patients with a more severe defect in the index patient of Family 3 (Fig. 4 and Table 1). The index patient of Family 2 displays minimal decreased amplitudes in response to a single photopic flash with moderately reduced flicker responses (Fig. 4 and Table 1). Photopic responses of the index patient of Family 3 are more altered with the amplitude of the b-wave which is significantly reduced in response to both the single flash as well as flicker (Fig. 4 and Table 1).

#### Discussion

In this work, we have used WES to identify a novel nonsense variant in *SLC24A1* in a family previously reported to us as affected with icCSNB (Family 1). In addition, targeted NGS has led to the identification of three other novel variants in the same gene in two other unclassified CSNB families (Family 2 and 3).

The index patient and his affected brother of Family 1 carry a homozygous SNV in *SLC24A1* (c.2401G > T). Localized in exon 7, this variant is predicted to result in a truncated protein (p.(Glu801\*)) or in nonsense-mediated mRNA decay (NMD). The variant co-segregates with the phenotype as the affected brother is also homozygous and both non-affected parents are heterozygous for this variant. Interestingly, a *SLC24A1* disease-causing variant



*Fig. 1. SLC24A1* novel disease-causing variants in congenital stationary night blindness (CSNB). (a) Corresponding pedigrees of patients with CSNB with *SLC24A1* disease-causing variants and co-segregation analysis. Indexes patients are individual II.1 of Family 1, II.4 of Family 2 and IV.2 of Family 3. Patients included for whole-exome sequencing are marked with a red arrow. Patients included for targeted next-generation sequencing are marked with a black arrow. Patients used for co-segregation analysis by Sanger sequencing are marked with a blue arrow. Square symbol = male, round symbol = woman, filled symbol = affected, unfilled symbol = unaffected, double line = consanguinity, barred = deceased. (b) Sequence electropherograms of CSNB patients with *SLC24A1* disease-causing variants, their relatives and controls. The underlined letters correspond to modifications of sequence induced by the different disease-causing variants. For Family 1, affected patient II.2 displays the same profile as the unaffected mother I.2; for Family 3, the non-affected brother IV.1 displays the same profile as the control.



*Fig.* 2. Conservation and positioning of SLC24A1 disease-causing variants. (a) Evolutionary conservation of Ser990 in other orthologs shown in green. Amino acid substitution is highlighted in red. The position of the amino acid is shown in black numbers. (b) Topological model of SLC24A1. Transmembrane domains (TM) are identified by numbers 0-10. Cleavage of the signal peptide, including TM 0, is illustrated by a cross. Two novel disease-causing variants reside in TM 4 and 7, one localizes in the intracellular loop between the two predicted ion exchanger domains and the last one was identified at the C-terminus of the protein. The disease-causing variants previously described resides in TM3.

had already been described in CSNB (c.1613 1614del, p.(Phe538Cysfs\*23)). However, this disease-causing variant was not associated with the incomplete form but with the Riggs-type of CSNB (26). On the clinical data on which we did not have access before genetic screening, myopia or nystagmus were not present and visual acuity was normal. In addition, it was noted that scotopic ERG responses were severely reduced, while photopic responses were in the normal range. These basic phenotypic descriptions are actually more in accordance with the Riggs-type of CSNB than with icCSNB. However, the misdiagnosis from the referring clinician may have come from some icCSNB cases with disease-causing variants in CACNA1F described with reduced scotopic a-wave (40, 41), but in these cases, photopic responses were also altered. Unfortunately, the patient was no longer available for retesting to confirm a Riggs-type of CSNB. Indeed, if we had access to these clinical details before genetic testing, SLC24A1 would have been a good candidate to screen first before the unbiased WES approach. In Family 2, we analyzed targeted NGS data of one index male and performed co-segregation analysis in his unaffected mother and one of his unaffected sisters, which led to the identification of compound heterozygous disease-causing deletions in SLC24A1 (c.1691\_1693delTCT and c.3291 3294delATCT). Localized in exons 2 and 10, these disease-causing variants are predicted to result in a deletion of a phenylalanine (p.(Phe564del)) and a longer protein at the C-terminus (p.(Val1099Glufs\*31)),

respectively. In Family 3, we analyzed targeted NGS data of one index male in a family with consanguinity. We found a homozygous missense variant in SLC24A1 (c.2968A > C), localized in exon 9, which co-segregates with the phenotype, resulting in the replacement of a serine by an arginine (p.(Ser990Arg)). For the index patients of Family 2 and 3, ERGs were not conclusive as scotopic as well as photopic responses were altered. In general, Riggs-ERG is characterized by a drastically reduced scotopic a-wave and b-wave due to rod dysfunction and normal photopic responses (20). This ERG phenotype is found in most of the Riggs-type of CSNB described, with disease-causing variants in *RHO*, GNAT1 and PDE6B (22, 23, 25-28, 32). However, in some cases with disease-causing variants in GNAT1, photopic responses were normal to moderately decreased in amplitudes (30, 31). Moreover, in the same family with the same SLC24A1 disease-causing variant, two indexes showed normal cone responses while they were modestly reduced in two other affected patients (26). It is unclear why photopic responses may be abnormal in Riggs-ERG as genes underlying the Riggs-type of CSNB are specifically expressed in rods. Some explanations may come from the fact that depending on the publications, not all ERGs were recorded according to the ISCEV current recommendations. Another explanation may come from interaction between rod and cone circuitry at the level of the inner retina or in rare cases the possibility for mild rod photoreceptor degeneration that may induce additional cone degeneration. However,



*Fig. 3.* Fundus photograph and spectral-domain optical coherence tomography (SD-OCT) scan of congenital stationary night blindness patients with SLC24A1 disease-causing variants (a) and (b) fundus photographs of patient II.4 of Family 2 and patient IV.2 of Family 3, respectively. (c) and (d) Normal SD-OCT scans in both the patients.

in a Slc24a1 knock-out mouse model, recently published, despite slow rod degeneration, the number of cones was maintained as well as cone responses to light stimulations, indicating that cone-mediated vision is not compromised in this mouse model (42). Further investigations are needed to address this point. However, it is important to notice that myopia and nystagmus were also absent in index patients of Family 2 and 3 and that their visual acuity was normal. These data demonstrate that the ERG phenotype may diverge from the general characterization for the Riggs-type of CSNB but that absence of other ocular abnormalities such as reduced visual acuity, myopia or nystagmus are consistent findings upon clinical examination. On the contrary, if the phenotype may vary among icCSNB patients even within the same family, all affected patients have reduced visual acuity and variable degrees of abnormal refractive errors and nystagmus (43). In conclusion, if a CSNB phenotype is suspected in a patient on the basis of night blindness and no sign of retinal degeneration on retinal examination and if the ERG recording does not allow the distinction between Riggs and Schubert-Bornschein CSNB, variants in genes underlying Riggs-type of CSNB may cause the disease in case of normal visual acuity and absence of major refractive errors or nystagmus. Of note, 50 patients with unclear phenotype screened in our laboratory for disease-causing variants in SLC24A1 did not reveal any disease-causing variant (data not shown), indicating that this phenotype or gene defect is rare or underdiagnosed.

As mentioned above, in 2010, Riazuddin et al. reported the first case of Riggs-CSNB associated with an autosomal-recessive inheritance. They found a homozygous 2 bp deletion in exon 2 of *SLC24A1* 

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(c.1613\_1614del), which is predicted to result in a frameshift leading to either truncated protein (p.(Phe538Cysfs\*23)) or NMD (26). SLC24A1 is a Na/Ca<sup>2</sup>-K exchanger which is present in the retina at the plasma membrane of the rod outer segments (42, 44, 45). Under dark conditions, guanosine 3',5'-cyclic phosphate (cGMP)-gated channels are opened and mediate an influx of cations, including Ca<sup>2+</sup>, (the so-called 'dark-current') into the cell (46). SLC24A1 balances this Ca<sup>2+</sup> current by exchange of one Ca<sup>2+</sup> against four Na<sup>+</sup> and one K<sup>+</sup> (42). Illumination and activation of the phototransduction cascade closes cGMP-gated channels, leading to a lowering of intracellular free  $Ca^{2+}$ . Finally, this low cellular Ca<sup>2+</sup> concentration activates a negative feedback loop which results in the termination of the phototransduction and subsequently in the re-opening of cGMP-gated channels (45).

Recently, Vinberg et al. described a mouse model lacking Slc24a1 which mimics the pathophysiology of the Riggs-CSNB described in human (42). They confirmed localization of SLC24A1 to the plasma membrane of rod outer segments. Slc24a1-/- mice revealed a slow progressive retinal degeneration and displayed a less sensitive and slower rod-mediated vision with drastically reduced ERG a-wave amplitude under dark-adaptation. Therefore, despite dramatically decreased rod response amplitudes, rods are viable and still contribute to visual function and rod-mediated synaptic signaling. Moreover, light adaptation of rods is not significantly compromised indicating that there is an unknown mechanism for  $Ca^{2+}$  to be extruded from rod outer segments in the absence of SLC24A1, even if this mechanism is too slow to provide normal feedback to the phototransduction



*Fig.* 4. Full-field electroretinogram of patient II.4 of Family 2 and patient IV.2 of Family 3, as an example. (a) Dark-adapted 0.01 electroretinogram (ERG). (b) Dark-adapted 3.0 ERG. (c) Dark-adapted 3.0 exciliatory potentials. (d) Light-adapted 3.0 ERG. (e) Light-adapted 3.0 flicker ERG. Scotopic responses are severely reduced for both patients. Patient II.4 of Family 2 shows relatively conserved photopic responses whereas photopic ERG is altered in patient IV.2 of Family 3.

cascade in the time scale of dim flash responses. The capability of rods to regulate  $Ca^{2+}$  influx in the absence of SLC24A1 together with the reduced 'dark-current' observed explain why there is no severe rod degeneration in these mouse model and are consistent with the stationary nature of the disorder. In our study, none of our patients demonstrated retinal degeneration at the time of the first investigation but this retinal degeneration has been reported to be slow and progressive in the *Slc24a1* knock-out mouse model (42). Therefore, re-investigation of the patients with *SLC24A1* disease-causing variants may be useful to see if the retinal degeneration can also be observed. Moreover, a novel homozygous truncating disease-causing variant has been reported in *GNAT1* 

(c.904C > T, p.(Gln302\*)) in an 80 year-old patient who displays marked peripheral pigmentary deposits resembling bone spicule on fundus examination (47). This last observation is more in accordance with a slow and late onset form of retinitis pigmentosa than CSNB. Moreover, *Gnat1* lacking mouse model showed a subtle and slow thinning of the retina (48).

Structurally, SLC24A1 is predicted to contain two clusters of five transmembrane domains (TM) that are separated by a large hydrophilic loop located in the cytosol. One putative TM does not span the membrane and precedes the second cluster of TM. An additional TM is present at the N-terminal of the protein but it is a signal peptide that can be cleaved. Therefore, N- and

	Dark-ada 0.01 ER(	apted G b-wave	Dark-ada 3.0 ERG	apted i a-wave	Dark-ada 3.0 ERG	apted b-wave	Light-ao 3.0 ER(	dapted 3 a-wave	Light-ad 3.0 ERG	apted b-wave	Light-ada 30 Hz flicker ER	pted G b-wave
	Amp (µV)	IT (ms)	Amp (µV)	IT (ms)	Amp (µV)	IT (ms)	Amp (µV)	IT (ms)	Amp (µV)	IT (ms)	Amp (µV)	IT (ms)
Controls $(n = 8)$	$297.9 \pm 83.4$	$63.13 \pm 3.29$	$226.4 \pm 54.6$	$16.88 \pm 0.52$	$479.4 \pm 116.1$	$44.94 \pm 2.35$	$25.3 \pm 5.0$	$16.25 \pm 0.93$	$105.6 \pm 26.7$	27.31 ± 0.88	$65.5 \pm 10.6$	$25.19 \pm 0.80$
II.4 of Family 2	6.6	50.0	88.9	17.0	57.6	35.0	19.3	16.5	62.7	26.5	39.8	25.5
IV.2 of Family 3	RN	NR	26.1	22.5	17.3	43.0	11.0	17.5	23.4	31.5	19.3	29.0
Amp, amplitud€	ss; IT, implicit ti	imes; NR, non-	-recordable.									

Table 1. Amplitudes and implicit times of electroretinogram responses of patient II.4 of Family 2 and patient IV.2 of Family 3, as an example

Amplitudes (in microvolts) and implicit times (in millisecond) of a-and b-waves in both patients compared with eight non-affected individuals (controls). Values for controls are given as mean ± standard deviation (SD) C-terminus are extracellular. The two TM clusters are proposed to constitute the two ion exchanger domains (45, 49). The novel disease-causing variant identified by WES is located in the intracellular loop located between these two predicted ion exchanger domains (Fig. 2b). Moreover, the deletion of the phenylalanine in position 564 as well as the replacement of a serine by an arginine in position 990 identified by targeted NGS are located in TM 4 and 7, respectively and could alter the ion exchange function (Fig. 2b). In particular, the Ser990 is analogous to Ser552 present in NCKX2 that appears to be critical for cation binding and transport (50, 51). It is hypothesized that the complete or even partial loss of the ion exchange function would result in abnormal levels of intracellular Ca<sup>2+</sup> concentrations that could potentially interfere with the proper functioning of the rod photoreceptors, resulting in the CSNB phenotype (26). Concerning the frameshift disease-causing variant, it is predicted to change the last amino acid of the protein and to elongate the protein by 29 novel amino acids. It is likely that the elongated protein presents structural changes which may alter the cation binding affinity or the interaction with other proteins, influence its intracellular localization or modify its stability. It has been shown that SLC24A1 interacts with the  $\alpha$ -subunit of cGMP-gated channel (52). Whether the extended SLC24A1 protein alters the interaction with the cGMP-gated channel needs to be tested. Alternatively, the elongated SLC24A1 protein may be mislocalized, leading to complete loss of function as found for other mutants implicated in CSNB (53, 54). Finally, the extended SLC24A1 protein may be misfolded and, thus, become subject to degradation.

Here, we showed that NGS techniques such as WES and targeted NGS are unbiased methods to identify gene defects leading to CSNB, helping clinicians to classify the subtype of the disorder where clinical diagnosis is unclear. Moreover, we identified four novel disease-causing variants in SLC24A1 in three different families, expanding the disease-causing variants spectrum for this gene. This report is only the second one which documents SLC24A1 defects leading to CSNB. considering the fact that the previous one described only one disease-causing variant in one family. Together with the knock-out mouse model, this article reinforces the assertion that SLC24A1, when mutated, is implicated in the Riggs-type of CSNB.

#### Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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# II. Identification and functional characterization of LRIT3, a novel molecule implicated in cCSNB

This chapter represents the main subject of my thesis and was specifically supported by the Fondation pour la Recherche Médicale (FRM) in partnership with the Fondation Roland Bailly and by the Fédération des Aveugles et Amblyopes de France. This word led to two awards, three publications, four paper presentations, and three poster presentations at national and international meetings.

## Publications:

1) Zeitz, C., Jacobson, S.G., Hamel, C.P., Bujakowska, K., Neuille, M., Orhan, E., Zanlonghi, X., Lancelot, M.E., Michiels, C., Schwartz, S.B., Bocquet, B., Congenital Stationary Night Blindness, C., Antonio, A., Audier, C., Letexier, M., Saraiva, J.P., Luu, T.D., Sennlaub, F., Nguyen, H., Poch, O., Dollfus, H., Lecompte, O., Kohl, S., Sahel, J.A., Bhattacharya, S.S. & Audo, I. (2013) Whole-exome sequencing identifies *LRIT3* mutations as a cause of autosomal-recessive complete congenital stationary night blindness. *Am J Hum Genet*, **92**, 67-75.

2) Neuille, M., El Shamieh, S., Orhan, E., Michiels, C., Antonio, A., Lancelot, M.E., Condroyer, C., Bujakowska, K., Poch, O., Sahel, J.A., Audo, I. & Zeitz, C. (2014) *Lrit3* deficient mouse (*nob6*): a novel model of complete congenital stationary night blindness (cCSNB). *PLoS One*, **9**, e90342.

3) **Neuille, M.**, Morgans, C.W., Cao, Y., Orhan, E., Michiels, C., Sahel, J.A., Audo, I., Duvoisin, R.M., Martemyanov, K.A. & Zeitz, C. (2015) LRIT3 is essential to localize TRPM1 to the dendritic tips of depolarizing bipolar cells and may play a role in cone synapse formation. *Eur J Neurosci*, **42**, 1966-1975.

## Paper presentations:

1) **Neuille, M.** - De l'identification à la caractérisation fonctionnelle d'une nouvelle protéine impliquée dans la cécité nocturne congénitale stationnaire de forme complète (cCSNB) - Autumn Meeting of the Société de Génétique Ophthalmologique Francophone (SGOF) (December 7-8 2012) – Gand (Belgium).

2) **Neuille, M.**, El Shamieh, S., Orhan, E., Michiels, C., Bujakowska, K.M., Poch, O., Sahel, J.A., Audo, I. & Zeitz, C. - A novel mouse model for complete Congenital Stationary Night Blindness (cCSNB) – Annual meeting of the Association for Research in Vision and Ophthalmology (ARVO) (May 4-8 2014) – Orlando (FL, USA).

3) **Neuille, M.**, Morgans, C.W., Michiels, C., Audo, I.S., Duvoisin, R.M. & Zeitz, C. - LRIT3 is essential to localize TRPM1 to the dendritic tips of depolarizing bipolar cells and may play
a role in cone synapse formation - Joint Meeting of the Société de Génétique Ophthalmologique Francophone (SGOF) and the section of genetics of the German Association of Ophthtalmology (DOG) (December 5-6 2014) – Giessen (Germany).

4) **Neuille, M.**, Morgans, C.W., Orhan E., Michiels, C., Sahel, J.A., Audo, I.S., Duvoisin, R.M. & Zeitz, C. - LRIT3 is essential to localize TRPM1 to the dendritic tips of depolarizing bipolar cells and may play a role in cone synapse formation - Annual meeting of the Association for Research in Vision and Ophthalmology (ARVO) (May 3-7 2015) – Denver (CO, USA).

### Poster presentations:

1) Zeitz, C., Jacobson, S., Hamel, C., Bujakowska, K., **Neuille, M.**, Orhan, E., Zanlonghi, X., Sahel, J.A., Bhattacharya, S., Audo, I. & CSNB group – Whole exome sequencing identifies mutations in *LRIT3* as a cause for autosomal recessive complete congenital stationary night blindness – Annual meeting of the Association for Research in Vision and Ophthalmology (ARVO) (May 5-9 2013) – Seattle (WA, USA).

2) **Neuille, M.**, Michiels, C., Audo, I., Sahel, J.A. & Zeitz, C. - From identification to functional characterization of LRIT3, a novel protein implicated in complete congenital stationary night blindness (cCSNB) - Journées de l'Ecole Doctorale Physiologie et Physiopathologie (May 16-17 2013) – Paris (France).

3) **Neuille, M.**, El Shamieh, S., Orhan, E., Michiels, C., Bujakowska, K.M., Poch, O., Sahel, J.A., Audo, I. & Zeitz, C. - A novel mouse model for complete Congenital Stationary Night Blindness (cCSNB) - Young Researchers in Life Science Conference (May 22-24 2013) – Paris (France)

### Awards:

1) Travel grant for the Annual meeting of The Association for Research in Vision and Ophthalmology (ARVO) (May 4-8 2014) – Orlando (FL, USA).

2) Best presentation award at the Joint Meeting of the Société de Génétique Ophthalmologique Francophone (SGOF) and the section of genetics of the German Association of Ophthalmology (DOG) (December 5-6 2014) – Giessen (Germany).

# A. Whole-Exome Sequencing Identifies *LRIT3* Mutations as a Cause of Autosomal-Recessive Complete Congenital Stationary Night Blindness

Whole-exome sequencing of one simplex cCSNB case lacking mutations in the known genes led to the identification of a missense mutation (c.983G>A, p.(Cys328Tyr)) and a nonsense mutation (c.1318C>T, p.(Arg440\*)) in *LRIT3*, encoding leucine-rich-repeat (LRR), immunoglobulin-like, and transmembrane-domain 3 (LRIT3). Subsequent Sanger sequencing of 89 individuals with CSNB identified another cCSNB patient harboring a nonsense mutation (c.1151C>G, p.(Ser384\*)) and a deletion predicted to lead to a premature stop codon (c.1538\_1539del, p.(Ser513Cysfs\*59)) in the same gene. LRIT3 antibody staining revealed a punctuate-labeling pattern in the outer plexiform layer of the human retina resembling the dendritic tips of bipolar cells; similar patterns have been observed for other proteins implicated in cCSNB. The exact role of this LRR protein in cCSNB remains to be determined.

### Whole-Exome Sequencing Identifies *LRIT3* Mutations as a Cause of Autosomal-Recessive Complete Congenital Stationary Night Blindness

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Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous retinal disorder. Two forms can be distinguished clinically: complete CSNB (cCSNB) and incomplete CSNB. Individuals with cCSNB have visual impairment under low-light conditions and show a characteristic electroretinogram (ERG). The b-wave amplitude is severely reduced in the dark-adapted state of the ERG, representing abnormal function of ON bipolar cells. Furthermore, individuals with cCSNB can show other ocular features such as nystagmus, myopia, and strabismus and can have reduced visual acuity and abnormalities of the cone ERG waveform. The mode of inheritance of this form can be X-linked or autosomal recessive, and the dysfunction of four genes (*NYX, GRM6, TRPM1*, and *GPR179*) has been described so far. Whole-exome sequencing in one simplex cCSNB case lacking mutations in the known genes led to the identification of a missense mutation (c.983G>A [p.Cys328Tyr]) and a nonsense mutation (c.1318C>T [p.Arg440\*]) in *LRIT3*, encoding leucine-rich-repeat (LRR), immunoglobulin-like, and transmembrane-domain 3 (LRIT3). Subsequent Sanger sequencing of 89 individuals with CSNB identified another cCSNB case harboring a nonsense mutation (c.1151C>G [p.Ser384\*]) and a deletion predicted to lead to a premature stop codon (c.1538\_1539del [p.Ser513Cysfs\*59]) in the same gene. Human LRIT3 antibody staining revealed in the outer plexiform layer of the human retina a punctate-labeling pattern resembling the dendritic tips of bipolar cells; similar patterns have been observed for other proteins implicated in cCSNB. The exact role of this LRR protein in cCSNB remains to be elucidated.

Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous group of retinal disorders caused by mutations in genes implicated in the phototransduction cascade or in retinal signaling from photoreceptors to adjacent bipolar cells.<sup>1</sup> Most of the individuals affected by CSNB show a characteristic electroretinogram (ERG) in which the b-wave amplitude is smaller than the a-wave amplitude in the dark-adapted bright-flash condition.<sup>2</sup> This electronegative waveform can be divided in two subtypes, incomplete CSNB (icCSNB) (CSNB2A [MIM 300071] and CSNB2B [MIM 610427]) and complete CSNB (cCSNB) (CSNB1A [MIM 310500], CSNB1B [MIM 257270],<sup>3</sup> CSNB1C [MIM 613216]), and CSNB1E [MIM 614565]). icCSNB is characterized by a reduced rod bwave and substantially reduced cone responses, indicative of both ON and OFF bipolar cell dysfunction. icCSNB has been associated with mutations in *CACNA1F* (MIM 300110), *CABP4* (MIM 608965), and *CACNA2D4* (MIM 608171), genes coding for proteins important for the continuous glutamate release at the photoreceptor synapse.<sup>1</sup> cCSNB is characterized by a drastically reduced rod-b-wave response due to ON bipolar cell dysfunction, as well as specific cone ERG waveforms.<sup>4</sup> cCSNB has been associated with mutations in *NYX* (MIM 300278), *GRM6* (MIM 604096), *TRPM1* (MIM 603576), and *GPR179* (MIM 614515), genes expressed and localized in ON

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#### Figure 1. LRIT3 Mutations and the Associated Electroretinographic Phenotype

(A) Family A (left) and family B (right), each with an affected individual who is a compound heterozygote for mutations in *LRIT3*, and cosegregation analysis in available family members. The cCSNB-19018-affected index individual (II.1 of family A) used for whole-exome next-generation sequencing is marked with a red arrow. The mother of I.2 of family A is deceased, which is highlighted by a crossed line. Females and males are depicted by circles and squares, respectively. Filled and unfilled symbols indicate affected and unaffected status, respectively. ERGs of a normal ("unaffected") subject are compared with those of individuals II:1 from family A and II:1 from family B. Normal ERGs have a rod-driven waveform, a mixed (combined) rod and cone response elicited by a bright flash of light, and two conedriven responses at different frequencies of stimulation (1 and 29 Hz). The ERGs from affected individuals II:1 from family A and II:1 from family A and II:1 from family B are typical of cCSNB and differ from a normal ERG as follows: there is a mixed response with a preserved negative component of the waveform (a-wave [from photoreceptors]) but a reduced positive component (b-wave [from postreceptoral retinal activity]), a cone-driven 1 Hz response with an unusual waveform, a cone-driven 29 Hz flicker ERG with a typical broadened trough and a mildly delayed implicit time, and no detectable rod-driven waveform.

(B) *LRIT3* (RefSeq NM\_198506.3) structure containing four coding exons. Different mutations identified in persons with cCSNB are depicted. Filled and unfilled boxes represent coding regions and UTRs of *LRIT3*, respectively. The start codon is indicated by an arrow. (C) The translated amino acid sequence of LRIT3 (RefSeq NP\_940908.3), predicted domains, and the different LRIT3 alterations of two persons with cCSNB are indicated. The exact start and end of the domains vary depending on the different prediction programs used.

bipolar cells. To date, more than 300 mutations have been identified in the genes underlying icCSNB and cCSNB.<sup>5–24</sup> Mutations in many genes associated with CSNB have been identified through a candidate gene approach comparing the human phenotype to similar phenotypes observed in knockout or naturally occurring animal models,<sup>10–16,25</sup> but techniques using massively parallel sequencing of all human exons have recently been successful in identifying mutations in genes underlying heterogeneous diseases, including Leber congenital amaurosis and, more recently, CSNB.<sup>18,26,27</sup> Thus, to rapidly identify the gene defect of

another autosomal-recessive-cCSNB-affected family (family A, Figure 1A), previously excluded for known cCSNB-associated gene defects, we sequenced the index nonconsanguineous affected female after whole-exome enrichment (IntegraGen, Evry, France). Research procedures were conducted in accordance with institutional guidelines and the Declaration of Helsinki; institutional-review-board approvals were obtained from the participating universities and the national Ministries of Health of each participating center. Prior to genetic testing, informed consent was obtained from all CSNB-affected

Table 1. LR/T3 Mutatio	ns and Predic	cted Functic	onal Consequences in	Individuals with c	CSNB		
Index Affected Individuals	Origin	Exon	Nucleotide Mutation	Allele State	Protein Alteration	Frequency	<b>Conservation and Prediction</b>
Family B: CH6126 (II.1)	France	4	c.1151C>G	heterozygous	p.Ser384*	0/380 alleles	truncated protein
		4	c.1538_1539del	heterozygous	p.Ser513Cysfs*59	0/376 alleles	truncated protein
Family A: 19018 (II.I)	USA	4	c.1318C>T	heterozygous	p.Arg440*	0/348 alleles	truncated protein
		4	c.983G>A	heterozygous	p.Cys328Tyr	0/380 alleles; 1 out of 10,757 alleles in the Exome Variant Server database	PolyPhen-2: predicted to be probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00)
							SIFT: predicted to be damaging with a score of 0.00; conserved except in alpaca (Phe)
							KD4v: predicted to be deleterious

individuals and their family members. Ophthalmic examinations were performed on all subjects: a full-field ERG incorporated the International Society for Clinical Electrophysiology of Vision standards and methodology previously described.<sup>28–30</sup> Exons of DNA samples were captured and investigated as shown before with in-solution enrichment methodology (SureSelect Human All Exon Kits version 3, Agilent, Massy, France) and next-generation sequencing (NGS) (Illumina HISEQ, Illumina, San Diego, CA, USA). Image analysis and base calling were performed with Real Time Analysis software (Illumina).<sup>18</sup> Geneticvariation annotations were performed by an in-house pipeline (IntegraGen), and results were provided per sample or family in tabulated text files. After very stringent criteria were used for excluding variants observed in dbSNP 132, HapMap, 1000 Genomes Project, and internal (IntegraGen) variant-detection databases, the results were further filtered so that only compound heterozygous or homozygous variants in coding regions remained. This allowed us to reduce the number of variants from 4,267 to 0 indels and from 50,807 SNPs to 21 homozygous variants in 13 genes and 30 compound heterozygous variants in 10 genes. To determine the most likely disease-causing gene defect for this cCSNB-affected family, we investigated missense changes with bioinformatic tools to predict the pathogenicity of the mutations and the conservation of affected amino acid residues (PolyPhen-2,<sup>31</sup> SIFT,<sup>32</sup> KD4v,<sup>33</sup> and the USCS Human Genome Browser). Those genes harboring the selected variants were assessed for eye and retinal expression with the use of the UniGene database, the retinal gene-expression profile database provided by Siegert et al.,<sup>34</sup> and the in-house rd1 mouse expression database (courtesy of Thierry Leveillard). On the basis of these criteria, the only selected variants were compound heterozygous mutations (c.983G>A [p.Cys328Tyr] and c.1318C>T [p.Arg440\*]) in exon 4 of LRIT3 (RefSeq accession number NM\_198506.3), which encodes leucine-rich-repeat (LRR), immunoglobulin-like, and transmembrane-domain 3 (LRIT3) (Figures 1A-1C; family A). For the c.983G>A variant, the G and A were found 46× and 53×, respectively, and for c.1318C>T, the C and T were present  $70 \times$  and  $54 \times$ , respectively, indicating that both variants were present heterozygously. Both variants were absent in more than 340 control chromosomes, and only c.983G>A (p.Cys328Tyr) was found at a very low frequency (1 out of 10,757 alleles, indicating that only one person was heterozygous for this substitution) in the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project Exome Variant Server (EVS, 15.06.2012). The cystein at amino acid position 328 is highly conserved; only alpaca show a different amino acid residue (Phe) (USCS Human Genome Browser 15.06.2012) (Table 1). PolyPhen-2, SIFT, and KD4v predicted this variant to be probably damaging (Table 1). The second mutation, c.1318C>T (p.Arg440\*), was absent in all publically available databases, including the EVS. Cosegregation analysis revealed that the unaffected father and brother were heterozygous for the p.Cys328Tyr substitution. Subsequent Sanger sequencing of seven fragments covering the four exons and flanking intronic regions of LRIT3 (RefSeq NM\_198506.3) (detailed conditions will be communicated on request) in 89 individuals (with cCSNB and unclassified CSNB) of various ethnic origins and from different clinical centers in Europe, the United States, Canada, Israel, and India (CSNB study group) detected one additional cCSNB-affected person, who carried compound heterozygous disease-causing mutations (c.1151C>G [p.Ser384\*] and c.1538\_1539del [p.Ser513Cysfs\*59]) in exon 4 (Figures 1A-1C; family B). Both variants cosegregated with the phenotype, were absent in more than 370 control chromosomes, and were not described in the current EVS database (Figure 1A, family B). The frequencies of LRIT3 polymorphisms found in our individuals with CSNB are provided in Table S1, available online. On the basis of all of the above evidence, we conclude that mutations in LRIT3 lead to cCSNB.

In family A, used for the whole-exome NGS approach (Figure 1A, family A), the index case (II.1) had visual blurring and night-vision disturbances from childhood. When she was 4 years old, spectacles for myopia were prescribed and strabismus surgery was performed in the right eye. A diagnosis was not specifically made, but the individual recalls being told that she had a progressive blinding disease with high myopia. Laser treatment of retinal tears occurred when she was 25 years old. When she was 45 years of age, an ERG was taken for the first time (Figure 1A, family A). The ERG features were those of cCSNB: undetectable responses to a dim flash under darkadapted conditions (rod, Figure 1A, family A), a negative waveform in the mixed rod-cone response in the darkadapted state, and an unusual square-shape appearance of the a-wave in the cone ERG<sup>1,14,18</sup> (29 Hz, Figure 1A, family A). In the detectable ERGs, amplitudes were abnormally reduced, a result that might be associated with high myopia.<sup>35</sup> Visual acuities were 20/80 (-26.00 D sphere) in the right eye and 20/30 (-27.00 D sphere) in the left eye. Eye pressures were normal, and the fundus appearance was that of myopia (1 Hz, Figure 1A). Other than undergoing eye-muscle and laser surgery (see above), the index case had arthroscopic knee surgery in her 40s. In family B, the affected person was diagnosed with high myopia at the age of 2 years. Prominent night blindness was also noticed at that age, and later on, visual acuity was found to be decreased. She had no photoaversion or loss in the peripheral visual field. At the time of presentation, 9 years of age, visual acuities were 20/40 (-7.00 D sphere) in the right eye and 20/50 (-8.00 D sphere) in the left eye. Lenses were transparent, and the fundus appearance was that of myopia with a tilted optic disc. Fundus autofluorescence was normal. An optical-coherence-tomography-3 scan of the maculae disclosed a normal photoreceptor layer. Light sensitivity was found moderately decreased over the whole visual field. She had an electronegative ERG mixed rodcone response in the dark-adapted state (Figure 1A, family B). In addition, there were moderately reduced cone 30 Hz flicker responses, an atypical waveform in the light-adapted state (1 Hz, Figure 1A, family B), and no ERG responses to dim stimuli in the dark-adapted state (rod, Figure 1A, family B); all of these findings are compatible with the diagnosis of cCSNB (Figure 1A, family B).

To date, only little information is available on the expression, localization, and function of *LRIT3*. It maps to chromosomal region 4q25 and contains four exons, the first of which was only recently identified and codes for a protein with 679 amino acids.<sup>36</sup> An available expressed-sequence-tag (EST) profile (from the UniGene EST Profile Viewer) indicates that the gene is expressed in the brain and the eye. Real-time-PCR experiments and subsequent Sanger sequencing of amplified real-time-PCR products confirmed the expression of *LRIT3* in the human retina (commercially available cDNA from Clontech, Saint-Germain-en-Laye, France) by giving a signal of  $\Delta C_T = 6.33$  ( $C_T \ LRIT3 = 24.96$ ) in relation to  $\beta$ -actin (*ACTB* [MIM 102630]) ( $C_T \ ACTB = 18.63$ ) (primers Table S2).

To immunolocalize the exact location of LRIT3 in the retina, we used a validated human LRIT3 antibody (Figures S1 and S2) in human retina from a cryosectioned eye and performed immunostainings. LRIT3 localization could be detected in the outer plexiform layer (OPL) (Figures 2A and 2B, green). Immunofluorescence was analyzed with a confocal microscope (FV1000 fluorescent, Olympus, Hamburg, Germany). Colocalization studies with an antibody against a mouse Goa (Millipore, Molsheim, France), a specific ON bipolar cell marker (Figures 2A and B, red), demonstrated that human LRIT3 antibody reveals a characteristic synaptic punctate labeling at the dendrites of depolarizing bipolar cells. Comparing this immunostaining with the immunostaining from other molecules implicated in cCSNB, we conclude that the dotted punctate labeling (arrows) represents multiple rod bipolar cell dendritic tips that invaginate a rod spherule and that the innermost punctate labeling organized in rows (arrowheads) represents labeling of cone bipolar cell tips that invaginate the foot of a cone pedicle (Figure 2B).<sup>37,38</sup> Fainter green fluorescence signal was also detected in other retinal layers and especially in the photoreceptor layers. In these latter layers, a comparable faint signal was also detected in human retina with only the use of secondary antibodies, indicating that this signal is not specific to LRIT3. Future studies on wild-type and knockout mice retinas with a mouse LRIT3 antibody are warranted for further validating whether LRIT3 is present in retinal layers other than the OPL.

LRIT3 (RefSeq NP\_940908.3) is predicted to belong to the LRR protein family, which contains a signal peptide (amino acids 1–19) and four LRR domains (amino acids 82–104, 106–128, 130–152, and 154–176), which are flanked by cysteine-rich LLRNT (amino acids 20–61) and LRRCT (amino acids 201–253) motifs. Furthermore, an immunoglobulin-like (Ig-like) domain (amino acids



Figure 2. LRIT3 Immunohistochemistry in Human Retinal Sections

(A) LRIT3 signal (green) in the OPL double labeled with an ON bipolar cell marker against Go $\alpha$  (red) was detected in the human retina by confocal microscopy. A strong signal with the human LRIT3 antibody was detected in a punctate manner at presumable dendritic tips of the ON bipolar cells (16 stacks of the confocal-microscopy image were chosen). The following abbreviations are used: PHR, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; and GCL, ganglion cell layer.

(B) A  $3.5 \times$  zoom of (A) focuses on LRIT3 staining at presumable dendrites of the bipolar cells (three stacks of the confocal-microscopy image were chosen). The scale bar represents 20  $\mu$ m.

254–344), a fibronectin type III (FNIII) domain (amino acids 484–574), and a transmembrane (TM) domain (amino acids 583–633) were annotated (UniProtKB/Swiss-



#### Figure 3. Genes Underlying CSNB

Different forms of CSNB in humans are classified according to their electroretinographic features, mode of inheritance, clinical phenotype, and mutated genes. Affected individuals discussed herein show a complete type of Schubert-Bornschein ERG. Genes are indicated in italics and are underlined. Chromosomal locations are given in parentheses. The following abbreviations are used: cCSNB, complete CSNB; icCSNB, incomplete CSNB; AR, autosomal recessive; and AD, autosomal dominant.

Prot and SMART). The C-terminal region is highly conserved and might harbor a PDZ-binding motif. More specifically, according to Tian et al.<sup>39</sup> and to publicly available motif research programs (ELM: The Database of Eukaryotic Linear Motifs), the C-terminal amino acid residues "ESQV" and "RPEYYC," respectively, are good candidates to represent such PDZ-binding motifs (Figure 1C). In addition, LRIT3 contains a serine-rich region (amino acids 373-433) and the two cysteine residues, Cys275 and Cys328, predicted to form disulfide bonds (UniProtKB/ Swiss-Prot) (Figure 1C). Recent in vitro studies have suggested that LRIT3 can regulate a fibroblast-growth-factor receptor, FGFR1.<sup>36</sup> Although FGFR1 is expressed in many tissues, including the retina (UniGene EST Profile Viewer), a role for FGFR1 in bipolar cells has not yet been reported.<sup>40</sup> The mutation spectrum described here affects different LRIT3 domains, including the Cys328 residue, which is predicted to form a disulfide bond in the Ig-like domain. Disulfide bonds have been shown to be important for folding and stability of some proteins and especially in proteins secreted to the extracellular medium.<sup>41</sup> The p.Cys328Tyr substitution might thus lead to accumulation of misfolded protein in the endoplasmic reticulum, as has been illustrated for substitution p.Cys203Arg, which affects a disulfide bond in the green opsin and leads to anomalous trichromacy.<sup>42</sup> Interestingly, cysteine substitutions have also been described in the protein encoded by NYX (RefSeq NM\_022567.2), another gene associated with cCSNB (Figure 3). We and others found that NYX missense mutations c.143G>A and c.144C>G, respectively, lead to substitutions p.Cys48Tyr (associated with CSNB without myopia)<sup>6</sup> and p.Cys48Trp (associated with CSNB with isolated myopia),<sup>43</sup> respectively. The Cys48 residue was predicted to be important for protein

stabilization via disulphide bridging. Because both substitutions are predicted to probably destabilize the protein structure, the phenotypic variability still needs to be elucidated. However, it is striking that most of the individuals with mutations in genes underlying the complete form of CSNB show high myopia. It has been suggested that altered cone signal resulting from mutations in genes important for the ON pathway might participate in myopia.<sup>43</sup> Blurred images seen by the affected persons might create an aberrant retinal signal before the local eye can react to retinal blur and might therefore induce myopia.<sup>44–47</sup>

The three other mutations represent two nonsense mutations and a frameshift, which are located in the last exon. Thus, it is likely that mutant mRNA products escape nonsense-mediated decay. The p.Ser384\* truncated protein is predicted to lack the serine-rich, FNIII, and TM domains and the PDZ-binding motifs; the p.Arg440\* altered protein is predicted to lack the FNIII and TM domains and the PDZ-binding motifs; and the p.Ser513-Cysfs\*59 truncated protein is predicted to lack the TM domain and the PDZ-binding motif. Given that all altered proteins are predicted to lack the TM motif, they might lead to a protein that cannot be targeted to the membrane and that therefore cannot function at its predicted site. Considering the different domains present in LRIT3, the protein resembles most LRIT1 and LRIT2 of unknown function, as well as SALM1, SALM2, and SALM3 of the synaptic-cell-adhesion-molecule protein family. These proteins contain LRRs; LLRNT and LRRCT motifs; Ig-like, FNIII, and TM domains; and an intracellular C-terminal PDZ-binding motif, which interacts within the SALM protein family with PSD-95, an abundant postsynaptic scaffolding protein.48,49 Compared to the SALM family, in which proteins always have six LRRs, LRIT3 contains only four. This protein family has been shown to be important in synapse formation. More precisely, SALM1 has been implicated in clustering NMDA receptors, and an altered SALM1 lacking PSD-95 binding fails to cluster these receptors. SALMS2 is targeted to and stabilized at excitatory synapses via its interaction with PSD-95, where it recruits NMDA and AMPA receptors to promote excitatory synaptic maturation. SALM1, SALM2, and SALM3 form homomeric and heteromeric complexes with each other.<sup>49</sup> In ON bipolar cells, TRPM1 represents the channel that opens after light stimulation, leading to the ERG bwave, which is severely reduced in individuals with cCSNB.<sup>14,50,51</sup> We and others have recognized low-surface TRPM1 localization in overexpressing mammalian cells.<sup>52</sup> Studies by Pearring et al. showed that the presence of NYX located in the dendritic tips of ON bipolar cells is necessary for the dendritic-tip localization of TRPM1.<sup>38</sup> A mouse model lacking NYX led to the absence of dendritic-tip labeling of TRPM1. The authors suggested that NYX is localized to the ON bipolar cell dendritic tips, subsequently interacts with TRPM1, and thereby establishes the correct TRPM1 localization. They also mention that another ancillary protein would be needed to interact with intracellular scaffolding complexes to hold the TRPM1 channels to the bipolar cell synapse. Given the fact that LRIT3 has a PDZ-binding motif, it might be the missing molecule interacting with scaffolding proteins to bring TRPM1 to the surface of the cell, and thereafter, NYX and LRIT3 hold the channel in this form. Confirming this hypothesis will require in vivo and in vitro studies.

Including in this study, mutations in five genes (*NYX*, *GRM6*, *TRPM1*, *GPR179*, and *LRIT3*) have been implicated in cCSNB (Figure 3).<sup>8–11,14–16,18,19,25</sup> These genes code for nyctalopin, metabotropic glutamate receptor 6, transient receptor potential cation melastatin 1, G-protein-coupled receptor 179, and LRIT3, respectively. All localize postsynaptically to the photoreceptors in the retina in ON bipolar cells.<sup>19,37,38,53</sup> Further functional studies will eventually clarify the exact role of LRIT3 within the ON bipolar cell pathway, which will also improve our understanding of the overall visual signal transduction through the retina.

#### Supplemental Data

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

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#### Web Resources

The URLs for data presented herein are as follows:

ELM: The Database of Eukaryotic Linear Motifs, http://elm.eu.org/ GenCards, http://www.genecards.org

Gene Expression Profile, http://www.fmi.ch/roska.data/

- KD4v: Comprehensible Knowledge Discovery System for Missense Variants, http://decrypthon.igbmc.fr/kd4v/
- National Center for Biotechnology Information (NCBI), http:// ncbi.nlm.nih.gov/
- NCBI UniGene EST Profile Viewer, http://www.ncbi.nlm.nih.gov/ UniGene/ESTProfileViewer.cgi?uglist=Hs.308127
- NHLBI Exome Sequencing Project Exome Variant Server, http:// evs.gs.washington.edu/EVS/
- Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/

PyMOL, http://pymol.sourceforge.net/

SMART, http://smart.embl-heidelberg.de/

Sorting Intolerant from Tolerant (SIFT), http://sift.bii.a-star.edu.sg/ UniProtKB/Swiss-Prot, www.uniprot.org

USCS Human Genome Browser, http://genome.ucsc.edu/

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# B. Lrit3 deficient mouse (nob6): a novel model of complete congenital stationary night blindness (cCSNB)

The purpose of this study was to characterize a commercially available *Lrit3* knockout mouse model and to establish whether this animal model may serve as a reliable model for human cCSNB.

