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Identification of a new mutant allele, *Grm6^{nob7}*, for complete congenital stationary night blindness

HAOHUA QIAN¹, RUI JI², RONALD G. GREGG^{2,3}, and NEAL S. PEACHEY^{4,5,6}

¹Visual Function Core, National Eye Institute, National Institutes of Health, Bethesda, Maryland

²Department of Biochemistry and Molecular Genetics, University of Louisville, Louisville,

Kentucky ³Department of Ophthalmology and Visual Sciences, University of Louisville, Louisville,

Kentucky ⁴Cole Eye Institute, Cleveland Clinic, Cleveland, Ohio ⁵Louis Stokes Cleveland VA

Medical Center, Cleveland, Ohio ⁶Department of Ophthalmology, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, Ohio

Abstract

Electroretinogram (ERG) studies identified a new mouse line with a normal a-wave but lacking the b-wave component. The ERG phenotype of this new allele, *nob7*, matched closely that of mouse mutants for *Grm6*, *Lrit3*, *Trpm1*, and *Nyx*, which encode for proteins expressed in depolarizing bipolar cells (DBC). To identify the underlying mutation, we first crossed *nob7* mice with *Grm6^{nob3}* mutants and measured the ERGs in offspring. All the offspring lacked the b-wave, indicating that *nob7* is a new allele for *Grm6*: *Grm6^{nob7}*. Sequence analyses of *Grm6^{nob7}* cDNAs identified a 28 base pair insertion between exons 8 and 9, which would result in a frameshift mutation in the open reading frame that encodes the metabotropic glutamate receptor 6 (*Grm6*). Sequencing both the cDNA and genomic DNA from exon 8 and intron 8, respectively, from the *Grm6^{nob7}* mouse revealed a G to A transition at the last position in exon 8. This mutation disrupts splicing and the normal exon 8 is extended by 28 base pairs, because splicing occurs 28 base pairs downstream at a cryptic splice donor. Consistent with the impact of the resulting frameshift mutation, there is a loss of mGluR6 protein (encoded by *Grm6*) from the dendritic tips of DBCs in the *Grm6^{nob7}* retina. These results indicate that *Grm6^{nob7}* is a new model of the complete form of congenital stationary night blindness, a human condition that has been linked to mutations of *GRM6*.

Keywords

Congenital stationary night blindness; Electroretinogram; Metabotropic glutamate receptor 6

Introduction

Mutations in *GRM6*, which encodes the metabotropic glutamate receptor 6 (mGluR6), underlie one form of complete congenital stationary night blindness (cCSNB; Dryja et al.,

2005; Zeitz et al., 2005). While rod-mediated vision is severely impaired in patients with cCSNB due to *GRM6* mutations, their deficits in visual acuity or other measures of cone-mediated vision are more variable and may be normal (Dryja et al., 2005; Zeitz et al., 2005; Godara et al., 2012; Sergouniotis et al., 2012; Bijveld et al., 2013). mGluR6 is the glutamate receptor used by depolarizing bipolar cells (DBC; Masu et al., 1995), and *GRM6* mutations impair vision when DBCs can no longer modulate transient receptor potential melastatin 1 (TRPM1) channel activity in response to changes in extracellular glutamate (Morgans et al., 2010).

Our understanding of the impact of abnormal mGluR6 activity on retinal function has been expanding through the availability of mouse *Grm6* mutants. Masu et al. (1995) characterized the retina of *Grm6^{tmNak/tmNak}* mice, referred to here as *Grm6^{-/-}*. They noted that retinal structure was intact, despite the loss of the electroretinogram (ERG) b-wave component, which reflects DBC activity (Kofuji et al., 2000). Two additional mutants were discovered when no b-wave (nob) mice were identified during screening of mice generated in a N-ethyl-N-nitrosourea mutagenesis program (*Grm6^{nob3}*; Pinto et al., 2007) or mouse colonies at the Jackson Laboratory (*Grm6^{nob4}*; Maddox et al., 2008). While normal retinal structure was observed in both *Grm6^{nob3}* (Pinto et al., 2007) and *Grm6^{nob4}* mice (Maddox et al., 2008), differences in receptive field abnormalities of retinal ganglion cells (RGCs) between the two mutants were noted. These studies indicate the value of mouse models of night-blinding disorders and of allelic series for further understanding human disease. Here, we report the discovery and initial characterization of a new nob mouse mutant (*nob7*), which involves a novel spontaneous mutation in *Grm6*.

Materials and methods

Mice

All procedures used in animal experiments were approved by the Institutional Animal Care & Use Committees of the institutions involved and conform to the principles regarding the care and use of animals adopted by the American Physiological Society and the Society for Neuroscience. Homozygous *nob7* mice were identified in the course of ERG studies of C57BL/6J mice conducted at the National Eye Institute. After establishing that *nob7* was inherited as an autosomal recessive trait, affected mice were transferred to Cleveland Clinic for further analysis including a cross to *Grm6^{nob3}* (*nob3*) mice (Maddox et al., 2008). C57BL/6J mice were used as controls.

