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Electrodiagnosis of dichromacy

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ABSTRACT

Retinal and cortical signals initiated by a single cone type can be recorded using the spectral compensation (or silent substitution) paradigm. Moreover, responses to instantaneous excitation increments combined with gradual excitation decreases are dominated by the response to the excitation increment. Similarly, the response to a sudden excitation decrement dominates the overall response when combined with a gradual excitation increase. Here ERGs and VEPs were recorded from 34 volunteers [25.9 ± 10.4 years old (mean ± 1 SD); 25 males, 9 females] to sawtooth flicker (4 Hz) stimuli that elicited L- or M-cone responses using triple silent substitution. The mean luminance (284 cd/m^2) and the mean chromaticity (x = 0.5686, y = 0.3716; CIE 1931 color space) remained constant and thus the state of adaptation was the same in all conditions. Color discrimination thresholds along protan, deutan, and tritan axes were obtained from all participants. Dichromatic subjects were genetically characterized by molecular analysis of their opsin genes. ERG responses to L-cone stimuli were absent in protanopes whereas ERG responses to M-cone stimuli were strongly reduced in deuteranopes. Dichromats showed generally reduced VEP amplitudes. Responses to cone-specific stimuli obtained with standard electrophysiological methods may give the same classification as that obtained with the Cambridge Colour Test and in some cases with the genetic analysis of the L- and M-opsin genes. Therefore, cone-specific ERGs and VEPs may be reliable methods to detect cone dysfunction. The present data confirm and emphasize the potential use of cone-specific stimulation, combined with standard visual electrodiagnostic protocols.

1. Introduction

Visual electrodiagnosis has been long used in diseases of the central nervous system (Arden, 1967) because it allows non-invasive recordings of neural activity initiated by photoreceptor stimulation *in vivo*. The International Society of Clinical Electrophysiology of Vision (ISCEV) has standardized several procedures that are periodically updated, to evaluate the functional integrity of the visual system (Bach et al., 2013; Hood et al., 2012; McCulloch et al., 2015; Odom et al., 2016). Retinal and cortical electrophysiological recordings are often obtained with white light stimuli. In the electroretinogram (ERG), short flashes elicit an initial negative a-wave, representing the activation of the photoreceptors and OFF-bipolar cells (photopic), followed by a larger positive b-wave related to the activation of ON-bipolar cells and Müller cells (Berson, Gouras, & Gunkel, 1968; Berson, Gouras, Gunkel, & Myrianthopoulos, 1969; Brown, 1968; McCulloch et al., 2015). Visual evoked cortical potentials to a single-flash stimulation generate earlier (minor) and later (major) components (Odom et al., 2016). The standard clinical protocols are useful for the differential diagnosis of several diseases affecting specific groups of cells in the retina such as cones or rods, or specific visual processes related with either the amplitude or time to peak of cortical responses. However, these clinical procedures do not take into account the contribution of cone subtypes (i.e. S-, M- or L-cones) to the signals. Additionally, they are not designed to access specific post-receptoral mechanisms, such as the ON- and OFF-pathways.

Previous studies have demonstrated that retinal photopic responses using long-duration flash stimulation show an initial negative

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component followed by a positive component (resembling but not completely equivalent to the a- and the b-waves of the short-flash ERGs) to stimulus onset while the response to light decrement shows only a positive component which is called the d-wave (Sieving, 1993; Sustar, Hawlina, & Brecelj, 2006). These ON- and OFF-responses are also elicited with rapid-on and rapid-off sawtooth modulation around the mean luminance, respectively. They are less burdening for the observers than long-flashes and thus less disturbed by blinks and squints. These electrophysiological protocols can be useful to evaluate asymmetrical impairment of ON- and OFF- post-receptoral visual pathways (Alexander, Barnes, Fishman, & Milam, 2002; Alexander, Fishman, Barnes, & Grover, 2001; Barboni et al., 2013; Pangeni, Lämmer, Tornow, Horn, & Kremers, 2012; Tsai, Barboni, et al., 2016). However, these signals are initiated by a combined and in-phase stimulation of all cone types. Since it is well known that some visual conditions affect differently specific cone types (Kellner & Foerster, 1992; Nathans, Piantanida, Eddy, Shows, & Hogness, 1986), it is more informative to access specific cone contributions to the post-receptoral ON- and OFF-mechanisms.

The spectral compensation paradigm (Estévez & Spekreijse, 1982) allows the stimulation of the L- or the M-cone while silencing the other types of photoreceptors (the amount of excitation of non-stimulated photoreceptors is held constant in stimulus and background). The stimulus method can be combined with square wave or sawtooth modulation and has been implemented in visual electrophysiology (Brainard, Calderone, Nugent, & Jacobs, 1999; Kremers, 2003). It was already demonstrated that ERG responses to selective L-cone excitation increments and decrements are similar in shape to those obtained with luminance increments and decrements respectively. On the other hand, ERG responses to M-cone isolating increments and decrements are antagonistic: the responses to M-cone excitation increments resemble those to L-cone (and luminance) decrements and *vice versa*. Additional evidence that this is caused by L/M opponency comes from the finding that the responses in protanopes to M-cone isolating stimuli are not sign-inverted relative to the L-cone driven responses in trichromats (Kremers et al., 2014; McKeefry et al., 2014; Tsai, Jacob, et al., 2016).

The L-/M-opponency is also present in VEP responses (Barboni et al., 2017), pupillary reflexes (Murray, Kremers, McKeefry, & Parry, 2018; Woelders et al., 2018) and psychophysical data (Parry, McKeefry, Kremers, & Murray, 2016). Moreover, subjects with congenital color vision deficiencies show near to absent VEP and ERG responses when the absent cone type is selectively stimulated (Barboni et al., 2017; Crognale, Rabin, Switkes, et al., 1993, Crognale, Switkes, Rabin, et al., 1993; Kremers et al., 2014; McKeefry et al., 2014; Tsai, Barboni, et al., 2016).

The above-described studies indicate the potential use of these non-invasive electrodiagnostic procedures to precisely investigate the sites of visual impairment. The aim of the present study is to compare individual data obtained from different methods in order to investigate cone-specific function and dysfunction. To reach the aim we recorded cone driven ERGs and VEPs from a large group of subjects with normal color vision as well as subjects with hereditary color vision deficiency and compared the individual electrophysiological data with each other, with psychophysical color vision thresholds and with molecular analysis of the opsin genes, as previously proposed (Crognale, Teller, Motulsky, & Deeb, 1998). Parts of these results were presented during the 24th symposium of the International Color Vision Society (ICVS 2017) in Erlangen (Germany).

2. Materials and methods

2.1. Participants

The experiments adhered to the tenets of the Declaration of Helsinki and were approved by the ethics committee of the University Hospital, University of São Paulo, Brazil (CEP-HU/USP 156.826). Signed informed consent was obtained from each subject after explanation of the nature and possible consequences of the study. Participants were 34 volunteers separated into three groups according to their color discrimination thresholds obtained with the Cambridge Colour Test (CCT): 23 trichromats [23.2 ± 7.9 years old (mean ± 1 SD); 15 males and 8 females]; five protanopes (34.2 ± 18.7 years old; 4 males and 1 female); and six deuteranopes (29.5 ± 6.4 years old; all males).

