Neuroscience

Alterations in cortical excitability during pain: A combined TMS-EEG Study

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Abstract

Transcranial magnetic stimulation (TMS) has been used to examine the inhibitory and facilitatory circuits during experimental pain and in chronic pain populations. However, current applications of TMS to pain have been restricted to measurements of motor evoked potentials (MEPs) from peripheral muscles. Here, TMS was combined with electroencephalography (EEG) to determine whether experimental pain could induce alterations in cortical inhibitory/facilitatory activity observed in TMS-evoked potentials (TEPs). In Experiment 1 (n = 29), multiple sustained thermal stimuli were administered over the forearm, with the first, second and third block of stimuli consisting of warm but non-painful (pre-pain block), painful heat (pain block) and warm but non-painful (post-pain block) temperatures respectively. During each stimulus, TMS pulses were delivered while EEG (64 channels) was simultaneously recorded. Verbal pain ratings were collected between TMS pulses. Relative to pre-pain warm stimuli, painful stimuli led to an increase in the amplitude of the frontocentral negative peak ~45ms post-TMS (N45), with a larger increase associated with higher pain ratings. Experiments 2 and 3 (n = 10 in each) showed that the increase in the N45 in response to pain was not due to changes in sensory potentials associated with TMS, or a result of stronger reafferent muscle feedback during pain. This is the first study to use combined TMS-EEG to examine alterations in cortical excitability in response to pain. These results suggest that the N45 TEP peak, which indexes GABAergic neurotransmission, is implicated in pain perception and is a potential marker of individual differences in pain sensitivity.

eLife assessment

This **valuable** study provides **solid** evidence that acute experimental pain induces changes in cortical excitability and GABAergic neurotransmission. The findings will be of interest to researchers interested in the brain mechanisms of pain and may lay the ground for future studies on acute pain-related excitability changes as probed by TMS and EEG.

Introduction

Pain is a complex subjective experience, and understanding how pain is processed remains a challenge (Apkarian, 2021). Several neuroimaging techniques have been applied to disentangle these complexities: functional magnetic resonance imaging has helped us understand where in the brain pain is processed (Reddan & Wager, 2018), while electroencephalography (EEG) has contributed to our understanding of the temporal sequence of pain processing (Ploner & May, 2018). Another useful technique that has been used to examine the role of inhibitory and facilitatory neural circuits in pain has been transcranial magnetic stimulation (TMS) (Chang et al., 2018; Schabrun & Hodges, 2012). However, a limitation of TMS studies has been the recording of output from the muscle, a signal that could be influenced by many intermediate (subcortical, spinal, peripheral) factors, and which restricts investigations to the motor system only. Here, we use a combined TMS-EEG measure to record output of TMS directly from the cortex and from multiple brain regions, in pain-free and tonic pain conditions.

When TMS is delivered over the primary motor cortex (M1), a magnetic pulse induces an electrical current in underlying cortical tissue that, if the intensity is sufficient, activates corticomotor pathways, inducing a motor evoked potential (MEP) in a target muscle. The magnitude of the MEP serves as an index of the excitability of the pathway between M1 and the muscle. Past systematic reviews on studies using TMS during acute experimental pain (Bank, Peper, Marinus, Beek, & Van Hilten, 2013; Burns, Chipchase, & Schabrun, 2016; Chowdhury et al., 2022; Rohel et al., 2021) have shown a reduction in corticomotor excitability during pain and after pain resolution, with stronger reductions in corticomotor excitability associated with lower acute pain severity (Chowdhury et al., 2022). It has been hypothesised that this reduction in MEP amplitude is an adaptive mechanism that restricts movement in the pain-afflicted area, to protect the area from further pain and injury (Hodges & Tucker, 2011).

While previous findings show promise for the use of the TMS to discover and validate potential biomarkers for pain, limitations exist when using TMS alone. First, MEP responses to TMS reflect the net sum of cortical, spinal, and peripheral activity within the corticomotor pathway. As such, the precise origin of the pain mechanism cannot be localized. Further, measurement of MEPs restricts investigations to M1. One way of overcoming the limitations of using TMS alone is by combining TMS and EEG. TMS-EEG is an increasingly popular tool that allows for the non-invasive investigation of cortical excitability, connectivity and plasticity *directly* from the cortex (i.e. without influence of subcortical, spinal and peripheral processes), as well as from regions *outside M1* (Farzan et al., 2016). TMS-EEG also provides an index of the activity of specific neurotransmitter circuits within the cortex. For example, TMS-evoked potential (TEP) peaks that occur at ~45ms and 100ms post-stimulation are linked to $GABA_A$ and $GABA_B$ neurotransmission respectively (Premoli et al., 2014), while the TEP

peak ~60ms post-stimulation is linked to glutamatergic neurotransmission (Belardinelli et al., 2021). Overall, TMS-EEG provides additional spatial and temporal information about cortical activity over TMS alone, making it ideal for understanding the brain mechanisms involved in pain perception.

TMS-EEG has already shown potential to serve as a biomarker for the development and prognosis of various neurological and psychiatric conditions (for reviews see (Kallioniemi & Daskalakis, 2022; Tremblay et al., 2019)). However, the applicability of TMS-EEG to pain research is yet to be established. While GABAergic processes indexed by TMS-EEG have been hypothesised to be involved in pain (Barr, Farzan, Davis, Fitzgerald, & Daskalakis, 2013), direct evidence is scarce. Two studies (Ye et al., 2022; Che et al, 2019) examined whether the potential analgesic effects of repetitive TMS (rTMS) over the dorsal prefrontal cortex are associated with plasticity in TEPs. These studies separately measured TEPs and ratings to painful stimuli, before and after rTMS, with one finding that increases in pain thresholds following rTMS were associated with changes in TEPs that index GABAergic processes (Ye et al., 2022). However, no study has investigated whether cortical activity is altered in direct response to pain.

The aim of the present study was to use TMS-EEG to determine whether acute experimental pain induces alterations in cortical inhibitory and facilitatory peaks observed using TEPs. We used a tonic heat pain paradigm (Furman et al., 2020; Granot, Granovsky, Sprecher, Nir, & Yarnitsky, 2006), in which multiple thermal stimuli were applied over the right extensor carpi radialis brevis (ECRB) muscle via a thermode. For each thermal stimulus, the temperature increased from a neural baseline of 32°C to either a warm non-painful or a painful (46°C) temperature, with this temperature maintained for 40 seconds. During this time, TMS was administered to the left M1 with concurrent EEG to obtain TEPs from 63 scalp channels, and MEPs from the ECRB muscle (see Figure 1). Verbal pain ratings were obtained between pulses. It was hypothesized that TEP peaks that index GABAergic processes, including the peaks at ~45 and 100ms after TMS, would increase in response to painful stimuli relative to warm non-painful stimuli.

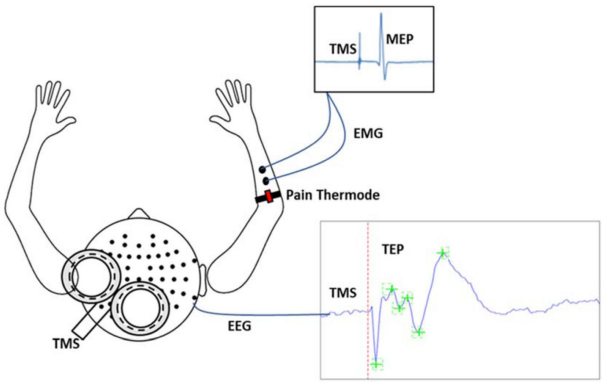

Figure 1.

Schematic of experimental apparatus.

This consisted of transcranial magnetic stimulation (TMS) during concurrent electroencephalography (EEG) to simultaneously record motor evoked potentials (MEPs) and TMS-evoked potentials (TEPs). MEPs were recorded using electromyographic (EMG) electrodes placed over the distal region of the extensor carpi radialis brevis (ECRB) while thermal pain was delivered over the proximal region of the ECRB.

Results

Experiment 1 – Does acute pain alter cortical excitability?

In Experiment 1 (n = 29), we determined whether painful thermal stimuli induced alterations in TEP peaks relative to a non-painful baseline. The protocol (Figure 2) consisted of three blocks of stimuli, in chronological order: pre-pain, pain, and post-pain blocks. The

pre-pain and post-pain blocks each consisted of six 40s thermal stimuli (20s interstimulus interval) delivered at a non-painful temperature (calibrated to each participant's warmth detection threshold), while the pain block consisted of six 40s thermal stimuli delivered at 46°C. The pre-pain/pain/post-pain design has been commonly used in the TMS-MEP pain literature, as many studies have demonstrated strong changes in corticomotor excitability that persist beyond the painful period (for a review see (Chowdhury et al., 2022)). As such, if we had used an alternative design with blocks of warm stimuli intermixed with blocks of painful stimuli, the warm stimuli blocks would not serve as a valid non-painful baseline.

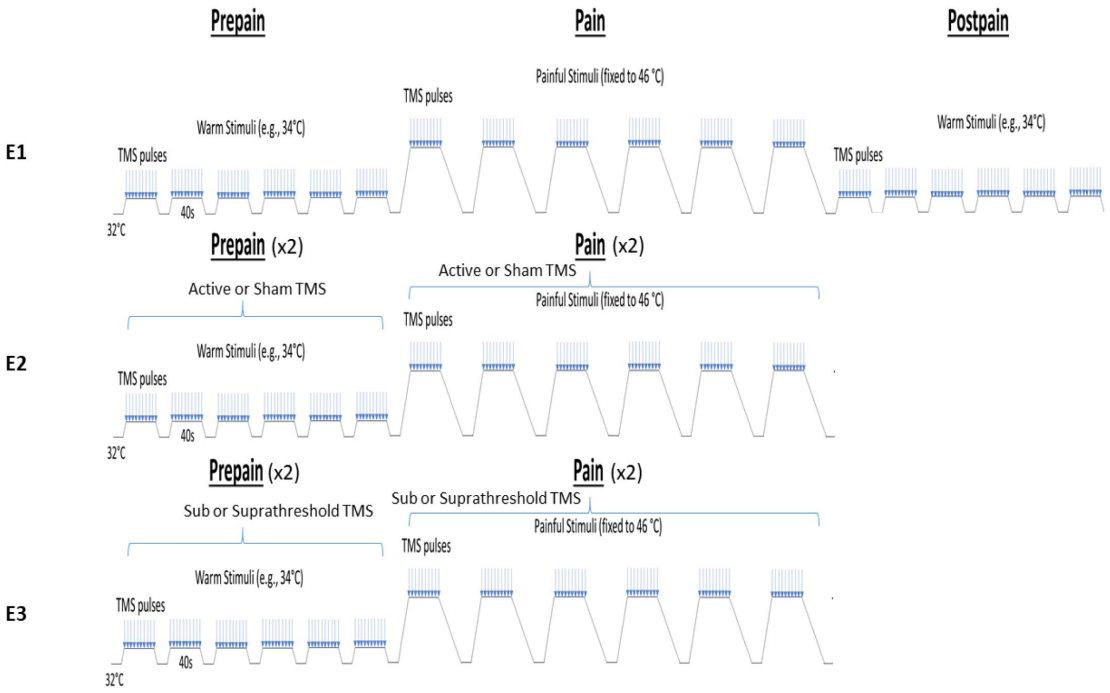

Figure 2.

