Substantial restoration of night vision in adult mice with congenital stationary night blindness

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Complete congenital stationary night blindness (cCSNB) due to mutations in TRPM1, GRM6, GPR179, NYX, or leucine-rich repeat immunoglobulin-like transmembrane domain 3 (LRIT3) is an incurable inherited retinal disorder characterized by an ON-bipolar cell (ON-BC) defect. Since the disease is non-degenerative and stable, treatment could theoretically be administered at any time in life, making it a promising target for gene therapy. Until now, adeno-associated virus (AAV)-mediated therapies lead to significant functional improvements only in newborn cCSNB mice. Here we aimed to restore protein localization and function in adult Lrit3–/– mice. LRIT3 localizes in the outer plexiform layer and is crucial for TRPM1 localization at the dendritic tips of ON-BCs and the electroretinogram (ERG)-b-wave. AAV2-7m8-Lrit3 intravitreal injections were performed targeting either ON-BCs, photoreceptors (PRs), or both. Protein localization of LRIT3 and TRPM1 at the rod-to-rrod BC synapse, functional rescue of scotopic responses, and on-responses detection at the ganglion cell level were achieved in a few mice when ON-BCs alone or both PRs and ON-BCs, were targeted. More importantly, a significant number of treated adult Lrit3–/– mice revealed an ERG b-wave recovery under scotopic conditions, improved optomotor responses, and on-time ON-responses at the ganglion cell level when PRs were targeted. Functional rescue was maintained for at least 4 months after treatment.

INTRODUCTION

Congenital stationary night blindness (CSNB) is a heterogeneous group of non-progressive rare inherited retinal disorders (IRDs).2 The most frequent type of CSNB is the Schubert-Bornschein-type, which is due to a disruption of the signal transmission between photoreceptors (PRs) and ON-bipolar cells (ON-BCs).12 CSNB can be further subdivided into the incomplete CSNB (icCSNB) and complete CSNB (cCSNB) forms.3 Here we focus on the latter one. cCSNB-affected subjects are mainly characterized by impairment of night vision, decreased visual acuity, severe myopia, nystagmus, and sometimes strabismus. cCSNB is mostly a non-degenerative disease with normal fundus. Clinically, it can be diagnosed by full-field electroretinogram (ERG) recording showing an isolated ON-BC defect.1 At low light intensities in dark-adapted (DA, scotopic) conditions, the b-wave is absent. With a brighter flash, the a-wave is normal, representing normal rod and cone function, while the b-wave remains absent in keeping with a transmission defect between PRs and ON-BCs. In light-adapted (LA, photopic) conditions, the responses to a single flash reveal a sharply arising b-wave with no oscillatory potentials and variable but often decreased b/a ratio indicating cone ON-BC dysfunction.1 This is in accordance with the expression of the genes mutated in patients with cCSNB including NYX,10 TRPM1,9,14 GRM610,11 GPR179,12,13 and leucine-rich repeat immunoglobulin-like transmembrane domain 3 (LRIT3).14 These genes code for proteins localized in the outer plexiform layer (OPL) affecting signal transmission between PRs and ON-BCs.2 Several mouse models of cCSNB have been described. All display an absence of ERG b-wave under both scotopic and photopic conditions.2,15 While the scotopic phenotype is similar to those of patients, the b-wave under photopic conditions is only reduced in patients. Herein, we focus on cCSNB due to mutations in LRIT3 coding for the LRIT3 protein using the respective mouse model (nob6 also called Lrit3–/–).14,16 Lrit3–/– mice are characterized by the absence of LRIT3 in the OPL, a lack of the ERG b-wave under both scotopic and photopic conditions, altered optomotor responses under scotopic conditions, and abolished ON-responses at the RGC level.16 The outer nuclear layer (ONL) is generally well preserved.14 Only some disorganized synaptic contacts of ON-BC at the cone pedicle but not at the rod spherule without ultrastructural alterations in cone terminals, horizontal cells, or synaptic ribbons were described.17 LRIT3 is crucial for the correct localization of TRPM1 at the dendritic tips of all ON-BCs, cone synapse formation, and/or maintenance18 and is essential for the localization of nystatin, encoded by NYX.19 Several gene therapies for IRDs have been developed over the years.20 However, treatment for CSNB patients is yet unavailable and gene replacement therapy might...
be the most promising approach to treat this disorder. cCSNB represents a non-progressive disorder, in which retinal morphology is well preserved. Genes underlying this disorder have been identified and specific targeting of ON-bipolar cells in primate retinas with adeno-associated virus (AAV)-vectors has been demonstrated. Due to this stable non-degenerative condition in mice and human, treatment could be, in theory, administrated at any time during the course of life. However, recent findings revealed functional restoration mainly in very young mice. In mice lacking Nyx and treated at 2 days of age (P2) with targeting only the ON-BCs, partial functional rescue of the b-wave under scotopic conditions using an intravitreal AAV-mediated gene replacement approach has been obtained. Similar observations were made in mice lacking Lrit3: targeting of rod PR at P5 or P35 resulted in more significant restoration at P5 compared to P35. After gene therapy in adult mice lacking Grm6, mGluR6 and other proteins of the same cascade were localized at the dendritic tips of ON-BCs in the absence of functional restoration. In all models, localization of TRPM1 at the dendritic tips of ON-BCs was observed, while no restoration of the b-wave under photopic conditions could be obtained. Here, we aimed to achieve a robust functional rescue in mature Lrit3 mice using different AAV-promoter combinations targeting ON-BCs, both rod and cone PRs, or the OPL.

