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# Single-trial averaging improves the physiological interpretation of contact heat evoked potentials

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ABSTRACT

Laser and contact heat evoked potentials (LEPs and CHEPs, respectively) provide an objective measure of pathways and processes involved in nociception. The majority of studies analyzing LEP or CHEP outcomes have done so based on conventional, across-trial averaging. With this approach, evoked potential components are potentially confounded by latency jitter and ignore relevant information contained within single trials. The current study addressed the advantage of analyzing nociceptive evoked potentials based on responses to noxious stimulations within each individual trial. Single-trial and conventional averaging were applied to data previously collected in 90 healthy subjects from 3 stimulation locations on the upper limb. The primary analysis focused on relationships between single and across-trial averaged CHEP outcomes (i.e., N2P2 amplitude and N2 and P2 latencies) and subject characteristics (i.e., age, sex, height, and rating of perceived intensity), which were examined by way of linear mixed model analysis. Single-trial averaging lead to larger N2P2 amplitudes and longer N2 and P2 latencies. Age and ratings of perceived intensity were the only subject level characteristics associated with CHEPs outcomes that significantly interacted with the method of analysis (conventional vs single-trial averaging). The strength of relationships for age and ratings of perceived intensity, measured by linear fit, were increased for single-trial compared to conventional across-trial averaged CHEP outcomes. By accounting for latency jitter, single-trial averaging improved the associations between CHEPs and physiological outcomes and should be incorporated as a standard analytical technique in future studies.

## 1. Introduction

Contact heat and laser evoked potentials (CHEPs and LEPs, respectively) represent recruitment of thinly myelinated A-delta fibres in the periphery, conduction in the spinothalamic tract, and are associated with the perception of pain (Chen et al., 2001; Haefeli et al., 2013b; Jutzeler et al., 2016; Kramer et al., 2009). While neural activity of CHEPs and LEPs may not directly reflect central processing of nociception and pain (Mouraux and Iannetti, 2018, 2009), both measures are reliably employed to assess the function of the spinothalamic pathways and pain perception (Chen et al., 2001; Haefeli et al., 2013a; Jutzeler et al., 2016; Kramer et al., 2012b) within subjects (Hu and Iannetti, 2019).

The amplitudes and latencies of nociceptive evoked potentials are dependent on numerous factors, including stimulation location (Granovsky et al., 2005; Haefeli et al., 2013b), age (Chao et al., 2007; Granovsky et al., 2016; Jutzeler et al., 2016), and sex (Chen et al., 2006; de Tommaso et al., 2017; Granovsky et al., 2016; Staikou et al., 2016; Truini et al., 2005). Location related variability is primarily attributable to differences in peripheral conduction distances (Magerl and Treede, 1996; Truini et al., 2005), temporal dispersion (Iannetti et al., 2006; Kramer et al., 2013), and receptor density gradients (Atherton et al., 2007; Perretti et al., 2003; Ragé et al., 2011). In

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addition, behavioral relevance of stimulus location further influences waveforms parameters of evoked potentials, as more proximal locations tend to result in larger and earlier responses (Bufacchi et al., 2016; Bufacchi and Iannetti, 2018; Sambo et al., 2012; Sambo and Iannetti, 2013). Microstructural changes within the somatosensory nervous system associated with aging give rise to lower amplitude and longer latency of nociceptive evoked potentials in older adults (Jacobs and Love, 1985; Lauria et al., 1999). Sex differences, while variably reported (Chen et al., 2006; de Tommaso et al., 2017; Staikou et al., 2016; Truini et al., 2005), tend to provide objective evidence that women are more sensitive to noxious stimulation compared to men (Granovsky et al., 2016), even after adjusting for relevant subject characteristics (e.g., height) (Jutzeler et al., 2016). Collectively, these findings demonstrate the inherent value of CHEPs and LEPs to depict biologically relevant information underlying noxious heat stimulation applied in the periphery.

The standard acquisition of either CHEPs or LEPs involves the repetitive application of noxious stimuli at long inter-stimulus intervals, from which waveforms are conventionally averaged for visual inspection and evaluation (Chen et al., 2006; Kramer et al., 2013). While effective in increasing signal to noise ratios, a major disadvantage of this approach is that trial specific variations in amplitude, latency, and morphology of evoked potentials within a subject, between stimuli are minimized and distorted from across trial averaging (Mouraux and Iannetti, 2008). An important example of such inter-trial variability is latency jitter, which describes the variation in latencies of N2 and P2 waveforms. The conventional analysis of across trial averaging, without accounting for latency jitter, can distort evoked potential components, and thus a major concern is that this practice removes biologically relevant information. As an alternative, single trial averaging techniques have been developed to extract waveform characteristics from evoked potentials generated in response to individual stimuli (Hatem et al., 2012; Hu et al., 2011, 2010; Huang et al., 2013; Mayhew et al., 2006; Warbrick et al., 2009). Single trial averaging tends to increase LEP amplitudes (Hu et al., 2011; Warbrick et al., 2009), improving the clarity of evoked potential waveforms by accounting for trial to trial variations. Despite these advances in signal processing (Mouraux and Iannetti, 2008), relatively few studies have adopted single trial approach to analyze and interpret CHEPs (Warbrick et al., 2009).

The present study addressed the hypothesis that the single-trial compared to across trial averaging approach would better capture physiological relevant information in responses to noxious stimulation, specifically by accounting for latency jitter in N2P2 waveforms. This in turn will strengthening the relationships between CHEP outcomes, subject characteristics, and stimulus location. Single-trial analysis was performed on previously published CHEPs data using an established technique (Hatem et al., 2012; Hu et al., 2011, 2010) and compared to outcomes from conventional averaging (Jutzeler et al., 2016).

## 2. Methods

To address our hypothesis, we utilized a large CHEPs dataset previously published by Jutzeler et al., 2016 (Jutzeler et al., 2016). This study focused on normative CHEP outcomes, based on conventional averaging only. For comparative purposes, the results of conventional averaging are included here again. All procedures were in accordance with the Declaration of Helsinki and approved by the local ethics board 'Kantonale Ethikkommission Zurich, KEK' (ref. number: EK-04/2006, cinicaltrial.gov number: NCT02138344). Participants provided written informed consent.

### 2.1. Subjects

One hundred and five neurologically healthy subjects were recruited through online and printed advertisements. Inclusion criteria comprised age of 18–80 years and native language being either English or German. Exclusion criteria included pregnancy, intake of any medication (except birth control), and any obvious neurological condition.

### 2.2. Study protocol

CHEPs were recorded after thermally stimulating the C4 (shoulder), C6 (base of the thumb), and C8 dermatomes (base of *digitus minimus*). Normal baseline stimulations (35 °C baseline, ramped to a peak temperature of 52 °C, at a rate of 70 °C/s) were employed to record CHEPs, described previously (Haefeli et al., 2013a; Jutzeler et al., 2015; Kramer et al., 2012b). During the acquisition of CHEPs, subjects were lying in a supine position with eyes open. In order to minimize ocular artefacts, subjects were instructed to focus on a point on the ceiling, minimize blinking, as well as to remain relaxed and quiet during testing. Traces contaminated with muscle or blink artefacts were excluded in real time and additional stimuli were applied to record 15 artefact free traces per location. Contact heat stimuli were applied with an inter-stimulus time interval that randomly varied between 8 and 12 s (Haefeli et al., 2013a; Jutzeler et al., 2015; Kramer et al., 2012b). Cued by an auditory signal two seconds post stimulus, subjects were instructed to rate the perceived pain of each stimulus from 0 (no pain) to 10 (most unbearable pain). The auditory cue also provided an opportunity for participants to blink and therefore, avoid blinking during heat stimulations. The CHEPs thermode was slightly repositioned after each stimulus within the dermatome tested to reduce receptor fatigue or sensitization by overheating the skin (Granovsky et al., 2005).

