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Reduced habituation of the retinal ganglion cell response to sustained pattern stimulation in multiple sclerosis patients

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ABSTRACT

Objective: Sustained pattern stimulation in normal subjects induces adaptive changes in pattern electroretinogram (PERG), an effect that has been interpreted as a response of glial cells and retinal ganglion cells (RGC). The aim of this study was to compare the effect in normal subjects and in multiple sclerosis patients without previous optic neuritis.
Methods: PERGs were elicited by a 7.5 Hz pattern stimulus, presented continuously over 152 s. Response cycles were averaged in 20 packets of 60 events each and amplitude and phase of the 2nd harmonic response was measured. Adaptive changes are expressed as amplitude reduction over the full examination time.
Results: In normal subjects PERG amplitude declined progressively to a plateau (dA = −0.46 μV, SE = 0.09 μV); in patients the effect size was severely reduced (dA = −0.20 μV, SE = 0.04 μV). No significant difference was found in mean amplitude.
Conclusions: The results show reduced RGC habituation in patients, suggesting an abnormal gain and sensitivity control in the inner retina, even in absence of clinical optic neuritis. Recent findings in astrocyte biology and indications drawn from a mathematical model point to a key role of glial cells in this process.
Significance: The proposed methodology may have implications in the assessment of MS patients and in understanding the pathophysiology of neurological and retinal disorders.

1. Introduction

The pattern electroretinogram (PERG) is a signal of inner retinal origin (Maffei and Fiorentini, 1981; Baker et al., 1988) evoked by a structured light stimulus consisting of a reversing pattern of luminance, purposely made to produce a pure contrast stimulus with no residual flicker. When such stimulation is administered over a prolonged time (i.e. 100 s or more) adaptive changes may occur in the inner retina function (Porciatti and Ventura, 2009), and may be characterized in terms of PERG amplitude variation, usually consisting in a reduction according to an exponential law, an effect often described by the term habituation. An opposite, increasing trend was in some cases also observed. In both cases an "energy budget model" may be invoked to explain such findings (Porciatti and Ventura, 2009). The model is based on an equilibrium equation between energy supply and demand, taking also into account the buffering role of glial cells. Accordingly the peak amplitude represents a specific index of retinal ganglion cells (RGC) activity and the plateau amplitude follows a dynamic equilibrium between RGC activity, metabolic demand, and available energy supply.

A recent study (Fadda et al., 2009) investigated whether sustained flicker stimulation (SFS) may also induce adaptive changes: the results indicated that the light-adapted normal flicker ERG (FERG), a signal supposed to originate from partly different retinal sources (Baker et al., 1988), did not show habituation under SFS. This finding support the hypothesis that the adaptation process observed in sustained pattern stimulation (SPS) paradigm is a specific indicator of RGCs activity, which has a direct connection with glial cells metabolism. These last cells, and astroglia in particular, are known to play a key role in neuron metabolism (Magistretti, 2011), and to be affected by multiple sclerosis (MS) pathology (Walter et al., 2012). Based on such considerations the present study assumed that PERG response is a specific indicator of RGCs

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activity, including metabolic adaptive changes, and aimed at comparing habituation of PERG of normal subjects with that of MS patients, excluding the cases of previous optic neuritis.

2. Methods

2.1. Subjects

Eight normal subjects (5 males and 3 females, mean age 40.2, standard deviation 11.8 years) and fourteen MS patients (8 males and 6 females, mean age 37.1, standard deviation 12.0 years) were included in the study. Normal volunteers were free from ocular or systemic diseases, had normal corrected Snellen acuity of 20/20 or better and refractive errors within ±3 sph and ±1 cyl diopters. Patients had refractive errors equal to or less than 2 spherical and 1 cylindrical diopters, no concomitant ocular (e.g. cataract, glaucoma) or systemic (e.g. diabetes) disorders which may affect PERG response. MS was diagnosed in each patient according to the 2010 revised McDonald criteria (Polman et al., 2011). Details of demographic and clinical features of MS patients are given in Table 1.

Preliminary experiments done in our laboratory showed that subjects' accommodation could be effectively ensured by maintaining throughout the experiment a sharp focus on the small fixation mark (composed by a bright annulus centered by a dark spot, total angular subtended: 0.5 degrees) at the center of the stimulator. So all control subjects and patients were asked to report whether during the recording there was a blurring of the fixation mark details.

