Development of a Chromatic Pupillography Protocol for the First Gene Therapy Trial in Patients With *CNGA3*-Linked Achromatopsia

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See the appendix for the members of the RD-CURE Consortium.

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Purpose. To establish a feasible and sensitive pupillographic protocol to assess outer and inner retinal function for the first gene therapy trial in achromatopsia patients (ACHM) with mutations in *CNGA3*.

Methods. Twenty-seven *CNGA3*-ACHM patients and 22 age-matched control subjects were tested using chromatic pupillography. Three different protocols were established to assess the pupillary light reflex parameters and to create the final protocol. In the individual protocols, various stimulus parameters (i.e., intensity, duration, wavelength, adaptation states) were applied to evaluate the impact of these stimuli on the pupillary response in untreated ACHM patients.

Results. In the light-adapted conditions, *CNGA3*-ACHM patients showed significantly reduced maximal amplitudes compared with the control group when using a 1-second high intensity (28-lux corneal illumination) blue or red stimulus ($P < 0.005$). In the dark-adapted conditions, *CNGA3*-ACHM patients unexpectedly revealed significantly increased maximal amplitudes when stimulating with red (1 second) or blue (4 ms and 1 second) stimuli of low intensity (0.01-lux corneal illumination; $P < 0.05$). Pupil responses of *CNGA3*-ACHM patients after high intensity (28 lux) red and blue 1-second stimuli were within the normal range.

Conclusions. Chromatic pupillography demonstrated significant reduced pupil responses to stimuli addressing primarily cone function, an increased sensitivity to rod-favoring stimuli and evidence for disinhibition of intrinsically photosensitive retinal ganglion cells in *CNGA3*-ACHM patients. A final protocol was established based on these findings. These conclusions may be useful for the objective assessment of efficacy gained by gene therapy or other innovative interventions in this hereditary retinal disorder.

Keywords: chromatic pupillography, achromatopsia, CNGA3, ipRGC

Achromatopsia (ACHM) is an inherited autosomal recessive congenital retinal disease, with a prevalence of one in 30,000.[1] The most characteristic symptom is the inability to discriminate colors due to the loss of cone photoreceptor function. Achromatopsia patients also suffer from severely reduced visual acuity, nystagmus, and photophobia.[2] Rarely, individuals have incomplete ACHM, in which one or more cone types may be partially functioning.[3] The clinical findings of incomplete ACHM are similar to the complete form, but milder. Fundus examination is usually normal, and electrophysiological testing demonstrates absent cone responses and normal or near-normal rod function.[2–5]

To date, mutations in six genes have been shown to be associated with ACHM: *CNGA3* and *CNGB3* encode the α and β subunits of the cGMP-gated cation channel,[6] *GNAT2* the α subunit of the cone-specific G-protein transducing,[7] *PDE6C* and *PDE6H* code for the α‘ and γ subunits of the cone-specific cGMP phosphodiesterase,[8,9] and *ATF6* encodes an unfolded protein response regulator.[10–12] All genes are expressed in the cone photoreceptor cells and the first five genes are mandatory for the cone phototransduction cascade. Mutations in *CNGB3* and *CNGA3* are the most common causes of ACHM, accounting for 50% and 30% of all cases, respectively.[6] Mutations in *GNAT2*, *PDE6C*, *PDE6H*, and *ATF6* are rare, and found in less than 10% of ACHM patients in total.[8] Studies in various animal models of ACHM have shown that gene therapy is a viable treatment with the potential to restore cone function.[13–16]

Table 1. Patient Characteristics

Protocol	Number of Patients	Age ± SD (Sex F/M) Patients	Visual Acuity Patients	Number of Control Group	Age ± SD (Sex F/M) Control Group
1	9	39 ± 11 (2F; 7M)	0.14 ± 0.03	10	37 ± 15 (4F; 6M)
2	14	42 ± 10 (6F; 8M)	0.19 ± 0.05	8	43 ± 11 (4F; 4M)
3	4	22 ± 5 (1F; 3M)	0.20 ± 0.04	4	25 ± 5 (2F; 2M)

SD, standard deviation; F, female; M, male.

Gene therapy trials in ACHM patients are in progress, but to control the effectiveness of treatment, sensitive testing procedures are required that can detect small changes in the activity of the retina. Previous studies have shown that the pupil light reflex is more sensitive for the detection of residual activity of cones and rods in the advanced stages of hereditary retinal degenerations like retinitis pigmentosa in comparison to a standard full-field ERG where responses from photoreceptors may be extinguished in the late stage of the disease.[2,17]

Pupillography is a noninvasive and objective method, which allows pupillary responses elicited by cones, rods, and intrinsically photosensitive retinal ganglion cells (ipRGCs) to be studied by changing the intensity, wavelength (color), and duration of the light stimulus.[18] Previous studies have been based on the assumption that L and M cones can be stimulated selectively using wavelengths beyond 620 nm of high-intensity, because rods, ipRGCs, and S-cones remain insensitive to such stimulus conditions. It has also been suggested that pupillary responses to a low-intensity blue light stimulus may be a marker of rod activity.[19] On the other hand, the pupillary responses to a high-intensity blue light stimulus are driven by S-cones and ipRGCs. Intrinsically photosensitive RGCs are considered to determine the sustained postillumination pupil response (PIPR).[20,21]

In complete ACHM, the only functional outer photoreceptors are rods, which – in normal retinas – are more sensitive than cones and become saturated at higher levels of illumination. Patients suffering from ACHM are extremely light sensitive and color blind; they complain of glare. To the best of our knowledge, there are only two studies that have investigated the pupil light reflexes to colored light stimuli in patients with ACHM and they only represented a minor subgroup of their study groups.[20,22] Furthermore, their study protocols were not designed specifically for ACHM patients.

