Targeting ON-bipolar cells by AAV gene therapy stably reverses

LRIT3-congenital stationary night blindness

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Keywords: congenital stationary night blindness, CSNB, ON-bipolar cells, *LRIT3*, AAV, animal model

Significance

Canine models of inherited retinal diseases have informed and advanced AAV gene therapies targeting specific cells, including photoreceptors in the outer retina, for treatment of blinding diseases in human patients. However, therapeutic targeting of diseases such as congenital stationary night blindness (CSNB) that exhibit defects in ON-bipolar cells (ON-BCs) of the mid-retina remains under-developed. Using a new *LRIT3* mutant canine model of CSNB exhibiting ON-BC dysfunction, we tested the ability of a cell-specific AAV capsid and promotor to specifically target ON-BCs for gene delivery. Notably, subretinal injection of AAV-*LRIT3* vector demonstrated safety and efficacy with robust and stable rescue of ERG signals and night vision up to 1 year, paving the way for clinical trials in CNSB patients.

Abstract

AAV gene therapies aimed at curing inherited retinal diseases to date have typically focused on photoreceptors and retinal pigmented epithelia within the relatively accessible outer retina. However, therapeutic targeting in diseases such as congenital stationary night blindness (CSNB) that involve defects in ON-bipolar cells (ON-BCs) within the mid-retina has been challenged by the relative inaccessibility of the target cell in intact retinas, the limited transduction efficiency of these cells by existing AAV serotypes, poor availability of established ON-BC-specific promoters, and absence of

33 appropriate patient-relevant large animal models. Here, we demonstrate safe and effective ON-BC
 34 targeting by AAV gene therapy in a recently characterized naturally-occurring canine model of CSNB,
 35 *LRIT3*-CSNB. To effectively target ON-BCs, new AAV capsid variants with ON-BC tropism and ON-
 36 BC specific modified GRM6 promoters were adopted to ensure cell-specific transgene expression.
 37 Notably, subretinal injection of one vector, AAV^{K9#4}-*sh*GRM6-c*LRIT3*-WPRE, significantly recovered
 38 rod-derived b-wave in all treated eyes (6/6) of adult dogs injected at 1-3 years of age. The robust
 39 therapeutic effect was evident 7 weeks post-injection and was sustained for at least 1 year in all
 40 treated eyes. Scotopic vision was significantly improved in treated eyes based on visually-guided
 41 obstacle course navigation. Restoration of *LRIT3* signals was confirmed by immunohistochemistry.
 42 Thus, we report on the first ON-BC functional rescue in a large animal model using a novel AAV
 43 capsid variant and modified promoter construct optimized for ON-BC specificity, thereby establishing
 44 both proof-of-concept and a novel translational platform for treatment of CSNB in patients with defects
 45 in photoreceptor-to-bipolar signaling.

46 Introduction

47 The vision process begins with photon capture by outer segments of rods and cones,
 48 photoreceptors within the outer retina. Subsequent release of neurotransmitters by photoreceptor
 49 synaptic terminals – rod spherules and cone pedicles – transfers this information to bipolar cell
 50 dendritic processes in the outer plexiform layer. Notably, distinct cell classes and signaling pathways
 51 allow for day and night vision. Specifically, night vision requires signaling from rod photoreceptors to
 52 ON-bipolar cells (ON-BCs), and activation of the mGluR6 signaling cascade at the dendritic tips of
 53 ON-BCs. In the dark, glutamate is released from photoreceptor terminals, binds to the mGluR6
 54 receptor, and activates the alpha subunit of a G-protein, G α . Dissociation of G-protein subunits
 55 culminates in the closure of TRPM1 channels, the key regulator of the mGluR6 signaling cascade¹.
 56 Several molecules, including LRIT3 and NYX, are critical in localizing TRPM1 to the ON-BC dendritic
 57 tip membrane². Inherited defects in the pathway, from photon capture, phototransduction,
 58 neurotransmitter release and mGluR6 signaling cause non-progressive vision impairing disorders,
 59 termed congenital stationary night blindness (CSNB), that are characterized by the absence of night
 60 vision from birth.

61 The genetically heterogeneous causation of CSNB³ underlies the spectrum of clinical
 62 presentations, broadly classified using electroretinography (ERG) as Riggs⁴ and Schubert-
 63 Bornschein⁵ forms of CSNBs. The Riggs type is caused by defects in the photoreceptors and is
 64 associated to date with four genes, *RHO*, *GNAT1*, *PDE6B*, and *SLC24A1*, in decreasing order of
 65 prevalence³. The Schubert-Bornschein type results from erroneous photoreceptor-to-bipolar cell
 66 signaling and is further subdivided into incomplete and complete CSNB⁶. Incomplete CSNB is
 67 associated with partial blockage of photoreceptor-to-bipolar cell signaling that results in some residual
 68 rod function. Defects in three genes, *CACNA1F*, *CABP4*, and *CACNA2D4*, all expressed pre-
 69 synaptically in the photoreceptor terminals are associated with incomplete CSNB³. Complete CSNB
 70 (cCSNB) is the most common form and patients exhibit characteristic ERG features including
 71 extinguished scotopic b-wave due to ON-BC dysfunction. Defects in five genes, *NYX*^{7,8}, *TRPM1*⁹⁻¹¹,
 72 *GRM6*^{12,13}, *GPR179*^{14,15}, and *LRIT3*¹⁶, all historically thought to localize post-synaptically in the ON-BC
 73 dendritic tips, are associated with cCSNB.

