Chapter 25
Gene Therapy Restores Missing Cone-Mediated Vision in the CNGA3$^{-/-}$ Mouse Model of Achromatopsia

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25.1 Introduction

Mutations in the genes CNGA3 and CNGB3 that encode either of the two types of cone cyclic nucleotide-gated (CNG) subunits account together for approximately 75% of all cases of complete achromatopsia (Kohl et al. 2005), a hereditary, autosomal recessive disorder characterized by lack of cone photoreceptor function. The complete unresponsiveness of cones in achromatopsia has grave consequences for vision, particularly with respect to the densely cone-packed human fovea. In addition to the lack of color discrimination, achromats suffer from very poor visual acuity, pendular nystagmus, and photophobia (Kohl et al. 1998).

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We have previously shown that genetic inactivation of CNGA3 in mice – in close agreement with the human phenotype – leads to selective loss of cone-mediated light responses (Biel et al. 1999) accompanied by morphological, structural, and molecular changes, and finally results in cone cell death (Michalakis et al. 2005).

Here, we set out to design a curative gene replacement strategy using recombinant adeno-associated viral vectors (rAAV) to restore cone function in the CNGA3$^{-/-}$ mouse model.

25.2 Materials and Methods

25.2.1 rAAV Production Vectors and Subretinal rAAV Injections

293T cells were cotransfected with a viral vector (pAAV2.1-mBP-CNGA3) that expresses mouse CNGA3 under control of a 0.5-kb mouse SWS opsin promoter (Akimoto et al. 2004), pAdDeltaF6 (Auricchio et al. 2001) and pAAV2/5Y719F (Petrs-Silva et al. 2009) plasmids followed by iodixanol-gradient (Grieger et al. 2006) purification, ion exchange chromatography (HiTrap Q ÄKTA Basic FPLC system, GE Healthcare, Germany), and by further concentration using Amicon Ultra-4 Centrifugal Filter Units (Millipore, Germany). Physical titers were determined by qPCR (LightCycler 480, Roche Applied Science, Germany). 1–1.5 μL rAAV particles were injected into the subretinal space using the NanoFil subretinal injection Kit (WPI, Germany) with a 34-gauge beveled needle. The procedure was monitored immediately following the injections using scanning laser ophthalmoscopy (Seeliger et al. 2005) and optical coherence tomography (Fischer et al. 2009). All procedures concerning animals were approved by local authorities (Regierungspräsidium Tübingen and Regierung von Oberbayern).

25.2.2 Electrophysiological Analysis

ERG analysis was performed 6, 10, and 11 weeks after injection according to procedures described elsewhere (Seeliger et al. 2001; Tanimoto et al. 2009). Spike trains of retinal ganglion cells were recorded extracellularly with commercial planar multielectrode arrays (Multi Channel Systems, Reutlingen, Germany). During recordings, the retina was continuously superfused with Ames medium, buffered with 22 mM $NaHCO_3$ and 5% CO_2/95% O_2 (pH 7.4), and maintained at 35°C. To visually stimulate the retina, the screen of a CRT monitor was focused with standard optics onto the photoreceptor layer, covering the recorded piece of retina. Periodic flashes were produced by switching the monitor display every 1 s between black and white, with a contrast (white−black)/(white + black) = 0.97. Overall, light level was controlled with neutral density filters in the light path.

25.2.3 Immunohistochemistry

The immunohistochemical procedure and antibody dilutions were described previously (Michalakis et al. 2005). Laser scanning confocal micrographs were collected using a LSM 510 meta microscope (Carl Zeiss, Germany).

25.2.4 Behavior

The experiment was performed at 111.0 ± 2.2 lux. The day after habituation to the water (21 ± 1°C, made opaque by the addition of nontoxic white dye) mice were trained for 6 trials to associate a red rectangle with a stable visible platform that was placed in a swimming pool (120 cm in diameter, 70 cm high, white plastic) filled with water up to a depth of 30 cm. The position of the platform was changed from trial to trial in a pseudorandom order to avoid association of the platform with distal spatial cues. On the following day, the animals had to discriminate between two visible platforms. One platform was stable (marked with the red rectangle; correct choice) and the other platform sank when a mouse climbed onto it (marked with a green rectangle; incorrect choice). Trials were terminated if the mouse climbed on one of the two platforms.

25.3 Results

We produced viral vector particles that drive expression of the mouse CNGA3 cDNA under control of a 0.5-kb-fragment of the mouse blue opsin (S-opsin) promoter (Akimoto et al. 2004) with a Y719F-modified AAV5 capsid (AAV5-mBP-CNGA3) that results in higher resistance to proteasomal degradation (Petrs-Silva et al. 2009). We delivered $6–9 \times 10^9$ rAAV genomic particles into the subretinal space within the central to ventral part of the retina of 12–14-day-old CNGA3$^{-/-}$ mice and monitored the procedure immediately following the injections using scanning laser ophthalmoscopy (Seeliger et al. 2005) and optical coherence tomography (Fischer et al. 2009).

