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Evidence for associative plasticity in the human visual cortex

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ABSTRACT

Background: Repetitive convergent inputs to a single post-synaptic neuron can induce long-term potentiation (LTP) or depression (LTD) of synaptic activity in a spike timing-dependent manner.
Objective: Here we set a protocol of visual paired associative stimulation (vPAS) of the primary visual cortex (V1) in humans to induce persistent changes in the excitatory properties of V1 with a spike timing rule.
Methods: We provided convergent inputs to V1 by coupling transcranial magnetic stimulation (TMS) pulses of the occipital cortex with peripheral visual inputs, at four interstimulus intervals of −50/-25/+25/+50 ms relative to the visual evoked potential (VEP) P1 latency. We analysed VEP amplitude and delayed habituation before and up to 10 min after each vPAS protocol.
Results: VEP amplitude was reduced after vPAS+25. Delayed VEP habituation was increased after vPAS-25 while it was reduced after vPAS+25.
Conclusions: We provide evidence that associative bidirectional synaptic plasticity is a feature not only of the sensorimotor but also of the human visual system.

Introduction

Evidence for lasting changes in the excitability of the human primary visual cortex (V1) has been provided by means of interventions producing repeated cortical activation by a single targeted input, based on either peripheral visual tetanic stimulation [1] or repetitive transcranial magnetic stimulation (rTMS) [2–6]. These kinds of interventions replicate in vivo the experimental phenomenon of activity-dependent synaptic plasticity [7]. Moreover, interventions acting on the depolarizing threshold of cortical cells, based on transcranial direct current stimulation (tDCS), have also shown a modulatory effect on V1 excitability [8,9]. Other attempts with 1 Hz and theta-burst rTMS protocols [10] or tDCS [11] failed to modulate visual acuity and phosphene threshold, respectively.

In humans, the easiest way of testing V1 excitability is by measuring the amplitude of visual evoked potentials (VEPs) after peripheral visual stimulation. Like for other sensory modalities [12], VEP amplitude is subject to the phenomenon of habituation, which consists in a response decrement as a result of repeated stimulation, a short-term plastic change that is a common feature of any kind of sensory stimulation [13]. Habituation of VEPs is hypothesised to depend on various “tonic” non-specific and motivational circuits, including the brainstem monoaminergic nuclei and the ascending thalamocortical loops, and/or activity of intracortical inhibitory circuits [14]. Hence, VEP amplitude and habituation are used to infer the mass activity and plastic properties of visual cortical neurons, which may be modified at short as well as long-term in many physiological and pathological conditions, including hyperventilation [15], heterotopic pain conditioning [16] and light deprivation [17].

It is reasonable to think that most plastic changes in the living brain, including in the visual cortex, are not simply mediated by repeated post-synaptic activation through a single pathway, but rather by the convergence of multiple afferents on a given post-synaptic target. Indeed, changes in the activity of neural circuits

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related to mechanisms of associative synaptic plasticity are considered a prominent phenomenon of functional adaptation [18,19].

Following the original Hebb's postulate [20], repetitive concomitant sub-threshold and supra-threshold inputs to a single post-synaptic neuron can induce long-term potentiation (LTP) or depression (LTD) of synaptic activity in a spike timing-dependent manner [21]. This essential process of associative plasticity is known as spike timing-dependent plasticity (STDP) and has been replicated, in its conceptual paradigm, at several levels spanning from single synapses to the complexity of the intact human brain, through protocols of paired associative stimulation (PAS) [22,23].

A widely-studied PAS protocol capable of producing plastic changes in the human primary motor cortex (M1) relies on mechanisms of sensorimotor interaction and is based on repetitive pairing of sensory peripheral nerve stimulation with transcranial magnetic stimulation (TMS) of M1 [24,25]. As in the concept of STDP, the long-term effect of this PAS protocol on M1 excitability critically depends on the interval between sensory and motor stimulation, within a narrow time window of few milliseconds. Indeed, facilitatory effects are obtained when somatosensory cortex activation precedes M1 stimulation of about 5 ms, while inhibitory effects are produced when sensory afferent input follows M1 activation of about 10 ms [26]. Suppa and colleagues [27] approached the study of plasticity in visuomotor integration processes by introducing a similar PAS protocol coupling V1 activation, achieved through light stimulation, with TMS of ipsilateral M1.

Having in mind the idea that STDP may represent a basic mechanism for learning in different areas of the brain, in the present study we investigated the possibility of inducing persistent changes in the excitatory properties of V1 through mechanisms of associative plasticity. This was obtained by using a TMS-based protocol pairing cortical magnetic to peripheral visual stimulation. In our experimental paradigm, visual pattern reversal presentation and TMS are hypothesised to produce convergent inputs on V1 pyramidal neurons, paralleling the classical synaptic models of STDP [18]. Thus, we translated for the first time the PAS method from the sensorimotor to the visual system.

Methods

Participants

Twenty-eight healthy volunteers were recruited (mean age: 29.1 ± 6.4 (SD) years; range: 21–43; M/F: 13/15).

For all participants, exclusion criteria were: a) best-corrected visual acuity of <8/10; b) regularly taking medication except for contraceptive pills; c) history of neurological disorders including migraine and chronic sleep deprivation, systemic hypertension, diabetes or other metabolic disease, and autoimmune disease. Subjects with any contraindication to TMS were also excluded. All subjects were right-handed. Female participants were always recorded mid-menstrual cycle. All participants were given a complete description of the study and they provided written informed consent. The study was approved by the local Ethics Committee and was conducted in accordance with the Declaration of Helsinki.

Experimental design

We recorded pattern-reversal VEP (PR-VEP) and determined VEP amplitudes and habituation at three times: before (T0), immediately after (T1), and 10 min (T2) after a visual paired associative stimulation (vPAS) procedure (Fig. 1A).