RESULTS

Immunolocalization studies on unaffected patients and mouse retinas showed Lrit3 protein in the OPL, between PRs and ON-BCs. It is a matter of debate whether this correlates with postsynaptic localization at the dendritic tips of ON-BCs or/and a presynaptic localization at the synapse of PRs. To revert the phenotype of nob6 mice (later referred as Lrit3−/−) toward normal function, we used the following two promoter constructs: a 200 bp enhancer of Grm6, which has been previously shown by us to drive expression of GFP to ON-BCs, and the hGRK promoter, which drives expression in both rod and cone PRs. These two constructs were encapsidated in the AAV2-7m8 serotype and injected either alone or together at a ratio of 1:1. Mice injected with the Grm6 promoter construct will be named Lrit3−/−-BC-Lrit3, mice treated with the GRK promoter construct will be named Lrit3−/−-PR-Lrit3, and mice injected with both constructs will be named Lrit3−/−-OPL-Lrit3 (Figure 1).

Protein localization of LRT3 at the rod-to-rods BC synapse

In the Lrit3−/− mice, LRT3 synthesis and localization is abolished in the OPL in both rod-to-rods BC and cone-to-cone ON-BC synapses (Figure 2A; arrows and arrowheads, respectively). The proper localization of LRT3 following treatment was investigated using immunolocalization studies. All treated retinas, Lrit3−/−-BC-Lrit3, Lrit3−/−-OPL-Lrit3, and Lrit3−/−-PR-Lrit3, injected at P30, displayed LRT3 immunostaining in the OPL, which is absent in untreated Lrit3−/− retinas (Figure 2A; Figure S1A). In addition, Lrit3−/− mice lack mGluR6 at the cone-to-cone ON BC synapse and TRPM1 in both rod-to-rods BC and cone-to-cone BC synapses (Figure 2). After treatment, the majority of LRT3 staining appeared at most likely rod-to-rods BC synapses (Figure 2A, arrow), while presumably cone-to-cone BC synapse staining (Figure 2A, arrowheads) remained spared. These observations were confirmed with co-staining studies using mGluR6, a marker for both synapses; PKCa, a marker of rod BCs; and cone arrestin or PNA, markers for the cone-to-cone BC synapse. While co-staining of LRT3 with mGluR6 at the rod-to-rods BC is present (Figure 2A, arrows; Figure S1B), LRT3 and mGluR6 remained absent at the cone-to-cone BC synapse in treated retinas (Figures 2A and 2B; Figure S1B). Thinning or remodeling of the ONL in the Lrit3−/− or treated mice was absent.

Treatment results in TRPM1 localization at the dendritic tips of rod BCs

To ensure correct ON-BC signal transmission, the correct localization of TRPM1 at the dendritic tips of ON-BCs is essential. In the Lrit3−/− mouse model, in which TRPM1 is still present in the cell bodies of ON-BCs, the dendritic tip staining at the ON-BCs is abolished (Figure 2C, TRPM1). Localization of TRPM1 following treatment that targets either PRs or ON-BCs was investigated using immunolocalization studies. In all treated retinas, TRPM1 was localized at the dendritic tips of rod BCs (Figure 2C, TRPM1). TRPM1 staining at the presumed dendritic tips of cone ON-BCs was undetectable. These results indicate that restoration of the signaling cascade was selective for the rod-to-rods BC synapse.