We utilized several different methods to genetically and functionally characterize a commercially available Lrit3 knockout mouse model. We confirmed that the insertion of a Bgeo/Puro cassette in the knock-out allele introduces a premature stop codon, which presumably codes for a non-functional protein. The mouse line did not harbor other mutations present in common laboratory mouse strains or in other known cCSNB genes. Lrit3 mutant mice exhibit a no b-wave (nob) phenotype, with lack of b-waves or severely reduced b-wave amplitudes in the scotopic and photopic electroretinogram (ERG), respectively. Optomotor tests revealed notably decreased optomotor responses in scotopic conditions. No obvious fundus autofluorescence or histological retinal structure abnormalities were observed. However, spectral domain optical coherence tomography (SD-OCT) revealed thinned inner nuclear layers in these mice and thinning of the retinal part containing inner plexiform layer, ganglion cell layer and nerve fiber layer. This murine phenotype was noted at both 6 weeks and at 6 months of age. The stationary *nob* phenotype of mice lacking *Lrit3*, which we named nob6, confirmed the previously reported findings in patients carrying LRIT3 mutations and is similar to other cCSNB mouse models. This novel mouse model will be useful for investigating the pathogenic mechanism(s) associated with LRIT3 mutations and clarifying the role of LRIT3 in the ON-bipolar cell signaling cascade.

# *Lrit3* Deficient Mouse (*nob6*): A Novel Model of Complete Congenital Stationary Night Blindness (cCSNB)

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#### Abstract

Mutations in LRIT3, coding for a Leucine-Rich Repeat, immunoglobulin-like and transmembrane domains 3 protein lead to autosomal recessive complete congenital stationary night blindness (cCSNB). The role of the corresponding protein in the ON-bipolar cell signaling cascade remains to be elucidated. Here we genetically and functionally characterize a commercially available Lrit3 knock-out mouse, a model to study the function and the pathogenic mechanism of LRIT3. We confirm that the insertion of a Bgeo/Puro cassette in the knock-out allele introduces a premature stop codon, which presumably codes for a non-functional protein. The mouse line does not harbor other mutations present in common laboratory mouse strains or in other known cCSNB genes. Lrit3 mutant mice exhibit a so-called no b-wave (nob) phenotype with lacking or severely reduced b-wave amplitudes in the scotopic and photopic electroretinogram (ERG), respectively. Optomotor tests reveal strongly decreased optomotor responses in scotopic conditions. No obvious fundus autofluorescence or histological retinal structure abnormalities are observed. However, spectral domain optical coherence tomography (SD-OCT) reveals thinned inner nuclear layer and part of the retina containing inner plexiform layer, ganglion cell layer and nerve fiber layer in these mice. To our knowledge, this is the first time that SD-OCT technology is used to characterize an animal model for CSNB. This phenotype is noted at 6 weeks and at 6 months. The stationary nob phenotype of mice lacking Lrit3, which we named nob6, confirms the findings previously reported in patients carrying LRIT3 mutations and is similar to other cCSNB mouse models. This novel mouse model will be useful for investigating the pathogenic mechanism(s) associated with LRIT3 mutations and clarifying the role of LRIT3 in the ON-bipolar cell signaling cascade.

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#### Introduction

Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous group of non-progressive retinal disorders caused by mutations in genes implicated in the phototransduction cascade or in retinal signaling from photoreceptors to adjacent bipolar cells [1]. These disorders can be associated with other ocular abnormalities, including reduced visual acuity, myopia, nystagmus and strabismus. Most of the individuals affected with CSNB show a characteristic electroretinogram (ERG) response, named Schubert-Bornschein, in which the bwave amplitude is smaller than that of the a-wave in the darkadapted bright flash condition [2]. This electronegative waveform can be divided in two subtypes, incomplete (ic)CSNB and complete (c)CSNB [3]. cCSNB is characterized by a drastically reduced rod b-wave response due to ON-bipolar cell dysfunction, and specific cone ERG waveforms [4]. cCSNB has been associated with mutations in NTX [5,6], GRM6 [7,8], TRPM1 [9–11] and GPR179 [12,13], genes expressed in the inner nuclear layer (INL) of the retina [6,14–17] and coding for proteins localized at the dendritic tips of ON-bipolar cells [12,14,15,17–20]. Recently, we have identified mutations in *LRIT3*, a gene coding for a Leucine-Rich Repeat (LRR), immunoglobulin-like and transmembrane domains 3 protein, that lead to cCSNB [21]. The corresponding protein also localizes at the dendritic tips of ON-bipolar cells in the retina [21].

Animal models have been shown to be an excellent tool for identifying and elucidating the pathogenic mechanism(s) of gene defects underlying CSNB [15,18,22–32]. Clinically, the pheno-types of these models can be assessed as in patients by performing full-field electroretinography, fundus autofluorescence imaging (FAF), and optical coherence tomography (OCT). In addition, the

retinal structure can be investigated *post mortem* in affected animals and compared to unaffected littermates. This aspect is valuable for a better assessment of histological changes since access to human retinas remains extremely difficult. Various mouse models have been design or are naturally occurring for Schubert-Bornschein type of CSNB with dysfunction in molecules important for the signaling from the photoreceptors to the adjacent bipolar cells. Six mouse models with four different gene defects, *Nyx* (no b-wave (*nob*)), *Grm6* (*nob3* and *nob4*), *Trpm1* and *Gpr179* (*nob5*) have already been published for cCSNB [12,15,18,25,26,32–36].

These mouse models were helpful in dissecting the ON-bipolar cell signaling cascade. In darkness, the glutamate neurotransmitter released by the photoreceptors binds to the metabotropic glutamate receptor 6 (GRM6/mGluR6) [16,37]. This binding leads to the activation of the  $\alpha$ -subunit of the G-protein,  $G_0 \alpha$ [38,39], which results in the closure of the non-selective cation channel TRPM1. When the photoreceptors are stimulated by light, the deactivation of the G-protein in the ON-bipolar cells is responsible for the opening of TRPM1, resulting in the formation of the ERG b-wave [15,25,32]. GRM6 and NYX interact with TRPM1 and are essential for its localization at the dendritic tips of ON-bipolar cells [23,24]. In addition GPR179 is essential for the action of the G-protein downstream of mGluR6 via the correct localization of Regulator of G protein Signaling proteins (RGS) [20], GPR179 also interacts with both mGluR6 and TRPM1 and its correct localization is mediated through mGluR6 [40]

The exact role of LRIT3 in this cascade remains to be elucidated. As previously described, NYX is essential for the correct localization of TRPM1 [23]. However, NYX alone would not be sufficient to bring TRPM1 at the dendritic tips of ONbipolar cells in the retina. Mouse and human NYX are mainly extracellular proteins [41,42] that lack an intracellular PDZbinding domain important for binding to scaffolding proteins involved in membrane trafficking [43]. Thus, another transmembrane protein is needed to interact with the scaffolding proteins for TRPM1 localization [23]. As LRIT3 resembles proteins of the SALM family, in particular containing a PDZ-binding motif, our hypothesis is that LRIT3 might interact with scaffolding proteins to bring TRPM1 to the cell surface and thereafter LRIT3 together with NYX might hold the channel in this form [21]. We have not so far been able to confirm this hypothesis or to study the pathogenic mechanism(s) of LRIT3 defects underlying cCSNB by in vitro experiments due to the lack of an antibody able to detect human LRIT3 at the cell surface of transfected cells and the lack of a characterized mouse model for Lrit3, which will be named nob6.

The aim of this study was to characterize a commercially available *Lrit3* mouse model and to establish whether this animal would be a reliable model for human cCSNB.

#### **Materials and Methods**

#### Ethics statements

All animal procedures were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the French Minister of Agriculture (authorization A-75-1863 delivered on 09<sup>th</sup> November 2011). All efforts were made to minimize suffering.

#### Lrit3 cDNA sequence

We deposited at GenBank the experimentally validated cDNA sequence of *Lrit3* (BankIt1682729 *Lrit3* KF954709), which

corresponds to the mouse Lrit3 cDNA sequence, which was updated on  $10^{\text{th}}$  December 2013 (NM\_001287224.1).

#### Animal Care

Three 129/SvEv-C57BL/6 heterozygous knock-out mice for Lrit3 of each sex were obtained from a company (TF2034, Taconic, Hudson, NY). These mice were intercrossed (Centre d'Exploration et de Recherche Fonctionnelle Expérimentale CERFE, Evry, France) to produce wild-type  $(Lnt3^{+/+})$ , heterozygous ( $Lrit3^{nob6/+}$ ) and mutant ( $Lrit3^{nob6/nob6}$ ) offspring. To follow up the phenotype at different ages, the two time points, six weeks and six months, were selected. For the optomotor test, we used seven  $Lrit3^{+/+}$ , eleven  $Lrit3^{nob6/+}$ , nine  $Lrit3^{nob6/nob6}$  mice of six weeks and nine Lrit3<sup>+/+</sup>, eight Lrit3<sup>nob6/+</sup> and nine Lrit3<sup>nob6/nob6</sup> mice of six months. The same animals were used for ERG recordings, FAF, and Spectral-Domain Optical Coherence Tomography (SD-OCT) except for one six weeks Lrit3<sup>+/+</sup> and one six weeks Lrit3<sup>nob6/nob6</sup> who died during FAF. For histology, two animals of each genotype for both ages were used. Mice were housed in a temperaturecontrolled room with a 12-h light/12-h dark cycle. Fresh water and rodent diet were available ad libitum.

#### Polymerase chain reaction (PCR) genotyping for Lrit3

DNA was isolated from mouse tails with 50 mM NaOH after incubation at 95°C for 30 min. Two couples of primers were designed to amplify wild-type (wt) or mutant allele independently (HOT FIREPol, Solis Biodyne, Tartu, Estonia): mLrit3\_4aF and mLrit3\_4aR for the wt allele, mLrit3\_3F and mLrit3\_CasR for the mutant one (Table S1). PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized using the Gel Doc XR+ system (Bio-Rad, Hercules, CA).

### Genotyping for common mutations found in laboratory mouse strains

The genotyping for the  $Crb1^{rd8}$  mutation was carried out by qPCR Taqman on genomic DNA with probes specific for wt or mutant allele, respectively (Table S2). The presence of common mutations in laboratory strains  $Pde6\beta^{rd1}$ ,  $Gnat2^{cpfl3}$ , and c.230G>T p.Arg77Leu in Tyr were investigated by direct Sanger sequencing. The following primers were used: Pde6b\_7-8F and Pde6b\_7-8R for the substitution in rd1 (Gotaq DNA Polymerase, Promega, Madison, WI, USA), Pde6b\_G2shortF and Pde6b\_G1shortR for the insertion in rd1 (HOT FIREPol), Gnat2\_6F and Gnat2\_7R for the muration in cpfl3 (HOT FIREPol) and Tyr\_F and Tyr\_R for p.Arg77Leu in Tyr (HOT FIREPol) (Tables S3, S4, S5). Subsequently, PCR products were Sanger sequenced with a sequencing mix (BigDyeTerm v1.1 CycleSeq kit, Applied Biosystems, Courtabœuf, France), analyzed on an automated 48capillary sequencer (ABI 3730 Genetic analyzer, Applied Biosystems), and the results interpreted by applying a software (SeqScape, Applied Biosystems).

#### Genotyping for genes with mutations underlying cCSNB

DNA of six founder mice were used to sequence the flanking intronic and exonic sequences of *Grm6*, *Gpr179*, *Nyx*, *Lrit3* and *Trpm1* as well as intron 2 of *Grm6* and intron 1 of *Gpr179* (HOT FIREPol). The corresponding primers, fragment sizes and annealing temperatures used are reported in Tables S1 and S6, S7, S8, S9. Identified variants were evaluated in respect to the conservation (UCSC Genome Browser: http://genome.ucsc.edu/), pathogenicity predictions (Sorting Intolerant from Tolerant (SIFT): http://sift.bii.a-star.edu.sg/, and PolyPhen-2: http://



**Figure 1. Construction of the** *Lrit3* **knock-out allele and genotyping.** (A) Wild-type (wt) *Lrit3* allele comprises 4 exons. In the knock-out (ko) construction, exons 3 and 4 were replaced by a selection cassette with only 21 bp of exon 3 still remaining. For genotyping, mLrit3\_ex4aF and mLrit3\_ex4aR were designed to only amplify the wt allele, whereas mLrit3\_ex3F and mLrit3\_CasR were designed to only amplify the ko allele. (B) After migration on 2% agarose gel, *Lrit3<sup>+/+</sup>* mice exhibited a single fragment at the expected length of 602 bp, *Lrit3<sup>nob6/nob6</sup>* exhibited a single fragment at the expected length of 377 bp and *Lrit3<sup>nob6/+</sup>* mice exhibited both fragments. Legends: WT: wild-type allele; Mut: mutant allele. doi:10.1371/journal.pone.0090342.g001

genetics.bwh.harvard.edu/pph2/) and presence in mouse strains used to generate the *Lrit3* mouse model (Ensembl Genome Browser: http://www.ensembl.org/index.html).

#### Electroretinography

Electroretinography was performed according to Yang and coworkers with some modifications [44]. After overnight dark adaptation, mice were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg). Eye drops were used to dilate the pupils (0.5% mydriaticum, 5% neosynephrine) and anesthetize the cornea (0.4% oxybuprocaine chlorhydrate). Body temperature was maintained at 37°C through the use of a circulating hot water heating pad. Upper and lower lids were retracted to keep the eyes open and bulging. Contact lens electrodes for mice (Mayo Corporation, Japan) were placed on the corneal surface to record ERG. Needle electrodes placed subcutaneously in cheeks served as reference and a needle electrode placed in the back served as ground. Recordings were made from both eyes simultaneously. The light stimulus was provided by a 150 Watt xenon lamp in a Ganzfeld stimulator (Multilinear Vision, Jaeger Toennies, Germany). Responses were amplified and filtered (1 Hz-low and 300 Hz-high cut off filters) with a 1 channel DC-/AC-amplifier. Eight levels of stimulus intensity ranging from  $0.0006 \text{ cd.s/m}^2$  to 60 cd.s/m<sup>2</sup> were used for the dark-adapted ERG recording. Each scotopic ERG response represents the average of five responses from a set of five flashes of stimulation. To isolate cone responses a 10-minute light adaptation at 20 cd/m<sup>2</sup> was used to saturate rod photoreceptors. Six levels of stimulus intensities ranging from 0.3 cd.s/m<sup>2</sup> to 60 cd.s/m<sup>2</sup> were used for the light-adapted ERGs. The light-adapted ERGs were recorded on the same rod-suppressive white background as for the light adaptation. Each cone photopic ERG response represents the average of twenty responses to a set of twenty consecutive flashes. The major components of the ERG were measured conventionally. The awave amplitude was measured from the baseline to the a-wave trough and the b-wave amplitude was measured from the onset of the flash stimulus to the a-wave trough and the b-wave peak, respectively.

#### Optomotor response

Optomotor test was performed as previously described [45]. After overnight dark adaptation, mice were placed on a grid platform (11.5 cm diameter, 19 cm above the bottom of the drum) surrounded by a motorized drum (29 cm diameter) that could be revolved clockwise or anticlockwise at two revolutions per minute. Vertical black and white stripes of a defined spacial frequency were presented to the animal. Spatial frequencies tested were 0.063, 0.125, 0.25, 0.5 and 0.75 cycles per degree. The stripes



**Figure 2. wt and ko** *Lrit3* **cDNAs and RT-PCR.** (A) ko cDNA comprised the remaining 21 bp of exon 3 and only the first 8 bp of the selection cassette which leads to a premature stop codon. mLrit3\_RT\_ex2F and mLrit3\_RT\_ex3R were designed to only amplify the wt cDNA whereas mLrit3\_RT\_ex2F and mLrit3\_RT\_ex2F and mLrit3\_RT\_ex2F and mLrit3\_RT\_ex3R were designed to only amplify the supposed ko cDNA. (B) After amplification and migration on agarose gel, *Lrit3<sup>+/+</sup>* mice exhibited a single fragment at 539 bp, *Lrit3<sup>nob6/nob6</sup>* mice exhibited a 443 bp fragment and *Lrit3<sup>nob6/+</sup>* mice exhibited both fragments. Legends: WT: wild-type cDNA; Mut: mutant cDNA. (C) Sequence of the ko LRIT3 protein. This 206 amino acid protein are supposed to lack its Ig-like, Serine-rich, fibronectin III, transmembrane and PDZ-binding domains. doi:10.1371/journal.pone.0090342.q002

were rotated for 1 min in each direction with an interval of 10 sec between the two rotations. Animals were videotaped using a digital video camera for subsequent scoring of head movements. Tests were initially performed under scotopic conditions, using the night shot function of the camera. Mice were then subjected to two lamps of 60 Watt each for 5 min and photopic measurements were performed. Head movements were scored only if the angular speed of the movement corresponded to that of the drum rotation. Head movements in both directions were averaged to obtain the number of head movements per minute.

#### FAF

Photographs of the eye fundus and autofluorescence were obtained with a scanning laser ophthalmoscope (SLO) (HRA1, Heidelberg, Germany). Mouse pupils were dilated by the ocular instillation of 0.5% mydriaticum and 5% neosynephrine.

#### SD-OCT

Mice were anesthetized by isoflurane inhalation and maintained under anesthesia via a mask. Eye drops were used to dilate the pupils (0.5% mydriaticum, 5% neosynephrine) and eye dehydration was prevented by regular instillation of sodium chloride drops. SD-OCT images were recorded for both eyes using a spectral domain ophthalmic imaging system (Bioptigen, Inc., Durham, NC, USA). We performed rectangular scans consisting of a 1.4 mm by 1.4 mm perimeter with 1000 A-scans per B-scan with a total B-scan amount of 100. Scans were obtained first while centered on the optic nerve, and then with the nerve displaced either temporally/nasally or superiorly/inferiorly. SD-OCT scans were exported from InVivoVue as AVI files. These files were loaded into Image] (version 1.47; National Institutes of Health, Bethesda, MD) where they were registered using the Stackreg plug-in. If the optic nerve was placed temporally/nasally, three Bscans at the level of the nerve were averaged and measurements were performed 500 µm away from the optic disc, on each side. In the case where the optic nerve was placed superiorly/inferiorly, 3 B-scans placed 500  $\mu$ m away from the optic disc were averaged to perform the measurements. We measured the thickness of outer nuclear layer (ONL), INL and a complex comprising inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fiber layer (NFL) that we called IPL+GCL+NFL [46].



**Figure 3. Scotopic ERG phenotype.** Dark-adapted ERG series were obtained from representative  $Lrit3^{n+/+}$  (black line),  $Lrit3^{nob6/+}$  (blue line) and  $Lrit3^{nob6/nob6}$  (red line) littermates. (A) At 6 weeks of age. The scale indicates 100 ms and 200  $\mu$ V. Values to the left of the row of waveforms indicate flash intensity in log cd.s/m<sup>2</sup>. (B) Amplitude of the major components of the dark-adapted ERG with increasing flash intensity at 6 weeks of age. The b-wave component is absent in  $Lrit3^{nob6/nob6}$  mice and therefore this data is not plotted. (C) Implicit time of the major components of the dark-adapted ERG with increasing flash intensity at 6 weeks of age. The b-wave component is absent in  $Lrit3^{nob6/nob6}$  mice and therefore this data is not plotted. (D) At 6 months of age. The scale indicates 100 ms and 200  $\mu$ V. Values to the left of the row of waveforms indicate flash intensity in log cd.s/ m<sup>2</sup>. (E) Amplitude of the major components of the dark-adapted ERG with increasing flash intensity in log cd.s/ m<sup>2</sup>. (E) Amplitude of the major components of the dark-adapted ERG with increasing flash intensity in log cd.s/ m<sup>2</sup>. (E) Amplitude of the major components of the dark-adapted ERG with increasing flash intensity at 6 weeks component is absent in  $Lrit3^{nob6/nob6}$  mice and therefore this data is not plotted. (D) At 6 months of age. The scale indicates 100 ms and 200  $\mu$ V. Values to the left of the row of waveforms indicate flash intensity in log cd.s/ m<sup>2</sup>. (E) Amplitude of the major components of the dark-adapted ERG with increasing flash intensity at 6 weeks component is absent in  $Lrit3^{nob6/nob6}$  mice and therefore this data is not plotted. (D) At 6 months of age. The scale indicates 100 ms and 200  $\mu$ V. Values to the left of the row of waveforms indicate flash intensity in log cd.s/ m<sup>2</sup>. (E) Amplitude of the major components of the dark-adapted ERG with increasing flash intensity at 6 months of age. The b-wave component is

absent in *Lrit3<sup>nob6/nob6</sup>* mice and therefore this data is not plotted. (F) Implicit time of the major components of the dark-adapted ERG with increasing flash intensity at 6 months of age. The b-wave component is absent in *Lrit3<sup>nob6/nob6</sup>* mice and therefore this data is not plotted. doi:10.1371/journal.pone.0090342.g003

#### Preparation of retinas for RNA

Mice were killed by  $CO_2$  administration and cervical dislocation. Eyes were removed and dissected in PBS to collect one retina of each mouse. Retinas were soaked in RNA latter (Qiagen, Venlo, The Netherlands) and stored at  $-80^{\circ}$ C until used.

#### RT-PCR

Total RNA was isolated from retinas of 6 weeks old mice using a kit (RNeasy Mini Kit, Qiagen) and 500 ng were used to synthesize cDNA with a reverse transcriptase (SuperScript II, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Two couples of primers were designed to amplify wt or mutant cDNA independently: mLrit3\_RT\_ex2F 5' GTGGAGCTG-CAGTACCTCT 3' and mLrit3\_RT\_ex3R 5' GCTGACAT-CATCACGGACG 3' for the wt cDNA, mLrit3\_RT\_ex2F and mLrit3\_RT\_CasR 5' GCTCGAAGCTTATCGCTAGT 3' for the mutant one. Amplification was carried out by a DNA polymerase (HOT FIREPol).

#### Preparation of retinal sections for histology

Mice were killed by  $CO_2$  administration and cervical dislocation. Eyes were removed, fixed in Davidson's fixative (22% formalin 37%, 33% absolute ethanol, 11% glacial acetic acid) at room temperature for 3 h, dehydrated and embedded in paraffin. Sections of 5-µm-thickness were cut on a microtome (HM 340E, Microm Microtech, Francheville, France), mounted onto Superfrost plus glass slides (Thermo Fisher Scientific, Waltham, MA, USA), dried in an oven and stored at room temperature until used.

#### Histology

Retinal sections for histology were hematoxylin-eosin colored by an automaton (HMS 70, Microm Microtech), dried and mounted with a non-aqueous medium (Diamount, Diapath, Martinengo, BG, Italy). Slides were then 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).

#### Statistical analyses

Statistical analyses were performed using the statistical software (SPSS, version 19.0 Inc, Chicago, Illinois, USA). Kruskal-Wallis's test was used to compare head movements per minute in optomotor test and retinal layer thickness in SD-OCT among the three genotypes. *Post-hoc* comparisons were used to compare the genotypes two by two when the Kruskal-Wallis's test permitted to reject the hypothesis H0. These *post-hoc* analyses were also applied to compare results obtained from mice at six weeks and six months of ages. The number of animals used for the different phenotyping experiments and groups are described above (Animal Care). Tests were considered as significant when p<0.05.

