Brain potentials evoked by intraepidermal electrical stimuli reflect the central sensitization of nociceptive pathways

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Abstract

Central sensitization (CS), the increased sensitivity of the central nervous system to somatosensory inputs, accounts for secondary hyperalgesia, a typical sign of several painful clinical conditions. Brain potentials elicited by mechanical punctate stimulation using flat-tip probes can provide neural correlates of CS, but their signal-to-noise ratio is limited by poor synchronisation of the afferent nociceptive input. Additionally, mechanical punctate stimulation does not activate nociceptors exclusively. In contrast, low-intensity intra-epidermal electrical stimulation (IES) allows selective activation of type-II Aδ mechano-heat nociceptors (II-AMHs), and elicits reproducible brain potentials. However, it is unclear whether hyperalgesia from IES occurs and co-exists with secondary mechanical punctate hyperalgesia, and whether the magnitude of the EEG responses evoked by IES within the hyperalgesic area is increased. To address these questions, we explored the modulation of the psychophysical and EEG responses to IES by intra-epidermal injection of capsaicin in healthy human subjects. We obtained three main results. First, the intensity of the sensation elicited by IES was significantly increased in participants who developed robust mechanical punctate hyperalgesia after capsaicin injection (i.e., responders), indicating that hyperalgesia from IES co-exists with punctate mechanical hyperalgesia. Second, the N2 peak magnitude of the EEG responses elicited by IES were significantly increased after the intra-epidermal injection of capsaicin in responders only. Third, a receiver-operator characteristics analysis showed that the N2 peak amplitude is clearly predictive of the presence of CS. These findings suggest that the EEG responses elicited by IES reflect secondary hyperalgesia, and therefore represent an objective correlate of CS.

New & Noteworthy

Secondary mechanical punctate hyperalgesia is a cardinal sign of central sensitization (CS), an important mechanism for chronic pain. Our study demonstrates that hyperalgesia from intra-epidermal electrical stimulation coexists with mechanical punctate hyperalgesia and elicits electroencephalographic (EEG) potentials that predict the robust occurrence of punctate hyperalgesia in a human experimental model of CS. These findings inform clinical development of EEG-based biomarkers of CS.

Keywords:

central sensitization; secondary hyperalgesia; mechanical punctate stimulation; intraepidermal electrical stimulation; EEG.

Introduction

Central sensitization (CS) refers to the increased sensitivity of the central nervous system to somatosensory inputs. CS accounts for the enhanced painful percepts elicited by nociceptive stimulation of the skin surrounding a site of tissue injury (secondary hyperalgesia) (Ringkamp et al. 2013), and it has been suggested to be an important contributor to several chronic pain states (Ji et al. 2003; Latremoliere and Woolf 2009). A cardinal sign of CS is secondary hyperalgesia to nociceptive punctate mechanical stimuli, also known as secondary mechanical punctate hyperalgesia. Such punctate stimuli, when delivered using flat-tip probes, preferentially activate the free-nerve endings of type-I Aδ mechano-heat nociceptors (I-AMH) (Magerl et al. 2001). CS is typically established by an intense activation of C-fibre skin nociceptors: the resulting afferent barrage to the dorsal horn results in a hetero-synaptic facilitation of I-AMH inputs, which substantiates secondary mechanical punctate hyperalgesia (Geber et al. 2007; Ziegler et al. 1999).

Secondary mechanical punctate hyperalgesia has been quantified by measuring the brain activity using non-invasive functional neuroimaging techniques, like functional magnetic resonance imaging (fMRI) (Lee et al. 2008) and magnetoencephalography (MEG) (Maihofner et al. 2010). Given that secondary hyperalgesia is a well-established surrogate model for centrally generated hyperalgesia in chronic pain patients, such neural correlates have potential clinical and pharmaceutical applications. However, fMRI and MEG are costly and not readily available. In contrast, electroencephalography (EEG) is more affordable and routinely used in clinical practice. Moreover, previous studies have shown that punctate stimulation causing pin-prick-like pain can elicit EEG potentials, whose amplitudes reflect subjective reports of secondary mechanical punctate hyperalgesia (Davies et al. 2010;

90 Iannetti et al. 2013). However, there are technical and physiological constrains that may
91 hamper clinical translation of pinprick-evoked potentials. First, the mechanical stimulus is
92 generated by hand-held probes. The use of hand-held probes is operator dependent, which
93 limits reproducibility of stimulus delivery. Second, given that the force exerted is driven
94 passively by a weighted cylinder (Magerl et al. 2001), the probe needs to be held
95 perpendicularly to both the skin and the ground, in order to ensure that a consistent force is
96 applied. This limits the number of body territories that can be effectively stimulated.
97 Pneumatically driven (Kohlloffel et al. 1991) or solenoid-powered (Davies et al. 2010)
98 mechanical devices have also be described: they circumvent some of the difficulties
99 associated with the use of hand-held probes. However, any device that relies on mechanical
100 stimulation to activate cutaneous nociceptors remains limited by a crucial factor, the
101 variability in skin compliance. This limits the synchronicity of nociceptor activation,
102 introduces high variability of spatial and temporal summation at central synapses, and thus
103 makes the estimation of response latency and amplitude difficult. Third, when using
104 mechanical probes, the spatial location of the stimulated spot is typically changed between
105 trials, which further increases the variability of the afferent nociceptive input. Lastly, and
106 most importantly, mechanical punctate stimulation activates intra-epidermal nociceptive
107 nerve endings *preferentially*, but not selectively. Indeed, at higher stimulus intensities the
108 dermis and subcutaneous tissues are more likely to become temporarily deformed, which
109 may result in a certain degree of activation of deeper Aβ afferents (Treede et al. 2002).

110 A possible alternative to punctate stimulation is the selective activation of Aδ nociceptors by
111 simple and affordable concentric electrodes that are designed to deliver currents exclusively
112 to the epidermal skin layers, where the free nerve-endings of nociceptors ramify (Inui and

Kakigi 2012; Inui et al. 2002). Psychophysical, behavioural and electrophysiological data indicate that, when used at low-intensity of current, intra-epidermal electrical stimulation (IES) activates Aδ nociceptors *selectively*, i.e. without coactivating Aβ afferents (Mouraux et al. 2010). Still, it remains to be determined whether the psychophysical and EEG responses evoked by IES are increased in the presence of secondary mechanical punctate hyperalgesia. This question is physiologically pertinent: given the evidence that IES predominantly activate type-II AMHs (Mouraux et al. 2010; Treede and Magerl 2000), the observation that EEG responses to IES are increased would imply that hyperalgesia from IES is also mediated by this class of nociceptive afferents.

Here, we explored whether IES evoked potentials hold promise as an objective neural correlate of secondary hyperalgesia. We intra-epidermally injected capsaicin in right hand of healthy subjects to induce a state of CS. Participants were classified in responders and non-responders based on whether or not they developed robust secondary mechanical punctate hyperalgesia. We then tested (1) whether subjects who developed secondary mechanical hyperalgesia also developed secondary hyperalgesia from nociceptive-specific IES. We also (2) explored whether the magnitude of the EEG responses to nociceptive IES delivered to the secondary hyperalgesic area was significantly increased and (3) quantified the sensitivity and specificity of the EEG responses elicited by IES for detecting the presence of secondary hyperalgesia in our study cohort.

Materials and Methods

Participants

Fourteen healthy right-handed volunteers participated in this study. All participants were pain-free, not taking any medication and did not have any history of severe allergic reactions to chilli peppers at the time of testing. They all gave signed written informed consent, and the experimental procedures were approved by the UCL Research Ethics Committee. Before the electrophysiological recording, the experimental setup and the psychophysical rating task were clearly explained to the participants, who were also familiarized with the sensation elicited by IES. Data from two participants were discarded because no clear event-related potential (ERP) could be identified, and the data from the remaining twelve participants (22-39 years, 7 female) were analysed.

