

Gene Therapy for Color Blindness

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Achromatopsia is a rare congenital cause of vision loss due to isolated cone photoreceptor dysfunction. The most common underlying genetic mutations are autosomal recessive changes in *CNGA3*, *CNGB3*, *GNAT2*, *PDE6H*, *PDE6C*, or *ATF6*. Animal models of *Cnga3*, *Cngb3*, and *Gnat2* have been rescued using AAV gene therapy; showing partial restoration of cone electrophysiology and integration of this new photopic vision in reflexive and behavioral visual tests. Three gene therapy phase I/II trials are currently being conducted in human patients in the USA, the UK, and Germany. This review details the AAV gene therapy treatments of achromatopsia to date. We also present novel data showing rescue of a *Cnga3*^{-/-} mouse model using an rAAV.CBA.CNGA3 vector. We conclude by synthesizing the implications of this animal work for ongoing human trials, particularly, the challenge of restoring integrated cone retinofugal pathways in an adult visual system. The evidence to date suggests that gene therapy for achromatopsia will need to be applied early in childhood to be effective.

INTRODUCTION

Achromatopsia is a rare (1 in 30,000 to 80,000) congenital visual condition characterized by diminished or absent cone photoreceptor function [1]. Patients have severely reduced visual acuity (~20/200), nystagmus and photophobia [2]. Causative autosomal recessive mutations affect genes of the cone phototransduction cascade, *CNGA3*, *CNGB3*, *GNAT2*, *PDE6H*, *PDE6C*, (Table 1) [3–8] or the transcription factor *ATF6* [9]. A subset of patients have unidentified causative mutations [10]. Patients typically show complete cone function loss, as shown by absent cone-isolating electroretinogram (ERG[†]) recordings [2]. An incomplete phenotype occurs more rarely, but is not clearly correlated to the underlying mutation [1]. Achromatopsia affects all three classes of photoreceptor and hence, by definition, relates to genes

that are expressed in all cone subtypes, as listed above. Conversely, blue monochromatism, tritanopia, and red-green color blindness (deuteranopia and protanopia) are caused by mutations in genes specific to certain subtypes of cone photoreceptors; an important difference to achromatopsia. The blue cone opsin gene is found on chromosome 7 and the red and green opsins are both on the X-chromosome. For instance, Bornholm disease can affect the green or red opsin gene only, or blue cone monochromatism can affect both [11]. Tritanopia (loss of the S-cone opsin) is rare because unlike the X-linked diseases, a recessive variant requires a mutation on both alleles. More commonly tritanopia results from a dominantly inherited S-opsin mutation that leads to progressive loss of the S-cones, similar to the rod loss caused by rhodopsin gene mutations in retinitis pigmentosa.

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†Abbreviations: ERG, electroretinogram; bp, base pairs; AAV, adeno-associated Virus; gp, genome particle; OMR, optomotor response; CBA, chicken beta actin; IHC, immunohistochemistry; TKO, triple knock out; fMRI, functional magnetic resonance imaging; *CNTF*, Ciliary Neurotrophic Factor.

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Table 1. Summary of the genes known to cause achromatopsia.

Gene	Full name	Location	Gene size (kB)	Exons	CDS length (bp)	First report	Percentage of international cases (Kohl, 2004)	Percent of Netherlands cases (Thiadins, 2009)
<i>CNGB3</i>	cyclic nucleotide gated channel beta 3	8q21.3	170	19	2430	(Kohl, 2000)	47	87
<i>CNGA3</i>	cyclic nucleotide gated channel alpha 3	2q11.2	53	9	2085	(Kohl, 1998)	23	5
<i>GNAT2</i>	G protein subunit alpha transducin 2	1p13.3	16.6	9	1065	(Kohl 2002; Aligianis, 2002)	2	0
<i>PDE6C</i>	phosphodiesterase 6C	10q23.33	69	22	2577	(Chang, 2009)	Undiscovered	Not tested
<i>PDE6H</i>	phosphodiesterase 6H	12p12.3	9.6	4	252	(Kohl, 2012)	Undiscovered	Undiscovered
<i>ATF6</i>	Activating Transcription Factor 6	1q23.3	197.8	17	2013	(Kohl, 2015)	Undiscovered	Undiscovered

