Intravitreal delivery of a novel AAV vector targets ON bipolar cells and restores visual function in a mouse model of complete congenital stationary night blindness

Miranda L. Scalabrino1, Sanford L. Boye1, Kathryn M. H. Fransen3, Jennifer M. Noel4, Frank M. Dyka1, Seok Hong Min1, Qing Ruan1, Charles N. De Leeuw6,7, Elizabeth M. Simpson6,7, Ronald G. Gregg5, Maureen A. McCall3,4, Neal S. Peachey8,9,10 and Shannon E. Boye1,2,*

1Department of Ophthalmology and, 2Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, FL 32610, USA, 3Department of Ophthalmology and Visual Sciences, 4Department of Anatomical Sciences and Neurobiology and, 5Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, KY 40202, USA, 6Centre for Molecular Medicine and Therapeutics at the Child and Family Research Institute, University of British Columbia, Vancouver, BC, Canada V5Z 4H4, 7Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada V6T 1Z3, 8Louis Stokes Cleveland VA Medical Center, Cleveland, OH 44106, USA, 9Cole Eye Institute, Cleveland Clinic, Cleveland, OH 44195, USA and 10Department of Ophthalmology, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH 44195, USA

*To whom correspondence should be addressed at: University of Florida, PO Box 100284, Gainesville, FL 32610, USA. Tel: +1 3522739342; Fax: +1 3523922062; Email: shannon.boye@eye.ufl.edu

Abstract

Adeno-associated virus (AAV) effectively targets therapeutic genes to photoreceptors, pigment epithelia, Müller glia and ganglion cells of the retina. To date, no one has shown the ability to correct, with gene replacement, an inherent defect in bipolar cells (BCs), the excitatory interneurons of the retina. Targeting BCs with gene replacement has been difficult primarily due to the relative inaccessibility of BCs to standard AAV vectors. This approach would be useful for restoration of vision in patients with complete congenital stationary night blindness (CSNB1), where signaling through the ON BCs is eliminated due to mutations in their G-protein-coupled cascade genes. For example, the majority of CSNB1 patients carry a mutation in nyctalopin (NYX), which encodes a protein essential for proper localization of the TRPM1 cation channel required for ON BC light-evoked depolarization. As a group, CSNB1 patients have a normal electroretinogram (ERG) a-wave, indicative of photoreceptor function, but lack a b-wave due to defects in ON BC signaling. Despite retinal dysfunction, the retinas of CSNB1 patients do not degenerate. The Nyx<sup>−/−</sup> mouse model of CSNB1 faithfully mimics this phenotype. Here, we show that intravitreally injected, rationally designed AAV2(quadY-F +T-V) containing a novel 'Ple155' promoter drives either GFP or YFP_Nyx in postnatal Nyx<sup>−/−</sup> mice. In treated Nyx<sup>−/−</sup> retina, robust and targeted Nyx transgene expression in ON BCs partially restored the ERG b-wave and, at the cellular level, signaling in ON BCs. Our results support the potential for gene delivery to BCs and gene replacement therapy in human CSNB1.
Introduction

Retinal ON bipolar cells (ON BCs) depolarize in response to light-induced reduction of photoreceptor glutamate release and form the primary excitatory conduit between photoreceptors and retinal ganglion cells (RGCs). Mutations in genes that encode proteins involved in the ON BC metabotropic glutamate receptor 6 (mGluR6) G-protein-coupled signaling cascade are responsible for various forms of complete congenital stationary night blindness (CSNB1) (1). Patients with CSNB1 present in early childhood with impaired night vision and can be diagnosed by ERG, where they display a distinctive absence of the ON BC-driven b-wave, while maintaining a normal photoreceptor a-wave (2). CSNB1 patients also exhibit other phenotypes including severe myopia, strabismus and nystagmus (3). Mouse models harboring mutations in homologous CSNB1 genes such as Grm6 (4–8), the non-selective cation channel TRP melastatin 1 (Trpm1) (9–12), the orphan G protein receptor (Gpr179) (13,14) and two leucine-rich repeat proteins, nyctalopin (Nyx) (15–17) and Lrit3 (18,19), faithfully recapitulate the no b-wave ERG phenotype seen in CSNB1 patients and collectively will be referred to as ‘nob’ mice. These proteins are all localized to the dendritic tips of ON BCs, and their mouse models have been used to interrogate the role of each in the mGluR6 cascade (Fig. 1). From these studies, we know that in the absence of light, glutamate released from photoreceptors binds to the metabotropic mGluR6 (20,21) and activates the alpha subunit of a G protein, Gαo. Dissociation of G-protein subunits and culminates in closure of the TRPM1 cation channel (24–26). NYX is required for the correct localization of TRPM1 (27), whereas GPR179 mediates channel sensitivity (28). LRT3 is also important for the correct localization of TRPM1 (27,29,30). Despite the disruption in ON BC signaling, mutations in these cascade proteins have no effect on retinal morphology in CSNB1 patients or respective mouse models (1). This suggests that they are good candidates for gene replacement therapy.