74 A series of cCSNB mouse models with ON-BC defects are categorized as ‘nob’ (no b-wave)
 75 mice³. Consistent with the non-progressive nature of CSNB, *Lrit3* deficient mice (*Lrit3*^{-/-}, *Lrit3*^{nob6}) have
 76 an intact outer nuclear layer (ONL)¹⁷, and the observed cone-specific disorganization of synaptic
 77 contacts support a role for LRIT3 in the transsynaptic communication between cones and ON-BCs¹⁸.
 78 As with other nob mouse models, *Lrit3*-deficient *nob6* mice only partially recapitulate the phenotype of

LRIT3-cCSNB human patients, due to unexpected loss of photopic b-waves in addition to expected loss of scotopic b-waves (**Table S1**)^{16,17}.

We have previously characterized a naturally-occurring canine model of CSNB with striking night blindness and normal day vision. Full-field ERGs showed a predominant loss of scotopic b-waves, consistent with Schubert-Bornschein type cCSNB¹⁹. A subsequent genome-wide association study and whole-genome sequencing identified an exonic 1bp insertion in *LRIT3*²⁰, the leucine-rich repeat Immunoglobulin-like transmembrane domain 3 gene associated with cCSNB in both patients¹⁶ and the murine model¹⁷. The canine *LRIT3* is predicted to have a signal peptide, two LRR (Leucine-rich repeats), two LRR type domains, and one each of LRRCT (LRR C-terminal), IGc2, FN3, and transmembrane domains. The canine *LRIT3* disease variant generated a premature stop codon, giving rise to a truncated *LRIT3* predicted to lack IGc2, FN3, and C-terminal transmembrane domains²⁰. *In vitro* studies showed subcellular expression and distribution of the truncated *LRIT3* was comparable to wild type (WT) *LRIT3*, indicating although non-functional it is unlikely to be toxic. Moreover, immunohistochemistry (IHC) analysis showed punctate *LRIT3* labeling in putative ON-BC dendritic terminals co-labeled with Go α in WT, but markedly decreased levels in the mutants²⁰, suggesting *LRIT3* augmentation might be an effective approach for therapeutic intervention. To target ON-BCs more effectively in mature retina, we screened a small pool of novel AAV capsid variants for ON-BC tropism in adult dogs. Herein we report the first ON-BC functional rescue in a large animal model using a novel AAV variant and a modified promoter, both designed for ON-BC specificity. We establish proof-of-concept and provide a translational platform for treatment of CSNB driven by defects in photoreceptor-to-bipolar signaling.

Results

Validation of new AAV capsid variants

Two AAV capsid variants, AAV^{K9#4} and AAV^{K9#12} were selected following a screen for ON-BC targeting in dogs (**Fig. S1, S2**) and tested in a non-human primate (NHP) using a long version of modified mGluR6 (*GRM6*) promoter (*IgGRM6*)²¹. The AAV^{K9#4} and AAV^{K9#12} capsids were each selected based on their superior capacity for ON-BC specific expression when injected via subretinal and intravitreal routes, respectively. *In vivo* cSLO imaging in WT dogs injected with AAV^{K9#4}-*IgGRM6-sfGFP* subretinally in one eye and AAV^{K9#12}-*IgGRM6-sfGFP* intravitreally in the contralateral eye (3x10¹³ vg/mL), showed strong sfGFP fluorescent signal in the subretinally-treated bleb area and in the central to mid-peripheral retina of the intravitreally-injected eye (**Fig. 1A**). Further, IHC confirmed tropism of both vectors to ON-BCs (**Fig. 1B, C**). Quantification of Go α -positive cells showed that

intravitreal AAV^{K9#12} transduced 34-86% (central), 0-45% (mid-periphery), and 0-3% (periphery) of ON-BCs (**Fig. 1C, D**). Transduction efficiency with AAV^{K9#4} in the subretinal bleb area was more homogeneous, ranging from 57% (periphery) to 80% (center) (**Fig. 1B, D**).

To assess applicability of the selected vectors in both subretinal and intravitreal routes of delivery, reporter constructs AAV^{K9#4}-*IgGRM6-sfGFP* and AAV^{K9#12}-*IgGRM6-tdTomato* were injected in a cynomolgus macaque. In one eye, both vectors were injected via routes per the canine study whereas injection routes were switched in the contralateral eye (i.e., AAV^{K9#4} intravitreally and AAV^{K9#12} subretinally) (**Fig. S3**). cSLO autofluorescence imaging performed 6 weeks post-injection showed distinct spatial pattern of sfGFP fluorescence between the two eyes. With subretinal injection, both vectors led to transgene expression in ON-BCs throughout the bleb/treated area with a higher transduction rate with AAV^{K9#4} (93%) than with AAV^{K9#12} (80%). Intravitreal injection of either vector led to expression of reporter proteins in ON-BCs in limited areas including the fovea and punctate areas as detected by cSLO. At the level of the fovea/perifovea, transgene expression in ON-BCs was more potent with AAV^{K9#12} than with AAV^{K9#4} when injected intravitreally.

Development of AAV-LRIT3 vectors and assessment of safety

To test effective targeting of ON-BCs in the canine CSNB model, therapeutic vectors harboring WT canine *LRIT3* were constructed. For efficient transgene expression in ON-BCs, two ON-BC-specific promoters were selected – the *IgGRM6* promoter (2.2.kb) used in prior testing with reporter genes, and its abbreviated version *shGRM6* (0.7kb), both developed and validated by Lu et al. for increased and specific transduction of ON-BCs compared to the commonly used 200En+SV40p promoter in mouse and NHP retinas²¹. Of the two, the *IgGRM6* promoter was reported to have greater transduction efficiency but reduced packaging capacity due to its length. To alleviate this challenge, two versions of the expression cassette were constructed. Cassette A utilized the *IgGRM6* promoter upstream of WT canine *LRIT3* cDNA and polyA signal (**Fig. 2A**). Cassette B instead utilized the *shGRM6* promoter, and to stabilize transgene expression, a Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was added in between the canine *LRIT3* and polyA signal (**Fig. 2B**). Each expression cassette was then packaged into AAV^{K9#4} and AAV^{K9#12} capsids, generating four AAV vector variants.