#### Results

#### Genetic characterization of the Lrit3 deficient mouse

To obtain an *in vivo* tool to study the pathogenic mechanism(s) of cCSNB due to mutations in *LRIT3*, common databases were checked for the existence of commercially available mice lacking functional LRIT3. Indeed, *Lrit3* knock-out mouse line (LEXKO-2034) was generated by a company (Lexicon Pharmaceuticals,

The Woodlands, TX, USA) and a basic phenotype description was given. For this line no obvious phenotype had been noted (behavior, hematology, endocrinology, immunology, cardiology, radiology, fertility, ophthalmology). Since the ophthalmic examination was also inconspicuous and no information on the phenotyping protocol was available, we wanted to elucidate if these mice represent a model for cCSNB. Six 129/SvEv-C57BL/6 mice heterozygous knock-out for Lrit3 of both sexes were obtained from a company (Taconic, Hudson, NY, USA) and three breeding pairs were established. For the knock-out allele, exons 3 and 4 are deleted and replaced by a Bgeo/Puro cassette, with only 21 bp of exon 3 remaining (Figure 1A). These mice were intercrossed and the offspring was genotyped by size specific PCR strategies. While  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  exhibited a single fragment at 602 bp and 377 bp respectively, Lrit3<sup>nob6/+</sup> mice revealed both fragments (Figure 1B). Lrit3 mice used in these experiments were free of common mutations in Tyr [47], Crb1 [48], Pde6ß [49,50] and Gnat2 [51] frequently found in laboratory strains and associated with different eye phenotypes (data not shown). Since already three naturally occurring mouse models for cCSNB have been described (Nyx [34], Gpr179 [12] and Grm6 [35]), we excluded these mutations and mutations in all genes underlying cCSNB (Nyx, Grm6, Trpm1, Gpr179 and Lrit3) by a direct sequencing approach. Only 129/SvEv and C57BL/6 strain specific non-pathogenic variants were detected (Tables S10, S11).

#### Validation of the Lrit3 deficient mouse

*Lrit3* deficient mice were validated at transcript level by performing RT-PCR experiments with subsequent direct sequencing of the amplicons (Figure 2A–B). A 539 bp and a 443 bp fragment was obtained for the wt and the mutant cDNA, respectively. Heterozygous mice exhibited both fragments confirming the PCR genotyping for *Lrit3* model (Figure 2B). The knock-out allele for *Lrit3* produced a transcript including 21 bp of exon 3 and the first 8 bp of the selection cassette (c.611\_2046de-linsGGCCATAG), which leads to a premature stop codon (p.Phe204Trpfs\*3) (Figure 2A). So, if a protein is produced, it would code for a short 206 amino acid lacking presumably the Immunoglobulin-like (Ig-like), Serine-rich, fibronectin III, transmembrane and PDZ-binding domains (Figure 2C).

## Functional characterization of mice lacking functional LRIT3

**Electroretinography.** ERG responses of  $Lrit3^{+/+}$ ,  $Lrit3^{nob6/+}$ and  $Lrit3^{nob6/nob6}$  mice were recorded at six weeks and at six months under scotopic and photopic conditions and increasing flash intensities. At six weeks of age, under scotopic conditions, which are dominated by rod-pathway function,  $Lrit3^{+/+}$  mice showed normal responses with the classic positive deflection of the b-wave. As expected, with increasing flash intensities, amplitudes of both a-wave and b-wave increased whereas implicit times of both waves shortened (Figure 3A–C). ERG responses of heterozygous mice were undistinguishable from the  $Lrit3^{+/+}$  responses (Figure 3A–C). In contrast,  $Lrit3^{nob6/nob6}$  mice were lacking b-wave on their ERG responses, while a-waves were comparable in amplitude or implicit time to  $Lrit3^{nob6/+}$  and to  $Lrit3^{+/+}$  mice (Figure 3A–C). This led to an electronegative ERG waveform in  $Lrit3^{nob6/nob6}$  mice, in which the b-wave was absent while the awave was preserved, indicating a signal transmission defect



**Figure 4. Photopic ERG phenotype.** Cone-mediated ERG series were obtained from representative  $Lrit3^{n/4+}$  (black line),  $Lrit3^{nob6/+}$  (blue line) and  $Lrit3^{nob6/nob6}$  (red line) littermates. (A) At 6 weeks of age. The scale indicates 100 ms and 100  $\mu$ V. Values to the left of the row of waveforms indicate flash intensity in log cd.s/m<sup>2</sup>. (B) Amplitude of the a-wave with increasing flash intensity at 6 weeks of age. (C) Amplitude of the b-wave with

increasing flash intensity at 6 weeks of age. (D) Implicit time of the a-wave with increasing flash intensity at 6 weeks of age. (E) Implicit time of the bwave with increasing flash intensity at 6 weeks of age. (F) At 6 months of age. The scale indicates 100 ms and 100  $\mu$ V. Values to the left of the row of waveforms indicate flash intensity in log cd.s/m<sup>2</sup>. (G) Amplitude of the a-wave with increasing flash intensity at 6 months of age. (H) Amplitude of the b-wave with increasing flash intensity at 6 months of age. (I) Implicit time of the a-wave with increasing flash intensity at 6 months of age. (J) Implicit time of the b-wave with increasing flash intensity at 6 months of age. (J) Implicit time of the b-wave with increasing flash intensity at 6 months of age. (J) Implicit time of the b-wave with increasing flash intensity at 6 months of age. (J) Implicit time of the b-wave with increasing flash intensity at 6 months of age.

between rod photoreceptors and ON-bipolar cells, whereas the phototransduction in rod photoreceptors is not affected. At 6 weeks of age, under photopic conditions, which reflect cone circuitry function,  $Lrit3^{+/+}$  mice showed normal ERG responses with the classic positive deflection of the b-wave. As expected, with increasing flash intensities, the amplitudes of both, the a-wave,



**Figure 5. Optomotor responses.** The number of head movements per minute was obtained in scotopic conditions with spatial frequencies from 0.063 to 0.75 cpd and compared using Kluskal-Wallis statistical test in representative  $Lrit3^{+/+}$  (black box),  $Lrit3^{nob6/+}$  (blue box) and  $Lrit3^{nob6/nob6}$  (red box) littermates. The star indicates a significant test (p<0.05). (A) At 6 weeks of age. (B) At 6 months of age. doi:10.1371/journal.pone.0090342.g005

when measurable, and the b-wave increased, whereas implicit times of both waves shortened (Figure 4A–E). Responses in  $Lrit3^{nob6/+}$  mice were not different from those of  $Lrit3^{+/+}$ (Figure 4A–E). In contrast, ERG responses for  $Lrit3^{nob6/nob6}$  were very variable and showed larger a-wave amplitudes, shorter bwave amplitudes and longer implicit times than for  $Lrit3^{+/+}$  mice (Figure 4A–E). These results are in keeping with cone-mediated pathway dysfunction in these mice. The *nob* phenotype observed in mutant mice seemed to be stationary since no difference was observed between responses obtained at six weeks (Figures 3A–C and 4A–E) and six months (Figures 3D–F and 4F–J). This new *nob* mouse model was named *nob6*.

**Optomotor test.** We tested optomotor responses of  $Lrit3^{+/+}$ , Lrit3<sup>nob6/+</sup> and Lrit3<sup>nob6/nob6</sup> mice at 6 weeks and 6 months of age under scotopic and photopic conditions at increasing spatial frequency. Under scotopic conditions, for all the genotypes at both ages, a maximum number of head movements per minute was reached at 0.125 cpd. The number of head movements per minute decreased then with increasing spatial frequency but the exact visual acuity was not evaluable because the zero was never reached. Responses in heterozygous mice were globally undistinguishable from the  $Lrit3^{+/+}$  responses (Figure 5A–B). However, optomotor responses in Lrit3<sup>nob67nob6</sup> mice were statistically decreased at all spatial frequencies and at both ages (from p < 0.001 to p = 0.032) (Figure 5A-B). Moreover, no statistical differences were observed for the mutant mice responses between 6 weeks and 6 months. Analyses of the optomotor responses under photopic conditions were not conclusive and therefore the data are not shown.

**FAF.** To validate if *Lrit3<sup>nob6/nob6</sup>* mice represent indeed a good model for a stationary not progressive night blindness disease, which is not associated with striking fundus abnormalities, we performed FAF. In the retinal pigment epithelium (RPE) of mammals, lipofuscin (LF) accumulates as a result of outer segment

renewal. LF levels increase with age and can be modified in case of photoreceptor/RPE diseases [52,53]. This dynamic process can be imaged through FAF. None of the six weeks or six months old mice whatever their genotypes exhibited changes in FAF (data not shown) in keeping with the absence of photoreceptor/RPE disease.

**SD-OCT and histology.** We compared retinal morphology and thickness for ONL, INL and IPL+GCL+NFL obtained by histology and SD-OCT analysis in Lrit3<sup>+/+</sup>, Lrit3<sup>nob6/+</sup> and Lrit3<sup>nob6/nob6</sup> at six weeks and six months of age (Figure 6 and 7A). Examination of histological retinal cross sections showed normal nuclear and synaptic layers among the three genotypes (Figure 6). SD-OCT images exhibited no changes in laver thickness according to the quadrant (dorsal, ventral, temporal or nasal) in each animal. No statistical differences were observed between Lrit3<sup>+/+</sup> and Lrit3<sup>nob6/nob6</sup> mice in ONL thickness, suggesting normal photoreceptors in the retina of mutant mice (Figure 7B-C). ONL thickness in Lrit3nob6/+ mice was slightly reduced compared to Lrit3<sup>+/+</sup> and Lrit3<sup>nob6/nob6</sup> mice at 6 weeks (p<0.001) (Figure 7B). However, thickness of INL and IPL+GCL+NFL was statistically decreased in mutant mice compared to  $Lrit3^{+/+}$  and heterozygous (from p<0.001 to p = 0.024) (Figure 7B–C). Thickness of ONL and INL but not IPL+GCL+NFL was also slightly decreased at six months compared to six weeks but these changes were similar in the three genotypes (from p < 0.001 to p = 0.025) (Figure 7B–C).

#### Discussion

In this work, we have genetically characterized a commercially available *Lrit3* mouse lacking *Lrit3* and determined the impact of this knock-out model on visual function. Our results show that mice lacking *Lrit3* display similar abnormalities as patients with cCSNB due to *LRIT3* mutations and other mice with mutations in



**Figure 6. Retinal anatomy of**  $Lrit3^{nob6/nob6}$  **mice.** Retinal sections of representative  $Lrit3^{+/+}$ ,  $Lrit3^{nob6/+}$  and  $Lrit3^{nob6/nob6}$  littermates were compared by light microscopy at 6 weeks and 6 months of age. Scale bar, 40  $\mu$ m. doi:10.1371/journal.pone.0090342.g006



**Figure 7. SD-OCT retinal nuclear layers measurements.** (A) ONL, INL and IPL+GCL+NFL thickness were obtained by SD-OCT and compared using Kluskal-Wallis statistical test in representative  $Lrit3^{+/+}$  (black box),  $Lrit3^{nob6/+}$  (blue box) and  $Lrit3^{nob6/nob6}$  (red box) littermates. The star indicates a significant test (p<0.05). (B) At 6 weeks of age. (C) At 6 months of age. doi:10.1371/journal.pone.0090342.g007

genes implicated in the retinal ON-pathway, most strikingly lacking the scotopic ERG b-wave. Here we describe in detail this novel *nob* mouse, which we subsequently called *nob6*.

nob6 denotes a mouse mutant carrying the Lrit3 knock-out (ko) allele homozygously. In this ko model, exons 3 and 4 were deleted and replaced by a Bgeo/Puro cassette, with only 21 bp of exon 3 still remaining, which was confirmed at genomic and RNA levels. This construction contains a premature stop codon in the first 8 bp of the cassette (c.611\_2046delinsGGCCATAG), which is predicted to lead to a truncated 206 amino acid LRIT3 protein (p.Phe204Trpfs\*3). A functional antibody against the mouse LRIT3 protein needs to be developed to validate this prediction.

If the truncated LRIT3 protein was indeed formed, it would lack several domains, including the transmembrane domain and the PDZ-binding motif that we previously hypothesized to be crucial for LRIT3 function. Our theory would be that LRIT3, with its PDZ-binding motif, might be the missing transmembrane protein, which brings the TRPM1 channel to the cell surface by interacting with scaffolding proteins [21,23,43]. Subsequently, the channel opens during light stimulation, allowing the depolarization of ONbipolar cells, which results in the signal transmission and the ERG b-wave [15,25,32]. Without its transmembrane domain and its PDZ-binding motif, LRIT3 could not fulfill its function. TRPM1 would not localize at the dendritic tips of ON-bipolar cells and thus would be inactive. ON-bipolar cells would not depolarize upon light stimulus resulting in the absence of a b-wave. Visual signal transmission would be compromised, leading to night blindness. Interestingly, the *nob6* allele resembles the deletion (c.1538\_1539del leading to p.Ser513Cysfs\*59) and nonsense mutations (c.1318C>T leading to p.Arg440\* and c.1151C>G leading to p.Ser384\*) in *LRIT3* identified in cCSNB patients [21]. These three human mutations, located in exon 4, are predicted to lead to truncated proteins lacking the transmembrane domain and the PDZ-binding motif. Thus, the *nob6* mouse represents in fact a reliable model to study the pathological mechanism(s) associated with autosomal recessive complete CSNB due to *LRIT3* mutations.

Functional characterization of nob6 mouse revealed a stationary nob phenotype as found in patients with cCSNB due to LRIT3 mutations [21], in patients with cCSNB due to other gene defects of the same cascade [5-13] and in other mouse models of cCSNB [12,15,18,25,26,32,33,35,36,54]. In scotopic conditions, nob6 mice defined a selective absence of the ERG b-wave, with a preserved a-wave component. The preserved a-wave indicates that photoreceptors respond normally to light, and the lack of the b-wave localizes the defect to synaptic transmission from the photoreceptors to the ON bipolar cells or to signaling within them. Cone-mediated pathways are also affected since implicit times of both a- and b-waves were delayed and amplitude of b-wave markedly reduced in photopic conditions. Therefore, visual dysfunction in nob6 mice affects both rod- and cone- ON-bipolar systems. Optomotor tests revealed that, under scotopic conditions, the number of head movements was strongly decreased in nob6 mice even if the visual acuity is not really measurable [45]. However, we can suppose that, by increasing more the spatial frequency, the optomotor response in mutant mice would reach the zero before wild-type and heterozygous mice and therefore the visual acuity of nob6 mice would be decreased in scotopic conditions. Moreover, there were no indications for photoreceptor degeneration in this mouse model. Retina morphology investigated by fundus autofluorescence and histology was unsuspicious for all genotypes at the different ages. Similar findings have been reported for other mouse models of cCSNB [12,18,26,33,35,36]. However, all steps providing the retinal sections such as fixation, dehydration or cutting are potential sources for significant and variable alteration in dimensions, especially for subtle changes. Thus, an *in vivo* analysis of the retinal structure as SD-OCT may be helpful to detect fine morphological changes without being biased by handling procedures [55]. Moreover, in patients, histology is not possible because it is difficult to have access to human tissues and SD-OCT is used in practice. Indeed, interestingly Godara and co-workers showed that, despite a normal retinal structure using light and electronic microscopy in nob mouse mutants carrying mutations in genes coding for postsynaptic proteins, three patients with cCSNB and GRM6 mutations exhibited a reduced retinal thickness in the extrafoveal region upon SD-OCT examination [56]. This thinning was the result of inner retinal defects, as opposed to photoreceptor loss, and involves GCL. Similarly, as we observed in the nob6 mice, a normal ONL thickness was measured whereas the inner retinal layer thickness, comprising GCL plus IPL, was reduced. These results support the emerging view that although cCSNB may be considered primarily as a stationary disorder, small structural changes in the retina of patients may be detectable [56]. Thus, this study is the first one using SD-OCT technology to characterize a mouse model with cCSNB. Mutant mice showed no photoreceptor degeneration as measured by the ONL thickness. Interestingly, as Godara and co-workers, we found a small but significant thinning of the inner retina (INL and IPL+GCL+NFL) but it remains unknown if this thinning is functionally relevant. Therefore, it would be interesting to study other patients with cCSNB and the respective mouse models by applying SD-OCT to investigate if this inner retinal thinning is a common finding. We also observed a thinning of ONL and INL between the two time-points but we assumed that this is an ageing consequence because it was observed in the three genotypes and in the same proportions. A slight reduction in ONL thickness was surprisingly detected in heterozygous mice but that phenomenon remains unexplained. In general our data show that the wild-type and heterozygous mice are very similarly, which is what we expected for an autosomal recessive mode of inheritance.

To conclude, this study describes a novel mouse model for human cCSNB associated with *LRIT3* mutations. It creates the basis to clarify the role of LRIT3 in the ON-bipolar cell signaling cascade, presents a tool to confirm putative interactions with various components of this cascade and to elucidate the pathogenic mechanism(s) of cCSNB.

#### **Supporting Information**

Table S1 Primers used for amplification and sequencing of the flanking intronic and exonic sequences of *Lrit3* (sequence of reference was purchased by the company Taconic) Sequences 5'-3', size of PCR products and annealing temperatures are indicated.

(DOCX)

Table S2 Primers used for qPCR Taqman on Crb1 (NM\_133239.2) c.3481delC p.Arg1161Glyfs\*48 is present in rd8 mouse. Sequences 5'-3' are indicated. (DOCX)

**Table S3** Primers used for amplification and sequencing of *Pde6β* (NM\_008806.2) c.C1041>A p.Tyr347\* and Xmv-28 insertion in intron 1 in are present in *rd1* mouse. Sequences 5'-3', size of PCR products and annealing temperatures are indicated. (DOCX)

Table S4 Primers used for amplification and sequencing of *Gnat2* (NM\_008141.2) c.517G>A p.Asp173Asn is present in *cpfl3* mouse. Sequences 5'-3', size of PCR products and annealing temperatures are indicated. (DOCX)

**Table S5 Primers used for amplification and sequencing of** *Tyr* **(NM\_011661.4)** We tested the c.230G>T p.Arg77Leu mutation. Sequences 5'-3', size of PCR products and annealing temperatures are indicated. (DOCX)

**Table S6 Primers used for amplification and sequencing of the flanking intronic and exonic sequences of** *Grm*δ (NM\_173372.2) Sequences 5'-3', size of PCR products and annealing temperatures are indicated. (DOCX)

Table S7 Primers used for amplification and sequencing of the flanking intronic and exonic sequences of *Gpr179* (NM\_001081220.1) Sequences 5'-3', size of PCR products and annealing temperatures are indicated. (DOCX)

Table S8 Primers used for amplification and sequencing of the flanking intronic and exonic sequences of Nyx (AY114303.1) Sequences 5'-3', size of PCR products and annealing temperatures are indicated. (DOCX) Table S9 Primers used for amplification and sequencing of the flanking intronic and exonic sequences of *Trpm1* (NM\_001039104.2) Sequences 5'-3', size of PCR products and annealing temperatures are indicated. (DOCX)

Table S10 Benign *Lrit3* variants identified in founder mice of *Lrit3* line (sequence of reference was purchased by the company Taconic) het: variant found heterozygously; hom: variant found homozygously. (DOCX)

Table S11 Benign *Trpm1* (NM\_001039104.2) variants identified in founder mice of *Lrit3* line het: variant found heterozygously; hom: variant found homozygously. (DOCX)

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#### **Author Contributions**

Conceived and designed the experiments: CZ IA JAS. Performed the experiments: MN EO CM AA MEL CC KB. Analyzed the data: MN OP SES CZ IA. Wrote the paper: MN CZ.

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# C.LRIT3 is essential to localize TRPM1 to the dendritic tips of depolarizing bipolar cells and may play a role in cone synapse formation

The purpose of this study was to describe the localization of LRIT3 in the mouse retina and compare the localization of known components of the mGluR6 signaling cascade in wild-type and *Lrit3*<sup>nob6/nob6</sup> retinas to better understand the function of LRIT3.

An anti-LRIT3 antibody was generated. Immunofluorescent staining of LRIT3 in wild-type mice revealed a specific punctuate labeling in the outer plexiform layer (OPL), and this staining pattern was notably absent in *Lrit3<sup>nob6/nob6</sup>* mice. LRIT3 did not co-localize with ribeye or calbindin, but it did co-localize with mGluR6. TRPM1 staining was severely decreased at the dendritic tips of all depolarizing bipolar cells in *Lrit3<sup>nob6/nob6</sup>* mice. mGluR6, GPR179, RGS7, RGS11 and  $G_{\beta5}$  immunofluorescence was absent at the dendritic tips of cone ON-bipolar cells in *Lrit3<sup>nob6/nob6</sup>* mice, but it was present at the dendritic tips of rod bipolar cells. Furthermore, PNA labeling was severely reduced in the OPL in *Lrit3<sup>nob6/nob6</sup>* mice. This study confirmed the localization of LRIT3 at the dendritic tips of depolarizing bipolar cells in mouse retina and demonstrated the dependence of TRPM1 localization on the presence of LRIT3. In addition, the disrupted localization of several components of the ON-bipolar cell signaling cascade and PNA in *Lrit3<sup>nob6/nob6</sup>* mice suggests a possible additional function of LRIT3 in cone synapse formation. These results also suggest that the regulation of the mGluR6 signaling cascade may differ between rod and cone ON-bipolar cells.



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### LRIT3 is essential to localize TRPM1 to the dendritic tips of depolarizing bipolar cells and may play a role in cone synapse formation

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#### Abstract

Mutations in *LRIT3* lead to complete congenital stationary night blindness (cCSNB). The exact role of LRIT3 in ON-bipolar cell signaling cascade remains to be elucidated. Recently, we have characterized a novel mouse model lacking *Lrit3* [*no b-wave 6*, (*Lrit3<sup>nob6/nob6</sup>*]], which displays similar abnormalities to patients with cCSNB with *LRIT3* mutations. Here we compare the localization of components of the ON-bipolar cell signaling cascade in wild-type and *Lrit3<sup>nob6/nob6</sup>* retinal sections by immunofluorescence confocal microscopy. An anti-LRIT3 antibody was generated. Immunofluorescent staining of LRIT3 in wild-type mice revealed a specific punctate labeling in the outer plexiform layer (OPL), which was absent in *Lrit3<sup>nob6/nob6</sup>* mice. LRIT3 did not co-localize with ribeye or calbindin but co-localized with mGluR6. TRPM1 staining was severely decreased at the dendritic tips of all depolarizing bipolar cells in *Lrit3<sup>nob6/nob6</sup>* mice. mGluR6, GPR179, RGS7, RGS11 and Gβ5 immunofluorescence was absent at the dendritic tips of cone ON-bipolar cells in *Lrit3<sup>nob6/nob6</sup>* mice, while it was present at the dendritic tips of rod bipolar cells. Furthermore, peanut agglutinin (PNA) labeling was severely reduced in the OPL in *Lrit3<sup>nob6/nob6</sup>* mice. This study confirmed the localization of LRIT3 at the dendritic tips of depolarizing bipolar cells in mouse retina and demonstrated the dependence of TRPM1 localization on the presence of LRIT3. As tested components of the ON-bipolar cell signaling cascade and PNA revealed disrupted localization, an additional function of LRIT3 in cone synapse formation is suggested. These results point to a possibly different regulation of the mGluR6 signaling cascade between rod and cone ON-bipolar cells.

