Human Gene Therapy

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Six Years and Counting: Restoration of Photopic Retinal Function and Visual Behavior Following Gene Augmentation Therapy in a Sheep Model of CNGA3 Achromatopsia (DOI: 10.1089/hum.2018.076)
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Six Years and Counting: Restoration of Photopic Retinal Function and Visual Behavior Following Gene Augmentation Therapy in a Sheep Model of *CNGA3* Achromatopsia

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SHORT TITLE: Gene therapy in day blind sheep - 6 year follow up

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ABSTRACT

Achromatopsia causes severely reduced visual acuity, photoaversion and inability to discern colors due to cone photoreceptor dysfunction. In 2010, we reported on day blindness in sheep caused by a stop codon mutation of the ovine *CNGA3* gene, and began gene augmentation therapy trials in this naturally occurring large animal model of *CNGA3* achromatopsia. The purpose of this study was to evaluate long-term efficacy and safety results of treatment, findings that hold great relevance for clinical trials in *CNGA3* achromatopsia patients that started recently. Nine day blind sheep were available for long-term follow-up. These were treated unilaterally with a single subretinal injection of an AAV5 vector carrying either the mouse (n=4) or human (n=5) *CNGA3* transgene under control of the 2.1-Kb red/green opsin promoter. The efficacy of treatment was assessed periodically with photopic maze tests and electroretinographic (ERG) recordings for up to 74 months post-operatively. Safety was assessed by repeated ophthalmic examinations and scotopic ERG recordings. The retinas of three animals that died of unrelated causes >5 years post-treatment were studied histologically and immunohistochemically using anti-hCNGA3 and anti-red/green cone opsin antibodies. Passage time and number of collisions of treated sheep in the photopic maze test were significantly lower at all follow-up exams as compared with pre-treatment values ($P=0.0025$, $P<0.001$, respectively). ERG Critical Flicker Fusion Frequency and flicker amplitudes at 30 and 40Hz showed significant improvement following treatment ($P<0.0001$) throughout the study. Ophthalmic examinations and rod ERG recordings showed no abnormalities in treated eyes. Immunohistochemistry revealed the presence of CNGA3 protein in red/green opsin positive cells (cones) of treated eyes. Our results show significant, long-term improvement in cone function, demonstrating a robust rescue effect up to six years following a single treatment with a viral vector that provides episomal delivery of the transgene. This unique follow-up duration confirms the safe and stable nature of AAV5 gene therapy in the ovine achromatopsia model.

Key words: Cones; day blindness; electroretinography

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INTRODUCTION

Congenital achromatopsia is a hereditary vision disorder with worldwide prevalence of approximately 1:30,000.[1] Cone photoreceptor function is primarily affected, and in cases of complete achromatopsia, only rod vision is present. As a result, patients suffer from severely reduced visual acuity and absent color perception, as well as day blindness, nystagmus and photophobia.[1, 2]

To date, mutations in six genes –*CNGA3*, *CNGB3*, *GNAT2*, *PDE6C, PDE6H* and *ATF6*– have been identified as causes of achromatopsia.[2] However, most cases are caused by mutations in either *CNGB3* or *CNGA3*, encoding for the cyclic nucleotide-gated ion channel subunits β or α, respectively, in cone photoreceptors.[3, 4] Particularly in Israel and possibly also in China, *CNGA3* mutations are the most common cause for achromatopsia and the prevalence reaches 1:5000 in the Jerusalem region in Israel.[5-7]

In 2010, our group reported on a novel disorder of congenital day blindness in sheep, caused by a C→T substitution leading to the appearance of a stop codon in the ovine *CNGA3* gene.[8, 9] This new, naturally-occurring large animal model of *CNGA3* achromatopsia was thoroughly characterized and investigated by means of visually-guided behavior testing, electroretinography (ERG), and histological as well as immunohistological (IHC) techniques.[10]

Starting in the summer of 2011, affected *CNGA3*-mutated sheep underwent unilateral gene augmentation therapy using adeno-associated virus 5 (AAV5) vectors carrying either the mouse or the human *CNGA3* transgene under control of the cone-specific 2.1-Kb red/green opsin promoter.[11] This treatment resulted in restoration of cone-mediated vision, demonstrated by both ERG and behavioral maze tests, that at the time of our original report was maintained for three years.[12]

The purpose of this study was to provide further follow up of the treated animals and to investigate the efficacy of gene augmentation therapy up to six years following a single subretinal treatment by means of functional assessment as well as histological and IHC assays. With the advent of clinical trials in *CNGA3* achromatopsia patients (NCT02935517 and NCT02610582), this long-term efficacy and safety follow-up holds great

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relevance for the development of treatments for this debilitating disease. Furthermore, it provides evidence that, at least in this large animal model, episomal delivery of the transgene provided by the AAV5 vectors allows for a long-term, stable effect that appears to resemble that seen in AAV vector treated eyes of RPE65 mutant dogs that serve as a canine model for Leber's congenital amaurosis (LCA).[13-18]

MATERIALS AND METHODS

Study design

Starting in the summer of 2011 nine affected sheep (5 females and four males, aged 4-10 months), homozygous for the *CNGA3* mutation, were treated unilaterally with a subretinal injection of an AAV5 vector containing either the normal mouse (n = 4) or human (n = 5) *CNGA3* transgene under control of a 2.1-Kb red/green opsin promoter.[12] Fellow eyes served as untreated control. For over six years since then, efficacy of treatment was evaluated by periodic maze testing and ERG recordings, and safety by ophthalmic examination and fundus photography. Age at the time of surgery, the transgene delivered and follow-up examinations for each animal are presented in Table 1. Three animals were sacrificed due to unrelated causes and their retinas used for histology and IHC studies.

