Dystrophin Is Required for Proper Functioning of Luminance and Red–Green Cone Opponent Mechanisms in the Human Retina

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Purpose. Visual information is processed in parallel pathways in the visual system. Parallel processing begins at the synapse between the photoreceptors and their postreceptoral neurons in the human retina. The integrity of this first neural connection is vital for normal visual processing downstream. Of the numerous elements necessary for proper functioning of this synaptic contact, dystrophin proteins in the eye play an important role. Deficiency of muscle dystrophin causes Duchenne muscular dystrophy (DMD), an X-linked disease that affects muscle function and leads to decreased life expectancy. In DMD patients, postreceptoral retinal mechanisms underlying scotopic and photopic vision and ON- and OFF-pathway responses are also altered.

Methods. In this study, we recorded the electroretinogram (ERG) while preferentially activating the (red–green) opponent or the luminance pathway, and compared data from healthy participants (n = 16) with those of DMD patients (n = 10). The stimuli were heterochromatic sinusoidal modulations at a mean luminance of 200 cd/m^2. The recordings allowed us also to analyze ON and OFF cone-driven retinal responses.

Results. We found significant differences in 12-Hz response amplitudes and phases between controls and DMD patients, with conditions with large luminance content resulting in larger response amplitudes in DMD patients compared to controls, whereas responses of DMD patients were smaller when pure chromatic modulation was given.

Conclusions. The results suggest that dystrophin is required for the proper function of luminance and red–green cone opponent mechanisms in the human retina.

Keywords: retina, electroretinography, magnocellular/parvocellular, Duchenne muscular dystrophy, dystrophin

Dystrophin is a large protein[1,2] responsible for connecting intra- and extracellular protein complexes.[3] The gene responsible for its transcription (*DMD*) is located at Xp21 on the short arm of the X chromosome. It has seven promoters, three of which are responsible for the transcription of the full-length dystrophin (Dp427), while the other four are needed for the intragenic transcriptions of smaller gene products: Dp260, Dp140, Dp116, and Dp71. Nonsense mutations in the *DMD* gene cause Duchenne muscular dystrophy (DMD), an X-linked lethal disorder that affects 1 among 3500 to 5000 males.[4–9] The severity of the symptoms in DMD, however, varies depending on the location of the mutation(s).

Nonsense mutations in the full-length dystrophin (Dp427) result in a decreased life expectancy,[10] progressive muscle degeneration and weakness,[1] impaired cognitive performance,[11] and altered retinal physiology.[12] Additional dysfunction of smaller gene products may lead to increased cognitive deficits[13–15] and/or abnormal retinal physiology.[16–20]

The first reports on altered retinal activity in DMD patients with a detectable deletion in the *DMD* gene showed a reduction or an absence of the scotopic b-wave.[17,18] It has also been reported that, in addition to rod-driven signals, photopic responses in the ON pathway were abnormal in DMD patients.[19] More recently, we showed an abnormal photopic retinal ON activity in DMD patients with genetic mutations in both the full Dp427 protein and smaller gene products, including Dp260.[16]

In this study, we investigated the physiology of the retina with electroretinography (ERG) in response to heterochromatic stimulation at two different temporal frequencies. Stimulation at 12 Hz in ERG is thought to mainly reflect red–green opponency when both red and green are modulated in

Investigative Ophthalmology & Visual Science

Table 1. Characteristics of the DMD Patients

N	Age	Cort	Mutation	Affected Dystrophin Isoforms
1	15	9	51–52	Dp427 / Dp260 / Dp140
2	16	7	46–47	Dp427 / Dp260 / Dp140
3	29	13	46–55	Dp427 / Dp260 / Dp140
4	14	9	55	Dp427 / Dp260 / Dp140
5	16	4	48–52	Dp427 / Dp260 / Dp140
6	24	18	43–45	Dp427 / Dp260 / Dp140
7	18	10	44–47	Dp427 / Dp260 / Dp140
8	17	10	3–7	Dp427
9	18	9	3–7	Dp427
10	12	5	3–15	Dp427
Ave	18	9		
SD	5	4		

Cort, years of corticosteroid intake; mutation, mutated exons; ave, average; SD, standard deviation.

counterphase, which is related to processing in the parvocellular pathways. Heterochromatic (red–green) 36-Hz flicker ERGs is thought to reflect luminance processing related to the magnocellular pathway. In the patients, the responses to 12 Hz containing only isoluminant red–green stimulation are decreased, indicating that the red–green opponent channel is affected. On the other hand, stimulation containing both luminance and chromatic modulation led to increased responses in the patients, suggesting additional input from the luminance channel. We speculate that the previously reported imbalance between ON and OFF activities may underlie this altered function of the luminance channel.