A well reported achromatopsia population lived on the South Pacific island of Pingelap [12]. A genetic bottleneck following Typhoon Lengkeki in 1775 created a founder effect and over 10 percent of the island population had complete achromatopsia [13]. The story is notably recorded in *The Island of the Colorblind* by the neurologist and author, Oliver Sacks. The causative cyclic nucleotide gated channel beta 3 subunit (*CNGB3*) gene mutation was subsequently genotyped [13]. *CNGA3* was the first achromatopsia gene to be identified [3] and since then five other autosomal (non-X-linked) genes have been discovered [4–9]. The coding sequences of all the genes causing achromatopsia are under 2,600 base pairs (bp), making them all small enough to package in an adeno-associated Virus (AAV) gene therapy vector [14]. Indeed, phase I clinical trials to treat achromatopsia using an AAV vector to deliver *CNGA3* have already started in Germany (NCT02610582), *CNGA3* in the USA and Israel (NCT02935517), *CNGB3* in the USA (NCT02599922), and *CNGB3* in the UK (NCT03001310) (Table 2).

This review will examine the examples of successful AAV rescue in animal models of achromatopsia, the registered human gene therapy trials and the potential barriers still to be overcome; particularly the reactivation of the dormant retinofugal pathway.

PRE-CLINICAL RESCUE OF ANIMAL MODELS

Eight different animal models of achromatopsia have been treated using AAV vectors utilizing either species-

specific or cross-species transgenes. There are also two instances of AAV transgene restoring normal spectral sensitivity in dichromatic or monochromatic animal models. After summarizing these preclinical experiments, we also present original data from our own experience with gene therapy for *Cnga3* achromatopsia in mice.

The first successful restoration of color vision using gene therapy in an animal model of achromatopsia delivered *Gnat2* cDNA to the *Gnat^{cpfl3}* mouse. The transgene was packaged in AAV5 under control of a fragment of human red cone opsin gene promoter that expresses in L/M-cones but not S-cones [15]. The vector was delivered by subretinal injection at a 10^{10} genome particle (gp) dose. Restoration of cone function was demonstrated on light adapted (photopic) electroretinography (ERG) and in a optomotor response (OMR) test. This work showed proof of principle for gene therapy to restore cone function in achromatopsia caused by a naturally occurring missense mutation in *Gnat2*.

Two canine models of *Cngb3* achromatopsia (missense in exon 6 or full deletion of *Cngb3*) were subsequently rescued using a AAV5 vector expressing human *CNGB3* [16]. The transgene was expressed with the same fragment of human red-green opsin promoter (PR2.1). The vector was delivered by transvitreal subretinal injection at a 10^{12} gp dose. Treatment improved both photopic electrophysiological response and functional vision in a photopic maze task. This study was the first to rescue a larger, cone-enriched model of achromatopsia and confirmed that human coding sequence could also rescue an animal model with the orthologous

gene defect. Dogs receiving treatment after a year of age however gained little benefit, which was discussed by the researchers as possibly relating to retinal cone pathway remodeling during the period of visual development.

A *Cnga3* knock-out mouse model had been previously developed by targeted deletion of exon 7 [17]. The first gene therapy rescue of this *Cnga3* mouse model also led to an improvement in cone-isolating ERG test steps and showed improved visually-mediated water maze behavior [18]. This study showed that restored cone signaling was integrated into higher cortical function, not just tectally-mediated optomotor responses.

The *Cnga3* knockout mouse model was rescued using a mouse *Cnga3* transgene under control of a 0.5 kB mouse short wavelength opsin promoter. This was packaged in a mutant AAV5^{Y719F} capsid with 6x10⁹ gp injected into the subretinal space of 2-week-old mice [18]. A modest cone ERG rescue was observed, approximately 40 to 50 percent. The magnitude of the ERG rescue is likely to have been diminished because the mouse blue opsin promoter only expresses in S-cones and a subset of M-cones [19].

To explore this promoter question further, the same research group subsequently rescued a different *Cnga3* mouse model (*cpfl5*) using the ubiquitous chicken beta actin (CBA) promoter [20]. This *cpfl5* mouse model of achromatopsia results from a missense mutation in exon 5 of the *Cnga3* gene. Modified CBA-*Cnga3* vector restored cone ERG responses to 80 percent of the wild type levels three weeks after treatment, more than was gained with the first S-cone promoter [18]. This difference in promoter efficacy in restoring normal electrophysiology to all subpopulations of cones may be a significant factor in the human trials currently underway. The *cpfl5* model of *Cnga3* achromatopsia also had a slow cone degeneration; a phenotype that is also seen in human patients. AAV rescue largely prevented cone degeneration and the transgene rescued disrupted cone opsin trafficking [20].