### 2.3. Stimulating device and recording set up

A contact heat stimulator was employed to deliver stimulation (Pathway, Medoc, RamatYishai, Israel). The CHEP thermode surface (diameter: 27 mm) consists of a heating thermo-foil covered with a layer of thermo-conductive plastic. The nominal heating rate of this device is 70 °C/ s, with a cooling rate of 40 °C/ s.

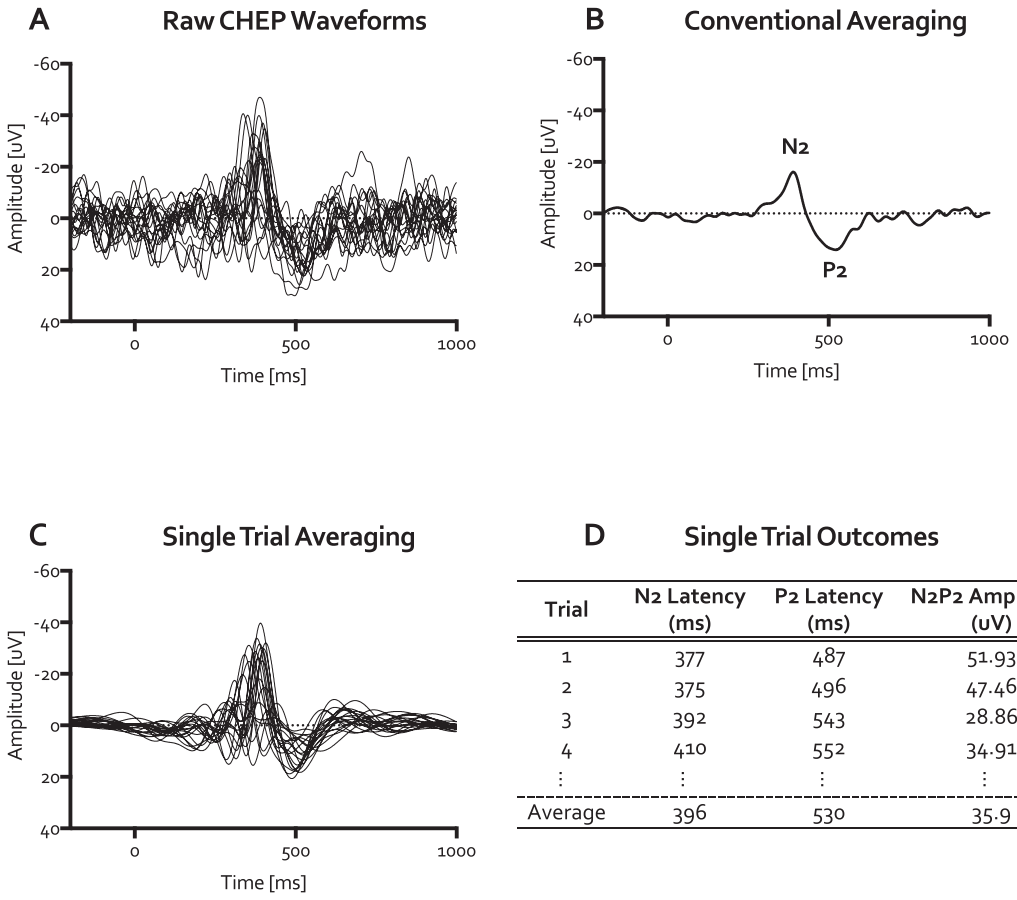
Cortical responses to the noxious heat were recorded with 9 mm Ag/AgCl surface disk electrodes filled with conductive adhesive gel. Scalp recording sites were prepared with Nuprep (D.O. Weaver & Co. Aurora, CO) and alcohol. Electrodes were positioned in accordance with the International 10–20 system. Both N2 and P2 were acquired from an active vertex recording electrode (Cz) referenced to linked earlobes (A1-A2). The rationale for a reduced electrode set up arose from the fact that consistent negative and positive potentials, labelled N2 and P2, are reliably detected at Cz (Chen et al., 2006; Granovsky et al., 2016; Haefeli et al., 2013a; Jutzeler et al., 2015; Kramer et al., 2012b). All signals were sampled at 2000 Hz using a preamplifier (20000x, bandpass filter 1 - 300 Hz, ALEA Solutions, Zurich, Switzerland). Data were recorded with 100 ms pre-trigger and a one second post-trigger in a customized LabView (V1.43 CHEP, ALEA Solutions, Zurich, Switzerland) program.

### 2.4. Conventional averaging

Filtered CHEPs from 15 artefact free trials were averaged and visually inspected for N2 and P2 waveforms. To ensure adequate signal to noise ratio for the determination of waveform parameters, averaged amplitudes of less than 10 uV were excluded from further analysis (Jutzeler et al., 2016).

### 2.5. Single trial analysis

Single trial analysis was performed using an openly available program (Hu et al., 2011, 2010). In brief, single trial averaging using a combination of wavelet filtering and multiple linear regressions to determine waveform parameters (N2/P2 amplitudes and latencies) from individual trials (Hu et al., 2011, 2010). Wavelet filtering was employed




| Trial | N2 Latency (ms) | P2 Latency (ms) | N2P2 Amplitude (uV) |
|---|---|---|---|
| 1 | 377 | 487 | 51.93 |
| 2 | 375 | 496 | 47.46 |
| 3 | 392 | 543 | 28.86 |
| 4 | 410 | 552 | 34.91 |
| ⋮ | ⋮ | ⋮ | ⋮ |
| Average | 396 | 530 | 35.9 |

**Fig. 1.** Representative traces of conventional averaging and single trial averaging analysis methods. A) Individual contact heat evoked potential (CHEP) waveforms, filtered and re-referenced. B) Conventional averaging of individual CHEP waveforms, from which N2 and P2 outcome are derived. C) Individual CHEP waveforms following single trial analysis, via wavelet filtering and multiple linear regression with dispersion term (Hu et al., 2011, 2010). D) Averaged CHEP outcomes determined from single trial analysis.

to enhance the signal to noise ratio and facilitate the estimation of latency and amplitude of single trial evoked potential peaks (Hu et al., 2011, 2010; Kramer et al., 2013). To perform an unbiased single trial analysis, an automated approach using multiple linear regression with a dispersion term (MLRd) is then implemented (Hu et al., 2011). The dispersion term enhances the ability of multiple linear regressions to detect changes in waveform morphology and provides a more accurate measure of latency and amplitude of single trial evoked potential peaks (Hu et al., 2011). Waveform peaks were automatically detected within a 100-millisecond window of the wavelet-filtered average N2 and P2. Individual trial N2 and P2 waveform parameters were then averaged together, to provide the single trial analysis outcomes for each participant (Fig. 1).

### 2.6. Statistical analysis

CHEP outcomes from conventional averaging and single-trial averaging were examined using linear mixed effects models. For each CHEP outcome (N2P2 amplitude, N2 latency, P2 latency), fixed effects of dermatome (C4, C6, C8), and analysis method (conventional vs single trial averaging) were assessed in a linear mixed model, with random effects of participants. Subject characteristics (age, sex, height, and rating of perceived intensity) were included in these linear mixed models, examining the overall effect of analysis method and dermatome on CHEP outcomes. Subsequent models with an interaction term between each subject characteristic and analysis method were included to explore of influence of analysis method on the relationships between CHEP outcomes and subject characteristics. Significant interactions were followed up via linear mixed models for each analysis method, to determine respective differences in CHEP outcomes and the relationship to subject characteristics between conventional and single-trial averaging. Based on preliminary linear mixed model analysis, multiple linear regressions between age, rating of perceived intensity, and CHEP outcomes were further explored for both analysis methods. Bonferroni correction was applied to adjust for multiple comparisons. An alpha level of 0.05 was used for all statistical tests. R Statistical Software (version 3.5.3, MacOS 10.14.6 Mojave) was used for all statistical analyses and producing all plots (R Core Team, 2019; Wickham, 2016).