To study in more detail how ACHM affects the pupil response, we investigated pupillary responses driven by rods/ipRGCs to stimuli of different duration, intensity, and wavelength, as well as the effect of the state of adaptation. To the best of our knowledge, this is the first study investigating pupillary responses in a comparable sample size of patients with *CNGA3*-ACHM. The main purpose of this study was to establish a feasible and sensitive pupillographic protocol for ACHM patients with mutations in *CNGA3*. Such a protocol may lead to a better understanding of the mechanisms triggering retinal sensitivity in these patients and could be used in the assessment of treatment efficacy in ACHM patients after gene therapeutic intervention.

Methods

Subjects

Twenty-seven ACHM patients with confirmed mutations in the *CNGA3* gene (for genotypes see Supplementary Table S1) and 22 healthy control subjects were tested using chromatic pupillography. In three cases (RD004, RD007, and RD024), incomplete ACHM was diagnosed based on the clinical findings of psychophysical and electrophysiological tests, while other patients presented with complete ACHM.

Pupillography

Pupillography was carried out with the Compact Integrated Pupillograph (CIP; AMTech, Dossenheim, Germany). The stationary CIP measured the pupil diameter with an infrared line camera. The head of the examined subject was positioned and the pupil focused with a camera; consecutive recordings were then performed automatically by the CIP (spatial resolution 0.05 mm, temporal resolution 0.004 ms). While one eye was stimulated, the consensual pupillary response of the fellow eye was recorded. Protocols were determined by predefined stimuli varying in color, intensity, duration, adaptation state, and interstimuli intervals (see below). In each session, measurements were repeated at least four times, artifact measurements were deleted and an average value was calculated out of four good-quality pupillograms. These pupillary responses were compared with those of age-matched controls.

Three different pupillographic protocols were developed and adjusted one after the other in the course of the study and applied to different individual ACHM patients.

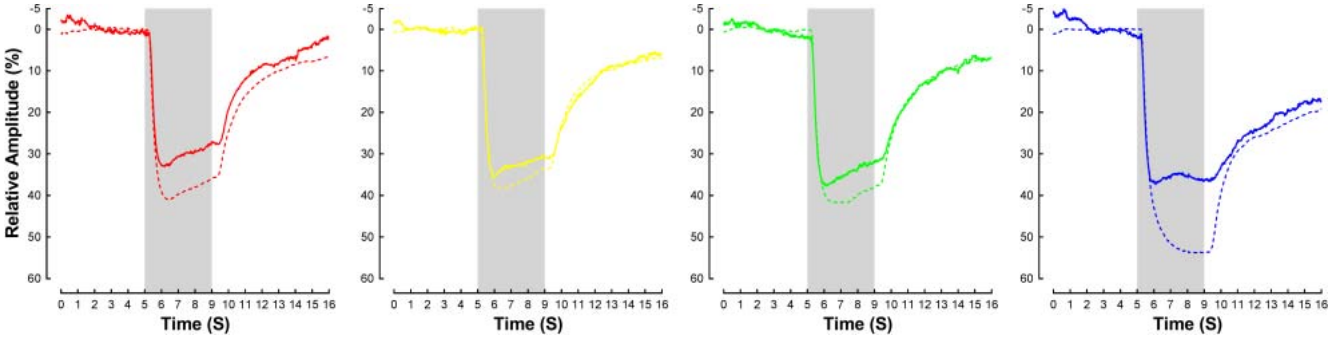

Figure 1. Protocol 1: Mean relative amplitude (%) versus time (seconds); stimulation with red (605 nm), yellow (562 nm), green (518 nm), or blue (420 nm) light of 28-lux corneal illumination, stimulus duration 4 seconds; *dotted line*: normals ($n = 10$); *solid line*: ACHM ($n = 9$).

Table 1 shows the characteristics of the subjects enrolled in the study.

Protocol 1

Four wavelengths (red: 605 nm, yellow: 562 nm, green: 518 nm, and blue: 420 nm) with a stimulus intensity of 28-lux corneal illumination and a stimulus duration of 4 seconds were presented under mesopic background illumination.

Protocol 2

Restricted to red (605 nm) and blue (420 nm) light, eight different stimulus intensities were used: 0.01, 0.03, 0.1, 0.316, 1, 3.16, 10, and 31.6 lux. Stimulus duration was 1 second and interstimulus intervals were used to allow the pupil to return to its initial size.

Protocol 3

Two adaptation states were compared for all stimulus characteristics described below: light adaptation (10 cd/m^2, 10 minutes) and dark adaptation (20 minutes). An interstimulus interval of at least 10 seconds for the low-intensity stimuli and of 40 seconds for the high-intensity stimuli were used and guaranteed that the pupil had returned to its initial pupil size before the next stimulus started. Parameters of the stimuli were as follows for both adaptation states:

1. Long-wavelength (605 nm; red) light stimulus: 4 ms, 0.01 lux.
2. Long-wavelength (605 nm; red) light stimulus: 1 second, 0.01 lux.
3. Long-wavelength (605 nm; red) light stimulus: 1 second, 28 lux.
4. Short-wavelength (420 nm; blue) light stimulus: 4 ms, 0.01 lux.
5. Short-wavelength (420 nm; blue) light stimulus: 1 second, 0.01 lux.
6. Short-wavelength (420 nm; blue) light stimulus: 1 second, 28 lux.