Electroretinography

After overnight dark adaptation, mice were anesthetized (ketamine: 80 mg/kg; xylazine: 16 mg/kg), their pupils were dilated (1% tropicamide and 2.5% phenylephrine HCl eyedrops) and the corneal surface was anesthetized (1% proparacaine HCl). ERGs were recorded from the corneal surface using a stainless steel wire. Needle electrodes placed in the cheek and tail served as reference and ground leads, respectively. Responses to strobe light flashes were amplified (0.03–1000 Hz) and stored using an LKC (Gaithersburg, MD) UTAS E-3000 signal averaging system. ERGs were recorded under stimulus conditions that allow the

response properties of rod- and cone-driven components to be defined using published protocols (Gregg et al., 2007).

Sequencing

Total RNA was isolated from retinas using Trizol reagent as per the manufacturer's protocol (Life Technologies, Carlsbad, CA). cDNA was synthesized from total RNA using the Superscript II reverse transcriptase as per the manufacturer's protocol (GE Biosystems). The entire coding region of *Grm6* was amplified with mGrm6-ex01F (5'-CAATGTCTTGCGCCTGTTTG-3') and mGrm6-ex10R (5'-GTGGAGGTCTTCTTGAGGCT-3'), which spanned the open reading frame encoded by exons 2–10. The PCR fragment was cloned and then sequenced on an Applied Biosystems (Carlsbad, CA) 3130XL Sequencer (using a 50 cm array and POP7 polymer). The causative mutation was confirmed to be present in genomic DNA.

Immunohistochemistry

Dissected retinas were immersion fixed for 15 min in 4% (w/v) para-formaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), then washed in PB, cryoprotected through a graded sucrose series, and frozen in OCT (Sakura Finetek, Torrence, CA):20% sucrose (2:1; Barthel & Raymond, 1990). 16- μ m sections were cut on a cryostat, mounted onto Super-Frost glass slides, air-dried, and stored at -80°C .

Sections were brought to room temperature, washed in PB saline (PBS) for 5 min, PBX (PBS containing 0.5% (v/v) Triton X-100) for 5 min, and then incubated in a blocking solution (PBX containing 5% (v/v) normal goat serum) for 1 h. Primary antibodies were diluted in the blocking solution and incubated on retinal sections at room temperature overnight. Primary antibodies and dilutions used were: anti-mGluR6 (1:1000; Cao et al., 2011) and anti-CACNA1F (1:1000; BD Biosciences). After incubation with the primary antibody, sections were washed three times in PBS for 5 min each, and subsequently incubated with fluorescently labeled secondary antibodies (1:1000 in blocking solution) at room temperature for 1 h. Secondary antibodies were Alexa 488 goat anti-rabbit and Alexa 555 goat anti-mouse (Invitrogen, Carlsbad, CA). Slides were then washed three times in PBS and coverslipped with Immunomount (ThermoShandon, Pittsburgh, PA). Sections were imaged on an Olympus (Center Valley, PA) FV1000 confocal microscope, using a 60 $\times$ oil objective (1.45 NA). Images shown are maximum projections of confocal stacks, adjusted for contrast and brightness with Fluoview software.

Results and discussion

The *nob7* mutant was discovered during ERG testing of mice anticipated to be wild type. Fig. 1 compares representative ERGs obtained from control and *nob7* littermates. Under dark-adapted conditions (Fig. 1A, left), the control response is dominated by the cornea positive b-wave. In response to high luminance stimuli, the b-wave is preceded by the negative polarity a-wave, reflecting the light-induced closure of cation channels along rod photoreceptor outer segments (Penn & Hagins, 1969). In comparison, the b-wave component is absent from responses of *nob7* mice, and the a-wave is followed by a negative

polarity component, slow PIII, that is generated by Kir4.1 channel activity in Müller glial cells (Kofuji et al., 2000; Wu et al., 2004) and is normally masked by the larger amplitude b-wave (Samuels et al., 2010).

Control and *nob7* ERGs also differ under light-adapted conditions (Fig. 1A, right). Under these conditions, the ERG represents activity of the cone pathway (Peachey et al., 1993). The control cone ERG is dominated by the positive polarity b-wave and high frequency oscillatory potentials, both of which reflect activity through the DBC-pathway (Sharma et al., 2005; Shirato et al., 2008). In contrast, cone ERGs of *nob7* mice are electronegative.

The *nob7* ERG phenotype is inherited in an autosomal recessive fashion and is distinct from that of mouse models for proteins involved in presynaptic aspects of photoreceptor-to-bipolar synaptic transmission, where the b-wave is reduced but still present (Pardue & Peachey, 2014). Instead, the *nob7* ERG phenotype closely matches that of mouse mutants for *Trpm1* (Morgans et al., 2009; Koike et al., 2010; Peachey et al., 2012a), *Nyx* (Pardue et al., 1998), *Grm6* (Masu et al., 1995; Pinto et al., 2007; Maddox et al., 2008), *Gpr179* (Peachey et al., 2012b), and *Lrit3* (Neuillé et al., 2014). Fig. 1B compares the responses obtained from *nob7* and *nob3* mice. There is an excellent agreement between the responses of the two mutants under both dark- (left) and light-adapted (right) conditions.