In a pilot study for evaluating initial tolerability, each vector was injected in control dogs via the intended route, i.e., AAV^{K9#4} subretinally and AAV^{K9#12} intravitreally, at doses considered high-end for intraocular injections (up to 1x10¹³ vg/mL) (**Table S2**). The two AAV^{K9#12} vectors were administered intravitreally as intended. The two AAV^{K9#4} vectors were administered via the intended subretinal route, although some intravitreal vector leakage occurred during subretinal injections and were recorded. Importantly, no acute or delayed adverse effects were observed for up to 10 weeks post-

injection in any of the control eyes as determined by a complete ophthalmic examination including indirect ophthalmoscopy and RetCam® fundus imaging.

Restoration of ERG b-wave following subretinal AAV^{K9#4}-shGRM6-cLRIT3-WPRE

To test therapeutic efficacy, the four AAV-*LRIT3* vectors were each injected via the intended route in CSNB dogs (**Table 1**). Of the 8 eyes/4 dogs injected in Cohort 1, two eyes that received subretinal injection of AAV^{K9#4}-shGRM6-cLRIT3-WPRE (**Fig. 3A**) showed significant scotopic b-wave recovery as early as 8 weeks post-injection, which remained stable at 51 weeks post-injection (**Fig. 3B, Fig. S5A**). Of these two eyes, a more dramatic ERG recovery was observed in the eye that received both subretinal delivery (150 µL) and inadvertent intravitreal leakage (200 µL) (left eye, CHACUG) compared to the eye with subretinal delivery only (left eye, K10) (**Fig. 3B**). None of the other vectors resulted in measurable ERG recovery and were hence deemed “non-therapeutic” and subsequent studies were focused on the “therapeutic” vector (AAV^{K9#4}-shGRM6-cLRIT3-WPRE).

Based on the greater b-wave recovery observed with concomitant intravitreal leakage, the effect of combined subretinal and intravitreal injections of the therapeutic vector was tested in Cohort 2. Two dogs received a combined subretinal and intravitreal injection in one eye and subretinal only in the other (**Table 1**). This time, there was no significant difference between the ERG b-wave recovery between the different routes of injection (subretinal + intravitreal vs. subretinal only), both resulting in comparable rod ERG b-wave rescue in one of the two dogs (**Fig. S5B**, K11). Across both cohorts, the eyes subretinally injected with the therapeutic vector had a rod-derived ERG b-wave amplitude that was significantly increased compared to eyes treated with other vectors/routes at each time point. The effect was evident as soon as 7 weeks post-injection ($p < 0.005$) and thereafter remained robust until at least 64 weeks post-injection ($p < 0.001$) (**Fig. 3C₁**). There was no consistent effect on cone-derived ERG associated with specific vectors and injection routes (**Fig. 3C₂, Fig. S6**).

Improved night vision in the eyes treated with subretinal AAV^{K9#4}-shGRM6-cLRIT3-WPRE

To evaluate the effect of gene therapy on scotopic visual function, injected animals were tested for their ability to navigate in an obstacle avoidance course under scotopic and photopic conditions, for each eye using an occluder. Because CSNB does not affect day vision, testing was done under the three dimmest scotopic conditions (0.003 lux, 0.009 lux, and 0.03 lux) to assess night vision while a photopic condition (65 lux) was used as the control. The CSNB control eye (**Fig. 4, Fig. S7**: white bar) had increased transit times and more collisions at the dimmest light intensity (0.003 lux) as expected from the disease¹⁹. The phenotype was less prevalent as the light intensity was increased under scotopic conditions (0.009 and 0.03 lux) and normalized at the photopic condition (65 lux). All six CSNB eyes subretinally injected with the therapeutic vector (AAV^{K9#4}-shGRM6-cLRIT3-WPRE) recovered night vision with significantly faster transit times and fewer collisions at the dimmest

light intensity (**Fig. 4, Fig. S7**: solid orange bar) (**Movie S1, S2**) consistent with the observed ERG recovery. Interestingly, eyes subretinally injected with the vector containing the longer promoter (AAV^{K9#4}-*IgGRM6-cLRIT3*) which did not result in ERG recovery, showed a modest trend of visual rescue at 0.003 lux (**Fig. S7**, solid blue bar). All other vectors injected intravitreally (**Fig. S7**, hatched orange bar) showed no improvement in night vision (**Movie S1, S2**). Strikingly, the recovered night vision was sustained until at least 43 weeks post-injection (**Movie S2**), indicating stability of the therapeutic effect. All CSNB eyes, regardless of the injections, demonstrated normal day vision at 65 lux (**Movie S1, S2**), as expected.