Statistical Methods and Analysis

For the statistical analysis, JMP 11.2.0 (SAS Institute, Inc., Cary, NC, USA) was used. A normal distribution was assumed in the basic population, thus ANOVA and 2-sided *t*-tests were performed for statistical analysis. Data are shown in terms of intra-individual normalized values to baseline and the 95% confidence interval for each time-point. The average out of four pupillograms was used to calculate the initial diameter, the baseline, the absolute amplitude, and the relative amplitude per subject. Baseline pupil diameter was defined as the median pupil diameter over a 5-second period before the light stimulus. The relative amplitude was calculated: [1-(absolute pupil diameter/baseline)] × 100. Maximal amplitude was defined as the maximum relative amplitude after stimulus onset.

Additionally, for the third protocol, the PIPR was calculated for the 1-second stimulus conditions. For this purpose, the relative amplitudes at each time-point were summarized and divided by 250 (temporal resolution: 4 ms) representing the area over the curve. Starting time-point was 2 seconds after stimulus offset (t = 8 seconds) and the endpoint was the end of the measurements (t = 16 seconds).

The investigation was planned and performed according to the tenets of the Declaration of Helsinki 1975 (1983 revision). The study protocol was approved by the Ethics Committee of the Medical Faculty at the University of Tübingen prior to the

Table 2. Protocol 1: Mean Relative Maximal Amplitudes (%) ± SD for ACHM Patients and Controls, *P* Values; Stimulation With Red (605 nm), Yellow (562 nm), Green (518 nm), or Blue (420 nm) Light and 28 lux Corneal Illumination, Stimulus Duration 4 Seconds

	Red			Yellow			Green			Blue		
Pupil Parameters	**ACHM**	**Controls**	***P* Value**	**ACHM**	**Controls**	***P* Value**	**ACHM**	**Controls**	***P* Value**	**ACHM**	**Controls**	***P* Value**
Baseline, mm	4.06 ± 0.82	6.33 ± 0.9	**<0.001**	4.23 ± 0.90	6.36 ± 0.96	**<0.001**	4.2 ± 0.8	6.27 ± 0.97	**<0.001**	3.88 ± 0.8	6.22 ± 0.94	**<0.001**
Relative maximal amplitude, %	34.97 ± 7.54	41.83 ± 4.94	**0.03**	36.75 ± 4.77	39.48 ± 5.9	0.286	38.75 ± 5.19	42.96 ± 5.86	0.117	39.63 ± 7.33	55.07 ± 5.3	**<0.001**

Bold values indicate statistical significance ($P < 0.05$).

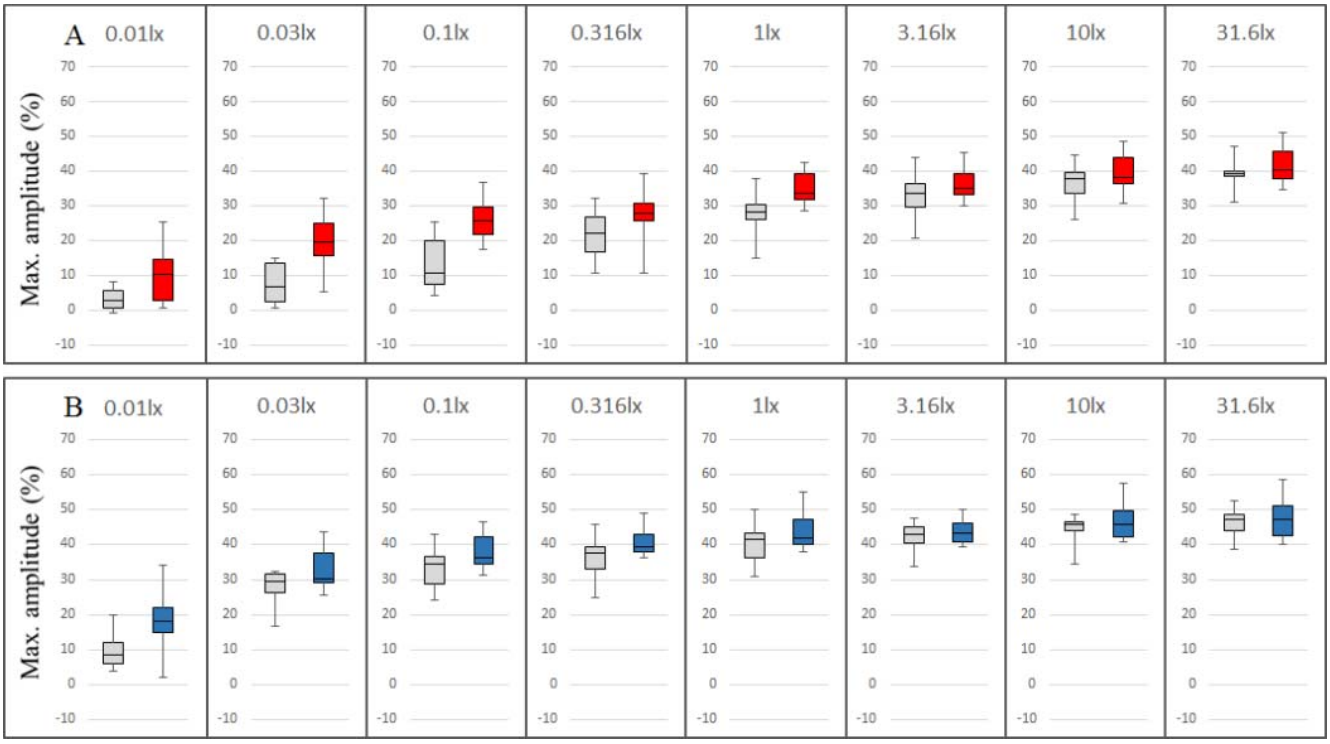

Figure 2. Protocol 2: Box plots of the maximal relative amplitude (%) for different stimulus intensities (0.01–31.6 lux). **(A)** Red stimulation; *red* = ACHM ($n = 14$), *gray* = normals ($n = 8$). **(B)** Blue stimulation; *blue* = ACHM ($n = 14$), *gray* = normals ($n = 8$).

start of the study. Furthermore, all participants were informed about the aims of the study, the study protocol and gave written informed consent prior to the start of the research experiments.