One group of 14 subjects (Sample1 - mean age: 28.6 ± 6.0 (SD) years; range: 23–43; M/F: 5/9) was tested with all the interstimulus intervals of the vPAS procedure (see below for details). After the analysis of the results, an additional group of 14 subjects (Sample 2 - mean age: 29.5 ± 7.0 (SD) years; range: 21–42; M/F: 8/6) was tested with the two shortest inter-stimulus intervals that produced significant changes in cortical excitability (−25/+25 ms in relation to VEP latency), in order to confirm the timing-dependent effects of the vPAS protocol.

All recordings were performed in the afternoon (between 14:00 and 18:00) by the same investigators (G.M. and F.R.) and numbered anonymously for offline analysis.

VEP recording

Subjects were seated fully relaxed in front of a monitor (diagonal: 19''; aspect ratio: 16:9; refresh rate: 60 Hz), in an acoustically isolated room with dimmed light and a uniform luminance field of ~5 cd/m^2. To obtain a stable pupillary diameter, each subject adapted to the ambient room light for 10 min before VEP recording.

The stimulation paradigm consisted of a full-field checkerboard pattern (contrast: 80%, mean luminance: 200 cd/m^2) generated on a monitor and reversed in contrast at a rate of 3.1/s (Fig. 1B). At the viewing distance of 114 cm, the single check edges subtended a visual angle of 15', while the checkerboard subtended an angle of 23°. Subjects were instructed to fix the center of the screen, marked by a small dot. VEPs were elicited by right monocular stimulation, with the left eye covered by a patch to maintain stable fixation.

VEPs were recorded from the scalp through Ag–AgCl cup electrodes positioned at Oz (active electrode) and Fz (reference electrode) points of the 10/20 International System; a ground electrode was placed on the right forearm. Signals were amplified with a Digitimer™ D360 amplifier (Digitimer Ltd, Welwyn Garden City, UK) (band-pass 0.05–2000 Hz, gain 1000) and recorded with a CED™ Power 1401-3 device and associated software Signal™ v5.08 (Cambridge Electronic Design Ltd, Cambridge, UK).

Six-hundred consecutive sweeps of 300 ms were collected using a sampling rate of 4000 Hz. All acquired traces were low-pass 100 Hz filtered and analysed off-line. Artefacts were automatically rejected if the signal amplitude exceeded 200 μV. After correcting the signal offline for DC drift, trials were partitioned into six sequential averaged blocks of at least 95 artefact-free sweeps. Thereafter, we identified the three major VEP latencies (N1, P1 and N2) and their respective peak-to-peak amplitudes (N1–P1 and P1–N2), that we used to calculate the habituation as the slope of the linear regression line for the six blocks.

Transcranial magnetic stimulation (TMS)

TMS was delivered through a Magstim™ 200^2 magnetic stimulator (The Magstim Company Ltd, Whitland, Carmarthenshire, UK) generating a monophasic magnetic pulse with a maximal stimulator output (MSO) of 1.2 T, connected to a figure-of-eight coil with an external diameter of 9 cm. Stimulation intensity was expressed as percentage of the MSO. Since, in previous reports, not all subjects experience the vision of phosphenes even bearing a 100% MSO [28], we decided to set the stimulation intensity to 120% of the individual motor threshold [5]. To this purpose, the coil was positioned over the left motor area of the first dorsal interosseous muscle for determining the resting motor threshold (RMT) using single TMS pulses with the same procedure described elsewhere [25]. In our subjects (n = 28), the mean intensity of stimulation corresponding to 120% RMT was 49.8 ± 8.0% of MSO (range 40–65%). The stimulation intensity of 120% RMT was then used to deliver TMS over V1, by placing the center of the coil over the Oz position and orienting the coil vertically (its handle pointing upward) [2,5] (Fig. 1C). This coil orientation generates a posterior-to-anterior induced current across the interhemispheric fissure.