Positive long-lasting effect of AAV-mediated LRT3 expression on the ERG b-wave

In Lrit3−/− animals, the transmission of the visual signal between PRs and ON-BCs is disrupted as shown by the absence of the b-wave on the ERG under scotopic and photopic conditions (Figure 3). ERG recordings performed 2 months after treatment on Lrit3−/−-BC-Lrit3...
mice injected at P30 revealed a partial b-wave under scotopic conditions, while the photopic b-wave was absent (Figures 3A, left, and 3B). Highest restoration was found at the lowest flash intensity. The amplitude of the b-wave corresponded to 45% compared to the b-wave amplitudes of \( Lrit3^{+/+} \) mice (Figure 3C). However, these results were obtained in a statistically non-significant number of \( Lrit3^{-/-}-BC-Lrit3 \) mice (\( n = 2 \)). Similarly, ERG recordings in \( Lrit3^{-/-}-OPL-Lrit3 \) mice treated at P30 also presented a b-wave under scotopic conditions (Figure 3A, middle) corresponding to 45% of the b-wave amplitude of \( Lrit3^{+/+} \) mice at the lowest light intensity (Figure 3C). As for \( Lrit3^{-/-}-BC-Lrit3 \) mice, the scotopic b-wave was the highest at low flash intensities and the restoration was only observed under scotopic conditions (Figures 3A–3C). Only a few treated mice (\( n = 2 \)) revealed this functional restoration. Strikingly, better restoration was obtained in \( Lrit3^{-/-}-PR-Lrit3 \) animals (\( n = 6 \)) treated at P30 under scotopic conditions (Figure 3A, right). The amplitude of the scotopic b-wave at the flash intensity of \(-2.5 \log \text{ cd} \times \text{s/m}^2\) improved to 58% compared to the b-wave amplitude of \( Lrit3^{+/+} \).
mice (Figure 3A). To assess the duration of this partial rescue, we made a follow-up ERG recording up to 4 months post-injection. In all animals presenting a b-wave, independently of the construct used, the b-wave was still recordable and of sustained amplitude (no statistically significant change) 4 months after treatment (Figure 3D). The number of treated mice versus the number of mice presenting a scotopic b-wave for every construct is mentioned in Table 1. The functional rescue was obtained in 25% of PR-Lrit3 injected mice, 10% of OPL-Lrit3 injected mice, and ~6% of BC-Lrit3 injected mice (Table 1).

**Presence of ON-BC signaling reveals ON-responses in RGCs in treated mice**

As previously described, in Lrit3−/− mice ON-responses are also abolished at the level of retinal ganglion cells (RGCs) (Figure 4). Correct localization of LRIT3 is mandatory for TRPM1 localization and...
Table 1. Experimental data on the treatment procedure

| Date of injection | Construct used | Viral preparation | Total number responding | Responding mice | Experimentator |
|-------------------|----------------|-------------------|-------------------------|-----------------|---------------|
|                   |                |                   | Female | Male | Female | Male | Experimentator |
| 03/06/2019        | PR-Lrit3       | Same              | 4      | 2    | 1 (71% b-wave rescue) | 0               |
| 01/11/2019        |                |                   | 2      | 6    | 1 (49% b-wave rescue) | 0               |
| 06/12/2019        | PR-Lrit3       | Same              | 0      | 4    | 0      | 1 (44% b-wave rescue) | 0               |
| 04/01/2020        |                |                   | 2      | 4    | 1 (56% b-wave rescue) | 0               |
| 15/11/2016        |                |                   | 1      | 4    | 2      | 0    | 0               |
| 27/02/2017        |                |                   | 0      | 0    | 2      | 1 (50% b-wave rescue) | 0               |
| 10/04/2018        |                |                   | 0      | 3    | 0      | 0               |
| 11/05/2018        | BC-Lrit3       |                   | 2      | 2    | 4      | 0    | 0               |
| 25/05/2018        |                |                   | 5      | 0    | 0      | 0               |
| 10/04/2018        |                |                   | 0      | 3    | 0      | 0               |
| 12/04/2019        |                |                   | 3      | 2    | 1      | 0    | J.V.            |
| 27/05/2019        |                |                   | 1      | 4    | 0      | 1 (40% b-wave rescue) | 0               |
| 03/07/2019        | OPL-Lrit3      | same              | 5      | 5    | 0      | 1 (45% b-wave rescue) | 0               |