Experimental design

The experimental design is summarised in Figure 1 (upper panel). Experiments were conducted in a silent and temperature-controlled room. Throughout the experiment participants sat on a comfortable chair with the hands resting on a table in front of them. Participants were instructed to keep their gaze fixed on a black cross (2 × 2 cm) placed centrally in front of them, at a distance of 1.5 m, ~20^{o} below eye level. To induce CS, capsaicin was injected intra-epidermally on the right hand dorsum (Ziegler et al. 1999). IES were delivered in two separate blocks, one before ('pre-capsaicin') and one after capsaicin injection ('post-capsaicin'). In the post-capsaicin block, IES were delivered only after capsaicin-induced spontaneous pain had resolved. In each block we delivered 20 stimuli on the left hand dorsum and 20 stimuli on the right hand dorsum, in pseudo-random order, with an inter-stimulus interval (ISI) of 8-12 s (rectangular distribution). Therefore, there were four conditions: (1) Pre-capsaicin, right hand (PreRH); (2) Pre-capsaicin, left-hand (PreLH); (3) Post-capsaicin, right-hand (PostRH); and (4) Post-capsaicin, left-hand (PostLH).

Three seconds after the stimulus onset, subjects were asked to state whether the stimulus was delivered on the right or the left hand, and to provide ratings of the perceived intensity of pinprick pain, using a numerical scale ranging from 0 (no pinprick sensation) to 100 (the most intense pinprick sensation imaginable).

Intra-epidermal electrical stimulation (IES)

IES consisted of two constant-current square-wave pulses delivered in rapid succession, as described in (Inui et al. 2002; Mouraux et al. 2010). Each pulse lasted 500 µs, and the inter-pulse interval was 10 ms (DS7, Digitimer, UK). Stimuli were delivered using a stainless steel concentric bipolar needle electrode, consisting of a needle cathode (length: 0.1 mm, Ø: 0.2 mm) surrounded by a cylindrical anode (Ø: 1.4 mm) (Inui et al. 2002; Mouraux et al. 2010). By gently pressing the device against the skin, the needle electrode was inserted into the epidermis. Two electrodes were applied, one on the dorsum of each hand. Once the electrodes were fixed, the thresholds for stimulus perception were determined for each hand and each subject, using an adaptive staircase procedure. The final intensity of the IES for the experiment was set to twice the perceptual threshold, to ensure selective stimulation of skin nociceptors (Mouraux et al. 2010).

After the thresholding procedure, we delivered a few stimuli at the intensity determined above, to familiarize the participant with the elicited sensation. If the participant reported a different perceived intensity on two hands, the location of the electrodes were adjusted on each participant until the reported intensities on both hands were similar, and then the thresholding procedure was repeated and the new stimulus intensity was determined.

Capsaicin injection

To induce CS, we injected intra-epidermally a 10 mM solution of capsaicin (40 µg in a 12.5 µl volume of normal saline containing 0.16% Tween 80; for details, see (LaMotte et al. 1991). The capsaicin solution was injected at an angle of approximately 15^{o} to the skin surface, using a 27-gauge disposable needle. The injection site was ~1.5 cm away from the IES electrode on the right hand dorsum. Therefore, IES was delivered on the skin area of secondary hyperalgesia away from the injection site where the skin would have been numbed by the local neurotoxic effects of capsaicin (LaMotte et al. 1992).

Capsaicin-induced spontaneous pain and secondary hyperalgesia assessment

Spontaneous pain intensity after capsaicin injection was recorded using a numerical rating scale ranging between 0 (no pain) and 100 (worst pain imaginable). Participants were required to rate verbally the intensity of spontaneous pain every 10 s during the first 3 minutes and then every 30 s until the pain intensity ratings were less than 5 out of 100.

The development of mechanical hyperalgesia in the skin area surrounding the injection site was confirmed by punctate mechanical stimulation of the skin adjacent (within 1 cm) to the external circumference of the concentric IES electrode, using a flat-tip punctate probe (256 mN). This probe comprises a stainless steel wire tip (Ø: 0.25 mm) attached to a mounted weight (256 mN) that glides smoothly within a hollow handheld cylindrical tube. When applied perpendicularly to the skin, the weight of the probe rested entirely on the wire tip, thus exerting a constant force of 256 mN. More details and a depiction of the punctate probe can be found in (Iannetti et al. 2013), as well as in the manufacturer website (MRC Systems GmbH; http://www.mrc-systems.de/en/products/pinprick). The same mechanical stimulus was applied to the corresponding position of the left hand, to obtain a baseline for quantifying the effect of secondary hyperalgesia, as follows. Participants were asked to

report the intensity of punctate stimulation of the right hand (capsaicin-injected) and of the left hand (control), using a numerical rating scale that ranged between 0 (no pinprick sensation) and 100 (the most intense pinprick sensation imaginable). For each hand, punctate stimuli were applied three times with an inter-stimulus interval of approximately 5 sec, after the spontaneous pain induced by the capsaicin injection in the right hand had decreased to less than 5 out of 100 (Figure 2). For every individual, the mean ratings of the sensations elicited by the three stimuli was obtained for each hand and condition. The intensity of secondary hyperalgesia was quantified as the ratio of the subjective ratings of the pinprick sensation elicited by mechanical stimulation of the right and the left hands (Right/Left). Participants were considered to have developed robust secondary hyperalgesia from punctate stimuli if the ratio was ≥2, and were thus classified as responders. All other participants were classified as non-responders. This ratio was chosen based on a previous EEG study, which showed that an approximately two-fold increase (+93%) in pinprick sensation elicited by punctate stimulation after capsaicin sensitisation was associated with significant increases in the evoked EEG response (Iannetti et al. 2013).

EEG recording

The EEG was recorded using 31 Ag-AgCl electrodes placed on the scalp according to the International 10-20 system, and referenced to the nose. Ocular movements and eye blinks were recorded using two surface electrodes, one placed over the right lower eyelid, the other placed approximately 1 cm lateral to the lateral corner of the right orbit. Signals were amplified and digitized using a sampling rate of 1,024 Hz (SD32, Micromed, Italy).

Behavioural data analysis

225 Single trial ratings of the sensation elicited by IES were first normalized between 0 and 100,
226 for each participant (the minimum value was set to 0 and the maximum value was set to
227 100). This procedure mitigates the differences in the range of values on the numerical rating
228 scale with which individuals reported the intensity of pinprick pain elicited by IES (Huang et
229 al. 2013). Normalized stimulus intensity ratings were subsequently averaged across trials for
230 each condition, resulting in four average values for each participant (PreRH, PreLH, PostRH,
231 PostLH).

232 To test whether capsaicin injection had an effect on the perceived IES intensity, we
233 performed a three-way ANOVA, with the following experimental factors: Group (two levels:
234 Responders, Non-Responders), Session (two levels: Pre-capsaicin, Post-capsaicin), and Hand
235 (two levels: Injected [Right], Control [Left]).

236 Where effects were significant, post-hoc analyses were performed to define their direction
237 and possible interactions. Two-way repeated-measures ANOVA for the main and interaction
238 effects of Session and Hand were performed to define the effects of capsaicin injection on
239 the intensity of the sensation elicited by IES, within each group. The statistical threshold of
240 the post hoc analyses was Bonferroni corrected accounting for the number of comparisons
241 ($P = 0.05/2 = 0.025$).

242

243 ***EEG data analyses***

244 EEG data analyses were performed using Letswave (www.nocions.org) (Mouraux and
245 Iannetti 2008) and Matlab (The MathWorks, Natick, MA). Continuous EEG recordings were
246 segmented into epochs using a time window of 2 s (-0.5 to 1.5 s relative to the stimulus

onset). Each epoch was baseline corrected (baseline interval ranging from -0.2 to 0 s), and band-pass filtered (1–30 Hz). Artefacts produced by eye blinks or eye movements were subtracted using a validated method based on independent component analysis (Jung et al. 2000). In all data sets, independent components related to eye movements had a large electrooculogram channel contribution and a frontal scalp distribution. In addition, epochs with amplitude values exceeding ±100 μV were rejected from further analysis. These epochs constituted 0.6 ±1.8% (mean ±SD across all conditions and participants) of the total number of epochs. Remaining epochs were then averaged for each condition, resulting in four average ERP waveforms for each participant.

The N2-P2 complex was measured at the vertex (Cz), and it was defined as the largest negative-positive deflection occurring after stimulus onset. The amplitude of both the N2 and P2 peaks were calculated for each condition and participant, and tested for the effect of capsaicin injection, using the same three-way ANOVA described for the behavioural data (Figure 1, lower panel). As two peaks (N2 and P2) were tested, the statistical threshold, P = 0.05/2 peaks = 0.025, was determined by Bonferroni correction accounting for the number of peaks. Where effects were significant, the same post-hoc analyses described for the behavioural data (i.e., two-way repeated measures ANOVA) were performed for each group, and the same statistical threshold, Bonferroni corrected (P = 0.05/2 groups = 0.025), was used to determine the significance of the post-hoc results. The latency of the N2 and P2 peaks were analysed using the same procedure.