Adeno-associated virus (AAV) has demonstrated remarkable success both in clinical trials for inherited retinal disease and in a multitude of proof of concept experiments (31–37). Retinal pigment epithelium and photoreceptors are efficiently targeted by various AAV serotypes following delivery to the subretinal space. RGCs are efficiently transduced following vitreal delivery (38,39). Transduction of inner nuclear layer cell types (e.g. ON BCs) within non-degenerate retinas and selective transgene expression in ON BCs has proved challenging. This is due to the inability of most AAV serotypes to efficiently traffic to cells of the inner retina. To date, AAV has been used only to deliver xenogeneic microbial opsins, an artificial iGluR6-based optical switch, or melanopsin-derived light sensors to ON BCs, all with the goal of conferring light sensitivity to second-order neurons in mouse models lacking photoreceptors (40–44). In all instances, transgene expression was driven in ON BCs by a Grm6 enhancer sequence fused to the SV40 promoter. Because these studies were conducted in rodent models exhibiting advanced photoreceptor degeneration, it is unclear whether the Grm6/SV40 promoter would be suitable for driving selective expression in ON BCs in non-degenerate, stationary models such as nob mice.

To this end, we developed a novel AAV vector and cellular promoter combination capable of driving efficient and targeted transgene expression in ON BCs of non-degenerate mouse retina following intravitreal injection. We show that AAV2 (quadY-F+T-V)-Ple155 mediates transgene expression in ON BCs and restores ON BC signaling in C57BL/6J wild type (WT) and in the Nyx<sup>nob</sup> mouse model of CSNB1. This is the first demonstration that an inherent genetic defect of ON BCs can be corrected with AAV-based gene therapy. Our results have implications for the development of gene therapies for NYX and other forms of CSNB1 and for non-invasive optogenetic manipulation of ON BC signaling.

Results

AAV2(quadY-F+T-V)-Ple155 drives robust and targeted transgene expression in ON BCs

We previously identified an AAV2-based capsid variant capable of transducing the majority of retinal cells following intravitreal injection in mouse (39). We combined this variant with our recently described human MiniPromoter, ‘Ple155’, that promotes transgene expression in BCs of single-copy knock-in mice (45), and evaluated its ability to drive GFP expression in BCs of C57BL/6J (WT) and Nyx<sup>nob</sup> mice following either subretinal or intravitreal injection (Fig. 2). In adult WT mouse retina, AAV2 (quadY-F+T-V)-Ple155-mediated GFP expression was targeted to BCs. Transduction by subretinal delivery was inefficient (Fig. 2A) compared with intravitreal injection in either WT or Nyx<sup>nob</sup> retina (Fig. 2B and C). AAV-mediated GFP expression colocalized with protein kinase C alpha (PKCα) (Fig. 2A/C), a marker of rod BCs, as well as Purkinje cell protein 2 (PCP2) (Fig. 2B), a marker of rod and a subset of cone ON BCs and the source gene for the Ple155 MiniPromoter design (45). GFP expression was observed...
in middle retina, with fluorescence localized to the somas and dendritic tips of ON BCs. GFP was also seen in the axon terminals of the ON BCs and was noted in some (albeit very few) RGCs in the inner retina. Intravitreal injection results in RGCs being exposed to a high concentration of vector particles. Even photoreceptor-targeted promoters such as GRK1 mediate ‘off-target’ GFP expression in RGCs when incorporated into AAV vectors delivered intravitreally at a high titer (39). Expression in ON BCs was widespread throughout the retinas of intravitreally injected mice (Fig. 2D). In contrast to the transduction achieved with an AAV2-based vector, intravitreal injection of AAV8(Y733F)-Ple155-GFP failed to promote transduction of ON BCs (Supplementary Material, Fig. S1).

We compared the relative abilities of Ple155 and the previously described Grm6/SV40 enhancer/promoter combination to drive transgene expression exclusively in ON BCs in a non-degenerative retina. AAV8(Y733F)-Grm6/SV40-hChR2-eGFP delivered subretinally to WT mice promoted off-target GFP expression primarily in photoreceptors (Supplementary Material, Fig. S2) (40). For its increased specificity relative to Grm6/SV40 in non-degenerative retina, the Ple155 promoter was incorporated into all further experiments.

**Intravitreally injected AAV2(quadY-F+T-V)-Ple155 restores Nyctalopin expression to Nyxnob ON BCs**

Toward development of a clinically relevant approach, we examined the expression pattern of Nyctalopin (Nyx) following postnatal delivery with our AAV2(quadY-F+T-V)-Ple155 vector. We used the identical YFP_Nyx fusion cDNA used to generate a transgenic mouse that rescued the Nyxnob phenotype (46). Intravitreal injection in P2 Nyxnob mice with AAV2(quadY-F+T-V)-Ple155-YFP_Nyx (Fig. 3A) on the dendritic tips of ON BCs (Fig. 3B and C). These data confirm reports of NYX localization to synaptic sites (15,17,46–49). There was also expression of AAV-mediated YFP_NYX in ON BC soma and axon terminals. These locations are likely to be ectopic expression due to over-expression by AAV within some ON BCs. The number of transduced ON BCs per P2-treated retina was quantified relative to the total number of ON BCs (determined by anti-PCP2 co-expression) in representative cross sections by three independent observers. The mean percentage of ON BC transduction in P2-treated Nyxnob mice was 21.5%.

