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Mapping cortical responses to somatosensory stimuli in human infants with simultaneous near-infrared spectroscopy and event-related potential recording

Multimodal cortical pain responses in infants

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Abstract

Near-infrared spectroscopy (NIRS) and electroencephalography (EEG) have recently provided fundamental new information about how the newborn brain processes innocuous and noxious somatosensory information. However, results derived independently from these two techniques are not entirely consistent, raising questions about the relationship between haemodynamic and electrophysiological responses in the study of touch and pain processing in the newborn. To address this, we have recorded NIRS and EEG responses simultaneously for the first time in the human infant following noxious (time-locked clinically required heel lances) and innocuous tactile cutaneous stimulation in 30 newborn infants. The results show that both techniques can be used to record quantifiable and distinct innocuous and noxious evoked activity at a group level in the newborn cortex. Noxious stimulation elicits a peak haemodynamic response that is 10 fold larger than that elicited by an innocuous stimulus (HbO_2: 2.0 vs. 0.3 μM) and a distinct nociceptive-specific N3P3 waveform in electrophysiological recordings. However, a novel single-trial analysis revealed that haemodynamic and electrophysiological responses do not always co-occur at an individual level, although when they do (64% of noxious test occasions), they are significantly correlated in magnitude. These data show that while haemodynamic and electrophysiological touch and pain brain activity in newborn infants is comparable in group analyses, important individual differences remain. These data indicate that integrated and multimodal brain monitoring is required to understand central touch and pain processing in the newborn.

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Significance Statement

Processing of touch and pain in the developing newborn brain can be studied using near-infrared spectroscopy (NIRS) and electroencephalography (EEG). However, the relationship between haemodynamic and electrophysiological responses to somatosensory stimuli in the newborn is not known. We recorded NIRS and EEG responses simultaneously and found that haemodynamic and electrophysiological touch and pain brain activity in newborn infants is comparable in group analyses; however, single-trial analysis revealed that these responses do not always co-occur in individual trials. This important variability suggests that integrated and multimodal brain monitoring is required to understand central touch and pain processing in the newborn.

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1 Introduction

Newborn infants are exposed to a wide range of cutaneous sensory stimuli in the first few days of life. Most of these are innocuous mechanical stimuli, such as touch or light pressure, although noxious skin breaking procedures are also given to neonates requiring hospital care. Little is known about how the developing newborn cortex processes these stimuli but cortical activation by noxious and innocuous mechanical stimulation has been recorded at the cotside using near-infrared spectroscopy (NIRS) (Bartocci et al., 2006; Slater et al., 2006) and EEG (Fabrizi et al., 2011; Slater et al., 2010a). Although these recordings offer great potential for investigating the postnatal development of human cortical somatosensory and pain networks, the data from these techniques has not been entirely consistent, raising the question of whether the two techniques are measuring the same integrated cortical activity following cutaneous noxious and innocuous stimulation.

Studies using NIRS have reported a clear haemodynamic response over the contralateral primary somatosensory cortex (SI) following noxious heel lance and noxious venepuncture in newborn infants (Bartocci et al., 2006; Slater et al., 2006). The haemodynamic response to heel lance was observed in single trials, was clear from 25 weeks gestational age, and increased with age. It was also smaller in sleeping compared to awake infants at the same age. By contrast, no response was detected following innocuous mechanical stimulation using von Frey hairs at intensities sufficient to elicit visible foot withdrawal (Slater et al., 2006), although a small response was reported following skin disinfection (Bartocci et al., 2006).

Studies using EEG have reported clear event-related potentials (ERPs) at the vertex following both noxious and innocuous stimulation (Fabrizi et al., 2011; Slater et al., 2010a, 2010b). The EEG response time-locked to noxious heel lance consists of two ERPs, the last of which is nociceptive specific (N3P3; N150-P260 and N420-P560), while the response following innocuous tactile stimulation consists of only the first ERP (N2P2). It is possible that these waveforms are preceded by an earlier somatosensory evoked potential (SEP; also referred to as the N1P1; for a review, see

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(Vanhatalo and Lauronen, 2006)), but this has not been reported yet. Both ERPs can be observed in single trials, but unlike haemodynamic responses, they only begin to appear reliably from around 37 weeks of age (Fabrizi et al., 2011). Furthermore, in contrast to the haemodynamic response, the second waveform is larger in sleeping infants, while the nociceptive-specific waveform is not dependent on sleep state (Slater et al., 2010a).

Some discrepancies between sensory evoked neural and haemodynamic responses in infants may be due to methodological differences; for instance, the criteria for detecting a tactile haemodynamic response may have been too stringent in previous studies, given the likely low signal to noise ratio (Slater et al., 2006). Likewise, the presence of a haemodynamic response in preterm infants in which ERPs are rarely observed could be related to different patterns of EEG activity in this age group, such as delta brushes (Fabrizi et al., 2011). Nevertheless, the differences raise the possibility that the two techniques are not measuring (either directly or indirectly) the same integrated somatosensory cortical activity.

To address this issue, we have recorded NIRS and EEG simultaneously in individual healthy term babies. Haemodynamic activity was recorded from the contralateral SI as it contributes, at least in part, to the generation of the adult noxious event-related potential (Hu et al., 2014; Valentini et al., 2012), and is therefore likely to contribute to the infant ERP. We predicted that with improved recording and analysis techniques, cortical responses to both innocuous and noxious mechanical skin stimulation could be quantified in neonates using both NIRS and EEG. Furthermore, we predicted that this would also be true in single trials so that evoked haemodynamic and electrophysiological activity, when recorded simultaneously in individual trials, would always co-occur and be correlated. To test these hypotheses we developed a novel goodness of fit (GOF) analysis for the evoked haemodynamic activity that allowed comparison and correlation with the principal component analysis (PCA) of evoked EEG activity.

2 Materials and Methods

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2.1 Participants

Thirty-six healthy term infants were recruited from the postnatal ward and special care baby unit at a location which will be identified if the article is published. Infants were not eligible for inclusion in the study if they were born in poor condition, had congenital malformations, or were receiving analgesics at the time of study. All infants and their mothers were well at the time of study. Infant demographics and clinical details are shown in Table 1.

Ethical approval for this study was given by the [Author University] ethics committee. Informed written parental consent was obtained before each study. The study conformed to the standards set by the *Declaration of Helsinki*.

2.2 Experimental protocol

Each infant received at least one of 3 types of stimuli: noxious, control, or tactile.

2.2.1 Noxious stimulus

21 infants were studied during a clinically-required routine heel lance for the purpose of obtaining blood samples. No heel lances were performed solely for the purpose of the study, and all samples were taken by a nurse or doctor using a lancet (Tenderfoot; ELITech UK Ltd, Berkhamsted, UK). The heel area was cleaned at least 30s prior to the lance, and the heel was not squeezed until at least 30s after the lance to enable the assessment of cortical responses to the lance stimulus alone.

2.2.2 Control stimulus

To control for the tactile (due to the placement of the lancet onto the heel) and auditory (due to the audible 'click' that is produced when the spring-loaded blade is released) aspects of the lance, all infants also received a control stimulus at least 2 minutes prior to the heel lance. This involved placing the lancet onto the heel in a 90-degree rotated position compared to lance so that when it

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was triggered the blade was released away from the foot. As with the noxious stimulus, care was taken to minimise other stimulation during the 30s prior to and following the control stimulus.

2.2.3 Tactile stimulus

A separate group of 15 infants, and one of the infants that were studied during lance, received tactile stimulation which consisted of a gentle tap to the heel using a custom-made tendon hammer. All infants received 10 taps with a mean inter-stimulus interval of 72 s (S.D. 16s), except for 3 infants who received fewer taps due to limited time. For the infant that received tactile stimulation in addition to lance, tactile stimulation was given prior to the control stimulus and the heel was lanced last.

All stimuli were time-locked to the EEG and NIRS recordings using a movement transducer attached to the lancet or tendon hammer (Worley et al., 2012).

2.2.4 Infant wellbeing

Throughout the experiment, care was taken to ensure the wellbeing and comfort of the babies and their families. Following hospital policy, comfort care was used rather than sucrose. Parents were always present during the study and were able to hold their baby if they wished. Babies were fed on demand. EEG and NIRS sensors were placed gently on the head by a trained clinical physiologist; handling of the babies was otherwise kept to a minimum throughout the experiment and nearly all infants were still asleep prior to the noxious heel lance (see Section 2.3.2). Parents were informed that they could stop the experiment at any time.

2.3 Multimodal recording

Cortical activity was measured with simultaneous NIRS and EEG recordings in 30 infants. In the remaining 6 infants only NIRS recordings were undertaken due to limited time.

2.3.1 NIRS recording

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Haemodynamic activity was recorded at a sampling rate of 5 Hz using the NIRO-200NX (Hamamatsu Photonics K.K., Hamamatsu City, Japan), which uses three wavelengths (735, 810 and 850 nm, with an output power of less than 2mW). Light absorption was converted into changes in [HbO_2] and [HHb] at the outset using the modified Beer-Lambert law with a differential pathlength factor of 4.39 (Wyatt et al., 1990).