To test the predictive value of ERP amplitude for the presence of central sensitisation, we plotted the receiver operating characteristic (ROC) curves obtained using the interaction term (i.e. (PostRH-PreRH)-(PostLH-PreLH)) calculated for the N2-wave and P2-wave peak

amplitudes. The true positive rate (Sensitivity) is plotted against the false positive rate (100-Specificity) for different cut-off values of the interaction terms. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold for the interaction term. Above each of these thresholds the individual is predicted to be a responder, and vice versa. If interaction terms had perfect classification performance, their ROC curves would pass through the upper left corner (100% sensitivity, 100% specificity). The closer the ROC curve is to the upper left corner, the higher the overall accuracy of the interaction term is in distinguishing responders and non-responders (Zweig and Campbell 1993). The Area Under Curve (AUC) is typically used to quantify the classification performance. An AUC value of 0.5 corresponds to a random classification (i.e. to a useless test), whereas an AUC of 1.0 indicates that the test performs perfectly. We calculated the AUC for the interaction terms obtained from the amplitude of the N2 and P2 peaks, to assess their sensitivity and specificity for detecting the presence of a CS state. We tested whether the AUC size of each measure was significantly greater than 0.5 (Hanley and McNeil 1982).

Results

Six out of twelve participants developed robust secondary hyperalgesia on the capsaicin-treated hand and were therefore classified as responders (Figure 2, upper panel).

Capsaicin-induced spontaneous pain

The time courses of the capsaicin-induced pain for all subjects are shown in the lower panel of Figure 2. In the first few seconds after the injection, capsaicin induced a very intense

sensation of burning pain, which decreased exponentially over time (Lee et al. 2008; Magerl et al. 1998). The time course of spontaneous pain ratings for each subject was summarised as area-under-curve (AUC). The AUC for responders and non-responders were compared using a two-sample t test. The result showed no significant difference in capsaicin-induced spontaneous pain between the two groups (T_{10} = 0.39, P = 0.70). This observation suggests that both groups perceived the conditioning stimulus (i.e. the intra-epidermal injection of capsaicin) similarly.

Psychophysics of intra-epidermal stimulation of the area of secondary mechanical punctate hyperalgesia

All subjects correctly reported whether the IES was delivered to the right or to the left hand, in all trials. The three-way ANOVA on the subjective ratings of perceived IES intensity showed a two-way interaction between Group and Hand ($F_{1,10}$ = 9.02, P = 0.01), and more importantly, a clear three-way interaction between Group, Session and Hand ($F_{1,10}$ = 59.27, P = 0.000016) (Figure 3). No other significant effects were detected (Table 1). This finding indicates that right hand stimulation was perceived as more painful than left hand stimulation in the responders, but only after capsaicin was injected in the right hand. The results of all post hoc two-way ANOVAs are shown in Table 2. Both responders ($F_{1,5}$ = 49.79, P = 0.001) and non-responders ($F_{1,5}$ = 15.19, P = 0.01) showed significant interactions between Session and Hand, but in opposite directions – the responders had clearly increased ratings on their treated hand after capsaicin injection, while the non-responders showed mildly decreased ratings on their treated hand after capsaicin injection (Figure 3).

The results demonstrate a clear secondary hyperalgesia from both IES and mechanical punctate stimulation after capsaicin injection.

ERP waveforms

ERPs elicited by IES stimuli showed a clear N2-P2 complex maximal at electrode Cz, in all four conditions of each group. Grand-average waveforms and scalp maps at N2 and P2 peak latencies are shown in Figure 4. The ERP amplitude increased after capsaicin injection in the right hand of the responders, compared with all other conditions. Statistical comparisons of peak amplitude and latency of the N2 and P2 waves across different conditions and groups are reported below, and summarized in Tables 1 and 2.

N2 peak amplitude. The three-way ANOVA of N2 peak amplitudes showed a three-way interaction between Group, Session and Hand ($F_{1,10}$ = 7.84, P = 0.019). No other significant effects were detected (Table 1). Hence, N2 peak amplitudes at Cz were greater following right-hand IES compared to left-hand IES in the responders, but only when IES were delivered to the hand where capsaicin had been injected (i.e. the right hand). Post hoc two-way ANOVAs (Table 2) revealed that only responders showed an interaction between Session and Hand ($F_{1,5}$ = 15.15, P = 0.011) indicating increased N2 amplitudes on their treated hand after capsaicin injection. Figure 5 shows the single-subjects N2 peak amplitudes, as well as the statistical results.

P2 peak amplitude. The three-way ANOVA of P2 peak amplitudes showed that there was a two-way interaction between Group and Session ($F_{1,10}$ = 11.13, P = 0.008). This effect was caused by an overall increased P2 amplitude in the post-capsaicin session of responders, but

a decreased P2 amplitude in the post-capsaicin session of non-responders. No other significant effects were detected (Table 1). Post hoc two-way ANOVAs (Table 2) showed that there was a trend for an interaction between Session and Hand which, however, did not survive correction for multiple comparisons in responders ($F_{1,5}$ = 9.77, P = 0.026): in this group, P2 amplitudes in the post-capsaicin session were, compared to the pre-capsaicin session, increased following right hand stimulation and slightly decreased following left hand stimulation.

N2 peak latency. The three-way ANOVA of N2 peak latencies showed a main effect of Hand ($F_{1,10}$ = 7.41, P = 0.022). No other significant effects were detected (Table 1). Post hoc two-way ANOVAs (Table 2) fail to detect any effects in either responders or non-responders that survived correction for multiple comparisons.

P2 peak latency. The three-way ANOVA on the P2 peak latencies did not detect any significant effect. Therefore, post hoc analyses were not performed.

ROC curves. The ROC curves obtained from N2 and P2 peak amplitudes are plotted in Figure 6. The AUC (±standard error) for N2 and P2 were 0.92 ±0.09 and 0.72 ±0.16, respectively. Only the AUC for N2 was significantly greater than 0.5 (N2: P = 0.016; P2: P = 0.200). This suggests that the N2 peak amplitude has adequate sensitivity and specificity for detecting the presence of CS induced by intra-epidermal injection of capsaicin.

Discussion

Developing a biomarker for secondary hyperalgesia, a cardinal symptom of central sensitization (CS), would be useful for both drug discovery and clinical therapy. Such a biomarker would help analgesic drug discovery in early phase trials, facilitate diagnosis of neuropathic pain, and allow objective monitoring of drug treatments in patients.

IES is a technically simple and inexpensive method to selectively stimulate type II Aδ skin nociceptors (Inui and Kakigi 2012; Inui et al. 2002; Mouraux et al. 2010). Importantly, IES elicits clear time-locked EEG responses, thus allowing quantification of CS. However, mechanical punctate hyperalgesia is known to be mediated by I-AMH units, rather than II-AMH units (Magerl et al. 2001). Given that IES selectively activates II-AMH units (Mouraux et al. 2010), we tested (1) whether secondary hyperalgesia from IES co-exists with secondary mechanical punctate hyperalgesia, and (2) whether such hyperalgesia is reflected in a corresponding increase in EEG responses.

We obtained several interesting results. First, the intensity of the sensation elicited by IES was significantly increased after intra-epidermal injection of capsaicin in those participants who developed robust mechanical punctate hyperalgesia – clearly showing that hyperalgesia from IES occurs and coexists with mechanical hyperalgesia. Second, the peak amplitude of the N2 wave elicited by IES was significantly increased in responders, similarly to the intensity of the sensation elicited by IES. This increased respons only occurred when IES were delivered to the hand where capsaicin was injected. Third, ROC analysis showed that the N2 peak amplitude offers the ability to predict the presence of CS with high sensitivity and specificity. These findings suggest that the EEG responses elicited by IES reflect secondary hyperalgesia and thus are a reliable neural correlate of CS.