Intravitreal injections in P30 WT and Nyxnob mice (Fig. 3D and E) produced targeted expression in ON BCs, although the percentage was markedly lower compared with the P2 injections.

**Nyttalpin gene replacement in Nyxnob mice partially rescues the electroretinogram (ERG) b-wave**

We evaluated the extent of ON BC function within the entire retina using the ERG. In Nyxnob mice treated at P2, ERGs were recorded at P32. ERG b-wave responses from eyes injected with AAV2(quadY-F+T-V)-Ple155-YFP_Nyx were compared with untreated contralateral eyes or eyes injected with control vector, AAV2(quadY-F+T-V)-Ple155-GFP. The dark-adapted ERG responses recorded from untreated Nyxnob control eyes (Fig. 4Ai, black tracings) were identical to previous reports (16,46). These ERGs had an overall negative polarity. In comparison, dark-adapted ERG responses in all P2 AAV-treated Nyxnob eyes had a clear positive component (Fig. 4Ai, black tracings). Arrows indicate the ERG response components restored by AAV treatment. These data reflect a rescue of signaling through rod ON BCs. Rescue was most easily seen in response to low-luminance stimuli (e.g. –3.6 to –2.4 log cd s/m²), where the untreated Nyxnob ERG is essentially flat. Figure 4B summarizes the magnitude of the rescue of the dark-adapted b-wave to the –2.4 log cd s/m² stimulus. There was no measurable b-wave response from untreated or AAV-GFP-treated eyes (Fig. 4B). ERG improvements were significant (P < 0.000001). As might be expected from the limited expression in Nyxnob mice injected with AAV2(quadY-F+T-V)-Ple155-YFP_Nyx at P30, we found no rescue of their ERG b-wave (data not shown, n = 5 retinas).

The dark-adapted responses indicate that AAV treatment rescued function in rod ON BCs. To determine if cone ON BCs were similarly affected, we recorded light-adapted ERGs with stimuli superimposed upon a steady rod-desensitizing field. Again, cone ERGs recorded from Nyxnob control eyes were identical to previous reports (16,46,50). Figure 4Aii illustrates the light-adapted ERG responses from a mouse with a large rescue effect. Arrows indicate the response component restored by AAV treatment. As in the example, the ERGs in AAV-treated eyes included components that resemble WT, including a waveform ‘deflection’ (oscillatory potential). None of these were present in untreated eyes. Finally, the magnitude of the light-adapted ERG correlated with the magnitude of dark-adapted b-wave (r = 0.92).
Gene replacement restores TRPM1 localization to dendritic tips of ON BCs and completely rescues ON BC TRPM1 channel gating

We previously showed that the expression of TRPM1 is lost in Nyxnob mice (27). Although the function of NYX is still incompletely understood, it is clearly required to localize TRPM1 to the membrane of ON BC dendritic tips. The expression of YFP_NYX and TRPM1 (Fig. 5) in whole-mount retina from an AAV-treated Nyxnob mouse shows that many ON BCs that express YFP_NYX (green) also express TRPM1 (red). As previously noted for TRPM1, expression is found both in ON BC cell bodies as well as on their dendritic tips (Fig. 5A). Co-expression is appreciable in single ON BCs (Fig. 5B), with robust YFP_NYX and TRPM1 expression.
ON BCs expressing YFP_NYX were targeted in retinal slices for whole-cell patch-clamp recordings and subsequently filled with sulforhodamine for morphological identification (Fig. 6A). Exogenous application of capsaicin, which directly gates the TRPM1 channel, resulted in robust outward currents in WT and AAV-treated Nyxnob rod ON BCs (Fig. 6B). In contrast, only a small outward current was evoked in Nyxnob rod ON BCs lacking YFP_NYX expression (Fig. 6B). This outward current is similar to residual currents recorded in Trpm1−/− ON rod BCs (28). The summary data show that the response amplitudes of Nyxnob rod ON BCs expressing AAV-mediated YFP_NYX are similar to WT and differ significantly from rod ON BCs without YFP_NYX, some of which were recorded in the same retinas (Fig. 6C).

