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Stimulus Novelty, and Not Neural Refractoriness, Explains the Repetition Suppression of Laser-Evoked Potentials

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Wang AL, Mouraux A, Liang M, Iannetti GD. Stimulus novelty, and not neural refractoriness, explains the repetition suppression of laser-evoked potentials. *J Neurophysiol* 104: 2116–2124, 2010. First published June 30, 2010; doi:10.1152/jn.01088.2009. Brief radiant laser pulses selectively activate skin nociceptors and elicit transient brain responses (laser-evoked potentials [LEPs]). When LEPs are elicited by pairs of stimuli (S1–S2) delivered at different interstimulus intervals (ISIs), the S2-LEP is strongly reduced at short ISIs (250 ms) and progressively recovers at longer ISIs (2,000 ms). This finding has been interpreted in terms of order of arrival of nociceptive volleys and refractoriness of neural generators of LEPs. However, an alternative explanation is the modulation of another experimental factor: the novelty of the eliciting stimulus. To test this alternative hypothesis, we recorded LEPs elicited by pairs of nociceptive stimuli delivered at four ISIs (250, 500, 1,000, 2,000 ms), using two different conditions. In the *constant* condition, the ISI was identical across the trials of each block, whereas in the *variable* condition, the ISI was varied randomly across trials and single-stimulus trials were intermixed with paired trials. Therefore the time of occurrence of S2 was both less novel and more predictable in the constant than in the variable condition. In the constant condition, we observed a significant ISI-dependent suppression of the biphasic negative–positive wave (N2–P2) complex of the S2-LEP. In contrast, in the variable condition, the S2-LEP was completely unaffected by stimulus repetition. The pain ratings elicited by S2 were not different in the two conditions. These results indicate that the repetition-suppression of the S2-LEP is not due to refractoriness in nociceptive afferent pathways, but to a modulation of novelty and/or temporal predictability of the eliciting stimulus. This provides further support to the notion that stimulus saliency constitutes a crucial determinant of LEP magnitude and that a significant fraction of the brain activity time-locked to a brief and transient sensory stimulus is not directly related to the quality and the intensity of the corresponding sensation, but to bottom-up attentional processes.

INTRODUCTION

Brief radiant heat pulses, generated by infrared laser stimulators, can be used to selectively excite Aδ- and C-fiber free nerve endings in the superficial skin layers (Bromm and Treede 1984) and evoke a number of responses in the human electroencephalogram (EEG; laser-evoked potentials [LEPs]) (Carmon et al. 1976).

Interestingly, despite the fact that high-energy laser pulses simultaneously activate Aδ and C nociceptors, and thereby elicit a dual sensation of first (Aδ-related) and second (C-related) pain, LEPs appear only in a time window compatible with the faster conduction velocity of Aδ fibers (Aδ-LEPs; Bromm and Treede 1984). However, avoiding the concomitant activation of Aδ nociceptors (i.e., selectively activating C nociceptors) leads not only to the disappearance of the perception of first pain and the Aδ-LEP, but also to the appearance of brain responses in a time window compatible with the slower conduction velocity of unmyelinated C fibers (C-LEPs; reviewed in Plaghki and Mouraux 2005).

Aδ-LEPs and C-LEPs have similar morphology and topography and are similarly modulated by attention and state of arousal (Mouraux and Plaghki 2006). Both responses consist of a large negative–positive complex maximal at the scalp vertex: N2–P2, peaking at 200–350 ms for Aδ-LEPs (Bromm and Treede 1984) and at 800–1,000 ms for C-LEPs (Bragard et al. 1996) when stimulating the hand dorsum. Several studies have suggested that Aδ-LEPs and C-LEPs share common cortical generators, reflecting a combination of activity originating from bilateral operculo-insular regions (secondary somatosensory cortices, insula) and the anterior cingulate cortex (Garcia-Larrea et al. 2003). For these reasons, it has been suggested that the lack of a C-LEP following an Aδ-LEP is due to refractoriness of these common cortical generators. Information relevant to test this refractoriness hypothesis has been gathered by studies investigating the effect of interstimulus interval (ISI) on Aδ-LEPs elicited by pairs or trains of laser stimuli. These studies have reported that the cortical response elicited by the repeated stimulus is significantly reduced and that this reduction is stronger at shorter ISIs (Raij et al. 2003; Truini et al. 2004, 2007). These observations were interpreted as evidence for "refractoriness" of synaptic transmission in the nociceptive afferent pathway and LEP generators (Truini et al. 2004) and as evidence that "only the earliest of a series of somatosensory volleys elicits cerebral responses synchronous enough to yield ERPs" (Truini et al. 2007), thus supporting the hypothesis that Garcia-Larrea (2004) christened "first come, first served".

However, we have recently suggested an alternative explanation: that the effect of ISI on the magnitude of Aδ-LEPs results from the modulation of novelty (i.e., the difference in one or more physical dimensions with respect to previously occurring stimuli; Naatanen and Picton 1987) and/or temporal uncertainty (i.e., the uncertainty of when a stimulus will occur; Klemmer 1956). Indeed, both of these factors are known to greatly affect the magnitude of vertex potentials elicited not only by nociceptive stimuli, but also by stimuli belonging to different sensory modalities (Clark et al. 2008; Hauck et al. 2007; Loveless 1986; McCarthy and Donchin 1976; Schafer

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and Marcus 1973). This alternative explanation was put forward to clarify why, when pairs of laser stimuli are presented using ISIs that vary randomly from trial to trial, the magnitude of the Aδ-LEP elicited by the repeated stimulus is entirely unaffected by stimulus repetition, even at ISIs as short as 280 ms (Mouraux et al. 2004). If this hypothesis is correct, the repetition-suppression of Aδ-LEPs that was observed, for example, by Truini et al. (2004, 2007), would be explained by the fact that when stimuli are presented using a short and *constant* ISI, the second stimulus becomes less novel and its time of occurrence more predictable (Mouraux and Iannetti 2008b).

