An optimal protocol for measurement of corticospinal excitability, short intracortical inhibition and intracortical facilitation in the *rectus femoris*

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Running head: Intracortical inhibition and facilitation in the knee extensors

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ABSTRACT

The study aimed to determine the optimal application of single- and paired-pulse transcranial magnetic stimulation (TMS) in the rectus femoris. Twenty-nine male adults participated in the study, which involved 5 separate experiments. Experiments 1 to 3 assessed the effect of conditioning stimulus (CS) intensity (60, 70, 80 and 90% active motor threshold, AMT), contraction strength (5, 10, 20 and 50% maximum voluntary contraction, MVC), and inter-stimulus interval (ISI, 2-5 ms for short-interval intracortical inhibition, SICI and 10-15 ms for intracortical facilitation, ICF) on SICI and ICF. In Experiment 4, 30 measurements of corticospinal excitability (CSE), SICI and ICF were recorded, with the minimum number of consecutive measurements required as a probability of falling within the 95% CI determined. In Experiment 5, within- and between-day reliability of CSE, SICI and ICF was assessed. The results suggest that for SICI, a CS of 70% AMT, ISI of 2 ms, and contraction strength of 5 or 10% MVC induces the greatest level of inhibition. Negligible differences in ICF were seen across stimulus variables. The minimum number of measurements required to obtain an accurate estimate of CSE, SICI and ICF was 21, 18 and 17, respectively. Using the optimal stimulus variables and number of measurements, CSE, SICI and ICF can be measured reliably both within- and between-days (intraclass correlation coefficient, ICC ≥ 0.87, ≥ 0.74, and ≥ 0.61, respectively). The current findings can be used to guide future investigations using single- and paired-pulse TMS to elicit responses in the rectus femoris.

Key words: transcranial magnetic stimulation, paired-pulse, knee extensors

INTRODUCTION

Transcranial magnetic stimulation (TMS) over the motor cortex is a safe and non-invasive technique that permits the quantitative assessment of intracortical and corticospinal activity in humans (Kobayashi and Pascual-Leone 2003). At a sufficient intensity, single-pulse TMS induces descending volleys which travel through pyramidal tract neurons and spinal motor neurons to evoke an electromyographical (EMG) response in a target muscle (Goodall et al. 2014). The amplitude of the compound EMG response, termed the motor evoked potential (MEP), can be used to quantify corticospinal excitability (CSE). Paired-pulse TMS paradigms can be used to examine intracortical inhibitory and facilitatory circuits. Specifically, when a subthreshold conditioning stimulus (CS) precedes a suprathreshold test stimulus by an interval of 1-5 ms, inhibitory circuits mediated by gamma-aminobutyric acid type A ($GABA_A$) interneurons are activated, resulting in a reduction in the size of the MEP (short-interval intracortical inhibition, SICI) (Kujirai et al. 1993). In contrast, paired-pulse TMS at a longer inter-stimulus interval (ISI; 10-15 ms) facilitates the MEP response (intracortical facilitation, ICF). While the mechanisms of ICF are less clear, it has been suggested that MEP facilitation could be due to activation of glutamate mediated N-methyl-D-aspartate excitatory interneurons (Liepert et al. 1997; Nakamura et al. 1997).

The stimulus variables used to measure SICI and ICF can be manipulated in order to maximise activation of inhibitory and facilitatory intracortical interneurons and thereby augment the level of inhibition and facilitation induced by paired-pulse TMS. Specifically, the subthreshold CS intensity (O'Leary et al. 2015; Sidhu et al. 2013b; Vucic et al. 2009), suprathreshold test pulse intensity (Temesi et al. 2017), ISI (Ortu et al. 2008) and the contraction strength used during paired-pulse TMS measurements (Ortu et al. 2008; Ridding et al. 1995; Zoghi and Nordstrom

2007) have all been shown to influence the degree of inhibition and/or facilitation. While these stimulus variables have been systematically optimised in upper limb muscle groups (Ortu et al. 2008), no study exists examining the optimal configuration used to elicit SICI and ICF in the knee extensors. Given the differences in intracortical circuits between upper and lower limb muscles (Chen et al. 1998), using stimulus variables optimised in the upper limb might not be appropriate when investigating responses to paired-pulse TMS in lower limb locomotor muscles. At present, much heterogeneity exists between studies in the stimulus variables applied when measuring SICI and ICF in the knee extensors. For example, the conditioning stimulus intensity applied when taking measures of SICI and ICF varies between studies, with some studies applying a conditioning stimulus intensity of 70% (Thomas et al. 2017b) active motor threshold (AMT) or 90% (Latella et al. 2017; O'Leary et al. 2016) resting motor threshold (RMT) when measuring both SICI and ICF. Similarly, inconsistencies exist in the ISI used when measuring SICI, with studies using either a 2 (Brownstein et al. 2017) or 3 ms (O'Leary et al. 2016; Thomas et al. 2017b) ISI for SICI, and an ISI of, 12, (Latella et al. 2017) 13 (Thomas et al. 2017b) or 15 ms for ICF (Luc-Harkey et al. 2017; O'Leary et al. 2016). Such methodological issues make comparisons between investigations problematic.