Schematic of the experimental protocols.

In Experiment 1, participants experienced three blocks of stimuli: a pre-pain, pain, and post-pain block, with each block consisting of multiple heat stimuli delivered 40s at a time, and during which TMS measurements (indicated by blue arrows) and verbal pain ratings were obtained. The pre-pain and post-pain blocks involved thermal stimuli delivered at the warm threshold (i.e., the temperature that leads to any perceived change in skin temperature from baseline). In the pain block, thermal stimuli were delivered at 46°C. For Experiment 2, the post-pain block was excluded, and an additional sham TMS condition was intermixed within both the pre-pain and pain blocks. For Experiment 3, the post-pain block was also excluded, and an additional subthreshold TMS condition intermixed within both the pre-pain and pain blocks.

Prior to the test blocks, we measured warmth, cool, and pain detection thresholds to ascertain whether: a) participants could perceive increases or decreases in the thermode temperature relative to a neutral baseline of 32°C, and b) the pain detection threshold was below 46°C. All participants were able to detect increases or decreases in temperature from baseline. The mean (± SD) cool and warmth detection threshold was 28.6 ± 1.9°C and 35.1 ± 1.5°C respectively. All participants reported a heat pain threshold that was above their warmth detection threshold and below the test temperature of 46°C. The mean heat pain threshold was 41.2 ± 2.8°C.

All participants reported 0/10 pain during the pre-pain and post-pain blocks, and pain ratings varying between 1-10 during the pain block. Figure 3 shows the mean pain ratings for the ten pain measurements of each of the six painful stimuli delivered during the pain block (~4 seconds in between pain measurements). A 6 (stimulus number: 1-6) x 10 (timepoint:1-10) Bayesian repeated measures ANOVA revealed anecdotal evidence (i.e., no conclusive evidence) of a difference in pain between six thermal stimuli (BF_{10} = 2.86). However, there was very strong evidence of a difference in pain ratings between ten timepoints (BF_{10} = 6.1^{30}). There was also strong evidence of an interaction between stimulus number and timepoint, suggesting the time course of pain across the 40 seconds heat stimulus differed across six thermal stimuli (BF_{10} = 19.6). Overall, there was no conclusive evidence for pain differing *between* successive stimuli, however there was evidence that pain fluctuated *during* each 40 second stimulus.

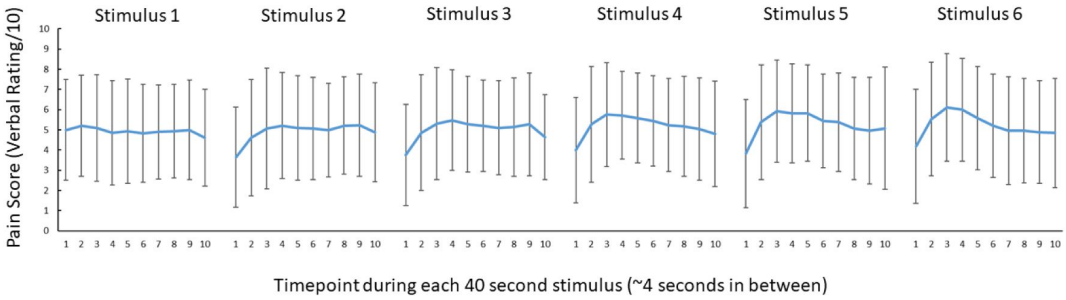

Figure 3.

No conclusive evidence of a difference in pain ratings between successive 46°C 40s thermal stimuli.

Mean (± SD) pain ratings during the 6 thermal stimuli delivered during the pain block (heat stimuli delivered at 46°C) of Experiment 1. 10 pain ratings were collected over each 40 second stimulus ~ every 4 second.

The mean resting motor threshold (RMT) and test intensity of TMS was (mean ± SD) 70.7 ± 8.5% and 77.7 ± 9.2% of maximum stimulus output respectively. Three participants were excluded from the MEP analysis due to EMG software failure – these participants were still included in the TEP analysis. A Bayesian repeated-measures ANOVA was run to compare MEP amplitudes between pre-pain, pain and post-pain conditions. There was anecdotal evidence of a difference in MEP amplitude between blocks (BF_{10} = 1.015) (Figure 4A). A Bayesian correlation test was also run to determine whether the mean pain rating (across blocks and timepoints) was associated with the change in MEP amplitude during pain as a proportion of pre-pain. These MEP change values were log-transformed as they were not normally distributed according to a Shapiro-Wilk Test (W = .572, p < .001). There was strong evidence for a positive relationship (r_{26} = 0.54, BF_{10} = 11.17) (Figure 4B). such that participants who showed a larger reduction in MEP amplitude during pain reported lower pain ratings.

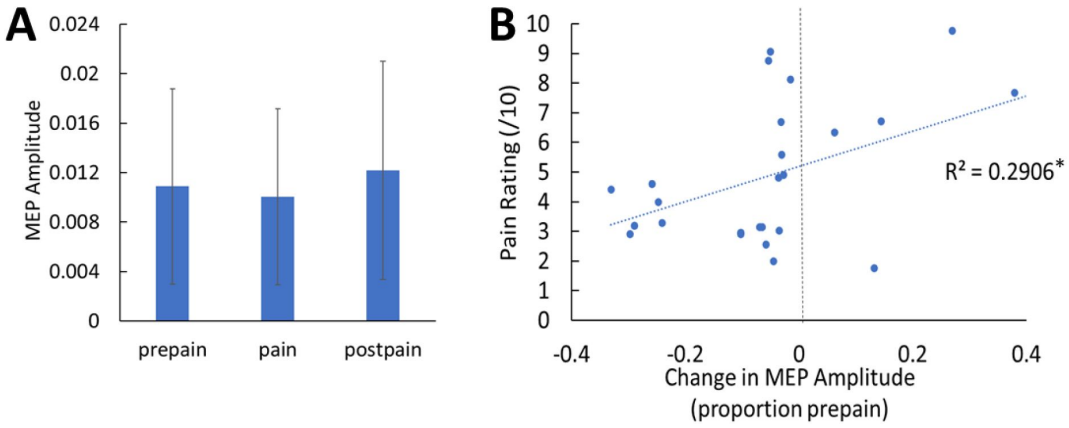

Figure 4.

No conclusive evidence of MEP amplitude differences between conditions; however individual pain sensitivity was predicted by changes in MEP amplitude.

A: Mean (± SD) MEP amplitude during the pre-pain, pain and post-pain blocks of Experiment 1. B: Individual-level Relationship between change in MEP amplitude during pain (proportion of pre-pain) and mean verbal pain rating provided by each participant.

One participant was excluded from the TEP analysis due to failure to save the recording during the experiment, though this participant was still included in the MEP analysis. One participant had missing post-pain data as the TMS coil had overheated during this portion of the experiment – their data were still included for the pre-pain vs. pain comparison. Figure 5 shows the grand average TEPs for all 63 channels, across pre-pain, pain and post-pain conditions, as well as the scalp topographies at timepoints where TEP peaks are typically observed – N15, P30, N45, P60, N100 and P180ms (Farzan et al., 2016). Source reconstruction using a co-registered template brain model was also conducted to characterise source activity at each timepoint (Figure 5). For the N15 and P30 peaks, there was higher current density in the left motor areas, consistent with previous studies suggesting that TMS evoked activity at 15 and 30ms after the TMS pulse reflect early excitation of motor areas ipsilateral to the stimulated region (Farzan & Bortoletto, 2022). For the N45 and P60 peaks, there was higher current density in the left motor and somatosensory areas at 45 and 60ms after the TMS pulse, consistent with previous studies showing a sensorimotor origin a these timepoints (Ahn & Fröhlich, 2021), however higher current density was also present in the left parietal and right sensorimotor areas. For the N100 and P180 peaks, there was higher current density in the central regions, mostly contralateral to the stimulated cortex. Overall, we found consistencies in the source localization with previous studies, including a sensorimotor origin of early peaks from 15-60ms. However, we did not directly compare source activity between conditions due to the inaccuracies involved in source estimation in the absence of co-registered magnetic resonance imaging (MRI) scans (Brodbeck et al., 2011; Michel & Brunet, 2019) and EEG electrode location digitization (Shirazi & Huang, 2019).

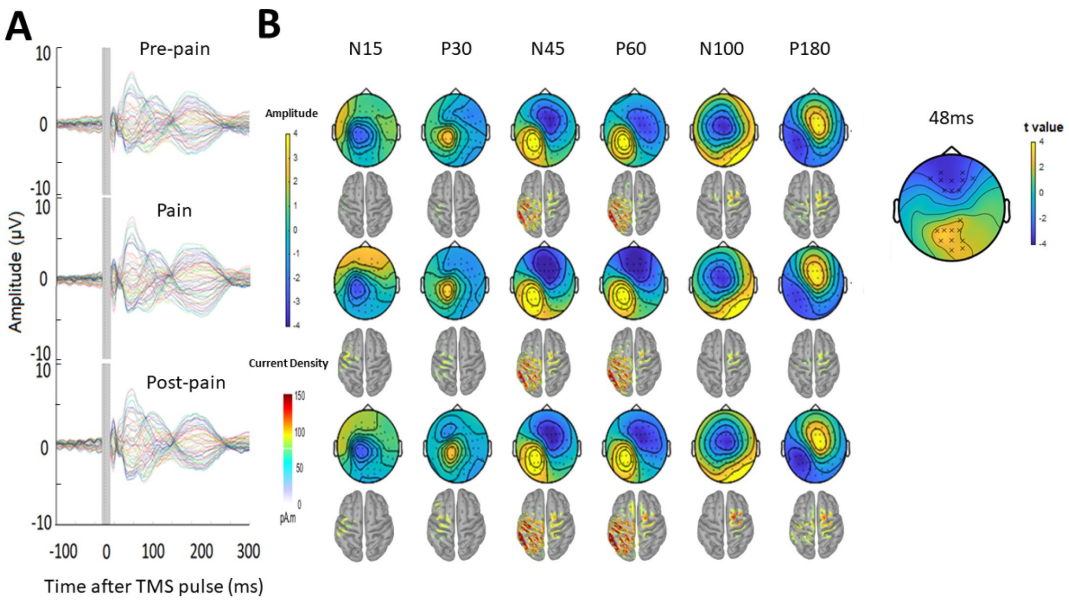

Figure 5.

Pain led to increased negative and positive amplitude in frontocentral and parietal-occipital sites respectively, 43-90ms after the TMS pulse.