At 10 weeks posttreatment, clear signs of a functional restoration of cone photoreceptor function were found in Ganzfeld electroretinograms (ERGs) (Fig. 25.1). No differences between the treated eyes (TE), untreated eyes (UE), or wild-type (wt) eyes were detectable at dim-light levels (Fig. 25.1a, top row), demonstrating regular rod function. A prominent rescue effect on cones was found in the light adapted (photopic) part of the protocol (Fig. 25.1a, bottom), in which rods are nonresponsive due to desensitization.

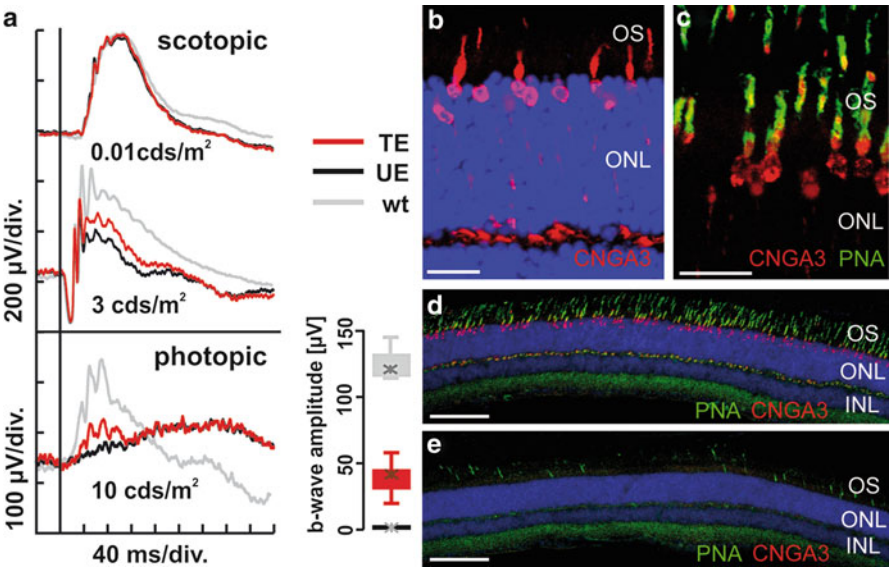

Fig. 25.1 Restoration of cone-mediated ERG, establishment of cone CNG channel, and delay of cone degeneration in treated CNGA3$^{-/-}$ cones. (**a**) Single flash ERG. Scotopic rod system response (*top*): no difference between the treated eye (TE), untreated eye (UE), and the wt eye. Mixed rod/cone system response (*center*): amplitude increase in the TE relative to the UE indicative for cone system function improvement. Photopic conditions (traces *bottom left*, corresponding box plot *bottom right*): substantial restoration of cone system function. (**b**–**c**) Cone-specific expression of CNGA3 (*red*) in a treated area of a CNGA3$^{-/-}$ retina. (**c**) Colabeling with the cone marker peanut agglutinin (PNA) indicates presence of rescued CNGA3 in cone outer segments (COS, PNA, *green*; CNGA3, *red*). (**d**–**e**) The treatment preserves a high number of cones. Retinal slices of age-matched treated and untreated CNGA3$^{-/-}$ mice were stained with the cone marker PNA (*green*) and anti-CNGA3 (*red*). Scale bars mark 20 µm in (**a**–**b**) and 100 µm in (**c**–**d**). In (**a**, **c**–**d**), nuclei are stained with Hoechst dye (*blue*). *INL* inner nuclear layer; *ONL* outer nuclear layer; *OS* (photoreceptor) outer segments

Following ERG measurements, eyes were removed, fixed, cryo-sectioned, and processed for immunohistochemistry. We found expression of CNGA3 in cone photoreceptors within the injected, but not the untreated part of the retina (Fig. 25.1b, c). The CNGA3 protein was specifically expressed in cones and localized throughout the cone photoreceptor (Fig. 25.1b). The CNGA3 protein that was produced as a result of our therapy was able to restore COS expression and localization of CNGB3 (not shown) and to reduce the degenerative process in the retina. In line with this, high numbers of cones were still present in the ventral retina of treated but not untreated (age-matched) CNGA3$^{-/-}$ mice (Fig. 25.1d, e).