Restoration of LRIT3 in the outer plexiform layer of canine CSNB retina

To localize LRIT3 transgene expression in canine retinas injected with AAV vectors, IHC was performed using antibodies against LRIT3 as well as a variety of pre- and post-synaptic markers. Dogs with the best therapeutic response remain in life and are being followed to assess potential lifetime stability of functional rescue. However, one dog with only modest scotopic b-wave recovery in the eye that was subretinally injected with the therapeutic vector (**Fig. 3A, 3B**; K10, left eye) was sacrificed for immunohistochemical analysis of the retina along with controls. The LRIT3 signals were detected in the outer plexiform layer (OPL) in WT canine retina (**Fig. 5A, D, G**). In this group, LRIT3 labeling appeared to be associated with the synapses between the photoreceptor (PSD95) and second order neurons (Goα) (**Fig 5A**), present in synapses between rods (CtBP2) and rod ON-BCs (PKCα) (**Fig 5D**), and in synapses between cones (HCA) (**Fig 5G**) and all ON-BCs (Goα) (**Fig 5A**). This signal was greatly reduced with the CSNB phenotype²⁰. In the eye treated with subretinal injection of the therapeutic vector, within the bleb area, there was limited but distinct LRIT3 signal restored in the OPL, adjacent to both rod (**Fig. 5E**) and cone terminals (**Fig. 5H**). No signal was present outside of the bleb area in the same eye. The canine CSNB eye intravitreally injected with one of the non-therapeutic vectors (AAV^{K9#12}-*shGRM6-cLRIT3-WPRE*) (**Fig. S4, S5, S6**; dog CHACRY, left eye) had no evidence of punctate LRIT3 labeling (**Fig. 5C, F, I**). These findings indicate subretinal delivery of the therapeutic vector and expression of the transgene restores LRIT3 to the OPL and contributes to the structural functionality of the synapse by positioning itself to the normal location, at lower levels compared to WT.

Discussion

In a newly developed canine model of *LRIT3*-CSNB, we demonstrate safety and efficacy of an AAV gene therapy approach using novel AAV capsid variants and improved mGluR6 promoters, each designed to specifically target ON-BCs. Phenotypic reversal was assessed by ERG and vision-guided

behavior revealing functional restoration with 100% efficacy in all six eyes injected subretinally with the therapeutic vector AAV^{K9#4}-*shGRM6-cLRIT3*-WPRE, providing excellent proof-of-concept.

We based our approach on previously successful AAV gene therapies delivered to outer retinal cells (i.e., RPE, rod, cone) in canine IRD models²²⁻²⁸. Two novel AAV capsid variants that originated from a prior directed evolution screen conducted in WT dogs²⁹ were found to specifically target ON-BCs via intravitreal (AAV^{K9#12}) or subretinal (AAV^{K9#4}) injections in WT dogs. Subsequently, these same variants were shown to effectively transduce ON-BCs in NHP. These new variants constitute novel tools for gene therapy to treat patients with various forms of cCSNB, and for delivery of optogenetic channels to these mid-retinal cell populations in patients with advanced photoreceptor degeneration.

Combining each capsid with two different mGluR6 promoters, we tested four unique vector constructs, and identified a single “therapeutic vector” (AAV^{K9#4}-*shGRM6-cLRIT3*-WPRE) candidate. This therapeutic vector, when injected subretinally, consistently led to the significant and stable functional rescue in all six treated eyes. However, there were some noticeable variations in the degree of phenotypic rescue as quantified by recovery of scotopic b-wave amplitude. Possible injection-related factors affecting phenotypic rescue between eyes include inadvertent intravitreal leakage of vector during the subretinal injection procedure and/or location of the subretinal bleb relative to the area centralis.

The hypothesis that a combined subretinal and intravitreal delivery of the therapeutic vector provides superior efficacy to subretinal delivery alone remains to be confirmed. While the therapeutic vector was originally optimized for subretinal injection, its ability to transduce ON-BC via solely the intravitreal route remains to be tested. Nevertheless, in our experience, some degree of intravitreal deposition of vectors while performing subretinal injections is expected, and varying degrees of vector exposure interaction with vitreous humor is inevitable. It is therefore critical that vectors intended for subretinal delivery to be assessed both for toxicity risks and potential transduction benefits resulting from intravitreal leakage.

The current study was conceived prior to the report by Hasan et al. which proposed an alternative, pre-synaptic localization of LRIT3 in the mouse retina³⁰. While this localization may be mouse-specific, the gene therapy presented herein was designed for specific targeting of ON-BCs to deliver functional *LRIT3* in the canine *LRIT3*-CSNB model. The AAV vectors used were identified following a screen in WT dogs and selected for ON-BC tropism when administered by subretinal or intravitreal routes. Furthermore, the *shGRM6* promoter, derived as a modified version of the mGluR6 (*GRM6*) promoter, was developed and verified by Lu et al. for specific and increased ON-BC

expression in mouse and NHP retinas²¹. As well, in our study, subretinal injection of the vector-promoter-reporter constructs in NHP clearly demonstrated ON-BC expression.

While the exact cell of origin and species-specificity of LRIT3 expression in the retina is being resolved, functional rescue from specific targeting of ON-BCs via AAV gene therapy in the canine *LRIT3*-CSNB is striking and sheds light into the cellular origin as well as the optimal functional location of LRIT3. Hasan et al. reported ERG recovery in *Lrit3*^{-/-} mice receiving AAV-*Lrit3* targeted to the rod photoreceptors, but this was successful only with early treatment at P5, and with limited effect with late treatment at P30 (**Table S1**). Interestingly there was rod-specific transgene expression following the injections at either timepoints³⁰, suggesting that physical augmentation of LRIT3 in rods may not always be capable of restoring function in mature retina, thus limiting its translational potential. In a recent report by Varin et al., adult *Lrit3*^{nob6} mice were treated intravitreally using AAV2-7m8-*Lrit3*, targeting either ON-BCs or photoreceptors alone, or both (OPL) by interchanging the cell-specific promoters³¹. Restoration of LRIT3 and TRPM1 localization, and functional rescue were demonstrated in only a fraction (7-25%) of the treated mice (**Table S1**). While targeting of either or both cells resulted in a few responsive animals, rate and degree of functional recovery was greater with targeting the photoreceptors, followed by OPL and then ON-BCs, again pointing to potential species-specific differences. In our current work, functional recovery was achieved in adult *LRIT3*-CSNB dogs treated at ages ranging from 1.3 to 2.8 years, demonstrating therapeutic efficacy in mature retina with the CSNB phenotype in 100% (6/6) of eyes injected subretinally with the therapeutic vector targeting ON-BCs. Future work will evaluate if efficacy may be improved if *LRIT3*-CSNB animals are treated at a younger age, and if augmentation therapy is effective in animals older than 3 years of age.