The study adhered to the Tenets of the Declaration of Helsinki and was approved by the Institutional Review Board.

2.2. Apparatus and procedure

PERG was elicited by a sinusoidal grating of 1.7 cycles/degree spatial frequency and 90% contrast (mean luminance: 35 cd/m^2), modulated in counterphase at 7.5 Hz (15 reversals/s). Stimulus was administered continuously over 3 min and response recorded as a sequence of 20 partial averages (packets), each one obtained summing up 60 cycles (8 s average duration). Signals were monocularly derived, with an inter-ocular reference, by means of Ag–AgCl superficial cup electrodes taped over the skin of the lower eyelids, amplified (gain of 100 k, 1–250 Hz bandwidth, 6 dB/octave slope), digitized (12 bit resolution, 2 kHz sampling rate, 100 μV AC range) and averaged in synchronism with stimulus onset. The averaging time (i.e. the sweep duration) was locked to the stimulus temporal period (133 ms). Single sweeps exceeding a large amplitude voltage window (±30 μV) were rejected to minimize the effects of blinks or large eye movements, without introducing amplitude bias (Sieving et al., 1998).

A discrete Fourier analysis was performed in order to isolate the PERG second harmonic (2P), that is known to be the significant outcome of PERG experiments (Bach and Hoffmann, 2006; Holder et al., 2007). In brief a stimulus made of a balanced pattern of light bars modulated in counterphase at a given frequency (the first harmonic frequency) has the key feature to elicit a space averaged response in which the linear contributions from the corresponding retinal areas cancel each other. This cancellation highlights a second harmonic component in the emerging signal, arising from non linear effects, that was shown to be correlated to RGC activity. Results are expressed as vector data, consisting of peak-to-peak amplitudes (in μV) and phases (in degrees) for every packet. A second, asynchronous averaging channel, having a slightly detuned frequency (10% larger), was used to reject the signal and give an on-line estimate of the background noise in the signal band, as it appears after the same processing (Salgarello et al., 2008). Such in-band noise measures were similarly recorded as a sequence of 20 vectors.

2.3. PERG recording during SPS

All subjects fixated at the center of the pattern stimulus. The pupils were not dilated and their size was measured at the beginning of adaptation (see also later), at the end of adaptation, and just before the ERG recordings and at the end of the recordings.

The test presentation was preceded by a noise measurement procedure, performed in exactly the same conditions of the test, apart a blank paper covering the stimulator, having care to obtain a uniform field having the same mean luminance as test (35 cd/m^2). Following a short period (2–3 min) needed to reach stable conditions of light adaptation, electrode potentials and subject rest, a 'noise' response was measured. Recording consisted of 20 packets of 60 events each (160 s duration), with a protocol identical to the PERG protocol (see below). Then the pattern stimulus was started and presented continuously over 160 ± 11 s. Twenty packets of 60 events each were collected during one recording session. The first five cycles were discarded to eliminate the transients at the onset of stimulation. Data collection period of a single packet

Table 1
Demographic and clinical features of MS patients.

Pt. #, Gender, Age	Eye	Visual acuity	Afferent pupillary defect	Visual field perimetric indices[a]		Number of localization of MRI lesions[b]		CSF[c] analysis
				MD	PSD			
1, M, 46	RE	20/20	–	−0.56	0.77	7	P,J	+
2, M, 23	RE	20/20	–	−0.27	0.46	9	P,J	+
3, F, 53	LE	25/20	–	0.25	1.33	8	P,S	+
4, F, 26	LE	20/20	–	0.76	0.87	9	P,J,S	+
5, F, 51	RE	25/20	–	−1.35	0.92	6	P,J,I	+
6, M, 45	LE	25/20	–	−0.86	1.14	11	P,J,S	+
7, F, 32	LE	20/20	–	0.78	0.56	7	I,S	+
8, M, 45	RE	20/20	–	−1.24	0.76	10	P,I,S	+
9, F, 25	LE	20/20	–	−0.55	0.98	8	P,J,I	+
10, M, 55	LE	25/20	–	0.65	1.56	12	P,J,S	+
11, F, 25	RE	20/20	–	0.84	0.78	6	P,J	+
12, M, 28	RE	20/20	–	−1.28	0.42	8	P,J	+
13, M, 24	RE	20/20	–	−0.76	1.12	10	P,J	+
14, M, 41	LE	20/20	–	0.56	0.84	13	P,I,S	+

MD, mean deviation; PSD, pattern standard deviation; MRI, magnetic resonance imaging; CSF: cerebrospinal fluid.
[a] HFA 30-2: Humphrey field analysis 30-2.
[b] T^2 and/or gadolinium-enhancing lesion(s) in the MS-characteristic regions (periventricular, juxtacortical, infratentorial, or spinal cord).
[c] Isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index.

was on average 8 ± 0.5 s. The amplitude and phase data obtained from every packet were studied as temporal series, assuming a constant time interval for packet recording.