Results

Protocol 1

The pupillographic results from nine patients with *CNGA3*-ACHM were compared with those from 10 control subjects. Using yellow and green stimuli of 4-second duration and 28-lux intensity, the maximal amplitude in the patient group and the control group was comparable. Using red and blue stimuli of the same duration and intensity, the control group showed significantly higher maximal amplitudes (Fig. 1; Table 2). Therefore, in the second protocol only red and blue stimulation was used.

Protocol 2

The main focus in this protocol was to test different stimulus intensities ranging between 0.01 lux and 31.6 lux. The pupillographic results from 14 *CNGA3*-ACHM patients were compared with those from eight control subjects.

Red Light

Using red stimuli of 1-second duration and low intensities (0.01, 0.03, 0.1, 0.316, and 1 lux), *CNGA3*-ACHM patients revealed significantly higher maximal pupil amplitudes than the control group (Fig. 2A; Table 3). Using stimuli of higher intensities, the pupillary responses were comparable for the two groups.

Blue Light

Using blue stimuli of 1-second duration, *CNGA3*-ACHM patients had significantly higher maximal pupil amplitudes

Table 3. Protocol 2: Mean Maximal Relative Amplitudes (%), *P* Values; Stimulus Duration 1 Second, Stimulation With Red (605 nm), Blue (420 nm) Light, and 0.01-, 0.03-, 0.1-, 0.316-, 1-, 3.16-, 10-, and 31.6-lux Corneal Illumination

Maximal Relative Amplitude, %	0.01 lux	0.03 lux	0.1 lux	0.316 lux	1 lux	3.16 lux	10 lux	31.6 lux
Red light								
ACHM	10.16 ± 8.27	19.78 ± 7.88	25.95 ± 6.04	28.3 ± 7.19	34.92 ± 4.70	36.5 ± 4.68	39.43 ± 5.34	41.39 ± 5.01
Control	3.31 ± 3.55	7.6 ± 5.86	13.16 ± 7.67	21.68 ± 7.23	27.77 ± 6.53	33 ± 7.19	36.76 ± 5.99	39.14 ± 4.43
P value	**0.018**	**<0.001**	**0.002**	0.059	**0.022**	0.246	0.327	0.29
Blue light								
ACHM	18.29 ± 8.6	32.58 ± 5.46	38.01 ± 5.01	40.52 ± 3.99	43.73 ± 5.33	43.64 ± 3.55	46.53 ± 5.3	47.01 ± 5.49
Control	9.83 ± 5.59	27.59 ± 5.55	33.08 ± 6.45	35.83 ± 6.66	39.81 ±6.38	41.69 ± 4.86	44.13 ± 4.81	46.03 ± 4.73
P value	**0.013**	0.063	0.092	0.101	0.181	0.345	0.31	0.665

Bold values indicate statistical significance ($P < 0.05$).

Table 4. Protocol 3: Mean Maximal Relative Amplitudes (%) ± SD, PIPR From $t_0 = 8$ to $t_1 = 16$ Seconds, *P* Values; Stimulation With Red (605 nm) or Blue (420 nm) Light and 0.01 or 28 lux Corneal Illumination, Stimulus Duration 4 ms or 1 Second

	Red Light			Blue Light		
Pupil Parameter, Stimulus Condition	**ACHM**	**Controls**	***P* Value**	**ACHM**	**Controls**	***P* Value**
Light-adapted						
Baseline, mm	4.35 ± 0.55	7.04 ± 0.98	**<0.001**	4.23 ± 0.92	6.84 ± 1.19	**<0.001**
Maximal amplitude, 28 lux 1 s	29.97 ± 1.33	40.05 ± 3.24	**<0.001**	29.97 ± 5.95	50.64 ± 3.53	**0.002**
Dark-adapted						
Baseline, mm	5.64 ± 0.40	7.15 ± 1.1	**<0.001**	5.58 ± 0.35	7.12 ± 1.08	**<0.001**
Maximal amplitude, 0.01 lux 4 ms	–	–	–	13.12 ± 3.94	5.63 ± 0.9	**0.029**
Maximal amplitude, 0.01 lux 1 s	22.15 ± 5.52	11.31 ± 5.23	**0.029**	21.23 ± 7.05	11.52 ± 4.21	**0.031**
Maximal amplitude, 28 lux 1 s	39.96 ± 2.84	45.09 ± 3.55	0.07	48.6 ± 5.07	53.03 ± 2.82	0.191
PIPR (8–16 s), 0.01 lux 1 s	55.15 ± 34.38	5.62 ± 6.03	0.061	30.98 ± 33.25	10.06 ± 4.37	0.298
PIPR (8–16 s), 28 lux 1 s	87.19 ± 15.39	70.39 ± 10.48	0.128	238.97 ± 49.06	287.24 ± 18.23	0.142

Bold values indicate statistical significance ($P < 0.05$).

using 0.01-lux intensity ($P < 0.0134$), but at higher intensities the responses were comparable and the difference was statistically not significant (Fig. 2B; Table 3).

Protocol 3

In the third protocol, the adaptation status of the retina was taken into consideration. The pupillographic results from four patients with *CNGA3*-ACHM were compared with those from four control subjects after either light or dark adaptation.