The therapeutic vector was the only one of four vectors tested resulting in scotopic ERG b-wave functional rescue. However, of the three non-therapeutic vectors, AAV^{K9#4}-*IgGRM6-cLRIT3* injected subretinally in two dogs demonstrated modest improvement in scotopic vision-guided behavior parameters compared to controls despite having no detectable recovery of rod b-wave function. This indicates the *IgGRM6-cLRIT3* expression cassette may be functional albeit less efficient than the *shGRM6-cLRIT3*-WRPE cassette. In contrast, Lu et al. reported that the *IgGRM6* promoter was more efficient than *shGRM6*²¹, suggesting that other factors could affect the expression efficiency in our study, such as improved expression driven by WPRE or interference due to the expression cassette nearing maximum capacity, which was the case for *IgGRM6-cLRIT3*.

The mildly improved scotopic function in the eye receiving subretinal AAV^{K9#4}-*IgGRM6-cLRIT3* was only detectable by a vision-guided obstacle course navigation and not by ERG, indicating that the former outcome measure is more sensitive for detecting minor functional recovery with potential clinical relevance. This is consistent with the previous assessment of therapeutic outcome in Leber congenital amaurosis (LCA) patients receiving AAV therapy^{32,33}, and re-emphasizes the importance of

incorporating intuitive and straightforward vision-guided behavior as an outcome assessment of therapy. None of the vectors that utilized the AAV^{K9#12} capsid designed for intravitreal injection were therapeutically effective. While the initial screening of this AAV capsid variant was done in intact canine eyes, going forward pre-treatment of diseased eyes with vitrectomy or enzymatic vitreolysis³⁴ may improve access of the vector to the retinal layers.

We hereby demonstrate safety and efficacy of a novel AAV gene therapy designed to target ON-BCs in the canine *LRIT3*-CSNB model. Significant and consistent functional recovery was achieved up to 1.2 years post-injection as assessed by ERG and vision-guided behavior testing. This is the first patient-relevant data showing rescue of ON-BC function by gene augmentation therapy using a translationally suitable large animal model of *LRIT3*-CSNB. With intact retinal architecture and reliable and validated outcome measures available, the canine *LRIT3*-CSNB model has translational applicability for determination of optimal treatment strategy for CSNB patients. In addition, the model will serve more broadly as a platform to test therapeutic products which target OPL. As the evolving narrative for cell of origin of native *LRIT3* in mature retina is further refined, it will guide our quest to identify and test the most optimal therapeutic targets for the treatment of *LRIT3*-CSNB.

Materials & Methods

Ethics statement. The research was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, in full compliance with the USDA's Animal Welfare Act, Animal Welfare Regulations, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (IACUC# 803269), and Charles River Laboratories.

Animals. The *LRIT3*-CSNB model originated from a research colony that was used for disease characterization¹⁹ and mutation identification²⁰. The dogs in the current study are part of a newly established *LRIT3*-CSNB research line developed at the Retinal Disease Studies Facility (RDSF) of the University of Pennsylvania, and formed by outcrossing dogs from the original colony¹⁹ to WT dogs and to unrelated dogs that had been identified to harbor the *LRIT3* variant through molecular screening. Both control (WT and *LRIT3*-CSNB carrier) and affected dogs, including an *NPHP5*-LCA dog used in AAV vector screening, were bred at the RDSF or by a commercial laboratory animal supplier and maintained and housed at the RDSF under identical conditions of diet, medications, vaccinations, and ambient illumination with cyclic 12h ON-12h OFF. CSNB affected dogs were identified based on genotyping of the *LRIT3* genetic variant²⁰ and the characteristic loss of scotopic ERG b-wave¹⁹. A single adult male cynomolgus macaque housed at Charles River Laboratories,

315 Mattawan, MI, was used in this study to validate the ON-BC tropism of top performing variants
316 identified following a screen in WT dogs.

317 **Promoter and AAV capsid screen for bipolar cell targeting.** Eight different AAV capsid variants
318 originating from an AAV2-7-mer library that had undergone directed-evolution screens in dogs²⁹ for
319 their ability to target the outer retina after intravitreal injection were selected together with AAV2_{4YF+TV}
320 (used as a control) for this current study (**Table S3**). Each of these 9 capsids were used individually to
321 package a recombinant genome containing the ON-BC-specific promoter Ins4s-In3-200En-
322 mGluR500p (named thereafter *IgGRM6*)²¹ driving expression of sfGFP followed by a 3'-barcode
323 unique to that capsid. The vectors were diluted until the titers of all variants were equal, as confirmed
324 by quantitative real-time PCR, and then combined in equal ratios by adding equivalent volume of each
325 vector solution to a pool. A similar approach was used to produce a second pool containing the same
326 nine AAV capsids carrying sfGFP cDNA with the unique barcode but under control of the 4xGRM6³⁵
327 ON-BC-specific promoter. A third pool of the same AAVs was also made but with the Ple155³⁶ ON-
328 BC-specific promoter. The latter two pools showed lack of ON-BC specificity when injected intravitreal
329 or subretinal with AAV2_{4YF+TV} vector and were not developed further for this study.