Methods

Subjects

The participants included 10 DMD patients (mean age 18 ± 5 years) and 16 controls (mean age 17 ± 7 years), all of whom were males. The inclusion criteria were best-corrected visual acuity of 20/30 or better, absence of ophthalmologic diseases, normal fundus, and absence of any other disease that could affect the visual system (as so informed by the patients or their relatives). Table 1 shows the mutated exons for all DMD patients. Seven DMD patients presented genetic alteration between exon 30 and exon 56, which affects *DMD* gene products Dp427, Dp260, and Dp140. Three DMD patients had mutations upstream of exon 30, affecting only Dp427. Table 1 also indicates the age and the period of corticosteroid intake for all patients prior to testing (mean = 9 ± 4 years).

The experiments adhered to the tenets of the Declaration of Helsinki and were approved by the institutional ethics committee (CEP-HU/USP 156.826). Signed informed consent was obtained from the subjects and, when applicable, from their parents after explanation of the nature and possible consequences of the study.

Genetic Screening

Duchenne muscular dystrophy mutations were screened through the MLPA reaction (Multiplex Ligation-dependent Probe Amplification), allowing the detection of deletion or duplication occurring in this gene. The reaction was performed according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands) and the resulting products were separated by capillary electrophoresis. Analyses were done by the Human Genome and Stem Cell Research Center from the Institute of Biosciences using the GeneMarker software (www.mpla.com).

ERG Recordings

After the ophthalmologic examination, one eye was dilated with a drop of mydriaticum (0.5% tropicamide). Corneal ERG responses were acquired using a Dawson, Trick, Litzkow (DTL) fiber electrode attached at the outer to inner canthus of the eye. The reference and ground skin electrodes were attached to the ipsilateral temple and forehead, respectively. Signals were amplified 100,000×, filtered between 1 and 300 Hz, and sampled at 1024 Hz using the Roland Consult (Brandenburg, Germany) RetiPort system. At least 20 episodes, each lasting 1 second, were averaged.

The stimuli and full protocol details have been described previously by Kremers, et al.[21] Barboni, et al.[22,23] described a short version of the protocol that was used in the present work. Briefly, a Ganzfeld stimulator (Q450 SC; Roland-Consult) with red (638 nm; full width at half maximum [FWHM] = 19 nm) and green (523 nm; FWHM = 36 nm) light-emitting diode (LED) arrays was used. The mean luminance of the red and green LED arrays was 100 cd/m^2, resulting in a mean retinal illuminance of approximately 10^4 photopic Td (assuming 8-mm pupil diameter) with a yellow mean chromaticity of $x = 0.5675$, $y = 0.4201$ (Commission Internationale de l'Eclairage [CIE] 1931 coordinates).

Heterochromatic stimuli were generated by counterphase modulation of the output of the red and green LEDs. The red and green modulation contrast was varied to generate three different red fractions: $(R / (R+G))$. At $R / (R+G) = 0$, only the green LEDs were modulated with 100% contrast, while the red LEDs were not modulated and had a constant output of 100 cd/m^2. At the $R / (R+G) = 0.5$ condition, the red and green LEDs were modulated in counterphase with 50% contrast. At the $R / (R+G) = 1$ condition, only the red LEDs were modulated with 100% contrast; the output of the green LEDs was constant at 100 cd/m^2.

Figure 1B shows the modulation of the LEDs for each of the three conditions. Figure 1 also shows the resulting theoretical chromatic modulation (Fig. 1A) and the luminance modulation (Fig. 1C). When $R / (R+G) = 0$ or 1, the luminance modulation is large and of equal amplitude. At $R / (R+G) = 0.5$ there is no luminance output. The luminance modulation followed the green LEDs at $R / (R+G) = 0$ and the red LEDs at $R / (R+G) = 1$, resulting in a shift of the phase of the luminance modulation by 180° (Fig. 1E). The amplitude and the phase of the cone opponent system's estimated output was the same for all values of $R / (R+G)$ (Fig. 1D) because the chromatic modulation was equal in all three conditions.