Work by Pang *et al.* (2012) provides independent confirmation that the CBA promoter drives therapeutic levels of transgene expression in cones. This may be relevant to the RPE65 gene therapy clinical trials which also use the CBA promoter, because RPE65 is expressed in cones in some species [21].

Carvalho *et al.* (2011) explored the therapeutic age window for gene therapy in achromatopsia. A mouse model caused by a premature stop codon was rescued with human *CNGB3* transgene packaged in an AAV8 capsid. The use of a cone arrestin promoter fragment resulted in universal expression in all cone subpopulations, an alternative solution to the use of the ubiquitous CBA promoter. The subretinal vector rescued retinal function, as tested by ERG, at a range of ages, but again there were poor visual responses in mice treated at later ages. This

confirmed that there is an optimal therapeutic window at a younger age [22]. The limited benefit at later ages may be a result of slow retinal degeneration in this model or amblyopia. Cone outer segment abnormalities have also been noted in achromatopsia animal models and it has been shown that regenerating the outer segments with intravitreal ciliary neurotrophic factor just before achromatopsia gene therapy may improve the responses significantly in older *Cnga3* deficient mice [23].

Interestingly, a clinical trial using *CNTF* delivered by an encapsulated cell-based device aimed to see if achromatopsia in humans could be improved by a similar mechanism on outer segments found no evidence for improved cone function [24]. This trial showed a decline in rod function, which is a critical issue for an achromatopsia patient fully dependent on rod function. These cells do not generally decline in achromatopsia, although there are rare exceptions [25]. The importance of maintaining baseline rod function following achromatopsia gene therapy creates additional challenges compared to typical cone-rod dystrophies in which there is no residual normal population of cells to maintain.

Two serendipitous discoveries of day blindness in unrelated Awassi sheep flocks in Israel yielded two new large-animal models of *Cnga3* achromatopsia. Both models—a premature stop codon [26] and a missense mutation [27]—were rescued using the same human PR2.1-CNGA3 vector described by Komaromy *et al.* (2010). As expected, photopic ERG was restored. Treated animals were also able to use the restored cone function to navigate a maze in photopic conditions. A mouse *Cnga3* transgene also provided a similar rescue effect [26].

All the above studies delivered the vector by subretinal injection. The outer retina is difficult to target efficiently from intravitreal injections and the risk of off-target transduction of inner retinal cells is increased in an intravitreal approach [28]. Regarding other retinal conditions and the comparison of intravitreal vs subretinal delivery; the optimal details of different cell-type promoters, optimized viral capsid serotypes and target cells-types are beyond the scope of this review.

These animal studies have largely explored if the developing visual pathways of young adults benefit from gene therapy and the evidence suggests that the response to treatment gets less as the visual system matures. There is however evidence that plasticity of the mammalian cone visual pathways exists elsewhere. Two related papers explore cone opsin gene therapy in naive visual systems. Human L-opsin gene therapy produces trichromatic vision in Old World dichromatic adult primates. Multifocal-ERG and modified Cambridge color test show new spectral sensitivity in treated monkeys [29]. Secondly, a vector containing rat M-opsin restored M-cone ERG function in a rat model of congenital S-cone