Peripheral afferents mediating secondary hyperalgesia from IES

Whilst our observations clearly indicate that secondary hyperalgesia elicited by IES appears to co-exist with secondary hyperalgesia elicited by mechanical punctate stimuli, it remains unclear whether the two phenomena are mediated by similar populations of Aδ nociceptors. There is strong physiological evidence that secondary mechanical punctate hyperalgesia is mediated by I-AMH nociceptors. For example, Magerl and colleagues (2001) demonstrated that secondary mechanical punctate hyperalgesia still occurs in skin that was rendered devoid of II-AMH epidermal terminals by application of high concentrations of topical capsaicin (Magerl et al. 2001). In contrast, Mouraux and colleagues (2010) showed that both sensations and EEG responses elicited by IES were abolished in skin that was similarly treated with high-concentration capsaicin, suggesting that IES activates mostly II-AMH nociceptors (Mouraux et al. 2010). It follows that the secondary hyperalgesia from IES observed in this study is likely to be mediated mainly by II-AMH, rather than I-AMH nociceptors. However, further experiments are required to confirm whether hyperalgesia from IES and mechanical punctate stimulation are truly mediated by different populations of Aδ afferents. Nonetheless, it is plausible that, after capsaicin injection, inputs from both I-AMH and II-AMH nociceptors are heterosynaptically facilitated via a common central mechanism, and account for the co-existence of secondary hyperalgesia from IES and mechanical punctate stimulation (Ziegler et al. 1999).

Variability in capsaicin-induced secondary hyperalgesia

We observed considerable variability in the degree of punctate hyperalgesia that developed after intra-epidermal capsaicin injection. Only half of the subjects developed robust

402 punctate hyperalgesia (i.e. a two-fold increase of pain ratings when stimulating the injected
403 hand with respect to the control hand; Figure 2).

404 It is unlikely that this difference between responders and non-responders was related to the
405 strength of conditioning stimulus, i.e., the activation of C-nociceptors by intra-epidermal
406 injection of capsaicin. Indeed, both groups reported similar intensities and durations of
407 burning pain following intra-epidermal injection of capsaicin, which suggests that the
408 conditioning stimulus was similar for both groups. We note that the development of
409 secondary hyperalgesia can be highly variable even with a highly standardized electrical
410 conditioning stimulus, which suggests considerable differences in the development of CS
411 responses between individuals (Pfau et al. 2011). Furthermore, there is clear evidence that
412 genetic variability contributes to variability in hyperalgesic response following intra-
413 epidermal capsaicin injection (Tegeder et al. 2008).

414 *Brain potentials evoked by IES and central sensitization: advantages and limitations*

415 Previous studies have suggested that brain potentials elicited by punctate mechanical
416 stimulation may be recorded and employed as a potential objective correlates of the CS
417 states (Davies et al. 2010; Iannetti et al. 2013; Kohlloffel et al. 1991). However, as detailed in
418 the Introduction, evoked potentials elicited by punctate mechanical stimuli have significant
419 technical and physiological constrains that may hamper clinical translation.

420 In contrast, IES have several advantages over mechanical punctate stimulation. When
421 delivered at low currents, they are fully selective for Aδ nociceptors, and allow for accurate
422 timing and standardization of stimuli. The stimulating electrode is affordable and can be
423 affixed to any part of the body without difficulty.

424 The current results show that the amplitude of the ERP elicited by IES of the skin with
425 secondary hyperalgesia clearly reflects that the somatosensory system is centrally
426 sensitised. The amplitude of the N2 wave was significantly larger when IES were delivered to
427 the hand in which capsaicin injection resulted in a clear secondary hyperalgesia (Figures 4
428 and 5, Tables 1 and 2). Moreover, the areas under the ROC curves indicate that the change
429 in N2 peak amplitude was significantly predictive of the presence of secondary hyperalgesia
430 (Figure 6). This result suggests that the changes in N2 amplitude may be developed as a
431 potentially useful biomarker of CS.

432 Several limitations to IES remain. First, we were unable to isolate the early, contralateral N1
433 wave typically observed in the brain potentials evoked by nociceptive laser stimuli (Treede
434 et al. 1988; Valentini et al. 2012), most likely because of its lower signal-to-noise ratio.
435 Compared to the subsequent N2-P2 complex, the N1 wave has been shown to better reflect
436 the afferent nociceptive drive (Lee et al. 2009) and appears less susceptible to top-down
437 modulation, for example placebo manipulation (Martini et al. 2015). These characteristics
438 make the N1 wave a potentially more robust marker for central sensitisation. Second, the
439 selective activation of Aδ nociceptors by IES relies on the use of strictly low-intensity
440 currents. This limitation prevents recording stimulus response functions, as higher-intensity
441 currents necessarily entail a coactivation of tactile Aβ afferents, and therefore a loss of
442 specificity for Aδ fibre stimulation (Mouraux et al. 2010). Stimulus response functions are
443 particularly useful when assessing the analgesic potential of novel drugs as they can divulge
444 interactions between stimulus or pain intensity and dose effects. Recording of stimulus
445 response function using the brain response elicited by mechanical punctate stimuli is
446 similarly problematic because, as detailed earlier, when high forces are exerted the

mechanical punctate stimulus becomes less selective for Aδ fibre activation (Treede et al. 2002; van den Broeke et al. 2015). More recent data reveal that stimulus response functions can be constructed using IES, by varying the number of pulses delivered in quick succession (5 ms intervals) to normal skin – increasing the number of pulses increases the intensity of sensation and EEG amplitudes without changing reaction times or response latencies (Mouraux et al. 2014). Further experiments are required to ascertain if this remains the case after capsaicin-induced hyperalgesia. Moreover, although our present results suggest the potential usefulness of EEG responses to IES as an objective measure of CS, the small sample size used in the present study limits statistical power for detection of smaller effects. Future studies with large samples are needed to confirm the predictive value of IES brain potentials for the state of CS.

Conclusion

Our study demonstrates that secondary hyperalgesia to IES occurs in a well-recognized experimental model of CS, and that the subjective report was corroborated by increased evoked EEG responses. These findings suggest that EEG responses elicited by low-intensity IES, particularly the change in the peak amplitude of the N2 wave, can be used as an objective, physiological correlate of secondary hyperalgesia. Hence, IES evoked potentials hold promise as a low-cost non-invasive biomarker for CS that can be translated for clinical use with relative ease compared to existing techniques.

Acknowledgments

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Figure legends

Figure 1. *Upper panel*. Experimental design. The state of central sensitization was induced by intra-epidermal injection of capsaicin (red arrow on the timeline). Capsaicin-induced spontaneous pain lasted between 8 and 35 minutes, during which pain ratings were collected every 10 s during the first 3 minutes and then every 30 s until the pain intensity ratings were less than 5 out of 100 (red box). Psychophysical and EEG responses to IES were collected before capsaicin injection (i.e., pre-capsaicin session, green box) and after capsaicin induced spontaneous pain had disappeared (i.e., post-capsaicin session, blue box). The development of secondary hyperalgesia to punctate mechanical stimuli was assessed by the ratio of the subjective intensity ratings of the sensation evoked by stimulation of the right and the left hand (Right/Left) (purple arrow on the timeline). Participants were considered responders if the ratio was ≥2, and non-responders otherwise. *Lower panel.* Schematic of the statistical analysis. A three-way ANOVA, with the factors of Group (responders, non-responders), Session (pre-capsaicin, post-capsaicin) and Hand (left, right), was used to analyse both psychophysical and ERP responses. The three-way interaction (Group x Session x Hand) indicated the effect of central sensitization on these responses. Further *post hoc* two-way ANOVAs with the factors of Session and Hand were performed to define the effect within each group.

Figure 2. *Upper panel*. Participants were divided into two groups according to the ratio of probe ratings to punctate stimulation of the right and left hands: participants who rated the intensity of right hand stimulation as at least twice that of the left hand stimulation were

classified as responders. Participants were sorted by the ratio of reported intensity ratings, in descending order. *Lower panel*. Time course of capsaicin-induced pain ratings. Single participants are colour coded. Solid lines indicate responders. All participants rated the pain intensity between 90 and 100 at the moment of the injection. Pain ratings decreased fairly quickly over time. *Upper-right inset.* The comparison of the mean area under curve (AUC) between responders and non-responders revealed no significant difference (T_{10} = 0.39, P = 0.70). Coloured symbols indicate single-subject AUC data.