330 **GFP-barcoded library screening in canine retina.** Seven weeks following subretinal (1 eye) and
331 intravitreal (2 eyes) injections in two WT dogs, biopsies of neuroretina taken from eyes enucleated
332 after humane euthanasia were collected from locations in which sfGFP fluorescence was detected by
333 cSLO. RNA was purified from these samples and deep sequencing was performed to quantify the
334 relative extents to which each capsid was capable of delivering and expressing its transgene in the
335 canine neuroretina from both the vitreous and subretinal space. Quantification of AAV capsid
336 performance revealed that AAV^{K9#12} and AAV^{K9#4} outperformed other variants when delivered via the
337 intravitreal and subretinal routes, respectively, and thus were selected for further validation.

338 **AAV-LRIT3 viral constructs.** To identify the most optimal expression cassette for robust and stable
339 therapeutic gene expression, AAV expression cassette variants *IgGRM6-cLRIT3* and *shGRM6-*
340 *cLRIT3-WRPE* were constructed (**Fig. 2**). In both variants, the therapeutic gene consisted of full-
341 length canine *LRIT3* (*cLRIT3*) cDNA, with a canonical Kozak sequence incorporated at the
342 translational initiation site. The *cLRIT3* sequence was cloned from WT canine retinal cDNA, verified
343 by Sanger sequencing, and corrected for polymorphic changes in amino acids to match those of the
344 canine reference genome (CamFam3.1) and of the consensus across mammalian species. Improved
345 mGluR6 promoters developed by Lu et al. aimed at targeting ON-BCs²¹ were provided by Dr. Z-H Pan
346 of Wayne State University. The *cLRIT3* transgene was placed under the control of either the original
347 full-length promoter (Ins4s-In3-200En-mGluR500P²¹, *IgGRM6*) or the shortened promoter (200En-
348 mGluR500P²¹, *shGRM6*). For transcript stabilization, a WPRE sequence was added downstream of
349 the transgene with the *shGRM6* promoter. This was not possible with the *IgGRM6* promoter due to

350 packaging capacity limitations. The two construct variants were each packaged into AAV^{K9#4} and
351 AAV^{K9#12} capsids selected via directed-evolution for subretinal and intravitreal injections, respectively,
352 as described above. AAV vectors were produced by triple plasmid co-transfection purified and titered
353 as previously described³⁷.

354 **Vector administration and post-injection treatment.** Under general anesthesia, viral vectors were
355 delivered by subretinal or intravitreal injections in control and affected canine eyes (**Table S2** and
356 **Table 1** show animals, vectors, and injection details in control and CSNB dogs, respectively), as well
357 as in a single NHP. Viral vectors were diluted in sterile balanced salt solution (BSS) and delivered
358 100-200 μ L per eye. A transvitreal approach was used without vitrectomy via *pars plana* by using a
359 custom-modified subretinal injector with a 39-gauge polyimide cannula (Retinaject; Surmodics, Inc.,
360 Eden Prairie, MN)^{38,39} under direct visualization through Machemer magnifying lens (OMVI; Ocular
361 Instruments Inc., Bellevue, WA, USA) using an operating microscope. Subretinal delivery was
362 directed at the superotemporal quadrant aiming to form a large uniform bleb, encompassing
363 approximately 20-40% of the retina (**Fig S4**), which was immediately visualized and documented by
364 fundus photography (Retcam shuttle; Natus Medical, Inc., Pleasanton, CA). Intravitreal delivery was
365 performed by deposition of viral solution in the posterior vitreous just pre-retinally at four sites
366 horizontally across the visual streak. Anterior chamber paracentesis was performed immediately post-
367 injection to prevent increase in intraocular pressure. The post-injection antibiotic and anti-
368 inflammatory routine treatment plan has been described previously²³.

369 **Ophthalmic examination.** Ophthalmic examinations included slit-lamp biomicroscopy, tonometry,
370 indirect ophthalmoscopy, and fundus photography. Any abnormalities, including any development of
371 inflammation or lesions, were documented. The ophthalmic examinations were conducted pre-
372 injection, 1- and 2-day post-injection, weekly for the following 4 weeks, bi-weekly for additional 8
373 weeks, then every 12-16 weeks up to 64 weeks post-injection.

374 **Electroretinography.** Recordings were conducted at pre-injection, then post-injection time points of
375 7-8, 15-16, 29-38, and 51-64 weeks after vector delivery. Pupils were dilated with topical atropine
376 sulfate 1% (Akorn, Inc., Lake Forest, IL), tropicamide 1% (Akorn, Inc.), and phenylephrine 10%
377 (Paragon Biotech, Portland, OR). After induction with intravenous propofol (Zoetis, Kalamazoo, MI),
378 dogs were maintained under general inhalation anesthesia (isoflurane 2-3%; Akorn, Inc.). Full-field
379 flash electroretinography (ERG) was performed on both eyes using a custom-built Ganzfeld dome
380 fitted with LED stimuli from a ColorDome stimulator (Diagnosys LLC, Lowell, MA). After 20 min of dark
381 adaptation, rod and mixed rod-cone-mediated responses to single 4 ms white flash stimuli of
382 increasing intensities (-3.7 to 0.5 log cd.s.m⁻²) were recorded. After 5 min of white light adaptation
383 (10.6 cd.m⁻²), cone-mediated responses to a series of single white flashes (-2.7 to 0.5 log cd.s.m⁻²)
384 and to 29.4 Hz flicker stimuli (-2.7 to 0.2 log cd.s.m⁻²) were recorded. Finally, photopic long-flash

385 ERGs were recorded using 200 msec white stimuli of 400 cd.m⁻² on a rod-suppressing white
386 background of 10.6 cd.m⁻² to assess the ON- and OFF-pathways. Statistical significance was
387 calculated by an unpaired t-test using GraphPad (GraphPad Software, San Diego, CA).