Although near-infrared light travels diffusely through tissue, the sensitivity distribution associated with a given emitter-detector pair has a well-described shape that resembles a banana, with narrow ends at the emitter and detector and the highest depth sensitivity at the midpoint between the optodes (Arridge, 1995; Ferrari and Quaresima, 2012; Gervain et al., 2011; Lloyd-Fox et al., 2010a). A single emitter-detector pair was positioned according to the international 10/10 electrode placement system so that the midpoint was centred over either C1 or C2 (whichever was contralateral to the stimulation site) (Figure 1A), to provide access to the representation area of the heel in the primary somatosensory cortex (Figure 1B). The emitter was always placed towards the front of the head and the emitter-detector separation was kept constant at 4 cm using a commercial probe holder (Hamamatsu Photonics K.K., Hamamatsu City, Japan) which was held in place using an elastic net (Surgifix; FRA Production SpA, Cisterna d'Asti, Italy) and a baby hat. Figure 1B shows that with this separation, an emitter-detector pair centred over C1 in 40 week old infants is sensitive to large areas of SI including the heel area. Since NIRS is mainly sensitive to the microvasculature (Ferrari and Quaresima, 2012; Schroeter et al., 2006), the presence of the superior sagittal sinus in the midline region is unlikely to influence the evoked haemodynamic responses. The sensitivity maps in Figure 1B were produced using the 4D neonatal head model described in (Brigadoi et al., 2014).

2.3.2 EEG recording

Recording electrodes (disposable Ag/AgCl cup electrodes) were positioned according to a modified international 10/20 electrode placement system at Fp1, Fp2, Fz, F3, F4, Cz, C3, C4, CPz, CP3, CP4, T3, T4, T5, T6, O1, O2, and POz (Figure 1A). Although it was not always possible to apply the full set of

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electrodes, in the majority of infants at least 12 electrodes were used, and the Cz electrode was used in all recordings. Reference and ground electrodes were placed at FCz and on the forehead, respectively. Electrode-skin impedance was kept to a minimum by rubbing the skin with an EEG prepping gel (NuPrep gel; DO Weaver and Co, Aurora, CO, USA) and contact with the electrodes was optimised by applying conductive EEG paste (Ten20; DO Weaver and Co, Aurora, CO, USA, or Elefix; Nihon Kohden, Chessington, United Kingdom). Electrodes were held in place using an elastic net (Surgifix; FRA Production SpA, Cisterna d'Asti, Italy) and electrode leads were tied together to minimise electrical interference. EEG activity, from DC to 70 Hz, was recorded using the Neuroscan SynAmps2 EEG/EP recording system (Compumedics Neuroscan, Charlotte, NC, USA). A 50 Hz notch filter was used and signals were digitised with a sampling rate of 2 kHz and a resolution of 24 bit.

All EEG recordings were reported as normal by a clinical physiologist (A.L.) with respect to symmetry, synchronicity, absence of epileptiform activity, and background rhythms appropriate for age. The infants' sleep state was also classified as either 'awake' or 'asleep' using electrophysiological and behavioural data. 18 out of 21 infants who received a heel lance were classified as asleep prior to the heel lance; one infant was awake and two infants could not be classified.

2.4 NIRS analysis

Concentration changes of HbO_2 (Δ [HbO_2]), HHb (Δ [HHb]), and HbT (calculated as Δ [HbT] = Δ [HbO_2] + Δ [HHb]) were analysed using custom-written MATLAB (The Mathworks, Inc; Natick, MA, USA) scripts. Traces were band-pass filtered between 0.05 and 1 Hz (using a first order Butterworth filter) and were segmented into 40s epochs starting from 20s prior to each stimulus. Each epoch was then baseline-corrected using the pre-stimulus interval. HbO_2, HHb, and HbT traces were assessed for movement artefact; epochs containing large-amplitude movement artefact (defined as Δ [HbT] > 25 µM) were rejected, and epochs containing brief (< 3 s duration), low-amplitude (< 15 µM) artefactual spikes were interpolated (at the point of inflection, using piecewise cubic spline interpolation).

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213 Three lance and two control epochs were rejected for technical reasons (e.g. poor quality data due
214 to poor light shielding; failed time-locking of the stimulus to the NIRS recording) and a further three
215 lance and one control epochs were rejected due to large-amplitude movement artefact. Therefore,
216 15 lance and 18 control epochs were included in the final NIRS sample.

217 For touch, epochs were rejected if they contained movement artefact (> 5 μM; 18 trials). 5 epochs
218 were also rejected as outliers. Thus a total of 131 touch epochs from 16 participants were
219 considered in the final NIRS sample (Table 2).

220 To determine whether we could record a haemodynamic response to noxious, control and tactile
221 stimuli at a group level, we computed the group average Δ [HbO_2], Δ [HHb] and Δ [HbT] for each
222 stimulus type and performed z-tests at each time point from 0 to 20s post stimulation. In order to
223 account for multiple testing only significant segments of at least 1 second duration were considered
224 meaningful. This allowed us to identify when the hemodynamic response exceeded random baseline
225 noise. For touch, epochs were first averaged within individual participants, and then across
226 participants to give a grand average. Haemodynamic responses to each stimulus were characterised
227 from the group averages in terms of peak changes and latencies.

228 To compare the haemodynamic response to lance with the response to control or touch stimulation,
229 independent samples t-tests were then carried out at each time point from 0 to 20s post
230 stimulation. Corrections for multiple comparisons were carried out as described above.

231 In adults, a typical haemodynamic response consists of an increase in [HbO_2] and a concomitant,
232 lower amplitude decrease in [HHb], reflecting an increase in cerebral blood flow to the activated
233 region and the resulting over-supply of HbO_2 and displacement of HHb from the veins. However, in
234 infants the direction of the Δ [HHb] is not consistent across studies, with some studies reporting
235 decreases and others increases in [HHb], or inconsistent results (for a review, see (Lloyd-Fox et al.,
236 2010a). To determine the dominant peak of the HHb and HbO_2 responses for each trial in our data,

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we looked for changes that exceeded a threshold of 2 SD for at least 1 s from the mean baseline of the given trial between 1 and 6.5 s after stimulation.

For all tests, the threshold for significance was set at $\alpha = 0.05$.

2.5 Event-related potential analysis

Five lance and five control trials were removed from the analysis for technical reasons (e.g. because the EEG was not done or was of poor quality; or due to failed time-locking of the stimulus to the EEG recording). Therefore, 16 lance and 16 control trials were included in the final EEG sample.

For touch, five test occasions and a further three trials from three other test occasions were excluded for technical reasons (e.g. because the EEG was not done or was of poor quality). Thus a total of 106 touch trials from 11 participants were included in the final EEG sample (Table 2).

EEG traces were analysed using EEGLAB (Delorme and Makeig, 2004)and custom-written MATLAB (The Mathworks, Inc; Natick, MA, USA) scripts. Traces were band-pass filtered between 1 and 30 Hz (using a 2nd order bidirectional Butterworth filter), segmented into 1.7 second epochs starting from 0.6 seconds before the stimulus, and baseline-corrected using the pre-stimulus interval. Channels containing movement artefact (defined as activity exceeding ±100 μV) or high-frequency muscle activity were removed.

The analysis focussed on Cz. In order to correct for inter-trial and inter-subject latency jitter (Bromm and Scharein, 1982; Woody, 1967), traces were aligned by Woody filtering within time windows centred on the second and third waveforms: (1) 50-400 ms after stimulation for lance epochs or 50-300 ms for control and touch epochs; and (2) 350-600 ms after stimulation. The maximum allowed jitter correction was ±50 ms for lance and touch epochs, and ±75 ms for control epochs. This approach resulted in 2 aligned group averages per stimulus type. For touch trials, traces were first aligned and averaged within participants and the resulting traces were then aligned and averaged across individuals to give the grand average trace.

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To determine whether the waveforms exceeded random baseline noise, z-tests were carried out at each time point within the alignment windows (50-300 ms and 350-600 ms). We used the false discovery rate (Benjamini and Hochberg, 1995) to correct for multiple comparisons and assumed 30 independent tests per second, because data were lowpass filtered at 30 Hz.

The amplitude and latency of the negative (N) and positive (P) peaks of the two waveforms were obtained from the aligned group averages. Scalp topography maps were also created from the aligned group averages in order to display the scalp distribution of the negative (N) and positive (P) peaks of each waveform. For each peak, the average amplitude at Cz and at each of the other channels at the time of the given peak was plotted as a heat map. Channels that were excluded due to contamination by artefacts or not recorded were interpolated.

The peak-to-peak amplitude of the N and P peaks of the nociceptive-specific waveform in the lance trials was obtained by comparing each individual trace with the average and selecting the peaks that most resembled the average trace N and P peaks in terms of latency and morphology.

2.6 Within infant comparison of NIRS & EEG lance responses

After establishing the presence of haemodynamic and electrophysiological responses at the group level we investigated the relationship between the two measures at the single trial level. Specifically, we explored whether NIRS and EEG responses co-occurred within the same test occasion. To do so, we checked for the presence of an HbO_2 response and of the nociceptive-specific EEG waveform in each lance trial and then compared the two. For control trials, we assessed the presence of the second (non-nociceptive-specific) EEG waveform.