Having shown by electroretinography that treated cones acquired the ability to generate regular light-evoked signals and to activate respective bipolar cells, we examined next whether these signals are capable of exciting ganglion cells in a regular fashion. To this end, we performed multielectrode array recordings to

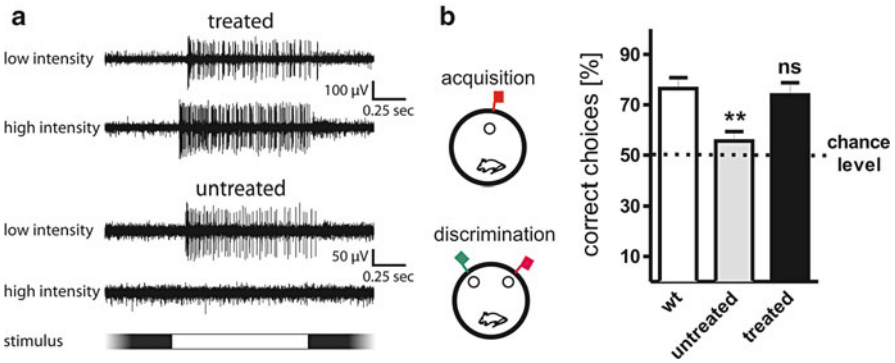

Fig. 25.2 Gene replacement therapy restores responsiveness of ganglion cells to photopic stimuli and enables cone-mediated central vision in CNGA3$^{-/-}$ mice. (**a**) Spike trains of different types of ON ganglion cells from treated (*top*) and untreated CNGA3$^{-/-}$ mice (*bottom*) in response to periodic flashes of light at two different intensity levels. Stimulus phase is indicated at the *bottom*. The spike trains obtained from the treated mice show reliable response patterns for both applied light intensities. By contrast, ganglion cells from untreated retinas do not respond to light flashes at the highest light level, which corresponds to photopic conditions. (**b**) Behavioral test for cone-mediated vision. Mice were trained to associate a red-colored cue with a stable visible platform (acquisition). Subsequently, the mice had to discriminate between two visible platforms (discrimination), a stable platform (positioned next to a red cue = correct choice) and a platform that sank when a mouse climbed onto it (positioned next to a green cue = incorrect choice). The graph shows the mean percentage of correct choices for 6 trials during the discrimination test. Statistical significance (*t*-test) of differences from comparisons with wild type is shown on top of bars (**$p < 0.01$; *ns* non significant)

measure the spiking activity of ganglion cells from isolated retinas of treated and untreated eyes of CNGA3$^{-/-}$ mice (Fig. 25.2a). As expected for a retina limited to rod function only, ganglion cells from untreated CNGA3$^{-/-}$ mice responded well at low light levels, but did not show any light-evoked activity under photopic conditions (Fig. 25.2a). Much in contrast, many neurons in treated regions displayed strong light-evoked activity for both low and high light levels (Fig. 25.2a). This indicates that transmission of cone signals to the inner retina was reestablished in the treated retinas.

Finally, we aimed at assessing whether the restoration of retinal cone-mediated signaling enabled treated CNGA3$^{-/-}$ mice to develop cone vision-guided behavior. We therefore designed a simple test for vision-guided behavior in mice that highly depends on cone-mediated vision under photopic light conditions. The mice were trained in a cued water maze to associate a red cue with a stable visible platform (day one). On day two, the mice had to discriminate between two randomly arranged visible platforms, a stable platform marked with a red cue (correct choice), and a platform that sank when a mouse climbed onto it marked with a green cue (incorrect choice). Wild-type mice were able to differentiate between the two platforms based on the visual cues and performed significantly above chance level (Fig. 25.2b). This indicates that wild-type mice were able to differentiate between the two cues.

Note that the mice may have used differences in the spectral identity, luminous intensity, or some combination of the two to discriminate between the two visual cues. The fact that cone-mediated vision is essential for stimulus discrimination, however, was confirmed by the fact that CNGA3$^{-/-}$ mice were not able to solve this task; their performance was not significantly different from the 50% chance level (Fig. 25.2b). Treated CNGA3$^{-/-}$ mice, on the other hand, performed significantly better than untreated CNGA3$^{-/-}$ mice (Fig. 25.2a). Moreover, treated CNGA3$^{-/-}$ mice showed no significant difference to the wild-type control mice in this test. This confirms that our gene replacement therapy is sufficient to restore cone-mediated visual behavior.

25.4 Discussion

Cone vision is the most important visual quality in daytime environment. Inherited diseases such as achromatopsia lead to dysfunction and later degeneration of cone photoreceptors and are currently untreatable. We here show that the principal subunit of the cone CNG channel (CNGA3) could successfully be produced in congenitally nonfunctional cone photoreceptors of CNGA3$^{-/-}$ mice. The electrophysiological recordings in combination with the behavioral data provide clear evidence that retinas with cones that are completely nonfunctional from birth can become capable of generating signals that higher visual centers can process in a way that permits the animal to successfully discriminate objects based on cone-mediated signals and take respective action. This proof-of-concept in mice is very promising and relevant for future human use of this kind of therapeutic strategy.

Although it will take some time until the results of long-term follow-up experiments are available, the preserved number of cones suggests that the treatment also ameliorates the progressive cone degeneration.

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