Measurements were repeated at two temporal frequencies: 12 and 36 Hz. The time-averaged luminance and chromaticity were constant for all stimuli, indicating that the state of adaptation was constant (assuming that the 12- and 36-Hz stimulus frequencies are too high for adaptation processes). S-cone input is assumed to be negligible, and the total mean luminance of 200 cd/m^2 is assumed to be too high for substantial rod input.[21] The full-field stimulation avoids stray light that can stimulate the rods in dark-adapted parts of the retina (Aher, Kremers, unpublished data, 2016).

We also performed ERG recordings to luminance ON and OFF stimulation using protocols described previously.[16] Briefly, a white LED array (chromaticity of x = 0.3116, y = 0.3277; CIE 1931 coordinates) was used to generate 4-Hz flicker rapid-ON sawtooth or rapid-OFF sawtooth stimulation at 100% contrast, with a mean luminance of 60 cd/m^2.

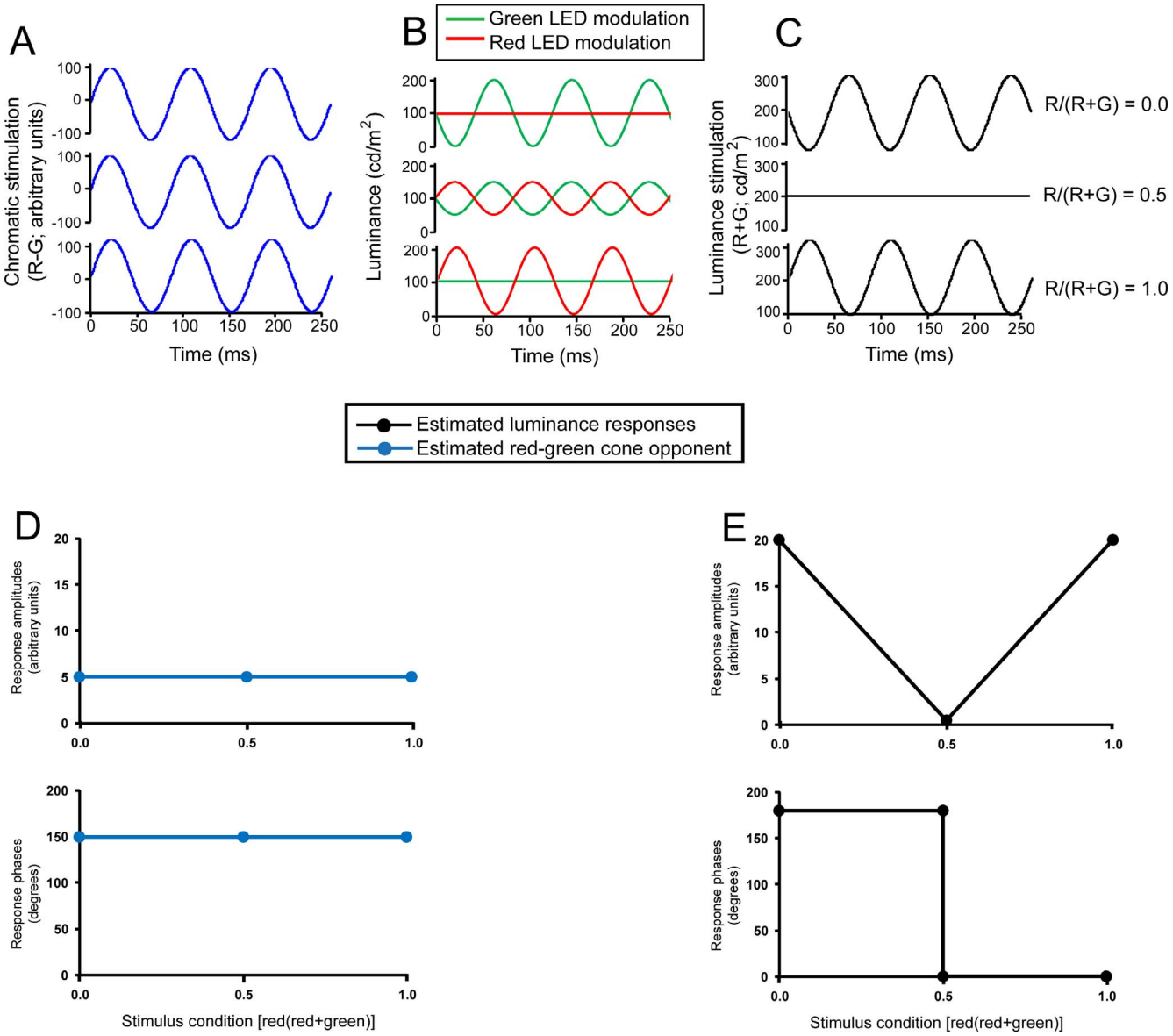

FIGURE 1. Modulation of the LEDs for each of the three conditions: 0.0, 0.5, and 1.0 (**B**). Chromatic (**A**) and luminance (**C**) stimulations. Estimated amplitude (*upper graphs*) and phase (*lower graphs*) responses for the cone opponent (**D**) and luminance (**E**) outputs.

Data Analyses

The recordings were Fourier analyzed using self-written software (MATLAB; The MathWorks, Natick, MA, USA). Noise was quantified by the average of the response amplitudes of the 11- and 13-Hz components and 35- and 37-Hz components for the 12- and 36-Hz stimulus conditions, respectively. Phase values were disregarded when the signal-to-noise ratio