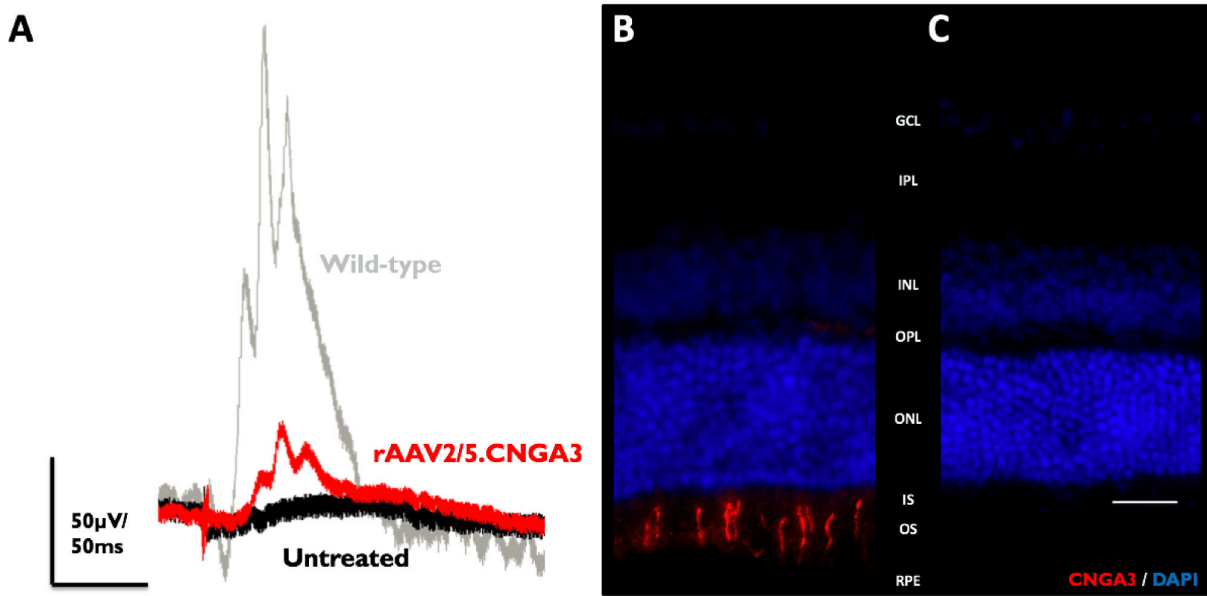


Figure 1. (A) Representative photopic ERG traces recorded from CNGA3 (red) treated eye and untreated eye (black trace) of a TKO mouse. The response of an untreated wildtype control is also shown (grey trace). Scale bar is 50µV by 50 ms. (B) Immunohistochemical staining for CNGA3 in retinas of TKO mice following injection with rAAV2/5.CBA.CNGA3 but no labelling in an area of distant to the injection in the same eye (C). Scale bar 50µm.

monochromatism [30].

These studies in animal models do, however, differ from complete achromatopsia in that both have residual pathways of cone subpopulation function. They therefore presumably have parvo-cellular retinofugal pathways and development of cortical areas such as the blobs in layers 2 and 3 of V1 and the V4 area that mediate color vision [31]. This perhaps proves indirectly that the failure to restore full color vision in the adult achromatopsia models has more to do with re-modelling of the cone visual pathway more than any intrinsic deficiencies in the cone photoreceptors themselves. The implications for human achromatopsia gene therapy are that the treatment will most likely need to be applied in early childhood to be maximally effective.

ORIGINAL DATA ON CNGA3 RESCUE IN MICE

Mouse models of achromatopsia remain heavily dependent upon rod function and the contribution from the non-image forming melanopsin photoreceptor pathway can also be significant [32]. To isolate any functional vision improvement mediated by cone rescue, we treated a triple knock out (TKO) mouse line that has almost no intrinsic light sensitivity [32]. The mouse is homozygous for targeted mutations in three genes: *Gnat1* (rod-specific alpha transducin), *Opn4* (ganglion cell specific melanopsin) and *Cnga3*. We demonstrated effective cone

transduction and rescue of cone ERG following gene therapy with an AAV vector expressing *Cnga3*.

Five to 6-week-old TKO mice received 1µl subretinal injections containing 1×10^9 genome particles of rAAV2/5.CBA.CNGA3. The mice received no injection in the fellow eye. Two to 3 months after injection, ERGs were recorded to assess restoration of visual function of transduced retinæ *in vivo*. Eyes were removed post-mortem, fixed in 4 percent paraformaldehyde, embedded and sectioned (18 µm) for immunohistochemistry (IHC) to detect and localize CNGA3 protein.

In vivo, treated eyes showed an approximately 20 percent improvement in photopic ERG b-wave amplitude (Figure 1). The restoration of a proportion of visual function in TKO mice shows that *CNGA3* transgene expression acts solely through cone photoreceptors. This is a finding that was not empirically evident from AAV rescue of other achromatopsia models that had other remaining photosensitivity pathways. The functional rescue supported findings by earlier groups that human transgene can successfully rescue mouse models of achromatopsia.

On IHC, the *CNGA3* signal localized to outer segments, with no *CNGA3* signal in the uninjected eyes (Figure 1). This demonstrated effective vector transduction of the cone photoreceptors and correct transport and localization of the transgene. It was interesting to note that *Cnga3* staining was not seen in rod photoreceptors, despite use of the ubiquitous CBA promoter. A reason

may be that *CNGA3* needs to form a 3:1 tetrameric protein with *CNGB3* to produce a functional cyclic GMP channel [33].