2.6.1 NIRS Goodness of Fit analysis

To establish whether an HbO_2 response was present in a given trial, we compared each epoch to a haemodynamic response function (HRF) template. This was modelled as the average Δ [HbO_2] of all lance epochs, as in section 2.4.1, and smoothed with an automatic 1-D wavelet denoising filter in

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MATLAB (threshold selection was based on Stein's Unbiased Risk Estimate and additional parameters included soft thresholding and no rescaling). This yielded a lance HRF template with latency to positive peak 3.4 s, positive peak amplitude 1.8 µM, latency to undershoot peak 9.8 s, and a ratio of undershoot amplitude to positive peak amplitude equal to 0.72 (Figure 2A). The same procedure applied to the control trials produced a control HRF template with latency to positive peak 2.2 s, positive peak amplitude 0.3 µM, latency to undershoot peak 12.6 s, and a ratio of undershoot amplitude to positive peak amplitude equal to 1.07. Each individual [HbO_2] trace was correlated with the HRF to determine the goodness of fit (GOF) between the observed and expected Δ [HbO_2]. NIRS epochs were classified as 'response present' if they exceeded a GOF threshold of 0.45, and 'response absent' otherwise.

2.6.2 EEG Principal Component Analysis

We then assessed the presence of the tactile and the nociceptive specific event related potentials (ERPs) in the same lance and control trials. This was done using Principal Component Analysis (PCA) (Slater et al., 2010a). The tactile and the nociceptive specific components were identified by conducting PCA in two time intervals: (1) 50-300ms post stimulation (lance and control trials separately) and (2) 350-700ms post stimulation (lance trials only). The tactile or nociceptive specific waveform was considered to be present in a given EEG epoch if the weight associated with the corresponding component exceeded a threshold of 0.1, and absent otherwise.

Using these criteria, responses were classified as present in 10/15 lance NIRS trials (GOF > 0.45) and 9/16 lance EEG trials (PC weight > 0.1). The accuracy of this classification is confirmed by the fact that a significant response is present in the group averages of the trials where the response was "present", but not in that of the trials where the response was "absent" (Figure 3). Finally, even though our method is preferable to using peak amplitudes because it takes into account the overall signal rather than a single data point that can be affected by noise, the GOF values [a] and PC weights [b] correlated well with the more traditional peak measures (Figure 2C-D).

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3 Results

3.1 Distinct haemodynamic responses to noxious heel lance and innocuous heel touch in newborn infants: group analysis

We first analysed and compared the haemodynamic response to lance and to innocuous touch of the heel. NIRS analysis was performed on 15 infants undergoing a clinically required heel lance (n=9 right heel). Consistent with previous studies (Bartocci et al., 2006; Slater et al., 2006), a significant increase of 2.3 ±2.9 µM in [HbT] was recorded over the contralateral primary somatosensory cortex. The response had a maximum peak at 3.2 s, and was followed by a significant undershoot at 10 s and a later decrease from 15.2 s (Fig. 4; Table 3).

The Δ [HbT] largely reflects the Δ [HbO_2], which had the same statistically significant response pattern (Fig. 4; Table 3). Since the HHb response was small and variable (with increases in the early HHb response in 6/15 trials; decreases in 4/15 trials; and no change in 5/15 trials), as is well known from other infant studies (Lloyd-Fox et al., 2010a), [HbO_2] changes (10/15 increases; 1/15 decreases; 4/15 no change) were used for subsequent analysis.

NIRS analysis was also performed on infants undergoing non-noxious control stimulus (18 infants, n=9 right side) or repeated touch of the heel (n=16 infants, 131 trials, mean=7 trials per infant, n=10 right side). As with lance stimulation, there was a significant early increase in [HbO_2] following both control and touch stimuli, although this increase was markedly lower in amplitude compared to lance (2.0 ± 2.2 µM following lance vs. 0.4 ± 0.6 and 0.3 ± 0.2 µM following control and touch stimulation, respectively; Fig. 5A; Table 3). For the control stimulus, this peak was followed by a significant undershoot peaking at 13.4 s. There was no significant early Δ [HHb] following either control or touch, although there was a late decrease in [HHb] peaking at 9.8 s following touch stimulation.

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The haemodynamic response to noxious heel lance was significantly larger than the response to innocuous mechanical skin stimulation. Independent samples t-tests confirmed that both the early increase in [HbO_2] and the ensuing undershoot were significantly larger following lance than either control or touch. No significant differences were observed in HHb responses (Fig. 5B).

3.2 Distinct EEG responses to noxious and innocuous heel stimulation in newborn infants: group analysis

We next analysed and compared the EEG response to lance and to innocuous touch of the heel. Sixteen infants receiving a heel lance were included in the EEG analysis. Heel lance evoked a clear event-related potential (ERP) consisting of a late N2P2 complex followed by an N3P3 complex. Both waveforms were significantly different from baseline (Fig. 6). Following alignment, the mean latencies of the N and P peaks of the first waveform were 139 and 202 ms, and the amplitudes were -5.0 ± 12.2 and 8.7 ± 16.6 μV, respectively. The mean N3 and P3 peaks were 385 and 554 ms in latency, and -12.8 ± 12.1 and 12.7 ± 17.1 μV in amplitude, respectively (Table 4). Scalp topography maps showed that the N peak of the second waveform and the P peaks of both waveforms were maximal at the vertex. The N peak of the first waveform was instead maximal at POz.

The EEG traces of 16 infants having a control stimulus and 11 infants having a total of 106 touches were also analysed. Both control and touch stimuli elicited a distinct late N2P2 complex. For both stimuli, the N and P peaks were significantly different from baseline (Fig. 7). For the control stimulus, the mean latencies of the N and P peaks following alignment were 93 and 189 ms, respectively, and the amplitudes were -5.1 ± 15.5 and 20.1 ± 20.1 μV. For the touch stimulus, the mean latencies and amplitudes of the N and P peaks were 147 and 248 ms and -9.1 ± 10.1 and 9.5 ± 8.4 μV, respectively (Fig. 7; Table 4). Scalp topography maps showed that the N and P peaks were maximal at the vertex for both the control and touch averages. The late N3P3 EEG response to noxious heel lance was not observed in response to innocuous mechanical skin stimulation.

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3.3 Simultaneous NIRS and EEG recordings in response to cutaneous stimulation in newborn infants: individual infant analysis

The results above show that both NIRS and EEG can be used to record quantifiable and distinct innocuous and noxious evoked activity at a group level in the newborn cortex. We next asked whether such distinct haemodynamic and electrophysiological responses to noxious and innocuous stimulation co-occur in individual trials. In all 18 infants that received a noxious heel lance and a matching control non-noxious stimulus (lancet rotated 90^{o}), artefact-free NIRS and EEG recordings were successfully obtained (Figure 8), but 4 lance trials were rejected for technical failure or movement artefact during the noxious procedure.

11/14 lance trials contained a cortical nociceptive response (3 HbO_2 only; 2 ERP only; 6 both), while on 3 trials no response was detected in either HbO_2 or ERP recordings, according to the stringent criteria described in the Methods. Thus, although cortical NIRS and EEG responses to noxious heel lance can be recorded simultaneously and in the majority of test occasions the two methods are consistent (present or absent together, 9/14, 64%; Table 5), they do not always co-occur.

The noxious evoked HbO_2 response showed less co-occurrence with the early N2P2 component of the noxious evoked EEG response (5/14, or 35.7% agreements), suggesting that the haemodynamic response was better related to the nociceptive-specific component of the lance ERP.

3 control trials were rejected for technical failure or movement artefact during stimulation. 11/15 test occasions contained a cortical touch response (3 HbO_2 only; 5 ERP only; 3 both), while 4 contained neither an HbO_2 nor an ERP response. Thus, although cortical NIRS and EEG responses to innocuous touch can be recorded simultaneously, the two methods are not consistent (present or absent together, 7/15, 47%) and they do not always co-occur.

3.4 Cortical NIRS and EEG responses to noxious heel lance and to innocuous control are correlated

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Despite the fact that they did not always co-occur, we predicted that the HbO_2 goodness of fit (GOF) values and the PC weights of the N3P3 nociceptive-specific waveform would be correlated when they did co-occur. In the 9 trials where the incidence of the two cortical measures agreed, a significant positive correlation between HbO_2 goodness of fit (GOF) values and the PC weights of the nociceptive-specific N3P3 waveform was found [c] (Pearson's r: 0.69, p=0.04; Fig.9). Similarly, there was a positive correlation between HbO_2 GOF values and the PC weights of the N2P2 waveform of the 7 control trials where the incidence of the two cortical measures agreed, although this was not significant [d] (Spearman's rho: 0.54, p=0.215).

4 Discussion

Here, we have recorded, for the first time, simultaneous haemodynamic and neurophysiological responses to innocuous control and tactile stimuli and to noxious heel lance in newborn human infants. We fully characterised haemodynamic responses in terms of both [HbO_2] and [HHb] changes and developed a method to identify the presence of a clear haemodynamic response to cutaneous stimuli on a single-trial basis. The magnitude of the haemodynamic responses reported here is in the same range as those reported elsewhere in infants, in response to somatosensory (Slater et al., 2006), visual (Meek et al., 1998), olfactory (Bartocci et al., 2000) and other stimulation (Lloyd-Fox et al., 2009, 2010b). As predicted, we observed significant haemodynamic responses to both noxious and innocuous stimuli that also elicited clear ERPs in a group of healthy term infants. Importantly, we also found significantly greater haemodynamic activation in the contralateral SI following lance compared to control or tactile stimulation, indicating selectivity of haemodynamic responses. However, our hypothesis that neural and haemodynamic responses would always co-occur in individual trials was not supported, although in the majority of noxious lance trials they did coincide and were positively correlated.