A: Grand-average TEPs during the pre-pain, pain and post-pain blocks of Experiment 1. The grey shaded area represents the window of interpolation around the TMS pulse. B: Scalp topographies and estimated source activity at timepoints where TEP peaks are commonly observed, including the N15, P30, N45, P60, N100 and P180. A cluster plot is also shown on the right comparing signal amplitude between the pain and pre-pain conditions at a representative timepoint (48ms) between 43-90ms, which is where significant amplitude differences were observed. The black stars demonstrate the presence of significant positive (yellow) or negative (blue) clusters.

Comparisons of TEP amplitude between conditions were based on the electrode-space data. However, as this is the first study investigating the effects of experimental pain on TEPs amplitude, there were no *a priori* regions or timepoints of interest to compare between conditions. A statistically robust starting point in these situations is to use a cluster-based permutation analysis (Frömer, Maier, & Abdel Rahman, 2018). This analysis was used to compare amplitudes between pre-pain and pain, and pre-pain and post-pain, at each timepoint and for each electrode. We found that during pain relative to pre-pain, there was a significantly larger negative amplitude (p = .021) at frontocentral electrodes and a significantly larger positive amplitude at parietal-occipital electrodes (p = .028), specifically between 43-90ms after the TMS pulse. No significant differences in TEP amplitude were found when comparing the pre-pain and post-pain conditions. As such, the subsequent TEP peak analyses were focused on the pre-pain vs. pain comparison, while the pre-pain vs. post-pain comparisons are presented in the supplementary material.

Figure 6A shows the grand average TEP waveform at the frontocentral electrodes ('AF3','AFz','AF4','F1','Fz','F2','F4','FC2','FC4') identified from the cluster analysis for the pre-pain vs. pain conditions (Supplementary Figure 1 shows the pre-pain vs. post-pain

comparison). Two peaks at ~45 and 85ms after the TMS pulse are visible in the time window where the significant cluster was detected. Given the approximate timing, these peaks are likely to be the N45 peak and an early N100 peak. The amplitude of these peaks was identified for each participant using the TESA peak function (Rogasch et al., 2017) with defined time windows of 40-70 and 75-95ms for the first and second peak respectively. These time windows were chosen to account for variation between participants in the latency of the first and second peak. Bayesian paired-sample t-tests showed very strong evidence that the first peak at ~45ms (BF_{10} = 57.21) and moderate evidence that the second peak at ~85ms (BF_{10} = 6.77) had larger amplitude during pain block compared to pre-pain block. Figure 6B shows the individual level relationship between the mean pain rating, and the difference in N45 and N100 amplitude between pain and pre-pain. There was strong evidence that participants who reported higher pain ratings also showed a larger increase in N45 peak amplitude during pain block (r_{26} = 0.52, BF_{10} = 10.64). There was anecdotal evidence for no association between pain ratings and changes in the N100 peak amplitude during pain (r_{26} = 0.24, BF_{10} = 0.48).

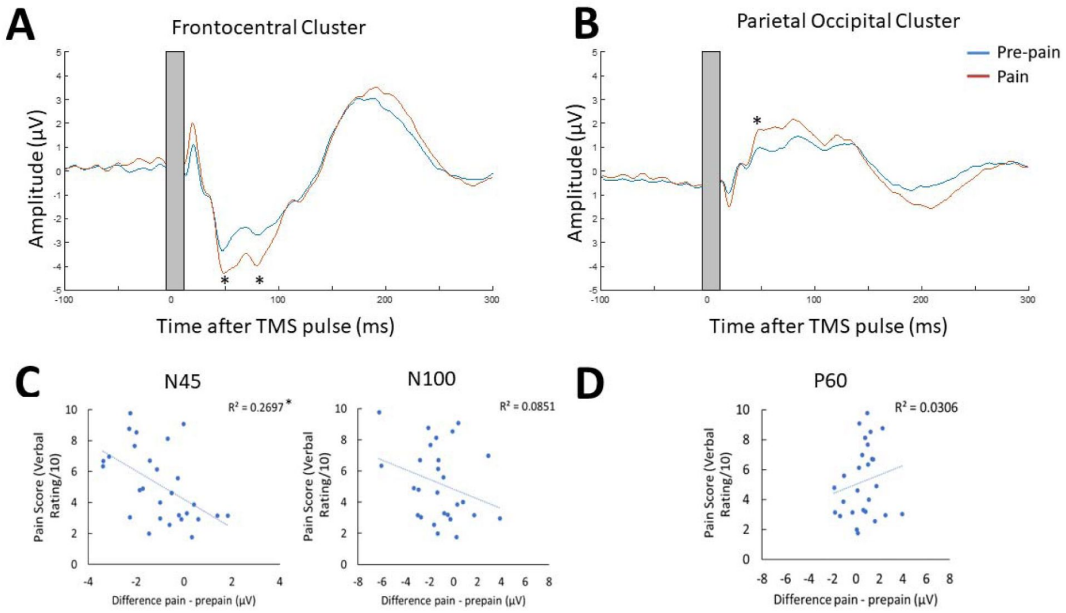

Figure 6.

Pain led to increases in N45, P60 and N100 peak amplitude, and individual pain sensitivity was predicted by changes in the N45 peak.

A: TEPs across pain and pre-pain condition for the frontocentral electrodes and parietal-occipital electrodes identified from the cluster analysis of Experiment 1. The grey shaded area represents the window of interpolation around the transcranial magnetic stimulation (TMS) pulse. For the frontocentral electrodes, two significantly stronger negative peaks were identified at ~45 and 85ms post-TMS. For the parietal-occipital electrodes, a significantly stronger positive peak was identified at ~50ms post-TMS. * indicates at least moderate evidence the alternative hypothesis that the amplitude is larger in pain vs. pre-pain (BF_{10} > 3). B: Individual-level relationship between change in peak amplitudes at ~45ms (N45), ~50ms (P60) and ~85ms (N100) post-TMS, and mean verbal pain ratings provided by each participant.

Figure 6C shows the mean TEP waveform of the parietal-occipital electrodes ('P1','PO3','O1','CPz','Pz','Pz','Oz','CP2','P2', 'PO4','O2','CP4','P4') identified from the cluster analysis for the pre-pain vs. pain conditions (Supplementary Figure 1 shows the pre-pain vs. post-pain comparison). One peak at ~50ms is visible in the time window where the significant cluster was detected. The approximate timing of this peak is consistent with the commonly identified P60. The amplitude of this peak was identified for each participant with a defined time window of 35 to 65ms. This time window was chosen to account for variation between participants in the latency of the peak. There was moderate evidence that the peak at ~50ms was stronger during pain block compared to pre-pain block (BF_{10} = 5.56). Figure 6D shows the individual level relationship between the mean pain rating and the difference in the P60 amplitude between pain and pre-pain. There was anecdotal evidence in favour of no relationship between pain ratings and changes in P60 amplitude during pain block (r_{26} = 0.21, BF_{10} = 0.407).

Experiment 2 – Does acute pain alter cortical excitability or sensory potentials?

Several studies have shown that a significant portion of TEPs do not reflect the direct cortical response to TMS, but rather auditory potentials elicited by the "clicking" sound from the TMS coil, and somatosensory potentials elicited by the "flicking" sensation on the skin of the scalp (Biabani, Fornito, Mutanen, Morrow, & Rogasch, 2019; Chowdhury et al., 2022; Conde et al., 2019; Rocchi et al., 2021). To separate the direct cortical response to TMS from sensory evoked activity, Experiment 2 (n = 10) included a sham TMS condition that mimicked the auditory/somatosensory aspects of active TMS to determine whether any alterations in the TEP peaks in response to pain were due to changes in sensory evoked activity associated with TMS, as opposed to changes in cortical excitability. A similar design was used to Experiment 1, with the inclusion of a sham TMS condition within the pre-pain and pain blocks, and exclusion of the post-pain block, since the aim was to identify the source of the pain effect from Experiment 1. The sham TMS condition was similar to a recent study (Gordon et al., 2021), involving the delivery of the TMS coil rotated 90 degrees to the scalp to simulate the auditory component associated with real TMS, and concurrent electrical stimulation beneath a sham coil to simulate the somatosensory component associated with real TMS (see Figure 7A).

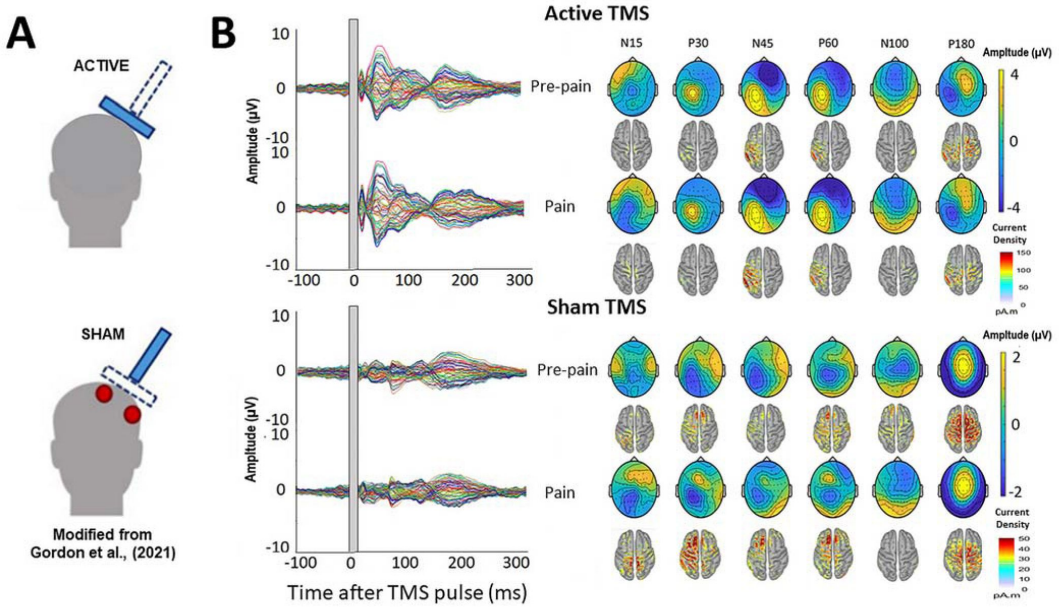

Figure 7.

TMS-evoked potentials for Active and Sham TMS.

A: Schematics showing the delivery of active and sham TMS of Experiment 2. Sham TMS involved scalp electrical stimulation (in red) beneath a sham coil (in dotted blue) to mimic somatosensory stimulation associated with active TMS, and concurrent delivery of active TMS 90 degrees to the scalp (in shaded blue) to mimic auditory stimulation associated with TMS. B: Left: TEPs during the pre-pain and pain blocks, for both active and sham stimulation. The grey shaded area represents the window of interpolation around the TMS pulse. Right: Scalp topographies and estimated source activity at timepoints where TEP peaks are commonly observed, including the N15, P30, N45, P60, N100 and P180.