Inclusion criteria were: best-corrected visual acuity of at least 20/20, the absence of ophthalmological diseases as well as systemic diseases that could affect the visual system (such as diabetes mellitus). The subjects were recruited among students and workers at the University of Sao Paulo (Brazil). When recruiting the volunteers, we especially looked for subjects with self-reported and/or known color vision deficiencies. Part of the study population participated in a previously published study (Aher et al., 2018).

2.2. Visual stimulation

A Ganzfeld bowl (Q450SC; Roland Consult, Brandenburg, Germany) equipped with six arrays of differently colored light-emitting diodes (LEDs) was used as a stimulator. As previously described (Barboni et al., 2017; Kremers et al., 2014; Tsai, Barboni, et al., 2016), four LED arrays were used: red (peak wavelength ± half width at half maximum = 638 ± 9 nm), green (523 ± 19 nm), blue (469 ± 11 nm), and amber (594 ± 8 nm). The luminance of each LED array was modulated with rapid-on or rapid-off sawtooth profiles. Mean luminance, temporal frequency, modulation depth, and phase of each LED were independently controlled by the RETiport software (Roland Consult, Brandenburg, Germany).

The luminance for the red, green, blue and amber diodes were 80, 40, 4 and 160 cd/m^2 respectively. The total mean luminance (284 cd/m^2; resulting in about 14,200 ph td and 5600 scot td assuming an 8 mm diameter pupil) and the mean chromaticity (x = 0.5686, y = 0.3716; CIE 1931 color space), and thus the state of adaptation, were the same for all stimulus conditions. The mean (yellow) chromaticity was chosen in order to obtain maximal and equal L- and M-cone contrasts. We may consider that inputs to the S-(L + M) pathway may affect VEP responses in trichromats. The temporal frequency was always 4 Hz.

The stimuli were triple silent substitution conditions (i.e. the excitation of three photoreceptor types, including the S-cones and the rods, was not modulated) thereby isolating the output of the L- or the M-cones. The cone contrasts in L- and M-isolating stimuli were 18% and 17% respectively. Cone contrast is defined as the Michelson contrast, e.g. ($E_L$max − $E_L$min)/($E_L$max + $E_L$min) in which $E_L$max and $E_L$min are the maximal and minimal L-cone excitation respectively. Two modes of temporal modulation of cone-excitation were used: rapid-on (increments) and rapid-off (decrements). In increments, each cycle consisted of an abrupt increment in cone excitation, to activate the ON mechanism, followed by a linear decrease in excitation (observe that this also includes a rapid deactivation of the OFF mechanisms). Decrement profiles were the opposite, to activate the OFF mechanism.

2.3. Electrophysiological recordings

ERG and VEP responses were simultaneously recorded using monocular stimulation of one randomly chosen dilated (one drop of 0.5% tropicamide) eye. For the ERG recordings, a DTL fiber electrode attached to the outer and the inner canthus of the eye was used as the active electrode and gold cup skin electrodes were used as reference and ground electrodes attached to the ipsilateral temple and the forehead, respectively. For the VEP recordings, gold cup skin electrodes were used as the active electrode (Oz position), as reference electrode (Fz position), and as the ground electrode (placed on the forehead and also used for the ERG recordings). The ERG and VEP signals were amplified 100,000×, filtered between 1 and 300 Hz, and sampled at 1024 Hz using the RetiPort system (Roland Consult, Brandenburg,

Germany). At least 20 episodes, each lasting one second, were averaged. The first two seconds of each recording were discarded to avoid onset artifacts. The impedances between the electrodes were below 5 kΩ.

2.4. Color vision test

Type of color vision (trichromatic, protanopic, or deuteranopic) was determined using the Cambridge Colour Test (CCT) (Regan, Reffin, & Mollon, 1994). The CCT is a psychophysical test that determines color discrimination thresholds along different directions in the CIE 1976 color space (Mollon & Reffin, 1989). The test was performed using a Sony FD Trinitron color monitor GDMF500T9 (Sony Electronics, Tokyo, Japan; 100 Hz temporal resolution and 800 × 600 spatial resolution) and a VSG 5 graphics card (Cambridge Research Systems, Rochester, United Kingdom) controlled by the CCT version 2.0 software (Cambridge Research Systems, Rochester, United Kingdom) (Regan et al., 1994).

The stimuli were pseudoisochromatic fields composed of discs with a given chromaticity varying in size (between 5.2 and 22.8 arcmin) and in luminance (between 7 and 15 cd/m^2). The chromaticity of the discs forming the background was constant (u′ = 0.1977, v′ = 0.4689; CIE 1976), while the chromaticity of the discs forming the target (a Landolt "C") was set at specific points in the CIE diagram and varied during the presentations.

In order to determine the thresholds for discriminating target and background chromaticities, a maximum excursion of 0.110 units and a minimum excursion of 0.002 units in CIE color space were used. A four-alternative (up, down, left, and right) forced choice (4AFC) procedure was used and the chromaticity of the target was changed according to staircase procedures in which step sizes decreased after a reversal in the direction in the CIE space. After 11 reversals, a threshold was calculated (as the means of the target chromaticities at the last 7 reversals). Thresholds were obtained for protanopic, deuteranopic, and tritanopic confusion axes. The three axes were tested in random order during a single session. Results are given as the difference between the chromaticity of the background and the average chromaticity of the threshold. The chromaticity difference of the final result was quantified by the multiplication of the threshold excursion by 10,000 (u' v'* 10^4). Only participants showing color discrimination thresholds below 100 along protan and deutan axes and below 150 along the tritan axis were considered trichromats.

2.5. Genetic analysis

The L and M opsin genes of the subjects with color vision deficiencies were screened by extracting DNA samples from buccal brushes using the Gentra Puregene Buccal Cell Kit (Gentra Systems, Inc., Minneapolis, Minn., USA), according to the manufacturer's protocol. Polymerase chain reactions (PCR) were performed to amplify a fragment of about 300 base pairs containing the exon 5 of the X-linked (L and M) opsin genes, using primer pairs and protocols described by Neitz and Neitz (1995). We used restriction endonuclease Rsa I (Invitrogen, Carlsbad, USA) to digest and separate the two genes that were observed in electrophoresis at 1.0% agarose gel. The restriction endonuclease Rsa 1 recognizes a specific nucleotide sequence (GT-AC), which is present in exon 5 of the L-gene, but not in the M-gene. Thus, the enzyme recognizes and cleaves the amplified exon 5 of the L-gene in two smaller fragments, while the M-gene, not recognized and cleaved by the enzyme, displays only one band (Neitz & Neitz, 1995).