### 2.7. Simulated data analysis

To further explore the effects of conventional averaging and single trial analysis on CHEPs outcomes, we performed a small simulation experiment. The goal here was to systematically manipulate the amplitudes and latencies of individual trial waveforms to determine the comparative effects on conventional averaging and single trial analysis, respectively. A single waveform was artificially increased in amplitude by 40%, and the latency was shifted 20 ms to the left. Thus, two waveforms (small and large) were used for our simulation. First, the small waveform was replicated, such that a 15-trial dataset contained only the small waveform. Then, a single large waveform trial was added, with a small waveform removed. This was repeated until only large waveforms remained. For each dataset, we performed conventional (across-trial) averaging and single trial analysis, and N2P2 outcomes were compared for each dataset. Findings from the simulation and example traces of waveforms can be found in Fig. 7.

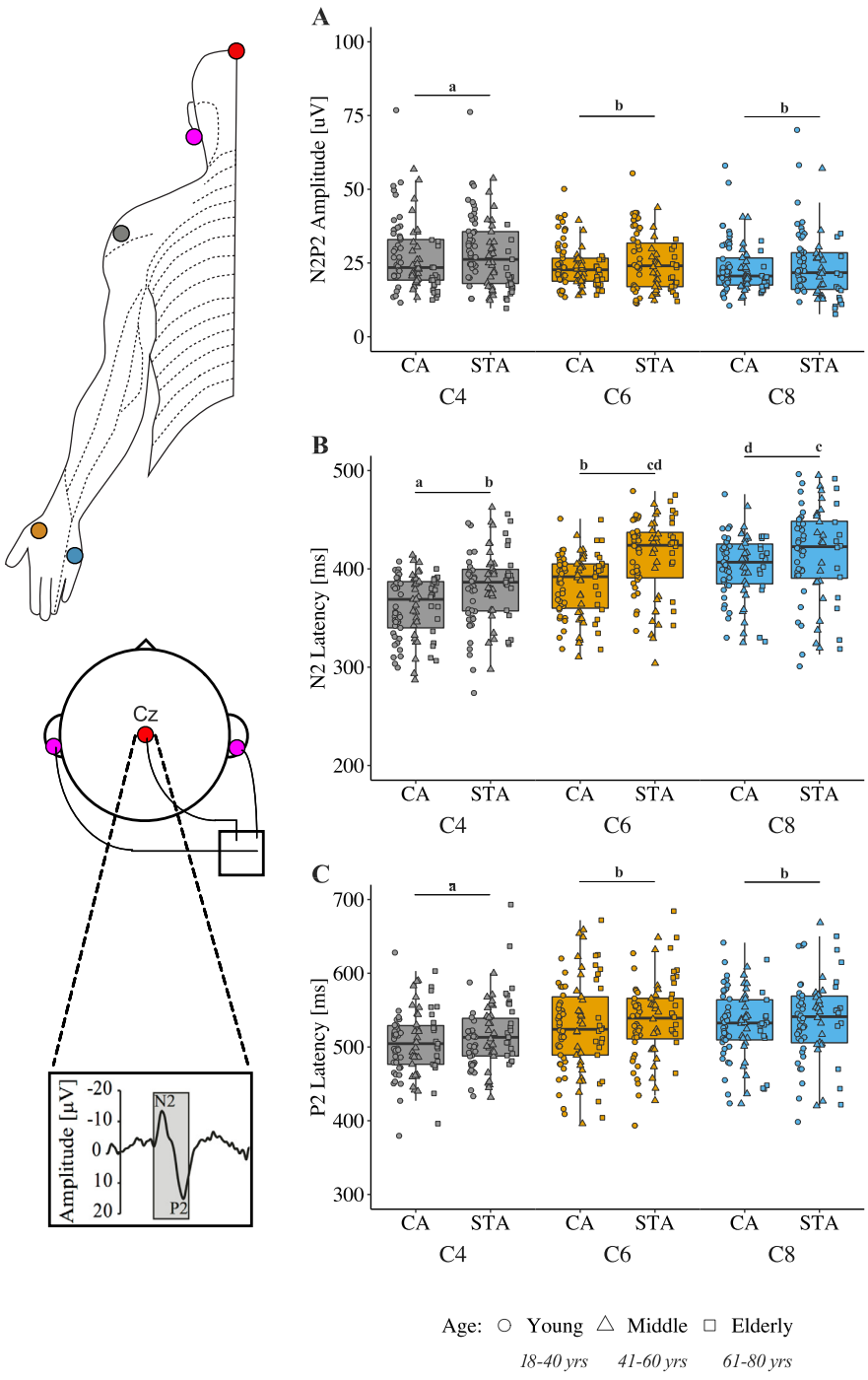



**Fig. 2.** Contact heat evoked potentials (CHEP) N2P2 amplitudes (A), N2 latencies (B), and P2 latencies (C) from cervical spine dermatomes (C4, C6, C8). Conventional averaging (CV) and single trial averaging (STA) analysis methods compared within each dermatome / CHEP outcomes. Age groups separated into young (18–40 yrs), middle (41–60 yrs), and elderly (61–80 yrs). Letters denote significant differences between dermatomes for both stimulation protocols, such that different letters correspond to significant differences between dermatomes. For panel B specifically, different letters denote significant differences between dermatomes and between analysis methods (i.e. a is significantly different from b, c, cd, and c; d is significantly different from c, but neither are significantly different from cd). Linear mixed models were adjusted for age, sex, and height, with a significance level of alpha <0.05.

## 3. Results

### 3.1. Cohort summary

Out of 105 neurologically healthy participants, four were previously excluded due to intolerance to heat stimuli and poor data quality (Jutzeler et al., 2016). An additional eight participants were excluded from the present analysis due to poor data quality (i.e., conventional averaged N2P2 amplitudes below 10uV or substantial artefacts that influenced single trial analysis). The remaining 93 individuals composed of 45 men and 48 women with a mean age 45.8 ± 16.8 years (range: 19–80 years) were included in the both analysis methods (conventional averaging and single trial analysis). Individual characteristics are summarized in Table 1. Summary N2P2 amplitude, N2 latency, and P2 latency following conventional and single-trial averaging are summarized in Table 2. Representative CHEPs (N2/P2) individual trial traces of conventional and single-trial averaging are illustrated in Fig. 1.

**Table 1**
Participant characteristics (mean ± standard deviation).

| Sex | Males ($N$ = 45) | Females ($N$ = 48) |
|---|---|---|
| Age (years) | 44.2 ± 15.4 | 47.2 ± 18.0 |
| Height (cm) | 168.1 ± 7.0 | 178.4 ± 7.1 |

**Table 2**
Contact heat evoked potential (CHEP) summary outcomes (mean ± standard deviation).

| CHEP Outcome | Analysis Method | Dermatome | | |
|---|---|---|---|---|
| | | *C4* | *C6* | *C8* |
| N2P2 Amplitude (uV) | CA | 27.0 ± 11.7 | 23.8 ± 7.4 | 23.2 ± 8.5 |
| | STA | 28.2 ± 12.2 | 25.2 ± 9.8 | 24.1 ± 11.4 |
| N2 Latency (ms) | CA | 361.4 ± 31.3 | 383.6 ± 31.1 | 402.3 ± 31.6 |
| | STA | 380.8 ± 37.9 | 414.6 ± 41.2 | 420.3 ± 53.8 |
| P2 Latency (ms) | CA | 504.4 ± 44.2 | 527.5 ± 61.2 | 530.0 ± 46.5 |
| | STA | 513.9 ± 43.8 | 536.8 ± 53.6 | 541.5 ± 62.4 |

CA - Conventional averaging.
STA – Single-trial averaging.