#### Introduction

The visual ON-pathway is initiated at the first retinal synapse between photoreceptors and ON-bipolar cells. In darkness, glutamate released by the photoreceptors binds to the metabotropic glutamate receptor 6 (GRM6/mGluR6) on the ON-bipolar cell dendrites (Nakajima *et al.*, 1993; Nomura *et al.*, 1994). This binding leads to the activation of the heterotrimeric G-protein,  $G_o$  (Nawy, 1999; Dhingra *et al.*, 2000), which results in the closure of the transient receptor potential melastatin 1 (TRPM1) cation channel (Morgans *et al.*, 2009; Shen *et al.*, 2009; Koike *et al.*, 2010). When the photoreceptor

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tors are stimulated by light, glutamate release is reduced, the cascade is deactivated and TRPM1 channels open, resulting in cell membrane depolarization.

Mutations in several genes disrupt synaptic transmission between photoreceptors and ON-bipolar cells, leading to complete congenital stationary night blindness (cCSNB) (Zeitz, 2007; Zeitz *et al.*, 2015). cCSNB has been associated with mutations in genes encoding proteins localized at the dendritic tips of ON-bipolar cells (Masu *et al.*, 1995; Morgans *et al.*, 2009; Orlandi *et al.*, 2012; Peachey *et al.*, 2012b; Orhan *et al.*, 2013), including nyctalopin, a leucine-rich repeat protein (Morgans *et al.*, 2006). Recently, we have identified mutations in *LRIT3*, a gene encoding the leucine-rich repeat, immunoglobulin-like and transmembrane domains 3 protein, leading to cCSNB (Zeitz *et al.*, 2013). The corresponding protein localizes at the dendrites of ON-bipolar cells in human (Zeitz *et al.*, 2013).

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The exact role of LRIT3 in the mGluR6 signaling cascade remains to be elucidated. It has been shown that nyctalopin binds to TRPM1 (Cao *et al.*, 2011; Pearring *et al.*, 2011) and is essential for its correct localization (Pearring *et al.*, 2011). Membrane transport of proteins such as TRPM1 involves cytoskeletal scaffolding proteins, many of which contain PDZ domains (Feng & Zhang, 2009). Nyctalopin, being mainly an extracellular protein, does not contain an intracellular PDZ-binding domain and is therefore probably not able to bring TRPM1 to the cell surface via scaffolding proteins (Pearring *et al.*, 2011). Interestingly, LRIT3 has a PDZ-binding motif and might fulfill this function (Zeitz *et al.*, 2013).

Mouse models for cCSNB have been helpful in dissecting the visual ON-pathway in bipolar cells (Cao *et al.*, 2011; Pearring *et al.*, 2011; Orlandi *et al.*, 2012, 2013). Seven mouse models with defects in four different genes have already been published (Masu *et al.*, 1995; Pardue *et al.*, 1998; Gregg *et al.*, 2003; Pinto *et al.*, 2007; Maddox *et al.*, 2008; Morgans *et al.*, 2009; Shen *et al.*, 2009; Koike *et al.*, 2010; Peachey *et al.*, 2012a,b). Recently, we have characterized a novel mouse model lacking *Lrit3* [*no b-wave 6* (*Lrit3<sup>nob6/nob6</sup>)*], which displays similar abnormalities to patients with cCSNB due to *LRIT3* mutations, with lacking or severely reduced b-wave amplitudes in the scotopic and photopic electroretinogram, respectively (Neuille *et al.*, 2014).

Here we describe the localization of LRIT3 in mouse retina and compare the localization of known components of the mGluR6 signaling cascade in wild-type and  $Lrit3^{nob6/nob6}$  retinas to better understand the function of LRIT3.

#### Materials and methods

#### Ethics statements

All animal procedures were performed according with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes and were approved by the French Minister of Agriculture (authorization A-75-1863 delivered on 9 November 2011).

#### Animal care

The generation and characterization of the *Lrit3* knock-out mouse has been described elsewhere (Neuille *et al.*, 2014) (http://www.ta conic.com/knockout-mouse/Irit3/tf2034). Ten wild-type (*Lrit3*<sup>+/+</sup>) and seven mutant (*Lrit3*<sup>nob6/nob6</sup>) 6- to 7-week-old male and female mice were used in this study. Mice were housed in a temperaturecontrolled room with a 12-h light/12-h dark cycle. Fresh water and rodent diet were available *ad libitum*.

#### LRIT3 antibody

To obtain an antibody to mouse LRIT3, the following procedure was performed by Eurogentec (Seraing, Belgium). Two peptides corresponding to amino acids 180–195 and 613–628 of mouse LRIT3 (NP\_001274153.1; peptide sequences: AVTPSRSPDFPPRR II and CTSKPFWEEDLSKETY, respectively) were synthesized and coupled to keyhole limpet haemocyanin (KLH). Two adult New Zealand White rabbits were immunized with both peptide–KLH conjugates. The rabbits received three more immunizations 7, 10 and 18 days later. Immune sera were collected 10 days after the final immunization and stored at -20 °C. We performed immunohistochemistry on *Lrit3*<sup>+/+</sup> and *Lrit3*<sup>nob6/nob6</sup> retinal sections with these two sera. One of them produced a specific punctate signal in the

outer plexiform layer of the  $Lrit3^{+/+}$  retinal sections that was absent on  $Lrit3^{nob6/nob6}$  sections. However, strong non-specific signal and noise were also present on the whole  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  sections (data not shown). To determine which of the two peptides resulted in the specific staining, we performed immunohistochemistry on  $Lrit3^{+/+}$  retinal sections after pre-incubation of the serum with either the N- or the C-terminal peptide. The specific signal was absent when the serum was pre-incubated with the peptide localized at the N-terminus but noise remained (data not shown). Subsequently, affinity purification was performed with this peptide to decrease noise and to obtain a more specific antibody.

#### Preparation of retinal sections for immunohistochemistry

Mice were killed by CO<sub>2</sub> administration and cervical dislocation. Eyes were removed and prepared following three methods. For method 1, we made two slits in a cross within the cornea and placed the eyeball in ice-cold 4% (w/v) paraformaldehyde in 0.12 M phosphate buffer, pH 7.2, for 1 h. After three 10-min washes with icecold phosphate buffered saline (PBS), we transferred the eyeball to ice-cold 30% sucrose. Finally, the lens was removed and the eyecup was embedded in OCT (Sakura Finetek, AJ Alphen aan den Rijn, the Netherlands) and frozen in a dry ice-cooled isopentane bath. For method 2, the anterior segment and lens were removed and the eyeсир was fixed in ice-cold 4% (w/v) paraformaldehyde in 0.12 м phosphate buffer, pH 7.2, for 20 min. The eyecup was washed three times in ice-cold PBS and cryoprotected with increasing concentrations of ice-cold sucrose in 0.12 M phosphate buffer, pH 7.2 (10%, 20% for 1 h each and 30% overnight). Finally, the eyecup was embedded in 7.5% gelatin-10% sucrose and frozen in a dry icecooled isopentane bath. For method 3, we made a hole just behind the ora serrata and placed the eyeball in 4% (w/v) paraformaldehyde in 0.12 M phosphate buffer, pH 7.2, for 5 min. We then removed the lens and the eyecup was again fixed for 20 min in paraformaldehyde at room temperature. The eyecup was washed three times in PBS and cryoprotected with increasing concentrations of ice-cold sucrose in 0.12 M phosphate buffer, pH 7.2 (10% for 1 h and 30% overnight). Finally, the eyecup was embedded in 7.5% gelatin-10% sucrose and frozen in a dry ice-cooled isopentane bath. Sections were cut at a thickness of 18 µm on a cryostat and mounted onto glass slides (Super-Frost; Thermo Fisher Scientific, Waltham, MA, USA). The slides were air dried and stored at -80 °C.

#### Immunostaining of retinal cryosections

Primary antibodies used for immunostaining and the respective tissue section preparations are listed in Table 1. The TRPM1 immunoreactive serum from a patient suffering from melanoma-associated retinopathy (MAR), the rat mGluR6 antiserum raised in sheep, the purified goat polyclonal antibody to mouse GB5 and the mouse RGS11 antibody raised in rabbit were used as previously described (Chen et al., 2003; Morgans et al., 2006, 2007; Xiong et al., 2013). Immunohistochemistry on retinal sections was performed following two protocols depending on the section preparation. For evecups embedded in OCT (method 1 under Preparation of retinal sections for immunohistochemistry), we used a previously published protocol (Morgans et al., 2006). Briefly, eyecup sections were blocked by incubation with antibody incubation solution (AIS) [3% (v/v) normal horse serum, 0.5% (v/v) Triton X-100, 0.025% (w/v) NaN3 in PBS] at room temperature for 60 min. Subsequently, the sections were incubated with primary antibodies in AIS for 1 h at room temperature. After washing in PBS, the sections were incubated with sec-

TABLE 1. Primary antibodies used in immunohistochemistry in this study

Antibody	Species	Dilution	Reference/source	Retinal section preparation
LRIT3	Rabbit	1:500	This paper	Method 2
TRPM1	Human	1:2000	Xiong et al. (2013)	Method 1
mGluR6	Sheep	1 : 100	Morgans et al. (2006)	Method 1
mGluR6	Guinea	1:200	AP20134SU-N Acris	Methods 2 and 3
GPR179	Mouse	1:200	Ab-887-YOM Primm	Method 1
RGS11	Rabbit	1:4000	Chen et al. $(2003)$	Method 1
Gβ5	Goat	1:100	Morgans et al. (2007)	Method 1
RGS7	Mouse	1:1000	sc-271643	Method 2
Lectin PNA 488 conjugate	Arachis hypogaea	1 : 1000	L21409 Life	Method 2
Lectin PNA 594 conjugate	Arachis hypogaea	1 : 1000	L32459 Life Technologies	Method 2
Cone arrestin	Rabbit	1:2000	ab15282 Abcam	Method 2
РКСа	Mouse	1:1000	P5704 Sigma-Aldrich	Method 2
$G_o lpha$	Mouse	1:200	MAB3073 Merck Millipore	Method 3
Calbindin	Mouse	1:5000	300 Swant	Method 2
ribeye	Mouse	1 : 10 000	612044 BD Biosciences	Method 2

ondary antibodies coupled to Alexa Fluor 488 or Alexa Fluor 594 (Life Technologies, Grand Island, NY, USA) at a dilution of 1:1000 in PBS for 1 h at room temperature. The slides were stained with 4',6-diamidino-2-phenylindole (DAPI) and subsequently cover-slipped with mounting medium (Aqua-Mount mounting medium; Thermo Fisher Scientific). For eyecups embedded in gelatinsucrose (method 2 and 3 under Preparation of retinal sections for immunohistochemistry), sections were blocked by incubation at room temperature for 60 min in 10% (v/v) donkey serum, 0.3% (v/ v) Triton X-100 in PBS. Subsequently, the sections were incubated with primary antibodies in blocking solution overnight at room temperature. After washing in PBS, the sections were incubated with secondary antibodies coupled to Alexa Fluor 488 or Cy3 (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:1000 in PBS for 1.5 h at room temperature. The slides were stained with DAPI and subsequently cover-slipped with mounting medium (Mowiol, Merck Millipore, Billerica, MA, USA). None of the secondary antibodies used gave recordable signal when used without primary antibodies (data not shown).

#### Image acquisition and quantification

Fluorescent staining signals were captured with a confocal microscope (FV1000, Olympus, Hamburg, Germany) equipped with 405-, 488- and 559-nm lasers.

For localization studies as well as for quantification of LRIT3, confocal images were acquired with a  $40 \times$  objective compatible with oil (lens numerical aperture: 1.3) imaging pixels of 310 and 77 nm in width and height for zoom 1 and 4, respectively, and

using a 0.52-µm step size. For localization, each image corresponds to the projection of three optical sections. For figures, brightness and contrast were optimized (ImageJ, version 1.49; National Institutes of Health, Bethesda, MD, USA). The method for quantification of LRIT3 on four independent Lrit3<sup>+/+</sup> retinal sections was adapted from a previously published protocol (Ramakrishnan et al., 2015), using Imaris (Bitplane, Zurich, Switzerland) and is illustrated in Supporting Information Fig. S1. Optical sections centered on the outer plexiform layer (OPL) were stacked to obtain a three-dimensional reconstruction of the entire retinal section thickness at this level. An initial region of interest (ROI) was drawn around the OPL (Fig. S1A and S1B). We used peanut agglutinin (PNA) staining as a marker to define the ROI of cone ON-bipolar cell dendritic tips (Fig. S1C). The mean intensity per voxel of LRIT3 staining at the dendritic tips of cone ON-bipolar cells was obtained by quantifying LRIT3 staining enclosed within this region. The ROI corresponding to LRIT3 staining at the dendritic tips of rod bipolar cells was formed by drawing spheres of 2-µm radius around LRIT3-labeled puncta that are not associated with PNA (Fig. S1D). The mean intensity per voxel of LRIT3 staining at the dendritic tips of rod bipolar cells was then measured. Finally, these two intensities were compared in the same retinal section.

For quantification of PNA, confocal images were acquired with a  $20 \times$  objective compatible with oil (lens numerical aperture: 0.85) imaging pixels of 621 nm in width and height, and using a 1.55- $\mu$ m step size. We reconstructed three  $Lrit3^{+/+}$  and three  $Lrit3^{nob6/nob6}$ retinas by assembling multiple confocal images of the same retina. For each Lrit3<sup>+/+</sup> mouse, a Lrit3<sup>nob6/nob6</sup> mouse of the same breeding pair was chosen and their retinas were processed in parallel and in the same way. Quantification of PNA in the OPL and in inner and outer segments (IS/OS) was adapted from a previously published protocol (Xu et al., 2012) and was performed with ImageJ (version 1.49). The background value was defined as the mean intensity per pixel in an ROI comprising the inner nuclear layer (INL) in which no PNA staining has been described. This value was then subtracted from the measurements of OPL and IS/OS intensities to give corrected measurements. For quantification of PNA in the OPL, the corrected mean intensity per pixel was measured for a segmented line of 30 pixels thickness drawn along the OPL. To obtain the number of PNA-labeled synaptic clefts between cones and corresponding cone ON-bipolar cells per millimeter of OPL (density of particles), the line was straightened and a threshold was applied to isolate each labeled structure. The same threshold was applied for the wild-type and the mutant retinas of the same pair. We then counted the number of labeled structures with a size between 4 and 45  $\mu$ m<sup>2</sup> and divided this value by the length of the selected line. The corrected mean intensity per pixel and the density of particles were compared between wild-type and mutant mice in each pair. To quantify the PNA staining in the IS/OS compartment, the corrected mean intensity per pixel was measured in an ROI surrounding these compartments. The values obtained were compared between Lrit3+/+ and *Lrit3<sup>nob6/nob6</sup>* mice of each pair.

#### Preparation of retinal lysates and Western blotting

Retinas were prepared according to a previously published protocol (Cao *et al.*, 2012). Mice were killed by  $CO_2$  administration and cervical dislocation. Whole retinas were removed from three  $Lrit3^{+/+}$  and three  $Lrit3^{nob6/nob6}$  mice and lysed by sonication in ice-cold PBS supplemented with 150 mm NaCl, 1% Triton X-100, and protease inhibitor (complete protease inhibitor tablets; Roche, Meylan, France). Supernatants were collected after a 15-min centri-

fugation step at 21 000 g at 4 °C and total protein concentration was measured by using a bicinchoninic acid protein assay kit (BCA Protein Assay Kit, Thermo Fisher Scientific). Sodium dodecylsulfate sample buffer (pH 6.8) containing 8 M urea was added to the supernatants and 8.5 µg of protein was subjected to 4-15% sodium dodecylsulfate polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were transferred onto nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked for 1 h at room temperature in 5% dry milk diluted in PBS and 0.1% Tween 20 and subjected to primary antibodies in 1% dry milk diluted in PBS and 0.1% Tween 20 overnight at 4 °C. After washing in PBS plus 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno-Research) in 1% dry milk diluted in PBS and 0.1% Tween 20, and subsequently bands were detected by using a chemiluminescent substrate (ECL2; Thermo Fisher Scientific). Primary antibodies were mouse anti-TRPM1 antibody raised in sheep diluted at 1:1000 (Cao et al., 2011) and anti-\beta-actin antibody raised in mouse diluted at 1: 10000 (MAB1501; Merck Millipore, Darmstadt, Germany). Band intensities were quantified by using ImageJ (version 1.49). For each lane, TRPM1 staining intensity was normalized to  $\beta$ -actin staining intensity and compared between three Lrit3<sup>+/+</sup> and three Lrit3<sup>nob6/nob6</sup> mice. The experiment was repeated seven times.

#### Statistical analyses

Statistical analyses were performed using SPSS Statistics (version 19.0, IBM, Armonk, NY, USA). A Student's *t*-test was used to compare TRPM1 protein amounts between  $Lrit3^{+/+}$  and  $Lrit3^{nob6/}$  mice, the intensity of LRIT3 staining at the dendritic tips of rod bipolar cells vs. cone ON-bipolar cells in  $Lrit3^{+/+}$  mouse, and PNA staining in the OPL and in the IS/OS complex between  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  mice. Tests were considered significant at P < 0.05.

#### Results

#### Validation of the LRIT3 antibody

Immunofluorescent staining of LRIT3 on *Lrit3*<sup>+/+</sup> mouse retinal sections revealed punctate labeling in the OPL (Fig. 1A, two pictures on the left and Fig. 1B left). On *Lrit3*<sup>nob6/nob6</sup> mouse retinal sections, this punctate labeling was absent, demonstrating the specificity of the LRIT3 labeling (Fig. 1A, two pictures on the right and Fig. 1B, right). Some non-specific signal remained in the inner plexiform and the ganglion cell layers (IPL and GCL, respectively) in both *Lrit3*<sup>+/+</sup> and *Lrit3*<sup>nob6/nob6</sup> mice (Fig. 1A, LRIT3 staining alone). The specific staining in the OPL probably represents the dendritic tips of rod bipolar cells (arrows, Fig. 1B, left).

## LRIT3 localizes at the dendritic tips of depolarizing bipolar cells in the mouse retina

To precisely localize LRIT3 in mouse retina, and particularly in the OPL, we performed co-immunolocalization studies with the anti-LRIT3 antibody and antibodies against ribeye, a component of the photoreceptor synaptic ribbon (Schmitz *et al.*, 2000; tom Dieck *et al.*, 2005), calbindin, which labels the post-synaptic processes of horizontal cells (Haverkamp & Wassle, 2000), protein kinase C $\alpha$ (PKC $\alpha$ ) to label rod bipolar cells (Negishi *et al.*, 1988; Greferath et al., 1990) and mGluR6 to label dendritic tips of ON-bipolar cells (Vardi et al., 2002; Morgans et al., 2007; Cao et al., 2008).

The LRIT3 puncta did not co-localize with ribeye but were nested within the arcs of the synaptic ribbons (Fig. 2A). LRIT3 staining was also adjacent to calbindin staining but without co-localization (Fig. 2B). Thus, LRIT3 was present in the OPL but did not localize presynaptically and was absent in the horizontal cells. PKC $\alpha$  was present in both the dendritic tips and the cell bodies of rod bipolar cells, whereas LRIT3 staining was solely concentrated in the OPL (Fig. 2C). Strong co-localization of LRIT3 and mGluR6 confirmed that LRIT3 is present at the dendritic tips of rod and cone ON-bipolar cells (Fig. 2D).

#### TRPM1 localization is disrupted at the dendritic tips of ONbipolar cells in Lrit3<sup>nob6/nob6</sup> retina

To test our hypothesis that LRIT3 is required for the correct localization of TRPM1, we performed immunohistochemistry on Lrit3+/+ and Lrit3<sup>nob6/nob6</sup> mouse retinal sections with serum from a MAR patient that was previously demonstrated to show specific TRPM1 immunoreactivity on mouse retinal sections (Xiong et al., 2013). On  $Lrit3^{+/+}$  retinal sections, the serum revealed punctate labeling in the OPL (Fig. 3A and C, left) that co-localized with mGluR6 staining (Fig. 3B and C, left), confirming the localization of TRPM1 at the dendritic tips of rod bipolar cells (arrows, Fig. 3B and C, left) and cone ON-bipolar cells (arrowheads, Fig. 3B and C, left). We also observed immunofluorescent labeling of bipolar cell bodies (asterisks) in the INL (Fig. 3A and C, left). In Lrit3<sup>nob6/nob6</sup> mice, the intensity of the TRPM1 puncta co-localizing with mGluR6 was dramatically decreased, but somatic staining (asterisks) seemed unchanged (Fig. 3A and C, right), suggesting that TRPM1 localization at the dendritic tips of ON-bipolar cells is dependent on LRIT3.

To investigate whether the decrease in TRPM1 localization at the dendritic tips of depolarizing bipolar cells is also reflected by a reduction of the total amount of TRPM1 protein, we compared TRPM1 protein levels in  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  retinas by Western blot analysis. TRPM1 protein was still present in the retina of  $Lrit3^{nob6/nob6}$  mice and the amount of protein was not significantly altered (Fig. 3D and E). These findings suggest that elimination of LRIT3 results in a severe reduction of TRPM1 at the dendritic tips of all ON-bipolar cells but does not significantly affect the total amount of TRPM1 protein.