Ethics and animal welfare

Experimental protocols were approved by the Volcani Center Institutional Animal Care and Use Committee and were conducted in accordance with principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. For electrophysiological recordings, animals were premedicated with acepromazine (Vetmarket Ltd, Shoham, Israel) and pethidine (Dolantina; Kern Pharma, Barcelona, Spain), induced with propofol (Diprofol; Taro Pharmaceutical Industries Ltd, Mount Pleasant, NY, USA) and diazepam (Ratiopharm, Ulm, Germany), and anesthetized and ventilated with isoflurane (Piramal Critical Care, Bethlehem, PA, USA).

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Visually guided behavior testing

Photopic maze navigation tests were conducted for each animal 0.5-2.0 months prior to gene augmentation treatment (baseline test), and up to 68.5 months post-operatively (Table S1). Mean (±SE) duration of behavioral follow up was 61.4 ± 1.3 months, with each animal tested 11 - 15 times. The test protocol has been previously described in detail.[12, 19] Briefly, animals were directed to pass through a 9-meter maze with two barrier obstacles. A group of sheep positioned at the end of the maze attracted the test animal to pass through the maze as quickly as possible. For each animal, a test consisted of two successive trials with the barriers randomly rearranged in their right-left orientation between trials to avoid a learning effect. Passage time and number of collisions with the obstacles were recorded for each trial. A trial lasted up to 30 seconds, and a passage time of 30 seconds was ascribed to animals that failed to transit the maze. The average illumination intensity at the testing area (mean ± SD) was 3352 ± 277 lux.

In addition, a subset of the maze tests (comprised of the last 5-6 tests for each animal) included additional trials with alternate patching of the treated and fellow untreated eyes, as described previously.[20]

ERG

ERG recordings were conducted 0.5-2.0 months prior to gene augmentation treatment and up to 73.6 months post-operatively. Mean duration of ERG follow up was 62.0 ± 1.8 months, during which an average of 7.7 ± 1.1 recordings were conducted in each animal (Table 1). The number of ERG follow-ups was smaller than the number of behavioral follow-ups as the former requires general anesthesia, which became increasingly risky as the animals aged, and was therefore performed more sparingly. Cone function was evaluated by full-field flash ERG as described previously.[10, 12, 19] Briefly, recordings were performed using a handheld mini Ganzfeld stimulator (HMsERG, Ocuscience, Henderson, NV, USA) with a bandpass of 0.3–300 Hz. Following 10 minutes of light adaptation (30 cd/m^2), responses to a series of flickers of increasing frequency (flashes presented at 10–80 Hz, with 128 responses averaged at each frequency) was recorded. These photopic flicker responses were recorded at four intensities (1, 2.5, 5, and

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10 cd·s/m^2). Responses of both treated and control eyes were recorded sequentially, in random order.

Flicker response amplitudes were measured between peak and trough. Where no flicker response was detected, a value of zero was inserted as the amplitude. The critical flicker fusion frequency (CFFF), or the highest frequency at which the eye could resolve flicker, was determined for each of the four intensities.

Rod function was evaluated post-operatively once in each animal, at least 7 days after the last photopic ERG recording (last column of Table 1), using a previously described protocol.[20] Briefly, under dark-adapted conditions, responses to 10 dim flashes (0.01 cd·s/m^2) presented at 0.5Hz were recorded and averaged to generate a single scotopic flash response. This was repeated every 4 min for a total of five scotopic flash responses recorded over 20 minutes of dark adaptation. Both eyes were recorded simultaneously.

Histology and IHC

Eyes were enucleated and fixed in Davidson solution. From each eye, three ~5 mm wide strips were cut along the naso-temporal plane, crossing the center of the optic nerve. The strips were dehydrated, and embedded in paraplast (Leica Biosystems, Nussloch, Germany). From each strip, 4-µm sections were cut and one section was stained with hematoxylin and eosin. For IHC, deparaffinized sections of the eyes were incubated in a decloaking chamber (Biocare Medical, Pacheco, CA, USA) with 10mM of citrate buffer (pH 6.0) at 125°C, blocked with phosphate-buffered saline solution containing 1% (w/v) bovine serum albumin, 0.1% (w/v) Triton X-100, and 10% (w/v) normal donkey serum, and subsequently incubated overnight at 4°C with anti-hCNGA3 antibody (goat polyclonal, 1:50; Santa Cruz Biotechnology, Inc, Dallas, TX, USA) and anti-red/green cone opsin antibody (rabbit polyclonal, 1:100; Chemicon International, Temecula, CA, USA). After washing, the corresponding secondary antibodies were applied for 1h at room temperature: rhodamine red-X-conjugated donkey anti-goat IgG and DyLight 488 donkey anti-rabbit IgG (1:250, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Nuclei were counterstained with 4,6-diamidino-2-phenylindole containing mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA). To determine the

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specificity of the antigen–antibody reaction, corresponding negative controls were exposed to secondary antibody alone.

Data evaluation and statistical analyses

For analysis of visual behavior and ERG, results were grouped into 21 and 12 follow-up groups, respectively, according to time (months) before/after treatment as detailed in Tables S1 and S2. The 68 and 74 months follow-up groups were not included in the ERG analysis since only one animal was recorded at each of these time points.

Visual behavior tests.

Results for navigation time and number of collisions of the two trials in each maze navigation test were averaged and were subjected as such or after log transformation to analysis of variance. For analysis of the overall data set, the statistical models included the effects of the transgene (mouse or human *CNGA3*), animal (n = 9) and follow-up time (months) from the treatment. For calculating the least squares mean (LSM) of the maze navigation time and number of collisions, the results were grouped into follow-up groups. For further analysis, the animal effect was replaced in the model with sex (male or female).