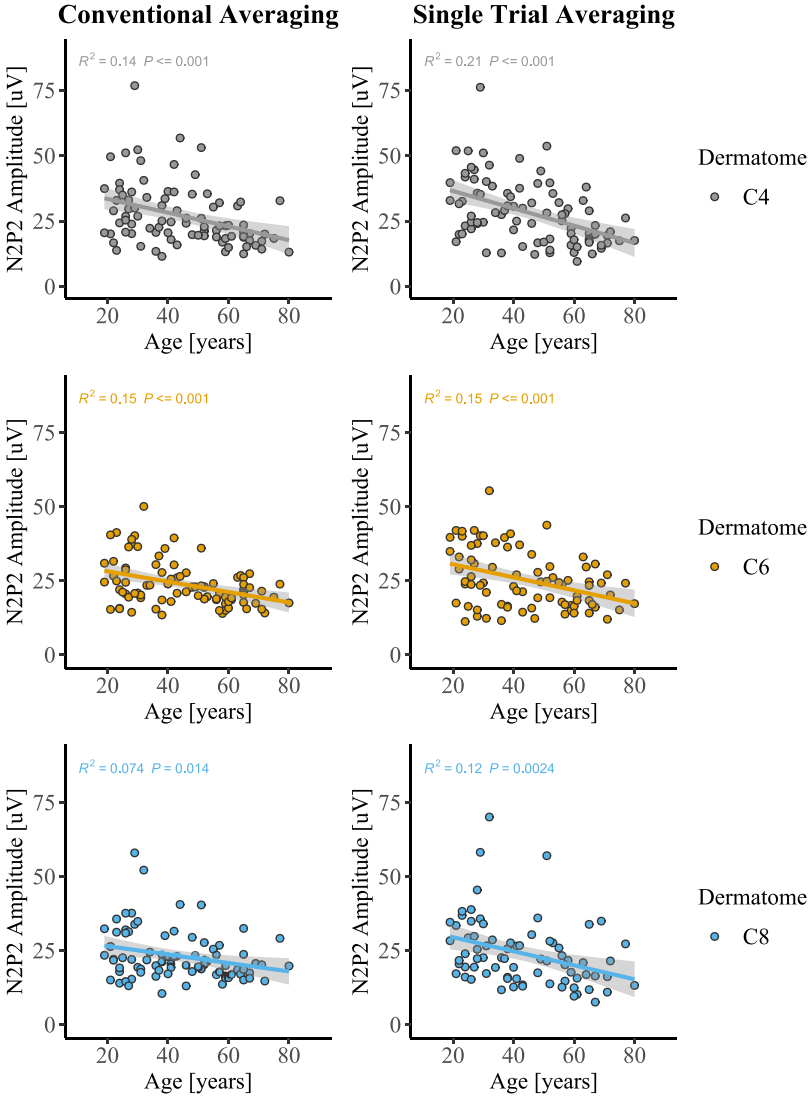



**Fig. 3.** Linear regressions between aging and N2P2 amplitude for each dermatome (C4, C6, C8) and analysis method (conventional averaging and single trial averaging). Strength of linear regression ($R^2$) is given for each dermatome, across analysis methods.

### 3.2. Main effects of analysis method

There were significant main effects of analysis method on N2P2 amplitude ($F_{(1408.3)} = 5.97$, $p<0.05$), N2 latency ($F_{(1413.7)} = 69.8$, $p<0.001$), or P2 latency ($F_{(1410.3)} = 6.37$, $p<0.05$). Overall, single-trial averaging resulted in larger N2P2 amplitudes and longer N2 and P2 latencies.

### 3.3. Main effects of stimulus location

There were significant main effects of stimulus location on N2P2 amplitude ($F_{(2420.7)} = 9.92$, $p<0.001$), and both P2 ($F_{(2428.7)} = 72.7$, $p<0.001$) and N2 latencies ($F_{(2422.4)} = 20.0$, $p<0.001$). Generally, latencies were longer, and amplitudes were smaller in C6 and C8 (i.e., hand) dermatomes compared to C4 (i.e., shoulder). Specific comparisons between dermatomes for CHEP outcomes are further outlined in Fig. 2.

### 3.4. Main effects of subject characteristics

There was a significant main effect of age on N2P2 amplitude ($F_{(1,87.4)} = 18.4$, $p<0.001$), N2 latency ($F_{(1,86.3)} = 4.72$, $p<0.05$), and P2 latency ($F_{(1,83.0)} = 8.00$, $p<0.01$). Our analysis also revealed a significant main effect of sex on N2 ($F_{(1,87.5)} = 7.06$, $p<0.01$) and P2

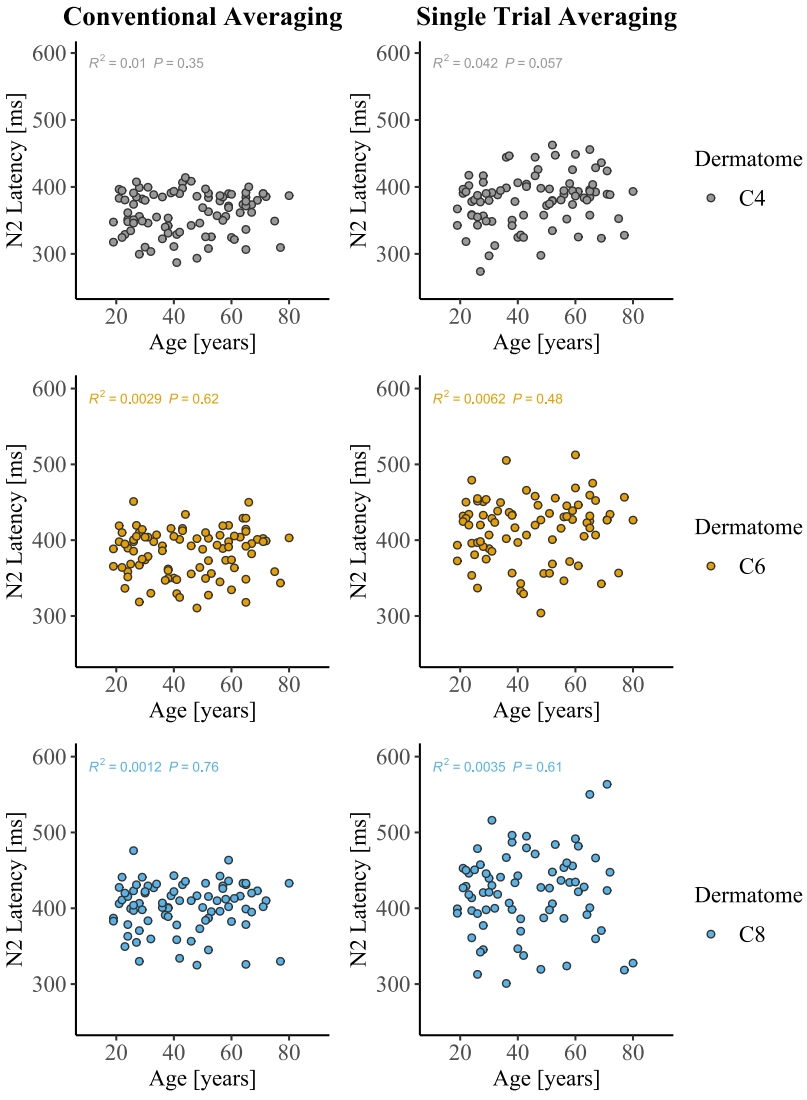



**Fig. 4.** Linear regressions between aging and N2 latency for each dermatome (C4, C6, C8) and analysis method (conventional averaging and single trial averaging). Strength of linear regression ($R^2$) is given for each dermatome, across analysis methods.