When two opsin genes were detected, we performed new rounds of PCRs to amplify the exons 2, 3, and 4 of both genes in order to search for polymorphisms. The primers and PCR conditions were described by (Neitz et al., 2004). PCRs were carried out in 50 µl reactions, using High Fidelity Platinum Taq Polymerase, 10× High Fidelity Buffer, 10 mM GeneAmp dNTPs (Applied Biosystems, Inc., Foster City, USA), $MgCl_2$ (Invitrogen, Carlsbad, USA), and 20 mM primers. PCR products were visualized by electrophoresis in 1.0% agarose gel and purified with Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK). Genetic sequencing was performed directly in both directions, using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) and the 3500 Applied Biosystems Sequencer. The electropherograms were visualized and aligned in BioEdit v7.0.9.0 (Hall, 1999).

2.6. Data analysis

Electrophysiological signals were analyzed offline using self-written MATLAB programs and Excel routines. Prior to further analysis, a notch 60 Hz offline filter was applied to the signals to reduce intrusion from the mains. Subsequently, the four cycles of each signal lasting 1000 ms (1024 points) were averaged in order to obtain a single cycle of 250 ms signal. Amplitudes (µV) and times to peak (ms) of each individual ERG responses were analyzed (see components analyzed in Fig. 2). Amplitude and time to peak of the first negative component (in the L-increment: N_{LI} and M-decrement: N_{MD} conditions) were analyzed by selecting the minimum in a 0 to 20 ms time window. The amplitude was calculated from the baseline (average voltage of the first five ms of the signal). The first positive component was analyzed for all ERG responses (P_{LI}, P_{LD}, P_{MI}, P_{MD}). The positive peak was the maximum between 25 and 50 ms (for P_{LI} and P_{MD}) and between 15 and 30 ms (for P_{LD} and P_{MI}). The positive amplitudes were calculated from the previously analyzed negative peak (for P_{LI} and P_{MD}) or from the baseline (for P_{LD} and P_{MI}) to the positive peak.

Total deviations, defined as sum of the absolute values of the differences between the baseline and the measured voltage at each time stamp of the signal between 50 and 150 ms after the sudden change in the stimulus (the average voltage of the first and the last five ms of the VEP signal), were used as a measure for VEP amplitude.

To obtain an estimate of the signal to noise ratio (SNR) the amplitude of the fundamental harmonic (4 Hz) was divided by the average amplitude of the neighboring frequencies (3 Hz and 5 Hz) provided by a Fast Fourier Transform (FFT). Responses with SNR (Meigen & Bach, 1999) less than two were considered to be non-significant, and the delay times of the response components were discarded. Meigen and Bach (1999) recommended an SNR of 2.82 for a significant signal. However, this recommendation was solely based on response amplitudes. We noticed that implicit times and phases were in the expected range when comparing to larger responses for responses with SNR down to 2.0.

We calculated the ratios between L- and M-cone driven responses (L_I/M_D and L_D/M_I), taking into account the previously described L/M opponency in ERG (Tsai, Jacob, et al., 2016) and VEP (Barboni et al., 2017) responses to cone isolating stimulus. The ratios were calculated by dividing the respective positive amplitudes for the ERGs and the total deviations for the VEP.

Statistical comparisons (SPSS, Statistical Package for the Social Sciences, Hong Kong, China) were performed using ANOVA and Bonferroni post hoc correction for multiple testing.

3. Results

The results (average and one standard deviation) of the psychophysical CCT are shown in Fig. 1. The upper graph shows averages (± one standard deviation) of color discrimination thresholds from 23 subjects. These values were within the normal range of color discrimination thresholds for the three axes (protan, deutan, and tritan) with no significant difference between thresholds along the protanopic (average = 41.6 ± 11.5) and the deuteranopic confusion lines (average = 41.0 ± 13.6) ($p = 0.89$). The color vision of these subjects was classified as normal trichromatic.

Five subjects showed (middle graph) significantly higher thresholds

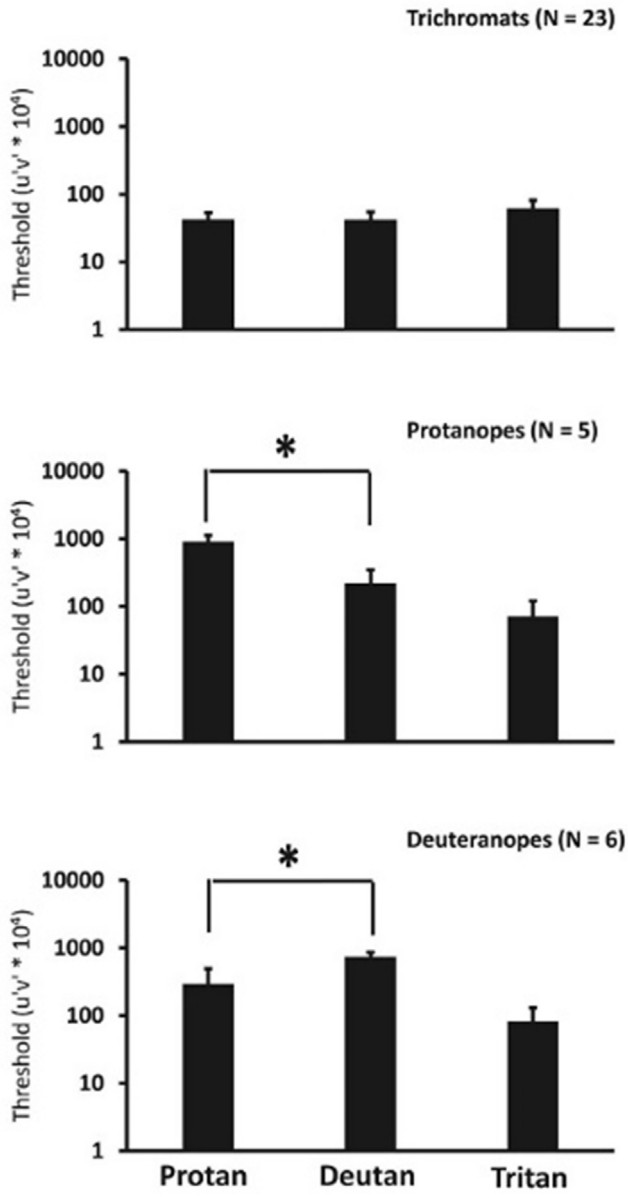

Fig. 1. Average (± one standard deviation) of color discrimination thresholds for the three groups of volunteers: trichromats (upper graph), protanopes (middle graph), deuteranopes (lower graph). Trichromats show low thresholds for all three axes: protan, deutan, and tritan. Protanopes showed significantly elevated protan thresholds and deuteranopes showed significantly higher deutan thresholds.

along the protanopic confusion lines compared to their thresholds along the deuteranopic confusion lines ($p = 0.001$) and they were classified as protanopes. The remaining six subjects showed (lower graph) thresholds along deuteranopic confusion line that were significantly higher ($p = 0.001$) than those along the protanopic confusion lines thresholds and the subjects were considered to be deuteranopes. All dichromats displayed higher thresholds along the two confusion lines protan ($p < 0.001$) and deutan ($p < 0.006$), but not along the tritan confusion line (protanopes $p = 0.9$ and deuteranopes $p = 0.4$) when compared to trichromats. Average results from the subjects with normal color vision as well as the individual results from the protanopes and the deuteranopes are summarized in Table 1.