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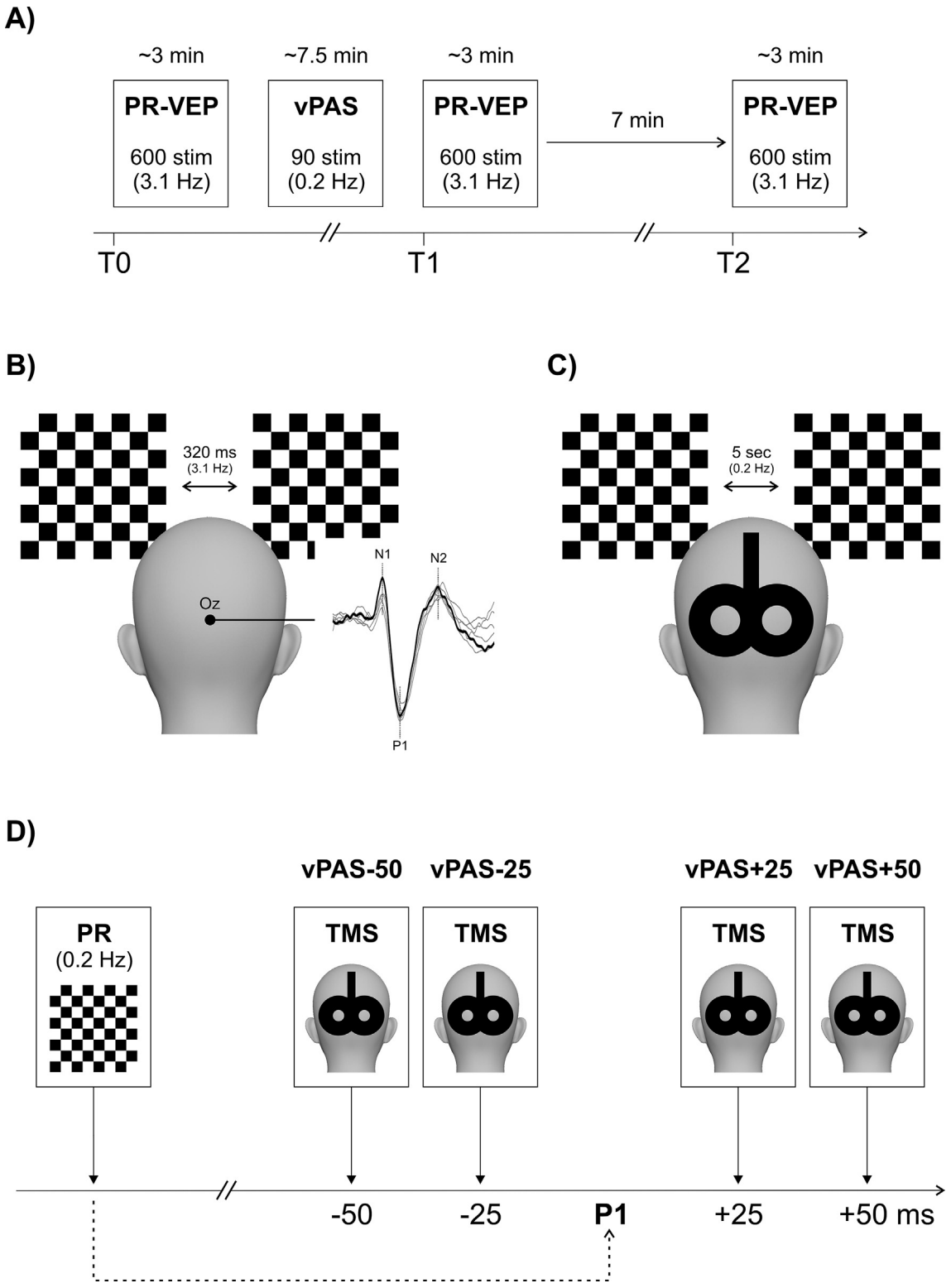

Fig. 1. A) Schematic representation of the experimental design of the study: pattern-reversal VEP (PR-VEP) (as detailed in panel B) are recorded at baseline (T0), immediately after (T1) and 10 min after (T2) the end of the visual paired associative stimulation (vPAS) protocol (as detailed in panels C and D). **B)** VEP recording procedure: 600 cortical responses generated by a checkboard pattern reversal, at a frequency of 3.1 Hz, are collected from the Oz point of the scalp. Latencies of N1, P1 and N2 waves and N1−P1 and P1−N2 peak-to-peak amplitudes are measured. **C)** vPAS procedure: the checkboard pattern reversal (90 stimuli at a frequency of 0.2 Hz) is associated with TMS over the Oz point of the scalp. **D)** Stimulus timing for the different vPAS protocols: after each pattern reversal (PR), TMS of the visual area is timed so that it precedes or follows cortical activation produced by peripheral visual stimulation, as indicated by the peak latency of the VEP P1 wave. Four interstimulus intervals, relative to P1 latency, are tested: −50, −25, +25 and + 50 ms.

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Visual paired associative stimulation (vPAS)

In designing our vPAS protocol, we adapted a paradigm commonly used for the study of sensorimotor associative plasticity [24], in order to try to induce persistent inhibition or excitation effects in V1 (Fig. 1D).

In the vPAS protocol, TMS of V1 is timed so that it precedes or follows cortical activation produced by peripheral visual stimulation, as indicated by the peak latency of the VEP P1 wave. Specifically, the vPAS protocol consists of 90 black and white checkerboard reversals coupled with subsequent TMS pulses over the visual occipital area (see above) delivered at a frequency of 0.2 Hz. Four interstimulus intervals between pattern reversal and TMS were a-priori chosen: P1 peak latency minus 50 ms (vPAS-50), P1 peak latency minus 25 ms (vPAS-25), P1 peak latency plus 25 ms (vPAS+25), and P1 peak latency plus 50 ms (vPAS+50) (Fig. 1D). For each participant, the vPAS sessions (vPAS-50, vPAS-25, vPAS+25, and vPAS+50) were performed in random order at $\geq$ 1-week intervals.

Statistical analysis

All recordings were analysed offline in a blinded fashion by a single investigator (G.C.) who was not blind to the order of the blocks. Data were analysed using the software JASP for Windows v0.9.2 (JASP Team, 2018). Sample size calculations were based on a previous study that examined the same evoked potentials in healthy volunteers [16], but with another conditioning paradigm, with a desired power of 0.80 and an alpha error of 0.05. Since a primary endpoint was to detect differences on habituation between the baseline and T1, we used the amplitude habituation of the N1–P1 VEP complex in the before vs after conditions to compute the sample size. The minimal required sample size was calculated to be 10 subjects.

A Kolmogorov-Smirnov test showed that latencies and amplitudes of VEP components had a Gaussian distribution. Repeated measures analysis of variance (rm-ANOVA) was performed to analyse the effects on 1st block VEP N1–P1 and P1–N2 Amplitudes and on the Slope of the linear regression line of amplitudes over the 6 blocks of traces, with 'Time' and 'Protocol' as independent variables. The sphericity of the covariance matrix was verified with the Mauchly Sphericity Test; in the case of violation of the sphericity assumption, Greenhouse-Geisser (G-G) epsilon (ε) adjustment was used. In rm-ANOVA, partial eta-squared (ηp^2) was used as measure of effect size. To identify the comparison(s) contributing to major effects, we performed post-hoc Fisher's least significant difference (LSD) tests. P values of less than 0.05 were considered statistically significant.