Figure 3. Subjective intensity ratings of the sensation elicited by the IES (Intra-epidermal Electrical Stimulation) of responders (left column) and non-responders (right column). *Upper panel:* to highlight the interaction between the factors Session and Hand, the subtracted ratings (Post *minus* Pre capsaicin injection) are shown for each hand. Colored dots indicate single subjects, and the black dots indicate the group average of each condition. Two-way ANOVA revealed that responders had a highly significant interaction between the factors Session and Hand. This reveals a capsaicin-induced increase of IES ratings (Post-Pre) on the right hand. In contrast, in non-responders the two-way ANOVA revealed a decrease of IES ratings on the right hand compared to those on the left hand. These differences in the capsaicin effect on IES ratings between responders and non-responders were confirmed by the three-way ANOVA, which revealed a highly significant triple interaction (Group x Session x Hand; the comparison between the left and the right columns). LH: left hand; RH: right hand. Lower panel: individual values (colored dots) and mean value (the black dots) of each condition. PreLH: Pre-capsaicin, left hand; PostLH: Post-capsaicin, left hand; PreRH: Pre-capsaicin, right hand; PostRH: Post-capsaicin, right hand.

Figure 4. Group-average ERP waveforms and scalp maps elicited by IES in responders (left panel) and non-responders (right panel). Waveforms at the channel Cz in different conditions are shown in different color. The ERP elicited by IES stimuli clearly increased after capsaicin injection only on the right hand in responders. Scalp maps at the N2 peak latencies show a central distribution, slightly lateralized to the hemisphere contralateral to the stimulated hand, maximal at the vertex (upper part of each panel). Scalp maps at the P2 peak latencies show a central distribution, maximal at the vertex (lower part of each panel). The color bar shows the ERP amplitude in scalp maps.

Figure 5. ERP amplitudes (N2) of IES of responders (left column) and non-responders (right column). *Upper panel:* to highlight the interaction between Session and Hand in each group, the subtracted ERP amplitudes (Post *minus* Pre capsaicin injection) are shown for each hand. Colored dots indicate single subjects, and the black dots indicate the group average of each condition. Two-way ANOVA revealed that responders had a significant interaction between the factors Session and Hand. This reveals a capsaicin-induced increase of IES ERP amplitudes (Post-Pre) on the right hand. In contrast, in non-responders the two-way ANOVA did not show any significant effect. These differences in the capsaicin effect on ERP amplitudes between responders and non-responders were confirmed by the three-way ANOVA, which revealed a significant triple interaction (Group x Session x Hand; the comparison between the left and the right columns). LH: left hand; RH: right hand. Lower panel: individual values (colored dots) and mean value (the black dot) of each condition.

PreLH: Pre-capsaicin, left hand; PostLH: Post-capsaicin, left hand; PreRH: Pre-capsaicin, right hand; PostRH: Post-capsaicin, right hand.

Figure 6. ROC curves and their corresponding area under curve (AUC) obtained using the interaction term for N2 peak amplitude (left panel) and P2 peak amplitude (right panel) as the predictive factor. Although both measures show predictive ability, only the AUC of N2 ROC was significantly greater than 0.5, indicating that it is therefore a predictor for the state of central sensitisation.

Table 1. Results of the three-way ANOVA of the psychophysical and EEG responses elicited by intraepidermal electrical stimulation (IES).

3-way ANOVA	Pain intensity ratings	ERP peak amplitude		ERP peak latency	
		N2	P2	N2	P2
Main effect of Group	$F_{1,10}$ = 0.20 P = 0.665	$F_{1,10}$ = 0.08 P = 0.778	$F_{1,10}$ = 0.15 P = 0.705	$F_{1,10}$ = 0.008 P = 0.930	$F_{1,10}$ = 1.51 P = 0.247
Main effect of Session	$F_{1,10}$ = 0.05 P = 0.833	$F_{1,10}$ = 0.26 P = 0.618	$F_{1,10}$ = 2.65 P = 0.134	$F_{1,10}$ = 0.50 P = 0.498	$F_{1,10}$ = 0.38 P = 0.553
Main effect of Hand	$F_{1,10}$ = 4.52 P = 0.059	$F_{1,10}$ = 1.60 P = 0.234	$F_{1,10}$ = 0.52 P = 0.487	$F_{1,10}$ = 7.41 **P = 0.022**	$F_{1,10}$ = 0.11 P = 0.742
2-way interaction Group x Session	$F_{1,10}$ = 3.04 P = 0.112	$F_{1,10}$ = 1.40 P = 0.265	$F_{1,10}$ = 11.13 **P = 0.008**	$F_{1,10}$ = 0.05 P = 0.827	$F_{1,10}$ = 0.53 P = 0.484
2-way interaction Group x Hand	$F_{1,10}$ = 9.02 **P = 0.013**	$F_{1,10}$ = 0.45 P = 0.517	$F_{1,10}$ = 1.42 P = 0.261	$F_{1,10}$ = 0.11 P = 0.751	$F_{1,10}$ = 0.0003 P = 0.987
2-way interaction Session x Hand	$F_{1,10}$ = 4.31 P = 0.065	$F_{1,10}$ = 0.19 P = 0.674	$F_{1,10}$ = 1.27 P = 0.286	$F_{1,10}$ = 4.33 P = 0.064	$F_{1,10}$ = 1.19 P = 0.301
3-way interaction Group x Session x Hand	$F_{1,10}$ = 59.27 **P = 0.000016**	$F_{1,10}$ = 7.84 **P = 0.019**	$F_{1,10}$ = 2.04 P = 0.184	$F_{1,10}$ = 0.37 P = 0.559	$F_{1,10}$ = 0.06 P = 0.813

Significant effects are highlighted in bold.

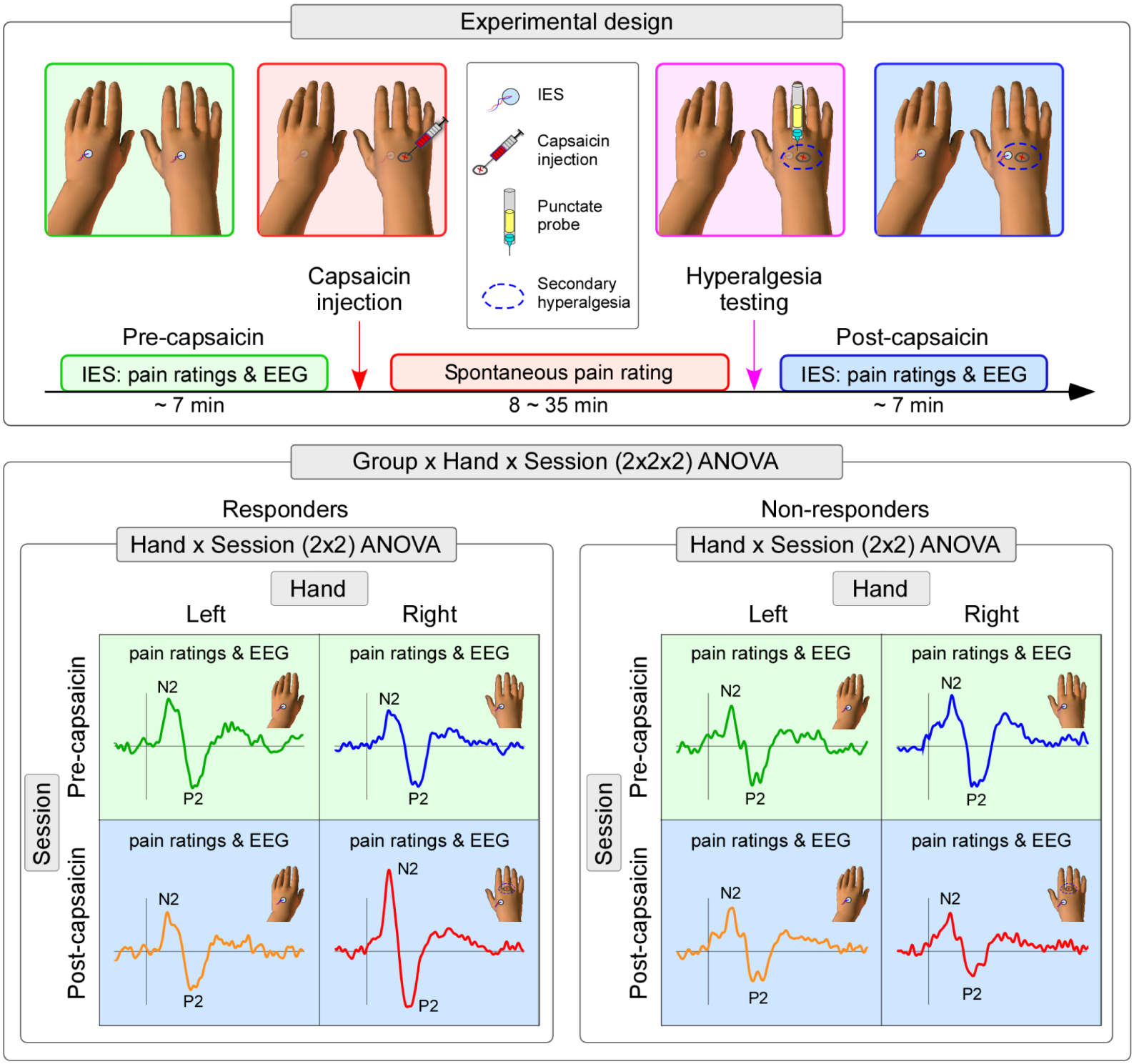
628 **Table 2.** Psychophysical and EEG responses elicited by IES for each condition, and results of