Genetic analysis was used to estimate the opsins' λ_{max} (nm) from each of the 11 dichromats. The estimate was based on the amino acid composition, at the sites that determine the spectral tuning (Asenjo, Rim, & Oprian, 1994). Table 1 shows the age and gender of the subjects, CCT results, expressed opsin gene with the respectively estimated λ_{max}, and the genetic sequencing of exons 2, 3, and 4.

All five protanopes (P1–P5; four males and one female) had one or more M-opsin genes and no L-genes. The genetic sequencing of exons 2, 3, and 4 showed that one protanope (P2) had one hybrid M-opsin gene resulting in an expected λ_{max} of 538 nm, instead of the normal λ_{max} of 532 nm. In addition, another protanope (P3) had at least two copies of the M-opsin gene: one normal and one hybrid, but both resulting in photopigments with expected λ_{max} values at 532 nm.

Deuteranopes D3 and D5 only had L-opsin genes with expected λ_{max} of 563 nm. Two deuteranopes (D1 and D2) had hybrid L-opsin genes resulting in photopigments with expected λ_{max} values at 556 and 559 nm, respectively. The remaining two deuteranopes (D4 and D6) had at least one copy of each (L- and M-) opsin gene. In one subject (D6) there were two L-genes: one normal with λ_{max} value at 563 nm and one hybrid with λ_{max} at 559 nm. Another deuteranope (D4) had one hybrid L-opsin gene resulting in a photopigment with expected λ_{max} at 556 nm as well as one hybrid M-opsin gene with expected λ_{max} at 532 that we hypothesize it has not been expressed (see discussion).

The absorption peaks (nm) of the opsins were estimated based on the amino acids located at spectral tuning sites according to Asenjo et al. (1994). A = alanine; I = isoleucine; L = leucine; M = methionine; S = serine; T = threonine; V = valine; Y = tyrosine.

Averaged electrophysiological traces are shown in Fig. 2. Sketches of the stimuli are shown in the left column. The average ERG and VEP responses of the trichromats are displayed in Fig. 2A. Their ERGs display negative and positive components depending on the stimulus type. The L-cone increment response has an initial negative peak (N_{LI}) around 18.6 ± 2.0 ms after the instantaneous increase in excitation followed by a positive peak (P_{LI}) after about 31.8 ± 3.1 ms. L-cone decrements elicited only one positive component (P_{LD}) at 20.3 ± 3.5 ms. The ERG responses elicited by M-cone isolating stimuli are smaller and delayed compared to those from the L-cone driven responses. Moreover, it shows more variability among healthy subjects. The positive peak of the M-cone increment (P_{MI}) protocol was found at about 23.8 ± 6.2 ms. M-cone decrements displayed mainly a positive component (P_{MD}) in which peak was found at 37.9 ± 10.4 ms. These results are in agreement with previous findings (Tsai, Jacob, et al., 2016).

The VEP responses of trichromats show a positive (main) component with a delay between 50 and 150 ms. The time to peak was similar for the four stimulus types.

The averaged responses of five protanopes are shown in Fig. 2B. They displayed strongly reduced ERG and VEP responses to L-cone incremental and decremental stimuli. The M-cone driven ERGs in the protanopes were quite similar to the L-cone driven responses and unlike the M-cone driven responses in the trichromats. Fig. 2C displays the average traces of the deuteranopes. Their responses to L-cone stimulation showed similar profiles as the average response of the trichromats, although with somewhat smaller amplitudes. ERG and VEP responses to M-cone isolating stimuli were reduced in the deuteranopes, although still measurable. This has been found before (Kremers et al., 2014; Tsai, Jacob, et al., 2016) when recording full-field ERGs.

We compared results from the responses driven by the dominant cone (defined as the cone whose stimulation results in the largest response, i.e. L-cones for trichromats and deuteranopes and M-cones for protanopes). Fig. 3 shows the average (± standard deviation) amplitudes (in μV) and implicit times (in ms) of ERG negative and positive components and VEP total deviations of the dominant cone responses. Fig. 3A shows that the ERG components are similar between trichromats (opened dots), protanopes (grey dots), and deuteranopes (black dots). There were no significant differences for increment amplitudes of the negative ($p = 0.077$) and of the positive ($p = 0.101$) component. Moreover, the positive components of responses to decrements were similar for the three groups ($p = 0.190$). The implicit times of the

Table 1
Control average and individual dichromatic CCT thresholds and genetic results.

			CCT			Genetic analysis		Amino acid at the spectral tuning sites							
Normal color vision (N = 23)	Age	Gender	Protan	Deutan	Tritan	Expressed opsin gene	Estimated λmax (nm)	Exon 2			Exon 3		Exon 4		
								65	111	116	153	180	230	233	236
AVE	*23.2*	*15 males*	*41.6*	*41.0*	*60.5*	*L*	*563*	*T*	*I*	*S*	*L*	*S*	*I*	*A*	*M*
SD	*7.9*	*8 females*	*11.5*	*13.6*	*20.7*	*M*	*532*	*I*	*V*	*Y*	*M*	*A*	*T*	*S*	*V*
Protanopes	Age		Protan	Deutan	Tritan	Expressed opsin gene	Estimated λmax (nm)	Exon 2			Exon 3		Exon 4		
								65	111	116	153	180	230	233	236
P1	36	male	1100	266	30	M	532	I	V	Y	M	A	T	S	V
P2	66	male	1100	422	155	M-hybrid	538	T	V	S	L	S	I	A	V
P3	23	male	658	144	64	M	532	I	V	Y	M	A	T	S	V
						M-hybrid	532	T	I	S	M	A	T	S	V
P4	21	female	752	99	45	M	532	I	V	Y	M	A	T	S	V
P5	25	male	965	168	63	M	532	I	V	Y	M	A	T	S	V
AVE	*34.2*		*915.0*	*219.8*	*71.4*										
SD	*18.7*		*202.2*	*128.5*	*48.8*										
Deuteranopes	Age		Protan	Deutan	Tritan	Expressed opsin gene	Estimated λmax (nm)	Exon 2			Exon 3		Exon 4		
								65	111	116	153	180	230	233	236
D1	33	male	227	849	29	L-hybrid	556	T	I	S	M	A	I	A	M
D2	24	male	377	687	145	L-hybrid	559	T	V	Y	L	S	I	A	M
D3	39	male	325	953	68	L	563	T	I	S	L	S	I	A	M
D4	24	male	364	1003	114	L-hybrid	556	T	I	S	L	A	I	A	M
						M-hybrid	532	I	V	Y	L	A	T	S	V
D5	33	male	282	469	78	L	563	T	I	S	L	S	I	A	M
D6	24	male	172	443	55	L	563	T	I	S	L	S	I	A	M
						L-hybrid	559	I	V	Y	L	S	I	A	M
						M	532	I	V	Y	M	A	T	S	V
AVE	*29.5*		*291.2*	*734.0*	*81.5*										
SD	*6.4*		*80.3*	*241.1*	*41.8*										

negative (p = 0.053) and positive (p = 0.070) ERG components to increments and of the positive ERG component to decrements (p = 0.160) were also similar.