latencies ($F_{(1,85.6)}$ = 10.32, $p$<0.01), as well as a main effect of rating of perceived intensity on N2P2 amplitude ($F_{(1480.6)}$ = 31.7, $p$<0.001). No significant main effect of height on any CHEP outcomes was found (N2P2: $F_{(1,85.8)}$ = 0.29, $p$ = 0.59; N2: $F_{(1,84.0)}$ = 1.23, $p$ = 0.27; P2: $F_{(1,81.4)}$ = 0.01, $p$ = 0.91).

### *3.5. Interaction effects between method of analysis, stimulation location, and subject characteristics*

There was a significant age by analysis method interaction effect for N2P2 amplitude ($F_{(1408.0)}$ = 4.60, $p$<0.05) and P2 latency ($F_{(1407.1)}$ = 8.55, $p$<0.01). These interactions suggest that the relationship between age and CHEP outcomes depends on the method of analysis. Linear mixed models performed separately for single-trial and conventionally averaged CHEPs revealed significant main effects of age for N2P2 amplitude for both analysis methods (conventional: $F_{(1,87.0)}$ = 13.7, $p$<0.001; single trial: $F_{(1,86.5)}$ = 21.8, $p$<0.001), while the effect of age was significant during single trial averaging only for P2 latency (conventional: $F_{(1,86.4)}$ =1.16, $p$ = 0.28; single trial: $F_{(1,81.8)}$ = 16.5, $p$<0.001). For both N2P2 amplitude and P2 latency, the beta coefficients for age from linear models were higher for single-trial averaging (N2P2: $ß$ = −0.31, $t$ = −4.8, $p$<0.001; P2: $ß$ = 0.84, $t$ = 2.5, $p$<0.05) compared to conventional averaging (N2P2: $ß$ = −0.25, $t$ = −4.3, $p$<0.001; P2: $ß$ = 0.27, $t$ = 0.83, $p$ = 0.40). Collectively, these observations suggest that the relationship between age and CHEPs is strengthened by single-trial analysis. Specific linear relationships between aging and CHEP outcomes for each dermatome and analysis method are further outlined in Figs. 3–5, while the relationships between rating of perceived intensity and N2P2 amplitude are outlined in Fig. 6.

There was a significant rating of perceived intensity by analysis method interaction for N2P2 amplitude ($F_{(1411.7)}$ = 4.24, $p$<0.05). This suggests that the relationship between N2P2 amplitude and rating of perceived intensity depends on the method of analysis. Separate linear mixed models reveal significant main effects of rating of perceived intensity on N2P2 amplitude for conventional and single trial averaged analysis methods (conventional: $F_{(1250.3)}$ =10.0, $p$<0.001; single trial: $F_{(1231.7)}$ = 15.9, $p$<0.001). The beta coefficients rating of perceived intensity from linear models was higher for single trial averaging ($ß$ = 1.74, $t$ = 3.53, $p$<0.001) compared to conventional averaging ($ß$ = 1.55, $t$ = 3.70, $p$<0.001). Similar to aging, these collective observations suggest that the relationship between rating of perceived intensity and N2P2 amplitude is strengthened by single trial analysis. There were no significant interactions for sex or height and analysis method for any CHEP outcomes. There were also no three-way interactions between subject characteristics, analysis, and dermatome for any CHEP outcomes.

There was no significant interaction between dermatome and analysis for N2 latency ($F_{(2406.4)}$ = 2.59, $p$ = 0.08). Pairwise comparisons

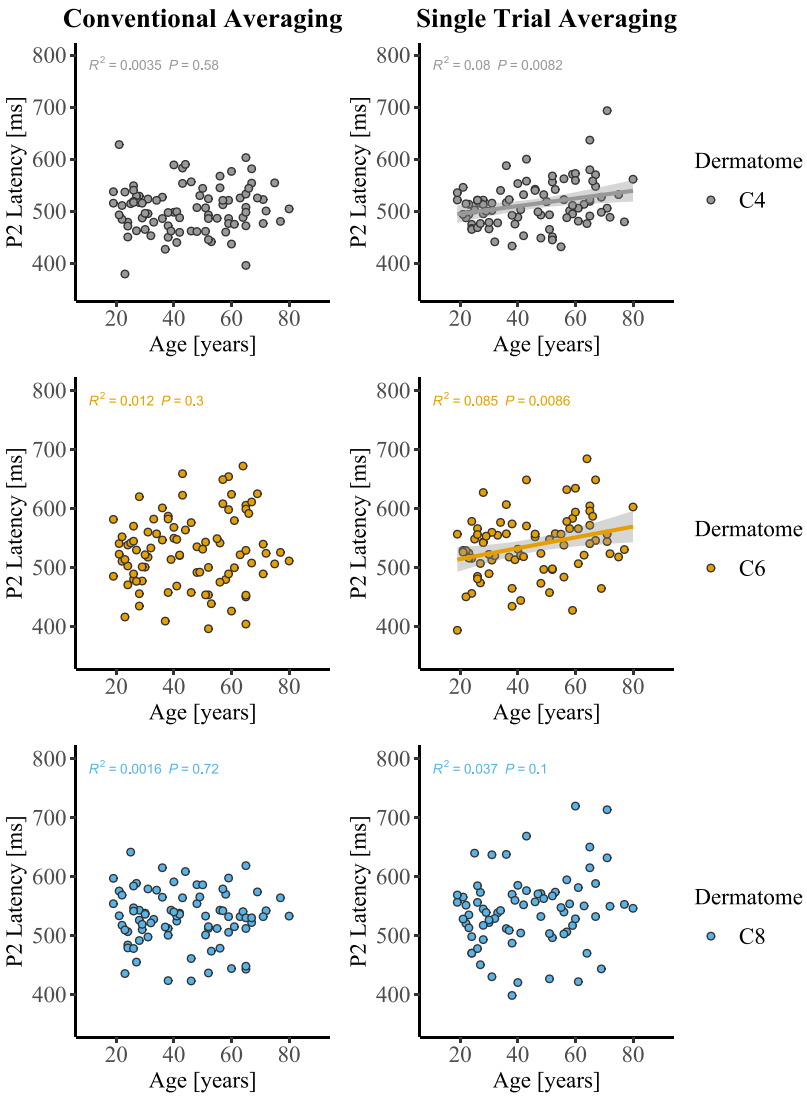



**Fig. 5.** Linear regressions between aging and P2 latency for each dermatome (C4, C6, C8) and analysis method (conventional averaging and single trial averaging). Strength of linear regression ($R^2$) is given for each dermatome, across analysis methods.

between analysis methods revealed significantly longer N2 latencies for single trial averaging compared to conventional averaging for C4 ($t = -8.4$, $p<0.001$, Cohen's $d = 0.56$), C6 ($t = -11.8$, $p<0.001$, Cohen's $d = 0.85$), and C8 ($t = -4.0$, $p<0.001$, Cohen's $d = 0.44$) dermatomes. Separate linear models calculated for conventional averaging and single trial averaging revealed differences between dermatomes. For single trial averaging, N2 latencies for C6 ($t = 5.8$, $p<0.001$) and C8 ($t = 6.5$, $p<0.001$) were significantly longer than C4, with no significant differences between C6 and C8 ($t = 1.06$, $p = 0.29$). This fits with increased peripheral conduction distance associated with stimulating the shoulder compared to stimulating the hand. Conventional averaging demonstrated significantly longer N2 latencies for C6 ($t = 9.2$, $p<0.001$) and C8 ($t = 16.5$, $p<0.001$) compared to C4, and also demonstrated a further significant increase for N2 latencies in C8 compared to C6 ($t = 8.4$, $p<0.001$) (Fig. 2).