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function to propagate the visual signal toward ON-ganglion cells. To confirm functional rescue following treatment, we used a 256 channel multi-electrode array (MEA-256) to record light-evoked responses in isolated retinas from Lrit3+/+ (3 animals, 6 retina explants), Lrit3–/– (6 animals, 6 retina explants), Lrit3–/–-BC-Lrit3 (1 animal, 1 retina explant), Lrit3–/–-OPL-Lrit3 (1 animals, 1 retina explant), and Lrit3–/–-PR-Lrit3 (4 animals, 4 retina explants) and assessed the potential ON-response at the level of ganglion cells. Lrit3–/– retina were untreated control samples, contralateral of the treated eye in the different conditions. In contrast to Lrit3+/+ retinas, untreated Lrit3–/– retinas display only a few ON-responses with small spike frequency and high temporal variability (4.5 ± 3 Hz and 1.3 ± 0.6 s; Figures 4A and 4B). Furthermore, in untreated Lrit3–/–/– retinas, 33% of the light-responsive ganglion cells displayed small amplitude ON-responses (ON + ON-OFF; 37/112 responsive electrodes; Figure 4C). In comparison, RGCs from Lrit3+/+/-BC-Lrit3 retinas also displayed small and variable ON-responses (7.9 ± 7.5 Hz and 1 ± 0.5 s; Figures 4A and 4B). Nonetheless, a higher fraction of RGCs displayed ON-responses, with ~45% of light-sensitive ganglion cells for Lrit3+/−-BC-Lrit3 (ON + ON-OFF: 34/75; Figure 4C). As expected, in Lrit3+/−-PR-Lrit3 and Lrit3+/−-OPL-Lrit3 retinas, peak firing rate for ON-responses were higher and closer to the stimulus onset (14.7 ± 9.5 Hz and 0.45 ± 0.4 s; 15.3 ± 12 and 0.74 ± 0.57, respectively; Figures 4A and 4B). Concerning the number of light-responsive RGCs displaying ON-responses, Lrit3+/−-PR-Lrit3 but not Lrit3+/−-OPL-Lrit3 retinas showed an increase in the proportion of ON-responses in the overall RGC population recorded (Lrit3+/−-PR-Lrit3: ~79%, 148/187; Lrit3+/−-OPL-Lrit3: ~36%, 41/113; Figure 4C). To establish whether the rescued ON-responses observed here were due to the restoration of the mGluR6 signaling, we performed experiments with bath application of the mGluR6 agonist (L-AP4) on our different conditions (Figure S2). Surprisingly, although L-AP4 blocked the ON-responses observed in Lrit3+/−-OPL-Lrit3 and Lrit3+/−-PR-Lrit3 retinas, with reduced firing rate and fewer electrodes recording ON-responses, it does not seem to affect the ON-responses recorded in Lrit3+/−-BC-Lrit3 retinas. More retina explants of these mice would be necessary for further understanding.

Presence of LRTI3 improves optomotor responses in treated mice
It has been shown that the transmission defect between PRs and ON-BGs in the Lrit3+/− mouse had an impact on the visual perception of these mice.16 Thus, treated animals with a partial functional rescue as determined by ERG recordings and MEA were subjected to measurements of optomotor responses as described before.18,27 For the two types of treated mice, Lrit3+/−-BC-Lrit3 and Lrit3+/−-OPL-Lrit3, optomotor reflexes improved compared to untreated Lrit3+/− mice. However, the animal number for each category is low and no statistical analysis could be conducted. Strikingly, the Lrit3+/−-PR-Lrit3 mice revealed a statistically significant improvement of the optomotor responses, compared to untreated Lrit3+/− mice (n = 8), for the two lowest spatial frequencies under scotopic conditions (n = 4, p = 0.006 for the first spatial frequency, and p = 0.03 for the second; Figure 5). Under photopic conditions, untreated Lrit3+/− mice presented diminished optomotor responses compared to Lrit3+/+ mice (p = 0.001), and no statistically significant improvement of these responses were observed in Lrit3+/−-PR-Lrit3 mice compared to untreated Lrit3+/− mice (Figure S3).
DISCUSSION

Over 2 million people worldwide are affected by IRDs, yet no treatment is available for most cases. An FDA approved gene therapy product (Luxturna, Sparks Therapeutics) has been available for 3 years to treat one of the most severe IRDs, Leber Congenital Amaurosis caused by mutations in RPE65 gene. This milestone opened the way to develop additional gene therapies for other well-characterized IRDs.28–32 Here we aimed to restore a cCSNB phenotype, another IRD, by analyzing the Lrit3−/− mouse model.