**AAV-mediated Nyx transcript is present in treated mice**

To determine the relative level of AAV-mediated YFP_Nyx transcript in early (P2) versus late (P30) treated Nyxrob mice, we performed quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) on RNA extracted from P2- and P30-treated Nyxrob retinas, as well as age-matched un.injected controls (retinas were extracted 30 days post-injection). Primers were designed to flank the 3' Nyx gene and 5' SV40 polyA signal with the probe binding within the first quarter of the amplicon (Table 1). Polymerase chain reaction (PCR) produced the appropriately sized amplicon in AAV-treated Nyxnob retina, but not in untreated Nyxrob or C57BL/6J retinas as expected (data not shown). AAV-mediated YFP_Nyx transcript was present in both P2- and P30-injected retinas and was normalized to Gapdh for comparison (Fig. 7). Despite the relatively low number of ON BCs expressing YFP_NYX in P30-treated mice (Fig. 3), YFP_Nyx transcript was relatively more abundant in P30- than P2-treated Nyxrob mice (Fig. 7). The presence of transcript in P30-treated sample suggests efficient transgene delivery and that the hurdle to achieving robust NYX expression in P30-treated Nyxrob mice is likely to be occurring post-transcription.

---

**Figure 5.** TRPM1 localization is restored to membranes of ON BC dendritic tips in treated Nyxnob mice. Top-down view of treated retina showing TRPM1 (red) and YFP_NYX (green, GFP antibody) in cell bodies (A, top row) and dendritic tips (A, middle row). Dendritic tip colocalization is also shown in retinal cross section (A, bottom row) and a representative ON BC at high magnification (B). OPL = outer plexiform layer, INL = inner nuclear layer. Scale bars = 10 μm (A) and 5 μm (B).
Discussion

Coordinated interaction of proteins within the mGluR6/GRM6 cascade ensures maintenance of a functional photoreceptor to ON bipolar synapse (Fig. 1). Binding of glutamate to GRM6 in the dark keeps the TRPM1 cation channel closed. In response to light, TRPM1 channels reopen, causing depolarization of ON BCs and the formation of the ERG b-wave. Proper localization of TRPM1 to the dendritic tip membrane depends on expression of NYX, although the TRPM1 protein remains expressed in the ON BC somas of Nyxnob mice (27,29). While there is conflicting evidence, TRPM1 expression also may require expression of GRM6 (7,29). Finally, the absence of LRIT3 also leads to an absence of TRPM1 in dendritic tips (30), but it is not known if this is a direct consequence or results from a change in NYX expression.

Our finding that AAV2(quadY-F+T-V)-Ple155-mediated YFP_NYX expression restored TRPM1 expression to the membrane of ON BC dendritic tips in AAV-treated Nyxnob mice is consistent with previous results (46). In addition, we show that this restoration of properly localized TRPM1 to the dendritic tips of rod BCs corresponds to full restoration of the rod BC’s response to TRPM1 agonist, capsaicin. At the level of the entire retinal response, AAV2(quadY-F+T-V)-Ple155-mediated YFP_NYX expression partially restores the ERG b-wave in treated Nyxnob mice. The mean b-wave amplitude in P2-treated eyes was ∼40 µV, while age-matched C57BL/6J responses are ∼675 µV (51). The fact that we observed full restoration of single-cell response amplitudes is consistent with our immunohistological results that ∼22% of ON BCs expressed AAV-mediated YFP_NYX. Taken together, the data indicate that the subset of infected ON BC cells are responsible for the b-wave response.

Table 1. qRT-PCR primers/probe

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH FWD</td>
<td>AATGGTGAAGGTCGGTGTG</td>
</tr>
<tr>
<td>GAPDH REV</td>
<td>GTGAGGTCTACTGAACATGTAG</td>
</tr>
<tr>
<td>GAPDH probe</td>
<td>6-FAM/TGCAATGG/ZEN/CAGGCCCTGTG/3IABkFQ</td>
</tr>
<tr>
<td>AAV-NYX FWD</td>
<td>CCATGCAGTGATGGTCTT</td>
</tr>
<tr>
<td>AAV-NYX REV</td>
<td>CATCAAGTACTTATCATGTCTGG</td>
</tr>
<tr>
<td>AAV-NYX probe</td>
<td>6-FAM/TTCTACCT/ZEN/CTCCGTCTGGCTCA/3IABkFQ</td>
</tr>
</tbody>
</table>

Probes are double-quenched using a 5’ 6-FAM fluorophore, 3’ IBFQ quencher and inner ZEN quencher, all trademark Integrated DNA Technologies.

Figure 6. ON BC signaling is restored following AAV treatment in Nyxnob mice. A representative rod BC expressing AAV-mediated YFP_NYX and properly localized TRPM1 was targeted for whole-cell patch-clamp recording and filled with sulforhodamine B (A). Capsaicin (10 µM), a TRPM1 agonist, was puffed onto the rod BC dendrites to gate the opening of the TRPM1 channel. Vhold = 50 mV. Representative current recordings from WT rod BC, AAV-treated and untreated rod BCs from Nyxnob mice reveal full restoration of ON BC signaling following treatment (B). Capsaicin was puffed onto rod BC dendrites for 200 ms and 1 s durations. Current responses were recorded from each cell and then averaged (C).