Figure 7B shows the grand average TEPs, scalp topographies and estimated source activity for active and sham TMS, across pre-pain and pain conditions. Figure 8 shows the mean TEP waveform for the frontocentral and parietal-occipital clusters identified from Experiment 1, across active and sham conditions. There was moderate evidence that the amplitude of the frontocentral N45 was increased during pain vs. pre-pain blocks for active TMS condition (BF_{10} = 3.26), and moderate evidence for no difference between pain and pre-pain blocks for sham TMS condition (BF_{10} = 0.309). When comparing pain and pre-pain blocks, there was, respectively, moderate and anecdotal evidence for no alterations in the frontocentral N100 for active (BF_{10} = 0.31) and sham TMS (BF_{10} = 0.42). There was anecdotal evidence for no alteration in the parietal occipital P60 for both active (BF_{10} = 0.786) and sham TMS (BF_{10} = 0.42). Overall, the results showed that the N45 peak was altered in response to pain for active but not sham TMS, suggesting the experience of pain led to an alteration in the excitability of the cortex, and not the auditory/somatosensory aspects of TMS.

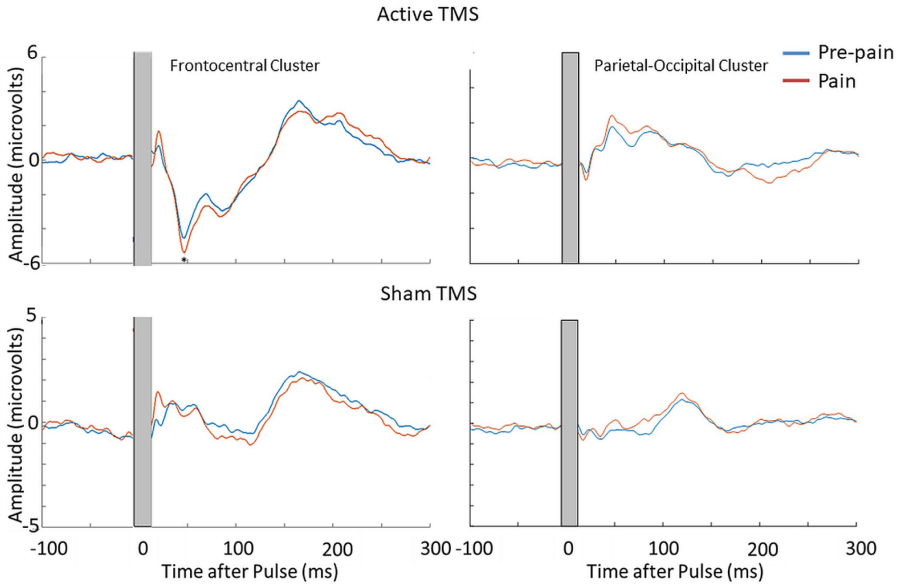

Figure 8.

Pain led to an increase in the N45 peak amplitude during active TMS but not sham TMS.

TEPs during pain and pre-pain blocks, across active and sham TMS conditions of Experiment 2, for the frontocentral electrodes (left) and parietal occipital electrodes (right) identified from the cluster analysis in the main experiment. A significantly stronger frontocentral negative peak was identified ~45ms post-TMS during pain compared to pre-pain, for the active TMS condition. * indicates at least moderate evidence the alternative hypothesis that the amplitude is larger in pain vs. pre-pain (BF_{10} > 3). The dotted line shows the timing of the peak.

Experiment 3 – Does acute pain alter cortical excitability or reafferent muscle activity?

Previous studies have shown that a significant portion of the TEP peaks at 45 and 60ms post-TMS reflect reafferent feedback from the muscle twitch in response to suprathreshold TMS applied over M1. This comes from MRI-informed EEG studies showing source localization of the N45 and P60 TEP peaks to the somatosensory areas, as well as correlations between MEP amplitude and N45/P60 amplitude (Ahn & Fröhlich, 2021; Petrichella, Johnson, & He, 2017). Indeed, Experiments 1 and 2 also showed localization of the N45 and P60 to sensorimotor areas. As such, Experiment 3 recruited a further ten participants to determine whether the pain-induced increase in the N45 peak was due to stronger reafferent feedback from muscle twitches. A design similar to Experiment 1 was used, with the inclusion of a subthreshold TMS condition (90% RMT) within the pre-pain and pain blocks.

Figure 9 shows the grand average TEPs, scalp topographies and estimated source activity for supra- and subthreshold TMS, across pre-pain and pain blocks. Figure 10 shows the mean TEP waveform for the frontocentral and parietal-occipital clusters identified from Experiment 1, across suprathreshold and subthreshold TMS conditions. When comparing the pain with pre-pain blocks, there was moderate evidence that the frontocentral N45 was increased during subthreshold TMS (BF_{10} = 3.05) and suprathreshold TMS (BF_{10} = 3.01). When comparing pain and pre-pain blocks, there was anecdotal evidence for no alterations in the frontocentral N100 peak during suprathreshold TMS (BF_{10} = 0.42) and subthreshold TMS (BF_{10} = 0.36). When comparing pain with pre-pain blocks, there was anecdotal evidence for an increase in the parietal occipital P60 peak during suprathreshold TMS (BF_{10} = 2.71) and anecdotal evidence for no alteration in P60 peak during subthreshold TMS (BF_{10} = 0.72). Overall, there was evidence that that the N45 peak was altered in response to both supra- and subthreshold TMS, suggesting the pain-induced increase in the N45 peak was not a result of stronger reafferent feedback from the muscle twitches.

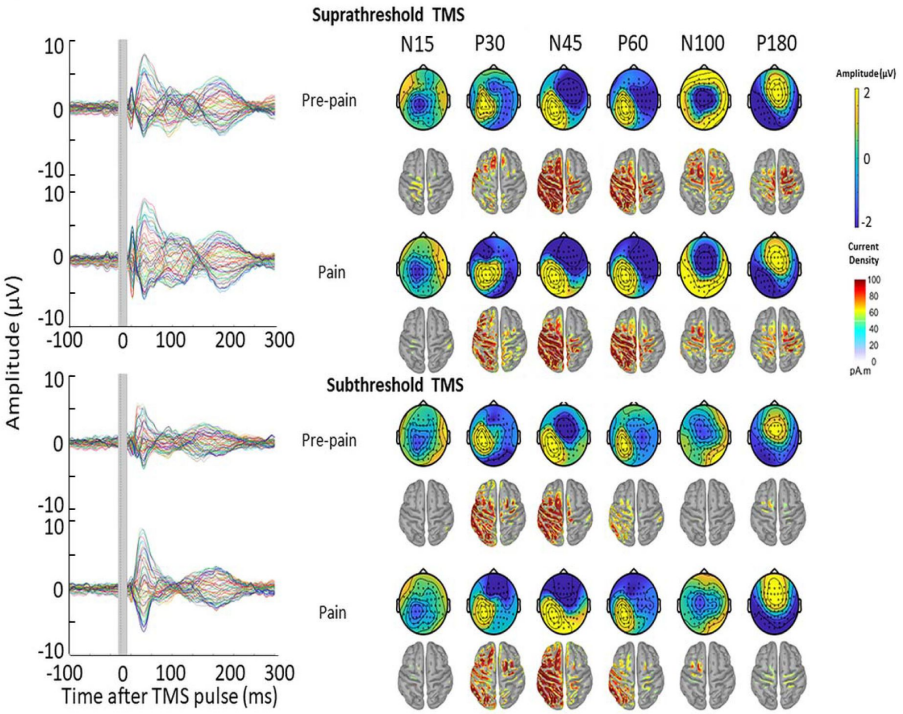

Figure 9.

TMS-evoked potentials for supra- and subthreshold TMS.

Left: TEPs during the pre-pain and pain blocks, for both supra- and subthreshold TMS of Experiment 3. The grey shaded area represents the window of interpolation around the transcranial magnetic stimulation TMS pulse. Right: Scalp topographies and estimated source activity at timepoints where TEP peaks are commonly observed, including the N15, P30, N45, P60, N100 and P180.

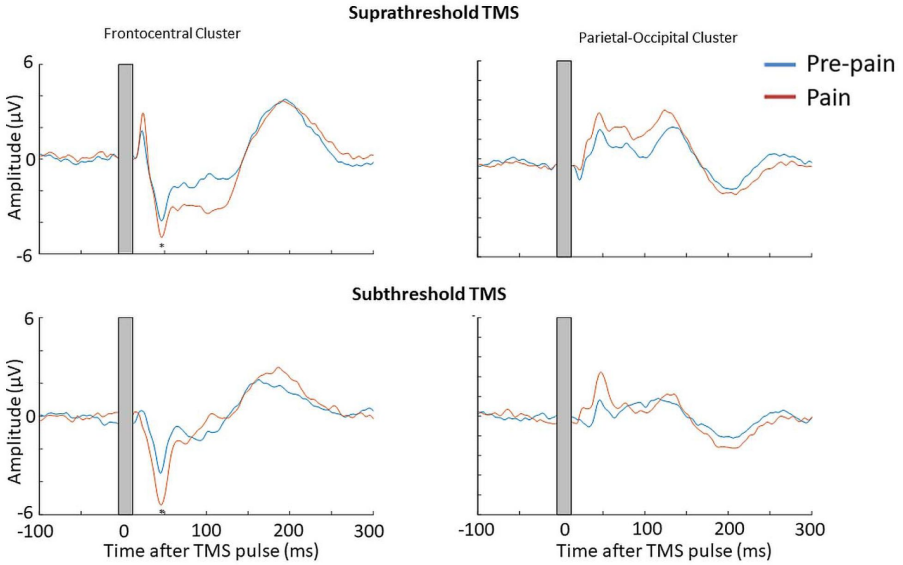

Figure 10.

Pain led to an increase in the N45 peak amplitude for both suprathreshold and subthreshold TMS.

TEPs during pain and pre-pain blocks of Experiment 3, across supra- and subthreshold TMS conditions, for the frontocentral electrodes (left) and parietal occipital electrodes (right) identified from the cluster analysis in Experiment 1. A significantly stronger frontocentral negative peak was identified ~45ms post-TMS during pain compared to pre-pain for both supra- and subthreshold stimulation. * indicates at least moderate evidence the alternative hypothesis that the amplitude is larger in pain vs. pre-pain ($BF_{10} > 3$). The dotted line shows the timing of the peak.

Discussion

The present study determined whether acute experimental pain induces alterations in cortical inhibitory and/or facilitatory activity observed in TMS-evoked potentials. Across three experiments, there was Bayesian evidence (varying between moderate to very strong) for an increase in the amplitude of N45 peak during painful stimuli compared to a non-painful baseline. Experiment 1 showed very strong evidence that a larger increase in N45 peak in response to pain was correlated with higher pain ratings. Experiment 2 showed that the increase in N45 peak during pain was not a result of alterations in sensory potentials associated with TMS pulses, but rather, changes in cortical excitability. Experiment 3 showed that the increase in N45 peak was not a result of stronger reafferent feedback from muscle twitches evoked by TMS during painful stimuli. While Experiment 1 showed moderate evidence for an increase in N100 and P60 peaks during pain relative to pre-pain baseline, this was not replicated in the follow-up experiments. Experiment 1 showed anecdotal evidence for group-level alterations in MEP amplitude during pain, however there was very strong evidence that a stronger reduction in MEP amplitude during pain was correlated with lower pain ratings.