Here we explicitly tested which one of these two hypotheses (novelty vs. neural refractoriness) explains the repetition-suppression of LEPs, by comparing the effect of stimulus repetition on the Aδ-LEPs elicited by pairs of laser stimuli (S1–S2) presented using two different conditions. In the *constant* condition the ISI was kept constant across all the trials of each block, whereas in the *variable* condition the ISI was varied randomly across all the trials of each block and single-stimulus trials were intermixed with paired trials (Fig. 1). Therefore both novelty and the temporal uncertainty of S2, compared with S1, were greatly reduced in the constant condition, whereas they were much less reduced in the variable condition.

METHODS

Subjects

Ten healthy volunteers (seven females and three males), aged from 22 to 50 yr (29 ± 8 yr, mean ± SD), participated in the study. All participants gave written informed consent and the local ethics committee approved the procedures.

Nociceptive stimulation

Noxious radiant-heat stimuli were generated by an infrared neodymium yttrium aluminum perovskite (Nd:YAP) laser with a wavelength of 1.34 μm (Electronic Engineering, Calenzano, Italy). At this short wavelength, the skin is very transparent to the laser radiation and, consequently, the laser pulses directly activate nociceptive terminals in the most superficial skin layers (Baumgartner et al. 2005; Iannetti et al. 2006). Laser pulses were directed to the dorsum of the right hand and a He–Ne laser pointed to the area to be stimulated. The laser pulse was transmitted via an optic fiber and focused by lenses to a spot diameter of about 8 mm (50 mm^2) at the target site. The duration of the laser pulses was 4 ms and its energy was 3.5 J. With these parameters, laser pulses elicit a clear pinprick sensation, related to the activation of Aδ skin nociceptors (Iannetti et al. 2006).

Experimental paradigm

Prior to the recording session, the experimental paradigm was explained to the participants. To familiarize the participants with the

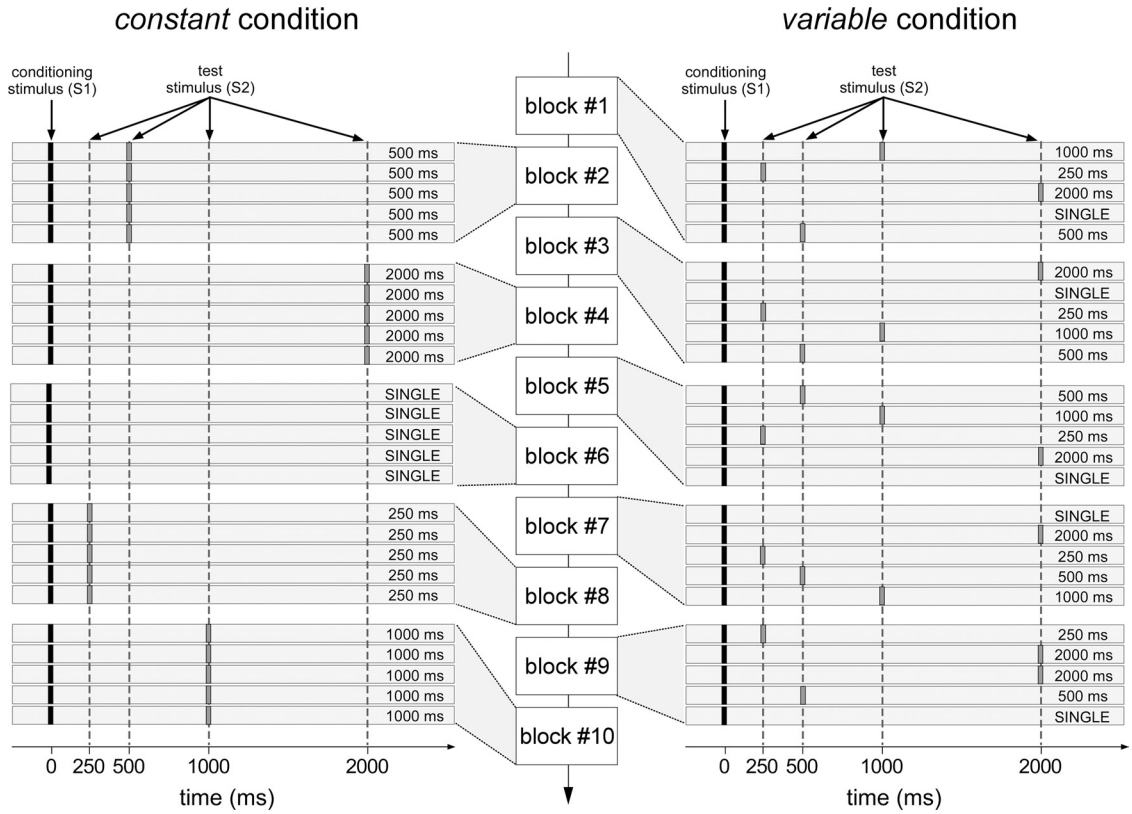

FIG. 1. Experimental design. Laser-evoked potentials (LEPs) were collected in a single recording session, comprising 10 blocks of stimulation. Laser pulses were delivered to the dorsum of the right hand, either as a single laser stimulus or as a pair of laser stimuli presented at an interstimulus interval (ISI) of 250, 500, 1,000, and 2,000 ms. In the *constant* condition (*left*) the ISI of the paired stimuli was *identical* across the trials of each block, whereas in the *variable* condition (*right*) the ISI was varied randomly across trials and single-stimulus trials were intermixed with paired trials. Therefore in the *variable* condition, the second laser pulse was both more novel and more unexpected, compared with the *constant* condition, because participants were not able to predict whether and when a second laser pulse would follow the first stimulus of each trial. Each condition comprised 5 recording blocks and the order of blocks was balanced across subjects using a Latin Square. Constant and variable blocks were presented in an interleaved fashion (*middle column*). In each block 30 trials were presented, with an intertrial interval ranging between 15 and 18 s. The energy of the laser stimulus was identical across trials and conditions. Paired laser stimuli were spatially displaced by about 20 mm using an automatic device controlling the laser beam (Lee et al. 2009).

nociceptive stimulus, a small number of laser pulses were delivered to the left-hand dorsum.