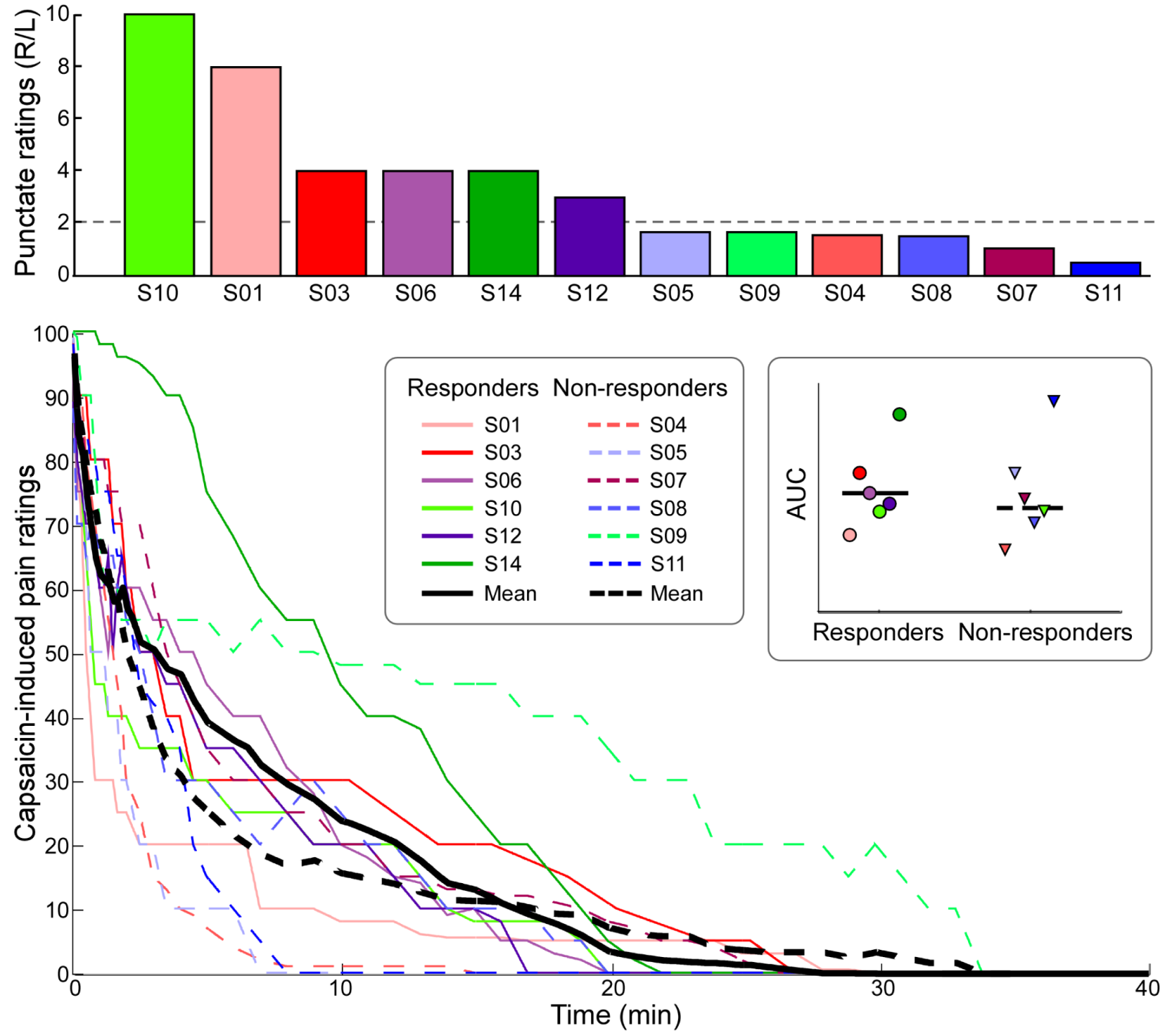
629 the post hoc two-way ANOVAs for each group.

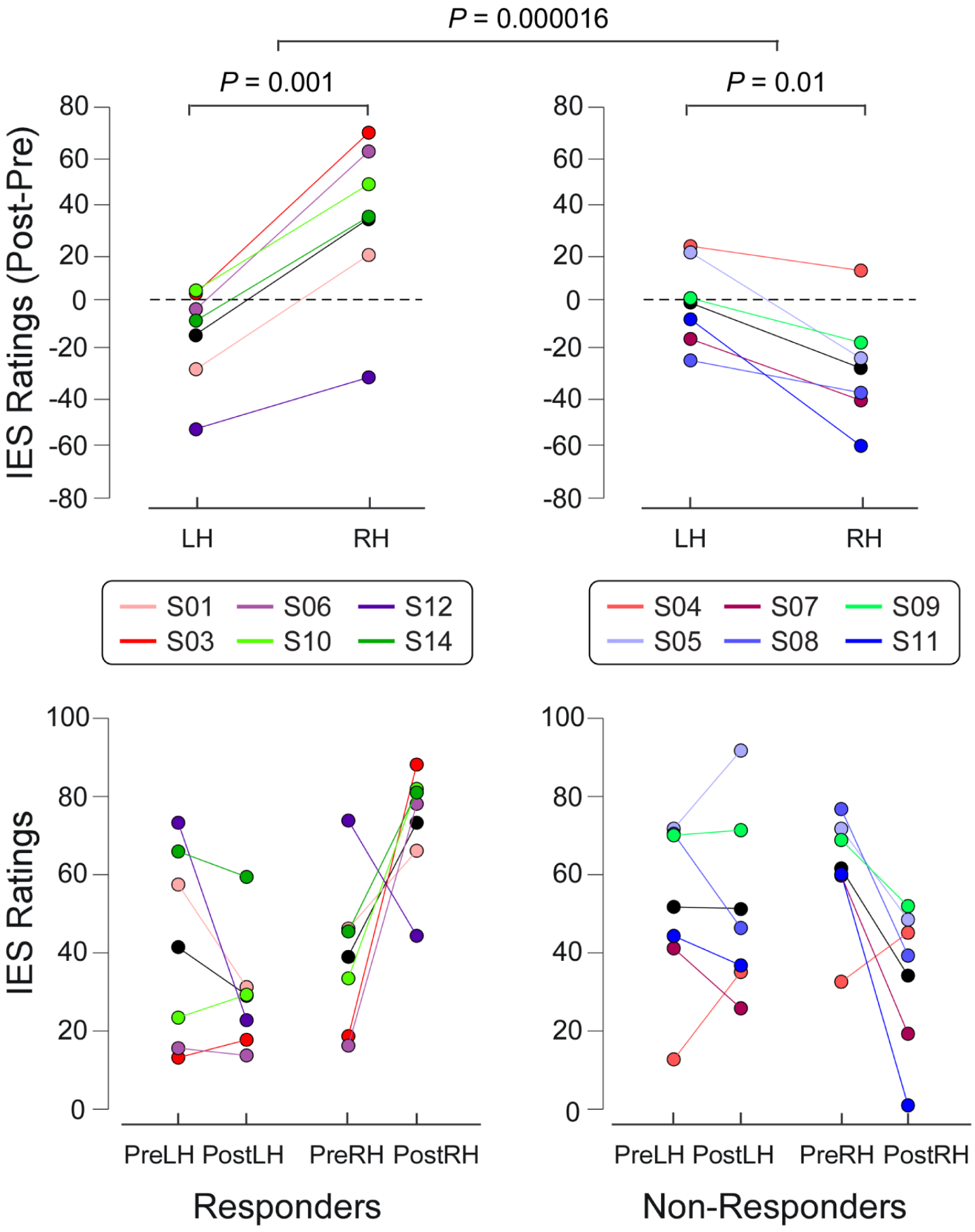
Group	PreRH (Mean ±SD)	PostRH (Mean ±SD)	PreLH (Mean ±SD)	PostLH (Mean ±SD)	Main Effect of Session (Pre vs. Post)	Main effect of Hand (LH vs. RH)	Interaction Session x Hand
Pain intensity ratings							
Responders	39.0 ±21.3	73.3 ±15.9	41.5 ±27.1	29.0 ±16.3	$F_{1,5}$ = 0.87 P = 0.394	**$F_{1,5}$ = 11.59 P = 0.019**	**$F_{1,5}$ = 49.79 P = 0.0009**
Non-Responders	61.5 ±15.7	34.1 ±20.0	51.6 ±23.6	51.1 ±25.3	$F_{1,5}$ = 2.90 P = 0.150	$F_{1,5}$ = 0.44 P = 0.535	**$F_{1,5}$ = 15.19 P = 0.011**
N2 peak amplitudes							
Responders	-8.6 ±4.4	-14.0 ±6.4	-9.9 ±5.4	-8.9 ±5.1	$F_{1,5}$ = 1.66 P = 0.254	$F_{1,5}$ = 2.55 P = 0.171	**$F_{1,5}$ = 15.15 P = 0.012**
Non-Responders	-12.9 ±5.5	-9.7 ±5.1	-10.0 ±6.2	-11.5 ±2.1	$F_{1,5}$ = 0.20 P = 0.676	$F_{1,5}$ = 0.14 P = 0.723	$F_{1,5}$ = 1.69 P = 0.250
P2 peak amplitudes							
Responders	7.9 ±2.3	10.6 ±2.5	8.3 ±3.5	7.6 ±1.4	$F_{1,5}$ = 4.32 P = 0.092	$F_{1,5}$ = 1.87 P = 0.230	$F_{1,5}$ = 9.77 P = 0.026
Non-Responders	10.7 ±3.6	7.4 ±4.2	10.8 ±3.2	7.9 ±3.8	$F_{1,5}$ = 7.42 P = 0.042	$F_{1,5}$ = 0.11 P = 0.755	$F_{1,5}$ = 0.03 P = 0.875
N2 peak latency							
Responders	165 ±29	136 ±16	168 ±29	178 ±47	$F_{1,5}$ = 0.68 P = 0.447	$F_{1,5}$ = 8.75 P = 0.032	$F_{1,5}$ = 5.94 P = 0.059
Non-Responders	162 ±38	147 ±47	169 ±44	175 ±39	$F_{1,5}$ = 0.08 P = 0.783	$F_{1,5}$ = 1.95 P = 0.221	$F_{1,5}$ = 0.78 P = 0.417