Results

Basic neurophysiological parameters

VEP recordings were obtained from all participants (n = 28); none of them reported adverse events due to rTMS. The latencies (N1, P1 and N2) and amplitudes (VEP N1–P1 and P1–N2) at baseline were not significantly different between experimental sessions ($P > 0.05$, tested in each experimental sample). At T0, before each vPAS session, all groups of slope data had negative values indicating a normal reducing response (habituation) to visual repetitive stimulations (Table 1).

Effects of vPAS on VEP parameters in the range of −50/+50 ms

A preliminary investigation of the vPAS effects was conducted in a group of 14 subjects using four interstimulus intervals corresponding to P1 latency −50/-25/+25/+50 ms.

The latencies (N1, P1, and N2) calculated on the 1st VEP block were not modified by vPAS protocols ($P > 0.05$; Table 1).

The rm-ANOVA model with N1–P1 VEP 1st block amplitude as dependent variable was not significant for the 'protocol' × 'time' interaction effect ($F_{2.52,32.75} = 2.149$, $\varepsilon = 0.420$, $P = 0.12$, corrected for violation of sphericity assumption).

The rm-ANOVA model with N1–P1 VEP amplitude slope as dependent variable was significant for the 'protocol' × 'time' interaction effect ($F_{6,78} = 4.708$, $P < 0.001$, $\eta p^2 = 0.266$). Post-hoc analysis showed that, immediately after vPAS-25, the slope of the linear trend in N1–P1 VEP amplitudes significantly increased from block 1 to block 6 (−0.45 vs −0.20; $P = 0.029$). Conversely, the slope of the linear trend in N1–P1 VEP amplitudes significantly decreased immediately after vPAS+25 (+0.11 vs −0.16; $P = 0.014$) (Table 1; Fig. 2). During the T2 recording session, the VEP amplitude linear trend was not different from that observed at T0 with all protocols (Table 1; Fig. 2).

The rm-ANOVA model with P1–N2 VEP 1st block amplitude or amplitude slope as dependent variable was not significant for the 'protocol' × 'time' interaction effect ($F_{2.89,37.55} = 2.006$, $\varepsilon = 0.481$, $P = 0.13$, corrected for violation of sphericity assumption; $F_{6,78} = 1.052$, $P = 0.40$, respectively) (Table 1; Fig. 3).

Effects of vPAS on VEP parameters at −25/+25 ms

In order to confirm the timing-dependent effects of the vPAS protocol observed in the range from −50 ms to +50 ms, the effect of vPAS was tested in an additional sample of 14 subjects using the two shortest inter-stimulus intervals (P1-25/+25 ms) that produced significant changes in the preliminary experiment.

In this new sample (n = 14), rm-ANOVA with N1–P1 VEP 1st block amplitude as dependent variable was not significant for the 'protocol' × 'time' interaction effect ($F_{2,26} = 2.410$, $P = 0.11$). Rm-ANOVA with N1–P1 VEP amplitude slope as dependent variable was significant for the 'protocol' × 'time' interaction effect ($F_{2,26} = 4.336$, $P = 0.024$, $\eta p^2 = 0.250$). At the post-hoc analysis, N1–P1 VEP amplitude slope was significantly increased immediately after vPAS-25 (−0.53 vs −0.25; $P = 0.048$), while it showed only a trend to reduce immediately after vPAS+25 (−0.18 vs −0.43; $P = 0.14$) and was significantly reduced at T2 after vPAS+25 (−0.09 vs −0.43; $P = 0.050$) (Table 1). Rm-ANOVA with P1–N2 VEP 1st block amplitude or amplitude slope as dependent variable was not significant for the 'protocol' × 'time' interaction effect ($F_{2,26} = 0.876$, $P = 0.43$; $F_{2,26} = 0.443$, $P = 0.65$, respectively) (Table 1).

Data were then analysed considering the pooled sample of 28 subjects who underwent the vPAS protocol with the interstimulus intervals of P1-25/+25 ms.

In this larger sample (n = 28), the latencies (N1, P1, and N2) calculated on the 1st VEP block were not modified by vPAS protocols ($P > 0.05$). Rm-ANOVA with N1–P1 VEP 1st block amplitude as dependent variable reached significance for the 'time' effect ($F_{2,54} = 6.433$, $P = 0.003$, $\eta p^2 = 0.192$). Post-hoc analysis (n = 28) showed that 1st block amplitude was significantly reduced immediately and at 10 min after vPAS+25 (T1: 8.6 vs 10.5; T2: 9.3 vs 10.5; $P < 0.001$ and $P = 0.017$ respectively) (Table 1; Fig. 2). Rm-ANOVA with N1–P1 VEP amplitude slope as dependent variable was significant for the 'protocol' × 'time' interaction effect ($F_{2,54} = 11.992$, $P < 0.001$, $\eta p^2 = 0.308$). Post-hoc analysis (n = 28) showed that, immediately after vPAS-25, the slope of the linear trend in N1–P1 VEP amplitudes significantly increased from block 1 to block 6 (−0.49 vs −0.22; $P = 0.003$). Conversely, the slope of the linear trend in N1–P1 VEP amplitudes significantly decreased immediately after vPAS+25 (−0.03 vs −0.30; $P = 0.015$) (Table 1; Fig. 2). During the T2 recording session, the VEP amplitude linear