Light Adaptation

In the light-adapted condition, the baseline pupil diameter in ACHM patients was significantly smaller than in the control group ($P < 0.001$). Although all patients' pupils showed a good dilatation in darkness, they did not reach the average dark-adapted level of the control subjects (Table 4). The difference in pupil size between the light- and dark-adapted conditions was larger in ACHM patients ($P < 0.05$; Table 5).

In the light-adapted condition, there were no recordable responses in both groups to stimulation with 0.01-lux long-wavelength (605 nm; red) and short-wavelength (420 nm, blue) stimuli of 4-ms and 1-second duration (data not shown).

With a high intensity (28 lux) red or blue stimulus of 1-second duration, assessing primarily cone function, all ACHM patients showed reduced maximal amplitudes compared with the control group ($P < 0.05$; Fig. 3; Table 4).

Dark Adaptation

The same steps were repeated after 20 minutes of dark adaptation. For red light, a 4-ms stimulus of 0.01 lux did not elicit any response in the control group; only one of the subjects saw the light but could not indicate the color of the stimulus. Unexpectedly, in the *CNGA3*-ACHM patient group every subject noticed the light and two of them showed pupil responses (data not shown). For blue light, a 4-ms stimulus of 0.01 lux elicited recordable pupil responses in all patients and the control subjects. The maximal pupil amplitude in the patient group was significantly larger than for the control group ($P < 0.05$; Fig. 4; Table 4). All subjects had light perception, but none of the control group was able to determine the color of the stimulus.

Using a 1-second red and blue stimulus of 0.01 lux, we also observed larger maximal pupil constrictions in patients with *CNGA3*-ACHM compared with the control group ($P < 0.05$) (Fig. 5; Table 4). The PIPR was somewhat elevated in ACHM patients (Fig. 5; Table 4). Surprisingly, not only to blue stimulation but also to red stimulation, a PIPR could be observed in ACHM patients. We obtained the same peak response for blue and red light in controls as well as in ACHM patients. For these stimulus conditions, all subjects in the control group were able to determine the stimulus color.

Pupil responses of *CNGA3*-ACHM patients after high intensity (28 lux) red and blue 1-second stimuli were within the normal range (Fig. 6; Table 4).

Table 5. Mean Difference ± SD in Pupil Size Between the Light-Adapted and Dark-Adapted Conditions for ACHM Patients and Control Subjects

Difference in Pupil Size Between the Light-Adapted and Dark-Adapted Conditions		
ACHM	**Controls**	***P* Value**
1.43 ± 1.01	0.07 ± 0.36	0.035

Discussion

There is significant interest in objective assessments of functional efficacy gained by gene therapy or other innovative interventions in hereditary retinal degeneration. In this patient group, electrophysiological measures may no longer be reliably recordable and subjective measures may be prone to patient performance, expectation, and motivation. The potential of pupillography has been examined infrequently in such interventional trials.[23] Maguire et al.[23] used a sinusoidal light stimulation in pupillography to establish safety and efficacy of gene transfer for Leber's congenital amaurosis. In their study, the pupillary light reflex confirmed an increased retinal sensitivity and also showed better function in the eye that received treatment compared with the function in the fellow eye.

In our study, we aimed to develop a pupillographic protocol with short- and long-wavelength stimulation, which is feasible to perform and sensitive to alterations in patients with *CNGA3*-ACHM. The aim is to use such a protocol in a planned gene therapy trial in this patient group. According to our knowledge, such a protocol for the application of chromatic pupillography in ACHM has not yet been established.

We developed the stimulus conditions by modifying stimulus wavelength, intensity, and duration as well as