Increased GABAergic activity during tonic pain

This study is the first to use TMS-EEG methodology to examine the direct cortical response to acute pain, extending previous studies that have used TMS to measure MEPs in response to pain (Chowdhury et al., 2022). The key finding was an increase in the amplitude of N45 peak in response to pain. This result was replicated across three experiments, providing robust evidence for the effect. Furthermore, we also accounted for major confounds that have caused significant data interpretation issues in the TMS-EEG literature in recent years, namely the contamination of TEPs by sensory potentials associated with TMS pulses (Biabani et al., 2019; Chowdhury et al., 2022) and the presence of reafferent feedback from muscle twitches (Ahn & Fröhlich, 2021).

The finding of a reliable increase in the amplitude of N45 peak during pain suggests a role for $GABA_A$ neurotransmission in pain processing, as previous work has shown that the amplitude of N45 peak is increased in response to $GABA_A$ agonists (Premoli et al., 2014). Our source reconstruction results suggest that around this timepoint, the current density was stronger in the sensorimotor area, consistent with the idea that N45 peak reflects GABAergic activity within the sensorimotor cortex (Farzan & Bortoletto, 2022). While it has been shown that reafferent muscle activity also contributes to N45 peak (Ahn & Fröhlich, 2021), Experiment 3 showed that pain increased the amplitude of N45 peak even during subthreshold TMS. Taken together, these findings suggest that the increased amplitude of N45 peak in response to pain reflect a stronger GABAergic activity within the sensorimotor cortex.

GABAergic neurons play a critical role in pain-related brain networks (Barr et al., 2013; Ong, Stohler, & Herr, 2019). They are involved in the generation of gamma oscillations (Buzsáki & Wang, 2012), which have been strongly implicated in pain perception (Barr et al., 2013; Li, Zhang, Zeng, Zhao, & Hu, 2023). Indeed, previous work has shown an increase in gamma oscillations in response to painful thermal stimuli comparable to the present study, across a wide range of brain regions such as the prefrontal (Schulz et al., 2015) and sensorimotor cortices (Gross, Schnitzler, Timmermann, & Ploner, 2007). It is therefore possible that increases in N45 peak during pain reflect increased sensorimotor gamma oscillations. Further multimodal work is required to confirm this finding.

While our findings are consistent with some studies that show increases in GABAergic activity in response to pain (Gross et al., 2007; Kupers, Danielsen, Kehlet, Christensen, & Thomsen, 2009; Schulz et al., 2015), other studies have also reported reduced GABAergic activity in response to experimental pain (Cleve, Gussew, & Reichenbach, 2015; De Matos, Hock, Wyss, Ettlin, & Brügger, 2017). Differences between studies can be attributed to the duration of the noxious stimulus (tonic pain lasting several seconds/minutes vs. transient pain stimuli lasting <1second). Indeed, pooled data have shown that the cerebral response to pain is highly dependent on the duration of the painful stimuli, as the adaptive response (to suppress or increase cortical activity) changes depending on the duration of pain (Chowdhury et al., 2022). This highlights the need for further work to replicate our findings using different durations of experimental and clinical pain.

Another finding of Experiment 1 was the increase in the amplitude of N100 peak, a marker of $GABA_B$ neurotransmission (Premoli et al., 2014), and the parietal-occipital P60 peak, a marker of glutamatergic neurotransmission (Belardinelli et al., 2021). However, this was not replicated in Experiments 2 and 3, potentially due to the smaller effect size. Nonetheless, we encourage further investigations of alterations in these peaks during pain, particularly P60 peak, as several magnetic resonance spectroscopy studies have reported increases in glutamate concentration during experimental pain (Archibald et al., 2020).

Predicting individual differences in pain using TEPs

Experimental pain models are useful tools to explore brain measures that may predict individual differences in pain sensitivity, with an ultimate goal of determining whether such measures explain why some people develop persistent pain. Experiment 1 showed that higher pain ratings were associated with a larger increase in N45 peak during pain. This analysis was not conducted in Experiments 2 and 3 due to smaller sample sizes and given the primary aims of Experiments 2 and 3 were to isolate the source of the group-level effect. Nonetheless, our results suggest that N45 peak is a potential marker of sensorimotor GABAergic activity and may be associated with individual differences in pain sensitivity. This is consistent with other studies measuring GABAergic responses to pain, showing associations between higher pain sensitivity and stronger sensorimotor gamma oscillations (Barr et al., 2013) and higher left somatosensory cortical GABA laterality (Niddam, Wang, & Tsai, 2021). However, the direction of this relationship likely depends on the duration of pain (Chowdhury et al., 2022). Our results have implications for understanding the development and maintenance of chronic pain. Further TMS-EEG studies are required to determine whether N45 peak is altered in chronic pain populations and whether N45 peak can explain why some individuals in the acute stages of pain transition to chronic pain.

The TEP vs. MEP response to pain

The present study showed that a larger reduction in MEP amplitude during pain was correlated with lower pain ratings, consistent with a recent systematic review (Chowdhury et al., 2022) and the idea that reduced corticomotor excitability is an adaptive mechanism that restricts movement in the pain-afflicted area, to protect the area from further pain and injury (Hodges & Tucker, 2011). The novelty of this study was the use of an experimental heat pain paradigm that has not yet been used in combination with TMS research, and a paradigm that controls for non-painful somatosensory stimulation.

The finding of a pain-induced increase in the amplitude of N45 peak, which indexes $GABA_A$ receptor activity, is consistent with TMS research showing pain-induced increases in short-interval intracortical inhibition (SICI) (Salo, Vaalto, Koponen, Nieminen, & Ilmoniemi, 2019; Schabrun & Hodges, 2012). SICI refers to the reduction in MEP amplitude to a TMS pulse that

is preceded 1-5ms by a subthreshold pulse, due to activation of $GABA_A$ receptors (Kujirai et al., 1993). Some studies have reported associations between SICI and the TEP N45 (Leodori et al., 2019; Rawji, Kaczmarczyk, Rocchi, Rothwell, & Sharma, 2019), suggesting the two may share common neurophysiological mechanisms. However, we also found that a larger reduction in MEP amplitude during pain was associated with less pain, while a larger increase in N45 peak during pain was associated with stronger pain ratings, suggesting that inhibitory processes mediating corticomotor excitability and the N45 peak during pain are distinct. Further work is required to disentangle the relationship between corticomotor excitability measured by MEPs and cortical activity measured by TEPs.

Study limitations

This study has some methodological limitations. Firstly, while there was no conclusive evidence for a difference in pain ratings between six thermal stimuli, there was evidence for fluctuations in pain ratings during each painful stimulus. This suggests the perceived pain intensity was not stable, which may have introduced noise in the TEP data. Future studies could use pain paradigms that can more effectively maintain a constant level of pain e.g., hypertonic saline infusion paradigms (Svensson, Cairns, Wang, & Arendt-Nielsen, 2003). Secondly, the use of verbal pain ratings prevented the characterisation of pain on a finer time scale. However, verbal ratings were used to eliminate potential contamination of MEPs introduced by using the hand for providing pain rating. Thirdly, while this study attempted to mimic the auditory and somatosensory potentials associated with TMS in the sham condition of Experiment 2, the facial/cranial nerve stimulation associated with active TMS was not accounted for (Mutanen, Mäki, & Ilmoniemi, 2013; Rogasch & Fitzgerald, 2013). However, we note that facial/cranial nerve twitches are more strongly associated with stimulation of lateral brain areas rather than central brain areas (Mutanen et al., 2013). Lastly, the increased N45 peak amplitude in response to pain may reflect increased alertness/arousal during pain. However, a recent study showed that higher alertness is associated with reduced TEP amplitude (Noreika et al., 2020), suggesting the increase in N45 peak amplitude is not related to pain-induced arousal.

Conclusion

This study is the first to use TMS-EEG methodology to examine alterations in cortical activity in direct response to acute pain. Findings across three experiments suggest that tonic heat pain leads to an increase in the amplitude of the frontocentral TEP N45 peak (associated with GABAergic neurotransmission), and that larger increases in this peak are associated with higher pain ratings. The findings suggest that TEP indices of GABAergic neurotransmission have the potential as predictive markers of pain severity.

Materials and Methods

Participants

Experiment 1 consisted of 29 healthy participants (18 males, 11 females, mean age; 26.24 ± 5.5). Participants were excluded if they had any contraindication to TMS such as pregnancy, mental implants in the skull, seizure, or if they reported a history of neurological or psychiatric conditions, or were taking psychoactive medication. Participants completed a TMS safety screen (Rossi, Hallett, Rossini, Pascual-Leone, & Group, 2009). Procedures adhered to the Declaration of Helsinki and were approved by the human research ethics committee of UNSW (HC200328). All participants provided informed written consent.

The sample size calculation was done in G*power 3.1.9.7 with 80% power. As there were no prior TMS-EEG studies, we used pooled data from our systematic review on TMS studies (Chowdhury et al., 2022) showing that the weighted effect size of changes in MEP amplitude in response to tonic experimental pain was 0.56. Using this value, 28 participants were required to detect a significant difference between pain and pre-pain blocks.

To determine the sample size of Experiments 2 and 3, we computed the effect sizes of the N45, P60 and N100 changes (pain vs. prepain) from Experiment 1 (Cohen's d_{RM} = 1.76, 0.99, 0.83 for N45, P60 and N100 respectively). With a power of 80%, the required sample size was 4-11 participants to detect a significant difference. Experiment 2 recruited a further ten healthy participants (four males, six females, mean age: 26.8 ± 5.9) and Experiment 3 consisted of ten healthy participants (four males, six females, mean age: 28 ± 5.9).

Experimental protocol

Participants were seated comfortably in a shielded room. They viewed a fixation cross to minimise eye movements. TMS was applied to left M1 while participants wore an EEG cap containing 63 scalp electrodes to record TEPs. Surface electromyographic (EMG) electrodes were placed over the distal region of the right ECRB to record MEPs. EMG signals were amplified (x 1000) and filtered (16 to 1000Hz), and digitally sampled at 2000 Hz (Spike2, CED). A thermode was attached over the proximal region of the right ECRB in close proximity to the EMG electrodes (Figure 1).The TMS coil was covered in a layer of foam (5mm thickness) to minimize decay artefacts (Rogasch et al., 2017). Participants also wore both foam earplugs and headphones to reduce any potential discomfort from the TMS click.