Fig. 3B shows the comparison of the total deviation of the VEP responses. In trichromats, VEPs to dominant (i.e. L-) cone increments were larger than those in protanopes to M-cone stimulation (p = 0.009 and those in deuteranopes to L-cone stimulation (p = 0.002). For decrement stimulation of the dominant cone, significantly lower total deviations were found for the deuteranopes (p = 0.042), but not for the protanopes (p = 0.182). There was no significant difference between the increment responses (p = 0.958) and decrement responses (p = 0.414) of trichromats, protanopes, and deuteranopes.

Fig. 4 shows the average (± standard deviation) amplitudes (in μV) and implicit times (in ms) of ERG negative and positive components and VEP total deviations as in Fig. 3, for responses driven by the non-dominant cone (M-cone for trichromats and deuteranopes and L-cone for protanopes). Amplitudes of the positive ERG components (Fig. 4A) were significantly higher for trichromats compared to protanopes (increments: p = 0.040; decrements: p = 0.009) and deuteranopes (increments: p = 0.009; decrements p = 0.003). Negative ERG components were absent in the responses obtained from most of the dichromats. Implicit times were not estimated because of the absent responses of the positive components in protanopes and deuteranopes.

Fig. 4B shows the same comparison for the VEP total deviations as shown in Fig. 3B, but now for the responses driven by the non-dominant cone type. In trichromats, VEPs to M-cone increments resulted in total deviation of 364 ± 166 μV while decrements resulted in larger total deviations (449 ± 238 μV) and opposite of what was found for the dominant (i.e. L-) cones (cf. Fig. 3B). Protanopes and deuteranopes showed smaller averaged total deviations to increments (protanopes p = 0.018 and deuteranopes p = 0.008) and decrements (protanopes p = 0.003 and deuteranopes p = 0.020). However, there were no significant differences between protanopes and deuteranopes for the total deviation in the responses to increments (p = 0.553) and to decrements (p = 0.282). In trichromats, VEPs always elicited responses above the noise level in both dominant and non-dominant cone responses. However, protanopes and deuteranopes showed measurable responses for the dominant cone, but more than half of the non-dominant cone responses (12 out of 22) were below the noise level, and therefore absent.

In Fig. 5 we plotted the ratios of the L- and M-cone driven responses in the ERGs and VEPs. The ratios were calculated considering the amplitudes of the positive peaks (ERG) and the total deviations (VEP) using L_I/M_D (A) and L_D/M_I (B). In order to identify the estimated λ_{max} (in nm) from each subject tested, different symbols were used.

ERG L/M ratios were 2.26 ± 1.72 (L_I/M_D) and 2.42 ± 2.31 (L_D/M_I). The ERGs were L-cone dominated in the majority of the trichromats (black opened circles), although some trichromats showed M-cone dominated responses with L/M ratios were close to unity. L/M ERG ratios of protanopes were lower than unity showing that their ERGs were M-cone dominated. L/M ERG ratios of the deuteranopes were larger than unity, indicating that the ERGs were L-cone dominated.

Stimuli activating the non-dominant cone always elicited measurable total deviation of the VEPs for the trichromats (Fig. 4B). Although there were no significant differences (p = 0.243 for L_I/M_D and p = 0.165 for L_D/M_I), the L-/M-cone VEP ratios were on average 1.20 ± 0.43 (L_I/M_D) and 0.84 ± 0.23 (L_D/M_I) for trichromats, smaller than one for protanopes (L_I/M_D = 0.85 ± 0.46 and L_D/M_I = 0.66 ± 0.9) and larger than one for deuteranopes (L_I/M_D = 1.31 ± 0.58 and L_D/M_I = 1.15 ± 0.78).

All protanopes with one M-gene with an estimated λ_{max} of 532 nm

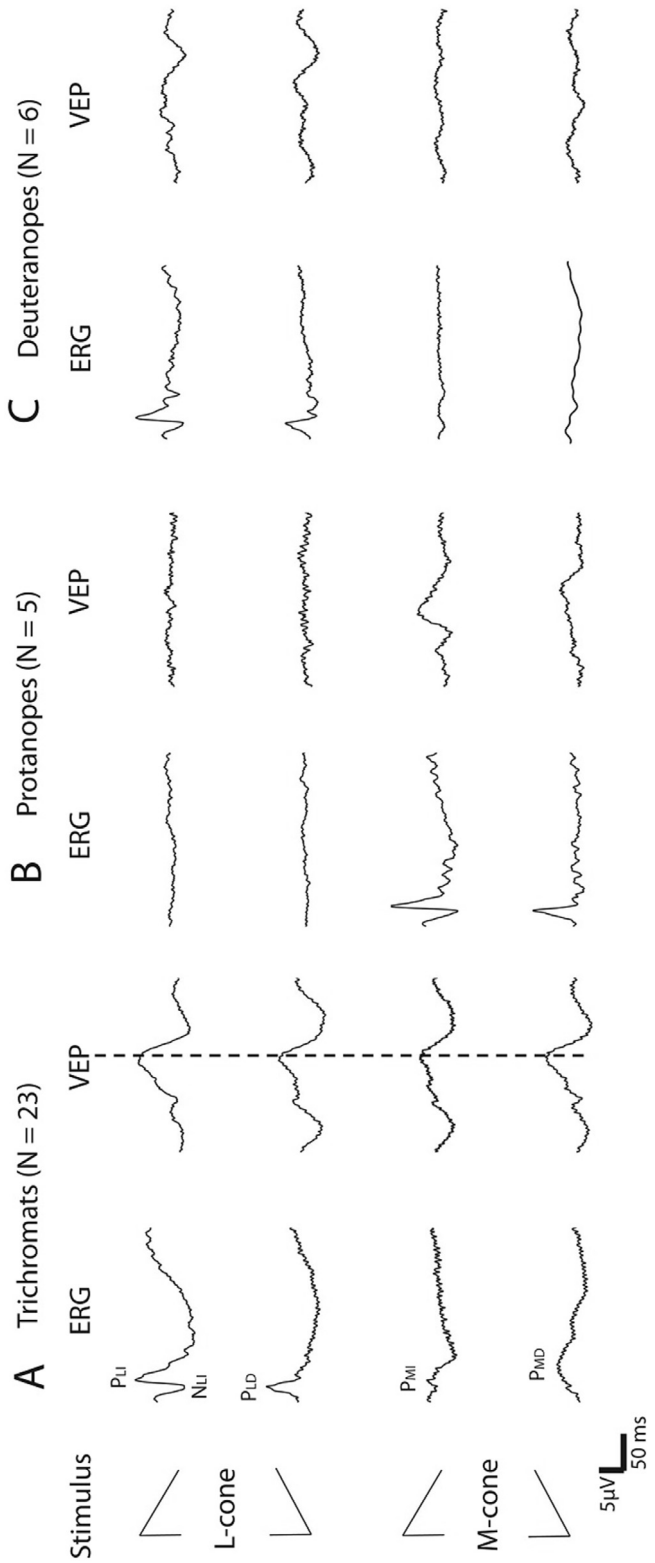

Fig. 2. Averaged ERG and VEP signals of trichromats (A), protanopes (B), and deuteranopes (C). On the left side, sketches of the stimulus profiles are shown. The two upper lines show the L-cone driven responses and the two lower lines show the M-cone driven responses. The scale for the amplitude and time is shown below. The signals of the normal color vision group show the components analyzed: N_{LI} and P_{LI}, P_{LD}, P_{MI}, and P_{MD}.