Figure 7. YFP_NYX mRNA is detectable in both early (P2)- and late (P30)-treated Nyxnob retinal extracts through qRT-PCR. Standard deviations were calculated from three replicate reactions performed for each sample.
Early (P2) and late (P30) intravitreal injection of AAV2(quadY-F +T-V)-Ple155 mediates robust and targeted GFP expression in both WT and Nyx<sup>−/−</sup> mice. In contrast, only early injection efficiently drives NYX expression in both strains. Not surprisingly, the ERG b-wave was not restored in Nyx<sup>−/−</sup> mice following the late treatment (data not shown). The treatment at P5 promoted relatively less expression than that seen following P2 treatment with ~3% of ON BCs expressing YFP_NYX (data not shown). These results suggest that supplementation of an ON BC-specific transgene is most effective when delivered, while BCs are still differentiating (52,53).

There are several possible reasons why it is difficult to express NYX in P30-treated mice. Optogenetic protein expression studies using intravitreal injection requires very high titer virus (41-44) to obtain widespread ON BC infection. Indeed, we observed that therapeutic effects were achieved when vector was delivered at 2.5 × 10<sup>13</sup> vg/mL but not at 3.8 × 10<sup>11</sup> vg/mL (data not shown). Thus, more viral particles may be required to make it through the barriers of the inner limiting membrane and plexiform layers to achieve sufficient transduction in the inner nuclear layer. However, the fact that AAV-mediated Nyx message was present in retinas injected at either P2 or P30 suggests that insufficient targeting of the transgene to ON BCs is not the cause. Indeed, transcript levels were higher in P30-treated mice, a result attributed at least in part to the larger number of ON BCs available for transduction in the fully differentiated, P30 rodent retina (52,53). Additionally, robust GFP expression mediated by AAV2(quadY-F+T-V)-Ple155 was achieved following the injection at P30. This suggests that AAV2(quadY-F+T-V) effectively transduced ON BCs at both ages, the Ple155 promoter was active and that the hurdle to achieving efficient NYX expression/therapy in older mice may be post-transcriptional.

Based on our results, it is clear that more work needs to be done to develop this strategy into a clinically translatable therapy. It is interesting to consider whether the window of therapy can be extended and if treatment, whether early or late, will resolve other phenotypes that accompany CSNB1. It is well known that the visual experience can impact synaptic function (54). For example, dark rearing disrupts GRM6 localization in cone ON BC dendritic tips (55). The absence of a functional ON pathway also affects eye development, leading to high myopia in CSNB1 patients (3) and increased susceptibility to form-deprivation myopia in mice (56). For example, dark rearing disrupts GRM6 localization in cone ON BC dendritic tips (55). The absence of a functional ON pathway defects (56,57). ON pathway defects also lead to reduced contrast sensitivity and cortical orientation selectivity (58). Taken together, it is tempting to speculate that expression of ON BC proteins such as NYX is developmentally regulated at the translational or post-translational level.

In conclusion, this study shows for the first time that postnatal delivery of an ON BC-specific gene via AAV is capable of restoring ON BC signaling in a mouse model of CSNB1. Further improvements are needed, however, to extend the window of therapy.

### Materials and Methods

#### Design and construction of ‘Ple155’ MiniPromoter

Overview methods on Pleiades Promoter design have been previously published (59). For the PCP2 gene, a 1652 bp MiniPromoter was derived from two alternative promoters, which are annotated in the UCSC Genome Browser (version GRCh38/hg38). The basal promoter sequence (Prom1) generates transcript variant 1 of transcript variant 1, generates the transcript variant 2. To avoid the conflict of two transcription start sites, the Prom2 sequence was added 3’ of the Prom1 sequence and in reverse orientation from the natural genomic configuration. Expression in the eye and brain of knock-in mice containing Ple155 driving lacZ had been characterized previously (45).

PCR primers were designed to amplify selected regulatory regions (Table 2). Briefly, human genomic DNA was amplified using PCP-X_OL_V1 and PCP-X_OR_V1, generating a contiguous 4500 bp PCR product containing all the regulatory regions of interest. To create the Ple155 MiniPromoter, two smaller regions were further amplified and a fusion PCR product generated as follows. Using the initial 4500 bp PCR product, primers PCP2-ABD_P1R_Miu1_V1 and PCP2-BD_P1L_V1 were used to amplify the Prom2 region. Primers PCP2-B_P2R_V1 and PCP2-B_P2L_SacI_V1 were used to generate the Prom2 region. Subsequently, both Prom PCR products were used along with primers PCP2-ABD_P1R_Miu1_V1 and PCP2-2B_P2L_SacI_V1 to generate a fusion PCR product containing the entire MiniPromoter, which was subcloned using the Miu/Sac sites in the multiple cloning site of the pEMS1313 backbone vector (59) to yield the pEMS1526 plasmid (45). Additional information and constructs are available from AddGene (www.addgene.org).