This is the first clearly documented quantitative analysis of both innocuous and noxious evoked haemodynamic activity in the infant human brain. Previous studies reported no HbT response to von

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406 Frey hair stimulation of the foot (Slater et al., 2006) which appears inconsistent with the present
407 results. Our finding here of a significant increase in both [HbO_2] and [HbT] following a similarly brief
408 innocuous mechanical stimulus suggests that the ability to detect a haemodynamic response does
409 not depend upon stimulus duration or which haemoglobin signal is measured. Instead, this
410 inconsistency is likely due to the smaller haemodynamic response and lower signal-to-noise ratio of
411 the tactile response relative to the noxious response, which was the focus of the earlier study. A
412 previous report of specific somatosensory haemodynamic responses in the infant cortex (Bartocci et
413 al. 2006) used longer duration skin wiping as a stimulus but the lack of detailed methodology and
414 analysis make it hard to compare with the current results.

415 4.1 Distinct tactile and nociceptive cortical responses at the group level

416 Our results show initial increases in [HbO_2] over the contralateral SI following lance, control, and
417 tactile stimuli (2.0, 0.4, & 0.3 µM, respectively). Furthermore, haemodynamic responses were
418 modulated such that it was possible to distinguish between noxious lance and the innocuous control
419 and tactile stimuli based on the magnitude of the [HbO_2] changes. The mean evoked noxious
420 response was almost ten times larger in amplitude than the response to innocuous stimulation. This
421 is a considerably greater difference than that reported in a previous NIRS study using innocuous and
422 noxious electrical stimulation in healthy adults (Yücel et al., 2015). Interestingly, other adult studies
423 using fMRI and diffuse optical tomography (a technique that uses NIRS to enable volumetric
424 monitoring of [HbO_2] & [HHb] changes), show that noxious and innocuous stimuli can only be
425 differentiated with respect to the shape and bilaterality, rather than magnitude, of responses
426 (Becerra et al., 2008, 2009; Chen et al., 2002).

427 This study also confirms the presence of a distinct late nociceptive N3P3 waveform in the infant
428 brain following noxious stimulation that is clearly separable from an earlier N2P2 wave associated
429 with innocuous mechanical stimulation (Fabrizi et al., 2011; Slater et al., 2010a; Verriotis et al.,

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2015). Thus we show here that both techniques are able to distinguish between painful and non-painful cortical activation, NIRS in terms of peak amplitudes and EEG in separable waveforms.

4.2 Interpreting the haemodynamic response

We did not find significant differences in [HHb] changes between stimuli; this could be due to the well-known variable nature of [HHb] changes in young infants (Lloyd-Fox et al., 2010a). In adults, increased oxygen consumption leads to regional overperfusion, resulting in a net increase in [HbO_2] and decrease in [HHb]. The variability in the Δ [HHb] seen in infants suggests that increased oxygen consumption does not always lead to regional overperfusion, perhaps due to immature vascular regulation or to greater metabolic demands of neurotransmission in unmyelinated white matter (Gervain et al., 2011; Kozberg et al., 2013; Meek, 2002).

In addition to the early changes in [HbO_2] and [HHb], we observed a significant [HbO_2] decrease from 7s onwards until the end of the 20s trace following lance, and a similar but shorter lasting decrease following control stimulation (Fig. 4 & 5; Table 3). This is consistent with the HbO_2 'undershoot' (and concomitant HHb 'overshoot') that has been observed in other NIRS studies (Boden et al., 2007; Gervain et al., 2011; Obrig et al., 2000; Steinbrink et al., 2006) as well as with the BOLD undershoot reported in fMRI studies, which has been observed for up to 60s (Arichi et al., 2012; Chen and Pike, 2009; Schroeter et al., 2006; Steinbrink et al., 2006). There was also a significant average [HHb] increase between 10 and 14s following lance, but this is difficult to interpret as either an over- or under- shoot because the direction of the early HHb response was variable. The physiological origin of an [HbO_2] undershoot and [HHb] overshoot has been explored in several studies, and is controversial due to the existence of evidence supporting different mechanisms (Chen and Pike, 2009; Schroeter et al., 2006; Steinbrink et al., 2006). The variable HHb response in our study, in addition to developmental maturation effects of neurovascular coupling and energy use on the haemodynamic response in young infants (Harris et al., 2011; Kozberg et al., 2013), makes it hard to associate our data with any of these mechanisms.

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455 The latencies of the peak haemodynamic responses to lance, control and tactile stimuli are relatively
456 short (3.4, 2.2, and 4.0 s, respectively for [HbO_2] changes), as reported elsewhere (Slater et al.,
457 2006). In adult fMRI studies, the canonical haemodynamic response function (HRF) to a stimulus is
458 typically modelled with a peak latency around 5-6s (Henson, 2004), though peak latencies of 4-5 s
459 have been reported (Steffener et al., 2010), while in infants haemodynamic responses tend to peak
460 later (Arichi et al., 2012; Meek, 2002). The shorter peak latencies reported here may be due to
461 differences in stimulus durations (~5-300ms vs 3.2 – 30s (Bartocci et al., 2006; Lloyd-Fox et al., 2009;
462 Meek et al., 1998; Taga et al., 2003; Wartenburger et al., 2007)), as well as age, type of stimulus,
463 technical parameters, and analysis methods (such as filtering methods). Another explanation is that
464 immature vascular regulation, and therefore neurovascular coupling, in the neonates may result in
465 reduced hyperemia relative to adults (Gervain et al., 2011; Harris et al., 2011; Kozberg et al., 2013;
466 Meek, 2002), leading to a shorter lasting increase in [HbO_2], and therefore a shorter peak latency.

467 It should be noted that in adults noxious stimuli can trigger a generalised sympathetic skin response
468 in addition to a cortical response (Yücel et al., 2015). While an effect of this autonomic response on
469 the haemodynamic changes observed in the present study cannot be conclusively ruled out without
470 monitoring superficial skin activity, the present results are likely to reflect cortical activity, for four
471 reasons. First, the results reported by Yücel and colleagues suggest that the superficial response
472 would be in the opposite direction to the [HbO_2] changes reported here, thus masking the cortical
473 response (Yücel et al., 2015). Second, consistent with other studies, haemodynamic responses to
474 noxious heel lance were in the same direction as responses to tactile stimuli, which are less likely to
475 trigger an autonomic skin response. Third, as neonates have a much thinner scalp and skull than
476 adults, the relative contribution of superficial skin activity to the measured signal would be much
477 smaller in neonates; indeed the optimal emitter-detector separation required to sample scalp
478 activity without cortical activity is 2.15 mm in term neonates vs. 8.4 mm in adults (Brigadoi and
479 Cooper, 2015). Finally, NIRS responses to heel lance are consistent with those reported in a previous
480 study in which simultaneously monitored ipsilateral responses were variable and often in the

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opposite direction, indicating that heel lance elicited localised rather than global haemodynamic changes (Slater et al., 2006).

4.3 Interpretation of simultaneous NIRS-EEG recordings

Several combined NIRS-EEG recordings have been recently undertaken in infants (Biallas et al., 2012; Roche-Labarbe et al., 2007, 2008; Telkemeyer et al., 2009) and there is great interest in developing probes for this purpose (Cooper et al., 2009; Wallois et al., 2012). Here we report the first combined EEG and NIRS analysis of infant somatosensory cortical activity. As predicted, we found that somatosensory stimuli elicit both neural and haemodynamic responses in a given group of infants. However, single-trial analysis showed that neural and haemodynamic responses do not co-occur in all trials. This is true following both innocuous control and noxious lance stimuli, although neural and haemodynamic responses co-occurred on a greater proportion of trials following lance. For both stimuli, an ERP was observed in the absence of a haemodynamic response over the contralateral SI on some trials, and a haemodynamic response was detected in the absence of an ERP on other trials.

In adults, a network of brain regions including the SI is implicated in the generation of the noxious laser evoked potential (Garcia-Larrea et al., 2003; Hu et al., 2014; Valentini et al., 2012). The co-occurrence of neural and haemodynamic responses in many of the trials suggests that the contralateral SI also contributes to the generation of the nociceptive-specific ERP in the maturing newborn brain, but is not reliably activated every time at this age. Similarly, the presence of a haemodynamic response in the absence of an ERP could be interpreted as intact involvement of the SI but insufficient involvement of the other generators contributing to the ERP. Alternatively, the presence of a clear HbO_2 response in the absence of an ERP could be explained by the sensitivity of the two techniques to slightly different populations of cells. NIRS can detect haemodynamic activity arising from highly metabolically active basket and cortical stellate cells which may well be invisible in scalp EEG (Wallois et al., 2012). Moreover, NIRS can detect haemodynamic activity arising from both synchronous and asynchronous neural activity, whereas EEG requires several square cm of

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synchronously active brain tissue in order to detect a response (Wallois et al., 2012). Thus a poorly organised neural response, as might be likely in the immature cortex, could generate metabolic demands that are sufficient to cause a detectable haemodynamic response, but not an ERP.

Similarly, the presence of an ERP in the absence of a haemodynamic response could be related to immature neurovascular coupling in this age group. Both the coupling between blood flow and neuronal activation, and the regulation of blood flow itself, might be less efficient at this age, such that the effects of increased oxygen consumption and increased blood flow cancel each other out and no haemodynamic response is observed (Harris et al., 2011). Therefore, even if the SI is one of the generators of the ERP, a haemodynamic response might not always be detectable in the immature brain. A lack of recruitment of pial arteries in early development (resulting in little or no initial hyperemia), but later vasoconstriction (resulting in an apparent inverted haemodynamic pattern), is one potential mechanism of the immature neurovascular coupling (Kozberg et al., 2013). Alternative explanations include masking of the haemodynamic response in some cases either due to autonomic sympathetic activity, known to follow noxious stimulation (Yücel et al., 2015), or to the sensitivity of the optodes to a large area of cortex, parts of which could be deactivated.