The protocol for each experiment is illustrated in Figure 2 (Furman et al., 2020; Granot et al., 2006). In Experiment 1, participants experienced three blocks of heat stimuli, in chronological order: pre-pain, pain, and post-pain block. Each block consisted of multiple heat stimuli delivered 40s at a time during which TMS measurements and verbal pain ratings were obtained. The thermode commenced at a baseline temperature of 32°C. The pre-pain and post-pain blocks consisted of six heat stimuli delivered at the warm threshold (the temperature that led to any detectable change in skin temperature from baseline). In the pain block, six heat stimuli were delivered at 46°C, which has been shown to produce lasting pain with a mean rating of ~5/10 (Furman et al., 2020). The inclusion of blocks with warm stimuli allowed for control for changes in cortical excitability due to non-painful somatosensory stimulation. The protocol for Experiment 2 were identical to Experiment 1 with two differences: the exclusion of the post-pain block (as the aim was to disentangle the source of the pain vs. pre-pain effect from Experiment 1) and the inclusion of a sham TMS condition intermixed within both the pre-pain and pain blocks. The protocol for Experiment 3 was identical to Experiment 1 with two differences: the exclusion of the post-pain block (as the aim was to disentangle the source of the pain vs. pre-pain effect from Experiment 1) and the inclusion of a subthreshold TMS condition intermixed within both the pre-pain and pain blocks.

Electrical stimulation setup (Experiment 2 Only)

Electrical stimulation was used to simulate the somatosensory component of active TMS. Prior to EEG setup, 8mm Ag/AgCl electrodes were placed directly over the scalp. "Snap on" lead wires were then clipped in place and connected to the electrical stimulator (Digitimer DS7AH, Digitimer Ltd., UK). To keep the electrodes and lead wires firmly in position, participants were fitted with a tight netted wig cap, which sat on top of the electrodes but underneath the EEG cap. Consistent with previous research (Chowdhury et al., 2022; Rocchi et al., 2021), and to minimize EEG artefacts caused by electrical stimulation, the stimulating

electrodes were not placed directly underneath the EEG electrodes. Rather, stimulating electrodes were positioned in the middle of the EEG electrode cluster located in closest proximity to the motor hotspot. This roughly corresponded to an anode position between FC1 and FC3 and a cathode position between C1 and C3. Scalp electrical stimulation was delivered using a 200 μs square wave via with a compliance of 200V.

Electroencephalography

EEG was recorded using a DC-coupled, TMS-compatible amplifier (ActiChamp Plus, Brain Products, Germany) at a sampling rate of 25000 Hz. Signals were recorded from 63 active electrodes, embedded in an elastic cap (ActiCap, Brain Products, Germany), in line with the 10-10 system. Active electrodes result in similar TEPs (both magnitude and peaks) to more commonly used passive electrodes (Mancuso et al., 2021). Recordings were referenced online to 'FCz' and the ground electrode placed on 'FPz'. Electrolyte gel was used to reduce electrode impedances below ~5kOhms.

Transcranial magnetic stimulation

Single, monophasic stimuli were delivered using a Magstim unit (Magstim Ltd., UK) and 70mm figure-of-eight flat coil. The coil was oriented at 45° to the midline, inducing a current in the posterior-anterior direction. The scalp site that evoked the largest MEP measured at the ECRB ('hotspot') was determined and marked. The RMT was determined using the TMS motor thresholding assessment tool, which estimates the lowest TMS intensity required to reliably induce an MEP (Awiszus and Borckardt, 2011), with the MEP amplitude threshold set at 50 microvolts (Rossini et al., 1994; Rothwell et al., 1999). The test stimulus intensity was set at 110% RMT.

Thermal pain

Thermal stimuli were delivered over the proximal region of the right ECRB using a contact heat stimulator (27-mm diameter Medoc Pathway CHEPS Peltier device; Medoc Advanced Medical Systems Ltd). Pain ratings were obtained after each TMS pulse using a verbal rating scale (0 = no pain, and 10 = most pain imaginable). Verbal ratings were collected rather than pain ratings provided on the computer by hand to avoid contamination of MEP measures from motor processes of hand movements. Verbal pain ratings have been shown to yield excellent test-retest reliability (Alghadir, Anwer, Iqbal, & Iqbal, 2018).

Quantitative sensory testing

Quantitative sensory testing was performed in line with a previous study (Furman et al., 2020). With the baseline temperature set at a neutral skin temperature of 32°C, participants completed three threshold tests: to report when they felt a temperature increase (warmth detection threshold), to report when they felt a temperature decrease (cool detection threshold); 3) to report when an increasing temperature first became painful (heat pain threshold). A total of three trials was conducted for each test to obtain an average, with an interstimulus interval of six seconds. Participants provided feedback for each trial by pressing a button (with their left hand) on a hand-held device connected to the Medoc Pathway. Temperatures were applied with a rise/decrease rate of 1°C/s and return rate of 2°C/s (initiated by the button click).

Matching task (Experiment 2 only)

As the aim of Experiment 2 was to perceptually match the somatosensory aspects of active and sham TMS, a 2-Alternative Forced Choice task was used to determine the electrical stimulation intensity that led to a similar flicking sensation to active TMS (Chowdhury et al., 2022). Participants received either electrical stimulation or active TMS in a randomized order and were asked whether the first or second stimulus led to a stronger flick sensation. The electrical stimulation intensity was then increased or decreased until participants could no longer judge the first or second stimulus as stronger. This intensity was then applied during the test blocks.

Test blocks

Experiment 1

The temperature of the thermode commenced at a neutral skin temperature (32°C). Participants were exposed to 18 sustained heat stimuli with a 20 second interstimulus interval. For each stimulus, a single temperature (rise rate of 1 °C/s, return rate of 2 °C/s) was applied over the proximal region of the ECRB for 40 seconds. Stimuli 1-6 (pre-pain block) were delivered at the participant's individually determined warmth detection threshold, Stimuli 7-12 at 46 degrees, and Stimuli 13-18 again at the participant's warmth detection threshold. Participants were not informed of the order of the warm and painful stimuli to minimize the influence of expectation of pain on TEPs and MEPs. During each 40 second stimulus, TMS pulses were manually delivered, with a verbal pain rating score obtained between pulses. As TMS was delivered manually, there was no set interpulse interval. However, the 40 second stimulus duration allowed for 11 pulses for each heat stimulus (hence 66 TMS pulses for each of the pre-pain, pain and post-pain blocks), and 10 verbal pain ratings between each TMS pulse (~ 4 seconds in between pain ratings). A test-retest reliability study by (Kerwin, Keller, Wu, Narayan, & Etkin, 2018) showed that 60 TMS pulses (delivered in the same run) was sufficient to obtain reliable TEPs (i.e., sufficient within-individual concordance between the resultant TEP peaks of each trial).

Experiment 2

Participants were exposed to 24 sustained heat stimuli (40 seconds each). Stimuli 1-6 and 7-12 consisted of warm stimuli (pre-pain block), while Stimuli 13-18 and 19-24 consisted of stimuli delivered at 46°C (pain block). Active or sham TMS was delivered during either Stimuli 1-6 or 7-12, with the order of active and sham randomly determined for each participant. The same applied for Stimuli 13-18 and 19-24. The active and sham TMS conditions were similar to that used in a recent TMS-EEG study (Gordon et al., 2021). Sham TMS involved the active TMS coil rotated 90 degrees to the scalp, and a sham coil (identical in shape/weight) placed underneath the active coil and tangentially over the scalp. The active TMS coil was then triggered with the electrical stimulation unit to simultaneously simulate the auditory and somatosensory components of active TMS respectively. Active TMS involved the delivery of the TMS coil placed tangentially over the scalp, and the sham TMS coil above the active coil rotated 90 degrees to the scalp (see Figure 9). The design allowed for 11 pulses for each heat stimulus and ten pain ratings (hence 66 TMS pulses for active pre-pain, sham pre-pain, active pain and sham pain blocks).

Experiment 3

Participants were exposed to 24 sustained heat stimuli (40 seconds each). Stimuli 1-6 and 7-12 consisted of warm stimuli (pre-pain block), while Stimuli 13-18 and 19-24 consisted of stimuli delivered at 46°C (pain block). Suprathreshold or subthreshold TMS (90% RMT) was delivered during either Stimuli 1-6 or 7-12, with the order of supra- and subthreshold TMS randomly determined for each participant. The same applied for Stimuli 13-18 and 19-24. The design allowed for 11 pulses for each heat stimulus and ten pain ratings (hence 66 TMS pulses for suprathreshold pre-pain, subthreshold pre-pain, suprathreshold pain and subthreshold pain blocks).

Data processing

Motor evoked potentials

The amplitude of each MEP was determined using a custom MATLAB script. The onsets and offsets of the MEPs were manually determined for each trial. In some participants, background EMG activity was observed due to placement of the thermode close to the EMG electrodes, which can influence MEP amplitude (Ruddy et al., 2018). To account for this, MEP amplitude was calculated by subtracting the root mean square (RMS) of background EMG noise from the RMS of the MEP window using a fixed window between 55 and 5ms before the TMS pulse (Chowdhury et al., 2023; Schabrun et al., 2017; Tsao et al., 2011).

TMS-evoked potentials

Pre-processing of the TEPs was completed using EEGLAB (Delorme & Makeig, 2004) and TESA (Rogasch et al., 2017) in MATLAB (R2021b, The Math works, USA), and based on previously described methods (Chowdhury et al., 2022; Mutanen et al., 2018; Rogasch et al., 2017). First, bad channels were removed. The period between -5 and ~14ms after the TMS pulse was removed and interpolated using the ARFIT function for continuous data (Neumaier & Schneider, 2001; Schneider & Neumaier, 2001). The exact interval was based on the duration of decay artefacts. Data was epoched 1000ms before and after the TMS pulse, and baseline corrected between -1000 and -5ms before the TMS pulse. Noisy epochs were identified via the EEGLAB auto-trial rejection function (Delorme, Sejnowski, & Makeig, 2007) and then visually confirmed. The fastICA algorithm with auto-component rejection used to remove eyeblink and muscle artefacts (Rogasch et al., 2017). The source-estimation noise-discarding (SOUND) algorithm was applied (Mutanen, Biabani, Sarvas, Ilmoniemi, & Rogasch, 2020; Mutanen et al., 2018), which estimates and supresses noise at each channel based on the most likely cortical current distribution given the recording of other channels. This signal was then re-referenced (to average). A band-pass (1-100Hz) and band-stop (48-52Hz) Butterworth filter was then applied. Any lost channels were interpolated.

Source localization

Source localization of TEPs was conducted using Brainstorm (Tadel, Baillet, Mosher, Pantazis, & Leahy, 2011). A template brain model (ICBM 152) was co-registered with the TMS-EEG data. Noise estimation was used to determine sensor weighting and regularization parameter of the current density construction. The forward model involved use of the Symmetric Boundary Element Method with the head having 3 compartments of fixed conductivities, implemented in OpenMEEG software (Gramfort, Papadopoulo, Olivi, & Clerc, 2010), and inverse model involved use of Minimum Norm Estimations.