#### Construction of AAV vectors

Recombinant AAV vector plasmids containing Ple155-GFP and Ple155-YFP_NYX were engineered as follows. NYX fused to enhanced YFP (YFP_NYX) was PCR amplified out of the original transgenic clone prGABAcpc1-YFP_NYX-rb456 (46). It was then inserted into a subcloning vector and fully sequenced. The YFP_NYX fragment was then cloned into pTR-mGrm6/SV40-hChr2-GFP, described in Dorodouchi et al. (40), using HindIII and NotI restriction enzymes. From there, YFP_NYX was moved via EagI restriction site into pTR-U6-SB (60) to generate pTR-CBA-YFP_NYX. To generate pTR-Ple155-YFP_NYX, the CBA plasmid was then digested with NotI and the resulting YFP_NYX fragment cloned into pTR-Ple155-GFP, resulting in pTR-Ple155-YFP_NYX. pTR-Ple155-YFP_NYX and pTR-Ple155-GFP were packaged into either AAV2(QuadY+F+T-V) or AAV8(Y733F). Additionally, pTR-mGrm6/SV40-hChr2-GFP packaged in AAV8(Y733F) was utilized (40). All vectors were packaged, purified and titered according to previously published methods (61,62). Resulting titers of each virus were as follows: AAV2(quadY-F+T-V) Ple155-hGFP, 4.8 × 10<sup>12</sup> vg/mL; AAV2(quadY-F+T-V) Ple155-YFP_NYX, 2.5 × 10<sup>12</sup> vg/mL; AAV8(Y733F)-Ple155-hGFP, 1.2 × 10<sup>13</sup> vg/mL; and AAV8(733F)-Gm6/SV40-hChr2-GFP, 2.9 × 10<sup>12</sup>.

---

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCP2-X_OL_V1</td>
<td>TTATTGGAAATGGGCCTATAGA</td>
</tr>
<tr>
<td>PCP2-X_OR_V1</td>
<td>TGCCATTTACAGTAAATCTA</td>
</tr>
<tr>
<td>PCP2-ABD_P1R_Miu1_V1</td>
<td>AGCTACGGGTGACCTGACCTTTC</td>
</tr>
<tr>
<td>PCP2-BD_P1L_V1</td>
<td>GGCACTTCATTGGGATAG</td>
</tr>
<tr>
<td>PCP2-B_P2R_V1</td>
<td>TTGCCAAGATGCTCC</td>
</tr>
<tr>
<td>PCP2-B_P2L_SacI_V1</td>
<td>GGTACAGGAGGAGAAGAC</td>
</tr>
<tr>
<td>PCP2-B_P2L_SacI_V1</td>
<td>AGCTGAGCTTCGACGATTGAAAGC</td>
</tr>
</tbody>
</table>

*Bold, sequence matching human genomic, italics, sequence matching Prom1; underlined, sequence matching Prom2; and black, non-genomic sequence containing restriction enzyme sites for subcloning.
Animals and injection methods

C57BL/6J (purchased from Jackson Laboratory) and Nyxnob mice on a C57BL/6J background were handled according to the statement for the use of animals in Ophthalmic and Vision Research of the Association of Research in Vision and Ophthalmology and the guidelines of the Institutional Animal Care and Use Committees at the University of Florida, University of Louisville and the Cleveland Clinic. One hour prior to P30 injections, eyes were dilated with 1% atropine, followed by 2.5% phenylephrine (both Akorn, Inc., Lake Forest, IL). Mice were then anesthetized with ketamine (72 mg/kg)/xylazine (4 mg/kg) (Phoenix Pharmaceutical, St. Joseph, MO). Trans-corneal subretinal injections of virus (1 µl) were performed using a 33-gauge blunt needle on a Hamilton syringe. Trans-scleral intravitreal injections (1 µl) were done using a 33-gauge beveled needle on a Hamilton syringe. P2 mice were anesthetized on ice, and virus was delivered intraocularly (0.5 µl) using a 34-gauge beveled needle on a Hamilton syringe. Vectors were delivered undiluted in all cases.

Electroretinogram

After overnight dark adaptation, mice were anesthetized with a solution of ketamine (80 mg/kg) and xylazine (16 mg/kg). Eye drops were used to dilate the pupils (1% tropicamide and 2.5% phenylephrine HCl) and to anesthetize the corneal surface (1% proparacaine HCl). A pair of stainless steel wire electrodes was used to record ERGs from each corneal surface. These active leads were referenced to a needle electrode placed in the cheek. A second needle electrode placed in the tail served as the ground lead. ERGs were recorded using an LKC (Gaithersburg, MD) UTAS E-3000 signal averaging system. Responses evoked by ganzfeld strobe flash stimuli were band-pass filtered (0.03–1000 Hz), amplified, and averaged and stored.