Two valid and reproducible recording sessions were obtained for each subject, based on the following protocol. As soon as a test was complete a quick signal assessment was made, based on the preliminary "blank" noise measurements (using the S/N ratio) and descriptive statistic on 2P amplitude and phase data of the packets (using the coefficient of variation, CV). For phase data the calculations of variance were performed with methods valid for circular distributions (Zar, 1999). The results were used to validate the single recordings according to acceptance levels (S/N > 2.8; amplitude CV < 20%; phase SE < 20°). If the amplitude and phase data of two successive sessions were consistent (i.e. response amplitude change <30%; phase changes <20°), they were averaged and used as a single entry for each subject. In about 90% of recordings these reproducibility criteria were met by the first two valid sessions, for the other 10% of the cases a third recording session was needed.

2.4. Statistical analysis and signal processing

In order to assess the overall statistical significance of PERG 2nd harmonic (2P) measures collected from the right or left eye of each subject, it was used the T^2 circular analysis (Victor and Mast, 1991), to reject the null hypothesis of only noise present. A statistical evaluation based on noise measurements was also used (Meigen and Bach, 1999; Fadda et al., 2010), because measurements obtained in a non-stationary state should be assessed in an individual and independent way, a condition that is not possible to meet with traditional methods based on sample scatter (see Fig. 1 or Fig. 2, bottom panels).

A smoothing procedure was also applied on raw data, consisting in a three-point adjacent vector average, that is the averaging was performed on vector Cartesian components and the result converted back in the usual amplitude and phase representation.

The exponential model used in previous studies to obtain a measure of the amplitude delta (Porciatti et al., 2005; Fadda et al., 2009) was not suitable for individual MS patient records, due to numerical instability problems when the extent of amplitude variation is reduced. A method suitable for both cases and controls was therefore needed. The preferred choice was to apply a linear regression on PERG amplitude data of every subject of the two groups, as a means to detect the declining trend hidden in noisy measures. The final outcome is given as dA, the equivalent amplitude delta over a 152 s time interval, expressed in μV. An additional method was also used to check the overall reliability of dA results. It is based on the direct difference (dV) of initial and final values, computed as the average of the first and last three samples of the time series.

Phase time courses where analyzed in a similar way, to obtain dP values.

2.5. Results

In all subjects, 2P signal component was well above the base noise level (S/N > 2.8) and adequately reliable (i.e. the standard error, SE, of amplitude was typically less than 20% of the average amplitude, and the phase SE was within ±20°). Noise level at the 2P frequency ranged from 0.05 to 0.14 μV.

Such indications of reliability of results, produced at recording time by the acquisition software, are confirmed by a successive assessment, based on the statistical methods cited in the former section.

In Fig. 1, the upper panels show the smoothed PERG amplitude and phase plot of two control subjects (A and B), while the lower panels show a Cartesian plot of the raw components of signal and noise and their confidence limits. In subject A a typical decreasing trend is easily recognized, showing limited oscillations around an exponential curve, obtained by a least-square data fitting procedure. Subject B is an extreme case of oscillatory pattern, but the exponential fit yields acceptable results as well. In both cases the trend can be detected in an equivalent way by a linear regression, producing the outcome value dA as described before. Values of amplitude delta for control subjects may be underestimated in this way, but the resulting bias is not detrimental to the purposes of this study. The effect of large oscillations, as found in subject B, has a limited effect too, due to the fast damping envelope and to the occurrence of two complete periods in the observation time.