## Other components of the ON-bipolar signaling cascade are mislocalized in Lrit3<sup>nob6/nob6</sup> retina

Interestingly, mGluR6 staining in Lrit3nob6/nob6 retina showed an apparently normal punctate labeling of the dendritic tips of rod bipolar cells (arrows, Fig. 3B and C), but immunofluorescence seemed nearly absent at the dendritic tips of cone ON-bipolar cells (arrowheads, Fig. 3B and C, left vs. right) with some mGluR6 immunofluorescence appearing instead over bipolar cell bodies in the INL. This dramatic decrease of staining at the dendritic tips of Lrit3<sup>nob6/nob6</sup> cone ON-bipolar cells was also observed for GPR179, RGS7, RGS11 and G<sub>β</sub>5 (arrowheads, Fig. 4), which are also components of the mGluR6 signaling cascade and normally localize at the dendritic tips of both rod and cone ON-bipolar cells (Morgans et al., 2007; Rao et al., 2007; Orlandi et al., 2012; Peachey et al., 2012b; Orhan et al., 2013). Co-immunostaining of mGluR6 with  $G_o \alpha$  (Fig. 5A) and with cone arrestin (Fig. 5B) confirmed these findings. While  $G_0 \alpha$  is a marker of the somas and dendrites of both rod and cone ON-bipolar cells (Vardi, 1998), cone arrestin labels cone photoreceptors including the cone pedicles (Sakuma et al.,

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FIG. 1. Validation of mouse LRIT3 antibody. (A) Representative confocal images of cross-sections of  $Lrit3^{h/+}$  and  $Lrit3^{nob6/nob6}$  retina stained with mouse LRIT3 antibody (green) with or without DAPI (gray). Scale bar, 50 µm. (B) 4 × zoom of A focused on outer plexiform layer (OPL). Arrows represent putative dendritic tips of rod bipolar cells, and arrowheads represent putative dendritic tips of cone ON-bipolar cells. Scale bar, 10 µm.



FIG. 2. Localization of LRIT3 in mouse retina. Representative confocal images of cross-sections centered on the OPL of  $Lrit3^{+/+}$  retina stained with antibodies against LRIT3 (green) and (A) ribeye (red), (B) calbindin (red), (C) PKC $\alpha$  (red) or (D) mGluR6 (red) and merge (yellow). Scale bar, 10  $\mu$ m.

1996), which are contacted by the dendritic tips of cone ON-bipolar cells. On *Lrit3<sup>+/+</sup>* retinal sections co-stained with  $G_o \alpha$ , mGluR6 was present as small puncta at the dendritic tips of rod bipolar cells (arrows, Fig. 5A, left) and as larger patches, where each patch represents the dendritic tips of multiple cone ON-bipolar cells contacting a single cone pedicle (arrowheads, Fig. 5A, left). On *Lrit3<sup>+/+</sup>* retinal sections co-stained with cone arrestin, mGluR6 was again normally present as small puncta at rod bipolar cell dendrites (arrows, Fig. 5B, left) and as larger patches at the dendritic tips of cone ON-bipolar cells (arrowheads, Fig. 5B, left). Interestingly, on *Lrit3<sup>nob6/nob6</sup>* sections, in the presence of unaltered  $G_o \alpha$  staining, cone-associated mGluR6 staining was absent whereas rod-associated mGluR6 puncta were still present (arrows, Fig. 5A, right). The cone

arrestin immunofluorescence showed that cone pedicles are present in the *Lrit3<sup>nob6/nob6</sup>* retina, but that mGluR6 in the close vicinity of cone arrestin was absent (Fig. 5B, right).

#### PNA staining is disrupted in the OPL in Lrit3<sup>nob6/nob6</sup> retina

We performed immunostaining on wild-type and mutant retinas with PNA, which binds specific glycosylated residues localized extracellularly to cone IS and OS, respectively, and at the synaptic cleft between cone pedicles and cone ON-bipolar cells located in the OPL (Blanks & Johnson, 1983, 1984; Wu, 1984) and was shown to overlap with GPR179 (Ray *et al.*, 2014). In wild-type mice, PNA staining overlapped with mGluR6 staining at the dendritic tips of



FIG. 3. Localization of TRPM1 and mGluR6 as well as TRPM1 protein quantification in  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  mice. Representative confocal images of cross-sections centered on OPL of  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  retina stained with antibodies against (A) TRPM1 (red) and (B) mGluR6 (green) and (C) merge (yellow). Arrows represent putative dendritic tips of rod bipolar cells, arrowheads represent putative dendritic tips of cone ON-bipolar cells and asterisks represent putative somas of ON-bipolar cells. Scale bar, 10 µm. (D) Western blots of three  $Lrit3^{+/+}$  and three  $Lrit3^{nob6/nob6}$  total retinal lysates probed with antibodies to TRPM1 (top) and  $\beta$ -actin (bottom). Bars on the left side of blots represent molecular weight in kDa. Arrows represent bands of interest. (E) Quantification of TRPM1 Western blotting data in D. For each lane, TRPM1 staining intensity was normalized to  $\beta$ -actin staining intensity. Values were plotted as the percentage of the level in  $Lrit3^{+/+}$ . Error bars represent standard deviations.



FIG. 4. Localization of GPR179, RGS11, RGS7 and G $\beta$ 5 in *Lrit3<sup>+/+</sup>* and *Lrit3<sup>nob6/nob6</sup>* retina. Representative confocal images of cross-sections centered on OPL of *Lrit3<sup>+/+</sup>* and *Lrit3<sup>nob6/nob6</sup>* retina stained with antibodies against (A) GPR179 (green), (B) RGS11 (red), (C) RGS7 (red) or (D) G $\beta$ 5 (green). Arrowheads represent putative dendritic tips of cone ON-bipolar cells. Scale bar, 10 µm.

cone ON-bipolar cells in the OPL (arrowheads, Fig. 6A–C, left). In contrast, PNA staining was dramatically decreased in the OPL on *Lrit3<sup>nob6/nob6</sup>* retinal sections (Fig. 6A–C, right). On low-power views of retinal sections, this decrease is obvious in the OPL with no apparent difference of staining intensity in the IS and OS of the cones (Fig. 6D). Quantitatively, PNA fluorescence intensity was significantly decreased (64%, P = 0.041) in the whole OPL of *Lrit3<sup>nob6/nob6</sup>* retinas compared with wild-type mice (Fig. 6E). More strikingly, the number of synaptic clefts stained with PNA between cone pedicles and corresponding cone ON-bipolar cells, per millimeter of OPL, was dramatically decreased with only 1% remaining (P = 0.008) in *Lrit3<sup>nob6/nob6</sup>* retinas compared with wild-type (Fig. 6F). In contrast, a measured 23% decrease of the PNA staining at the IS/OS complex in *Lrit3<sup>nob6/nob6</sup>* retinas compared with wild-type was not statistically significant (P = 0.515) (Fig. 6E).

### LRIT3 staining is similar at the dendritic tips of rod and cone ON-bipolar cells

As localization of several components of the cone to cone ON-bipolar cell synapse seemed to be disrupted in *Lrit3<sup>nob6/nob6</sup>* retina whereas their localization at the rod to rod bipolar cell synapse seemed to be unaffected, and because LRIT3 staining at the dendritic tips of cone ON-bipolar cells seemed to be more intense than that at the dendritic tips of rod bipolar cells, we quantified LRIT3 staining at the dendritic tips of rod and cone ON-bipolar cells in four *Lrit3<sup>+/+</sup>* mice. LRIT3 staining was 7% less intense at the dendritic tips of rod bipolar cells compared with the dendritic tips of cone ON-bipolar cells but this difference was not significant (*P* = 0.504).

#### Discussion

In this work, we have produced a specific antibody against mouse LRIT3, localized the corresponding protein in the mouse retina

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FIG. 5. Localization of mGluR6,  $G_0 \alpha$  and cone arrestin in  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  retina. Representative confocal images of cross-sections centered on OPL of  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  retina stained with antibodies against mGluR6 (red) and (A)  $G_0 \alpha$  (green) and merge (yellow) or (B) cone arrestin (green) and merge (yellow). Arrows represent putative dendritic tips of rod bipolar cells, and arrowheads represent putative dendritic tips of cone ON-bipolar cells. Scale bar, 10 µm.



FIG. 6. Localization and quantification of PNA in  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  retina. Representative confocal images of cross-sections centered on OPL of  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  retina stained with antibodies against (A) PNA (green) and (B) mGluR6 (red) and (C) merge (yellow). Arrowheads represent putative dendritic tips of cone ON-bipolar cells. Scale bar, 10 µm. (D) Representative confocal images of cross-sections of  $Lrit3^{+/+}$  and  $Lrit3^{nob6/nob6}$  retina stained with PNA. Scale bar, 50 µm. (E) Quantification of the intensity of PNA staining in the OPL and in the IS/OS complex in three independent  $Lrit3^{+/+}/Lrit3^{nob6/nob6}$  pairs of retina. Error bars represent standard deviations. Asterisks represent results that are significantly different (P < 0.05). (F) Quantification of the number of PNA-stained synaptic clefts between cone pedicles and corresponding cone ON-bipolar cells per millimeter of OPL in three independent  $Lrit3^{+/+}/Lrit3^{nob6/nob6}$  pairs of retina. Error bars represent standard deviations. Asterisks represent results that are significantly different (P < 0.05). (F) Quantification of the number of PNA-stained synaptic clefts between cone pedicles and corresponding cone ON-bipolar cells per millimeter of OPL in three independent  $Lrit3^{+/+}/Lrit3^{nob6/nob6}$  pairs of retina. Error bars represent standard deviations. Asterisks represent results that are significantly different (P < 0.05).

and studied how the localization of most of the known components of the ON-bipolar signaling cascade are affected in a mouse model of cCSNB lacking *Lrit3* (*Lrit3<sup>nob6/nob6</sup>*). Our results show that LRIT3 localizes at the dendritic tips of both rod and cone ON-bipolar cells, that the correct localization of TRPM1 is dependent on LRIT3 and that the localization of other partners is disrupted in *Lrit3<sup>nob6/nob6</sup>* retina particularly at the cone to cone ON-bipolar cell synapse.

### LRIT3 localizes at the dendritic tips of ON-bipolar cells in mouse retina

The punctate labeling observed in the OPL of wild-type retina with the mouse LRIT3 antibody was absent on  $Lrit3^{nob6/nob6}$  retina, demonstrating the specificity of this antibody and validating the knockout mouse model. This labeling resembles those obtained on human retina with an LRIT3 antibody (Zeitz *et al.*, 2013) as well as those obtained with antibodies directed against various components of the ON-bipolar signaling cascade on mouse retina such as mGluR6, GPR179, NYX, TRPM1, RGS7, RGS11, G $\beta$ 5 and R9AP (Masu *et al.*, 1995; Bahadori *et al.*, 2006; Morgans *et al.*, 2006, 2007, 2009; Rao *et al.*, 2007; Cao *et al.*, 2008, 2009; Jeffrey *et al.*, 2010; Orlandi *et al.*, 2012; Peachey *et al.*, 2012b; Orhan *et al.*, 2013). Colocalization studies with either ribeye or calbindin (Haverkamp & Wassle, 2000; Schmitz *et al.*, 2000; tom Dieck *et al.*, 2005) confirmed the absence of LRIT3 presynaptically in photoreceptors and in horizontal cells. In contrast, LRIT3 localizes at the dentritic tips of both rod and cone ON-bipolar cells where it co-localizes with mGluR6.

### TRPM1 localization at the dendritic tips of ON-bipolar cell is dependent on LRIT3

The cation channel TRPM1 is the endpoint of the ON-bipolar cell signaling cascade at the dendritic tips of ON-bipolar cells. TRPM1 opening during light stimulation results in the depolarization of ONbipolar cells and the formation of the ERG b-wave (Morgans et al., 2009; Shen et al., 2009; Koike et al., 2010). In some mouse models of cCSNB, TRPM1 staining is severely reduced or absent at the dendritic tips of depolarizing bipolar cells. This is the case in the Trpm1 knock-out mouse itself (Morgans et al., 2009; Koike et al., 2010), but also in three different Grm6 mutants: the Grm6 knockout model (Masu et al., 1995; Xu et al., 2012), the nob3 mouse [Grm6 splice mutation (Maddox et al., 2008; Cao et al., 2011)] and the nob4 mouse [Grm6 missense mutation (Pinto et al., 2007; Cao et al., 2011)]. Similarly, the TRPM1 staining at the dendritic tips was also absent in the nob mouse [Nyx deletion (Pardue et al., 1998; Gregg et al., 2003; Pearring et al., 2011)]. These results suggest that mGluR6 and nyctalopin, encoded by Nyx, are required for the correct localization of TRPM1. In contrast, other mouse models deficient for proteins participating in the same signaling cascade  $[Rgs7^{-/-}/Rgs11^{-/-}, nob5 \text{ (deficient for GPR179)] do not show such$ a mislocalization of TRPM1 (Orlandi et al., 2012; Ray et al., 2014). Therefore, these proteins probably play a role in regulating G-protein signaling, rather than a role in trafficking of TRPM1. Although it has been previously shown that nyctalopin is essential for the correct localization of TRPM1 at the dendritic tips of ONbipolar cells, it was suggested that nyctalopin alone is probably not able to bring the cation channel to the cell surface as it is mainly an extracellular protein (Pearring et al., 2011). We hypothesized that LRIT3 with its intracellular PDZ-binding domain might interact with scaffolding proteins to traffic TRPM1 to its correct localization and, together with nyctalopin, LRIT3 maintains TRPM1 at this localization (Zeitz et al., 2013; Neuille et al., 2014). Indeed, here we show that TRPM1 localization at the dendritic tips of both rod and cone ON-bipolar cells is dramatically decreased in Lrit3<sup>nob6/nob6</sup> mice, while the total quantity of TRPM1 was not significantly altered, indicating that localization but not the protein amount of TRPM1 is dependent on LRIT3. Previous studies have also investigated the localization and protein amount of TRPM1 in mouse models for cCSNB. Quantitative analysis of total intensity of TRPM1 in all retina layers in the knock-out model for mGluR6 was slightly lower compared with the wild-type mouse (15% reduction by immunostaining and 23% reduction by Western blot) (Xu et al., 2012). However the authors concluded that the main effect of eliminating mGluR6 is the severe reduction of TRPM1 localization at the dendritic tips of ON-bipolar cells (30% reduction in intensity and 60% lower number of puncta in the OPL) (Xu et al., 2012). However, this decrease of the total protein amount was not found in another study using the same mouse model (Ray et al., 2014). In the two other mouse models with mGluR6 mutations, nob3 and nob4, in addition to the lack of TRPM1 staining at the dendritic tips of ONbipolar cells, a moderate 30% reduction of TRPM1 levels in both mouse lines was described by Western blot analysis (Cao et al., 2011). In contrast, the absence of TRPM1 at the dendritic tips of ON-bipolar cells in nyctalopin mutant (nob) mice was accompanied with a severe reduction in total TRPM1 protein quantified by Western blot analysis (Pearring et al., 2011). The observation that the total amount of TRPM1 protein seems unchanged in the Lrit3<sup>nob6/nob6</sup> mouse compared with the nob mouse suggests that LRIT3 is necessary for correct trafficking of TRPM1, but that nyctalopin is necessary for both trafficking and either synthesis or stability of TRPM1. It has been shown that nyctalopin, mGluR6, GPR179 and TRPM1 form a macromolecular complex (Cao et al., 2011; Pearring et al., 2011; Orlandi et al., 2013; Ray et al., 2014). It is not clear if LRIT3 directly interacts with TRPM1 or via another component of the complex. Motif analysis suggests that the predicted intracellular PDZ-binding domain present in LRIT3 interacts with an as yet unknown scaffolding protein to bring TRPM1 to the surface (Zeitz et al., 2013).

#### LRIT3 may play a role in cone synapse formation

Surprisingly, we found that most of the known components of the ON-bipolar cell signaling cascade including mGluR6, GPR179, RGS7, RGS11 and GB5 are nearly absent at the dendritic tips of cone ON-bipolar cells in Lrit3<sup>nob6/nob6</sup> mice, whereas their staining at the dendritic tips of rod bipolar cells appears normal. In the Lrit3<sup>nob6/nob6</sup> mouse, some punctate mGluR6 labeling was present in the INL, possibly indicating a defect in trafficking mGluR6 in cone ON-bipolar cells. We also revealed a dramatic decrease of PNA staining at the synaptic clefts between cone pedicles and corresponding cone ON-bipolar cells in the Lrit3<sup>nob6/nob6</sup> mouse, whereas cone pedicles are still present. This decrease was qualitatively evident and was well reflected in the quantification of the number of PNAstained synaptic clefts per millimeter of OPL, while PNA staining was unchanged in IS and OS of cones. Together, these results suggest that LRIT3 may have an additional function in cone synapse formation. To our knowledge, this is the first report of the absence of PNA in the OPL in a mouse model of cCSNB. Indeed, PNA staining in the OPL of Grm6 knock-out, nob (Nyx), Trpm1 knockout and nob5 (Gpr179) retinal sections is not different from wild-type (Ray et al., 2014). However, alterations in ribbon synapse formation or bipolar dendrite invagination have been described previously in Grm6 mutants and GB5 knock-out mice (Rao et al., 2007; Cao et al., 2009; Ishii et al., 2009; Tsukamoto & Omi, 2014). Electron microscopy studies are needed to confirm if there is indeed an ultrastructural defect in Lrit3<sup>nob6/nob6</sup> retina, which might also explain the slight but significant thinning of inner retinal layers in the Lrit3<sup>nob6/nob6</sup> mouse that we previously highlighted (Neuille et al., 2014). We quantified LRIT3 staining at the dendritic tips of rod vs. cone ON-bipolar cells and showed that the staining is similar at the dendritic tips of both rod and cone ON-bipolar cells. These findings are in accordance with our previous LRIT3 staining on human retina, which revealed equal intensities at the dendritic tips of both rod and cone ON-bipolar cells (Zeitz et al., 2013; Fig. 2). Thus, the additional function of LRIT3 in cone ON-bipolar cells does not seem to be correlated with a difference in the amount of LRIT3 protein in these cells compared with rod bipolar cells. Rather, the mGluR6 signaling cascade may be differently regulated in rod and cone ON-bipolar cells.

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To conclude, this study reveals that several of the known components of the ON-bipolar cell signaling cascade display a disrupted localization in the *Lrit3<sup>nob6/nob6</sup>* mouse, a mouse model of cCSNB lacking *Lrit3*. In particular, TRPM1 is reduced at the dendritic tips of ON-bipolar cells, explaining the *no b-wave* phenotype and supporting our hypothesis that LRIT3 might bring TRPM1 to the cell surface. Moreover, LRIT3 may have an additional function in synapse formation as most of the known components of the cascade and PNA are absent at the cone to cone ON-bipolar cell synapse. Putative interactions between LRIT3 and partners of the cascade highlighted here have now to be confirmed to clarify the exact role of LRIT3 in the mGluR6 signaling cascade and to elucidate the pathogenic mechanism(s) of cCSNB.

#### Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. Method for the quantification of LRIT3 immunolabeling in the mouse retina OPL.

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#### Abbreviations

AIS, antibody incubation solution; BCA, bicinchoninic acid; cCSNB, complete form of congenital stationary night blindness; DAPI, 4',6-diamidino-2-phenylindole;  $G_{o}$ , heterotrimeric G-protein;  $G\beta 5/G\beta 5$ , G-protein beta 5; GCL, ganglion cell layer; Gpr179/GPR179, G-protein receptor 179; Grm6/GRM6/mGluR6, metabotropic glutamate receptor 6; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segments; KLH, keyhole limpet haemocyanin; Lrit3/LRIT3/LRIT3, leucine-rich repeat, immunoglobulin-like and transmembrane domains 3 protein; MAR, melanoma-associated retinopathy; *nob, no b-wave; Nyx*, nyctalopin; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; PBS, phosphate buffer saline; PDZ: PSD95, DLG1 and ZO1; PKC $\alpha$ , protein kinase C alpha; PNA, peanut agglutinin; R9AP, RGS9 anchor protein; Rgs7/RGS7, regulator of G-protein signaling 7; Rgs11/RGS11, regulator of G-protein signaling 11; ROI, region of interest; Trpm1/TRPM1, transient receptor potential melastatin 1 cation channel.

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## D.Ultrastructure of the rod and cone synapses in the *nob6* mouse model

#### 1. Rod synapses

Given that mice deficient in LRIT3 exhibit disrupted accumulation of synaptic proteins in the OPL, we evaluated the architecture of the synapses formed by rod and cone photoreceptors by transmission EM. These studies were performed in collaboration with Kirill Martemyanov (Department of Neuroscience, The Scripps Research Institute, Jupiter, FL, USA). Examination of the rod synapses revealed that retinas of *Lrit3<sup>nob6/nob6</sup>* mice had normally organized spherules formed by the axonal terminals of these neurons. They were regularly shaped and contained easily identifiable ribbons and mitochondria. Immediately adjacent to the ribbons we detected the lateral processes of horizontal cells and the deeply invaginating dendrites of rod BCs. The tips of the ON-BCs were positioned immediately below the ribbons and were properly aligned with respect to the orientation of horizontal cell processes (Figure 28A). On average 60% of wild-type rod-spherules from 520 rod synapses and 65% of knockout rod spherules from 436 rod synapses of *Lrit3<sup>nob6/nob6</sup>* mice contained the typical triad configuration containing invaginating ON-BC processes (Figure 28C). This finding demonstrates that the elimination of LRIT3 does not affect the structure of the rod-to-rod BC synapses.



Figure 28: Ultrastructure of rod and cone synapses in  $Lrit3^{nob6/nob6}$  mice. Representative electron microscopy images of rod (A) and cone (B) ribbon synapses a in  $Lrit3^{nob6/nob6}$  retina compared to a  $Lrit3^{+/+}$  retina. Rod spherules and cone pedicles are shown in yellow, invaginating dendrites of both rod and cone ON-BCs are shown in red, horizontal cell processes are shown in blue, and flat-contacting dendrites of cone OFF-BCs are marked with asterisks. Scale bar, 1  $\mu$ m. (C) Quantification of synaptic elements present in rod spherules. Synapses are classified as diads if they have identifiable ribbon with adjacent horizontal cell process. If spherules additionally have ON-BC process next to the synaptic ribbon, they are designated as triads. (D) Quantification of synaptic elements at the cone pedicles of  $Lrit3^{nob6/nob6}$  retinas compared to  $Lrit3^{+/+}$  retinas as a percentage of all counted synapses. In addition to diads and triads classified as described above, flat contacts at the base of the pedicle away from the ribbon were also distinguished and labeled as OFF type. Not otherwise classified contacts are labeled as NC. Error bars represent standard error of the mean (SEM) values.