The statistical model for analysis of the data subset collected when the maze test was conducted using alternate eye patching included the effects of transgene, animal nested within transgene, time after treatment (follow-up group), patched eye and vector x patch eye interaction. A p-value < 0.05 was considered significant. Results are expressed as LSM ± SE.

ERG.

The following photopic ERG parameters were analyzed for each eye: 30 and 40Hz flicker amplitudes, and CFFF values, at 10 cd·s/m^2. For scotopic recordings, b-wave amplitudes of the final (20 min) response were analyzed.

The collected data were analyzed with SPSS version 22.0 (IBM Corporation, Armonk, NY, USA), and with STATA, version 14.0 (StataCorp, College Station, TX, USA). We favored the linear mixed-effect model approach over repeated measures mixed-design

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since the linear mixed-effect model can tolerate some degree of imbalanced data, which was introduced by animals that were not included in each follow-up group or by noisy and unmeasurable responses in certain recordings. The fixed effects were transgene and follow-up groups while the random effect was the animal. A p-value < 0.05 was considered statistically significant.

RESULTS

Visually guided behavior testing

The association between time from treatment and maze navigation time is illustrated in Fig. 1A. Mean maze navigation time was > 25 sec in the pretreatment period (follow-up group 0), and within weeks after treatment significantly dropped to that of unaffected control animals, and remained statistically unchanged (p > 0.05) for the duration of the follow-up period. Analysis of the whole data set showed that for maze navigation time, time from treatment (follow-up group) had a significant effect (p = 0.001) while the effects of the transgene and animal were not significant (p > 0.05). Sex had no significant effect on navigation time (p > 0.05).

The association between time from treatment and number of collisions with obstacles during maze navigation is illustrated in Fig. 1B. The mean number of collisions was > 2 in the pretreatment period (follow-up group 0), and within weeks after treatment significantly dropped (< 0.5 collisions) and remained statistically unchanged for the duration of the follow-up period. Analysis of the number of collisions with obstacles during maze navigation demonstrated that time from treatment had a significant effect (p < 0.001). The transgene also had a significant effect on the number of collisions (p = 0.01) with an average of 0.4 and 0.1 collisions for animals treated with the human *CNGA3* and the mouse *CNGA3* transgenes, respectively. The specific animal did not have a significant effect on the number of collisions (p > 0.05).

Analysis of the subset of maze navigation tests conducted using alternate patching of eyes demonstrated that patching either the treated (right) or the untreated (left) eye had a significant effect (p < 0.0001) on navigation time. Average navigation time with the right treated eye was patched was 26.8 ± 0.7 seconds, significantly (p < 0.05) longer than

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the navigation time when the left untreated eye was patched (8.7 ± 0.7 sec) or when neither eye was patched (6.3 ± 0.7 sec). The transgene had no effect on navigation time in this subset of maze tests ($p = 0.18$). Eye patching also had a significant effect ($p < 0.0001$) on the number of collisions with obstacles during the navigation test. Animals collided with obstacles 3.9 ± 0.2 times when the treated eye was patched, and 0.3 ± 0.2 times when either the left eye, or neither eye, was patched. The transgene had a significant effect on the results of this subset of tests. When the treated right eye was patched, the number of collisions was 4.8 ± 0.3 and 3.1 ± 0.3 collisions for animals treated with the human and the mouse transgenes, respectively ($p < 0.05$). However, no difference ($p > 0.05$) was found between the two transgene treatment groups when the left eye, or neither eye, was patched (***See movie*** *in supplementary material*).

ERG

ERG recordings showed significant, long-term improvement in cone function following *CNGA3* augmentation therapy (Fig. 2). Representative flicker responses to 30-80 Hz stimuli (at 10 cd·s/m^2) are shown in Fig. 2A. Short- and long-term improvement of critical flicker fusion frequency (CFFF) at all four light intensities is shown in Fig. 2B. CFFF values of treated eyes in all eleven follow-up groups were significantly higher ($p < 0.0001$) than pre-operative values (follow-up group -1). On the other hand, CFFF values of the fellow untreated control eyes were not significantly different ($p > 0.05$) from pre-operative values (Table S3).

Responses to the 10 cd·s/m^2 30-80 Hz flicker stimuli, plotted as a function of the stimulus frequency, are shown in Fig. 2C. The 30 and 40 Hz flicker amplitudes of treated eyes in all eleven follow-up groups were significantly higher ($p < 0.006$) than pre-operative values (follow-up group -1). On the other hand, the 30 and 40 Hz flicker amplitudes of the fellow untreated control eyes were not significantly different ($p > 0.05$) than the pre-operative values in 8/11 of the follow-up groups (Table S3).

Analysis of rod responses revealed no significant differences between treated and fellow, untreated eyes ($p > 0.05$, data not shown).

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Safety, histology and IHC

Full autopsy of sheep 4954, euthanized 67 months post-operatively following post-parturition complications, showed no external pathologic findings. Excessive amounts of clear serous fluid without fibrin strands were found in the pericardiac sac. No pathological changes were seen in the myocard, endocardial valves and papillary muscle of the heart. Diffuse hyperemia was seen in all lung lobes. Consolidation and color changes were seen in the cranioventral lung lobe, affecting 10 - 15% of all lung volume. In the abdominal cavity there were chronic focal adhesions of the diaphragmatic liver lobes to the mid ventral diaphragm. Both kidneys were relatively soft (due to possible autolysis) and diffusely pale. There was focal, patchy, mild hyperemia of the serosa and mucosa of the duodenum and jejunum. No pathological findings were found in the brain and other organs. No abnormal histopathological findings were evidenced in the cerebrum, cerebellum and midbrain.