Data presented in the lower panels is not intended to study the time course of PERG but only to assess the reliability of measurements, using two different statistical approaches. Round points represent the 20 vectors of 2P PERG component, while triangle points are similar measurements for noise, obtained with the described on-line procedure. No smoothing or other processing was applied on such data. The two small confidence circles (solid-line) are centered on signal and noise average, radius is obtained by T^2_{circ} statistic (Victor and Mast, 1991). They indicate that average 2P amplitude is largely significant and that noise in the same band is randomly distributed around zero. This last result confirms that the on-line procedure used to extract noise is actually able to reject signal. The dashed-line circle, centered on origin, is the confidence limit for signal amplitude obtained by S/N statistic (Meigen and Bach, 1999; Fadda et al., 2010): this method has a reduced statistical power compared to the former, but is unique in allowing an independent assessment of each measurement. The results show that most single packets attain a significance level, while others are in a critical region. This is the reason why a moving average was necessary to obtain a reliable time course plot.

In Fig. 2, same scale PERG plots of two MS patients (C and D) are presented, showing a different amplitude pattern compared to controls. Here an exponential fitting on amplitude data does not produce reliable results, but an adaptation trend is still clearly seen and easily detected by a linear fitting procedure or by a direct measurement of amplitude difference between initial and final values, showing a reduced delta compared to controls A and B. Patient C has the highest value of delta in the MS group, while patient D is an average case. It may be noted that noise is somewhat greater compared to controls, a result that was generally found in all patients, but that was not significant in a comparison between the two groups. It may be argued that some marginal difference in noise level may be the consequence of difficulties found in patient preparation and in recording session management, compared to healthy volunteers. This higher value of noise may account for the somewhat higher (not significant) value of signal amplitude found in some patients, as it is the case for C and D versus A and B. This may be explained as a bias effect of noise, that may produce an over estimation of signal when a poor S/N ratio is present (Bach and Meigen, 1999). Phase is less stable compared to controls, but also in this case no significant difference was found.

The main results of the study are summarized in Fig. 3, where both delta measures (dA, dV) of the two groups are reported in a table and shown in a statistical plot. A two sample *T*-test on data in the table of fig. 3 confirmed that mean dA of controls (−0.46 μV, SE = 0.09 μV) is greater (in absolute value) than dA of MS cases (−0.20 μV, SE = 0.04 μV), with a *p*-value of 0.003. The same analysis made on mean amplitude data, showed no difference between the two groups, with controls' amplitude averaging at 1.03 μV, SE = 0.10 μV and cases' amplitude at 1.05 μV, SE = 0.14 μV (*p*-value 0.98).