Statistical Analysis

Bayesian inference was used to analyse the data using JASP software (Version 0.12.2.0, JASP Team, 2020). Bayes factors were expressed as BF_{10} values, where BF_{10}'s of 1-3, 3-10, 10-30 and 30-100 indicated "weak", "moderate", "strong" and "very strong" evidence for the alternative hypothesis, while BF_{10}'s of 1/3-1, 1/10-1/3, 1/30-1/10 and 1/100-1/30 indicated "anecdotal", "moderate", "strong" and "very strong" evidence in favour of the null hypothesis (van Doorn et al., 2021).

Pain ratings

A 6 (stimulus number: 1-6) x 10 (timepoint:1-10) Bayesian repeated measures ANOVA with default priors in JASP (r scale fixed effects = .5, r scale random effects = 1, r scale covariates = .354) was conducted on the pain ratings during the pain block. This was to assess differences in pain ratings between the six painful stimuli and whether pain ratings differed between the timepoints of each painful stimulus.

MEPs

A Bayesian one-way repeated measures ANOVA with default priors in JASP was performed to assess differences in MEP amplitudes between pre-pain, pain and post-pain blocks of Experiment 1. A Bayesian correlation analysis with default priors in JASP (stretched Beta prior width = 1) was run to determine whether the change in mean MEP amplitude during pain (as a proportion of pre-pain) was associated with the mean verbal pain rating score. Data were checked for assumptions of normally distributed data using a Shapiro-Wilk test. Where assumptions were violated, data were log-transformed.

TEPs

The grand-averaged signals for the pre-pain, pain and post-pain condition were obtained. For Experiment 1, a cluster-based permutation analysis was used to compare amplitude levels between pre-pain and pain, and pre-pain and post-pain, at each time-point and for each electrode. For all experiments, the mean TEP waveform of any identified clusters from Experiment 1 were plotted, and peaks were identified using the TESA peak function (Rogasch et al., 2017). Any identified peaks were then compared between conditions using Bayes paired sample t-tests with default priors in JASP (Cauchy scale = .707). A Bayesian correlation analysis with default priors in JASP was performed to determine whether the difference in identified peaks between pre-pain and pain blocks was associated with the mean pain rating score. Data were checked for assumptions of normally distributed data using a Shapiro-Wilk test. Where assumptions were violated, data were log-transformed.

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Editors

Reviewer #1 (Public Review):

The objective of this investigation was to determine whether experimental pain could induce alterations in cortical inhibitory/facilitatory activity observed in TMS-evoked potentials (TEPs). Previous TMS investigations of pain perception had focused on motor evoked potentials (MEPs), which reflect a combination of cortical, spinal, and peripheral activity, as well as restricting the focus to M1. The main strength of this investigation is the combined use of TMS and EEG in the context of experimental pain. More specifically, Experiment 1 investigated whether acute pain altered cortical excitability, reflected in the modulation of TEPs. The main outcome of this study is that relative to non-painful warm stimuli, painful thermal stimuli led to an increase on the amplitude of the TEP N45, with a larger increase associated with higher pain ratings. Because it has been argued that a significant portion of TEPs could reflect auditory potentials elicited by the sound (click) of the TMS, Experiment 2 constituted a control study that aimed to disentangle the cortical response related to TMS and auditory activity. Finally, Experiment 3 aimed to disentangle the cortical response to TMS and reafferent feedback from muscular activity elicited by suprathreshold TMS applied over M1. The fact that the authors accompanied their main experiment with two control experiments strengthens the conclusion that the N45 TEP peak could be implicated in the perception of painful stimuli. Perhaps, the addition of a highly salient but non-painful stimulus (i.e. from another modality) would have further ruled out that the effects on the N45 are not predominantly related to intensity/saliency of the stimulus rather than to pain per se.

Reviewer #2 (Public Review):

The authors have used transcranial magnetic stimulation (TMS) and motor evoked potentials (MEPs) and TMS-electroencephalography (EEG) evoked potentials (TEPs) to determine how experimental heat pain could induce alterations in these metrics. In Experiment 1 (n = 29), multiple sustained thermal stimuli were administered over the forearm, with the first, second, and third block of stimuli consisting of warm but non-painful (pre-pain block), painful heat (pain block) and warm but non-painful (post-pain block) temperatures respectively. Painful stimuli led to an increase in the amplitude of the fronto-central N45, with a larger increase associated with higher pain ratings. Experiments 2 and 3 studied the correlation between the increase in the N45 in pain and the effects of a sham stimulation protocol/higher stimulation intensity. They found that the centro-frontal N45 TEP was decreased in acute pain.

The study comes from a very strong group in the pain fields with long experience in psychophysics, experimental pain, neuromodulation, and EEG in pain. They are among the

first to report on changes in cortical excitability as measured by TMS-EEG over M1.

While their results are in line with reductions seen in motor-evoked responses during pain and effort was made to address possible confounding factors (study 2 and 3), there are some points that need attention. In my view the most important are:
1. The method used to calculate the rest motor threshold, which is likely to have overestimated its true value : calculating highly abnormal RMT may lead to suprathreshold stimulations in all instances (Experiment 3) and may lead to somatosensory "contamination" due to re-afferent loops in both "supra" and "infra" (aka. less supra) conditions.
2. The low number of pulses used for TEPs (close to ⅓ of the usual and recommended), lack of measures to mask auditory noise.
3. A supra-stimulus heat stimulus not based on individual HPT, that oscillates during the experiment and that lead to large variations in pain intensity across participants is unfortunate. So is the lack of report on measures taken to correct for a fortuitous significance (multiple comparison correction) in such a huge number of serial paired tests.

Reviewer #3 (Public Review):

The present study aims to investigate whether pain influences cortical excitability. To this end, heat pain stimuli are applied to healthy human participants. Simultaneously, TMS pulses are applied to M1 and TMS-evoked potentials (TEPs) and pain ratings are assessed after each TMS pulse. TEPs are used as measures of cortical excitability. The results show that TEP amplitudes at 45 msec (N45) after TMS pulses are higher during painful stimulation than during non-painful warm stimulation. Control experiments indicate that auditory, somatosensory, or proprioceptive effects cannot explain this effect. Considering that the N45 might reflect GABAergic activity, the results suggest that pain changes GABAergic activity. The authors conclude that TEP indices of GABAergic transmission might be useful as biomarkers of pain sensitivity.

Pain-induced cortical excitability changes is an interesting, timely, and potentially clinically relevant topic. The paradigm and the analysis are sound, the results are mostly convincing, and the interpretation is adequate. The following clarifications and revisions might help to improve the manuscript further.

1. Non-painful control condition. In this condition, stimuli are applied at warmth detection threshold. At this intensity, by definition, some stimuli are not perceived as different from the baseline. Thus, this condition might not be perfectly suited to control for the effects of painful vs. non-painful stimulation. This potential confound should be critically discussed.
2. MEP differences between conditions. The results do not show differences in MEP amplitudes between conditions (BF 1.015). The analysis nevertheless relates MEP differences between conditions to pain ratings. It would be more appropriate to state that in this study, pain did not affect MEP and to remove the correlation analysis and its interpretation from the manuscript.
3. Confounds by pain ratings. The ISI between TMS pulses is 4 sec and includes verbal pain ratings. Considering this relatively short ISI, would it be possible that verbal pain ratings confound the TEP? Moreover, could the pain ratings confound TEP differences between conditions, e.g., by providing earlier ratings when the stimulus is painful? This should be carefully considered, and the authors might perform control analyses.
4. Confounds by time effects. Non-painful and painful conditions were performed in a fixed order. Potential confounds by time effects should be carefully considered.
5. Data availability. The authors should state how they make the data openly available.

Author Response

Reviewer #1 (Public Review):

The objective of this investigation was to determine whether experimental pain could induce alterations in cortical inhibitory/facilitatory activity observed in TMS-evoked potentials (TEPs). Previous TMS investigations of pain perception had focused on motor evoked potentials (MEPs), which reflect a combination of cortical, spinal, and peripheral activity, as well as restricting the focus to M1. The main strength of this investigation is the combined use of TMS and EEG in the context of experimental pain. More specifically, Experiment 1 investigated whether acute pain altered cortical excitability, reflected in the modulation of TEPs. The main outcome of this study is that relative to non-painful warm stimuli, painful thermal stimuli led to an increase on the amplitude of the TEP N45, with a larger increase associated with higher pain ratings. Because it has been argued that a significant portion of TEPs could reflect auditory potentials elicited by the sound (click) of the TMS, Experiment 2 constituted a control study that aimed to disentangle the cortical response related to TMS and auditory activity. Finally, Experiment 3 aimed to disentangle the cortical response to TMS and reafferent feedback from muscular activity elicited by suprathreshold TMS applied over M1. The fact that the authors accompanied their main experiment with two control experiments strengthens the conclusion that the N45 TEP peak could be implicated in the perception of painful stimuli.

Perhaps, the addition of a highly salient but non-painful stimulus (i.e. from another modality) would have further ruled out that the effects on the N45 are not predominantly related to intensity/saliency of the stimulus rather than to pain per se.

We thank the reviewer for their comment on the possibility of whether stimulus salience influences the N45 as opposed to pain per se. However, we note that in Experiment 1, despite the same level of stimulus salience/intensity for all participants (46 degrees), individual differences in pain ratings were associated with the change in the N45 amplitude, suggesting that the results cannot be explained by stimulus intensity/salience.

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The authors have used transcranial magnetic stimulation (TMS) and motor evoked potentials (MEPs) and TMS-electroencephalography (EEG) evoked potentials (TEPs) to determine how experimental heat pain could induce alterations in these metrics.In Experiment 1 (n = 29), multiple sustained thermal stimuli were administered over the forearm, with the first, second, and third block of stimuli consisting of warm but non-painful (pre-pain block), painful heat (pain block) and warm but non-painful (post-pain block) temperatures respectively. Painful stimuli led to an increase in the amplitude of the fronto-central N45, with a larger increase associated with higher pain ratings. Experiments 2 and 3 studied the correlation between the increase in the N45 in pain and the effects of a sham stimulation protocol/higher stimulation intensity. They found that the centro-frontal N45 TEP was decreased in acute pain.

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1. *The method used to calculate the rest motor threshold, which is likely to have overestimated its true value : calculating highly abnormal RMT may lead to suprathreshold stimulations in all instances (Experiment 3) and may lead to somatosensory "contamination" due to re-afferent loops in both "supra" and "infra" (aka. less supra) conditions.*

The method used to assess motor threshold was the TMS motor threshold Assessment Tool (Awiszus et al., 2003). This was developed as a quicker alternative for calculating motor threshold compared to the traditional Rossini-Rothwell method which involves determining the lowest intensity that evokes 5/10 MEPs of at least 50 microvolts. The method has been shown to achieve the same accuracy of determining motor threshold as the traditional Rossini-Rothwell method, but with fewer pulses (Qi et al., 2011; Silbert et al., 2013). Therefore, the high RMTs in our study cannot be explained by the threshold assessment method. Instead, they are likely explained by aspects of the experimental setup that increased the distance between the TMS coil and the scalp, including the layer of foam placed over the coil, the EEG cap and the fact that the electrodes we used had a relatively thick profile.