was less than 3. The definition of signal-to-noise ratio was based on the methods and statistics introduced by Meigen and Bach,[24] which exclusively analyze amplitude data. Amplitudes (in microvolts) and phases (in degrees) of the first (fundamental) and second harmonic were statistically compared between controls and DMD patients.

In order to discover statistical differences within the different parameter settings, we have built a general linear model (GLM) to test the null hypothesis that all three test conditions (0.0, 0.5, 1.0) have equal results for both controls and DMD patients. A total of eight comparison GLMs were performed analyzing the first and second harmonic amplitudes and phases at the two test frequencies. In statistically significant cases, pairwise comparisons were applied with the Mann-Whitney *U* test. Spearman's rank correlation was used to verify the effect of age and corticosteroid intake on the ERG and to compare ON/OFF ampliture of the heterochromatic flicker ERGs ratios with the first harmonic amplitudes. A *P* value less than 0.05 was defined to indicate a significant difference.

Results

Representative recordings at 12 Hz (upper) and 36 Hz (lower) for all stimulus conditions are shown in Figure 2. At 12 Hz, the control subject (black) displayed similar response amplitudes and phases at the three conditions. The DMD patient (gray) showed higher amplitudes at $R / (R + G)$ 0 and 1 in comparison with the 0.5 condition. Furthermore, the response phases in extremes were approximately 180° apart. At 36 Hz, the responses measured at conditions 0 and 1 were large and