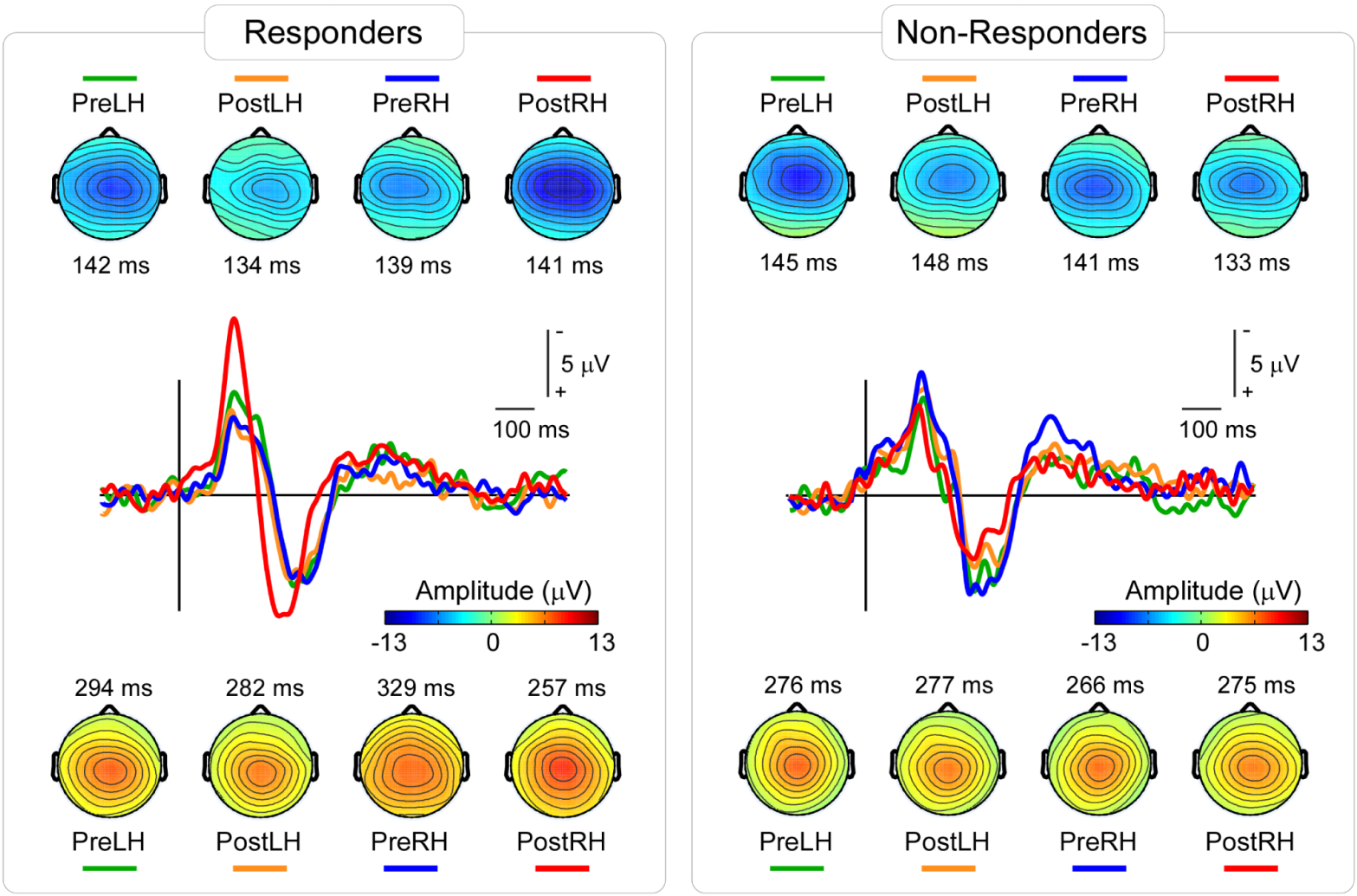
630 Significant effects are highlighted in bold.