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Table 1

	N1 lat (ms)	P1 lat (ms)	N2 lat (ms)	N1−P1 1st block amp (μV)	N1−P1 slope	P1−N2 1st block amp (μV)	P1−N2 slope
Sample 1 (n = 14)							
vPAS-50							
T0	93.0 ± 4.3	120.2 ± 6.3	163.3 ± 19.4	9.9 ± 6.1	−0.12 ± 0.42	8.8 ± 5.0	−0.16 ± 0.46
T1	93.5 ± 3.5	119.0 ± 5.9	163.8 ± 19.1	10.9 ± 8.0	−0.26 ± 0.44	10.4 ± 7.6	−0.22 ± 0.57
T2	91.1 ± 4.4	119.1 ± 6.8	160.5 ± 16.9	10.2 ± 6.6	−0.02 ± 0.38	9.5 ± 5.8	−0.19 ± 0.41
vPAS-25							
T0	94.1 ± 4.5	119.5 ± 7.0	160.1 ± 16.9	9.5 ± 5.2	−0.20 ± 0.45	9.2 ± 4.0	−0.34 ± 0.49
T1	92.0 ± 3.5	120.0 ± 7.7	160.1 ± 16.2	9.3 ± 5.2	**−0.45 ± 0.39***	8.7 ± 3.9	−0.42 ± 0.46
T2	92.6 ± 3.8	119.9 ± 6.1	160.1 ± 15.4	9.2 ± 4.9	−0.17 ± 0.22	8.5 ± 4.1	−0.17 ± 0.62
vPAS + 25							
T0	92.6 ± 3.6	119.4 ± 7.6	159.3 ± 16.4	10.2 ± 4.5	−0.16 ± 0.34	9.8 ± 3.3	−0.45 ± 0.24
T1	93.0 ± 3.3	120.3 ± 7.2	160.2 ± 15.9	7.9 ± 4.6	**+ 0.11 ± 0.27***	8.9 ± 3.0	−0.21 ± 0.50
T2	91.9 ± 3.9	119.8 ± 7.8	160.3 ± 15.5	9.5 ± 5.2	−0.36 ± 0.32	8.8 ± 2.8	−0.25 ± 0.42
vPAS + 50							
T0	92.5 ± 5.0	120.2 ± 7.2	162.3 ± 16.0	9.2 ± 4.9	−0.09 ± 0.35	8.0 ± 3.9	−0.10 ± 0.30
T1	92.8 ± 4.2	120.2 ± 9.4	161.4 ± 16.3	8.4 ± 5.0	−0.08 ± 0.31	8.2 ± 3.8	−0.21 ± 0.33
T2	92.6 ± 4.0	120.1 ± 8.6	161.1 ± 14.9	8.6 ± 4.8	−0.21 ± 0.42	8.7 ± 3.4	−0.28 ± 0.32
Sample 2 (n = 14)							
vPAS-25							
T0	91.3 ± 6.4	120.4 ± 5.5	167.2 ± 10.6	10.9 ± 6.0	−0.25 ± 0.46	8.4 ± 5.5	−0.13 ± 0.52
T1	92.6 ± 6.0	120.5 ± 5.8	168.3 ± 13.3	10.2 ± 5.5	**−0.53 ± 0.78***	8.8 ± 6.0	−0.34 ± 0.48
T2	92.3 ± 4.7	121.9 ± 4.2	170.0 ± 13.7	11.1 ± 6.6	−0.25 ± 0.56	8.9 ± 4.8	−0.06 ± 0.45
vPAS + 25							
T0	91.8 ± 5.4	122.1 ± 5.5	167.4 ± 9.9	10.8 ± 3.5	−0.43 ± 0.43	9.2 ± 3.6	−0.34 ± 0.28
T1	93.0 ± 5.0	121.1 ± 5.0	167.5 ± 10.7	9.4 ± 3.5	−0.18 ± 0.40	9.5 ± 5.1	−0.47 ± 0.35
T2	92.5 ± 4.8	122.9 ± 6.2	170.5 ± 11.5	9.0 ± 4.1	**−0.09 ± 0.35***	8.7 ± 4.2	−0.05 ± 0.35
Pooled sample (n = 28)							
vPAS-25							
T0	92.7 ± 5.6	119.9 ± 6.2	163.7 ± 14.3	10.2 ± 5.6	−0.22 ± 0.45	8.8 ± 4.8	−0.24 ± 0.51
T1	92.3 ± 4.8	120.3 ± 6.7	164.2 ± 15.1	9.7 ± 5.3	**−0.49 ± 0.61***	8.7 ± 5.0	−0.38 ± 0.46
T2	92.4 ± 4.2	120.9 ± 5.2	165.1 ± 15.2	10.2 ± 5.8	−0.21 ± 0.42	8.7 ± 4.4	−0.11 ± 0.53
vPAS + 25							
T0	92.2 ± 4.5	120.7 ± 6.6	163.3 ± 13.9	10.5 ± 4.0	−0.30 ± 0.40	9.5 ± 3.4	−0.40 ± 0.26
T1	93.0 ± 4.1	120.7 ± 6.1	163.9 ± 13.8	**8.6 ± 4.1***	**−0.03 ± 0.37***	9.2 ± 4.1	−0.34 ± 0.45
T2	92.2 ± 4.3	121.4 ± 7.1	165.4 ± 14.4	**9.3 ± 4.6***	−0.23 ± 0.36	8.8 ± 3.5	−0.15 ± 0.39

VEP parameters recorded with each vPAS intervention (vPAS-50, -25, +25, +50) at T0 (baseline), T1 (immediately after vPAS) and T2 (10 min after vPAS). Data are reported for the two samples of 14 subjects each and for the pooled sample of 28 subjects. Data are expressed as means ± SD. *: $P < 0.05$ compared with T0 at post-hoc comparisons.

trend was not different from that observed at T0 with both protocols (Table 1; Fig. 2). Rm-ANOVA with P1−N2 VEP 1st block amplitude or amplitude slope as dependent variable was not significant for the 'protocol' × 'time' interaction effect ($F_{2,54} = 0.735$, $P = 0.48$; $F_{2,54} = 0.804$, $P = 0.45$, respectively) (Table 1; Fig. 3).