### 3.6. Simulated data findings

N2 latencies were longer with single trial analysis compared to conventional (across-trial) averaging when a low number of large waveforms were included in datasets (Fig. 7A). As progressively more large waveforms were included, conventional averaging and single trial analysis N2 latencies converged (Fig. 7A). A similar trend was observed for P2 latencies, albeit with less convergence with progressively more large amplitude waveforms (Fig. 7B). N2P2 amplitude were most consistent between analysis methods with similar number of small and large amplitude trials, while extremes presented the largest differences between analysis methods (Fig. 7C). Overall, conventional averaging and single trial analysis are similar were similar when datasets contained mostly large waveforms with a few small waveforms. Conversely, analysis methods deviated substantially when datasets contained a few large amplitude trials.

## 4. Discussion

In the current study, single-trial averaging lead to larger amplitude CHEPs waveforms and longer latencies. Moreover, these subtle changes in N2P2 waveforms significantly changed the interpretation of CHEP outcomes compared to conventional (across-trial averaging) analysis. Single trial averaging revealed stronger associations with peripheral conduction distances, age, and rating of perceived intensity compared to conventional averaging. Overall, our results demonstrate the advantage of single trial averaging to capture biologically relevant information pertaining to the acquisition of CHEPs.

The application of single trial analysis to nociceptive evoked potentials dates back more than 25 years, to seminal research exploring time-shifted averaging of LEPs (Purves and Boyd, 1993). Subsequent approaches utilized wavelet transformation as a means of filtering indi-

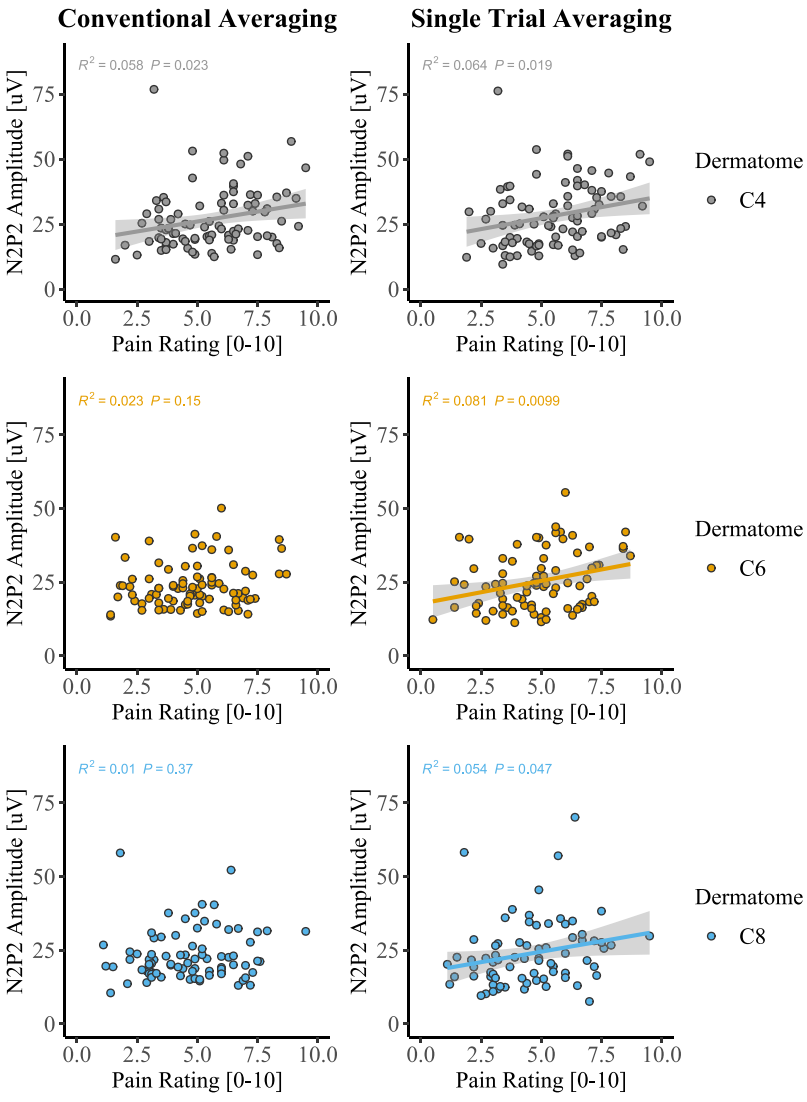



**Fig. 6.** Linear regressions between pain rating and N2P2 amplitude for each dermatome (C4, C6, C8) and analysis method (conventional averaging and single trial averaging). Strength of linear regression ($R^2$) is given for each dermatome, across analysis methods.

vidual evoked potentials (Mouraux and Plaghki, 2004) and incorporated automated peak detection by multiple linear regression (Mayhew et al., 2006), before ultimately arriving at the method employed in the current study (Hu et al., 2011, 2010). While demonstrating that single trial analysis is clearly possible, decidedly missing, to this point, has been evidence that single trial analysis improves the detection of biologically relevant aspects of nociception. The lack of this knowledge has likely, in part, contributed to limited uptake among researchers, for whom single trial analysis comes at the cost of increased analysis time and greater complexity compared to conventional averaging.

Towards shifting the discussion from theory to practice, our results demonstrate the inherent value of single trial analysis for nociceptive evoked potentials. This was evidenced by three key observations. The first is that single trial analysis nullified N2 latency differences between C6 and C8 stimulation sites, readily apparent with conventional averaging. We have reported significant differences between C6 and C8 stimulation sites previously based on an analysis of the same data (Jutzeler et al., 2016) and also observed trends towards similar differences in other, independent datasets that incorporating conventional averaging (Haefeli et al., 2013b). Based on matching peripheral conduction distances and marginal differences centrally, C6 (i.e., base of the thumb) and C8 (i.e., base of the 4th finger) stimulation should, from a neurophysiological perspective, yield similar latencies. The resolution of this discrepancy by single trial analysis suggests that differences between C6 and C8, as reported previously (Haefeli et al., 2013b; Jutzeler et al., 2016), are an artefact of conventional averaging. Specifically, stimulation of the C8 dermatome at the base of the 5th finger may yield more latency jitter due to a less evenly distributed activation of cutaneous thermo-nociceptors, owing to the anatomical structure of the skin area and size of the heat stimulator. These challenges may also result in unintentional stimulation of glabrous skin, known to result in longer CHEPs latencies (Hüllemann et al., 2019). The development of smaller, more effective contact heat stimulation devices (De Keyser et al., 2018) may improve the assessment of the C8 dermatome.