Serological tests for paratuberculosis and Visna/maedi virus in six sheep (Table 1) were negative. *Trueperella pyogenes* was isolated from the lungs of sheep 4954.

Serial ophthalmic examinations during this follow-up study revealed no significant differences between treated and untreated eyes. Histological H&E sections of the eyes of sacrificed animals (Table 1) were examined by a pathologist. Vascular congestion in the choroid and sclera, as well as evidence for focal areas of retinal atrophy, were seen in the treated eye of sheep 4056 (Figs. 3A-D). An area of local atrophy, as well as pigment granules, were seen in the treated retina of sheep 5187 (Fig. 3E). In addition, vascular congestion was noticed in the inner retina (Fig. 3F). In both animals these areas of atrophy and congestion were apparently located within the treated areas, as the blebs of sheep 4056 and 5187 were dorsal and ventral to the optic nerve heads, respectively.

IHC showed expression of the CNGA3 protein colocalized with red/green opsin in treated eyes of animals 5187 (treated with the human transgene), and 4056 and 3909 (treated with the mouse transgene) (Fig. 4A, B). No CNGA3 labeling was seen in the fellow, untreated eye of animal 3909, nor in the negative control (Figs. 4D, E). However, CNGA3 labelling was seen in the untreated control eyes of animals 4056 and 5187 (Fig. 4C).

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DISCUSSION

We report that in day blind sheep, gene augmentation therapy applied with a single subretinal injection of an AAV5 vector carrying either the mouse or human *CNGA3* transgene results in recovery of cone photoreceptor function, as demonstrated by both photopic behavioral maze tests and by ERG examinations (***see movie*** *in supplementary material*). At the time of writing, this recovery lasted an average of 62 months in our 9 study animals, and 73.6 months in the longest surviving animal, though data collection continues in the five surviving animals (Table 1).

CNGA3 achromatopsia is currently investigated using several animal models including mice and dogs.[21, 22] In mice, cone rescue following gene augmentation therapy using different AAV vectors was demonstrated for a period of 9 months.[15] To the best of our knowledge, gene augmentation therapy has not been reported in the canine *CNGA3* achromatopsia model, but it has been reported in the canine *CNGB3* achromatopsia model, where gene therapy using AAV5 was found to rescue cone function for at least 33 months.[23] Therefore, we believe that our ongoing study is currently the longest gene therapy follow up report published in any animal model of achromatopsia.

Alongside achromatopsia, gene therapy is being investigated in several other retinal diseases, where large animal models are used for long-term follow-up. Therapy using AAV2/5 in a canine model of *CNGB1*-deficient retinitis pigmentosa was found to rescue rod function for a period of 18 months,[24] and in a canine model of X-linked retinitis pigmentosa therapy using AAV2/5 was shown to arrest vision loss for a period of 2 years.[25] Gene augmentation therapy in an *RPE65* canine model of LCA showed restoration of rod and cone function during 4-11 years of follow-up.[13-18] These long-term studies in canine models of LCA have paved the way for clinical trials in patients, with follow-ups of 3-6 years reported in patients treated with AAV2/2,[13, 26, 27] and one year in patients treated with AAV2/4.[28] Based on these results, the FDA has recently approved Luxturna™ as a treatment for LCA. Similarly, positive results in our sheep model[12, 20] have paved the way for clinical trials in *CNGA3* achromatopsia patients (NCT02935517 and NCT02610582). As

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these clinical trials are ongoing, there is great importance for the long-term follow-up efficacy and safety results reported in the present study.

The question whether gene augmentation therapy based on episomal delivery of the transgene (as achieved with AAV vectors) will provide a stable rescue effect, or one that may diminish over time, is an open one. In contrast to insertion of the transgene into the host genome (as can be achieved with lentiviruses, for example), the DNA delivered by AAV vectors may degrade and become less effective, even in non-dividing cells like the photoreceptors. In the clinical arena, a number of reports on patients treated for *RPE65* LCA suggest some loss of treatment effects and failure to fully arrest disease progression a few years following treatment. [26, 27] However, even with the decline in benefit noted at 6 years post-treatment for RPE65 LCA patients[27] , vision function within the vector-treated area was still above the pre-treatment baseline. The stable effect seen to date in the sheep model of *CNGA3* achromatopsia may be due to delivery of a higher dose of the vector, the young age of the animals at time of treatment, and/or the fact that achromatopsia is largely non-progressive in nature,[1, 29, 30] such that an underlying degenerative process at the cellular level is not present. Nonetheless, our results reflect the need to study the benefit over a period even longer than 6 years in both animal models and human patients

The improvement in photopic visual behavior and ERG recordings of the treated eyes was significant throughout the study, and no time-dependent attenuation was noted in any of the parameters analyzed. Our IHC analysis also showed long-lasting effect of the treatment. Expression of the CNGA3 protein, colocalized with red/green opsin, was seen in the treated eyes of the animals sacrificed 58-73 months post-operatively. However, animals 4056 and 5187 that were treated in the right eye with a mouse and a human CNGA3 transgene, respectively, unexpectedly also showed suspected CNGA3 immunolabeling in the untreated left eye (Fig. 4). Nonetheless, results of our ERG recordings and behavioral tests did not reflect any photopic retinal function in the control eyes of these sheep. This may indicate that only a small number of cones were transfected in the contralateral eyes of animals 4056 and 5187, as our IHC finding is qualitative, rather than quantitative. Furthermore, studies in the RPE65 dog model of LCA have shown that on occasions there is no correlation between ERG rescue and numbers of surviving

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photoreceptors.[31] Admittedly, the last ERG recordings and maze tests of these two sheep were conducted 4-9 months prior to death and our IHC findings, but we do not believe that vector distribution and expression in the left eyes occurred in this short interval.