Discussion

In the present experiment, we reproduced in V1 the typical condition of associative stimulation by combining peripheral visual inputs with cortical activation by TMS. Since V1 pyramidal cells are considered the most likely source of VEPs [29–31], pattern reversal presentation and TMS are hypothesised to produce convergent inputs on V1 pyramidal cells, thus paralleling the classical synaptic models of STDP [18]. In our paradigm, since focal TMS was delivered at an intensity below phosphene threshold, but high enough (120% of motor threshold) to produce cortical depolarization, it is supposed to act by trans-synaptically depolarizing pyramidal cells below their spiking threshold. However, it should also be considered that the effects on VEP generators might be produced by TMS activation of surrounding V2 and V3 cortical circuits, in addition to direct V1 stimulation [32,33].

The main finding of our study is a timing-specific effect of our vPAS protocol on VEP habituation, that is dependent on the time interval between the stimuli converging in V1: habituation was increased using a repeated stimulation in which the magnetic stimulus precedes of 25 ms V1 activation by peripheral visual stimulation, while it was abolished with the magnetic stimulus following of 25 ms V1 afferent activation.

The analysis on VEP amplitude, measured in the first block of 100 stimuli, showed that vPAS-50, vPAS-25, and vPAS+50 leave unchanged baseline visual cortex excitability, while solely vPAS+25 significantly diminishes baseline cortical excitability, at least for 10 min. The direction of the effect of vPAS+25 is coherent with the post-pre spiking rule of STDP, where a conditioning input (TMS in our model) reduces post-synaptic initial baseline excitability (VEP 1st block amplitude in our case) when it repeatedly follows a supra-threshold post-synaptic input (light stimulation). This apparent unrelationship between the 1st block amplitude, i.e. the basic level of cortical excitability, and the degree of delayed habituation is not unusual. Indeed, in a habituation paradigm, early and late responses to a series of repetitive stimuli may behave differently because they are regulated by different mechanisms. In fact, according to the dual-process theory [13], facilitation/sensitization (increasing response) competes with habituation (decreasing response) to determine the final behavioural outcome. Sensitization occurs at the beginning of the stimulus session and accounts for the initial transitory increase in response amplitude. Habituation occurs throughout the recording session and accounts for the delayed response decrease. Several previous studies support differential effect of neuromodulatory and pharmacological interventions on early and late VEP amplitude blocks [15–17,34,35]. However, we must notice that, despite this differential effect, here we found that vPAS+25 significantly and durably reduces 1st block N1−P1 amplitude, and simultaneously induces lack of VEP

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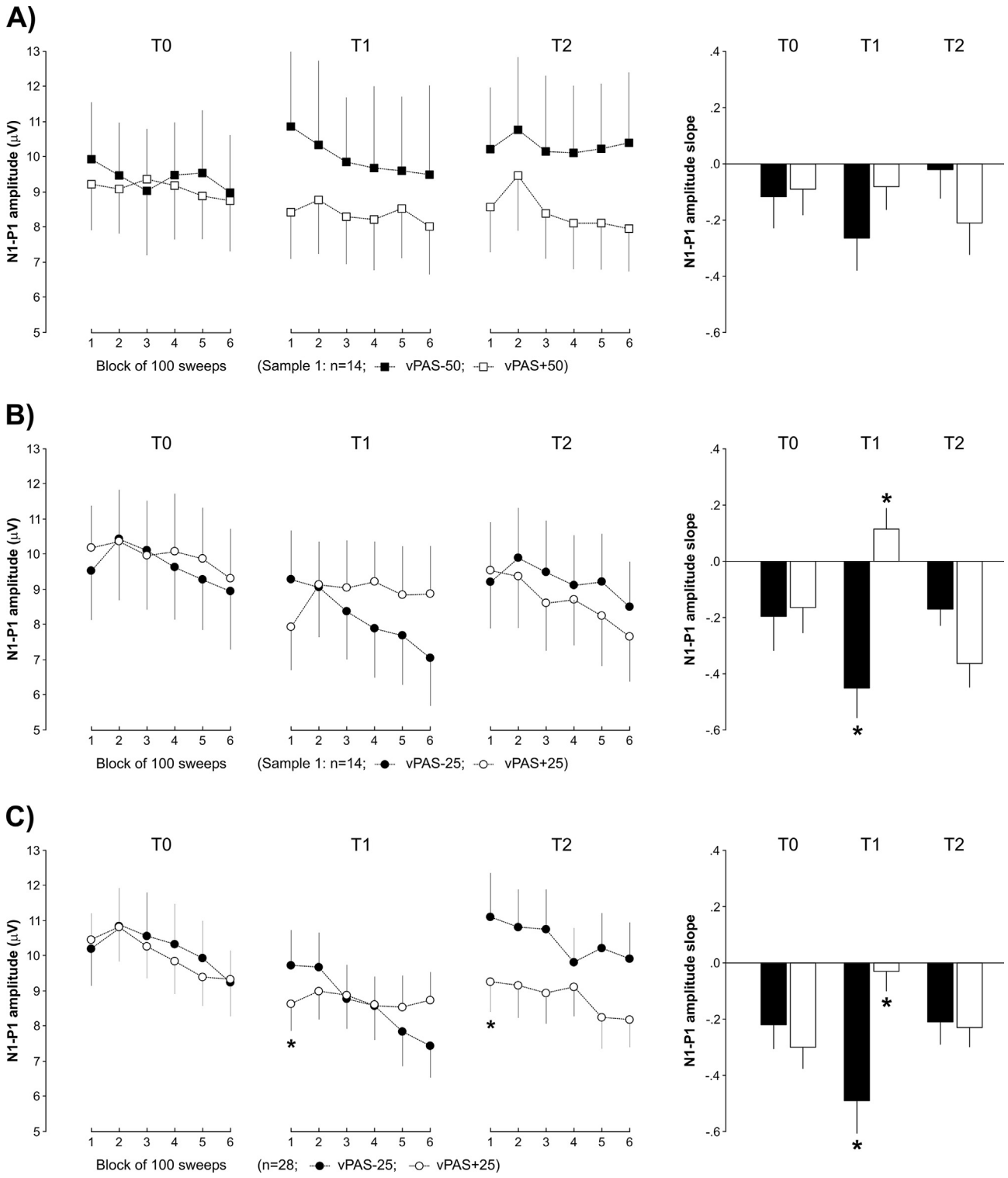