A schematic illustration of the experimental paradigm is shown in Fig. 1. EEG data were collected in a single recording session, comprising 10 blocks of stimulation. In each block 30 trials were presented, with an intertrial interval (ITI) ranging between 15 and 18 s. In each trial, laser pulses were delivered to the dorsum of the right hand, either as a single laser stimulus or as a pair of laser stimuli presented at an ISI of 250, 500, 1,000, or 2,000 ms. In the *constant* blocks (Fig. 1, *left*) the ISI of the paired stimuli was identical across all trials of each block, whereas in the *variable* blocks (Fig. 1, *right*) the ISI was randomly varied across trials and single-stimulus trials (one/five) were intermixed with paired trials (four/five). Therefore in the *variable* blocks, the second laser pulse was more novel compared with the *constant* condition and participants were not able to predict whether and when a second laser pulse would follow the first stimulus of each trial. The number of constant and variable blocks was equal and their order of presentation was balanced across subjects using a Latin Square. Constant and variable blocks were presented in an interleaved fashion. Participants were asked to report the number of perceived stimuli and to verbally rate the intensity of each perceived stimulus, using a scale ranging from 0 to 10, where 0 was defined as "no pain" and 10 was defined as "as painful as it could be" (Jensen et al. 1986). After each stimulus, the laser beam target was shifted by about 20 mm in a random direction to avoid nociceptor fatigue and sensitization. The laser beam was controlled by a computer that used two servo-motors (HS-422; Hitec RCD, Poway, CA; angular speed, 60°/160 ms) to orient the laser beam along two perpendicular axes (Lee et al. 2009).

EEG recording

Participants were seated in a comfortable chair and wore protective goggles. They were asked to focus their attention on the stimuli, relax their muscles, and keep their eyes open and gaze slightly downward. Acoustic isolation was ensured using earplugs and headphones. Both the laser beam and the controlling motors were completely screened from the view of the participants. The EEG was recorded using 30 Ag–AgCl electrodes placed on the scalp according to the International 10–20 system, using the nose as reference. To monitor ocular movements and eye blinks, electrooculographic (EOG) signals were recorded from two surface electrodes, one placed over the lower eyelid, the other placed 1 cm lateral to the outer corner of the orbit. The electrocardiogram was recorded using two electrodes placed on the dorsal aspect of the left and right forearms. Signals were amplified and digitized using a sampling rate of 1,024 Hz and a precision of 12 bits, giving a resolution of 0.195 μV digit^{-1} (System Plus; Micromed, Mogliano Veneto, Italy).

EEG analysis

Analyses were performed using Letswave (http://amouraux.webnode.com/letswave) (Mouraux and Iannetti 2008a) and Matlab (The MathWorks, Natick, MA). Continuous EEG recordings were segmented into epochs using a time window of 3.5 s (−0.5 to +3 s relative to the onset of the first stimulus). Each epoch was baseline corrected, using the time interval ranging from −0.5 to 0 s as reference, and band-pass filtered (1–30 Hz, fast Fourier transform filter). Electroculographic and electrocardiographic artifacts were subtracted using a validated method based on independent component analysis (Jung et al. 2000). In all data sets, independent components (ICs) related to eye movements had a large electrooculogram channel contribution and a frontal scalp distribution. Finally, epochs in which both laser stimuli were not perceived, epochs with pain ratings 2SDs above or below the average of the condition, and epochs containing artifacts exceeding ±100 μV were rejected from further analysis. The average absolute number of rejected epochs ranged, across blocks, between 0 and 0.24. For each subject, epochs were averaged according to trial category (SINGLE, 250, 500, 1,000, 2,000), yielding five average waveforms for the constant condition and five averaged waveforms for the variable condition. Because the overlap between two LEP responses elicited by laser stimuli delivered in pairs at short ISI causes noticeable signal distortions (Mouraux et al. 2004), to isolate the LEP elicited by the second stimulus (S2-LEP) from the LEP elicited by the first stimulus (S1-LEP), average waveforms obtained from single-stimulus trials (SINGLE-LEPs) were subtracted from the average waveforms obtained from paired stimuli at each ISI category for each subject (Luck 2005). Thus this procedure yielded four new average waveforms containing only the S2-LEP responses for each experimental condition.

The N2–P2 complex was measured at the vertex (Cz) referenced to the nose and defined as the largest negative–positive deflection occurring after stimulus onset. The N1 wave was measured at the central electrode contralateral to the stimulated side referenced to Fz (C3-Fz), following the procedure described by Hu et al. (2010). It was defined as the negative deflection preceding the N2 wave, which appears as a positive deflection in this montage. For each subject and ISI, the amplitudes of the N1 and N2–P2 waves of the S2-LEP were measured from the subtracted average waveforms. To examine changes of S2-LEP magnitude as a function of ISI, the amplitudes of the S2-LEP peaks were expressed as a percentage of the amplitude of the LEP peaks recorded in the SINGLE condition (i.e., the same procedure used by Truini et al. 2004, 2007). Statistical comparisons were performed using Prism 5.0 (GraphPad Software, San Diego, CA). A two-way, repeated-measures ANOVA was used to assess the main effects of the factors "ISI" (four levels: 250, 500, 1,000, and 2,000 ms) and "condition" (two levels: constant and variable), as well as their possible interaction, on the probability of S2 detection, rating of S2 perceived intensity, and on the latencies and amplitudes of the N1 wave and the N2–P2 complex of S2-LEP. When variance was not homogeneous, ANOVA results were reported after the Greenhouse–Geisser correction. When main effects were significant, Bonferroni post hoc tests were used to perform pairwise comparisons. Furthermore, to assess the distortion caused by signal overlap, we compared the latencies and amplitudes of the N2–P2 complex measured in the unsubtracted and subtracted LEP waveforms at each of the four ISIs, using paired-sample *t*-tests.