Boye *et al* studied guanylate cyclase-1 knockout mice treated subretinally in one eye with AAV and found vector genomes in the ipsilateral optic nerve and in the contralateral hemisphere of the brain.[32] Biodistribution of the viral vector was also investigated in nonhuman primates following subretinal delivery of AAV5 carrying the human rhodopsin kinase transgene.[33] Vector genome was recovered from the injected retinas, ipsilateral optic nerves and select parts of the brain, but no transgene expression was detected outside the retinal bleb.[33] Thus, it is possible that in our animals the vector reached the photoreceptors of the contralateral eye either through transneuronal passage from the ipsilateral to the contralateral retinal ganglion cells, possibly at the optic chiasma, or via systemic distribution. The latter route is less likely due to the dilution of the relatively small amount of vector (Table 1) in a 50 kg sheep, and its exposure to the systemic immune system. Obviously, biodistribution studies in the optic nerves and chiasm, brain and other organs are needed to establish the possible infection route.

We did not detect immunolabeling of CNGA3 in untreated control eyes during our previous gene augmentation sheep studies. [12, 20] Therefore, we consider the findings in the control eyes of 4056 and 5187 as preliminary results. Since we have only five surviving treated animals which represent a unique resource that allows such an ongoing long follow up, we feel it would be improper to sacrifice them to verify these findings and study the important implications of our IHC findings. Instead, we believe that a prospective study with a new set of experimental animals, as well as additional animal models, and the use of RNA assays, should be conducted. And obviously, additional PCR and IHC studies will be performed in the five surviving sheep should any of them die.

Another important issue, in addition to treatment efficacy, is that of safety. Repeated post-operative ophthalmic examinations of the sheep by a board-certified veterinary ophthalmologist showed no significant abnormalities in treated eyes during the study years. In addition, scotopic ERG recordings conducted at the last follow-up revealed

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no impairment of rod function in treated eyes. In two treated eyes, histopathology showed focal areas of retinal atrophy and vascular congestion (Fig. 3). However, despite these lesions, the last antemortem ERG recording and maze test of these eyes showed robust photopic function, not significantly different from that of the other experimental animals. Interestingly, the two animals in which these lesions were found (in their treated eyes) are the same animals in which IHC staining revealed CNGA3 protein expression the untreated eyes. However, our surgical records do not document any intravitreal reflux or any other intraoperative protocol deviations that can explain the inflammation or viral distribution in these animals. Nonetheless, it is possible that micro trauma to the RPE, caused as the cannula penetrated the sensory neuroretina (E. Averbukh, unpublished results), may be responsible for some of the pathology in the operated eyes.

Since our last report,[12] four sheep have been euthanized 58-73 months post-operatively. The animals were 5.5-6.3 years old at the time of death, and had been anesthetized 7-9 times for surgery and ERG recordings (Table 1). Two animals (3909 and 4954) were euthanized following post-parturition complications, and the other two (4056 and 5187) exhibited signs of a wasting disease few months before death. Though their behavior and appetite were unaffected, they lost weight and were classified as cachectic by a boarded specialist in small ruminant medicine. Serological testing for the two leading causes of wasting diseases in sheep (paratuberculosis and Visna/maedi) was negative. Autopsy did not revealed specific cause of death. The hyperemia noted in the lungs and intestines can result from premortem stress and agony. Combination of parturition and pneumonia caused by the isolated *Trueperella pyogenes*, a bacterium that can cause abscesses, mastitis, metritis and pneumonia in sheep, might explain the animals' extreme exhausted status that finally resulted in recumbency and euthanasia.

In conclusion, we report successful restoration of photopic vision in nine day blind sheep following gene augmentation therapy with AAV5 carrying the mouse/human CNGA3, lasting for over six years. We intend to continue monitoring the remaining five animals and perform periodic follow-up evaluation using ERG recordings and behavioral tests. We believe that it is essential to preserve these unique and precious animals that

currently represent the longest surviving patients of gene augmentation therapy reported in an animal model of achromatopsia.

ACKNOWLEDGEMENTS

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AUTHOR DISCLOSURE

WWH and the University of Florida have a financial interest in the use of AAV therapies, and own equity in a company (AGTC) that might, in the future, commercialize some aspects of this work. All other authors have no competing financial interests exist.

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Table 1: *Age, treatment, and follow-up of nine experimental day blind sheep treated with AAV5 vectors carrying either the mouse or human CNGA3 transgene*

Animal No.	Age at surgery (months)	Viral dose ($VG \cdot 10^{11}$)	Bleb volume (µl)	Sex	Transgene (m/h)	Post-op maze testing		Post-op ERG testing	
						Number of tests	Follow-op duration (months)	Number of tests	Follow-op duration (months)
*4056	4	12.0	500	M	m	15	68.5	8	62.9
£4019	6	14.4	600	F	m	15	66.4	10	73.6
*3909	6	6.5	500	F	m	14	59.2	7	55.0
£4855	6	7.1	600	M	m	15	60.9	8	62.3
£*4954	6	6.4	500	F	h	14	60.9	7	62.2
£4984	5	5.8	450	M	h	15	60.9	8	62.3
£4826	7	6.4	500	F	h	15	60.5	7	62
£4624	10	5.1	400	M	h	15	60.5	8	61.8
*5187	9	4.5	550	F	h	11	54.5	6	55.7

AAV, adeno-associated virus; ERG, electroretinography; VG, viral genomes; m, mouse; h, human *Animal sacrificed and studied histologically.