Fig. 2. N1−P1 VEP amplitudes over 6 blocks of 100 sweeps and slope of the linear regression line of amplitudes, recorded at baseline (T0), immediately after (T1) and 10 min after (T2) the end of the vPAS protocol. For each vPAS condition, the interstimulus interval indicates the time of the magnetic shock, expressed in ms, relative to the P1 latency (see methods). Panel (A) represents the effects of vPAS-50 (black squares and bars) and vPAS+50 (white squares and bars) in a group of 14 subjects; panel (B) represents the effects of vPAS-25 (black circles and bars) and vPAS+25 (white circles and bars) in the same group of 14 subjects; panel (C) represents the effects of vPAS-25 (black circles and bars) and vPAS+25 (white circles and bars) in the group of 28 subjects. Error bars represent standard error of the mean. *: significant difference with T0 at post-hoc comparisons ($p < 0.05$).

amplitude habituation. We argue that these results, on the one hand, point towards a stronger and persistent effect of vPAS+25 on parameters of cortical excitability and associative plasticity and, on the other hand, support, at least in part, the concept that the baseline level of cortical excitability can predict the effects of enhancing/reducing rTMS protocols, the so-called "context dependency" [36] or "ceiling effect" [37]. Following this concept, in systems with a normal-to-high level of basal activity, diminishing

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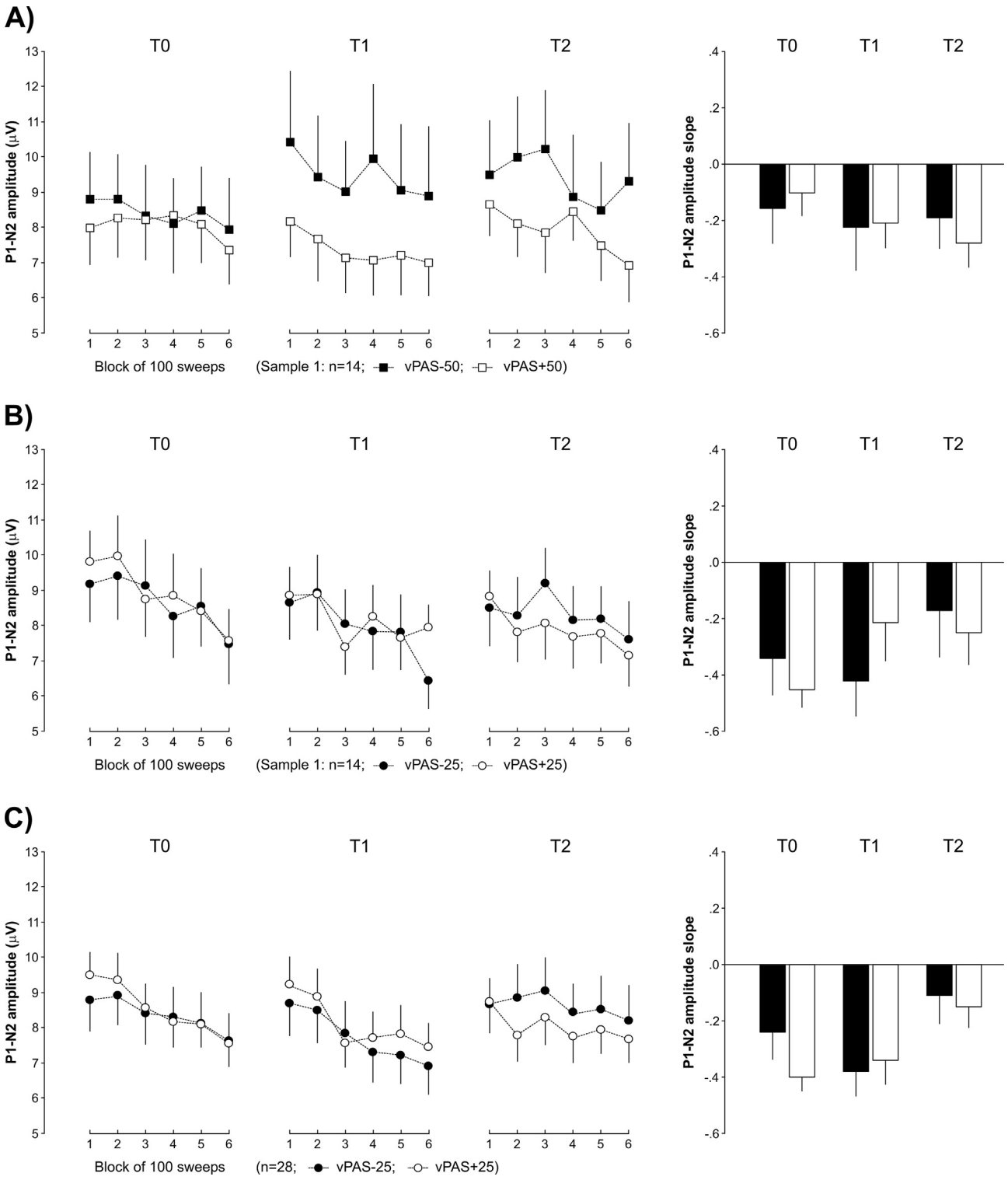