RESULTS

Probability of detection and pain ratings

Subjects described the perception of both the first and the second stimulus of the pairs as a distinct pricking, first-pain sensation, related to the activation of Aδ nociceptors. Two-way repeated-measures ANOVA revealed that there was a significant main effect of both the factor "ISI" [$F(3,27) = 5.942$, $P < 0.01$] and the factor "condition" [$F(1,9) = 10.76$, $P < 0.01$] on the probability of detecting the two stimuli of the pair as two distinct pinprick sensations, with a suggestion of interaction between these two effects [$F(3,27) = 2.488$, $P = 0.0702$] (Fig. 2, *top*). Post hoc comparisons revealed that at the 250-ms ISI, the probability of detecting S2 was significantly lower in the variable condition than that in the constant condition ($P < 0.001$). In contrast, there was a significant main effect of the factor "ISI" [$F(3,27) = 5.055$, $P = 0.0037$], but not of the factor "condition" [$F(1,9) = 0.0005$, $P = 0.9820$] on the S2 pain ratings, without interaction between them [$F(3,27) = 1.209$, $P = 0.3152$] (Fig. 2, *bottom*). Post hoc comparisons revealed that pain ratings at 250- and 500-ms ISIs were significantly higher than pain ratings at 1,000-ms ISI ($P < 0.05$).

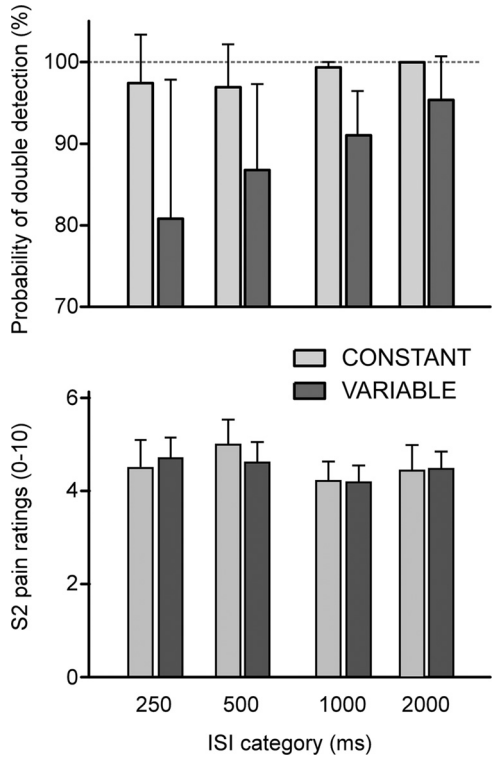

FIG. 2. Probability of detection and pain ratings. *Top*: *x*-axis, ISI category (ms); *y*-axis, probability of detecting the stimuli of each pair as 2 distinct pinprick sensations (%, means ± SE, $n = 10$). Two-way repeated-measures ANOVA revealed a significant main effect of both the factor "ISI" [$F(3,27) = 5.942$, $P < 0.01$] and the factor "condition" [$F(1,9) = 10.76$, $P < 0.01$] on the probability of detecting the 2 stimuli of the pair as 2 distinct pinprick sensations. Post hoc comparisons revealed that at 250-ms ISI, the probability of detecting the second laser stimulus (S2) was significantly lower in the variable condition (dark gray bars) than in the constant condition (light gray bars) ($P < 0.001$). *Bottom*: *x*-axis, ISI category (ms); *y*-axis, pain ratings elicited by S2 (0–10). Two-way repeated-measures ANOVA revealed a significant main effect of the factor "ISI" [$F(3,27) = 5.055$, $P = 0.0037$], but, crucially, not of the factor "condition" [$F(1,9) = 0.0005$, $P = 0.9820$] on the ratings of S2 pain ratings, without interaction between them [$F(3,27) = 1.209$, $P = 0.3152$].

LEPs elicited by single stimuli

All subjects displayed a clear N2–P2 complex in response to the SINGLE stimulus. In both the constant and the variable conditions the N2 and P2 waves were maximal at the vertex (Cz). The scalp distribution of the N2 wave extended bilaterally toward temporal electrodes, whereas the P2 was more centrally distributed. At electrode Cz, the average N2–P2 peak-to-peak amplitude, computed across subjects, was 32.9 ± 15.2 μV in the constant condition and 29.6 ± 15.5 μV in the variable condition. Latencies of the N2 and P2 peaks were 218 ± 37 and 386 ± 51 ms in the constant condition and 219 ± 28 and 359 ± 34 ms in the variable condition. Both amplitude and latencies of the N2–P2 complex elicited by single stimuli were not significantly different between conditions (N2–P2 amplitude: $P = 0.49$; N2 latency: $P = 0.91$; P2 latency: $P = 0.055$; paired *t*-test).

All subjects also displayed a clear N1 wave in response to the SINGLE stimulus. The N1 wave was maximal at the central electrode contralateral to the stimulated hand (C3), in both the constant and the variable conditions. At electrode C3, the average N1 peak amplitude, computed across subjects, was −4.69 ± 3.6 μV in the constant condition and −3.9 ± 2.1 μV in the variable condition. Latency of the N1 peak was 174 ± 30 ms in the constant condition and 178 ± 34 ms in the variable condition. Both amplitude and latency of the N1 peak elicited by single stimuli were not significantly different between conditions (N1 amplitude: $P = 0.43$; N1 latency: $P = 0.14$; paired *t*-test).

LEPs elicited by pairs of stimuli

SIGNAL OVERLAP. When laser stimuli were delivered in pairs, the overlap between the two LEP responses caused noticeable signal distortions. In particular, signal overlap led to a significant underestimation of the amplitude of the N2–P2 amplitude of S2-LEP when the ISI was 250 ms, both in the constant condition ($P < 0.05$, paired *t*-test) and in the variable condition ($P < 0.01$, paired *t*-test) (Fig. 3).