The Nyxnob ERG is comprised primarily of response components generated by photoreceptors (a-wave), Müller glial cells (slow PII) and hyperpolarizing BCs (50), but is missing the positive polarity component generated by depolarizing BCs (DBCs) (16). Because we were uncertain how AAV might impact the ERG waveform of treated mice, we repeated certain stimulus conditions multiple times for each mouse, to allow us to appreciate a consistent waveform change. With that exception, the recording protocol was comparable with that used for other mouse models. In each recording session, we began with dark-adapted responses to low-luminance stimuli and increased flash luminance from −3.6 to 2.1 log cd s/m². After dark-adapted recordings were complete, cone ERGs were isolated by superimposing stimuli upon a steady adapting field (20 cd/m²). Flash luminance ranged from −0.8 to 1.9 log cd s/m². All Nyxnob mice intracocularly injected at P2 with AAV-Nyx (n = 17) were analyzed by ERG.

Tissue preparation, immunohistochemistry and microscopy

Four weeks post-injection, the limbi of treated and untreated Nyxnob eyes were marked with ink at the 12 o’clock position, facilitating orientation. They were immediately enucleated, perfused with a needle through the cornea and stored overnight at 4°C in 4% paraformaldehyde. Cornea and lens were removed, and the eye cup was incubated in 30% sucrose solution for 3–4 h at 4°C before being cryoprotected in Optimal Cutting Temperature medium (Sakura Finetek USA, Inc., Torrance, CA). Twelve-micron sections were cut using a Leica cryostat (Leica CM3050 S, Wetzlar, Germany) and stored at −20°C. Retinal sections were immunostained according to previously described methods (63). Briefly, sections were brought to room temperature, rinsed with 1x phosphate-buffered saline (PBS), blocked with 0.5% Triton-X100 and 1% bovine serum albumin (BSA) for 1 h each and then incubated overnight at 4°C with primary antibodies in 3% Triton+1% BSA. Primary antibodies included rabbit polyclonal anti-GFP, which also labels YFP (1:1000, generously provided by Dr Clay Smith), 1:200 mouse monoclonal anti-PCP2 (Santa Cruz, Dallas, TX) or 1:500 anti-PCP2 (Santa Cruz). After incubation with primary antibody, slides were rinsed with 1X PBS and then incubated at room temperature for 1 h with respective Alexa Fluor secondary antibodies (1:500) in 1X PBS (Invitrogen, Eugene, Oregon). Slides were then rinsed with 1X PBS, mounted with 4',6-diamidino-2-phenylindole (DAPI)-containing SlowFade mounting media (Thermo Fisher Scientific, Waltham, MA) and coverslipped. High-magnification (20× or 40×) images were obtained via spinning disk confocal microscopy (Nikon Eclipse TE2000 microscope equipped with Perkin Elmer Ultraview Modular Laser System and Hamamatsu O-RC-A2 camera). Tiled images (10×) of eyecups were obtained with a Keyence BZ-9000 fluorescent microscope. Cell counts were performed on 40× images taken from four identical locations in optic nerve-containing eyecups. Far superior, superior/central, inferior/central and far inferior areas were sampled.

Retina pieces that were not sliced for patch-clamp recording were incubated in 4% paraformaldehyde for 20 min. Directly after the conclusion of patch-clamp experiments, retinal slices were incubated in 4% paraformaldehyde for 20 min at room temperature. The retina pieces were then rinsed in 1× PBS (3 × 5 min), incubated in blocking solution (0.5% Triton-X100 and 10% normal donkey serum) for 1 h and then incubated 5 days for flat-mount retina and 3 days for slices at 4°C with primary antibodies in blocking solution. Primary antibodies included 1:1000 rabbit polyclonal anti-GFP with conjugated Alexa 488 (Life technologies, Carlsbad, CA, Cat. Number A-21311) and 1:1000 sheep polyclonal anti-TRPM1 (generously provided by Dr Kirill Martemyanov, Scripps Research Institute, Jupiter, FL). After incubation with primary antibody, retina pieces were rinsed with 1X PBS and then incubated with 1:1000 donkey anti-sheep Alexa Fluor 546 secondary antibody in 1X PBS (Life technologies, Carlsbad, CA, Cat. Number A-21098). Slices were incubated at room temperature for 2 h, and flat-mount retina pieces were incubated at 4°C overnight. All the retina pieces were then rinsed with 1X PBS (6 × 10 min), mounted with DAPI-containing Vectashield medium (Vector Laboratories, Burlingame, CA, Inc., Cat. Number H-1200) and coverslipped. Two layers of paraﬁlm were used as a spacer around slices, and flat-mount pieces were mounted photoreceptor side up with duct tape used as a spacer surrounding the tissue. High-magnification (20× or 40× objective) images were obtained using confocal microscopy (Olympus Fluoview FV1000 microscope equipped with Hamamatsu EM Image CCD camera). Bipolar cells that had been previously recorded from and filled with sulforhodamine (see the following section) were identiﬁed by their bright red ﬂuorescence, which completely ﬁlled the cell as opposed to the weaker membrane-speciﬁc TRPM1 label.