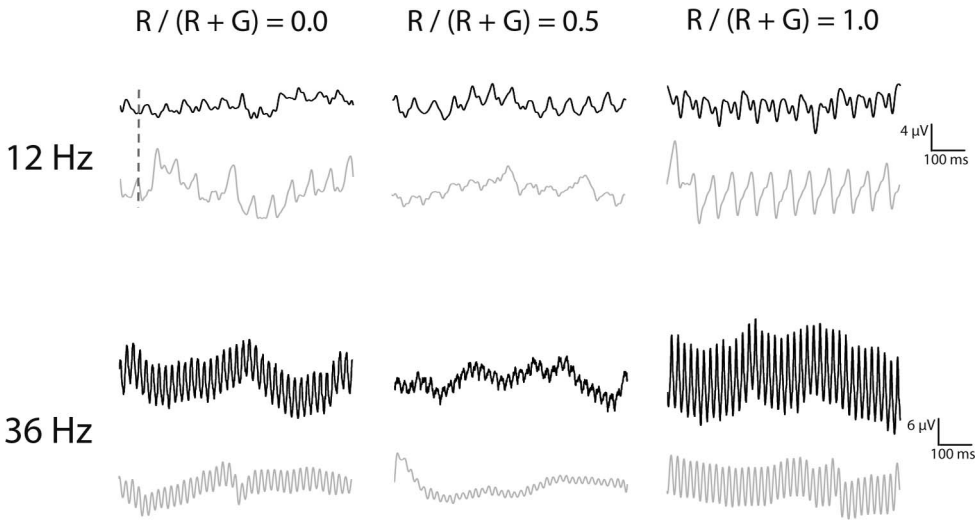

FIGURE 2. Individual recordings of representative subjects (*black*, a representative control; *gray*, a representative DMD patient) at 12 Hz (*upper signals*) and 36 Hz (*lower signals*). The scale and units are provided on the *right side*. A *dotted line* shows phase differences between the DMD patient and the control in condition $R / (R + G) = 0.0$ at 12 Hz.

approximately in counterphase relative to each other for the control and for the DMD patient. The response amplitudes at condition 0.5 were small.

Figure 3 shows the ERG amplitudes (upper plots) and phases (lower plots) at 12 Hz (left graphs) and at 36 Hz (right graphs). Solid and dotted lines connect the averages of control and DMD data, respectively. The gray dots represent the results of seven DMD patients with mutations at locations downstream to exon 30 and thus affecting Dp427, Dp260, and Dp140 (patients 1–7 in Table 1). The red dots are individual results of the three DMD patients with genetic mutations upstream of exon 30, who are deficient only in Dp427 (patients 8, 9, and 10 in Table 1). At 36 Hz, results from only two (of the three) DMD patients with genetic mutations upstream exon 30 are shown, due to poor responses from the third patient.

Table 2 shows averages and standard deviations at 12 and 36 Hz as well as the *P* values resulting from the GLM comparison between patient and control subject data. The GLM results, corrected for multiple comparison (Holm method) of the eight tests, showed statistically significant differences ($P < 0.05$) in only two conditions (12-Hz first harmonic amplitude and phase; $P \ll 0.05$ and $P \lll 0.05$, respectively). In order to discover differences between control and DMD within the different conditions, we applied pairwise statistical comparisons for each 12-Hz first harmonic amplitude and phase

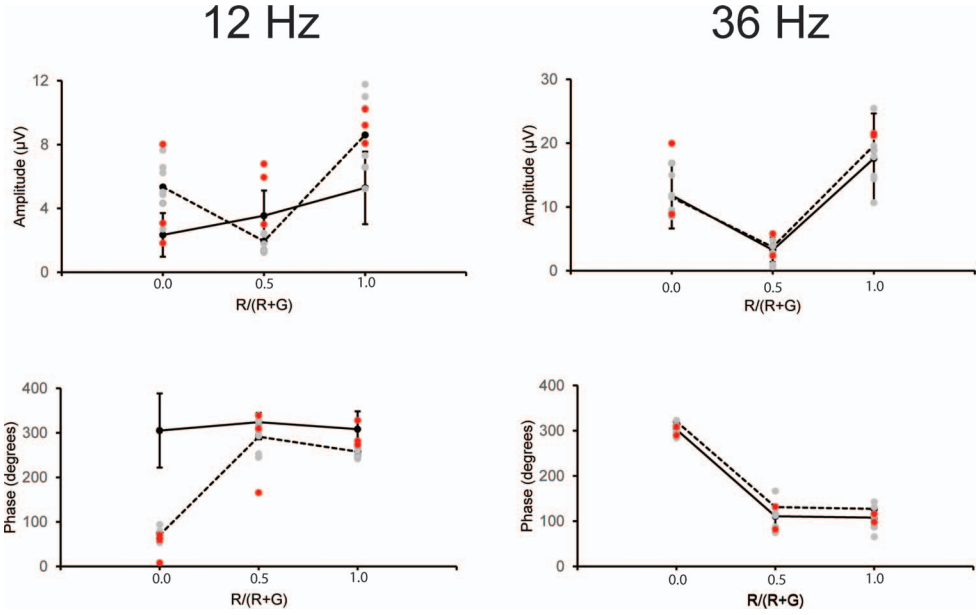

FIGURE 3. ERG results at 12 Hz (*left graphs*) and at 36 Hz (*right graphs*) for amplitude (*upper graphs*) and phase (*lower graphs*) responses. *Black dots* represent the average ± one standard deviation of the control group ($N = 16$). The gray dots are individual results of seven DMD patients downstream exon 30. The *drawn line* connects the averaged data of the control subjects; *dotted line* connects the averaged results of the DMD patients with mutation downstream of exon 30. The *red dots* are individual data of DMD patients with upstream 30 mutations. There were significant differences for all parameters at 12 Hz, while the results at 36 Hz were similar between controls and patients. All *P* values are provided in Table 2.