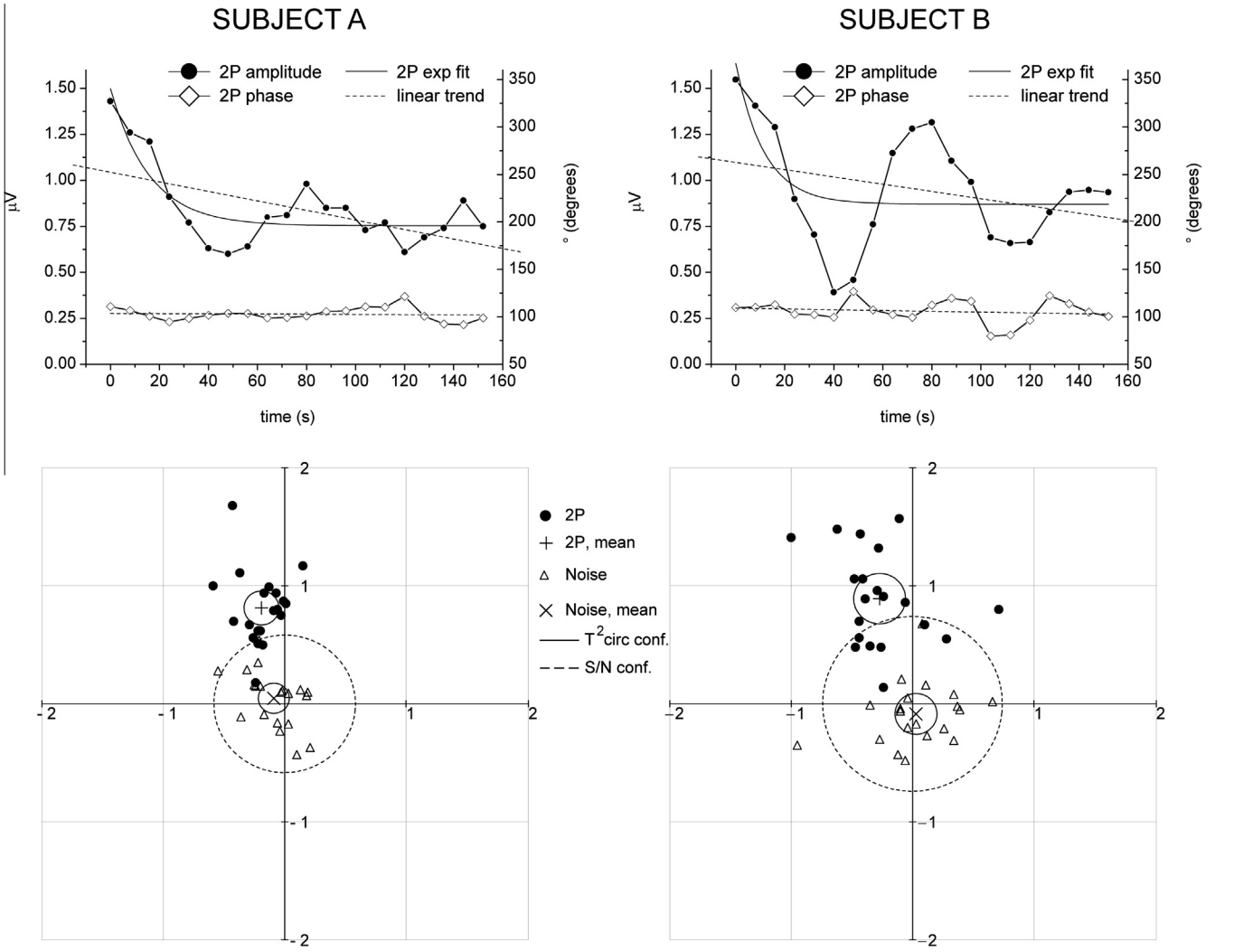

Fig. 1. The two upper panels show the time course of PERG 2nd harmonic amplitude and phase of control subjects A (an average example) and B (an extreme case of oscillatory pattern). Exponential fitting and trend lines are also shown. The two bottom panels show the corresponding raw components of 2P signal (●) and associated noise (Δ), in a sine–cosine plot.

Initial amplitude (V1, three point average) of the two groups was also considered and no significant difference was found. The results of *T*-test are that mean V1 of controls (1.42 μV, SE 0.12 μV) and mean V1 of cases (1.14 μV, SE 0.15 μV) have a non significant difference of 0.29 μV (*p*-value 0.21).

Variability of individual dA values was assessed considering the SE values produced by the linear regression. Even when the series variability is greater than the underlying delta, the SE of individual measurement is limited, averaging at 0.15 μV both for cases and controls. In a linear regression on a long temporal series slope value is more responsive to the long run trend than to short-time variations.

In the patient group no significant correlation was found between amplitude delta and initial amplitude, as it might be expected based on previous results (Porciatti and Ventura, 2009). It may be noted that, given the extremely reduced size of delta in patient's group, this correlation, if also present, would be hardly detectable and consequently it is not possible to affirm or deny a difference between the two groups in this respect.

An equivalent set of results for amplitude analysis are obtained using the values of dV obtained as direct difference between initial (V1) and final amplitude (V2). Mean dV of controls (−0.50 μV, SE = 0.04 μV) is greater (in absolute value) than dV of MS cases (−0.20 μV, SE = 0.06 μV), with a *p*-value of 0.001. Results appears slightly stronger using this last method, but this might be the effect of specific data, and the method based on linear regression is believed to be more robust in a general case.

Values of dP (delta on phase), obtained with the same linear regression method, were also considered. They range in a small interval around zero both for controls (mean = 3.2 deg, SE = 14.8 deg) and for MS cases (mean = 5.1 deg, SE = 9.1 deg); at the 0.05 level the two means or their difference are not significantly different from zero. Values of dP were found to have a negative correlation with the initial value of phase (R^2 = 0.34, p = 0.03), and a similar effect was also found in the control group, but with a poor significance, owing to the small sample size. This effect agrees with previous findings obtained for a large cohort of healthy subjects (Porciatti and Ventura, 2009).

Overall, the results related to phase analysis do not offer further ways to differentiate the group of MS cases from the control one.