#### 2. Cone synapses

Next, we assessed the morphology of the synapses formed by cone photoreceptors. As compared to rods, cone axons form substantially larger terminals that stratify in the lower sublamina of the OPL and contain multiple ribbons. Overall, this organization was preserved in  $Lrit3^{nob6/nob6}$  mice and well-formed cone pedicles were readily identified at the appropriate location (Figure 28B). However, vacuole-like structures were frequently observed in the knock-out terminals but not in the wild-type samples. The ribbons in  $Lrit3^{nob6/nob6}$  mice were normal both in number and morphology. Furthermore, these ribbons were properly positioned towards the edge of the terminal and contained adjacent horizontal cell processes. In contrast

to rod synapses, we observed a marked reduction in the number of invaginating contacts made by the cone ON-BCs. Quantification of 99-108 individual cone pedicles in wild-type and 82-123 pedicles in Lrit3<sup>nob6/nob6</sup> mice, respectively, confirmed this observation (Figure 28D). We found a dramatic reduction in the number of triads containing cone ON-BC processes in Lrit3<sup>nob6/nob6</sup> pedicles. While ~26% of cone terminals in wild-type retinas contained easily identifiable invaginating cone ON-BC processes immediately adjacent to the ribbons, only ~7% of  $Lrit3^{nob6/nob6}$  cone synapses had this organization. Consequently, the number of diads lacking cone ON-BC dendrites were increased in Lrit3<sup>nob6/nob6</sup> mice as compared to wild-type controls. We also observed a substantial increase in the number of flat contacts at the base of cone pedicles in these mice, typically observed for OFF-BCs. Because the identity of the BC types cannot be ascertained from only morphologic analysis by EM, these flat contacts may correspond to either OFF-BCs or cone ON-BC dendrites incompletely penetrating the cone pedicles and thus positioned farther away from the ribbons. However, despite these deficits we observed a number of normal cone ON-BC-like contacts with Lrit3<sup>nob6/nob6</sup> cone pedicles. To ensure preservation of some normal cone ribbon synapses we performed limited serial reconstruction. A representative panel of stacked sections through the same cone pedicle is presented in Figure 29 and provides evidence of a cone ON-BC dendrite entering deep into the cone terminal to position itself in immediate proximity to the ribbon (Figure 29). In summary, we detected substantial deficits in the organization of the cone synapses with quantitative loss of deeply invaginating contacts made by cone ON-BCs.



Figure 29: Serial reconstruction of a cone pedicle in a *Lrit3<sup>nob6/nob6</sup>* retina. Serial electron microscopy pictures of a cone pedicle in a *Lrit3<sup>nob6/nob6</sup>* retina. Cone pedicle is shown in yellow, invaginating dendrite of cone ON-BC is shown in red, and horizontal cell processes are shown in blue. Scale bar: 1 µm.

# **3. Localization of several ionotropic glutamate receptors and Pikachurin in the OPL**

In order to confirm the results obtained by EM, we studied the localization of some ionotropic glutamate receptors and Pikachurin in the OPL. We evaluated the localization of GluR1, an AMPA receptor present at the dendritic tips of type 3b and type 4 OFF-BCs, as well as GluR5, a kainate receptor present at the dendritic tips of type 3a, type 3b and type 4 OFF-BCs (61, 64). Pikachurin is a component of the dystrophin-glycoprotein complex (DGC), which localizes to the synaptic cleft of both rod and cone synapses (162, 163). PSD-95, a presynaptic marker for rod spherules and cone pedicles (164), was used to identify the position of photoreceptor axonal terminals. Both GluR1 and GluR5 antibodies showed a similar punctuate staining clustered in patches in the OPL regions of wild-type and

*Lrit3<sup>nob6/nob6</sup>* mice (Figure 30A and B). GluR1 staining was in close proximity with PSD-95 staining (Figure 30A) and both GluR5 and GluR1 positive dots were present at the same cone pedicles (Figure 30B). Thus, we conclude that targeting of ionotropic glutamate receptors to the dendritic tips of OFF-BCs was not affected by the loss of LRIT3. The pikachurin antibody showed a punctuate staining of two different types in the OPL of both wild-type and *Lrit3<sup>nob6/nob6</sup>* mice (Figure 30C): isolated dots were observed at the synaptic cleft of rod synapses and clustered dots were observed at the synaptic cleft of cone synapses (Figure 30C, arrowheads). Pikachurin staining was in direct contact to PSD-95 staining (Figure 30C). Thus, we conclude that the loss of LRIT3 has no effect on the localization of Pikachurin at the synaptic cleft of both rod and cone synapses.



Figure 30: Localization of ionotropic glutamate receptors GluR1 and GluR5 and Pikachurin in a *Lrit3<sup>nob6/nob6</sup>* retina. Representative confocal images of cross sections centered on the OPL of *Lrit3<sup>+/+</sup>* and *Lrit3<sup>nob6/nob6</sup>* retinas stained with antibodies against (A) GluR1 (red) and PSD-95 (green), (B) GluR1 (red) and GluR5 (green), and (C) Pikachurin (red) and PSD-95 (green). Arrowheads represent putative cone synapses. Scale bar, 10 µm.

# E. Functionality of ON- and OFF-pathways in the *nob6* mouse model

In light of our finding that LRIT3 plays a role in cone ribbon synapse formation (as confirmed by EM studies) and the observation that OFF-BCs make flat contacts at the cone pedicle, we hypothesized that loss of LRIT3 would influence the responses originating from OFF-BCs.

### 1. ON/OFF ERG

ON/OFF ERG studies were performed in collaboration with Stuart Coupland (Ottawa Hospital Research Institute, University of Ottawa, Ottawa ON, Canada). We tried to adapt an ERG protocol previously established in primate for mouse to separately measure cone driven ON- and OFF-responses at the level of BCs (161). We performed this study in the mouse model lacking *Lrit3* (*Lrit3<sup>nob6/nob6</sup>*) and in the mouse model lacking *Grm6* (*Grm6<sup>-/-</sup>*), a model of cCSNB that was previously shown to display intact OFF-responses but lacking ON-responses (83, 135, 136). Since the *Lrit3* mouse model is also a model for cCSNB, we hypothesized that *Lrit3* deficient mice should have similar responses to those of *Grm6* deficient mice.

Although we observed a high sweep to sweep variability in *Grm6* and *Lrit3* wild-type mice, in most cases we observed the same trend in the profile for both ON- and OFF-responses. Figure 31A and B show examples of ON- and OFF-response profiles, respectively, of a *Lrit3* wild-type mouse. In this mouse, the ramping rapid-ON-flicker stimulus resulted in a response that began with a relatively small negative component corresponding to the a-wave followed by a larger positive component corresponding to the b-wave (Figure 31A). The ramping rapid-OFF-flicker stimulus elicited a flicker response dominated by a positive component corresponding to the d-wave (Figure 31B). Interestingly, we observed a higher variability among the sweeps for the *Grm6*<sup>-/-</sup> mice than for wild-type animals, which was even higher for the *Lrit3*<sup>nob6/nob6</sup> mice. Because of this variability, we were not able to observe a trend in the ON- and OFF-response profiles in mutant mice (data not shown). Thus, we cannot make a conclusion based on these preliminary results



Figure 31: ON/OFF ERG in a *Lrit3*<sup>+/+</sup> mouse. Example of ERG recordings obtained in a wild-type mouse after ramping rapid-ON-flicker stimuli (A) and after ramping rapid-OFF-flicker stimuli (B). The left part corresponds to the right eye and the right part to the left eye. The scale indicates 50 ms and 20  $\mu$ V.

#### **2. MEA**

We subsequently evaluated the function of cone-driven ON- and OFF-pathways at the level of RGCs by MEA recordings. MEA studies were performed in collaboration with Serge Picaud (Institut de la Vision, Paris, France). Before the light stimulus, RGCs displayed a spontaneous spiking activity for all wild-type and *Lrit3<sup>nob6/nob6</sup>* tested retinas. Just after the light onset, RGCs from wild-type retinas showed a remarkable increase in their firing frequency that rapidly reached a maximum, then progressively returned to baseline (Figure 32A and C), reflecting ON-responses. Immediately after light offset, a similar response profile was observed in wild-type mice (Figure 32A and C), reflecting OFF-responses. In contrast, the spiking activity in the *Lrit3<sup>nob6/nob6</sup>* retinas remained at the baseline level after light onset, suggesting a total absence of light-evoked ON-responses (Figure 32B and D). At light offset, the firing frequency of RGCs in *Lrit3<sup>nob6/nob6</sup>* retinas markedly increased, suggesting the presence of intact light-evoked OFF-responses, although more time was required to achieve the maximum frequency and to return to baseline, as compared to wild-type retinas (Figure 32B and D).

To confirm these observations, two parameters were estimated for both ON- and OFFresponses: the maximum firing frequency and the time at which this maximum frequency was reached after the light onset (for ON-responses) or after the light offset (for OFF-responses). We demonstrated that the firing frequency was approximately six-fold increased in wild-type retinas after light onset, whereas the firing frequency was unchanged *Lrit3<sup>nob6/nob6</sup>* retinas after light onset (Figure 32E). We did not plot the time at which the maximum firing frequency was reached for ON-responses because ON-responses were only present in wild-type retinas. The maximum firing frequency for OFF-responses did not markedly differ between wild-type and *Lrit3<sup>nob6/nob6</sup>* retinas and was approximately 2.5 times higher than the spontaneous baseline activity (Figure 32F). However, the time at which this maximum was reached was increased in  $Lrit3^{nob6/nob6}$  retinas compared to wild-type retinas, suggesting that OFF-responses were delayed in  $Lrit3^{nob6/nob6}$  retinas (Figure 32G). Together, these results suggest that loss of LRIT3 results in the abolition of ON-responses in RGCs, whereas OFF-responses are still present but probably delayed and more sustained as compared to responses in wild-type retinas.



Figure 32: ON- and OFF-responses of RGCs in  $Lrit3^{nob6/nob6}$  retinas. Representative activity recording from  $Lrit3^{+/+}$  (A) and  $Lrit3^{nob6/nob6}$  RGC (C). The grey box corresponds to the light stimulus. Horizontal scale bar, 500 ms; vertical scale bar, 50  $\mu$ V. Representative raster plot (top) and peristimulus time histogram (bottom) from  $Lrit3^{+/+}$  (C) and  $Lrit3^{nob6/nob6}$  RGC (D). The grey box corresponds to the light stimulus. Horizontal scale bar, 1 s; vertical scale bar, 50 events/bin. Maximum firing frequency of ON- (E) and OFF-responses (F) in  $Lrit3^{nob6/nob6}$  retinas compared to  $Lrit3^{+/+}$  retinas, normalized to the spontaneous activity level. Error bars represent standard deviation (SD) values. (G) Time after the light stimulus offset for which the maximum firing rate frequency is reached for OFF-responses in  $Lrit3^{nob6/nob6}$  retinas compared to  $Lrit3^{+/+}$  retinas. Bars represent mean values.

# F. Analysis of mouse and human LRIT3 through mass spectrometry

The purpose of this study was to identify LRIT3 binding partners in the retina. The first step to achieve this objective was to optimize conditions for immunoprecipitation of mouse and

human LRIT3 with anti-mouse and anti-human LRIT3 antibodies, respectively, and subsequently to demonstrate that both proteins are detectable by mass spectrometry. We used a cell culture model overexpressing human or mouse LRIT3 for our preliminary studies in order to obtain sufficient protein concentration for detection by mass spectrometry. In the future, we will adapt the protocol to utilize mouse or human retinal samples for the immunoprecipitation experiment, rather than cell culture models. For Western blotting, we only used one tenth of each eluate, keeping the rest of the sample for mass spectrometry analysis. These experiments were conducted in collaboration with Manuela Argentini (Institut de la Vision, Paris, France) and the structural and functional proteomic/mass spectrometry platform at Institut Jacques Monod (Paris).

#### 1. Mouse LRIT3 immunoprecipitation

Proteins extracts from non-transfected cells and cells transfected with the mouse *Lrit3*-myc construct were immunoprecipitated with anti-Myc or anti-mouse LRIT3 antibodies and detected by Western blotting using an anti-Myc antibody. In lysates, we revealed a band between 70 and 100 kDa in transfected cells that is not present in non-transfected cells. The same band is present in both immunoprecipitated extracts from transfected cells and corresponds to the expected molecular weight of the LRIT3-Myc fusion protein (Figure 33).



Figure 33: Immunoprecipitation of the murine LRIT3-Myc fusion protein from transfected cell lysates with anti-Myc or anti-LRIT3 antibodies. The immunoprecipitated proteins were detected by Western blotting using the anti-Myc antibody. Non-transfected cells served as a control. Numbers indicate molecular weights. IP, immunoprecipitation

#### 2. Human LRIT3 immunoprecipitation

Proteins extracts from non-transfected cells and cells transfected with the human *LRIT3*-myc construct were immunoprecipitated with anti-Myc or anti-human LRIT3 antibodies. Our previous results showed that the anti-human LRIT3 antibody is able to detect LRIT3 by Western blotting (165), so we use both anti-Myc and anti-human LRIT3 antibodies for Western blotting. In lysates, we revealed a band between 70 and 100 kDa in transfected cells that is not present in non-transfected cells. The same band is present in both

immunoprecipitated extracts from transfected cells and corresponds to the expected molecular weight of the LRIT3-Myc fusion protein (Figure 34).



Figure 34: Immunoprecipitation of the human LRIT3-Myc fusion protein from transfected cell lysates with anti-Myc or anti-LRIT3 antibodies. Immunoprecipitated proteins were detected by Western blotting using anti-Myc or anti-LRIT3 antibodies. Non-transfected cells served as a control. Numbers indicate molecular weights. IP, immunoprecipitation; WB, Western blotting

### 3. Silver staining

Myc- and LRIT3-immunoprecipitated protein extracts from non-transfected cells and cells transfected with the human or the mouse *LRIT3*-myc construct were silver stained after electrophoresis in a polyacrylamide gel. The gel segments corresponding to molecular weights between 70 and 130 kDa were cut (Figure 35) for each sample and samples obtained from transfected cells were subjected to mass spectrometry analysis.



Figure 35: Silver staining of Myc- and LRIT3-immunoprecipitated protein extracts from non-transfected cells (NT) and cells transfected with the human (H) or the mouse (M) *LRIT3*-Myc construct before (left) and after (right) cutting of bands. Numbers indicate molecular weights. IP, immunoprecipitation.

### 4. Mass spectrometry

Protein bands from transfected cells were analyzed by mass spectrometry. Seven unique peptides were clearly identified for mouse LRIT3; of these, 2 were identified in the sample from the anti-Myc antibody immunoprecipitation, and 6 of these were identified in the sample

from the anti-mouse LRIT3 antibody immunoprecipitation (1 peptide in common). Similarly, 7 unique peptides were clearly identified for human LRIT3, 5 of which were identified in the bands corresponding to lysates immunoprecipitated with the anti-Myc antibody, and 4 of which were identified in the bands corresponding to lysates immunoprecipitated with the anti-human LRIT3 antibody (2 peptides in common).

### **III. Other projects**

Throughout my doctoral studies, I also contributed to several additional projects in the laboratory. The publications and presentations resulting from this work are listed below, but these projects will not be discussed in further details in this thesis manuscript.

### KIZ identification and characterization project

### Publication:

1) El Shamieh, S., **Neuille, M.**, Terray, A., Orhan, E., Condroyer, C., Demontant, V., Michiels, C., Antonio, A., Boyard, F., Lancelot, M.E., Letexier, M., Saraiva, J.P., Leveillard, T., Mohand-Said, S., Goureau, O., Sahel, J.A., Zeitz, C. & Audo, I. (2014) Whole-exome sequencing identifies *KIZ* as a ciliary gene associated with autosomal-recessive rod-cone dystrophy. *Am J Hum Genet*, **94**, 625-633.

### Paper presentation:

1) El Shamieh, S., **Neuille, M.**, Terray, A., Orhan, E., Condroyer, C., Demontant, V., Michiels, C., Antonio, A., Boyard, F., Leveillard, T., Mohand-Said, S., Goureau, O., Sahel, J.A., Zeitz, C. & Audo, I. – Whole exome sequencing identifies a new ciliary gene underlying autosomal recessive rod-cone dystrophy - Young Researchers in Life Science Conference (May 22-24 2013) – Paris (France).

### Poster presentation:

1) Audo, I., El Shamieh, S., **Neuille, M.**, Terray, A., Orhan, E., Mohand-Said, S., Leveillard, T., Goureau, O., Sahel, J.A. & Zeitz, C – Whole exome sequencing identifies a new ciliary gene in autosomal recessive rod-cone dystrophy – Annual meeting of the Association for Research in Vision and Ophthalmology (ARVO) (May 4-8 2014) – Orlando (FL, USA).

# Genome editing approaches applied to Rhodopsin mutations project

### Publication:

1) Orhan, E., Dalkara, D., **Neuille, M.**, Lechauve, C., Michiels, C., Picaud, S., Leveillard, T., Sahel, J.A., Naash, M.I., Lavail, M.M., Zeitz, C. & Audo, I. (2015) Genotypic and phenotypic characterization of P23H line 1 rat model. *PLoS One*, **10**, e0127319.

### mGluR6 gene therapy project

### Paper presentation:

1) Miranda de Sousa Dias, M., **Neuille, M.**, Orhan, E., Dalkara, D., Audo, I. & Zeitz, C – Gene replacement therapy for CSNB as a proof-of-concept for other inner retinal disorders - Joint Meeting of the Société de Génétique Ophthalmologique Francophone (SGOF) and the section of genetics of the German Association of Ophthtalmology (DOG) (December 5-6 2014) – Giessen (Germany).

### Poster presentation:

1) Miranda de Sousa Dias, M., Pugliese, T., **Neuille, M.**, Orhan, E., Michiels, C., Desrosiers, M., Sahel, J.A., Dalkara, D., Audo, I. & Zeitz, C – Gene for CSNB associated with *GRM6* gene defects - Annual meeting of the Association for Research in Vision and Ophthalmology (ARVO) (May 1-5 2016) – Seattle (WA, USA).

## **DISCUSSION AND PERSPECTIVES**

### I. Identification of gene defects in CSNB

To date, more than 300 different mutations in 12 genes have been identified in CSNB patients with normal fundus exams. However, some cases still have no molecular diagnosis, and there are several possible explanations for this: (1) the mutated gene(s) in these patients is not yet known to be involved in CSNB, (2) the gene defects are in regulatory elements or introns that have not previously been studied by WES, or (3) the phenotype of the patient is not well characterized. These unresolved cases highlight the importance of developing new tools to identify gene defects. In addition, unbiased next-generation sequencing techniques may also help to clarify unclear phenotype by identifying the underlying genetic mutation responsible for the patient's condition.

Our team recently identified *LRIT3* as a new gene implicated in cCSNB by applying WES approaches to an affected family that previously lacked a molecular diagnosis (165). Sanger sequencing in a second affected family identified mutations in the same gene, strongly suggesting LRIT3 as the causative gene in both cases. Working in collaboration with a research team in India, we also identified four novel mutations in SLC24A1 in three families leading to the Riggs-type of CSNB (166). Although one of these families had initially been diagnosed with icCSNB, Sanger sequencing for genes underlying icCSNB detected no mutations. By applying WES, we revealed a homozygous nonsense mutation in SLC24A1 in this family; upon further review of the clinical data, they were found to have phenotypes consistent with the Riggs-type of CSNB. Interestingly, the other two families with SLC24A1 mutations exhibited ERG traces that were quite different from the classic ERG description for the Riggs-type of CSNB. In all three of these cases, next-generation sequencing techniques not only enabled us to accurately identify the underlying gene defect, but also to properly reclassify the diagnosis of the icCSNB family and to clarify the diagnosis for the other two families. For those patients in whom no pathogenic mutations were found in coding exons, new approaches such as whole-genome sequencing have already been used to successfully identify intronic mutations involved in retinal diseases (167).

Efficient and comprehensive genotyping, as well as correct clinical characterization of CSNB patients and families, remain necessary to fully characterize genotype-phenotype correlations in this disease. These correlations are key to our improved diagnosis and genetic counseling

for CSNB and other inherited retinal disorders. However, further studies are required to uncover the function of proteins encoded by the identified genes in order to provide knowledge in physiology and physiopathology of the retina.

# II. Functional characterization of new gene defects identified in CSNB

At the time that *LRIT3* mutations were identified in cCSNB patients, the function of the LRIT3 protein remained to be discovered. To address this, we needed an animal model that accurately reproduced the human disease. Animal models (and particularly mouse models) are the most commonly used model for cCSNB because: (1) they occur spontaneously or are quite easy to generate, (2) their small size, short lifespan, high reproductive rate, and ease of handling make them convenient experimental subjects, (3) their retina is very similar to the human retina in structure and function, (4) it is possible to perform behavioral tests on mice, (5) their phenotype can be assessed by performing full-field ERG, FAF and SD-OCT in similar manner to human patients, and (6) structural and functional studies can be performed *post mortem* (75, 168).

Functional characterization of a mouse model lacking Lrit3 revealed a stationary no b-wave (nob) phenotype similar to that observed in cCSNB patients with *LRIT3* mutations (165). The phenotype was also similar to the phenotypes of cCSNB patients with gene defects in other members of the same molecular cascade (99, 100, 102-104, 107-109, 113) and to phenotype of other mouse models of cCSNB (22, 32-34, 109, 123, 132, 133, 138, 146). We called this mouse model *nob6*. *nob6* mice exhibit a selective absence of the scotopic b-wave, with a preserved a-wave component. Cone-mediated pathways are also affected, with markedly reduced b-waves in photopic conditions. Therefore, visual dysfunction in *nob6* mice affects both the rod and cone ON-bipolar systems. Moreover, the visual acuity of nob6 mice was decreased in scotopic conditions. Finally, there were no indications of photoreceptor degeneration in nob6 mice (75). The nob6 mice therefore represent a relevant cCSNB model in which to decipher the function of LRIT3 in the retina and the pathophysiologic mechanism(s) associated with LRIT3 mutations. An independent, chemically induced Lrit3 mouse model was recently described. This mouse model harbors a missense mutation (p.Leu134Pro) and displays an electroretinographic phenotype that is similar to nob6 mice (169).

We subsequently confirmed the localization of LRIT3 at the dendritic tips of ON-BCs in the mouse retina, suggesting that LRIT3 plays a role in the mGluR6 signaling cascade (Figure 36). We also showed that LRIT3 is important for the correct localization of the TRPM1 channel at the dendritic tips of ON-BCs (as also found for nyctalopin), suggesting that the pathophysiological mechanism associated with *LRIT3* mutations is probably the mislocalization of TRPM1 and the incapacity of ON-BCs to depolarize upon light stimulus. It has been hypothesized that LRIT3, which contains a PDZ-binding motif at its C-terminus, may interact with scaffolding proteins to bring TRPM1 to the cell surface and to maintain it there, through association with NYX (165). These findings suggest that LRIT3 interacts with one or more partners expressed by ON-BCs. More surprisingly, nob6 retinas exhibit near complete elimination of post-synaptic clustering of the mGluR6 cascade components at the dendritic tips of cone ON-BCs, (including mGluR6 itself, GPR179, RGS7, RGS11 and  $G_{\beta5}$ ) despite their undisrupted accumulation at the dendritic tips of rod BCs (160). These deficits suggest the potential involvement of LRIT3 in cone synapse formation and signaling to ONand OFF-BCs.