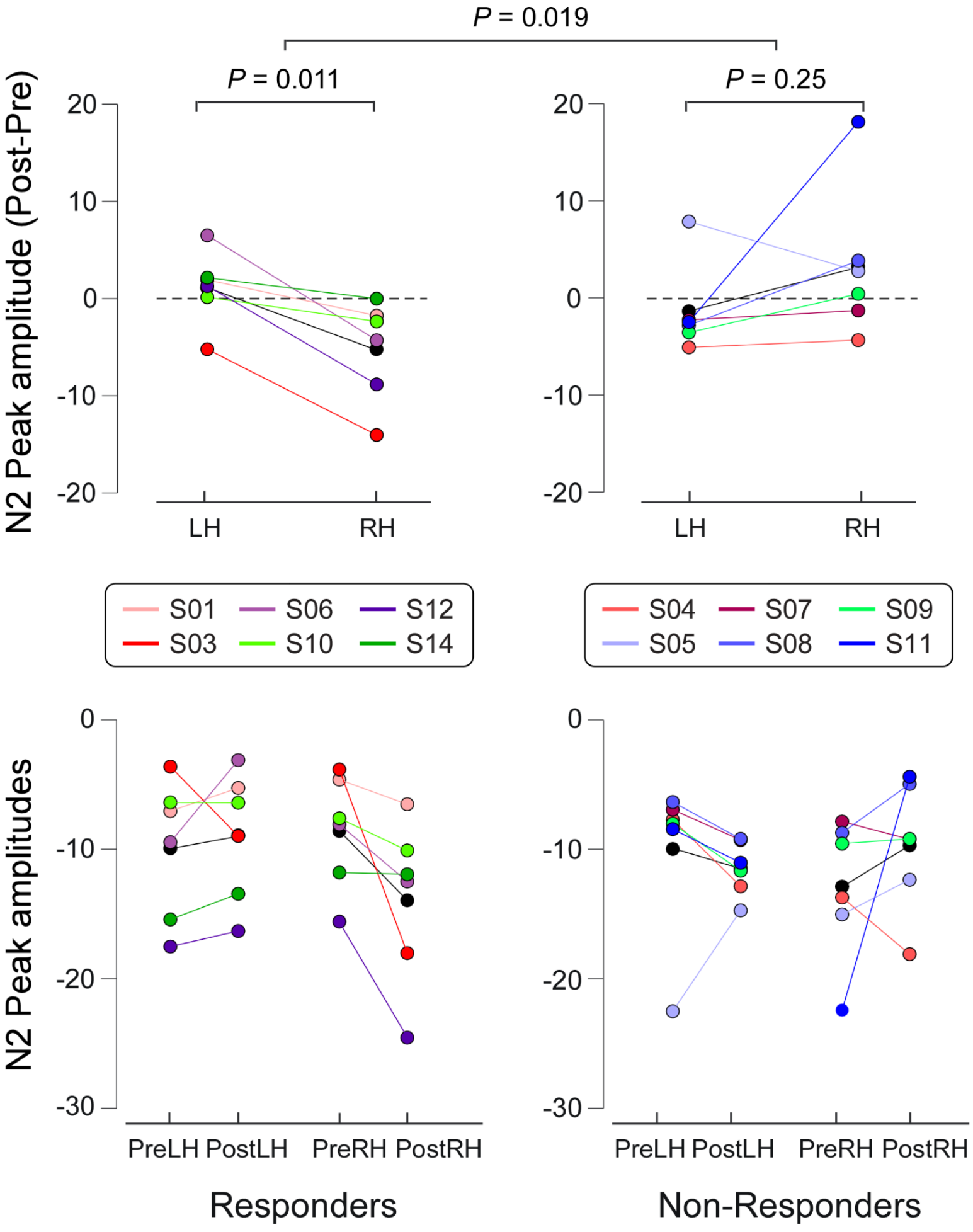
631

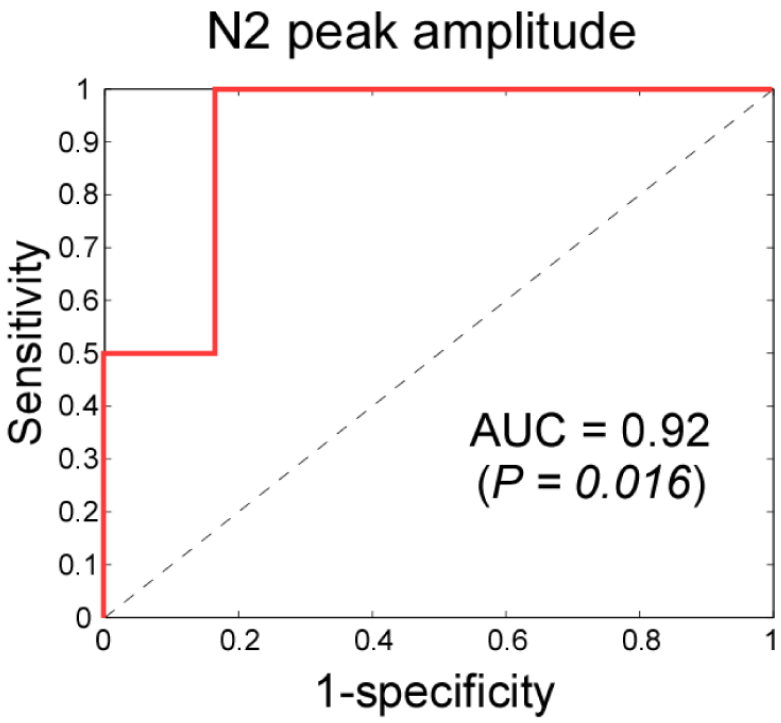

Experimental design
IES
Capsaicin injection
Punctate probe
Secondary hyperalgesia
Capsaicin injection
Hyperalgesia testing
Pre-capsaicin
IES: pain ratings & EEG
~ 7 min
Spontaneous pain rating
8 ~ 35 min
Post-capsaicin
IES: pain ratings & EEG
~ 7 min
Group x Hand x Session (2x2x2) ANOVA
Responders
Hand x Session (2x2) ANOVA
Hand
Left
Right
Session
Pre-capsaicin
Post-capsaicin
pain ratings & EEG
N2
P2
Non-responders
Hand x Session (2x2) ANOVA
Hand
Left
Right
Session
Pre-capsaicin
Post-capsaicin
pain ratings & EEG
N2
P2

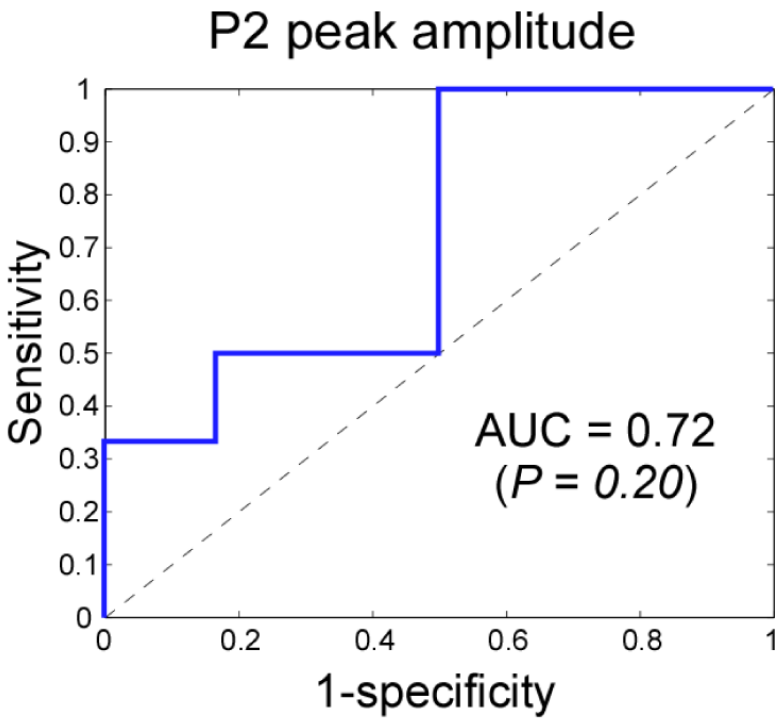

Punctate ratings (R/L)
10
8
6
4
2
0
S10
S01
S03
S06
S14
S12
S05
S09
S04
S08
S07
S11
Capsaicin-induced pain ratings
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
Time (min)
Responders
Non-responders
S01
S03
S06
S10
S12
S14
Mean
S04
S05
S07
S08
S09
S11
Mean
AUC
Responders
Non-responders


P = 0.000016
P = 0.001
P = 0.01
IES Ratings (Post-Pre)
80
60
40
20
0
-20
-40
-60
-80
LH
RH
S01
S06
S12
S03
S10
S14
S04
S07
S09
S05
S08
S11
IES Ratings
100
80
60
40
20
0
PreLH
PostLH
PreRH
PostRH
Responders
Non-Responders


Responders
PreLH
PostLH
PreRH
PostRH
142 ms
134 ms
139 ms
141 ms
5 µV
100 ms
Amplitude (µV)
-13
0
13
294 ms
282 ms
329 ms
257 ms
PreLH
PostLH
PreRH
PostRH
Non-Responders
PreLH
PostLH
PreRH
PostRH
145 ms
148 ms
141 ms
133 ms
5 µV
100 ms
Amplitude (µV)
-13
0
13
276 ms
277 ms
266 ms
275 ms
PreLH
PostLH
PreRH
PostRH


P = 0.019
P = 0.011
P = 0.25
N2 Peak amplitude (Post-Pre)
20
10
0
-10
-20
LH
RH
S01
S06
S12
S03
S10
S14
S04
S07
S09
S05
S08
S11
N2 Peak amplitudes
0
-10
-20
-30
PreLH
PostLH
PreRH
PostRH
Responders
Non-Responders


N2 peak amplitude
Sensitivity
1-specificity
AUC = 0.92
(P = 0.016)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
0.2
0.4
0.6
0.8
1


P2 peak amplitude
Sensitivity
1-specificity
AUC = 0.72
(P = 0.20)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
0.2
0.4
0.6
0.8
1