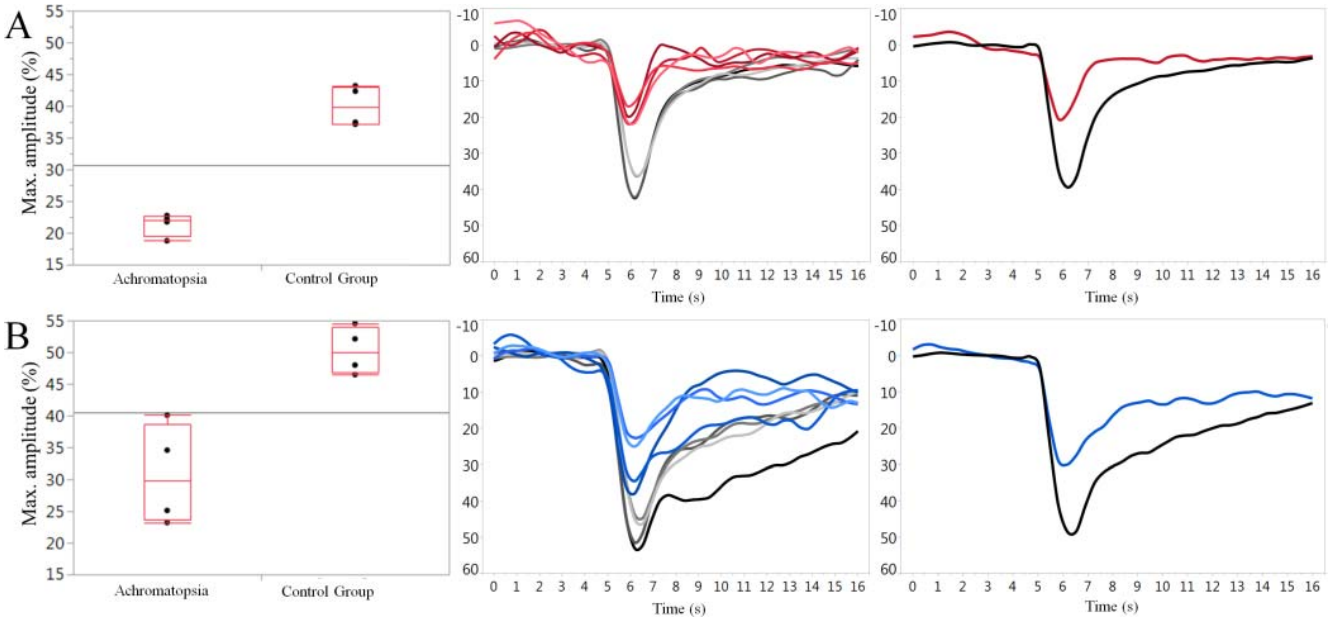

FIGURE 3. Protocol 3: Box plots of the maximal relative amplitude (%) and relative amplitude (%) versus time (seconds) for each subject and as a mean per group; stimulation after 10-minute light-adaptation; *black line*: normals ($n = 4$); *red/blue line*: ACHM ($n = 4$). (**A**) Stimulation with red (605 nm) light of 28-lux corneal illumination, stimulus length 1 second; (**B**) stimulation with blue (420 nm) light of 28-lux corneal illumination, stimulus length 1 second.

background adaptation in order to favor rod, cone, or melanopsin activation.

In the first protocol, we found out that blue and red light elicits significant differences in the maximal pupil amplitude between the control group and the patient group compared with stimulation with yellow and green light. Therefore, in the next steps only red and blue light stimuli were used.

In the second protocol, we found significant differences between the results of the control group and those of the patients when using low-stimulus intensities and shorter stimulus durations. Therefore, in the following protocol, we used 1 second and additionally 4-ms stimulus durations.

In the third protocol, we examined the effect of either 10-minute light adaptation or 20-minute dark adaptation. These two adaptation states elicited a noticeable difference in the baseline pupil diameter. Achromatopsia patients presented with smaller pupils after light and after dark adaptation compared to the control group, but the difference in pupil size between these two adaptation states was more pronounced in patients. This difference is probably related to the symptom of severe photophobia that patients experience during light exposure. The involvement of ipRGCs in photophobia has been described in mice.[24–26] It has been confirmed that the pupil diameter increases when ipRGC and rod excitation is at a minimum.[27] Furthermore, there is strong evidence of an inhibiting influence of S-cones on ipRGCs.[22,28] We speculate that ACHM patients with absence of cone function lack this cone-related inhibition on ipRGCs. The consequence would be an increased excitation of ipRGCs leading to a smaller baseline pupil diameter in photopic conditions. It is, however, not fully excluded that a disinhibition of the rod system in ACHM patients occurs also at a more distal circuitry level of the retina (e.g., in the outer plexiform layer).

In order to minimize the potential role of the smaller baseline diameter on our findings, we used relative amplitudes for our analyses of the light- and dark-adapted responses.

To examine primarily cone function, we used red and blue stimuli of high intensity (28 lux) with 1-second duration under light-adapted conditions. Pupillary responses from patients with ACHM were significantly reduced compared with those from the control group, which may be a consequence of the lack of cone function in this patient group, while there seems to be a relative saturation of rods in the light-adapted state. Under

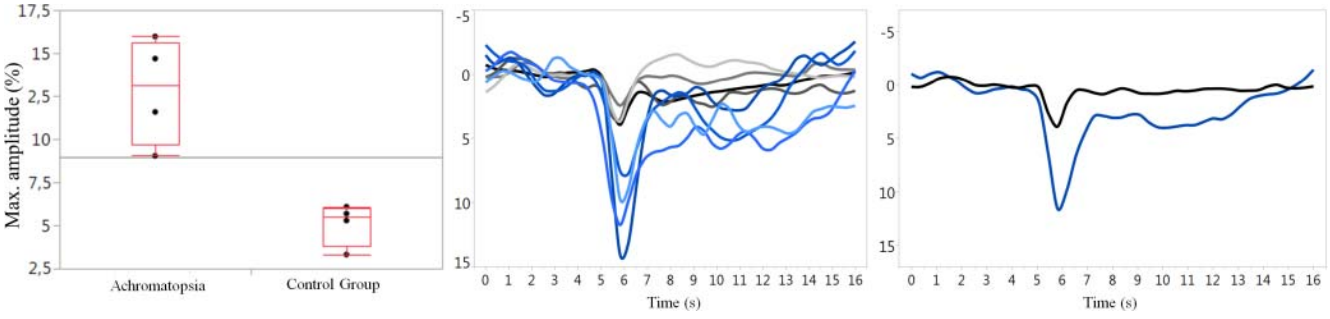

FIGURE 4. Protocol 3: Box plots of the maximal relative amplitude (%) and relative amplitude (%) versus time (seconds) for each subject and as a mean per group; stimulation after 20-minute dark-adaptation, with blue (420 nm) light of 0.01-lux corneal illumination, stimulus length 4 ms; *black line*: normals ($n = 4$); *blue line*: ACHM ($n = 4$).