Figure 36: Schematic drawing of major molecules important for the first visual synapse between photoreceptors and ON-BCs in light of discovery of LRIT3's role in the retina (adapted from (16))

EM studies revealed that rod spherules are normally organized in *Lrit3<sup>nob6/nob6</sup>* mice with correctly formed triads (Figure 28), indicating that LRIT3 has no role in the rod ribbon synapse formation. However, the results were strikingly different in cone pedicles. A dramatic decrease in the number of ON-BC dendrites deeply invaginating cone pedicles was found

(Figure 28), leading to an increased diad/triad ratio at cone synapses in Lrit3<sup>nob6/nob6</sup> mice. These results confirmed that LRIT3 plays an important role in cone ribbon synapse formation. However, we do not know yet if LRIT3 is important for synapse maintenance and/or synapse development. Structural defects of the first visual synapse have already been shown in some cCSNB mouse models or in mouse models deficient in protein components of the mGluR6 signaling cascade (30, 38, 44, 143, 144, 170) (Table 3). For example, rod spherules were completely normal in the Grm6 knock-out model (22), but cone pedicles showed a reduced number of invaginating ON-BC dendrites. However, this decrease was correlated with a reduced number of ribbons per cone pedicle (143). For the nob4 mouse model with a mutation in Grm6 (132), rod spherules and cone pedicles showed a reduced number of invaginating ON-BC dendrites (44, 144). The same phenotype is present in mice lacking Gnb5. Moreover, the decrease for rod spherules was observed from post-natal day 13 to 21, when retinal development is not totally finished, indicating that  $G_{\beta5}$  has a role in ribbon synapse development (38). Mice lacking Gnb3 exhibited a reduced number of invaginating ON-BC dendrites in rod spherules. Moreover, the number of rod triads in Gnb3 lacking mice was similar to that of wild-type mice at three weeks of age, suggesting that  $G_{\beta3}$  has a role in ribbon synapse maintenance (30). Finally, in mice lacking Gnaol (28), the number of ON-BC dendrites invaginating rod spherules was also decreased, but to a lesser extent than in mice lacking Gnb3 (170).

 $Lrit3^{nob6/nob6}$  mice differ from these models because the structural defect affects only cone synapses, but not the number of ribbons per cone pedicle. It would be interesting to perform an ultrastructural study at an earlier stage of retinal development to determine if LRIT3 function is important for the development and/or maintenance of the cone ribbon synapse. Recently, Cao *et al.* described a mouse model lacking *Elfn1* (144), which encodes a transmembrane protein (ELFN1) that has been shown to be specifically expressed by rods and to localize to rod synaptic terminals. ELFN1 interacts with mGluR6 at the dendritic tips of rod BCs and *Elfn1* deficient mice exhibit absence of mGluR6 at the dendritic tips of rod BCs, but staining at the dendritic tips of cone ON-BCs was normal. Moreover, mice lacking *Elfn1* showed a nearly complete absence of invaginating ON-BC dendrites in rod spherules. Functionally, mice lacking *Elfn1* lacked the ERG b-wave in response to a dim flash under scotopic conditions whereas a small b-wave was present in response to a bright flash. In both cases, the a-wave was normal. Photopic ERG is also undistinguishable between knock-out and wild-type mice. Together, these results indicate that ELFN1 is necessary for the proper development of rod ribbon synapses and the correct functioning of the primary rod-driven ON-pathway (144).

Given that cone ribbon synapses are affected in mice lacking *Lrit3*, it would be interesting to identify LRIT3 interacting partners in order to determine if trans-synaptic contacts between LRIT3 and proteins localized at the cone synaptic terminal exist (as shown for mGluR6 and ELFN1 in the rod ribbon synapses). Moreover, proteins of the dystrophin-glycoprotein complex (DGC) may be involved in interactions with pre- and post-synaptic complexes in order to maintain a stable adhesion between rod spherules and rod ON-BC dendrites (170), and it is possible that they play a similar role at the cone synapse. Dystroglycan (DG) is the key component of this complex and is composed of an extracellular a-subunit and a transmembrane  $\beta$ -subunit. The  $\alpha$ -DG has been shown to localize at retinal photoreceptor synaptic terminals where it interacts in a glycosylation-dependent manner with its ligand Pikachurin (162, 163). Mice lacking *Pikachurin* and conditional knock-out mice specifically lacking Dg in the retina display similar phenotypes. Structurally, these mouse models lack invaginating ON-BC dendrites in both rod spherules and cone pedicles. However, the invaginating dendrites remain in close vicinity to photoreceptor terminals and seem to help maintain the integrity of the ribbon synapse. Functionally, the ERG b-wave is reduced and delayed in both scotopic and photopic conditions in these mice (162, 163). We evaluated the localization of Pikachurin in the OPL of Lrit3<sup>nob6/nob6</sup> mice and showed that Pikachurin localizes properly to the synaptic cleft of both rod and cone synapses (Figure 30), indicating that Pikachurin is probably not directly involved in the maintenance of the cone ribbon synapse.

Mouse model	Rod ribbon synapses	Cone ribbon synapses	Comments	Reference
Grm6 <sup>-/-</sup>	Normal	Reduced number of invaginating		(143)
		ON-BC dendrites		
		Corresponding reduced number		
		of ribbons		
nob4	Reduced number of invaginating	Reduced number of invaginating		(44, 144)
	ON-BC dendrites	ON-BC dendrites		
Gnb5 <sup>-/-</sup>	Reduced number of invaginating	Reduced number of invaginating	Role for $G_{\beta5}$ in rod synapse	(38)
	ON-BC dendrites	ON-BC dendrites	development	
Gnb3 <sup>-/-</sup>	Reduced number of invaginating	n.a.	Role for $G_{\beta 3}$ in rod synapse	(30)
	ON-BC dendrites		maintenance	
Gnao1 <sup>-/-</sup>	Reduced number of invaginating	n.a.		(170)
	ON-BC dendrites			
nob6	Normal	Reduced number of invaginating		This thesis
		ON-BC dendrites		

 Table 3: Ultrastructural defects of photoreceptor ribbon synapses in knock-out mice with mutations in different components of the mGluR6 signaling cascade. ON-BC, ON-bipolar cell; n.a, non-available

By EM, we showed that the number of OFF-BC dendrites making flat contacts with cone pedicles was slightly increased in mice lacking *Lrit3* (Figure 28). However, we may be slightly overestimating this increase given that some of these dendrites, especially those close to ribbons, may actually be ON-BC dendrites that do not invaginate cone pedicles (but rather just lie in close vicinity to ribbons). This hypothesis could be confirmed by performing electron microscopy coupled with cone ON-BC staining using a ON-BC marker such as Goa (171). This particular configuration has already been highlighted in conditional Dg mutants and in mice lacking Pikachurin, as both corresponding proteins play a role in ribbon synapse maintenance. There are no invaginating ON-BC dendrites in these mouse models, but the dendrites are correctly formed and remain close to the ribbons, helping to maintain the organization and function of the synapse (162, 163). Thus, OFF-BC dendrites are normally present in the OPL of Lrit3<sup>nob6/nob6</sup> mice. Moreover, we demonstrated that the cellular localization of OFF-BC ionotropic glutamate receptors is similar in wild-type and Lrit3<sup>nob6/nob6</sup> mice (Figure 30). Our studies showed that at least type 3a, type 3b and type 4 OFF BCs make flat contacts at cone pedicles Lrit3 deficient mice, potentially allowing for both transient and sustained responses at light offset (65).

In our ERG studies, we tried to separately elicit cone driven ON- and OFF-responses originating from corresponding BCs in our *Lrit3* deficient mice in order to confirm that ON-responses are absent in this cCSNB mouse model and to evaluate the state of OFF-responses in these animals (161). We also used the *Grm6* mouse model as a control, since it has already been shown by ERG to have intact OFF-responses and absent ON-responses, which is in

accordance with the cCSNB phenotype (83, 135, 136). Despite the sweep-to-sweep variability, we identified a clear trend in the wild-type profiles of ON- and OFF-responses in wild-type mice with the adapted protocol. In comparison, the *Grm6* deficient mice demonstrated higher variability in the ERG studies, which was even higher for the *Lrit3* mouse model and which did not allow us to obtain reproducible profiles. Further efforts are thus needed to reduce the high variability observed in the ERG studies. This may be possible by increasing the sample size, by recording more sweeps, and/or by reducing the signal to noise ratio. Moreover, it would be interesting to remove the adaptation step in the future as it may negatively influence the responses by saturating both rods and cones. In order to obtain quantitative results on the functionality of ON- and OFF-pathways in mice lacking *Lrit3*, we decided to record ON- and OFF-responses at the level of RGCs by MEA in collaboration with Serge Picaud (Institut de la Vision, Paris, France).

Consistent with the fact that the Lrit3<sup>nob6/nob6</sup> cCSNB mouse model has signaling defect between photoreceptors and ON-BCs, Lrit3<sup>nob6/nob6</sup> mice exhibit no cone driven ON-responses as evaluated by MEA (Figure 32). This phenotype of no ON-responses is also observed in other mouse models of cCSNB, although authors in some reports described long-latency ONresponses with drastically reduced firing frequencies originating from OFF-pathway inputs that can be blocked by functional ON-pathways. Moreover, spontaneous activity of RGCs is increased in these mouse models (128, 129, 132, 133, 136). These results suggest that the mGluR6 signaling cascade is non-functional in mice lacking Lrit3, leading to an ON-defect and to a cCSNB phenotype. OFF-responses are present in *Lrit3<sup>nob6/nob6</sup>* mice and are similar in frequency to wild-type mice, which is in accordance with the cCSNB phenotype. However, our results demonstrated OFF-responses that are delayed and more sustained. Small OFFresponse abnormalities have been already shown in one of the other mouse models. Although the Grm6 mutants Grm6<sup>-/-</sup>, nob3 and nob4 displayed normal OFF-responses in frequency and latency (132, 133, 136), the Nyx mutant showed decreased OFF-responses with normal latencies (128, 129). A deeper analysis of the data is needed to confirm the presence of delayed OFF-responses in mice lacking Lrit3 and discuss the differences observed due to different gene defects.

The next step of this study will be to identify LRIT3 binding partners. These proteins might be localized post-synaptically in ON-BCs, pre-synaptically at the cone pedicles, or in the synaptic cleft at the cone synapses. We have shown in this thesis that we are able to successfully immunoprecipitate both human and mouse LRIT3 with the corresponding antiLRIT3 antibodies, and that we are able to detect both proteins by mass spectrometry. The protocol has now to be adapted for mouse and human retina in order to identify LRIT3 partners.

Our improved understanding of the function of LRIT3 will lead to a better knowledge of the retinal signaling pathway(s) at the first synapse. Furthermore, identification of LRIT3 binding partners may reveal other proteins that, when mutated, result in CSNB in human patients. Finally, our data will provide the basis for the development of comprehensive therapeutic approaches for CSNB and possible new approaches to monitor treatment efficacy.

### **III. Development of therapeutic approaches for CSNB**

Since CSNB is a stationary condition (rather than a degenerative process), gene therapy represents a promising therapeutic approach. The basic premise of gene therapy is to replace or "repair" a mutated, improperly functioning gene with a normal gene that can restore protein function when expressed in retinal cells (172). To achieve this goal, it is necessary to transfer the therapeutic gene to the affected cell type in a safe, efficient and stable manner, and with specificity to the appropriate cell type (173). In the case of the Riggs-type of CSNB and for icCSNB, the therapeutic gene must therefore be expressed in photoreceptors. Similarly, for cCSNB, the therapeutic gene must be expressed in ON-BCs.

Most gene therapy approaches developed today use viral vectors to deliver the therapeutic gene to a targeted cell population. Adeno-associated virus (AAV)-derived vectors are the preferred vectors for ocular gene therapy for several reasons: (1) they have low immunogenicity because viral coding sequences are absent from their genome, (2) a large numbers of AAVs exist with different preferential targets, and thus may be engineered in order to target certain cell types of interest with high specificity, and (3) they confer long-lasting transgene expression (172). However, AAV vectors only have a cloning capacity of about 4.7 kilobases (kb) in length, which might be insufficient for some large CSNB genes such as *SLC24A1*, *CACNA1F* or *TRPM1*. Despite this challenge, gene therapy remains a very promising approach for several gene defects underlying CSNB, and several gene therapy studies in CSNB mouse models have already been reported (156, 174). The most recent reports have utilized intravitreal injections for AAV delivery rather than subretinal injections in order to avoid complications associated with retinal detachment (172).

### A. Gene therapy for the dominant mode of inheritance

To date, only the Riggs-type of CSNB (resulting from mutations *RHO*, *GNAT1* and *PDE6B*) exhibits an dominant mode of inheritance (16). The dominant nature of the disorder implies that gene therapy approaches could be used to knockdown the mutant allele. Conley and coworkers recently used ribozymes to knockdown the G90D CSNB mutation in rhodopsin (174). Ribozymes are RNAs that possess a catalytic activity and that cleave RNAs in a sitespecific manner. The G90D mutation in rhodopsin leads to a constitutively active protein that suppresses rod sensitivity (116, 175). The ribozyme designed specifically for the G90D rhodopsin mutation was cloned into a recombinant AAV under the regulatory control of the mouse opsin promoter, resulting in its expression of in photoreceptors (173). The AAV was injected intravitreally at 4 weeks of age in G90D transgenic mice. An AAV coding for GFP was injected in the contralateral eye as a positive control. GFP-positive cells were found throughout the retina at 6 weeks post-injection and remained for up to 10 months. Functionally, the ribozyme designed to specifically target the G90D mutation was only able to slow the long-term loss of ERG function in this mouse model, but not to restore a normal phenotype (174). At 3 and 8 months post-injection, the amplitude of the scotopic b-wave was increased by almost 25 and 66% compared to AAV-GFP controls, respectively. However, in the same time, the amplitude of the scotopic b-wave was still decreasing in controls and, thus, the amplitude of the scotopic b-wave at 8 months post-injection in the treated retinas was even lower than in controls at 6 weeks post-injection. It is possible, but currently unknown, that slowing down the long-term loss of ERG function would be sufficient to improve the clinical phenotype of affected subjects.

Of note, this study utilized a specifically designed ribozyme that was developed to selectively recognize and act upon the mutated G90D allele. However, wide spread use of this approach would mean that a specific ribozyme must be developed for each disease-causing mutation on a case-by-case basis, which represents an inefficient therapeutic approach. An alternative approach would be to develop one optimal ribozyme that is able to knockdown both mutated and wild-type alleles non-selectively (independent of the pathogenic variants), coupled with concurrent supplementation of a knockdown-resistant wild-type gene to restore protein function. In this way, multiple different disease-causing mutations could be treated using the exact therapeutic approach, and this method could also be extended to other retinal disorders.

### **B.** Gene therapy for recessive or X-linked modes of inheritance

To date, all known forms of CSNB demonstrate autosomal recessive or X-linked modes of inheritance. The proteins encoded by the mutated genes underlying these disorders localize in photoreceptors and play roles in the phototransduction cascade (Riggs-type of CSNB), in glutamate release (icCSNB), or in the mGluR6 signaling cascade at the dendritic tips of ON-BCs (cCSNB) (16). Theses modes of inheritance imply that complementation by a wild-type copy of the causative gene would be sufficient to restore a normal phenotype.

Scalabrino and coworkers recently published the first study showing that postnatal delivery of an ON-BC specific gene via AAV is capable of restoring ON-BC signaling in a mouse model of cCSNB (156). Prior to that report, the transduction of cells located in the inner retinal layers (such as BCs) within non-degenerative retinas with successful selective expression of the therapeutic transgene had been extremely challenging. Scalabrino et al. used the Nyx deficient nob mouse model for their study. Nyx gene was packaged in an AAV2-based capsid variant capable of transducing the majority murine retinal cells following intravitreal injection (176) under the control of the human MiniPromoter "Ple155" that drives transgene expression in BCs (177). The AAV was injected intravitreally at post-natal day 2 or 30 (P2 or P30) in nob mice. An AAV coding for GFP was injected in the contralateral eye as a positive control. GFP expression was observed in ON-BCs and the fluorescence was localized in the somas, at the dendritic tips of ON-BCs and in their axon terminals. In the experimentally treated eyes, nyctalopin protein was observed at the dendritic tips of ON-BCs in nob retinas. The final percentage of transduction of the protein was more important with the P2 injection (21.5% of ON-BCs), suggesting that supplementation of an ON-BC-specific transgene is most effective when delivered while BCs are still differentiating (178). This therapeutic window therefore needs to be extended for potential future applications in humans, since delivery of gene therapy in utero is not feasible. In ON-BCs that were transduced, TRPM1 was relocated to the dendritic tips of ON-BCs and the cells responded to the TRPM1 agonist capsaicin with robust outward currents. At the level of entire retina, 30 days after the P2 injection, the transduction of nyctalopin partially restored the ERG b-wave in both scotopic and photopic conditions (156). Whereas the scotopic b-wave was totally absent in AAV-GFP controls, a scotopic bwave was clearly observed in the experimentally treated eyes. However, the amplitude of this "restored" b-wave was only 5.7% of typical wild-type amplitudes. It is possible that even such

a small increase in amplitude would be sufficient to rescue (or improve) the phenotype, and this remains to be determined.

Gene replacement therapy would be also the best therapeutic approach for CSNB due to *SLC24A1* mutations. However, the large size of the gene precludes it from being packaged into a single AAV vector. Although other approaches such as lentiviral vectors with a larger packaging capacity (up to 8 kb) or alternative delivery systems such as nanoparticles could circumvent this problem, to date AAV vectors still remain the most effective and safest tool for gene therapy. Interestingly, recent studies highlighted the use of AAV vectors to deliver larger genes to the retina by employing a dual AAV approach (179, 180). The general idea is to split the large gene into pieces, each less than 5 kb in length, and to package these into two separate AAV vectors. After co-transducing these gene fragments in to retinal cells, the full-length transgene is reconstituted. This dual AAV approach has already been shown to allow expression of full-length *ABCA4* and *MYO7B* transgenes in photoreceptors and RPE cells, improving the retinal phenotype in two mouse models of Stargardt's disease and Usher syndrome, respectively (179, 180).

*LRIT3* is an even better candidate for gene replacement therapy as compared to *SLC24A1*. The relatively small size of this gene would allow its packaging into a single AAV vector. Moreover, we already have a suitable and well-characterize mouse model with an *Lrit3* gene defect that reproduces the human cCSNB phenotype, making it an ideal model system in which to test *LRIT3* gene therapy approaches. Finally, Deniz Dalkara (Institut de la Vision) developed a genetic variant of AAV2, known as 7m8, which is capable of transducing multiple cell types in the retina including BCs after intravitreal injection (181). *Lrit3* can be placed under the control of a 200-base pair enhancer sequence of the mouse *Grm6* gene that has been shown to drive transgene expression specifically in ON-BCs in degenerative retina (84). The rescue of the cCSNB phenotype in these mice could subsequently be evaluated by analyzing the restored localization of previously mislocalized components of the mGluR6 cascade, and by performing a series of functional and clinical phenotyping studies including ERG, MEA and behavioral tests.

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### Abstract:

The first steps in vision occur when rod and cone photoreceptors transform light into a biochemical signal, which gets processed through the retina. Our group investigates genetic causes and mechanisms involved in inherited retinal diseases as congenital stationary night blindness (CSNB), which reflects a signal transmission defect between photoreceptors and bipolar cells. This thesis gives several insights on the retinal physiology at this first visual synapse. We identified four novels mutations in SLC24A1 underlying the Riggs-type of CSNB, which has a role in calcium balance in rods. We subsequently identified a novel gene, LRIT3, which is mutated in the complete form of CSNB. We delivered a knock-out mouse model lacking Lrit3 which displays a phenotype similar to patients. We confirmed the localization of LRIT3 at the dendritic tips of ON-bipolar cells, suggesting a role of LRIT3 in the mGluR6 signaling cascade. We showed that LRIT3 is necessary for the functional localization of TRPM1. We also revealed that LRIT3 has an additional role in formation of the cone synapse but with probably only a minor effect on OFF-pathway functionality. We finally succeeded in immunoprecipitating and detecting LRIT3 by mass spectrometry, opening the way for the identification of LRIT3 partners. Improving knowledge about retinal physiology and physiopathology will lead to a better diagnosis and genetic counseling of the patients and to the development of novel therapeutic approaches.

Keywords: retina, congenital stationary night blindness, *SLC24A1*, *LRIT3*, mouse model

# Identification et caractérisation fonctionnelle de défauts génétiques à l'origine de la cécité nocturne congénitale stationnaire

Résumé :

Le processus visuel débute lorsque les photorécepteurs transforment la lumière en un signal biochimique qui est ensuite traité et transmis via la rétine. Notre groupe s'intéresse à élucider les défauts génétiques et les mécanismes à l'origine de pathologies rétiniennes comme la cécité nocturne congénitale stationnaire (CNCS), conséquence d'un défaut de transmission du signal entre les photorécepteurs et les cellules bipolaires. Cette thèse apporte de nouvelles connaissances sur la physiologie de cette première synapse visuelle. Nous avons identifié quatre nouvelles mutations dans SLC24A1, un échangeur ionique intervenant dans l'homéostasie du calcium dans les bâtonnets, à l'origine de la CNSC de type Riggs. Nous avons également identifié LRIT3 comme étant un nouveau gène impliqué dans la forme complète de CNCS. Nous avons décrit un modèle de souris invalidé pour Lrit3 avec un phénotype visuel similaire à celui des patients. Nous avons confirmé la localisation de LRIT3 aux extrémités dendritiques des cellules bipolaires ON, suggérant un rôle dans la cascade de signalisation mGluR6. Nous avons montré que LRIT3 était nécessaire à la localisation fonctionnelle de TRPM1. Nous avons de plus démontré un rôle additionnel de LRIT3 dans la formation de la synapse du cône n'impactant probablement que faiblement les voies OFF. Nous avons également réussi à détecter LRIT3 par spectrométrie de masse, ouvrant la voie à l'identification de ses partenaires. La meilleur connaissance de la physiologie et de la physiopathologie rétinienne doit mener non seulement à un meilleur diagnostic et conseil génétique des patients mais également au développement de nouvelles approches thérapeutiques.

Mots clés : rétine, cécité nocturne congénitale stationnaire, SLC24A1, LRIT3, modèle de souris