4.4 Interpreting trials with no cortical response

It is clear from this study that the lack of a detectable NIRS or a detectable EEG response alone does not imply that noxious input was not processed in the cortex of these infants. Nonetheless, the proportion of infants having no detectable NIRS response to noxious heel lance (5/14) is larger than that reported elsewhere (1/18) (Slater et al., 2006). This discrepancy is probably due to the more stringent classification method used here, and to the large number of babies that were asleep leading to a reduced haemodynamic response (Slater et al., 2006). The proportion of infants with no EEG response is expected: a clear lance ERP is not always detected in neonates, as the ERP begins to appear more reliably just before the time of normal birth, and its incidence continues to increase

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with age until at least 45 weeks GA (Fabrizi et al., 2011) and in amplitude until at least one year of age (Verriotis et al., 2015).

However, in a small number of infants there was neither an EEG nor a NIRS response to somatosensory stimulation, be it noxious or innocuous, despite clear evidence of high quality artefact free recording and of behavioural reactivity (data not shown). It is likely that a lack of either response reflects genuine inter-trial and inter-individual variability in pain reactivity in infants. The reproducibility of within-subject responses in key somatosensory regions to tactile and painful stimuli is known to be not entirely stable in healthy adults (Taylor and Davis, 2009) and is likely to be less so in infants. But there is also increasing evidence of inter-individual differences in pain perception influenced by both genetic and epigenetic factors. A recent study has highlighted the significant variability of infant pain behaviour between groups of infants aged from 2 -12 months (Pillai Riddell et al., 2013) consistent with the considerable variability in facial expression following heel lance in preterm and term infants on a given study occasion (Slater et al., 2009).

Our ability to record and quantify single-trial haemodynamic and EEG responses increases the reliability of either method alone and will allow us to interrogate these responses in the maturing infant brain and to better interpret developmental changes in central pain processing.

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679

680 6 Figure Legends

681 Fig. 1: NIRS optode and EEG electrode locations (A) and NIRS sensitivity maps (B). A. NIRS optode
682 (black) and EEG electrode (blue circles) locations are presented on a schematic of the top view of the
683 head (left). The EEG reference electrode was placed at FCz (grey circle). The NIRS emitter and
684 detector were placed in a holder (pictured right) at a fixed distance of 4 cm, with the emitter
685 towards the front of the head (red dot in schematic). (B) The sensitivity map of the optodes is shown
686 at C1 (halfway between Cz and C3) in 2 mm thick sagittal (left) and coronal (right) slices taken from a
687 head model of 40 week old infants. The scale bar indicates the log of the normalised sensitivity (in
688 arbitrary units). A high sensitivity indicates that many photons pass through the given region on their
689 way to a detector. Red and black arrows indicate the emitter and detector locations. A, anterior; P,
690 posterior; L, left; R, right; ECT, extra-cerebral tissue; CSF, cerebrospinal fluid; GM, grey matter; WM,
691 white matter.

692

693 Fig. 2: HbO_2 goodness of fit (GOF) values and EEG waveform 3 PC weights correlate with peak Δ
694 [HbO_2] and waveform 2 amplitudes, respectively, in term infants having a noxious heel lance. A.
695 Haemodynamic response function (HRF) used for classifying trials according to the presence of an
696 HbO_2 response. B. The principal component (PC) used for classifying trials according to the presence
697 of the nociceptive-specific waveform 3 is shown in bold, overlaid onto the average EEG response for
698 clarity. C. NIRS HbO_2 GOF values are plotted against peak positive [HbO_2] changes, indicating a
699 positive correlation that is almost significant (Spearman's rho = 0.51, p=0.052, n=15). D. EEG
700 waveform 3 PC weights are plotted against the N3P3 amplitudes, indicating a significant positive
701 correlation (Spearman's rho = 0.81, p=0.0002, n=16).

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Fig. 3: Nociceptive-specific responses can be identified in average of NIRS (A) and EEG (B) trials classified as 'response present', but not in the average of those trials classified 'response absent'. A. Average (± SD) Δ [HbO_2] following noxious heel lance (t = 0 s) at the contralateral primary somatosensory cortex in 10 response present (left) and 5 response absent (right) trials. B. Average (± SD) event-related potential (ERP) at the vertex (Cz) following noxious heel lance (t = 0 ms) in 9 response present (left) and 7 response absent (right) trials, with topography maps at the negative (N) and positive (P) peaks. Time points that are significantly different from baseline are highlighted in grey.

Fig. 4: Average (± SD) haemodynamic response to a noxious heel lance (t = 0 s) at the contralateral primary somatosensory cortex in 15 term infants. [HbT], [HbO_2], and [HHb] changes are plotted separately (in green, red, and blue, respectively), and time points that are significantly different from baseline are highlighted in grey.

Fig. 5: Noxious stimulation elicits a more pronounced haemodynamic response than innocuous control or touch stimulation. A. Average (± SD) haemodynamic response to non-noxious control (left, n=18 infants) and touch (right, n = 131 touches from 16 infants) at the contralateral primary somatosensory cortex. B. Results of an independent samples t-test comparing lance (black traces; n=15 infants) and control (left) or touch (right). Average (± SD) [HbT], [HbO_2], and [HHb] changes are plotted separately (in green, red, and blue, respectively), and statistically significant differences from baseline (A) or between stimuli (B) are highlighted in grey.

Fig. 6: Average (± SD) event-related potential (ERP) at the vertex (Cz) following noxious heel lance (t = 0 ms) in 16 term infants when aligned to the second waveform (between 50-400 ms; left) and to

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727 the third waveform (between 350-600 ms; right), with topography maps at the negative (N) and
728 positive (P) peaks. Time points between 50-300 ms (left) and between 350-600 ms (right) that are
729 significantly different from baseline are highlighted in grey. Note that the group average responses
730 are from the same group of infants but look different because the individual trials have been aligned
731 differently. Grey arrows indicate the location of the third waveform when traces are aligned to
732 waveform 2 (left) and of the second waveform when traces are aligned to waveform 3 (right).

733

734 Fig. 7: Average (± SD) event-related potential (ERP) at the vertex (Cz) following innocuous control
735 (left; n = 16 term infants) and touch (right; n = 11 term infants having 106 touch trials) stimulation
736 when aligned to the second waveform (between 50-300 ms), with topography maps at the negative
737 (N) and positive (P) peaks. Time points between 50-300 ms that are significantly different from
738 baseline are highlighted in grey.

739

740 Fig. 8: Combined EEG and NIRS recordings can be successfully carried out in neonates in response to
741 cutaneous stimulation. Simultaneous EEG (left) and NIRS (right) recordings in a single term infant
742 following noxious heel lance (upper panel) and innocuous control stimulation (lower panel; stimulus
743 at t=0s), showing artefact-free EEG traces at Cz and NIRS traces at C1. Arrows indicate the presence
744 of a nociceptive-specific EEG waveform (red) following heel lance and an earlier EEG waveform
745 (green) following both stimuli. Clear increases in [HbT], [HbO_2], and [HHb] follow both noxious and
746 innocuous stimulation (right).

747

748 Fig. 9: Haemodynamic and EEG responses to innocuous control and to noxious heel lance are
749 related. Scatterplots show a strong positive correlation between haemodynamic and EEG responses

750 for the lance (left, n=9) and control (right, n=7) trials that were classified in the same way by the two

751 cortical measures.