388 **Obstacle course vision testing**

389 Vision-guided navigation was tested in an obstacle avoidance course as previously described⁴⁰. An
390 abbreviated protocol was used for this study under the three dimmest scotopic conditions (0.003 lux,
391 0.009 lux, and 0.03 lux) and under ambient photopic condition (65 lux, fluorescent room light). Each
392 eye was tested individually by having an Aestek opaque corneoscleral shield (Oculo-plastik Inc.,
393 Montreal, Canada) placed over the ocular surface of the contralateral eye after topical anesthesia
394 (proparacaine 0.5%). All eyes were tested three times under each light intensity, and the position of
395 the 5 panels was randomly changed between each of the 3 trials/eye/illumination. The contralateral
396 eye was tested with the same set of panel positions. The eye to be tested was randomly selected
397 before the session. Animals were first dark-adapted for 20 min to the lowest ambient illumination
398 (0.003 lux) before running through the course. After testing under scotopic conditions, room
399 illumination was increased to the photopic brightness to light-adapt animals for 10 min and animals
400 were tested under photopic conditions in the same manner. Two digital Sony Handycam DCR-
401 DVD108 cameras (Sony, San Diego, CA) located above the obstacle course recorded navigation
402 performance of the dogs. The infrared imaging function of the camera enabled recording under the
403 dimmest light conditions. An experienced observer who was masked to experimental design reviewed
404 all videos and measured for each trial total number of collisions and transit time in seconds between
405 first forward motion at course entrance and moment the animal completely passed through the exit
406 gate. Vision testing was repeated at least three times on different dates post-injection to ensure inter-
407 testing consistency. An unpaired t-test was carried out using GraphPad (GraphPad Software, San
408 Diego, CA) to analyze statistical significance in number of collisions and transit time under each
409 illumination level between groups with a cut-off of $p < 0.0001$.

410 **Immunohistochemistry (IHC).** Preparation and processing of tissues for retinal morphology and IHC
411 have been detailed previously^{20,23}. Antibodies used included GFP (Millipore cat# MAB3580; Abcam
412 cat# ab290), tdTomato (SICGEN cat# AB8181-200), LRIT3 (Sigma-Aldrich, cat# HPA013454), pre-
413 synaptic markers PSD95 (BioLegend cat#810401), goat anti-human CAR (custom) and CtBP2 (BD
414 Biosciences, cat# 612044), and post-synaptic markers PKC α (BD Biosciences, cat# 610107) and Goa
415 (Millipore cat# MAB3073). Slides were examined with a Nikon A1R confocal microscope (Nikon
416 Instruments Inc., Melville, NY). Digital images were captured and processed using the NIS Elements
417 (Nikon Instruments Inc., Melville, NY) and ImageJ software (National Institutes of Health).

418

Acknowledgements. We are grateful to Dr. Mineo Kondo of Mie University, Mr. Kazuhiko Sakai and Mr. Takehiro Aihara of Kitayama Labes, Japan, for providing foundation dogs for the establishment of the canine CSNB research colony. Additional foundation dogs were generous donations from Labcorp Drug Development, formerly Covance. The *IgGRM6* and *shGRM6* promoters were kindly provided by Dr. Zhuo-Hua Pan of Wayne State University. We also thank Ms. Courtney Spector, formerly of University of Pennsylvania for technical assistance, Dr. András Komáromy, Michigan State University, for procedural instructions, Dr. Leslie King, University of Pennsylvania, for critical review of the manuscript. This study was supported, in part, by Margaret Q. Landenberger Research Foundation (KM), NEI/NIH grant EY-006855 (GDA/KM), Foundation Fighting Blindness (GDA/WB), the Van Sloun Foundation for Canine Genetic Research (GDA), and the Sanford and Susan Greenberg End Blindness Outstanding Achievement Prize (GDA).

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522

Table 1. Injection details of efficacy testing in *LRIT3*-CSNB dogs

								Dose level						
	Dog ID	LRIT3 g'type	Sex	Age@ inj (y)	Eye	Route	Vector	vg/mL	mL	vg/eye	ERG rescue	PI months at tissue collection		
Cohort 1	K6	-/-	M	1.6	OD	IVT	AAV ^{K9#12} -lgGRM6-cLRIT3	1x10 ¹³	0.20	2.0 x 10 ¹²	N	13		
					OS	SR	AAV ^{K9#4} -lgGRM6-cLRIT3	1x10 ¹³	0.15	1.5 x 10 ¹²	N			
	CHACRY	-/-	M	2.8	OD	IVT	AAV ^{K9#12} -lgGRM6-cLRIT3	1x10 ¹³	0.20	2.0 x 10 ¹²	N	13		
					OS	IVT	AAV ^{K9#12} -shGRM6-cLRIT3-WPRE	1x10 ¹³	0.20	2.0 x 10 ¹²	N			
	CHACUG	-/-	M	2.8	OD	SR	AAV ^{K9#4} -lgGRM6-cLRIT3	1x10 ¹³	0.15	1.5 x 10 ¹²	N	n/a		
					OS	SR*	AAV ^{K9#4} -shGRM6-cLRIT3-WPRE	1x10 ¹³	0.15 +0.20(IVT)	3.5 x 10 ¹²	Y			
Cohort 2	K10	-/-	M	1.3	OD	IVT	AAV ^{K9#12} -shGRM6-cLRIT3-WPRE	1x10 ¹³	0.20	2.0 x 10 ¹²	N	13		
					OS	SR	AAV ^{K9#4} -shGRM6-cLRIT3-WPRE	1x10 ¹³	0.15	1.5 x 10 ¹²	Y			
	K11	-/-	M	1.6	OD	SR	AAV ^{K9#4} -shGRM6-cLRIT3-WPRE	1x10 ¹³	0.10	1.0 x 10 ¹²	Y	15		
					OS	SR*	AAV ^{K9#4} -shGRM6-cLRIT3-WPRE	1x10 ¹³	0.13 +0.12(IVT)	2.5 x 10 ¹²	Y			
	K16	-/-	M	1.3	OD	SR*	AAV ^{K9#4} -shGRM6-cLRIT3-WPRE	1x10 ¹³	0.10 +0.15(IVT)	2.5 x 10 ¹²	Y	n/a		
					OS	SR	AAV ^{K9#4} -shGRM6-cLRIT3-WPRE	1x10 ¹³	0.10	1.0 x 10 ¹²	Y			