Second, single trial analysis improved the relationship between CHEPs, specifically N2P2 amplitude and P2 latency, and age. Numerous studies have highlighted this relationship previously, generally confirming that nociceptive evoked potentials are smaller and longer with advanced age (Creac'H et al., 2015; Di Stefano et al., 2017; Granovsky et al., 2016; Lagerburg et al., 2015; Rosner et al., 2018; Truini et al., 2005). This is thought to primarily reflect a progressive loss of nociceptors in the periphery (Ceballos et al., 1999; Ochoa and Mair, 1969; O'Sullivan and Swallow, 1968; Yezierski, 2012) and a reduction in conduction velocity of the spinothalamic tract (Kakigi and Shibasaki, 1991), which are both paralleled by changes observed for other measures of pain (e.g., thresholds) (Chakour et al., 1996; Gagliese, 2009; Gibson and Farrell, 2004; Gibson and Helme, 2001). Across studies, however, the details of the relationship between age and

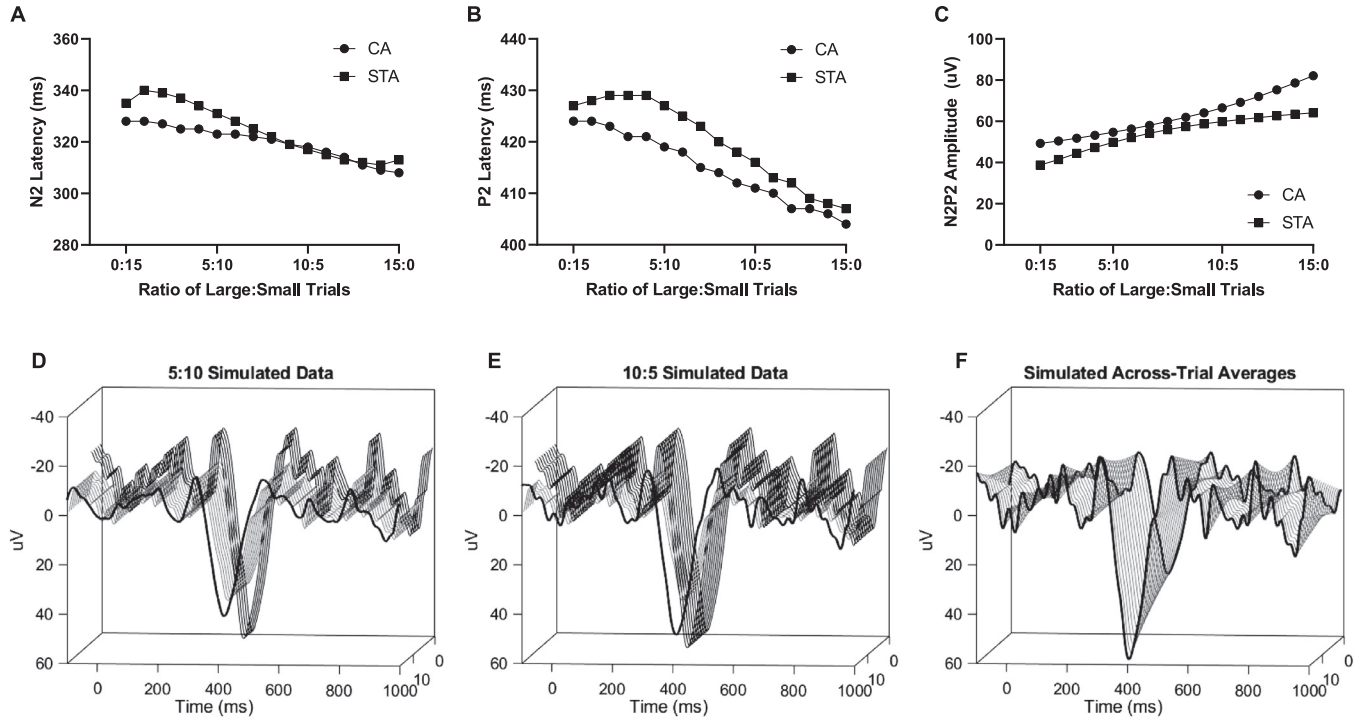



**Fig. 7.** Simulation study findings: a small waveform and large waveform (40% larger amplitude, 20 ms earlier latency) were used to create 16 datasets. The ratio of large to small trials is given in the x-axis of panels A, B, and C (e.g. 5:10 equate to 5 large waveforms and 10 small waveforms). Conventional averaging (CA) and single trial analysis (STA) N2P2 outcomes from each dataset were compared for each dataset. A) N2 latencies across simulated datasets. B) P2 latencies across simulated datasets. C) N2P2 amplitudes across simulated datasets. D) Individual trials from 5:10 simulated dataset, bold line is conventional (across-trial) average. E) Individual trials from 10:5 simulated dataset, bold line is conventional (across-trial) average. F) Across-trial averages from all simulation datasets, bold lines are the all small waveform and all large waveform datasets.

nociceptive evoked potentials are less consistent. For example, studies have reported an association for amplitude only (Frasson et al., 2020; Truini et al., 2005), while others observed location dependence (Creac'H et al., 2015; Granovsky et al., 2016; Rosner et al., 2018). Our observations suggest the optimal approach to detect genuine age related changes in nociceptive evoked potentials, unrelated to slight afferent desynchronization and increased latency jitter, is with single trial analysis.

Finally, ratings were more strongly correlated with CHEPs from single trial analysis compared to conventional averaging. As a general rule of thumb, N2P2 amplitudes are larger and latencies shorter when stimulations are, on average, more intense (Jutzeler et al., 2016; Kramer et al., 2013, 2012a; Linde et al., 2020), albeit within-subjects (Hu and Iannetti, 2019). While exceptions are commonplace and have a neuroanatomical basis (Kramer et al., 2016), the relationship between CHEPs and ratings reflects a number of important processes, including attention, arousal, saliency, and stimulus novelty (Iannetti et al., 2008; Le Pera et al., 2002; Madsen et al., 2014; Ronga et al., 2013). Such endogenous contributions of evoked potentials are thought to be more susceptible to latency jitter (Kutas et al., 1977; Legrain et al., 2002; Siedenberg and Treede, 1996). To this end, methods that better capture a relationship with pain, as was the case for single trial analysis, are highly desirable. The inherent value of single trial analysis is the more accurate portrayal of nociceptive evoked potentials. Similar to previous studies, we observed significantly larger N2P2 amplitudes (Hu et al., 2011) and longer N2 latencies (Warbrick et al., 2009) following single trial analysis compared to conventional averaging. Increased amplitudes are attributable to phase cancelation resulting from trial to trial waveform variability (i.e., latency jitter), which leads to "flattening" of the grand average waveform that serves the basis to interpret conventionally averaged CHEPs (Hu et al., 2011). Increased N2 latencies are attributed to distortions in waveform morphology introduced by conventional averaging (Mayhew et al., 2006; Warbrick et al., 2009). More specifically, large amplitude, individual waveforms (i.e., outliers), which tend also to be shorter (Hu et al., 2011; Iannetti et al., 2005), "pull" the grand average left, biasing interpretation of conventionally averaged latency. We demonstrated this phenomenon with simulated data. When a low number of large amplitude trials, with earlier latency, were included in a dataset with predominately small trials, conventional (across-trial) averaged N2 latencies progressively shifted left to a greater extent than single trial averaged latencies (Fig. 7A). This difference in N2 latency can be explained by the distortion or waveform morphology in conventional averaging, which puts greater weight on larger amplitude waveforms. In contrast, single trial averaging accounts for these differences in waveform morphology in the determination individual trial N2 latencies, which are subsequently averaged. We also provided an example of this difference between analysis methods as individual trials are added stepwise (Fig. 8), again demonstrated the effect of large amplitude trials influencing across trial averaging. In addition, the non-linear increase in N2P2 with conventional averaging provided further evidence of distortions of waveform morphology, which have previously reported (Mayhew et al., 2006; Warbrick et al., 2009).