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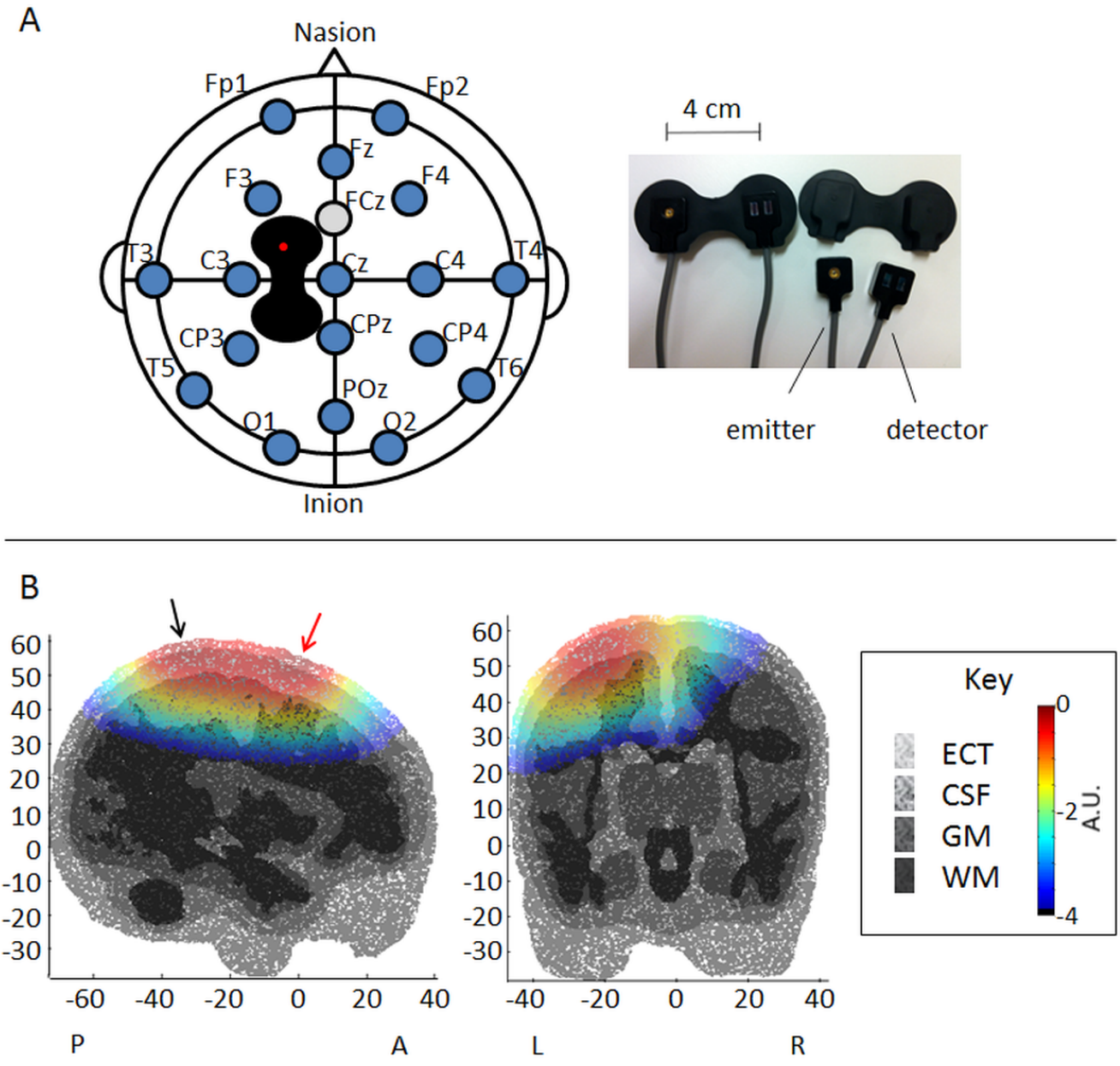

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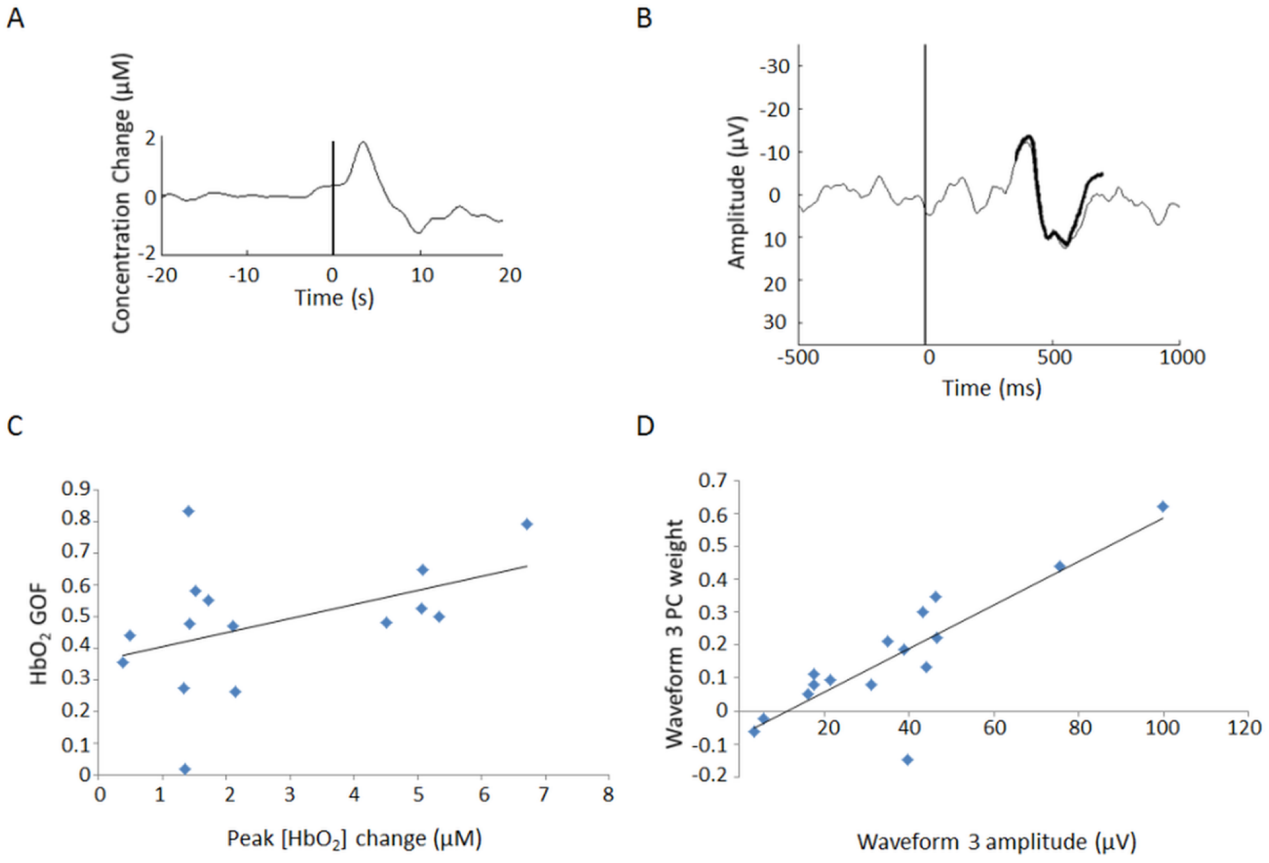

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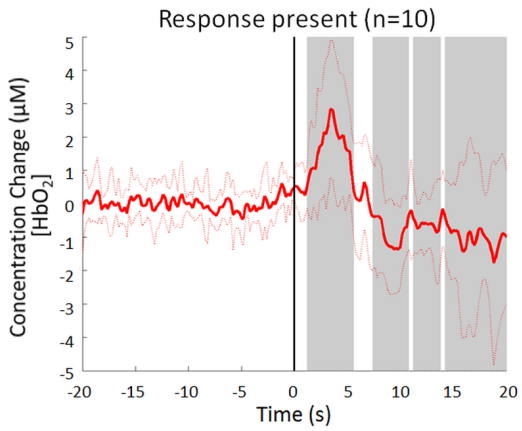

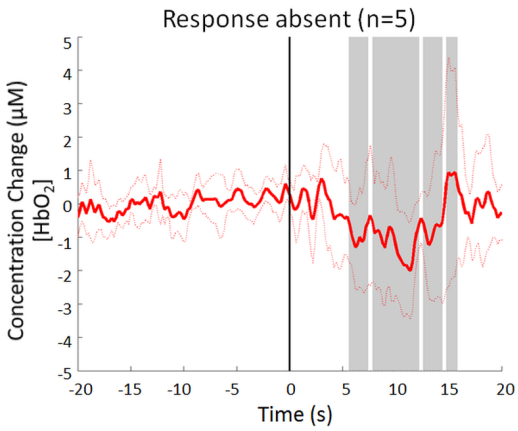

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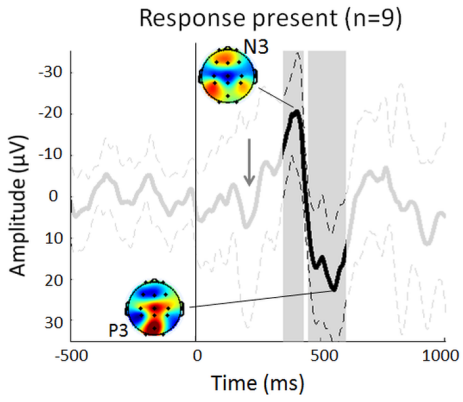