Awiszus, F. (2003). TMS and threshold hunting. In Supplements to Clinical neurophysiology (Vol. 56, pp. 13-23). Elsevier.

Qi, F., Wu, A. D., & Schweighofer, N. (2011). Fast estimation of transcranial magnetic stimulation motor threshold. Brain stimulation, 4(1), 50-57.

Silbert, B. I., Patterson, H. I., Pevcic, D. D., Windnagel, K. A., & Thickbroom, G. W. (2013). A comparison of relative-frequency and threshold-hunting methods to determine stimulus intensity in transcranial magnetic stimulation. Clinical Neurophysiology, 124(4), 708-712.

1. *The low number of pulses used for TEPs (close to ⅓ of the usual and recommended)*

We agree that increasing the number of pulses can increase the signal to noise ratio. During piloting, participants were unable to tolerate the painful stimulus for long periods of time and we were required to minimize the number of pulses per condition.

We note that there is no set advised number of trials in TMS-EEG research. According to the recommendations paper, the number of trials should be based on the outcome measure e.g., TEP peaks vs. frequency domain measures vs. other measures and based on previous studies investigating test-retest reliability (Hernandez-Pavon et al., 2023). The choice of 66 pulses per condition was based on the study by Kerwin et al., (2018) showing that optimal concordance between TEP peaks can be found with 60-100 TMS pulses delivered in the same run (as in the present study). The concordance was particularly higher for the N40 peak at prefrontal electrodes, which was the key peak and electrode cluster in our study.

Further supporting the reliability of the TEP data in our experiment, we note that the scalp topographies of the TEPs for active TMS at various timepoints (Figures 5, 7 and 9) were similar across all three experiments, especially at 45 ms post-TMS (frontal negative activity, parietal-occipital positive activity).

In addition to this, the interclass correlation coefficient (Two-way fixed, single measure) for the N45 to active suprathreshold TMS across timepoints for each experiment was 0.90 for Experiment 1 (across pre-pain, pain, post-pain time points), 0.74 for Experiment 2 (across pre-pain and pain conditions), and 0.95 for Experiment 3 (across pre-pain conditions). This suggests that even with the fluctuations in the N45 induced by pain, the N45 for each participant was stable across time, further supporting the reliability of our data. These ICCs will be reported in the next revision.

Hernandez-Pavon, J. C., Veniero, D., Bergmann, T. O., Belardinelli, P., Bortoletto, M., Casarotto, S., ... & Ilmoniemi, R. J. (2023). TMS combined with EEG: Recommendations and open issues for data collection and analysis. Brain Stimulatio, 16(3), 567-593

Kerwin, L. J., Keller, C. J., Wu, W., Narayan, M., & Etkin, A. (2018). Test-retest reliability of transcranial magnetic stimulation EEG evoked potentials. Brain stimulation, 11(3), 536-544.

> *Lack of measures to mask auditory noise.*

In TMS-EEG research, various masking methods have been proposed to suppress the somatosensory and auditory artefacts resulting from TMS pulses, such as white noise played through headphones to mask the click sound (Ilmoniemi and Kičić, 2010), and a thin layer of foam placed between the TMS coil and EEG cap to minimize the scalp sensation (Massimini et al., 2005). However, recent studies have shown that even when these methods are used, sensory contamination of TEPs is still present, as shown by studies that show commonalities in the signal between active and sensory sham conditions that mimic the auditory/somatosensory aspects of real TMS (Biabani et al., 2019; Conde et al., 2019; Rocchi et al., 2021). This has led many authors (Biabani et al., 2019; Conde et al., 2019) to recommend the use of sham conditions to control for sensory contamination. To separate the direct cortical response to TMS from sensory evoked activity, Experiment 2 (n = 10) included a sham TMS condition that mimicked the auditory/somatosensory aspects of active TMS to determine whether any alterations in the TEP peaks in response to pain were due to changes in sensory evoked activity associated with TMS, as opposed to changes in cortical excitability. Therefore, the lack of auditory masking does not impact the main conclusions of the paper.

Ilmoniemi, R. J., & Kičić, D. (2010). Methodology for combined TMS and EEG. Brain topography, 22, 233-248.

Massimini, M., Ferrarelli, F., Huber, R., Esser, S. K., Singh, H., & Tononi, G. (2005). Breakdown of cortical effective connectivity during sleep. Science, 309(5744), 2228-2232.

Biabani, M., Fornito, A., Mutanen, T. P., Morrow, J., & Rogasch, N. C. (2019). Characterizing and minimizing the contribution of sensory inputs to TMS-evoked potentials. Brain stimulation, 12(6), 1537-1552.

Conde, V., Tomasevic, L., Akopian, I., Stanek, K., Saturnino, G. B., Thielscher, A., ... & Siebner, H. R. (2019). The non-transcranial TMS-evoked potential is an inherent source of ambiguity in TMS-EEG studies. Neuroimage, 185, 300-312.

Rocchi, L., Di Santo, A., Brown, K., Ibáñez, J., Casula, E., Rawji, V., ... & Rothwell, J. (2021). Disentangling EEG responses to TMS due to cortical and peripheral activations. Brain stimulation, 14(1), 4-18.

> 1. *A supra-stimulus heat stimulus not based on individual HPT, that oscillates during the experiment and that lead to large variations in pain intensity across participants is unfortunate.*

The choice of whether to calibrate or fix stimulus intensity is a contentious question in experimental pain research. A recent discussion by Adamczyk et al., (2022) explores the pros and cons of each approach and recommends situations where one method may be preferred over the other. That paper suggests that the choice of the methodology is related to the research question – when the main outcome of the research is objective (neurophysiological measures) and researchers are interested in the variability in pain ratings, the fixed approach is preferrable. Given we explored the relationship between MEP/N45 modulation by pain and pain intensity, this question is better explored by using the same stimulus intensity for all

participants, as opposed to calibrating the intensity to achieve a similar of pain across participants.

Adamczyk, W. M., Szikszay, T. M., Nahman-Averbuch, H., Skalski, J., Nastaj, J., Gouverneur, P., & Luedtke, K. (2022). To calibrate or not to calibrate? A methodological dilemma in experimental pain research. The Journal of Pain, 23(11), 1823-1832.

> *So is the lack of report on measures taken to correct for a fortuitous significance (multiple comparison correction) in such a huge number of serial paired tests.*

Note that we used a Bayesian approach for all analyses as opposed to traditional frequentist approach. In contrast to the frequentist approach, the Bayesian approach does not require corrections for multiple comparisons (Gelman et al., 2000) given that they provide a ratio representing the strength of evidence for the null vs. alternative hypotheses as opposed to accepting or rejecting the null hypothesis based on p-values. As such, throughout the paper, we frame our interpretations and conclusions based on the strength of evidence (e.g. anecdotal/weak, moderate, strong, very strong) as opposed to referring to the significance of the effects.

Gelman A, Tuerlinckx F. (2000). Type S error rates for classical and Bayesian single and multiple comparison procedures. Computational statistics, 15(3):373-90.

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In Experiment 3, we also collected warmth ratings to confirm whether the pre-pain stimuli were perceived as different from baseline. We did not include this data initially in the first submission, but will do so in the supplemental material in our next revision. This data showed warmth ratings were close to 2/10 on average. This confirms that the non-painful control condition produced some level of non-painful sensation.

> 1. *MEP differences between conditions. The results do not show differences in MEP amplitudes between conditions (BF 1.015). The analysis nevertheless relates MEP differences between conditions to pain ratings. It would be more appropriate to state that in this study, pain did not affect MEP and to remove the correlation analysis and its interpretation from the manuscript.*

The interindividual relationship between changes in MEP amplitude and individual pain rating is statistically independent from the overall group level effect of pain on MEP amplitude. Therefore, conclusions for the individual and group level effects can be made independently.

It is also important to note that in the pain literature, there is now increasing emphasis placed on investigating the individual level relationship between changes in cortical excitability and pain as opposed to the group level effect (Seminowicz et al., 2019; Summers et al., 2019). As such, it is important to make these results readily available for the scientific community.

Summers, S. J., Chipchase, L. S., Hirata, R., Graven-Nielsen, T., Cavaleri, R., & Schabrun, S. M. (2019). Motor adaptation varies between individuals in the transition to sustained pain. Pain, 160(9), 2115-2125.

Seminowicz, D. A., Thapa, T., & Schabrun, S. M. (2019). Corticomotor depression is associated with higher pain severity in the transition to sustained pain: a longitudinal exploratory study of individual differences. The Journal of Pain, 20(12), 1498-1506.

> 1. *Confounds by pain ratings. The ISI between TMS pulses is 4 sec and includes verbal pain ratings. Considering this relatively short ISI, would it be possible that verbal pain ratings confound the TEP? Moreover, could the pain ratings confound TEP differences between conditions, e.g., by providing earlier ratings when the stimulus is painful? This should be carefully considered, and the authors might perform control analyses.*

It is unlikely that the verbal ratings contaminated the TEP response as the subsequent TMS pulse was not delivered until the verbal rating was complete and given that each participant was cued by the experimenter to provide the pain rating after each pulse (rather than the participant giving the rating at any time). As such, it would not be possible for participants to provide earlier ratings to more painful stimuli. We will make this part of the protocol clearer in the next revision of the manuscript.

> 1. *Confounds by time effects. Non-painful and painful conditions were performed in a fixed order. Potential confounds by time effects should be carefully considered.*

Previous research suggests that pain alters neural excitability even after pain has subsided. In a recent meta-analysis (Chowdhury et al., 2022) we found effect sizes of 0.55-0.9 for MEP reductions 0-30 minutes after pain had resolved. As such, we avoided intermixing pain and warm blocks given subsequent warm blocks would not serve as a valid baseline, as each subsequent warm block would have residual effects from the previous pain blocks.

At the same time, given there was no conclusive evidence for a difference in N45 amplitude between pre-pain and post-pain conditions of Experiment 1 (Supplementary Figure 1), it is unlikely that the effect of pain was an artefact of time i.e., the explanation that successive thermal stimuli applied to the skin results an increase in the N45, regardless of whether they are painful or not. We will make this point in our next revision.

Chowdhury, N. S., Chang, W. J., Millard, S. K., Skippen, P., Bilska, K., Seminowicz, D. A., & Schabrun, S. M. (2022). The Effect of Acute and Sustained Pain on Corticomotor Excitability: A Systematic Review and Meta-Analysis of Group and Individual Level Data. The Journal of Pain, 23(10), 1680-1696.

> 1. *Data availability. The authors should state how they make the data openly available.*

We will upload the MEP, TEP and pain data on the Open science framework at the time of the next revision.