£ Animal was tested serologically for paratuberculosis and Visna/maedi virus

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FIGURES LEGENDS

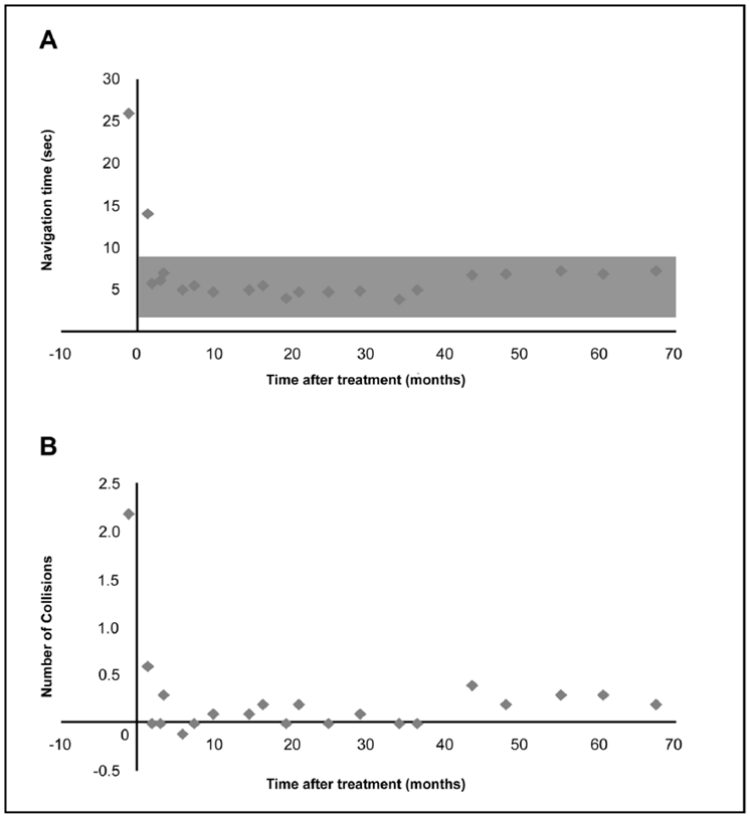

Figure 1. Long-lasting improvement in photopic maze testing of nine day-blind sheep that underwent gene augmentation therapy with the mouse or human *CNGA3* transgene. Results are presented as group's Least Square Mean. (**A**) Maze navigation time (seconds) as a function of time (months) after treatment. The group's average SE was 1.23 sec. Baseline results of normal, unaffected sheep (mean ± 2 SD) were determined previously[12] and are presented as a gray background. (**B**) Number of collisions into obstacles while navigating the maze as a function of time (months) after treatment. The group's average SE was 0.22 collisions.

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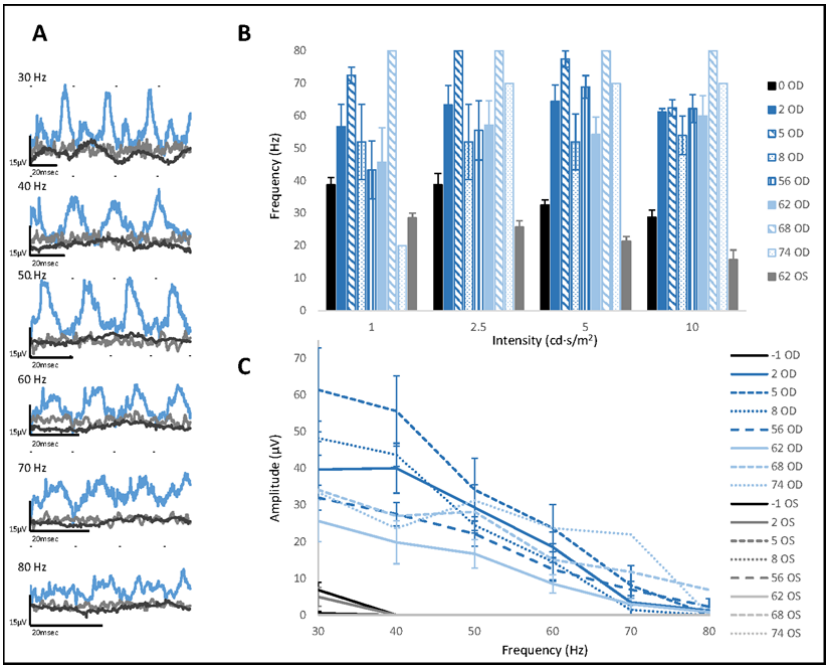

Figure 2. Long-lasting improvement in ERG recordings of nine day-blind sheep that underwent gene augmentation therapy with the mouse or human *CNGA3* transgene. (**A**) Cone flicker responses (30-80 Hz, 10 cd·s/m^2) of a representative *CNGA3*-mutant sheep eye treated with the mouse *CNGA3* transgene (sheep 4019). Black tracings: baseline, preoperative recordings of the treated eye; blue and gray tracings: recordings in the treated (*blue*) and untreated fellow (*gray*) eyes of the same sheep, 73.6 months postoperatively. (**B**) Critical flicker fusion frequency (CFFF) of *CNGA3*-mutant sheep treated with the *CNGA3* transgene. Black bars: baseline, preoperative CFFF of the treated eyes at four intensities (1, 2.5, 5, and 10 cd·s/m^2). Blue bars: post-operative CFFF of the treated eyes (key to bar colors, and the corresponding follow up interval groups (in moths), shown on the right.). Gray bars: CFFF of the untreated fellow eyes of the 62 months follow-up group. (**C**) Cone flicker amplitudes of *CNGA3*-mutant sheep treated with the mouse or human *CNGA3* transgene. Black lines: baseline preoperative flicker amplitudes of the treated and control eyes. Blue lines: post-operative amplitudes of the treated eyes (key to line colors, and the corresponding follow up interval groups (in months), shown on the right). Gray lines: flicker amplitudes of the untreated fellows eye at the same follow-up interval groups. Responses to the 10 cd·s/m^2 flicker stimulus are plotted as a function of the stimulation frequency (Hz) and results are presented as mean ± SE in µV.