EFFECT OF ISI AND PRESENTATION PARADIGM. Two-way repeated-measures ANOVA revealed that there was a significant main effect of the factor "ISI" [$F(3,27) = 4.549$, $P < 0.01$] and a trend for a main effect of the factor "condition" [$F(1,9) = 4.23$, $P = 0.055$] on the amplitude of the N2–P2 complex of the S2-LEP (Figs. 4 and 5). Importantly, there was significant interaction between these two factors [$F(3,27) = 3.577$, $P < 0.05$]. Post hoc comparisons revealed that the N2–P2 amplitude of S2-LEP at 250-ms ISI was significantly smaller in the constant condition than that in the variable condition ($P < 0.05$). In contrast, there was no significant effect of either "ISI" or "condition" on the peak latency of the N2–P2 complex of the S2-LEP ("ISI": N2 latency, $P = 0.065$; P2 latency, $P = 0.522$; "condition": N2 latency, $P = 0.650$; P2 latency, $P = 0.25$) and no interaction between these two factors (N2 latency: $P = 0.315$; P2 latency: $P = 0.439$).

Two-way repeated-measures ANOVA with Greenhouse–Geisser correction revealed that there were no significant main effects of either "ISI" [$F(3,27) = 1.854$, $P = 0.208$] or "condition" [$F(1,9) = 0.903$, $P = 0.370$] on the N1 amplitude of the S2-LEP and no significant interaction between these two factors [$F(3,27) = 1.605$, $P = 0.214$]. Finally, there was no significant effect of either "ISI" [$F(3,27) = 0.9407$, $P = 0.4311$] or "condition" [$F(3,27) = 0.7743$, $P = 0.3847$] on the peak latency of the N1 wave of S2-LEP and no significant interaction between these two factors [$F(3,27) = 0.0418$, $P = 0.9884$].

DISCUSSION

The present study shows that the context within which pairs of laser stimuli are presented crucially determines the effect of stimulus repetition on the elicited LEPs. When pairs of stimuli are presented using a paradigm in which the time of occurrence of the second stimulus is constant, i.e., nonnovel and highly predictable (*constant* condition), a strong ISI-dependent suppression of the N2–P2 complex of the LEP elicited by the second stimulus of the pair is observed. In contrast, when the same pairs of stimuli are presented using a paradigm in which the time of occurrence of the second stimulus is variable, i.e., more novel and highly unpredictable (*variable* condition), the amplitude of the N2–P2 complex of the LEP elicited by the second stimulus of the pair is entirely unaffected by stimulus repetition, even for ISIs as short as 250 ms. These results