Finally, individual amplitude data of controls and patients may be averaged to form two group means. Results are shown in Fig. 4, where data are fitted by a first order exponential decay. A different time course pattern of the two groups is evident in this global graphical representation, while no difference may be noticed in mean amplitude evaluated all over the time course. It may also

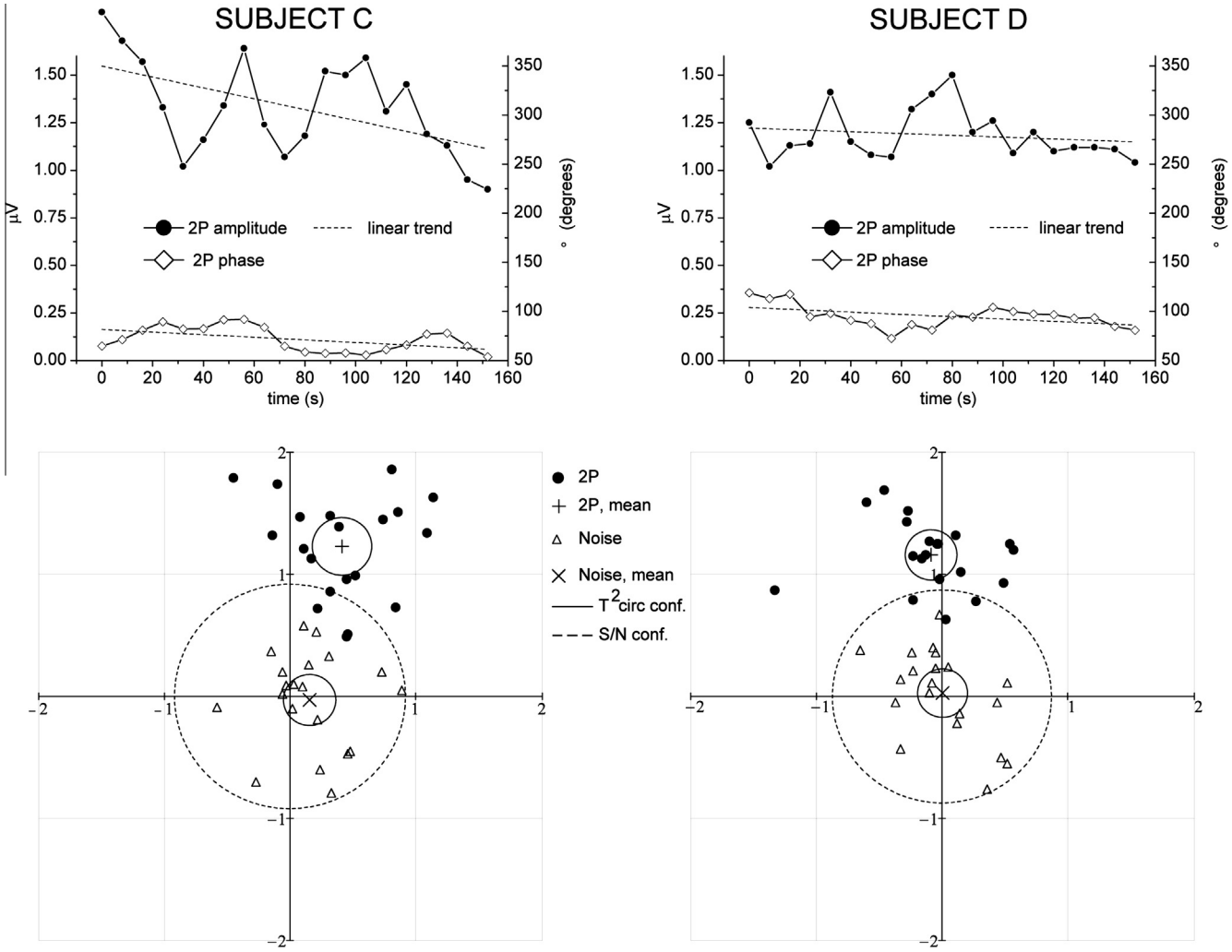

Fig. 2. The figure shows the results obtained for two MS cases, C (the max delta found in patients) and D (an average delta example). Data is shown in the same form previously described for Fig. 1. Exponential fitting of amplitude 2P time course is omitted.