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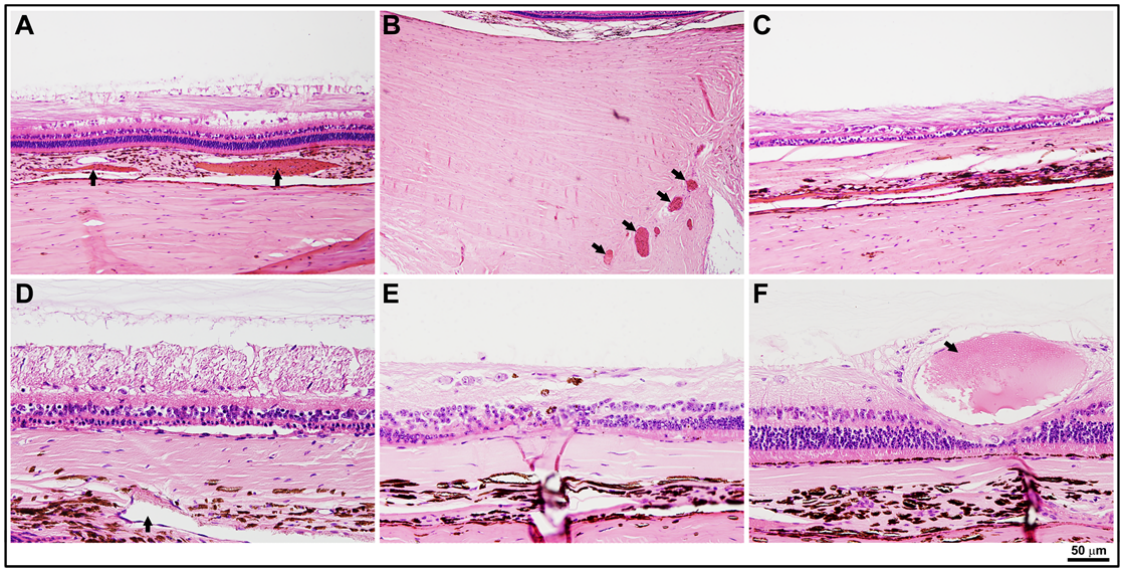

Figure 3. Histology of the posterior segment of the right eyes of sheep 4056 treated with the mouse *CNGA3* transgene, 73 months post-operatively. (**A**-**D**), and sheep 5187 treated with the human *CNGA3* transgene, 58 months post-operatively (**E, F**). Naso-temporal plane strip ventral to the optic nerve; vascular congestion in the choroid and sclera is marked with black arrows (**A-B**). Naso-temporal plane strip at the optic nerve level; retinal atrophy can be seen noticed (**C**). Naso-temporal plane strip dorsal to the optic nerve; vascular congestion in the choroid is marked with a black arrow, and retinal atrophy is evident (**D**). Naso-temporal plane strip ventral to the optic nerve; local atrophy and granules of pigment are seen in the retina (**E**). Naso-temporal plane strip dorsal to the optic nerve; vascular congestion in the inner retina is marked with a black arrow (**F**).

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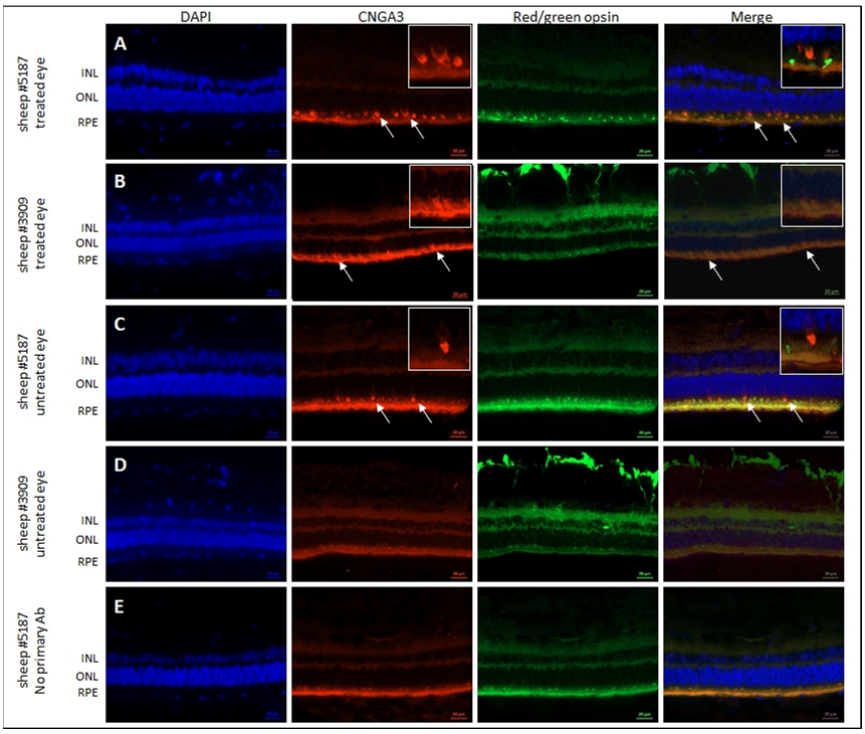