Table 2. Summary of ERG Parameters, Average ± Standard Deviation, and *P* Values of the GLM Comparisons at 12 Hz

First Harmonic	12 Hz			36 Hz		
Amplitude, μV	0.0	0.5	1.0	0.0	0.5	1.0
Control, $n = 16$	2.7 ± 2	3.5 ± 2	5.3 ± 2	11.5 ± 5	3.7 ± 2	19.6 ± 7
DMD downstream 30, $n = 7$	5.3 ± 2	1.9 ± 0.6	8.6 ± 2	11.8 ± 3	3.3 ± 2	17.6 ± 5
P value	0.020	0.016	0.027			
Phase, °	0.0	0.5	1.0	0.0	0.5	1.0
Control, $n = 16$	305 ± 83	324 ± 20	308 ± 40	320 ± 12	131 ± 24	127 ± 20
DMD downstream 30, $n = 7$	70 ± 13	291 ± 29	258 ± 10	303 ± 12	111 ± 29	108 ± 24
P value	0.001	0.020	0.005			

condition using the Mann-Whitney *U* test. The resulting six pairwise comparisons are shown in Table 2 with their respective *P* values corrected for multiple comparisons with the Holm method. All pairwise comparisons were statistically significant.

We did not find statistically significant correlation between the ERG results (response amplitudes and phases at all conditions) and age of the subjects (control subjects: $P > 0.05$ and $|R^2| < 0.57$; DMD patients: $P > 0.05$ and $|R^2| < 0.8$) or the duration of corticoid treatment in DMD patients ($P > 0.05$ and $|R^2| < 0.63$).

At 12 Hz, the response amplitudes and phases in the control group were relatively constant for the three conditions (Fig. 3 left, drawn line), indicating that the chromatic content in the stimulus determined the responses (Fig. 1D). However, the response amplitudes and phases measured in the patients depended strongly on $R / (R + G)$ in the stimulus. The amplitudes at $R / (R + G) = 0$ and 1 were larger than the controls for the DMD patients with downstream 30 genetic defects. On the other hand, these patients had lower amplitudes for $R / (R + G) = 0.5$, revealing a V-shaped response amplitude profile. In addition, their response phases were also changed, showing a phase shift of approximately 180° between conditions 0.0 and 1.0. One DMD upstream exon 30 patient exhibited the same response characteristics as those observed in the downstream 30 patients. Data from the other two were more intermediate between those of the control subjects and the patients with downstream exon 30 mutations.

At 36 Hz, the responses were similar in controls and DMD patients. Response amplitudes as a function of chromatic content displayed a V shape: Amplitudes at $R / (R + G) = 0.0$ and 1.0 are larger than those obtained at $R / (R + G) = 0.5$. The responses were approximately 180° phase shifted at conditions 0 and 1.

Figure 4 shows the response amplitudes for the extreme conditions [$R / (R + G) = 0$ (left graph) and $R / (R + G) = 1$ (right graph)] as a function of the ratio between b- and d-wave amplitudes of the ON and OFF responses, respectively (see the lower part of Figure 4 for traces of the ON and OFF components considered to calculate the ratio). The ratio was used to quantify the ON/OFF asymmetry. There is a negative