As the name implicates, cCSNB is present since birth, does not evolve over time, and represents a signal transmission defect between PRs and ON-BCs.2 In addition, the retinal structure is preserved, e.g., the morphology of PR and BCs are largely normal; hence, treatment should be applicable at adult ages.2 However, two previously reported gene therapy approaches for cCSNB described partial functional rescue mainly in newborn mice (injection at P2 or P5 versus injection at P30 or P35).22,23 Given that, a gene therapy approach in newborn patients is less feasible than in adults, combined with the non-progressive nature of cCSNB prompted us to investigate functional restoration in the mouse model Lrit3−/− at the adult stage of P30. Gene therapy for cCSNB is difficult as proteins involved are part of a complex cascade and the localization and function of all of these is not clearly elucidated. However, our study shows that it is indeed achievable to obtain a strong rescue after treatment, at the protein level and at the functional level, in adult CSNB mice, most likely due to the use of a highly specific AAV capsid (AAV2.7m8), which has already been shown to efficiently transduce all retinal layers.26

Figure 4. ON-responses in treated retinas using MEA-256 recordings

(A) Spike density function for all responsive ganglion cells displaying an ON component (ON only and ON-OFF) recorded on all treated retina (1 Lrit3−/−-BC-Lrit3 [34 RGCs], 1 Lrit3−/−-OPL-Lrit3 retina [41 RGCs], 4 Lrit3−/−-PR-Lrit3 retina [148 RGCs], 6 Lrit3−/− retina [37 RGCs], and 6 Lrit3+/+ retina [315 RGCs]). Light stimuli are indicated as a black bar (light intensity at 4.1011 photons/cm2/s) and light gray area, responses recorded for individual ganglion cells are displayed as gray line (average of 10 repetitions), and the peak firing rate amplitude and latency are overlaid with the traces as colored open circle. Lrit3−/− recording have a different scaling (upper left) than all other conditions (middle). (B) ON peak firing rate (up) and ON peak latency (bottom) for all RGCs with ON responses in the different conditions. Horizontal black bar represents the average value, and vertical black bar the mean ± SD. (C) Fraction of the recorded ganglion cells displaying ON, ON-OFF, or OFF profile of response. Surprisingly, only 5.75% ± 4% of wild-type cells displayed OFF responses. Unresponsive ganglion cells (where spontaneous activity is recorded without light-evoked spiking) are not shown here (Lrit3+/+ = 220 cells, Lrit3−/− = 249 cells, Lrit3−/−-PR-Lrit3 = 369 cells, Lrit3−/−-BC-Lrit3 = 50 cells, and Lrit3−/−-OPL-Lrit3 = 41 cells).
Previously, LRIT3 was thought to be localized post-synaptically at the dendritic tips of ON-BCs in the OPL due to the isolated ON-BC defect observed in cCSNB patients and other proteins involved in cCSNB, localizing at the ON-BCs dendritic tips. Now expression data and the partial restoration of function in LRIT3 KO mice when rods are targeted suggest PR expression of LRIT3 and presynaptic localization. Although despite many studies, the pre- or post-synaptic localization of LRIT3 has not been clearly elucidated, it is certainly essential at the OPL in human and mouse retina. Thus, our approach included transgene expression of LRIT3. To achieve this goal, we tested different vector-promoter combinations in LRIT3−/− mice. Our findings revealed partial restoration of the cCNSB phenotype when LRIT3 was expressed in either ON-BCs, photoreceptors, or both. Therefore, the exact LRIT3 expression in one or the other retinal cell type remains unresolved. In addition, even though RNA in situ studies were already done before showing a strong expression of LRIT3 in the ONL, we are aware of a dog model lacking LRIT3, in which the function was also restored after targeting ON-BCs. This latest argues in favor of an expression of LRIT3 in ON-BCs even though leakiness of the vectors ending up in expression of the transgene in other retinal cells cannot be completely excluded. Taken together, our data demonstrate that functional restoration is obtained in adult mice when LRIT3 is localized at the OPL.