Cases		Controls	
dA(μV)	dV(μV)	dA(μV)	dV(μV)
-0.43	-0.70	-0.32	-0.40
-0.14	-0.30	-0.98	-0.72
-0.08	-0.05	-0.38	-0.54
-0.23	-0.43	-0.32	-0.49
-0.08	-0.00	-0.58	-0.50
-0.23	0.01	-0.17	-0.28
-0.18	-0.28	-0.39	-0.51
-0.46	-0.30	-0.50	-0.52
-0.30	0.01		
-0.18	-0.21		
-0.04	-0.05		
-0.07	-0.05		
-0.04	-0.06		
-0.38	-0.37		

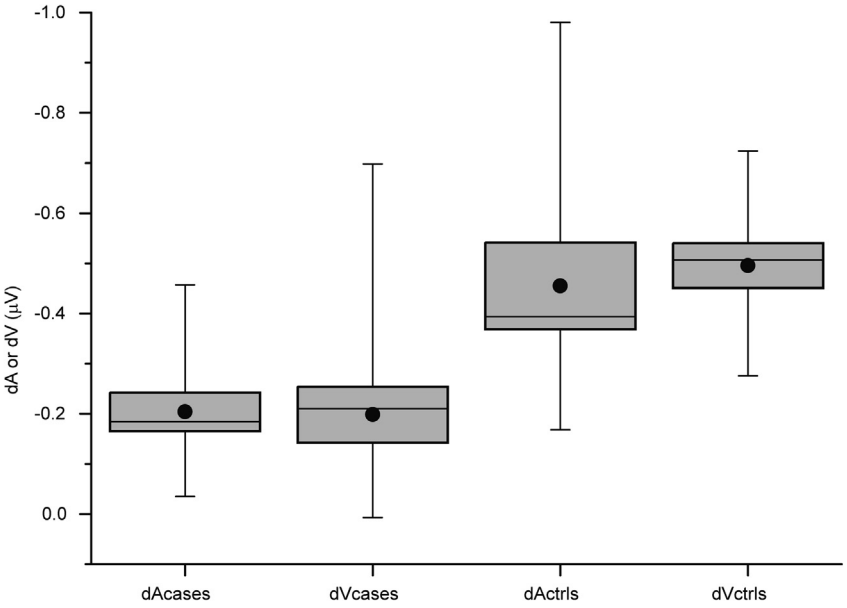

Fig. 3. Data table and statistical plot of delta measures (dA and dV) of the MS group ($n = 14$) and control group ($n = 8$).

be noted that initial amplitude of controls is greater than MS cases, but the difference do not attain significance, and therefore could not be used to discriminate MS cases.

An oscillatory component, also seen in individual records of control subjects, is retained in the group average, with an apparent period of about 80 s. The limited number of control cases prevents further speculation on this result, which might be the object of following studies. A time delay component could be eventually introduced in the energy budget model to account for this last effect.

3. Discussion

Our results show that PERG habituation, measured with the described methods, may be reliably detected in MS patients without optic neuritis, showing a reduced effect in presence of no significant difference of mean amplitude. While in healthy subject a first order exponential decay fits 2P PERG course, in MS patients only the average PERG amplitudes may be fitted by this mathematical model. A linear regression was therefore used to detect amplitude trends of PERG, using the equivalent reduction over a 152 s time as the outcome measurement. Equivalent results may also be obtained retrieving the amplitude delta by the simple difference of amplitude measures made at initial and final time. Unlike a previous work (Porciatti and Ventura, 2009), in our study all cases and controls showed a reduction of PERG amplitude and no case of increase was detected. In studies with a larger group of healthy subjects, spanning over a different age range, such cases will probably occur and could eventually be handled using the absolute value of amplitude variation as final measurement. The rationale of this approach, if needed, is that in controls a large amplitude variation, irrespective of its sign, indicates large adaptive changes.

Amplitude delta (dA or dV) was finally the only parameter of sustained PERG recordings to show a significant alteration in the MS group, pointing at similar alterations in the underlying temporal dynamics of the metabolic system feeding RGC cells.

This point may be further discussed with the help of the "energy budget model" previously cited. In summary the model sketches the energy exchanges between RGC, glia and blood vessels by means of a simple equilibrium equation:

$$\Delta\varepsilon = (b - n - g)\cdot\Delta t \tag{1}$$

where the energy variation $\Delta\varepsilon$ over the time Δt results from the balance of flows coming from blood (b), going to neurons (n) and being used in the inner workings of glia (g). It is also supposed that flows n and g are constant or linearly dependent from energy level ε, accordingly the stimulation state.