had L/M ERG and VEP ratios that were smaller than unity. The protanope with an additional hybrid M-gene with a λ_{max} at 532 nm (grey opened circles) and the protanope with one hybrid gene resulting in pigment with λ_{max} at 538 nm (grey filled triangle) had larger VEP ratios. The deuteranopes show ERG L/M ratios that were substantially larger than one whereas the VEP L/M ratios were closer to unity.

4. Discussion

The present data confirm (Barboni et al., 2017; Brainard et al., 1999; Crognale, Rabin, Switkes, et al., 1993, Crognale, Switkes, Rabin, et al., 1993; Jacob et al., 2015; Kremers et al., 2014; McKeefry et al., 2014; Tsai, Jacob, et al., 2016) and emphasize the potential use of cone isolating stimuli, combined with electrophysiological standard protocols (full-field ERG and VEP), to determine color vision type. The present study extends the previously obtained data by comparing psychophysical, genetic and electrophysiological ERG and VEP data obtained from the same individuals. Recently, ERGs to heterochromatic (red-green) modulation stimulus was shown to allow determining the type of color vision deficiency (Aher et al., 2018; Brainard et al., 1999; Kremers & Bhatt, 2016). The present data show that ERGs to cone selective stimuli can also be used to identify dichromacy.

As expected, and previously reported (Kremers et al., 2014), the ERG responses to the absent cones are absent or substantially smaller in dichromats. In addition, cone driven VEP signals are generally smaller (including to the dominant cone; see Fig. 3B) in dichromats (Barboni et al., 2017) and may also be delayed (Crognale, Rabin, Switkes, et al., 1993, Crognale, Switkes, Rabin, et al., 1993) depending on stimulus condition. This is not the case with the ERGs, where the responses to stimulation of the dominant cone have very similar amplitudes in dichromats and trichromats (see Fig. 3A). It might also be considered that individual variations such as macular pigment density, may explain the small but detectable responses in M-cone isolating conditions (Huchzermeyer & Kremers, 2017).

Previous reports indicated that the absent cone pigment may be replaced by the present cone pigment in dichromats without a change in the number of functional cones (Carroll, Neitz, Hofer, Neitz, & Williams, 2004; Kremers & Meierkord, 1999). This was demonstrated by studying ERG responses to high frequency stimulation of the dominant cone that is larger in dichromats than in trichromats (Kremers, Usui, Scholl, & Sharpe, 1999). ERG responses to high frequency stimuli reflect luminance responses and their amplitudes are correlated with the number of stimulated cones (Murray, Parry, Kremers, Stepien, & Schild, 2004). Here we found that responses to M-cone increments in protanopes were similar to those to L-cone increments (the dominant cone) in trichromats. Furthermore, response amplitudes to L-cone increments were not different in trichromats and deuteranopes. Thus, ERG responses to sawtooth cone isolating stimuli contradict the 'replacement model'. However, we previously suggested that the responses to 4 Hz sawtooth stimuli are probably a mixture of luminance and cone opponent responses (Tsai, Jacob, et al., 2016). The latter is absent in dichromats which may explain the relatively smaller ERG responses in dichromats.

It has been shown that pattern-onset cone-specific VEPs of the dominant cone (Rabin, Kryder, & Lam, 2016) are normal in anomalous trichromats. In the present study, the fact that the VEP responses to stimulation of the dominant cone type are substantially smaller in dichromats than in trichromats (Fig. 4B), indicates that, compared to the full field ERGs, the full field VEPs are even more strongly dominated by cone opponent activity. This is in agreement with previously proposed ideas (Barboni et al., 2017).

In trichromats, the ERG ratios were calculated from responses to pairs of opposite stimuli (i.e. to L_I/M_D and to L_D/M_I) because of the resemblance in the response waveforms. This was also found previously (Kremers et al., 2014; McKeefry et al., 2014; Tsai, Barboni, et al., 2016) and indicates the above-mentioned presence of cone opponency in the