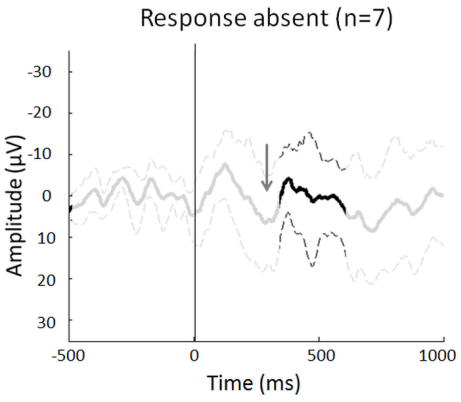

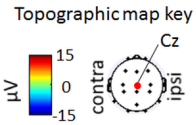

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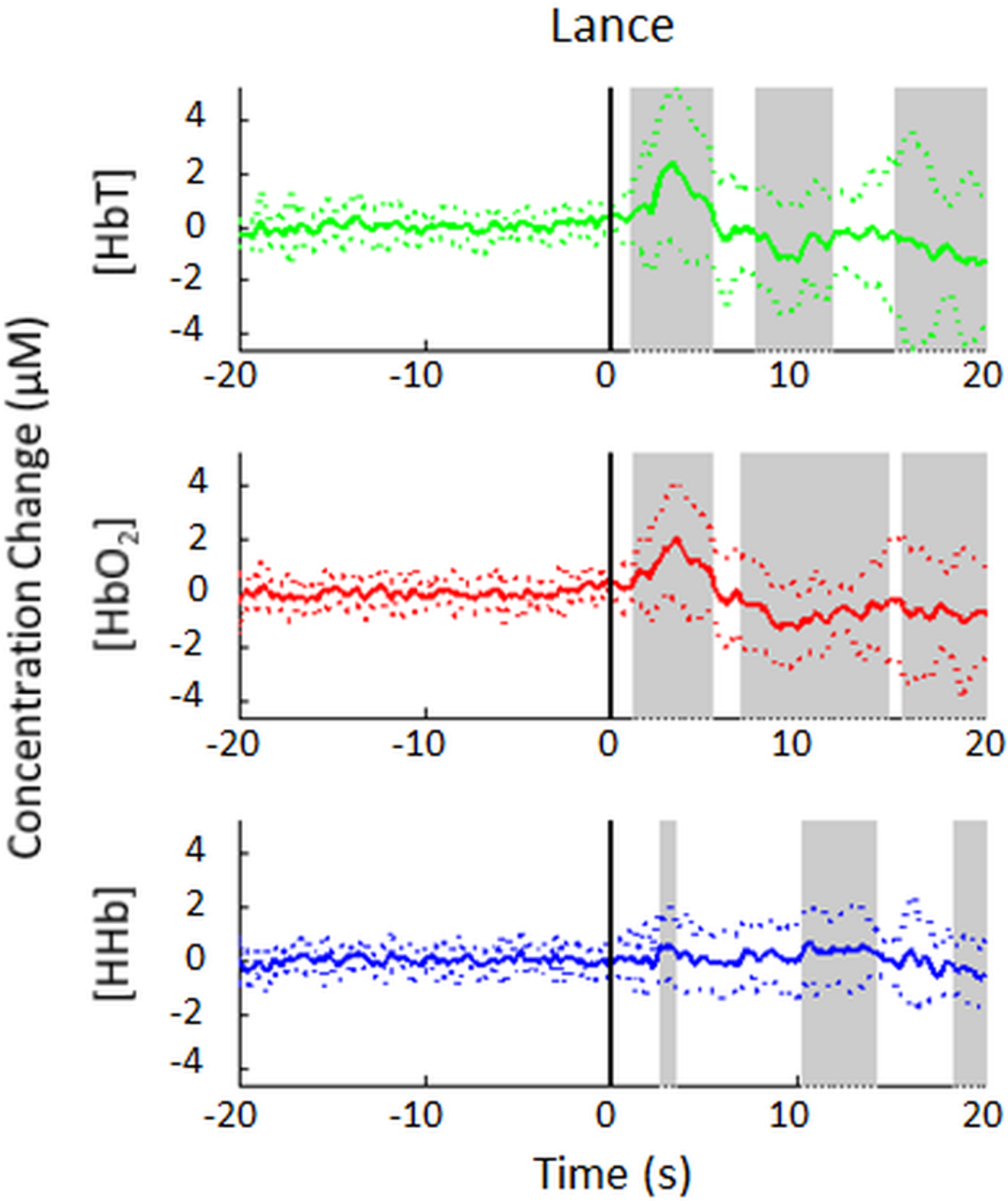

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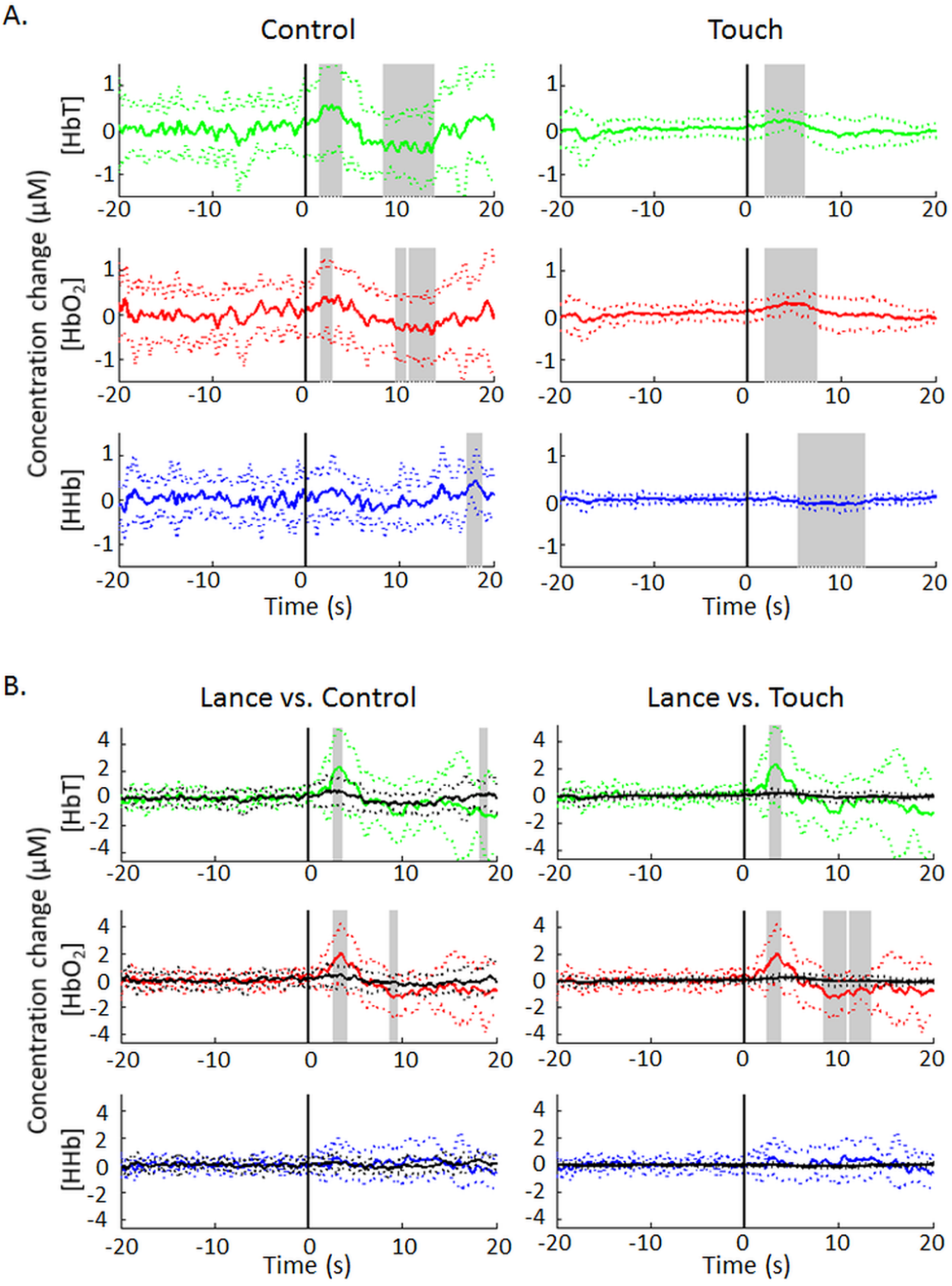

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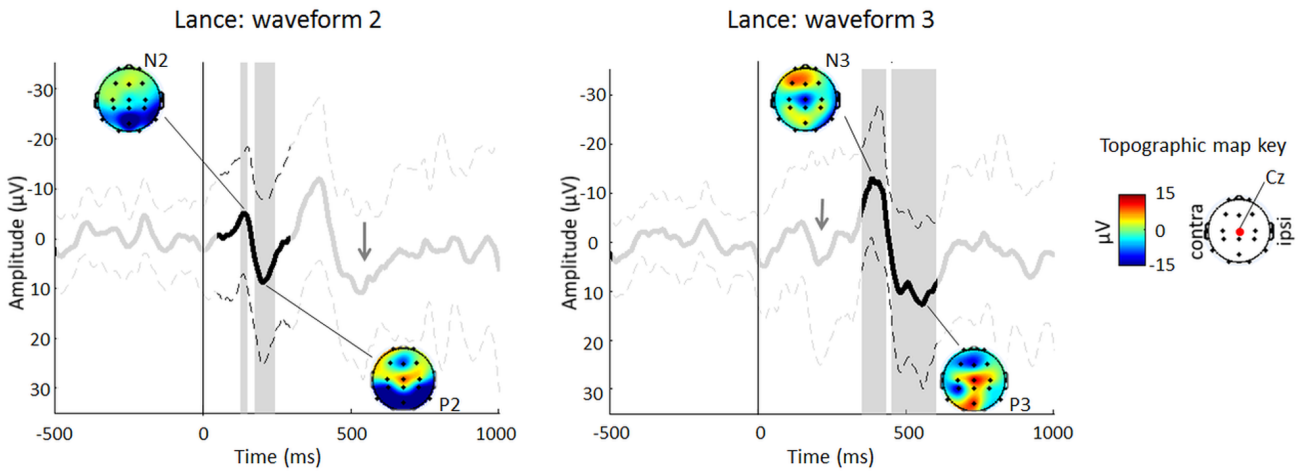