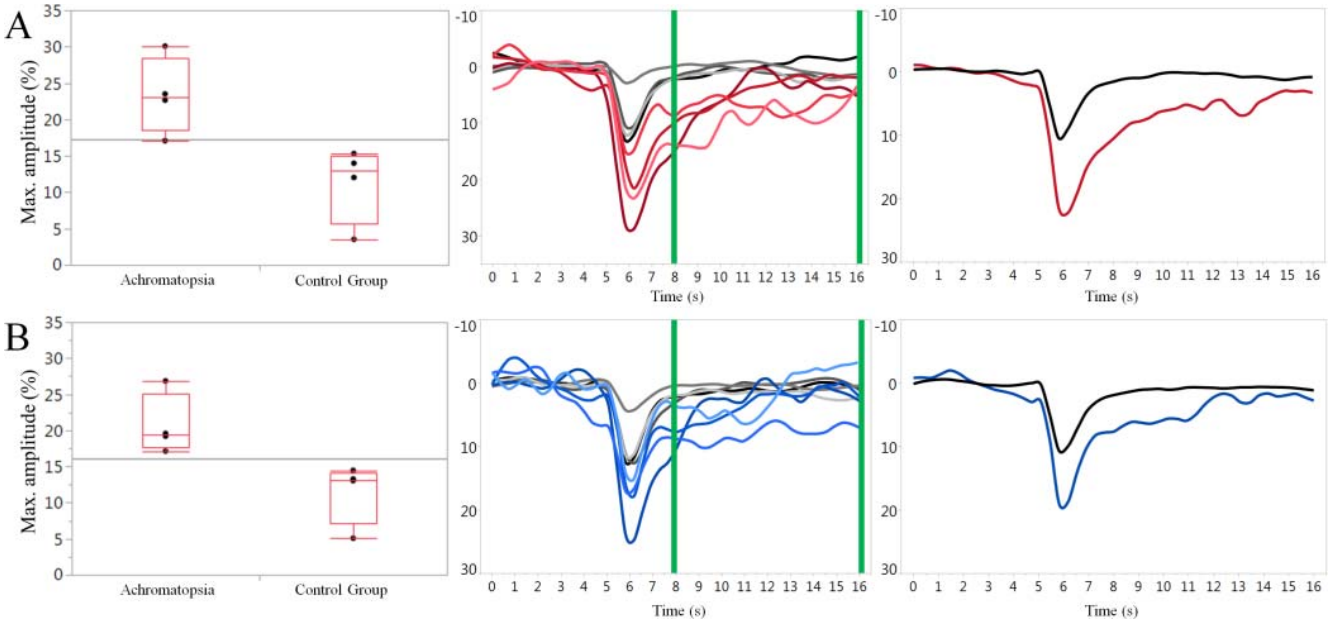

FIGURE 5. Protocol 3: Box plots of the maximal relative amplitude (%) and relative amplitude (%) versus time (seconds) for each subject and as a mean per group; stimulation after 20-minute dark-adaptation; *black line*: normals ($n = 4$); *red/blue line*: ACHM ($n = 4$); *green lines* give the timeframe within which the PIPR was calculated. **(A)** Stimulation with red (605 nm) light of 0.01-lux corneal illumination, stimulus length 1 second; **(B)** stimulation with blue (420 nm) light of 0.01-lux corneal illumination, stimulus length 1 second.

photopic conditions, smaller amplitudes were not unexpected in patients with ACHM due to the underlying defect.

After 20 minutes of dark adaptation, mainly rods were addressed by the application of red and blue stimuli of low intensity (0.01 lux) and a very short (4 ms) stimulus duration. We interpret our findings of an increased amplitude of the pupillary reaction in the patient group as a pronounced disinhibition of rod activity due to the lack of adequate (inhibitory) cone function. Additionally, there is the possibility that there might be some alteration of the rod-driven circuitry in ACHM. Reorganization of the retinal architecture as a consequence of retinal dysfunction and/or degeneration has been described by other authors in a number of photoreceptor retinal disorders.[29,30] This hypothesis is supported by the finding of ectopic synapses between rods and cone bipolar cells in the *Cnga3*$^{-/-}$ mouse model.[31] We assume that these anatomic connections between rods and cone bipolar cells may also contribute to the greater amplitude in ACHM patients compared with normals.[32]