Single-cell recordings

Mice were anesthetized with an intraperitoneal injection of Ring-er’s solution containing ketamine/xylazine (127/12 mg/kg, respectively) and killed by cervical dislocation. The eyes were enucleated and the retinas removed. Retinal slices were made and then placed in a recording chamber. Glass electrodes were ﬁlled with intracellular solution that contained Cs-glucosinate
solution (20 mM CsCl, 107 mM CsOH, 107 mM β-gluconic acid, 10 mM NaHEPES, 10 mM BAPTA, 4 mM ATP and 1 mM GTP). The intracellular solution contained 1% sulforhodamine to visualize the cell and classify its morphology (64). To block inhibitory inputs, the Ames bath solution was supplemented with the following: 1 μM strychnine, 100 μM picrotoxin and 50 μM 6-tetrahydropyridin-4-yl methylphosphinic acid. L-AP4 (4 μM) was added to the bath solution to saturate mGluR6 receptors. Capsaicin (10 μM), a TRPM1 agonist, was puffed onto the rod BC dendrites to gate the opening of the TRPM1 channel. Rod BC somas were targeted for whole-cell, patch-clamp recording. Rod BCs were voltage clamped at +50 mV (25,28,65). Capsaicin (10 μM) was puffed onto the rod BC dendrites for 200 ms and 1 s durations to gate the opening of the TRPM1 channel. Three to five responses were recorded from each cell and then averaged. Prism 5.04 software (GraphPad Software) was used to perform a Kruskal–Wallis test, with a Dunn’s post hoc test. Statistical significance was indicated as “P<0.01 and “P<0.001. Error bars report standard error of the mean. Additional methodological details are published (28).

mRNA quantification by real-time PCR

Treated and untreated eyes from Nyyx<sup>−/−</sup> mice and age-matched WT controls were enucleated 4 weeks post-injection and retinas immediately dissected from the eyecup and submerged in RNA Protect (Qiagen, Inc., Valencia, CA). Retinas (n=1 per condition) were homogenized in lysis buffer (Buffer RLT; RNeasy Protect Mini Kit; Qiagen, Inc.) plus βME for 45 s. RNA extraction was performed with a stabilization and purification kit (RNeasy Protect Mini Kit; Qiagen, Inc.). After measuring RNA concentration (Nanodrop, ThermoFisher, Wilmington, DE), 5ug of RNA was treated with DNase I to remove contaminating DNA (Ambion, Foster City, CA) and used in real-time PCR (Applied Biosystems TaqMan Gene Expression Master Mix and CFX96 PCR detection system interfaced with C1000 Touch thermal cycler; Bio-Rad Laboratories) to measure AAV-mediated Nyyx and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) transcripts in P2- and P30-treated Nyyx<sup>−/−</sup> retinas and untreated contralateral controls.

PrimeTime mixes containing primer pairs and probe (Integrated DNA Technologies (IDT), Coralville, IA) for each transcript are included in Table 1. AAV-mediated Nyyx primer pairs were designed to flank the 3’ Nyyx gene and 5’ SV40 polyA and generate an amplicon of 150 bps. Internal restriction sites within the amplicon were used to cut the PCR product and analyze via electrophoresis. If the IDT pre-made set is used, this is not an issue as there is a target-specific probe that can only anneal to sequence within the amplicon. Prior to experimental data collection, PrimeTime mixes were validated according to minimum information for publication of quantitative real-time PCR experiments standards (66). Results are the average of three replicate reactions and were calculated using the ΔΔC<sub>T</sub> method with signals normalized to Gapdh and expression relative to zero.

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We are grateful to the Retinal Gene Therapy Vector Laboratory at the University of Florida for AAV packaging. We also acknowledge Drs Clay Smith and Kirill Martemyanov for providing GFP and TRPM1 antibodies, respectively.

Conflict of Interest statement. S.E.B., S.L.B., F.M.D. and E.M.S. are co-inventors on provisional patent, ‘UF#14807–Highly Selective Transduction of ON Bipolar Cells from Intravitreal Delivered AAV Vector’. E.M.S. and C.N.D.L. are also co-inventors on US Patent 9,006,413 B2 ‘PCP2 Mini-Promoters’.

Funding

This work was supported by R01 EY024280 (S.E.B.), R01 140701 (M.A.M.), R01 EY12354 (R.G.G.), Genome British Columbia AGCP-CanEuCre-01 (E.M.S.), Foundation Fighting Blindness, Department of Veterans Affairs, and unrestricted grants to both the University of Florida and the University of Louisville from the Research to Prevent Blindness (RFB).

References


40. Mace, E., Caplette, R., Marre, O., Sengupta, A., Chaf, E5574, E5583. – 1163.


42. Mike, E., Caplette, R., Marre, O., Sengupta, A., Chaf, E5574, E5583. – 1163.