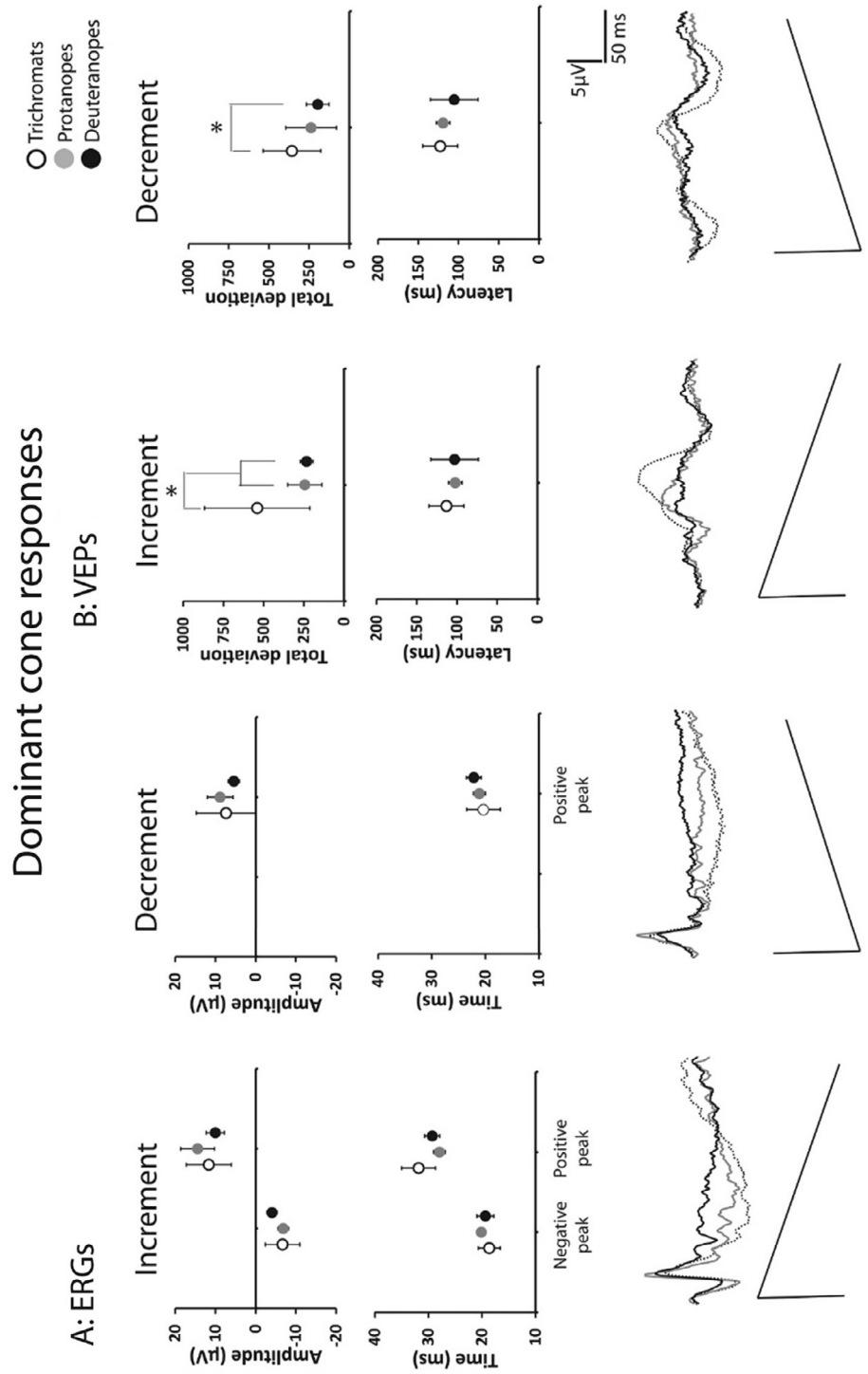

Fig. 3. Average (± one standard deviation) component responses of the ERG (A) and total deviation and latency responses of the VEP (B) for responses elicited by the dominant cone: L-cone for trichromats (open symbols) and deuteranopes (black filled symbols) and M-cone for the protanopes (grey filled symbols). Respective average signals are shown below. Significant differences are marked with an asterisk. For increment total deviation VEP responses trichromats were different from both protanopes and deuteranopes. For decrement VEP responses only trichromats and deuteranopes were significantly different.

responses. Interestingly, the L-/M-ratio is what is expected from a luminance reflecting response because cone-opponent retinal pathways have a balanced input from L- and M-cones (Lee, 1996; Smith, Lee, Pokorny, Martin, & Valberg, 1992). Although it is unclear how the luminance and cone-opponent pathways are 'translated' in an ERG response (Brainard et al., 2000; Cicerone & Nerger, 1989; Krauskopf, 2000; Kremers et al., 2000), the ERG ratios are as in the expected range. However, if the ERG responses are a mixture of luminance and cone-opponent responses, the ratios in the luminance reflecting responses may be larger than those obtained from the ERGs. Indeed ERG responses to full field sinewave stimuli have generally larger L/M ratios (Jacob et al., 2015).

VEP ratios to L_I/M_D and to L_D/M_I are around one in trichromats. Although most protanopes show VEP ratios below one (M-cone dominance) and most deuteranopes show VEP ratios above one (L-cone dominance), they are closer to the trichromatic range than ERGs. This

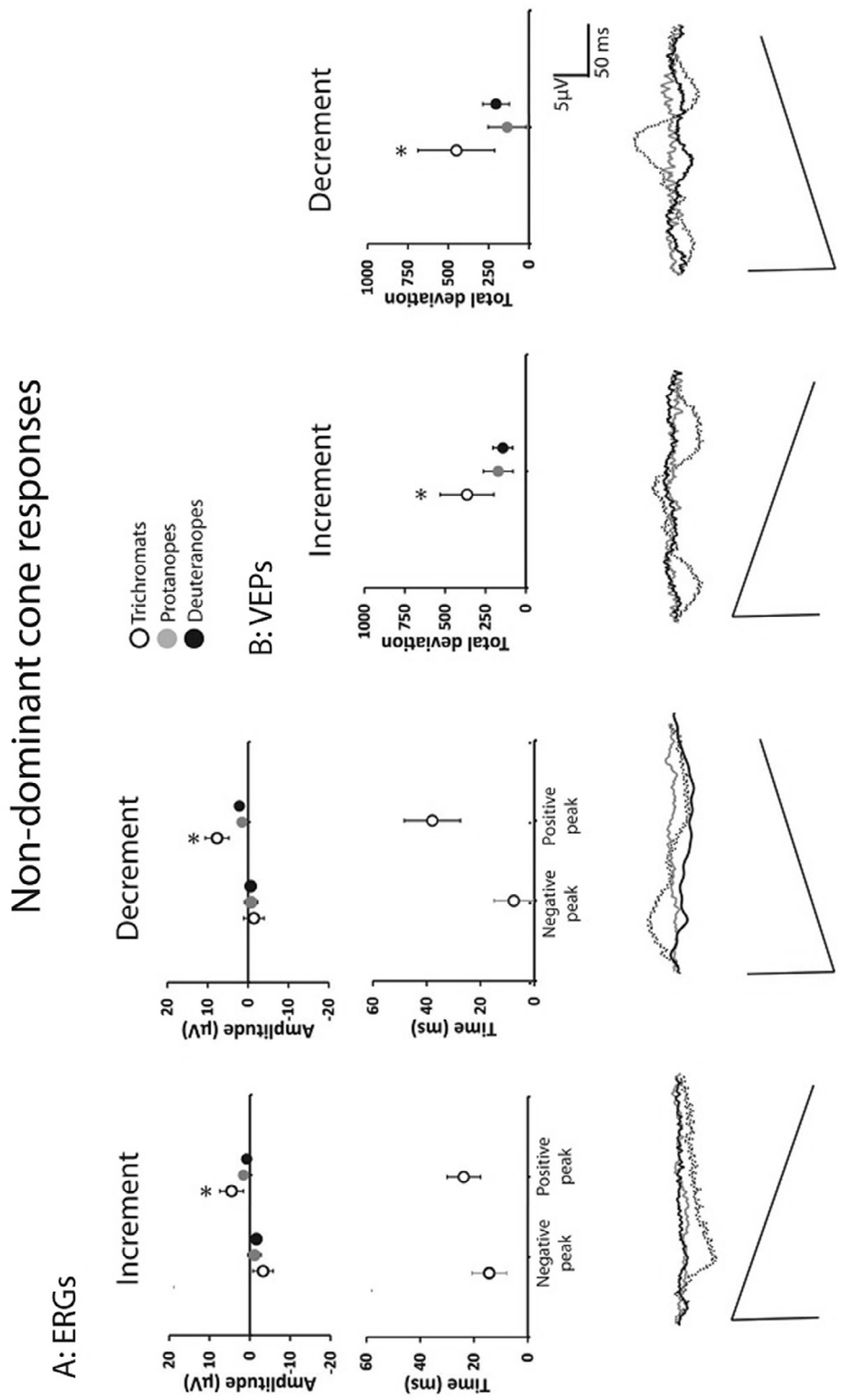

Fig. 4. Average (± standard deviation) component responses of the ERG (A) and total deviation responses of the VEP (B) for responses elicited by the non-dominant cone: M-cone for trichromats (open symbols) and deuteranopes (black filled symbols) and L-cone for the protanopes (grey filled symbols). Respective average signals are shown below. Significant differences are marked with an asterisk. Trichromats are different from protanopes and deuteranopes and there is no significant difference between protanopes and deuteranopes.

may be influenced by larger inter-individual variability of cone-driven VEP responses. The data may also indicate that cone opponent responses have larger input to the VEP. As mentioned above, this may also explain why VEPs to cone isolating sawteeth are generally smaller in the dichromats.