THE HUMAN RETINA IN ACHROMATOPSIA

Achromatopsia fundi are grossly normal. Yet, cone structure and density, as measured with high resolution adaptive-optics SLO, may show subtle changes. Studies of *CNGB3* and *CNGA3* patients show variable foveal appearance on OCT and cone density [34,35]. Notably, patients with *Gnat2* mutations may have almost normal cone density [34,36]. Thus, the causative mutation and cone count may limit the magnitude of any treatment effect and illustrates the overlap between achromatopsia and a slow cone dystrophy. Over half of adult *CNGB3* patients have some degree of foveal hypoplasia. Foveal cone density is less than normal adults, yet cone inner segments remain intact; the cone mosaic pattern is often irregular. Cones also have abnormal reflectance patterns, however, cone density has not been shown to decline with age and is likely a static feature throughout life [35]. This metric is likely not an important influence or barrier to selecting patient age in a trial. The use of intravitreal *CNTF* injections to regenerate cone outer segments as in the animal models may have a role in these patients [23]. One caution is that all these studies on cone mosaics rely on manual quantification by an unreported number of observers. It is unclear if these studies accommodated the inter-observer variability of manual cone mosaic analysis in achromatopsia [37].

Animal models of achromatopsia with cone degeneration show late-stage reduction in rod ERG [20]. However, the scotopic retinal electrophysiology of affected patients is generally normal [38]. Rod photoreceptor electrophysiology is not usually affected in achromatopsia, although there are rare exceptions of *CNGA3* missense changes leading to rod ERG reduction. This is presumed to be due to synaptic changes, since *CNGA3* is not expressed in rods. The canine models of achromatopsia however show normal rod function [16].

In one clinical study, adult patients with congenital achromatopsia viewed photopic and scotopic visual stimuli; functional magnetic resonance imaging (fMRI) recorded the cortical retinotopic representations of foveal function [39]. The study controlled for the effects of achromatopsia nystagmus on vision. Normal controls demonstrated a region of VI occipital cortex dedicated to the cone-specific fovea. In achromatopsia patients, this same rod-free foveolar representation in the visual cortex was activated by rod-isolating visual tests. Rod visual pathways had monopolized the unused cortical space normally dedicated to cones [39]. Thus, absence of cone visual input during childhood development remodels

and re-allocates retinofugal pathways and this fits with psychophysics studies which suggest that achromatopsia patients have faster rates of dark adaptation than normal [40].

ACHROMATOPSIA AND CIRCADIAN RHYTHM

The human circadian rhythm is a roughly 24-hour cycle; the internal body clock. It is endogenously generated, but modulated by external cues, such as light levels. Cones initiate the circadian response to light and melanopsin provides sustained input for ongoing response [41]. Cone photoreceptor electrophysiological response to light also varies by an intrinsic 24-hour cycle [42]. What effect does achromatopsia have on photopic entrainment and the sleep-wake cycle?

No evidence is available for the effect of achromatopsia on circadian rhythm in humans. A small study of patients with red-green color deficiency showed no effect on serum melatonin suppression by light. Melatonin is a signaling molecule secreted in a 24-hr circadian pattern by the pineal gland. It correlates with other markers of circadian rhythm [43]. Yet, mice lacking medium wavelength cones exhibit impaired photopic photo-entrainment and phase shifting [44]. More clinical data from achromatopsia patients is needed to explore circadian disruption.

GENE THERAPY CLINICAL TRIALS FOR ACHROMATOPSIA

As of June 2017, there are four registered clinical trials for gene therapy in achromatopsia, of which three have commenced. All trials use the human transgene driven by cone-specific promoter fragments and packaged in an AAV capsid; details of the vectors and study design are listed in Table 2. All vectors have been previously shown to rescue animal models of achromatopsia. Patients receive delivery of the vector by subretinal injection after vitrectomy. None of the constructs include regulatory elements that increase transgene expression such as WPRE [45] or intron/exon splice sites.