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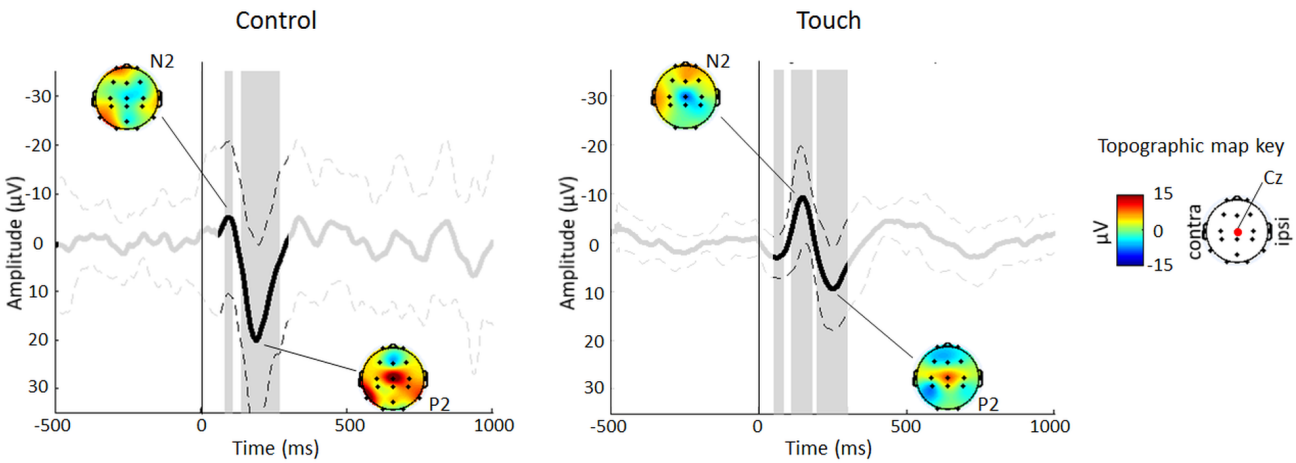

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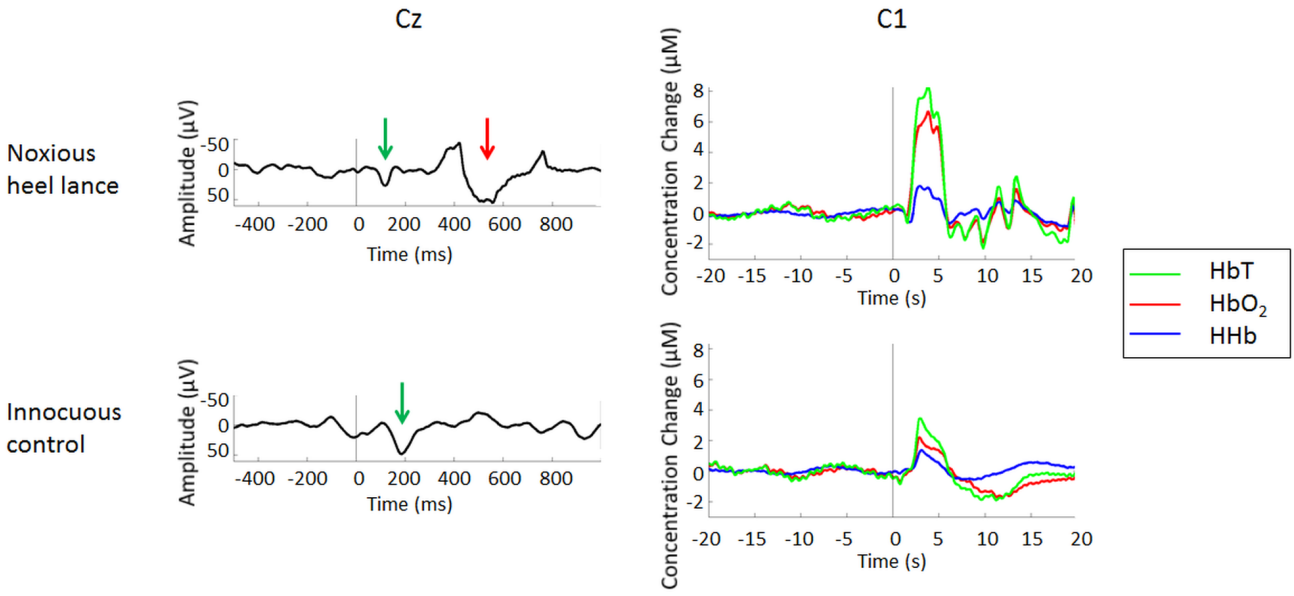

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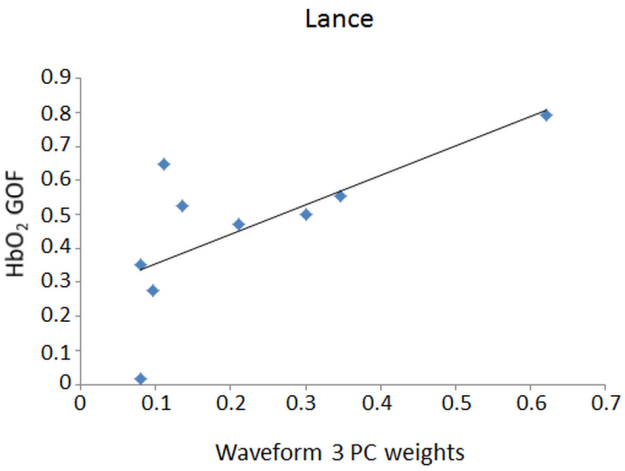

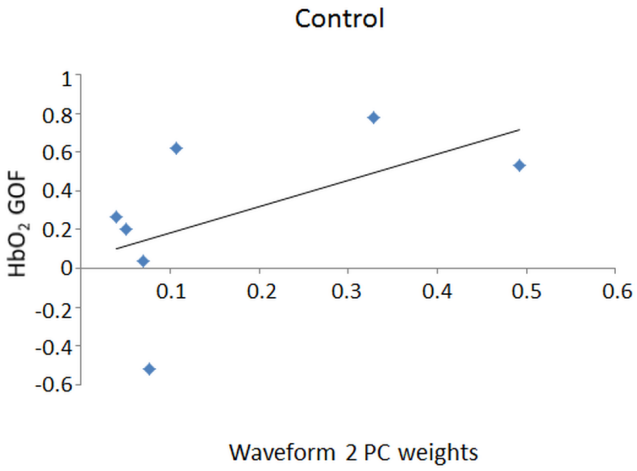

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Table 1: Demographic characteristics of participating infants

Demographic information	
No. infants	36
Age at birth (weeks)	39.0 (36.3-42.0)
Age at study (weeks)	39.2 (36.6-43.3)
Postnatal age at study (days)	2 (0-16)
No. female infants	15/36
No. infants receiving right heel stimulation	20/36
Weight at birth (g)	3257 (1920-4750)
No. caesarean deliveries	18/36

Data are median (range) or n/N and refer to number of infants unless otherwise indicated

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Table 2: Number of infants included in the analysis

	Lance	Control	Touch
Total sample included	17	20	16 (145)
NIRS accepted	15	18	16 (131)
EEG accepted	16	16	11 (106)

Data refer to number of infants; brackets indicate number of touches across all infants

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Table 3: Peak amplitude and latency of the grand average haemodynamic response to lance, control, and touch

		Early peak			Undershoot/overshoot		
		[HbT]	[HbO_2]	[HHb]	[HbT]	[HbO_2]	[HHb]
Lance							
	Amplitude, µM	2.3 ± 2.9	2.0 ± 2.2	0.5 ± 1.5	-1.3 ± 1.9	-1.3 ± 1.5	0.7 ± 1.4
	Latency, s	3.2	3.4	3.2	10	9	10.6
Control							
	Amplitude, µM	0.6 ± 1.1	0.4 ± 0.6	NS	-0.5 ± 0.8	-0.5 ± 0.6	NS
	Latency, s	2.8	2.2	NS	12.4	13.4	NS
Touch							
	Amplitude, µM	0.2 ± 0.3	0.3 ± 0.2	NS	NS	NS	-0.1 ± 0.2
	Latency, s	2.8	4.0	NS	NS	NS	9.8

Amplitude is shown as mean ± SD. Early peak refers to an initial response occurring between 1.0 and 6.5 s while Undershoot/overshoot refers to the next identifiable peak occurring after 6.5 s. NS = not significant.

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Table 4: Peak latency and amplitude of the mean lance, control, and touch ERPs

		Lance	Control	Touch
N1				
	Amplitude, μV	-5.0 ± 12.2	-5.1 ± 15.5	-9.1 ± 10.1
	Latency, ms	139	93	147
P1				
	Amplitude, μV	8.7 ± 16.6	20.1 ± 20.1	9.5 ± 8.4
	Latency, ms	202	189	248
N2				
	Amplitude, μV	-12.8 ± 12.1	--	--
	Latency, ms	385	--	--
P2				
	Amplitude, μV	12.7 ± 17.1	--	--
	Latency, ms	554	--	--

Data are mean ± S.D.

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Table 5: Classification of NIRS HbO_2 and EEG waveform 2 responses

		NIRS HbO_2		
		Present	Absent	
EEG N3P3	Present	**6**	2	**8**
	Absent	3	**3**	6
		9	5	14

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Table 6: Statistical Table

Location	Data structure	Type of test	Confidence interval
a (Fig.2C)	HbO_2 GOF values but not HbO_2 peak values are normally distributed	Spearman's rho	-0.03 – 0.86
b (Fig.2D)	N2P2 PC weights but not N2P2 amplitudes are normally distributed	Spearman's rho	0.39 – 0.98
c	N3P3 PC weights and HbO_2 GOF values both normally distributed	Pearson's r	0.15 – 0.96
d	HbO_2 GOF values but not N2P2 PC weights are normally distributed	Spearman's rho	-0.22 – 0.89