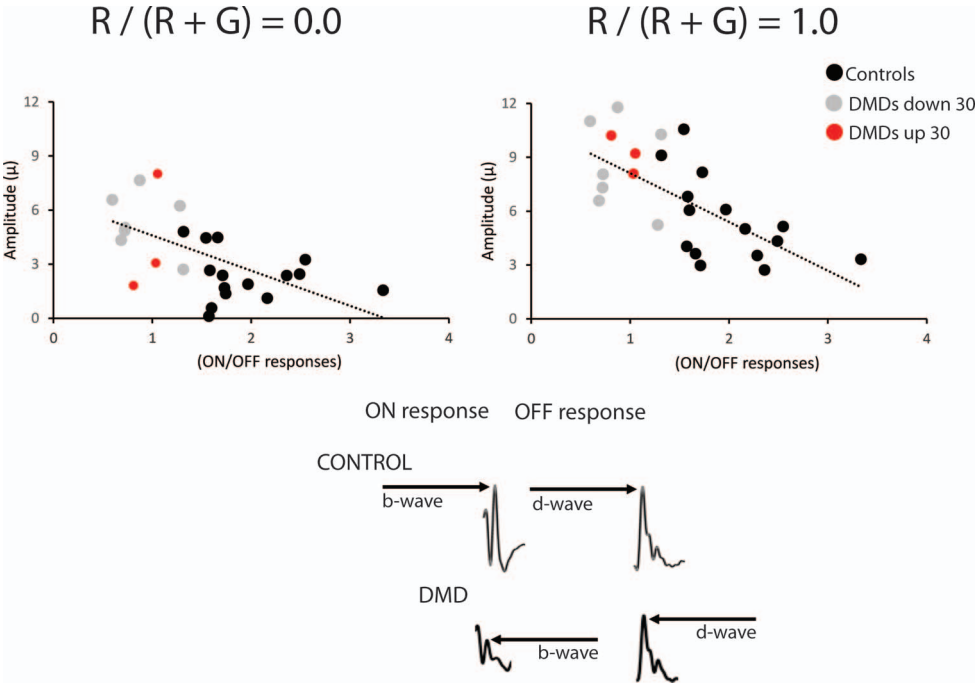

Figure 4. Correlation between the HFP amplitudes at 12 Hz for condition $R / (R+G) = 0$ (*left graph*) and $R / (R+G) = 1$ (*right graph*) and the ON/OFF response ratio. The ratio was calculated by dividing the amplitude of the b-wave obtained from recording luminance rapid-ON sawtooth ERG by the amplitude of the d-wave obtained from recording luminance rapid-OFF sawtooth ERG. Examples of the responses used for this calculation are shown in the *lower part* of the figure (typical control ON and OFF responses and typical DMD ON and OFF responses). A negative correlation between the ON/OFF response amplitude ratios and the response amplitudes to 12-Hz HFP stimuli is apparent: $R / (R+G) = 0$ ($P = 0.001$ and $R^2 = -0.67$) and $R / (R + G) = 1$ ($P = 0.0001$ and $R^2 = -0.69$).

correlation between the two conditions: $R / (R + G) = 0$ ($P = 0.001$) or $R / (R + G) = 1$ ($P = 0.0001$), but not for the $R / (R + G) = 0.5$ condition (data not shown).

Discussion

The ERG responses of DMD patients to 12-Hz red–green counterphase sine wave modulation differed from those of healthy control subjects. In the control group, these responses followed the chromatic content of the stimulus (see Fig. 1D); therefore amplitude and phase responses were relatively constant and independent of the red-to-green ratio of the stimulus. For conditions with large luminance content, response amplitudes of DMD patients (particularly with mutations downstream of exon 30) were larger than those of the controls, whereas responses of DMD patients were smaller than those of the controls where pure chromatic modulation was given. Moreover, their response phases were also not as constant as in controls. The response phases of DMD patients for conditions with large luminance contents [$R / (R + G) = 0.0$ and 1.0] were shifted by approximately 180° relative to each other (Fig. 3, left lower graph). This response profile was found for the controls at 36 Hz, where the responses are determined by the luminance modulation of the stimulus (see Fig. 1E). At 36 Hz, controls and DMD patients showed similar response properties.

The second harmonic amplitudes and phases were similar between controls and DMD patients at 12 and 36 Hz (data not shown). We previously proposed that the responses to 12-Hz luminance stimuli are, to a large extend, determined by a nonlinear interaction between two response mechanisms (called "sine-like" and "transient"; Pangeni et al.[25]). This would explain the large second harmonic component in the response. The unaltered presence of a second harmonic component in the DMD patients indicates that this interaction was not changed by the mutation of the *DMD* gene.