All trials are phase I/II and the primary outcome measure is safety. It is unclear whether the CNG-naïve immune system of null mutation achromatopsia patients may react to transgene proteins expressed in the cell membrane. Mutations in *CNGA3* and *CNGB3* account for 70 percent of mutations in human patients; both are channel proteins that insert into the cell membrane. The modest pro-inflammatory stimulus of retinal surgery and viral capsid proteins may potentiate any pro-inflammatory effects of these transmembrane proteins. None of the clinical trials have publicly recorded plans for routine

Table 2. Summary of the publicly registered clinical trials for gene therapy in Achromatopsia.

ClinicalTrials Identifier	Gene	Vector	Capsid	Promoter	Dose	Delivery route	Routine immunosuppression	Locations
NCT03001310	CNGB3	AAV2/8-hCARp. hCNGB3	AAV5	Human cone arrestin fragment	Unreported	Vitreotomy + subretinal injection	Unreported	United Kingdom
NCT02610582	CNGA3	rAAV/hCNGA3	rAAV8	Potentially, full length red-cone opsin promoter	low dose: $\leq 1 \times 10^{10}$ gp (n=3) intermediate dose: $\leq 5 \times 10^{10}$ vgp (n=3) high dose: $\leq 1 \times 10^{11}$ gp (n=3)	Vit + subretinal injection	Unreported	Tubingham, Germany
NCT02935517	CNGA3	rAAV2 \dagger YF-PR1.7-hCNGA3 ('AGTC-402')	AAV2 \dagger YF	PR1.7 (Fragment L-Opsin pr)	Three doses, unspecified	Vit + subretinal injection	Unreported	USA, Israel
NCT02599922	CNGB3	rAAV2 \dagger YF-PR1.7-hCNGB3	AAV2 \dagger YF	PR1.7 (Fragment L-Opsin pr)	Three doses, unspecified	Vit + subretinal injection	Unreported	USA

immunosuppression in treated patients.

Secondary outcomes for the trials include visual acuity, electrophysiology, and color vision function. It is possible that AAV transgene expression may rescue electrophysiology without providing integrated central visual functions like color or acuity. The trials are designed to initially test the safety profile in adult patients, but three of the trials plan to include patients as young as 6 years in later groups. By comparing older and younger patients, the trials will examine differences in integrated functional cone rescue between age groups. If the pattern observed in the animal trials holds true, there may be a difference in benefit due to patient age at time of treatment. Such a difference may arise from reorganization of cortical pathways [39] or foveal hypoplasia [35].

In the one other therapeutic trial registered for achromatopsia (ClinicalTrials.gov number, NCT01648452), six *CNGB3* achromatopsia patients received intraocular implants that released Ciliary Neurotrophic Factor (*CNTF*) [24]. *CNTF* provides neuroprotection to rod and cone photoreceptors in degenerative retinal disease [46]. Concurrent treatment with intravitreal *CNTF* in a canine model of *CNGB3* achromatopsia potentiated gene therapy rescue. *CNTF* improved ERG rescue in older animals, overcoming a notable inability of gene therapy alone to benefit most dogs over 1 year of age [23]. Limited evidence suggested that *CNTF* pre-treatment de-differentiated the cone photoreceptors by elongating the outer segments to facilitate CNGA3/B3 tetrameric protein assembly. As mentioned above, in the *CNTF* only trial, no functional improvement in cone function occurred. There are no current plans to include *CNTF* pre-treatment in any of the registered human gene therapy trials. This discussion may be reignited if the early trials show difficulty in rescuing cone function in older patients.

CONCLUSION AND OUTLOOK

Animal trials have demonstrated that gene therapy can restore the electrophysiological function of cones. The three most common causative mutations (*Cnga3*, *Cngb3*, and *Gnat2*) have all been rescued, often by multiple groups using different vectors.

There is some evidence that cone density declines with age. This metric is unlikely to affect the therapeutic window. Nonetheless, in a mouse model showing atypical cone degeneration, *Cnga3* re-expression halted cell loss and corrected opsin mislocalization.

The electrophysiological treatment effect is long-lasting; over 180 days in mice and over 3 years in sheep. The effect is maximal in younger animals and more modest in older mice and dogs; making age an important consideration in study design. *CNTF* potentiates cone

rescue in animals and is an example of the adjuvant therapies that may be needed to successfully treat older patients.

Optimally, treatment should occur in childhood (under 6 years). Thus, allowing functional cones to direct retinofugal pathway development. In such young patients there would be additional technical challenges to the injection surgery, but a greater potential for integrated functional rescue.

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