Here we tailor that model to the sole study of equilibrium conditions, a case where $\Delta\varepsilon/\Delta t = 0$ holds true (constant energy level) and consequently Eq. (1) simplifies to:

$$b = n + g \tag{1a}$$

In a rest state (no stimulation present) Eq. (1a) may be written:

$$b_0 = n_0 + G\cdot\varepsilon_0 \tag{2}$$

where it is assumed that n is constant and g is proportional to energy level, being G the rate of energy burning in glial processes. In a maximal, or critical, stimulation state Eq. (1a) becomes:

$$b_1 = N\cdot\varepsilon_1 + G\cdot\varepsilon_1 \tag{3}$$

where it is assumed that available energy is the factor limiting the flow in neurons (n), so that also n depends on ε, according N. Finally a useful expression for the ratio R of energy levels at rest and after stimulation may be obtained solving (2) and (3) for ε_0 and ε_1:

$$R = \frac{\varepsilon_0}{\varepsilon_1} = \frac{b_0 - n_0}{b_1}\cdot\frac{N+G}{G} \tag{4}$$

This formula highlight a connection between the habituation effect and the metabolic parameters (n_0,N,G) introduced by the model. In a condition of abnormal functional status of glial cells it may be expected that the term G is large compared to N (meaning inefficient energy processing) and n_0 is small compared to b_0 or b_1 (reduced neural activity). Given such assumptions the R value approaches unity (little energy variation), a condition that agrees with the observed reduced effect. In different words it may be said that when large amounts of energy are wasted in inefficient internal processing (large G) the room for the physiological adaptive effects is severely reduced.

The idea of astrocyte role as an energetic intermediary for the neuron is also supported by recent studies on biochemical reactions and transport mechanisms. The ANLS model (Astrocyte-Neuron Lactate Shuttle) (Pellerin and Magistretti, 2012) assumed that the astrocyte, that can also store glycogen, supplies the energy for neuron demands by increasing their uptake rate of glucose from the capillaries (Carmignoto and Gomez-Gonzalo, 2010). Other studies (Cambron et al., 2012; Dong et al., 2012) highlighted that the lack of β2AR in multiple sclerosis patients causes disorder of astrocyte glucose transportation and glycogenlysis, and decreases the energy supply to axons.

In conclusion the reported results show a reduced RGC habituation in MS patients, without associated differences in mean response amplitude and without optic neuritis diagnosis. These findings, discussed with the help of a mathematical model and

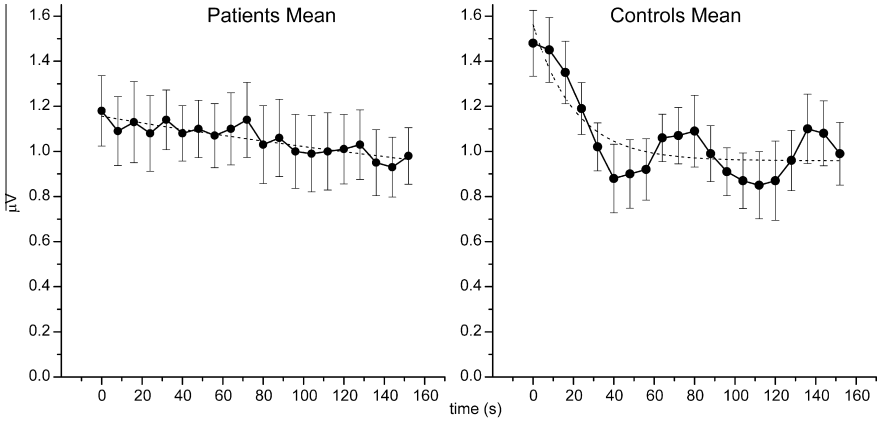

Fig. 4. The figure shows the plots of 2P amplitude average of the control group and the MS group, with SE bars. A visibly different time course pattern is highlighted by the fitting curves, while no difference may be noticed in mean amplitude. Initial amplitude of controls is greater but the difference do not attain significance.

recent research results on astrocyte metabolism, suggests an abnormal gain and sensitivity control in the inner retina of MS patients, which have been interpreted as reflecting alterations in the buffering mechanism of glial cells.

Conflict of interest

The authors report no financial or other conflict of interest relevant to the subject of this article.

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