The genetic information allowed estimating the expected λ_{max} of pigments of the dichromats (Asenjo et al., 1994). All subjects classified as protanopes according to their CCT thresholds and by their genetic results, showed L-/M-cone ratios smaller than unity in the ERGs and the majority showed L-/M-cone ratios smaller than one in the VEPs. L-cone

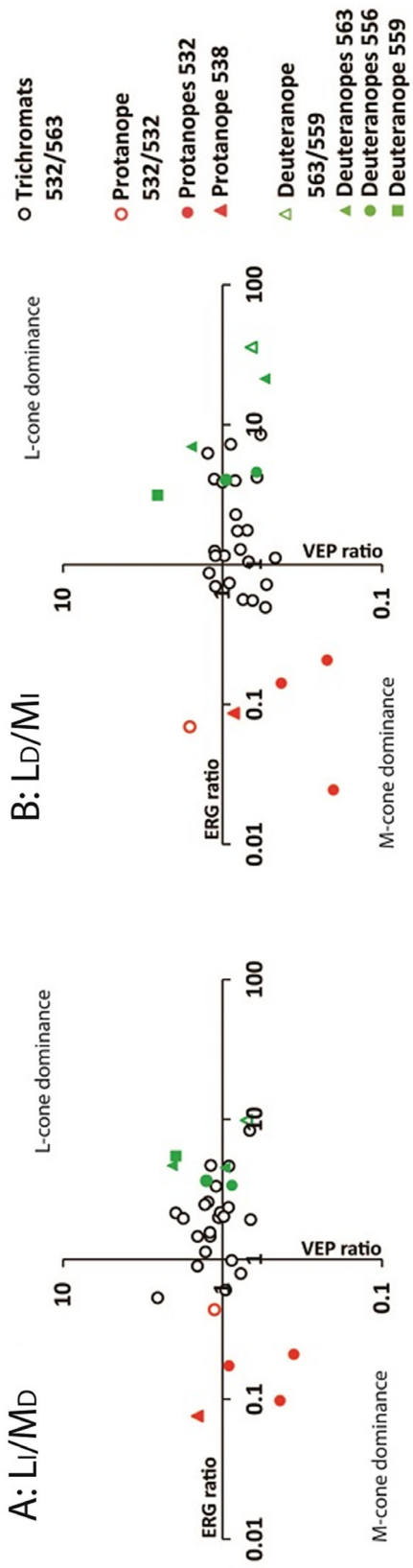

Fig. 5. ERG and VEP ratios for L_I/M_D (A) and for L_D/M_I (B). Black opened dots are results from 23 trichromats. Other red (protanopes) and green (deuteranopes) symbols are used depending on the estimated λmax of each dichromat subject. Ratios displayed on the right-upper part of the graphs indicate L-cone dominated responses. Ratios in the left-lower part of the graphs indicate M-cone dominated responses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

specific absence, as demonstrated here for protanopes, may be reliably identified using electrodiagnostic tools such as these protocols to measure cone-driven responses. Further investigations may consider characterizing L-cone driven ERGs and VEPs in anomalous trichromats, as previously demonstrated (Brainard et al., 1999; Crognale, Rabin, Switkes, et al., 1993, Crognale, Switkes, Rabin, et al., 1993), as well as subjects with acquired L-cone deficiency to verify potential use of these examinations in milder L-cone specific dysfunction.

Subjects were classified as deuteranopes according to their CCT thresholds. Four subjects had their phenotypes confirmed by their genetic results. The genetics of one deuteranope (D4) showed the presence of both L- and M-opsin genes. Although the L-opsin gene was hybrid with estimated $\lambda_{max} = 556$ and the M-opsin gene was hybrid with estimated λ_{max} of 532 nm, the subject was classified as deuteranope according to his CCT thresholds. The L-/M- ERG ratios of this subject are 3.64 for L_I/M_D (green filled circle on the L-cone dominance quadrant of Fig. 5A) and 4.02 for L_D/M_I (green filled circles on the x-axis of Fig. 5B). Therefore, the ERGs confirm the results of the CCT supporting the possibility of inactivating mutations in non-coding regions of the M-cone opsin gene that were not sequenced, as intron-exon junctions or the promoter regions, or due to the position of the genes in the chromosomal array, leading to his deuteranopic color vision phenotype (Hayashi, Motulsky, & Deeb, 1999; Neitz et al., 2004).

Congenital color deficiency classifications obtained with the CCT and with the genetic analysis of the opsin genes were previously shown to be in agreement with each other (Honnell et al., 2015). In the present study, the comparison is extended to a quantitative analysis of CCT scores, molecular genetics, and responses to cone-driven ERGs and VEPs. The present study confirms (Aher et al., 2018; Barboni et al., 2017) that specific cone-driven responses obtained with standard electrophysiological methods may give the same classification as that obtained with the CCT and in some cases with the genetic analysis of the L- and M-opsin genes.

Although the ERG is a worldwide non-invasive clinical tool for diagnosing inherited and acquired retinal diseases allowing direct assessment of the functional integrity of retinal cells (McCulloch et al., 2015; Perlman, 1983), its application may not be possible in young infants or in patients who refuse to accept ERG electrodes. Our data suggest that VEP recordings, though more variable, are possibly an alternative to investigate cone-specific deficiencies in these subjects, with consistent VEP responses provided by L- and M-cone isolating unpatterned full-field stimulation.

The use of chromatic stimuli to elicit ERG responses has long been considered to identify specific cone abnormalities (Copenhaver & Gunkel, 1959). For instance, Kellner and Foerster (1992) showed that color stimulation to record ERGs is efficient to identify cone-specific retinal dysfunction in several retinal conditions. The characteristics of stimulation generated by four primaries allowed for a silent substitution in three photoreceptor types including the rods and, therefore, to prevent a possible asymmetric influence from On- and Off-bipolar cells. ERG responses to increments and decrements have different morphologies, indicating that responses of On- and Off-pathways are not mirror-images of each other.

Currently, cone isolating stimuli can be programmed in commercially available instruments used in clinics to record ERG and VEP responses. The requirements are very simple: light sources with different spectral distributions (see the four LED arrays we used in the method section) and the possibility to independently set luminance, contrast and On- or Off-sawtooth modulation profiles. The present demonstration of the efficacy of cone-isolating stimulation to detect dichromacy may be extrapolated to anomalous trichromats and to acquired color vision deficiencies.

Competing interests

The authors declare that they have no competing interests.

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