To identify the *nob7* gene defect, we focused initially on genes expressed in DBCs. Given the close agreement between the ERG phenotypes of *nob7* and *nob3* mice, we crossed affected animals and examined the ERGs of compound heterozygote offspring. The ERG phenotype of *nob3/nob7* compound heterozygous mice was indistinguishable from either parental line (compare Figs. 1C and 1B). This result demonstrates that the *nob7* phenotype is caused by a mutation in the *Grm6* gene, and the allele will be hereafter referred to as *Grm6^{nob7}*.

To identify the *Grm6^{nob7}* mutation, we used reverse transcription PCR to clone and sequence the cDNA fragments representing the open reading frame for mGluR6, from RNA isolated from control and *Grm6^{nob7}* retinas. Sequence analyses of the cDNA clones from the *Grm6^{nob7}* retinas (Fig. 2) identified an insertion between exons 8 and 9 of the normal transcript (ENSEMBLE Transcript ENSMUST00000000631). This insertion is predicted to cause a frameshift mutation, which will result in early termination and truncation of the mGluR6 protein. This truncation occurs at amino acid 701, which is located in predicted transmembrane domain IV (Nakajima et al., 1993). In addition, seven amino acids are added to the carboxy terminus that are encoded by the insertion and prior to a stop codon that occurs in the insertion. Given the loss of several transmembrane domains and the intracellular carboxy region, this truncation would not be functional and likely the mRNA or protein if produced would be rapidly degraded.

BLAST comparison (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of the 28 base pair sequence inserted into the *Grm6^{nob7}* cDNA with the mouse genome database indicated that it was derived from the beginning of intron 8 of *Grm6*. Furthermore, the last base of exon 8, a G, was mutated to an A. This G is highly conserved at most splice donor sites because it establishes alignment with U1 snRNA during splicing. To determine if the mutation was

present in the genomic DNA, we PCR-amplified fragments from genomic DNA isolated from control and *Grm6^{nob7}* mice using primers located in exon 8 and exon 9. As shown in the partial sequence of this fragment, the last base of exon 8 contains a G to A transition (Fig. 2C). Alignment of the *Grm6^{nob7}* cDNA with the genomic DNA shows that a cryptic splice 28 base pair downstream from the normal site at the end of exon 8 was used (Fig. 2C). We predict that this mutation, which creates a frameshift mutation truncating the normal protein by 170 amino acids, would result in a null allele.

To evaluate the expression of mGluR6 on the dendritic tips of DBCs, we used immunohistochemistry and confocal microscopy. As shown in Fig. 3, mGluR6 is localized as discrete puncta in the OPL of control retinas, which are known to represent the dendritic tips of DBCs located in the outer plexiform layer (OPL). However, this staining is absent from the *Grm6^{nob7}* retina, confirming that the *Grm6^{nob7}* mutation results in a null allele. Null mutations also underlie the *Grm6^{nob3}* and *Grm6^{nob4}* models, in which more extensive anatomical studies reveal that overall retinal and synaptic structures are preserved (Pinto et al., 2007; Maddox et al., 2008). In contrast, immunohistochemical staining using an antibody to CACNA1F, a presynaptic marker (Schmitz et al., 2000), is comparable between control and *Grm6^{nob7}* retinal sections, indicative of preserved OPL structure.

The availability of animal models for human retinal diseases has facilitated the evaluation of experimental therapies for many conditions (Fletcher et al., 2011; Farrar et al., 2014), and the availability of allelic series provides resources with which to fully characterize a potential therapy. The *Grm6^{nob7}* mutant is a new model for cCSNB and will be useful in understanding this condition and evaluating experimental therapies for the *GRM6* form of cCSNB as they become available.

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Fig. 1. ERG characteristics of *nob7* mice. **(A)** ERGs of *nob7* mice lack the b-wave under dark-adapted (left) and light-adapted (right) conditions. **(B)** ERG waveforms of *Grm6^{nob7}* and *Grm6^{nob3}* mice have a comparable loss of the b-wave component. **(C)** Representative ERGs from two *Grm6^{nob3/nob7}* double heterozygotes.

Fig. 2.

A splice site mutation causes the *Grm6^{nob7}* phenotype. (A) The control and *Grm6^{nob7}* cDNA and genomic sequences are shown aligned in the region of interest. The *Grm6^{nob7}* cDNA (B) and genomic DNA (C) have a G to A mutation at the last nucleotide of exon 8. This is a highly conserved base at splice sites and its mutation results in the use of a cryptic splice site 28 base pairs downstream (arrows). This frameshift truncates the normal protein at amino acid 701 and the intron insertion results in the addition of seven amino acids.

Fig. 3.

Immunohistochemistry of control (top) and *Grm6^{nob7}* (bottom) retinas stained for mGluR6 (green) and CACNA1F (red). CACNA1F staining is comparable in both retinas and localizes to the outer plexiform layer where it opposes mGluR6 labeling in the control retina. In comparison, mGluR6 staining is not present in the *Grm6^{nob7}* retina. Scale bar indicates 10 μ m.