It became clear that using the PR-LRIT3 construct, a significantly better outcome was obtained, as 25% of mice injected with the PR-LRIT3 construct presented a strong scotopic ERG b-wave signal with significant restoration at the GC level upon MEA. Indeed, while the fraction of cells presenting ON-responses seems to increase using the LRIT3−/−-PR-LRIT3 condition, this is not the case for the other constructs, indicating a possible recruitment of more synapses with the expression of LRIT3 in photoreceptors. The amplitude of the rescue that we describe herein is greater than previously reported in age-matched mice, likely due to the highly efficient vector and promoter combination used. Yet, an even greater signal might be obtained by using subretinal injection, which still seems to be the ideal route of administration to target photoreceptors. Different reasons may account for the fact that restoration was only obtained in a significant number of animals when PR cells were targeted. One could be the predominant expression of LRIT3 in PRs, and thus targeting PRs re-establishes the normal pathway. However, our studies also showed partial restoration when ON-BCs were targeted, although in few animals. Delayed ON-responses at the level of RGCs were noted in these animals, which could potentially be due to the expression of LRIT3 in ON-BCs instead of PR where LRIT3 is probably naturally expressed, slowing down the process. However, the origin of these responses on the GC level is not fully understood since they could not be blocked by L-AP4. It may be a technical issue or of mechanistic origin. More treated mice with this construct would have been needed to make a real conclusion. The less efficient treatment with the BC-LRIT3 construct could also be explained by the fact that BCs are more difficult to target than PRs. This is especially the case in mature retinas (only 6% of injected mice presented a b-wave versus 25% for the PR-LRIT3 construct) while some functional rescue has already been described following BC targeting in pups. Again, since these observations were only noted in one animal, we cannot conclude on these hypotheses. In case of co-injection to target both ON-BCs and PRs, a stronger effectiveness was expected since both sides of the synaptic cleft were targeted; however, this was not the case. The effect of each construct might have been decreased by the co-injection, since each vector was diluted at 50% to have the same injection volume, compared to injection of only one construct.

Interestingly, function as measured by ERG and improved optomotor responses were only obtained under scotopic conditions, indicating that solely night vision was partially restored. In the study from

![Figure 5](https://example.com/figure5.png)
Secondary and possibly developmental effects might account for the lack of photopic restoration. Previously it was shown that cone synapses of Lrit3−/− mice have significantly less invaginating cone ON-BC dendrites compared to wild-type animals, indicating a role for LRT3 in the development and or maintenance of the cone synapse. Treatment of adult mice, where the synapse is fully formed, for LRT3 in the development and or maintenance of the cone synapse is not expected and was not studied. Furthermore, although our studies revealed LRT3 restoration of protein localization in treated animals, the staining was more present in the OPL close to rods than cones explaining a functional rescue solely under scotopic conditions. In addition, it did not seem that TRPM1 localization at the dendritic tips of cone ON-BCs was restored conversely to rod BCs. These observations confirm the probable greater remodeling capacity of these synapses compared to cone-to-cone BCs synapses, even in adult mice, as previously discussed by Wang and coworkers. This implication of CSNB proteins in synaptic development was also noticed in the Gmrgm8mNak mouse, in which invaginating dendrites of rod BCs are larger and often contain ectopic ribbons while the number of invaginating dendrites of cone ON-BCs and ribbons decrease at the cone pedicle, as observed in the Lrit3−/− mouse model. Other molecules implicated in the development of the ribbon synapse might be influenced at an early developmental stage by the absence of LRT3 and thus would explain the absence of rescue when treatment occurs at an adult stage. Treatment at a younger age using a promoter-targeting cone PRs and delivery of other molecules influenced by the absence of LRT3 might rescue the photopic phenotype in Lrit3−/− mice. Furthermore, it would be interesting to measure the function of cones or cone-BCs with transgenic LRT3 by patch-clamp recordings to see whether a functional rescue can be obtained, as previously described using a reporter gene. However, as there might be an influence of the reporter gene on the conformation and/or interactions of LRT3 and keeping in mind a future gene therapy approach for patients, we did not follow this approach.

Of note, even though they are the most used animal models, the photopic phenotype of cCSNB mice models is in general more severe than the one of patients or larger animal models. Indeed, no b-wave is measured in photopic conditions in any of the cCSNB mouse models while cone-driven responses are comparable between CNSB dogs, horses, and patients, e.g., mildly reduced; scotopic ERG responses are similar in all models. This difference of the cone ERG b-wave might be explained by the different cellular contribution that was noted between rodents and primates. For example, in the latter, OFF-BCs contribute to a large proportion of the photopic ERG b-wave responses while in the mouse photopic ERG, only a small contribution of the OFF-BCs was noted. Taken together, as different pathways could be implicated in the cone-driven responses in mice leading to a severer photopic phenotype and rendering a functional restoration under photopic conditions hardly achievable, mice models of CSNB might not be the ideal ones to assess cone-driven pathway restoration following treatment.