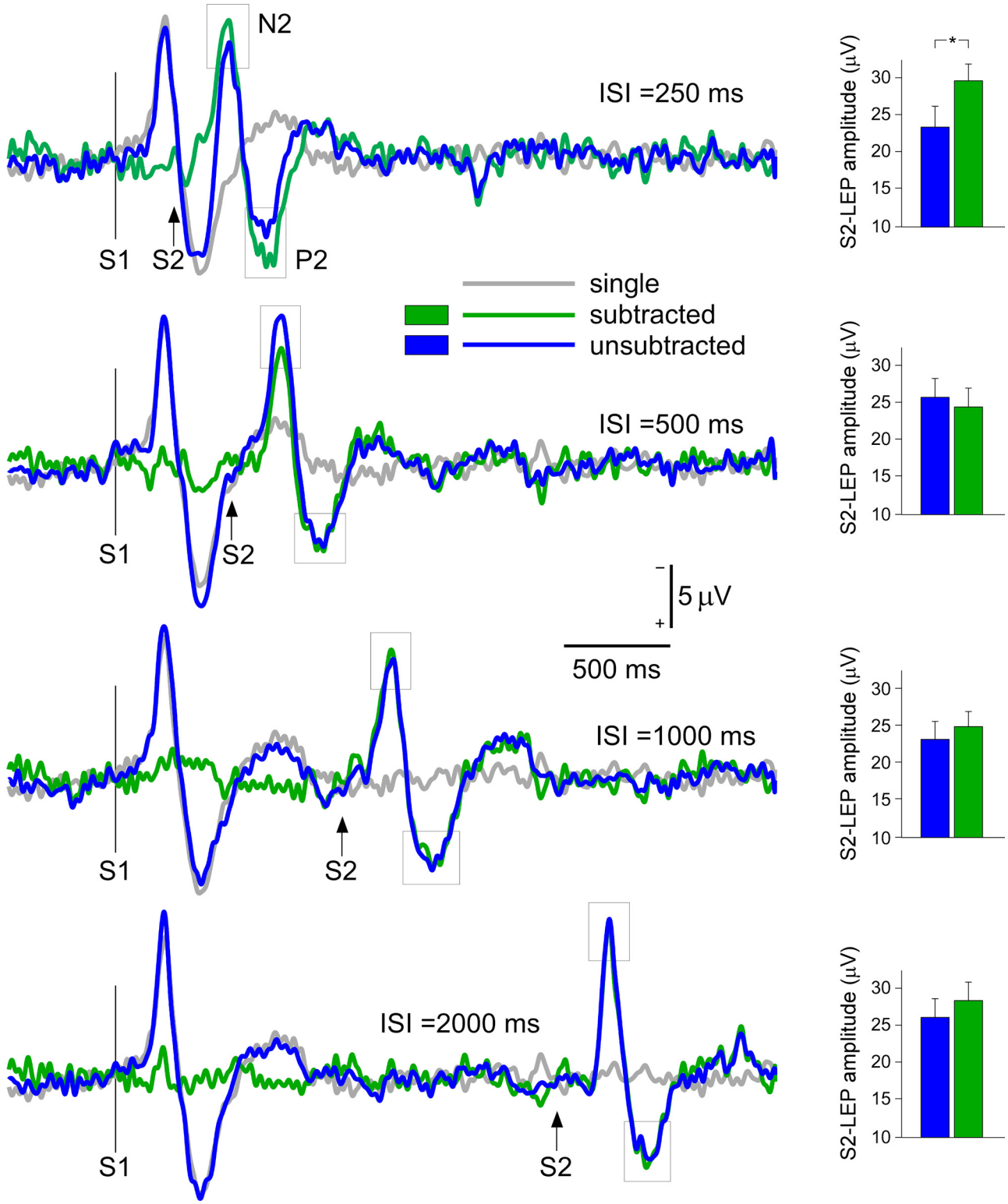

FIG. 3. Waveform overlap on event-related potentials (ERPs) elicited by paired laser stimuli. *Left*: laser stimuli elicit ERPs whose main constituent is a biphasic negative–positive wave (N2–P2), maximal at the vertex (Cz). The horizontal calibration bar represents time (500 ms). The vertical calibration bar represents amplitude (5 μV, negativity plotted upward). Each row represents the group-level average waveforms (Cz vs. nose reference, $n = 10$) elicited by pairs of laser stimuli presented at 4 different ISIs (250, 500, 1,000, and 2,000 ms). The vertical bars indicate the onset of the first (conditioning) laser stimulus (S1), whereas the arrows indicate the onset of the second (test) laser stimulus (S2). Blue waveforms represent the unsubtracted ERPs elicited by the 2 stimuli. Green waveforms represent the ERPs obtained by subtracting the ERP waveform obtained in the single condition (shown in gray) from the unsubtracted ERPs (in blue). *Right*: histograms display the average amplitude of the N2–P2 wave elicited by S2, in the unsubtracted (blue) and subtracted (green) waveforms (average of single-subject ERP amplitudes; error bars show variance across subjects, expressed as SE). Note how the signal overlap between the ERPs elicited by S1 and S2 causes a significant underestimation of the amplitude of the ERP elicited by the second stimulus at 250-ms ISI ($P < 0.05$, paired t-test).

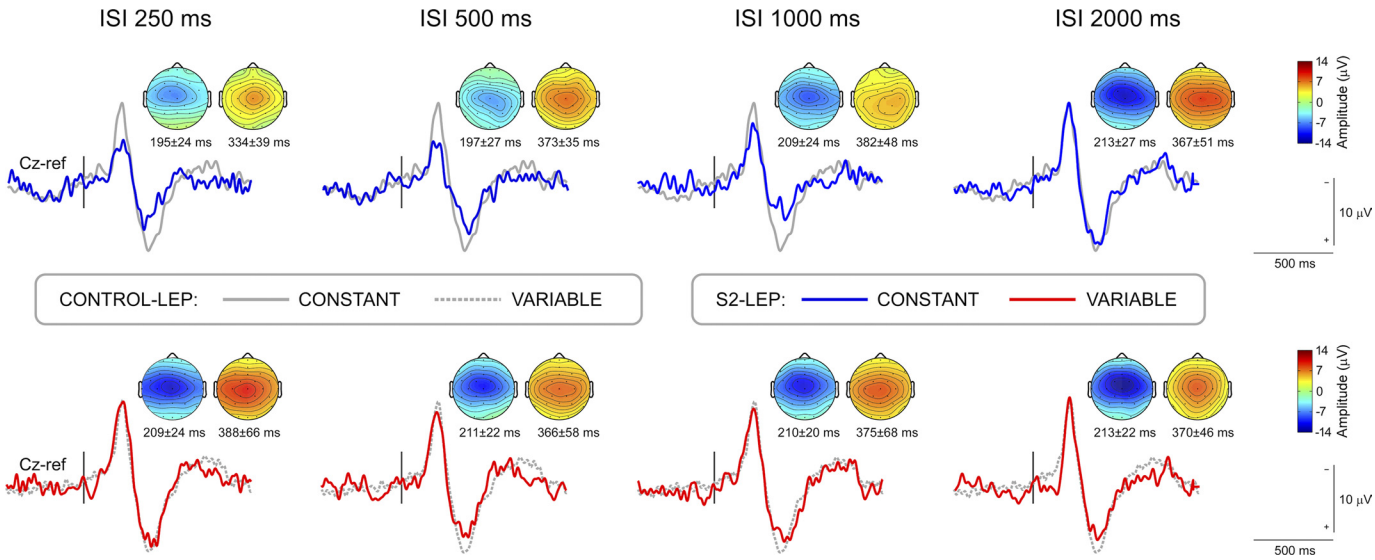

FIG. 4. Waveforms and scalp topography of LEPs elicited by pairs of laser stimuli delivered at different ISIs using constant and variable presentation paradigms. Group-average LEP waveforms (Cz vs. nose reference, $n = 10$) elicited by pairs of laser stimuli presented at ISIs of 250, 500, 1,000 and, 2,000 ms. Blue waveforms represent the LEPs elicited by the second stimulus of pairs presented using a *constant* ISI across trials (low stimulus novelty and temporal uncertainty; see Fig. 1, *left*). Red waveforms represent the LEPs elicited by the second stimulus of pairs presented using a *variable* ISI across trials (high stimulus novelty and temporal uncertainty; Fig. 1, *right*). Full and dashed gray waveforms represent the control ERPs elicited by single laser stimuli in the constant and variable conditions, respectively. The horizontal calibration bar represents time (500 ms). The vertical calibration bar represents amplitude (10 μV, negativity plotted upward). The topographies of the N2 and the P2 peaks are displayed in the corresponding scalp maps. Note that presenting pairs of laser stimuli at short ISI significantly reduces the S2-LEP amplitude only when pairs of stimuli are presented using a *constant* ISI across trials ["ISI" × "condition" interaction: $F(3,27) = 3.577$, $P < 0.05$].

indicate that refractoriness due to change in excitability of the afferent nociceptive pathways (Truini et al. 2004) cannot explain the previously observed reduction of the LEP elicited by the second stimulus of the pair at short ISIs and clearly exclude the "order of arrival" as a main determinant to LEP elicitation and magnitude. Instead, these results indicate that this reduction can be mainly explained by a modulation of the novelty of the eliciting stimulus. Since novelty is an important factor determining the saliency of any sensory stimulus, our results provide further support to the notion that the magnitude of the LEP response mostly reflects brain processes involved in saliency detection (Iannetti et al. 2008; Legrain et al. 2009), regardless of the "order of arrival" of the stimulus (Garcia-Larrea 2004).