Fig. 3. P1–N2 VEP amplitudes over 6 blocks of 100 sweeps and slope of the linear regression line of amplitudes, recorded at baseline (T0), immediately after (T1) and 10 min after (T2) the end of the vPAS protocol. For each vPAS condition, the interstimulus interval indicates the time of the magnetic shock, expressed in ms, relative to the P1 latency (see methods). Panel (A) represents the effects of vPAS-50 (black squares and bars) and vPAS+50 (white squares and bars) in a group of 14 subjects; panel (B) represents the effects of vPAS-25 (black circles and bars) and vPAS+25 (white circles and bars) in the same group of 14 subjects; panel (C) represents the effects of vPAS-25 (black circles and bars) and vPAS+25 (white circles and bars) in the group of 28 subjects. Error bars represent standard error of the mean.

neuromodulatory protocols can successfully reduce the initial level of neuronal excitability, as it is the case after vPAS+25; whereas, enhancing neuromodulatory protocols cannot further increase the already maximal baseline cortical excitability level. Therefore, the initial suppression of V1 excitability in this condition might account, at least in part, for the abolished delayed habituation of VEPs observed after vPAS+25, because of a "floor" effect on cortical excitability that cannot be further reduced by prolonged visual

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stimulation (blocks 2–6); however, the persistent suppression of V1 excitability at T2 without significant slope reduction suggests that this possible mechanism is not likely to be the sole cause of the vPAS effect on VEP habituation.

The inter-stimulus intervals that we have explored were based on findings of previous experiments that investigated STDP in single synapses [21,38] or sensori-motor PAS effects in the intact human brain [24,26]. Overall, it is required that converging inputs are enclosed in a narrow time window of 5–40 ms to produce their facilitatory or inhibitory effects. In our vPAS protocol, the shortest inter-stimulus interval that we tested is 25 ms, based on the consideration that the pattern reversal generates a P1 VEP wave that has a duration greater than the N20 wave of the somatosensory evoked potential: since the P1 wave is considered as the hallmark of V1 activation, we wanted to make sure that the magnetic stimulus did not overlap with the peripheral visual input on V1. Coherently with previous findings, the largest effects of vPAS were observed with the shortest inter-stimulus intervals of −25/+25 ms; a smaller increase of VEP habituation, not reaching statistical significance, was observed with the longer interval of −50 ms and no consistent effect at +50 ms. This possible asymmetry of the effects of the vPAS-50 protocol might be related to the fact that STDP mechanisms are effective within a narrow time window and the interval of 50 ms is likely to be at the limit of this window (as discussed above).

We have not explored in the present set of experiments if timing-specific effects are also produced by couples of stimuli separated by intervals shorter than 25 ms.

Interstimulus intervals in the same time range were explored in the study by Suppa and colleagues [27], testing visuo-motor associative plasticity: in this study facilitatory effects on M1 excitability were produced in a time window between visual and motor inputs of −60 to −20 ms (visual preceding) while inhibitory effects were produced in a time window of +20 to +40 ms (visual following), as estimated from the time required for VEP-induced visual afferent inputs to travel from V1 to M1 [27].

Since electrophysiological measures of brain excitability in human subjects usually suffer from high variability (e.g. in the case of the study on the classical PAS [39]), we replicated in an additional sample the vPAS protocol using the two shortest inter-stimulus intervals around to the P1 latency (i.e. P1-25/+25 ms) producing significant changes in the initial experiment. By pooling all data and obtaining a larger sample size for the −25/+25 ms time intervals, we were able to increase the statistical power in detecting the timing-dependent effects of the vPAS protocol and thus to improve the reliability of the results.

To speculate on the nature of the effects produced by vPAS conditioning, we take the phenomenon of habituation as a measure of short-term cortical plasticity and the amplitude of cortical potentials evoked by the first block of stimulation as an index of cortical excitability. Indeed, habituation is one the simplest forms of synaptic plasticity, characterized by a decrement of the post-synaptic response after repeated stimulation [13]: short-term modifications of neurotransmitter release and of post-synaptic receptor sensitivity might explain the initial reduction of post-synaptic excitability, while long-term habituation would involve mechanisms of LTD. Given that modulatory effects of vPAS are characterized by a reduction of V1 excitability after vPAS+25 and by a bidirectional modulation of VEP habituation with vPAS+25 and vPAS-25, we can hypothesise that our intervention does not only act by modifying cortical excitability, but also the propensity of visual cortical neurons to undergo phenomena of activity-dependent synaptic plasticity (i.e. the phenomenon of habituation). This is coherent with previous experimental data supporting the hypothesis that other interventions of neuromodulation acting on the spiking threshold of post-synaptic neurons, such as direct current stimulation, act on LTP with a similar mechanism of metaplasticity [40]. Evidence for metaplasticity in the human visual cortex was also provided using combined tDCS-rTMS approaches [41,42]. Moreover, since all the observed effects on habituation were limited to the first 10 min after stimulation, we can hypothesise that vPAS mainly affects mechanisms of short-term plasticity.

In conclusion, the results of our study are the first demonstration that it is possible to induce persistent changes in the excitatory properties of V1 through STDP-like mechanisms in the intact human brain. Extending our study from healthy subjects to patients with known altered cortical excitability, such as migraine between attacks [14] or photosensitive epilepsy [43], would offer a unique opportunity to investigate visual associative plasticity mechanisms under conditions where baseline habituation is absent.

Conflicts of interest

The authors declare no competing interests.

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