IVT, intravitreal injection; **OD**, right eye; **OS** left eye; **PI**, post-injection; **SR**, subretinal injection

*SR injection with notable IVT leakage

Figure 1: Targeting of canine ON-BCs with top performing AAV capsid variants.

A) cSLO images (BAF mode) showing GFP fluorescence 6.6 weeks after intravitreal injection of AAV^{K9#12}-*IgGRM6-sfGFP* (0.38 mL at 2×10^{13} vg/mL) in the right eye, and subretinal injection of AAV^{K9#4}-*IgGRM6-sfGFP* (0.15 mL at 3×10^{13} vg/mL) in the left eye. Dashed red line shows approximate location of retinal cryosections used for IHC and shown in B-C. **B-C**). Retinal cryosections showing native sfGFP fluorescence and Go α immunolabeling. **D**) Percentage of ON-BCs (Go α -positive) that co-express sfGFP transgene in various retinal locations (n=3 technical replicates/eye). OD, right eye; OS, left eye; S, superior; I, inferior; T, temporal; N, nasal; ONL, outer nuclear layer; INL, inner nuclear layer.

Figure 2. AAV expression cassette variants including GRM6 promoter variants and canine LRIT3 transgene.

Schematic diagram of **A**) AAV-*IgGRM6-cLRIT3* and **B**) AAV-*shGRM6-cLRIT3-WPRE* cassettes consisting of enhancers (Introns, 200En) and the GRM6 promoter driving canine *LRIT3*. Due to limitations in packaging capacity, vector A lacks WPRE while vector B lacks the Introns and a large part of the enhancers.

Figure 3. Subretinal AAV-*LRIT3* injection and ERG recovery in CSNB canine eyes.

A) Subretinal blebs in the left eye of dogs CHACUG and K10 that received subretinal injection of the therapeutic vector AAV^{K9#4}-shGRM6-cLRIT3-WPRE. Note the injection variables between the two eyes such as bleb location as well as intravitreal vector leakage (200 μ L) that occurred in CHACUG. +: canine fovea-like region. **B)** Rod-derived ERG b-wave recovery in treated eyes shown in A). Pre-injection ERG traces demonstrate typical CSNB phenotype with diminished b-wave in both eyes. The eyes receiving subretinal injection of the therapeutic vector show significant b-wave recovery as early as 7 weeks post-injection, with stability up to 64 weeks post-injection. **C)** Rod-derived ERG b-wave (C₁) and cone-derived 29Hz flicker response (C₂) pre- and post-injection over time, up to 64 weeks. The eyes subretinally injected with the therapeutic vector (n=6, orange bar) had significant (*p<0.005, **p<0.001) and stable rod-mediated ERG b-wave recovery compared to all other vector/routes (n=6, grey bar) at each post-injection time points. SR, subretinal injection.

A. Transit time

B. Number of collisions

Figure 4. Improved night vision in CSNB canine eyes treated with subretinal AAV^{K9#4}-shGRM6-cLRIT3-WPRE.

Visually-guided navigation tested per eye in an obstacle course in six *LRIT3*-CSNB dogs (12 eyes) injected with one of the AAV vector variants in each eye. **A)** The transit time was significantly reduced following subretinal injection of the therapeutic vector (AAV^{K9#4}-shGRM6-cLRIT3-WPRE) (6 eyes) compared to the eyes injected with all other vector/routes (6 eyes) or BSS-injected control CSNB eyes at the two dimmest scotopic conditions, 0.003 and 0.009 Lux (*p<.0001). The transition times at 65 Lux (photopic) were minimally affected by the CSNB phenotype and, as such, comparable across different therapeutic and control groups. The dotted lines define the 95% confidence interval for transit time in 4 WT control dogs. **B)** The number of collisions was significantly reduced at 0.003 and 0.009 Lux in eyes subretinally injected with the therapeutic vector (6 eyes) compared to CSNB eyes injected with all other vector/routes (6 eyes) or BSS (*p<.0001). No collisions are observed in WT dogs. While the bars represent the combined data of three available time points 18-50 weeks after the injection, there were no significant changes in vision testing outcomes over time. SR, subretinal; IVT, intravitreal.

Fig 5. Restoration of LRIT3 in OPL of canine CSNB retina treated with subretinal AAV-LRIT3.

WT and CSNB canine retinas immunolabelled with LRIT3 together with a variety of pre- and post-synaptic markers. WT canine retina (A, D, G) exhibits punctate LRIT3 signals in OPL adjacent to rod (D1) and cone (G1) terminals. LRIT3 signal is restored, albeit to a limited amount, in the bleb area of canine CSNB retina treated with subretinal AAV-LRIT3 and showing ERG recovery (B, E, H). Restored LRIT3 signal is present adjacent to the synaptic terminals of both rods (E, arrows) and cones (H, arrowhead). Canine CSNB retina treated with intravitreal AAV-LRIT3 without ERG recovery (C, F, I) lacked the LRIT3 signals in OPL. IVT, intravitreal; SR, subretinal.