MATERIALS AND METHODS

Ethical statement

All animal procedures were performed according to the Council Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010, on the protection of animals used for scientific purposes, with the National Institutes of Health guidelines and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. They were approved by the French Minister of National Education, Superior Education and Research (authorization delivered on January 21, 2019). When possible, all mice showing a restoration on ERG after treatment went through optomotor responses measurements due to the use of a specific vector and promoter combination.

AAV production

The production of recombinant AAVs was made by following the plasmid cotransfection method. Lysates were then purified using iodixanol gradient ultracentrifugation as previously described: 40% iodixanol fraction was concentrated and buffer exchanged using Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore, Billerica, MA, USA). Real-time PCR was used to titer the vector stocks for DNase-resistant vector genomes relatively to a standard.

Intravitreal injections

Mice were anesthetized by isoflurane inhalation (5% in oxygen for induction and 2% for maintenance). Intravitreal injections in the right eyes were P30. Pupils were dilated (0.5% mydriaticum) and a 33-gauge needle was passed through the sclera at the ora serrata level. 1 µL of a viral stock solution at a concentration of 1.73 × 10^14 vg/mL maximum was injected directly in the vitreous cavity. The left eyes were injected with PBS1X.
Immunolocalization studies

Animals were sacrificed by CO₂ inhalation followed by cervical dislocation. Eyes were removed and dissected to keep the posterior part of the eyes, which were then fixed in ice-cold 4% paraformaldehyde for 20 min. Subsequently, the eye cups were washed in ice-cold PBS and cryoprotected by increasing concentrations of sucrose (ranging from 10% to 30%) in water and 0.12 M phosphate buffer for 1 h at 4°C for 10% sucrose and 20% sucrose solutions and overnight at 4°C under agitation for the 30% sucrose solution. The eye cups were then embedded in 7.5% gelatin and 10% sucrose, and the blocks were frozen at −40°C in isopentane and kept at −80°C until cutting. Sections of 12 μm were generated using a cryostat (MICROM HM 560, ThermoFisher Scientific, Waltham, MA, USA) and mounted on glass slides (Superfrost Plus, ThermoFisher Scientific). Mouse retina sections were treated to decrease background noise (Antigen Retrieval Reagent, Biotechnne, Minneapolis, MN, USA) for 4 min at 92°C and subsequently blocked for 1 h at room temperature in PBS1X 10% Donkey Serum (v/v), 0.1% Triton X-100. Primary antibodies and the dilutions used were as follows: rabbit anti-LRIT3 (1:200, Neuillé et al., 2015), guinea pig anti-mGluR6 (1:15,000, AP20134SUN, Acris, Herford, Germany), rabbit cone-arrestin (1:2,000, ab15282, Abcam, Cambridge, UK), mouse anti-PKCζ (1:1,000, P5704 Sigma-Aldrich, Darmstadt, Germany), lecin PNA 594 conjugate (1:1,000, L32459, Life Technologies, Grand Island, NY, USA), and sheep anti-TRPM1 (1:500).⁴⁹ The sections were incubated with primary antibodies diluted in PBS1X 2% Donkey Serum (v/v) and 0.1% Triton X-100 for 1 h at room temperature. After washes with PBS1X 0.1% Triton X-100, the sections were incubated with anti-rabbit and anti-sheep secondary antibodies coupled with Alexa Fluor 488, or Cy3 (Jackson ImmunoResearch) along with 4’,6-diamidino-2-phenylindole (DAPI), all used at 1:1,000, for 0.5 h at room temperature. Subsequently, the sections were coverslipped with mounting medium (Mowiol, Merck Millipore, Billerica, MA, USA). Fluorescence images retinal sections were acquired with a confocal microscope (FV1000, Olympus). Images for figures were handled with the ImageJ software (ImageJ Software).