In addition, these results have far-reaching implications in the field of perceptual neuroscience. Indeed, the clear dissociation between pain ratings and LEP amplitude observed at short ISIs in the *constant* condition provides further support to the evidence, already obtained with both electrophysiological and functional magnetic resonance imaging (fMRI) methods in other sensory modalities (e.g., Chapman et al. 1981; Dowman 1996; Kulkarni et al. 2005; Seminowicz and Davis 2007), that a significant portion of the brain activity time-locked to a brief and transient sensory stimulus is not directly related to the quality and the intensity of the corresponding sensation, but is instead related to bottom-up attentional processes (e.g., orienting). Therefore this notion has to be taken into consideration when using transient sensory stimuli to investigate the neural basis of sensory perception, regardless of the imaging methods used.

Signal overlap

When sensory stimuli are presented in pairs and the ISI is shorter than the latency of the last ERP deflection elicited by

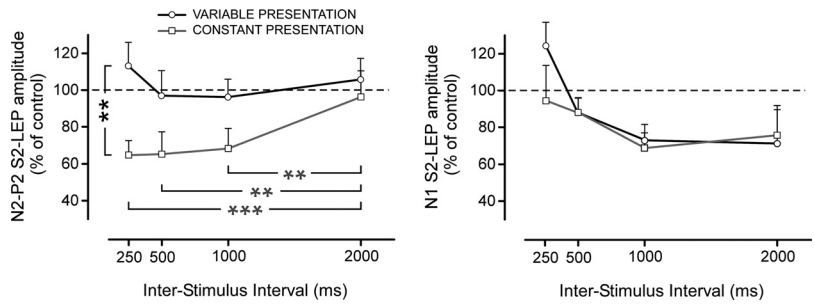

FIG. 5. Amplitude of LEPs elicited by pairs of laser stimuli delivered at different ISIs using constant and variable presentation paradigms. *x*-axis, ISI (ms); *y*-axis, amplitude of the S2-LEP, expressed as a percentage of the LEP elicited by a single laser stimulus (means ± SE, $n = 10$). Grey and black lines represent the constant and the variable presentation conditions, respectively. *Left*: N2–P2 amplitude. Two-way repeated-measures ANOVA revealed that there was a significant interaction between the experimental factors "ISI" and "condition" [$F(3,27) = 3.577$, $P < 0.05$]. Post hoc comparisons revealed that, in the constant condition, the N2–P2 amplitude of S2-LEP at 250, 500, and 1,000 ms was significantly lower than the N2–P2 amplitude of S2-LEP at 2,000-ms ISI (grey asterisks; $^{**}P < 0.01$; $^{***}P < 0.001$). Post hoc comparisons also revealed that the N2–P2 amplitude of S2-LEP at 250-ms ISI was significantly smaller in the constant than that in the variable condition (black asterisks; $^{**}P < 0.01$). *Right*: N1 amplitude. Two-way repeated-measures ANOVA revealed no main effect of either "ISI" [$F(3,27) = 1.854$, $P = 0.208$] or "condition" [$F(1,9) = 0.903$, $P = 0.370$].

the first stimulus plus the latency of the first ERP deflection elicited by the second stimulus, an overlap between the ERPs elicited by the first stimulus and the second stimulus of the pair is observed (Fig. 3) (Woldorff 1993). This overlap can be corrected by arithmetically subtracting the ERP elicited by a single stimulus, under the assumption that the ERP elicited by a single stimulus is identical to the ERP elicited by the first stimulus of the pair, and that ERP signals summate in a linear fashion (Luck 2005). As shown in Fig. 3, when examining LEPs elicited at ISIs comprised between 250 and 2,000 ms, the signal overlap significantly affects both peaks of the N2–P2 wave of the S2-LEP at 250-ms ISI and the N2 peak of the N2–P2 wave of the S2-LEP at 500-ms ISI. For this reason, at these intervals the estimates of the S2-LEP amplitude performed without taking into account the overlap with the S1-LEP are unreliable. Therefore the fact that Truini et al. (2004) measured the amplitude of the P2 peak of the LEP elicited by the second stimulus of a pair, delivered at an ISI of 250 ms without subtracting the LEP elicited by the first stimulus of the pair, may have led to an underestimation of the actual LEP amplitude and thus to an overestimation of the effect of stimulus repetition.

Challenging the "first come, first served" hypothesis

The observations that when laser stimuli are presented in pairs, the S2-LEP is significantly reduced at short ISIs (Raij et al. 2003; Truini et al. 2004, 2007), has been interpreted as a consequence of "neuronal refractoriness" of polysynaptic nociceptive afferent pathways and LEP generators (Truini et al. 2004) and as evidence that "only the earliest of a series of somatosensory volleys elicits cerebral responses synchronous enough to yield ERPs" (Truini et al. 2007), thus supporting the view that the first afferent volley obligatorily affects the processing of the second afferent volley ("first come, first served" hypothesis; Garcia-Larrea 2004). This "first come, first served" hypothesis has been also suggested as an explanation for the lack of a clear C-fiber LEP following an Aδ-fiber LEP (Garcia-Larrea 2004).

This neuronal refractoriness explanation interprets the observed response decrement as a consequence of basic neurophysiological mechanisms, related to a transiently reduced state of neuronal excitability (Hille 1991). However, this explanation is ruled out by experimental evidence both from our group and from other groups, showing that when pairs of sensory stimuli (both nociceptive and nonnociceptive) are delivered at variable ISIs, the ERP elicited by the second stimulus of a pair is either unaffected or even enhanced compared with the ERP elicited by the first stimulus (Budd and Michie 1994; Loveless et al. 1989; Mouraux et al. 2004; Ohman and Lader 1972; Wang et al. 2008). In the present study, we directly compared the ERPs elicited by pairs of stimuli with identical ISIs, but presented using variable versus constant conditions (Fig. 1), and confirmed this finding: when pairs of stimuli are presented using a variable condition, the S2-LEP is not reduced, compared with the control response (Figs. 4 and 5). Furthermore, behavioral results showing a lack of reduction (and, in fact, a slight but significant increase) of pain ratings at short ISIs (i.e., 250 and 500 ms) provide additional evidence against the neural refractoriness explanation. Therefore neural refractoriness and any form of "first come, first served" interaction are not likely to explain either the reduction of the S2-LEP that is systematically observed when ERPs are elicited by pairs of stimuli presented using a *short* and *constant* ISI (Angel et al. 1985; Armington 1964; Bourbon et al. 1987; Bromm and Treede 1987; Budd et al. 1998; Chapman et al. 1981; Davis et al. 1966; Fruhstorfer 1971; Hari et al. 1982; Lehtonen 1973; McEvoy et al. 1997; Nelson and Lassman 1968; Raij et al. 2003; Shipley and Hyson 1977; Truini et al. 2004, 2007) or the lack of a clear C-fiber LEP following an Aδ-fiber LEP (Bromm and Treede 1987). Indeed, a response decrement induced by stimulus repetition would constitute evidence supporting the "first come, first served" hypothesis (or the "refractoriness" hypothesis, or the "relative refractoriness of sensory networks generating LEPs"; Truini et al. 2007) *if and only if* the response decrement was still observed when pairs of stimuli were presented using an ISI randomly varied from trial to trial. In addition, the observation that the amplitude of the middle-latency N1 wave, which is thought to more closely reflect the incoming nociceptive input (Lee et al. 2009), was not modulated by ISI contributes evidence showing that neural refractoriness in the afferent pathways does not hold as a suitable explanation for the reduction of the S2-LEP observed when pairs of laser stimuli are presented at short and constant ISIs (Truini et al. 2004, 2007).