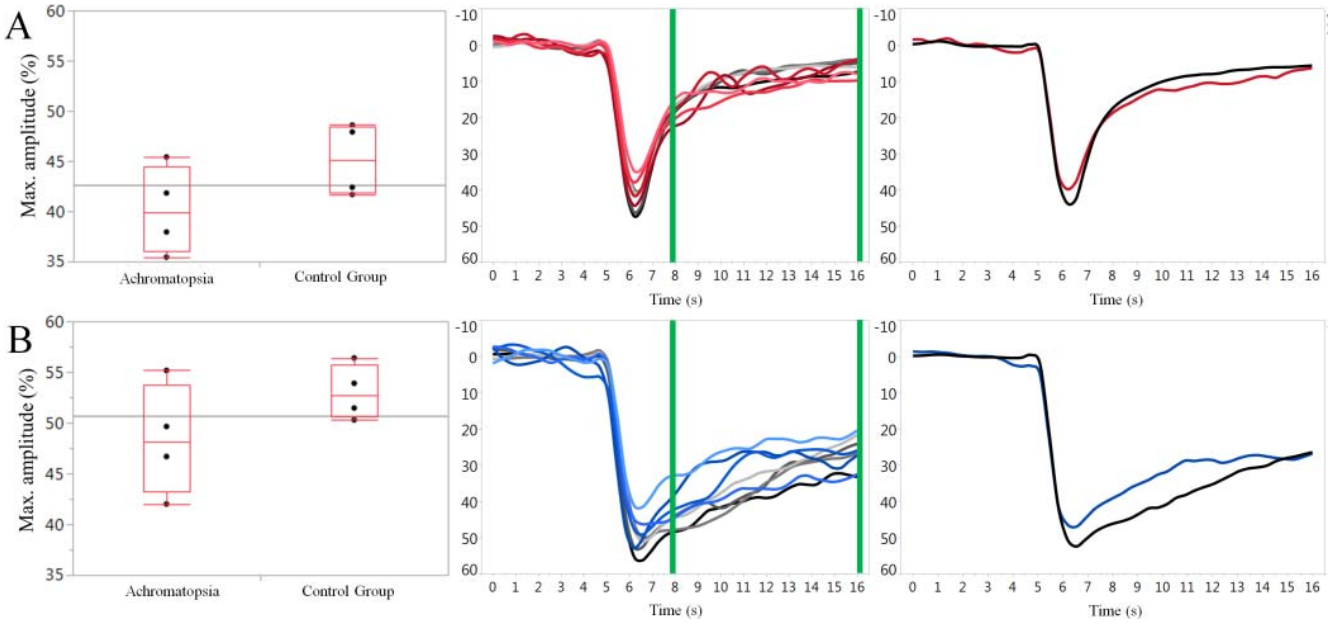

FIGURE 6. Protocol 3: Box plots of the maximal relative amplitude (%) and relative amplitude (%) versus time (seconds) for each subject and as a mean per group; stimulation after 20-minute dark-adaptation; *black line*: normals ($n = 4$); *red/blue line*: ACHM ($n = 4$); *green lines* give the timeframe within which the PIPR was calculated. **(A)** Stimulation with red (605 nm) light of 28-lux corneal illumination, stimulus length 1 second; **(B)** stimulation with blue (420 nm) light of 28-lux corneal illumination, stimulus length 1 second.

Table 6. Final Protocol

Stimulus Condition	Proposed Activated Receptors	Proposed Effect of Treatment Efficacy
Dark adaptation 20 min		
420 nm (blue), 0.01 lux, 4 ms	rods	hypersensitivity of rods↓: maximal pupil amplitude↓
420 nm (blue), 0.01 lux, 1 s	rods + cones (+ipRGCs)	maximal pupil amplitude↓ inhibition of ipRGCs↑: PIPR ↓
Light adaptation 10 min		
605 nm (red), 28 lux, 1 s	cones (+rods)	cone function↑: maximal pupil amplitude↑ latency ↓

Using red and blue stimuli of low intensity (0.01 lux) and a duration of 1 second, patients with *CNGA3*-ACHM again revealed significantly stronger pupil responses than healthy controls. We propose that in this case also cones are influencing the response in healthy subjects as all control subjects were able to recognize the correct color of the stimuli.

However, it might seem surprising that ACHM patients revealed a comparable red response in the absence of functional cones at all. But the absolute sensitivity of rods at 605 nm is very close to the long-wavelengths sensitivity of cones.[33] Furthermore, in recently published mice models rod origin of pupillary responses to low and medium red lights in cone-less *CNGA3*- mice could be confirmed.[34] It is thus not unexpected that stimulation with 605 nm elicits pupillary responses only driven by rods in ACHM patients which are nearly as strong as those to stimulation with 420 nm. Correspondingly, ACHM patients revealed the same phenomenon of similar pupillary peak responses to red and blue light stimulation as normal subjects, although they do not have functioning cones (complete ACHM). It might also be possible that during the transition between the conditions, when in normal controls cones start to be activated, there occurs not only an induction of disinhibition of rod activity due to absent cone function in ACHM patients, but also an additional interference with ipRGCs. This assumption is based on the following findings: we demonstrated a characteristic hypersensitivity of rods under dark adaptation conditions in ACHM patients compared with normal subjects (larger maximal constriction amplitudes in ACHM). Furthermore, in contrary to normal, ACHM patients showed a PIPR even to red stimulation (Table 4). This suggests a pronounced Melanopsin activation of ipRGCs that in normal controls preferentially occurs to short-wavelength stimulation in the blue spectrum range.[35–39] Both findings strengthen the hypothesis that in ACHM patients not only the blue but also the red pupillary responses were elicited via (hypersensitive and disinhibited) rods and ipRGCs in the absence of functioning cones. Recently, as mentioned above, it could be shown by specific gene defective mouse models, which selectively sublated the rod or cone function, that mouse rod photoreceptors have a major contribution not only to the pupil responses to blue light stimuli but also to low and medium red stimuli.[34] This finding perfectly corresponds to our results in humans.

In the last step of the third protocol, stimulating with high-intensity (28 lux) red and blue light of 1-second duration in the dark, there were no significant differences between the pupillary responses of the patients and those of the controls. This observation may be because in patients with ACHM the rods become saturated at higher levels of illumination, while on the other hand, ipRGCs are vigorously responding in the absence of the inhibition effect of S-cones.[22,28]

From the results of the three presented protocols, we were able to derive a final protocol for chromatic pupillography in the *CNGA3* gene therapy trial. This is described in Table 6.

After 20 minutes of dark adaptation, blue stimuli with an intensity of 0.01-lux corneal illumination and stimulus durations of 4 ms and 1 second are presented. By applying these two steps, we expect a normalization of the hypersensitive rod response (i.e., decreased maximal amplitude) and an enhanced inhibition of ipRGCs (i.e., decreased PIPR) due to restored retinal function as a marker for treatment efficacy. After 10 minutes of light adaptation (10 cd/m^2), a red stimulus of 28-lux intensity and 1-second duration is used to show enhanced cone function by improving pupil reaction parameters (i.e., increased maximal amplitude and decreased latency). The total examination time is approximately 45 to 50 minutes (including 30 minutes adaptation time).

In conclusion, we have introduced a feasible and sensitive protocol in *CNGA3*-ACHM, to be used for the objective assessment of efficacy of gene therapy or other innovative interventions in this hereditary retinal disorder.

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Appendix A

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