Electroretinogram

Mice were DA overnight before performing the ERG recordings. They were anesthetized by ketamine (80 mg/kg) and xylazine (8 mg/kg) and eye drops were used to dilate their pupils (0.5% mydriaticum 5% neosynephrine) and anesthetize the cornea (0.4% oxybuprocaine chlorohydrate). Mice corporal temperature was maintained through a heating pad along the test. Upper and lower eyelids were retracted to keep the eyes opened and bulging. Corneal lenses (Mayo Corporation, Japan) were applied on corneal surface to record the ERG. A reference electrode was placed on the nose while the ground electrode was placed above the tail. Recordings from both eyes were made in parallel to compare infected to non-infected eyes. All scotopic ERG were made first using six increasing light intensity of flashes ranging from 0.003 to 30.0 cd. s/m². Each flash corresponding to one light intensity results from the average of five traces originating from five flashes. To ensure a saturation of rod PIs and the recording of cone-driven responses, we performed a 10-min light-adaptation step at 20 cd/m². Following this light-adaptation step, photopic ERGs were recorded first at 3.0 cd. s/m² and at the same intensity; 5 Hz and 10 Hz flickers were also checked. All data were analyzed with GraphPad Prism v.6 (GraphPad Software, La Jolla, CA, USA). The b-wave amplitude was manually calculated from the peak of the a-wave to the peak of the b-wave.

MEA

After overnight dark adaptation, mice were sacrificed by CO₂ inhalation followed by cervical dislocation. Retinas were carefully dissected under dim red light and conserved in Ames medium (Sigma-Aldrich, St. Louis, MO, USA) oxygenated with 95% oxygen and 5% CO₂. Retinas were placed on a Spectra/Por membrane (Spectra Laboratories, Rancho Dominguez, CA, USA) previously coated with poly-D-lysine and gently pressed against an MEA (MEA256 100/30 iR-ITO, Multi Channel Systems MCS, Reutlingen, Germany) using a micromanipulator, with RGCs facing the electrodes. Retinas were continuously perfused with bubbled Ames medium at 34°C at a rate of 1 to 2 mL/min and left to rest for 45 min before the recording session. Under dark conditions, 10 repeated full-field light stimuli at a 450 nm wavelength were applied to the samples at 4.10¹¹ photons/cm²/s for 2 s with 10 s interval by using a Polychrome V monochromator (Olympus, Hamburg, Germany) driven by an STG2008 stimulus generator (MCS). Raw RGC activity recorded by MEA was amplified (gain 1,000–1,200) and sampled at 20 kHz by using MC Rack software (MCS). The resulting data were stored and filtered with a 200-Hz high-pass filter. Raster plots were obtained by using a combination of threshold detection, template matching, and cluster grouping based on principal component analysis using Spike2 v.7 software (CED, Cambridge, UK). Peristimulus time histograms were plotted with a bin size of 50 ms by 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 light onset (for ON-responses) and in an interval of 2 s after light offset (for OFF-responses). These values were adjusted 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 ± 5 SD, 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 with GraphPad Prism v.6 (GraphPad Software, La Jolla, CA, USA). Detailed description on experiments with bath application of the mGlur6 agonist L-AP4 will be provided upon request.

Optomotor test

Optomotor test was performed as described previously.¹⁶ Mice were DA overnight before the optomotor test. Eight wild-type animals and eight knockout animals of each lineage were studied along with the treated animals (PR-Lrit3 n = 4, BC-Lrit3 n = 2, OPL-Lrit3 n = 1). Mice were placed on a grid platform (11.5 cm diameter, 19 cm above the bottom of the drum) at the center of a motorized drum (29 cm diameter) covered by vertical black and white stripes of a defined spatial frequency (0.063, 0.125, 0.25, 0.5, and 0.75 cycles per degree). A 5 min break was made before the test so the animal could get used to its new environment. The stripes were rotated for 1 min clockwise
and 1 min counterclockwise at a speed of 2 rotations per min. An interval of 10 s was made after the first min. Each test was recorded with a digital infrared camera to count head movements of the mice. Tests were first performed under scotopic conditions and then in photopic condition after 5 min of light adaptation (two lamps of 60 Watts). Head movements in both directions were considered to obtain the number of head movements per minute.

**Statistical analysis**

Mann-Whitney statistical analysis was performed for the scotopic ERG b-wave persistence and optomotor responses significance evaluation. We chose an unpaired t test and non-parametric test because the groups were not related and a Gaussian distribution of the data with the small number of animals involved was not expected. The star indicates a significant test (p < 0.05; Figure 5).

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.05.008.

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**AUTHOR CONTRIBUTIONS**

J.V. wrote the manuscript; G.G. wrote the MEA part of the manuscript; J.V., N.B., C.J., M.D., C.R., M.M.D.S.D., M. Neuillé, and C.M. performed the experiments; J.V. and G.G. (MEA) analyzed the results; J.-A.S., S.P., and D.D. co-supervised the study; M. Nassisi, I.A., and D.D. gave their input in the study design and manuscript writing; C.Z. designed the study, supervised the experiments, and corrected the manuscript writing.

**DECLARATION OF INTERESTS**

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

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