Importantly, the behavioral results of the present study (Fig. 2) also corroborate the view that the "first come, first served" hypothesis cannot explain the response decrement induced by stimulus repetition. Indeed, we observed that, in the variable condition, the probability of perceiving the second stimulus as a separate percept is lower at short ISIs (Fig. 2). This finding confirms the general notion that distance in time is an important factor determining whether physically separated stimuli are integrated as a unified percept, but only when the interval between the two stimuli is varied from trial to trial (King and Calvert 2001; Meredith et al. 1987). This has already been observed in the nociceptive system: when two laser stimuli are delivered close in space using a short ISI varied from trial to trial, subjects are likely to perceive them as a single percept, whereas if two stimuli are delivered using a long ISI, subjects are likely to perceive them as two distinct percepts (Lee et al. 2009; Mouraux et al. 2004). Crucially, the magnitude of the N2–P2 wave is selectively reduced when the second stimulus of the pair is unperceived (Lee et al. 2009). Thus the already significant reduction of the amplitude of the N2–P2 wave of the S2-LEP in the constant condition (Figs. 4 and 5) would be even underestimated by the fact that S2 is less frequently perceived in the variable than in the constant condition (Fig. 2).

Supporting the saliency hypothesis

If neuronal refractoriness is ruled out, what is the mechanism underlying the response decrement repeatedly observed when stimuli are presented using a short and constant ISI? We believe that this reduction can be mainly explained by the modulation of the saliency of the eliciting stimulus (Iannetti et al. 2008; Mouraux and Iannetti 2009).

The saliency of a sensory stimulus is not simply defined by one of its physical dimensions, but by the contrast by which these physical dimensions differ relative to the surrounding (Itti and Koch 2001) and preceding sensory input (Kayser et al.

2005). Thus one of the main determinants of saliency is how different (i.e., novel) a sensory stimulus is, compared with the preceding input. In the constant condition, the intertrial interval (ITI) was long and randomly varied across trials (e.g., between 15 and 18 s), whereas the ISI was identical across trials (Fig. 1, *left*). Therefore S1 occurred after a long period without somatosensory stimulation, whereas S2 occurred systematically after a short and constant delay from the preceding somatosensory stimulus. Consequently, in the constant condition, the novelty of S2 was, within each block, much lower than the novelty of S1. In addition to this lower bottom-up novelty of S2, in the constant condition the time of occurrence of S2 was much less uncertain than the time of occurrence of S1 because the ISI was identical across trials. In contrast, in the variable condition, both the ITI and the ISI were randomly varied across trials (Fig. 1, *right*). Thus both bottom-up novelty (change in time of occurrence) and top-down cognitive expectations (uncertainty of time of occurrence) were different in the constant and variable conditions.

Both novelty and uncertainty are important factors contributing to the saliency of any sensory stimulus, i.e., to its ability to involuntarily attract attention. Our results indicate that the response decrement observed when stimuli are presented using a short and constant ISI (i.e., when stimuli are nonnovel and their occurrence is certain) reflects the notion that the magnitude of ERPs elicited by noxious stimuli is largely determined by the saliency of the eliciting stimulus, rather than by pain perception per se (Chapman et al. 1981; Dowman 1996; Iannetti et al. 2008). Notably, the magnitude of a large part of the blood oxygenation level dependent–fMRI response is also reduced when identical sensory stimuli are repeated at constant ISI (e.g., Grill-Spector et al. 2006), thus indicating that a large part of the brain response elicited by transient stimuli may be related to stimulus saliency and, possibly, attentional orientation.

What are the components of the LEP response that could be modulated by paradigms that reduce the novelty of the eliciting stimulus and thus its saliency? By comparing ERPs elicited by stimuli belonging to different sensory modalities we recently provided a quantitative assessment of the multimodal and modality-specific neural activities underlying the LEP response (Mouraux and Iannetti 2009). We observed that LEPs can be entirely explained by a combination of multimodal neural activities (i.e., activities also elicited by stimuli of other sensory modalities) and early somatosensory-specific, but not nociceptive-specific, neural activities (i.e., activities elicited by both nociceptive and nonnociceptive somatosensory stimuli). Iimportantly, regardless of the sensory modality of the eliciting stimulus, the magnitude of multimodal activities correlated with the subjective rating of stimulus saliency, indicating that these multimodal responses are probably involved in stimulus-triggered mechanisms of arousal or attentional reorientation. These findings suggest that the observed reduction of LEP magnitude consequent to the reduced stimulus saliency observed in the present and in previous studies (Iannetti et al. 2008; Raiji et al. 2003; Truini et al. 2004, 2007) may mainly reflect a modulation of multimodal neural activities whose magnitude is strongly reduced by the fact that less novel stimuli are also less prone to capture attention and thus less likely to elicit an orienting response.

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DISCLOSURES

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