It has been reported earlier by our group that these ERG protocols probably reflect indirectly the activity of the red–green cone opponent (parvocellular or P) pathway at 12 Hz, and of the luminance (magnocellular or M) pathway at 36 Hz in healthy subjects.[21–23] Since at both 12 and 36 Hz there are relatively small response amplitudes in condition $R / (R + G) = 0.5$, we assume that the phase difference between the green and the red stimulation might cancel out when the signals are recorded. Moreover at 12 Hz, the frequency at which the chromatic mechanism is driving the first harmonic responses, this cancellation appears independent of the ratio between the red and the green modulation. Although the ERGs do not reflect direct activity in the P and M pathways, the present results suggest that midget and diffuse bipolar cells are differently affected in DMD patients.

We propose two distinct significant effects of dystrophin deficiency on the responses to 12-Hz heterochromatic modulation. One is a diminished response when the luminance content in the stimulus was small [i.e., for $R / (R + G)$ equals 0.5]. These findings support the notion that dystrophin is required for the proper function of the red–green cone opponent mechanism (related to the parvocellular pathway) in the human retina and might explain the presence of red–green color vision deficiencies in DMD patients.[26,27]

Another is an augmented effect, as responses in DMD patients were larger to 12-Hz stimuli with large luminance content [i.e., when $R / (R + G)$ equals 0 or 1]. Though surprising, this could possibly be explained by a decreased destructive interaction between ON and OFF responses at this temporal frequency. To elaborate, previous reports[28–30] revealed that ON and OFF responses have about the same amplitude at 12 Hz under normal circumstances, but are 180° apart in phase. As a result, ON and OFF responses in control subjects may have cancelled each other out, resulting in a small total response to 12-Hz luminance modulation. An alternative explanation is that the luminance responses show a prominent second harmonic response at this frequency. We therefore suggested previously[25] that the minimum at 12 Hz is a result of a nonlinear interaction of two mechanisms that lead to a frequency doubled response at 12 Hz. Such a frequency doubled response was observed only in condition $R / (R + G) = 1.0$ at 12 Hz, indicating that the nonlinear interaction is more important when there is the red modulation. Dystrophin seems to disturb the synchronization of the red and green signals when a chromatic opponent mechanism is driving the response in the human retina. The evidence of an asymmetric defect of the ON ERG responses in DMD patients gives rise to the hypothesis that the increase in the amplitudes at 12 Hz in conditions $R / (R + G) = 0.0$ and $R / (R + G) = 1.0$ would be the result of this phase change that would not cancel red and green signals as observed in the controls.

Pillers et al.[12] showed that DMD gene products are present in the outer plexiform layer of the human retina. Although the function that dystrophins have there is unclear, they may play a role in the structural connection between photoreceptors and ON-bipolar cells,[31] given its presence in their invaginating contacts in the mouse retina.[32,33] In line with this, ON-luminance responses were found to be more affected than OFF responses in DMD patients.[16,19] We propose, therefore, that the imbalance of ON versus OFF responses in DMD patients may explain the larger 12-Hz luminance responses, as the ON and OFF responses would not cancel each other out. If this were true, it can be expected that there is a correlation between the amplitude ratios of the ON versus OFF responses and the amplitudes of the 12-Hz luminance responses in the present study.

We were able to test this hypothesis since the subjects who participated in the current study also underwent ERG recordings to luminance ON and OFF stimulation using protocols described previously.[16] In line with our hypothesis, there is a significant negative correlation between the ON/OFF asymmetric amplitudes with amplitudes of 12-Hz extreme conditions, but not for the intermediate condition.

In conclusion, our data show that dystrophin deficiency in the human retina leads to an increase in the ERG responses to 12-Hz luminance stimuli, probably caused by an imbalance between ON and OFF activity. In addition, changes in the activity of the postreceptoral chromatic mechanism are apparent. The number of subjects tested is a limitation of the study (seven DMD patients with genetic alteration downstream exon 30 and three DMD patients with genetic alteration upstream 30). Future studies are needed to provide additional information about the role that Dp427 and the subproducts of the *DMD* gene play in the human retinal electrophysiology. In addition, ERG measurements using white flicker at the frequencies used in our study will enable analysis of whether the alterations found in the present report are due to the heterochromatic content of the stimuli or determined by the frequency of the stimulation.

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