Figure 4. Immunohistochemistry of two representative day blind sheep retinas (5187 and 3909) following gene augmentation therapy with the human and mouse *CNGA3* transgene, respectively. Rows (**A**, **B**) In both treated eyes, anti-CNGA3 staining shows strong protein expression (*red*) in the photoreceptor layer, and colocalization with red/green opsin (*green*). Rows (**C**, **D**) Fellow, untreated control eyes. Anti-CNGA3 staining shows protein expression (*red*) in the photoreceptor layer of sheep 5187, but not in sheep 3909. (**E**) Negative control in which the secondary antibodies were applied without prior use of primary anti-CNGA3 or anti-red/green cone opsin antibodies. No CNGA3 expression was observed in the retina. The arrows in rows A, B and C point to cones expressing CNGA3. An enlarged view is shown as an inset in positively-stained sections. INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Nuclei are counterstained with 4',6-diamino-2-phenylindole (*blue*). Original magnification x40.

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Table S1: *Follow-up groups of visual behavior tests of nine experimental day blind sheep treated with AAV5 vectors carrying either the mouse or human CNGA3 transgene*

Animal No.	4056	4019	3909	4855	4954	4984	4826	4624	5187	
Follow up group (months)	Times of visual behavior test before or after treatment (months)									mean
-1	-0.5	-2.6	-2.6	-0.4	-0.4	-0.4	-0.8	-0.8	-0.4	-1.0
1.5		1.5	1.5				1.6	1.6	1.3	1.5
2	2.2			2.1	2.2	2.1	2.2	2.2		2.2
3				2.7	2.7	2.7	3.0	3.0		2.8
3.5	3.6	4.3	4.3	3.4	3.4	3.4			3.6	3.7
6	6.3			6.1	6.1	6.1	5.6	5.6		6.0
7.5		7.4	7.4	7.7	7.7	7.7	7.3	7.3	8.5	7.6
10	9.5			10.0	10.6	10.0	9.5	9.5	9.8	9.9
14.5	15.4	13.3	13.3	14.9	14.9	14.9	14.4	14.4		14.4
16.5		17.3	17.3	16.2	16.2	16.2	15.8	15.8	17.6	16.6
19.5	19.4	19.0	19.0					19.7		19.3
21	21.0	21.2	21.2							21.2
25	24.3	26.1	26.1	24.0	24.0	24.0	23.5	23.5	27.7	24.8
29	29.5	27.5	27.5						30.7	28.8
34	33.5			34.1	34.1	34.1	33.6	33.6		33.8
36.5	37.3	35.2	35.2	37.1	37.1	37.1	36.6	36.6	36.7	36.6
43.5		45.3	45.3	43.1	43.1	43.1	42.6		41.5	43.4
48	47.4	48.3	48.3	47.9		47.9	47.5	47.5	48.7	48.0
55	56.4	54.3	54.3	55.1	55.1	55.1	54.7	54.7	54.5	54.9
60.5	61.2	59.2	59.2	60.9	60.9	60.9	60.5	60.5		60.4
67.5	68.5	66.4								67.4

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Table S2: *Follow-up groups of ERG recordings of nine experimental day blind sheep treated with AAV5 vectors carrying either the mouse or human CNGA3 transgene*

Animal No.	4056	4019	3909	4855	4954	4984	4826	4624	5187	
Follow up group (months)	Times of visual behavior test before or after treatment (months)									mean
-1		-0.3	-0.3	-0.5	-0.5	-0.5	-0.5	-0.3	-2.4	-0.7
2	2.9	0.8	0.8	2	2	2	1.5	1.6	2.0	1.7
5	6.2	4.1	4.1	5.9						5.1
8					8.1	7.7	7.2	7.2	8.7	7.8
11	12.8	10.7	10.7							11.4
19	21.7	19.6	20	18.6		18.5		16.5		19.2
30	32.1	29.5	29.5							30.4
37				37.6	37.6	37.6	36.6	37.1	37.1	37.3
43				43.5	43.6	43.5	44.3	43.1	42.9	43.5
49	51.1	48.5	48.5	49.8	49.9	49.5	49.0	49.0	49.9	49.5
56	57.1	55.0	55.0	55.9	56.4	55.9	56.0	55.5	55.7	55.8
62	62.9	60.9		62.3	62.2	62.3	62.0	61.8		62.1
68		67.9								
74		73.6								

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Table S3: *P-values of CFFF and 30 and 40Hz flicker amplitudes for the 11 follow-up groups compared to the pre-operative values (follow-up group -1, approximately 1 month before treatment) in treated right (OD) and fellow, untreated control left (OS) eyes. Follow-up groups 68 and 74 were not included in this analysis since only one animal was recorded at each of these time points.*

Follow up group	CFFF		30Hz Flicker amplitudes		40Hz flicker amplitudes	
(months)	OD	OS	OD	OS	OD	OS
2	<0.0001	0.202	<0.0001	0.022	<0.0001	0.969
5	<0.0001	0.355	<0.0001	0.578	<0.0001	0.788
8	<0.0001	0.805	<0.0001	0.999	<0.0001	0.860
11	<0.0001	0.751	<0.0001	0.613	<0.0001	0.807
19	<0.0001	0.635	<0.0001	0.015	<0.0001	<0.0001
30	<0.0001	0.751	<0.0001	0.002	<0.0001	0.807
37	<0.0001	0.286	<0.0001	0.886	<0.0001	0.586
43	<0.0001	0.112	<0.0001	0.913	0.006	0.916
49	<0.0001	0.774	<0.0001	0.203	<0.0001	0.969
56	<0.0001	0.149	<0.0001	0.790	<0.0001	1.000
62	<0.0001	0.117	<0.0001	0.769	<0.0001	0.977