C6 CHEPs appear to have been more affected by single trial averaging compared to C8 (i.e., larger increase in C6 N2 latency), which may relate to physical differences in testing sites. On the dorsum of the hand, where stimulation yields more robust CHEPs compared to stimulation of the palmer surface (Haefeli et al., 2013b), the C8 site is smaller than C6. The size of the stimulation site is a major issue for contact heat because the thermode is comparatively large and needs to be subtly shifted after each stimulation whilst remaining in the target dermatome. This is important for clinical applications, which aim to assess segmental pathologies (Haefeli et al., 2013a; Kramer et al., 2012a). In C6, shifting the thermode is more likely to activate novel receptors, in turn producing more "large" amplitude responses in C6 (i.e., outliers), which ultimately leads

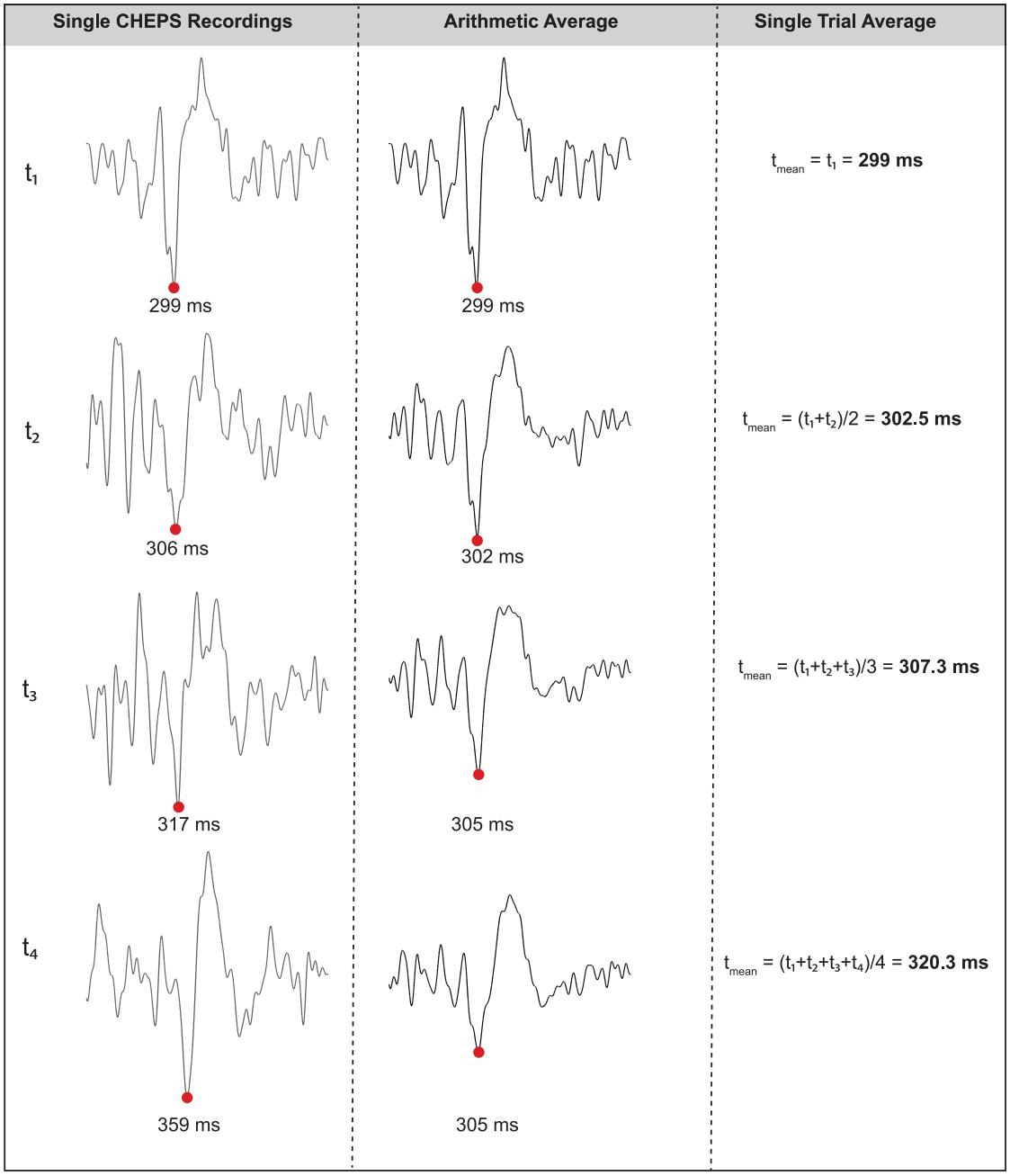



**Fig. 8.** Conventional (arithmetic) average and single trial averaged N2 latencies compared during stepwise addition of individual trials. N2 latency denoted in red. It can be observed that with subsequent trials, conventional averaging is pulled further left, due to differences in trial amplitude.

to a larger discrepancy between single trial and conventionally averaged CHEPs.

### *4.1. Limitations*

A major strength of our analysis is our sample size, which is large in comparison to previous studies applying single trial analysis (Hu et al., 2010; Kramer et al., 2016, 2013; Mayhew et al., 2006; Warbrick et al., 2009). Moreover, both men and women of varying ages were included. Nevertheless, there are limitations. Single trial analysis involves a series of signal processing steps, which ultimately improve signal clarity of the N2P2 waveform before extracting key amplitude and latency outcomes. Given there are a series of steps involved, these may, in part, also enhance the signal clarity of N2P2 waveforms. For example, conventionally averaged CHEPs were not wavelet filtered. As such, our findings are limited to a comparison of two overall methods of data analysis. Another important point is that our findings are limited to a comparison of two separate methods of CHEPs analysis, with no objective 'gold standard'. While we provide evidence and support for the use of single trial analysis, there remains no true 'gold standard' approach for CHEPs analysis. Our findings are also limited to healthy subjects. Further research is needed to determine if single trial analysis improves understanding of pathology in patient populations.

## 5. Conclusion

CHEPs provide a method to reliably and safely assess small diameter nociceptive afferents of the spinothalamic pathway that are typ-

ically involved in peripheral sensitization and chronic pain development. When optimal stimulation and data processing parameters are employed, CHEP outcomes demonstrate clear, robust age- and location-dependent changes. Our reported improved associations to aging and rating of perceived intensity when using single trial averaging suggest a better representation of the underlying physiology of the nociceptive system compared to traditional across-trial averaging. While conventional (across-trial) averaging offers convenience and ease of use, it may be more susceptible to waveform distortion and latency shifts when amplitude variation is present among individual trials are included in analysis. We recommend the use of single-trial averaging, with freely available software (Hu et al., 2011, 2010), to assess the nociceptive system using CHEPs in both clinical and research settings.

## CRediT authorship contribution statement

**Catherine R. Jutzeler:** Conceptualization, Methodology, Investigation, Data curation, Writing - review & editing, Funding acquisition, Validation, Visualization. **Lukas D. Linde:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Software, Visualization. **Jan Rosner:** Investigation, Writing - review & editing. **Michèle Hubli:** Investigation, Writing - review & editing. **Armin Curt:** Supervision, Writing - review & editing. **John L.K. Kramer:** Conceptualization, Writing - review & editing, Supervision, Resources, Project administration.

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## Data and code availability statement

Fully anonymized data will be shared at the request from any qualified investigator (please contact the *Corresponding Author*). The code to run the analysis as well as create the figures can be found on our Github repository (https://github.com/jutzca/CHEPs_STA).

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