Another pertinent question when attempting to optimise single- and paired-pulse TMS in the knee extensors is the number of pulses required to obtain an accurate estimate of CSE, SICI and ICF. During single- and paired-pulse TMS, the amplitude of the MEP demonstrates significant pulse-to-pulse variation due to constant fluctuations in CSE (Heroux et al. 2015; Kiers et al. 1993), as well as randomness in the firing of pyramidal tract neurons and spinal motor neurons (Pitcher et al. 2003) and desynchronization of action potentials (Magistris et al. 1998). This variability can be reduced by taking measurements when the muscle is in an active state (Darling et al. 2006). Nonetheless, consecutive measurements are required in order to

obtain a reliable and accurate estimation of CSE, SICI and ICF. Cuypers *et al*. (2014) and Bashier *et al*. (2017) suggested that at least 30 consecutive stimuli are required to obtain a reliable estimate of CSE in the relaxed first dorsal interosseous muscle. However, it is known that the variability in MEP amplitude differs according to the muscle under investigation (Brasil-Neto et al. 1992; Malcolm et al. 2006), and differences in corticospinal projections between upper and lower limbs could influence the pulse-to-pulse variability in MEP amplitude (Brouwer and Ashby 1990). Currently, the appropriate number of pulses in the active knee extensors remains unclear, with the majority of studies arbitrarily using 10-15 responses (O'Leary et al. 2015; Weier et al. 2012). Understanding the appropriate number of stimuli required during single- and paired-pulse TMS in the knee extensors is an important consideration in order to maximise the accuracy of intracortical and corticospinal measurements when assessing the neurophysiological effects of various acute and chronic interventions, such as fatiguing exercise, repetitive TMS, or strength training.

Assessing intracortical and corticospinal activity in the knee extensors is conceptually appealing given the key role of this muscle group in locomotion and sporting activity. Indeed, an increasing number of studies have used paired-pulse TMS to examine intracortical mechanisms involved in locomotion (Sidhu et al. 2013b), fatigue-induced alterations in intracortical activity (O'Leary et al. 2016; Thomas et al. 2017a; Verin et al. 2004), and neural adaptations to strength training (Weier et al. 2012), as well as the neurophysiology of movement disorders (Cantello 2002). As such, understanding the optimal methods used to measure CSE, SICI and ICF and the reliability of these measures could provide guidance for the design of experimental protocols, and mitigate the heterogeneity which currently exists between studies. Accordingly, the aims of the study were threefold: 1) to establish the optimal combination of stimulus variables (CS intensity, ISI and contraction strength) when measuring

SICI and ICF in the knee extensors, 2) to determine the minimum number of stimuli required to obtain an accurate estimation of CSE, SICI and ICF and 3) to assess the within-day and between-day reliability of CSE, SICI and ICF once the optimal stimulus variables and number of responses had been established.

METHODS

Participants

The study received ethical approval from the Northumbria University Faculty of Health & Life Sciences Ethics committee in accordance with the ethical standards established in the *Declaration of Helsinki*. Written informed consent was obtained from all participants prior to data collection. Twenty-nine young male adults participated in at least one experiment of the study. Participants were free of any cardiorespiratory, neurological or neuromuscular health disorders, had no metal plates in the head/brain, and were not taking any medication that might have interfered with the nervous system. All participants completed a TMS safety screening questionnaire prior to the data collection procedure (Keel et al. 2001). Participants were required to refrain from alcohol consumption and strenuous physical activity in the 24 h prior to data collection, and to abstain from caffeine consumption for the 12 h prior to each experimental visit.

Design

The study was divided into five experiments (Figure 1). During all experiments within the study, single- and paired-pulse TMS was delivered during tonic contractions. This is because

studies applying single- and paired-pulse TMS paradigms in the knee extensors are commonly related to locomotor activities (Sidhu et al. 2013b; Thomas et al. 2017a; Thomas et al. 2017b), and it is thus recommended that assessment of corticospinal and intracortical activity be conducted during contraction in order to provide a better reflection of neurophysiological processes occurring during motor activity (Gruet et al. 2013; Kalmar 2018).

Experiments 1-3 aimed to determine the optimal stimulus variables used to measure SICI and ICF in the *rectus femoris* by investigating the effects of CS intensity, contraction strength and ISI, respectively, on the level of inhibition and facilitation. Experiment 4 assessed the minimum number of measurements required to obtain an accurate estimate of CSE, SICI and ICF using the optimal stimulus variables determined from Experiments 1-3. Using the optimal stimulus variables and number of measurements obtained from Experiments 1-4, Experiment 5 assessed the within- and between-day reliability of CSE, SICI and ICF. Each experiment was separated by between three and five weeks.

Instrumentation

Torque and electromyography recordings

A calibrated load cell (MuscleLab force sensor 300, Ergotest technology, Norway) was used to measure isometric knee extensor force (N) during voluntary and stimulated contractions. The load cell was fixed to a custom built chair and strapped with a non-compliant cuff to the participant's right leg, superior to the ankle malleoli. Knee and hip angle were measured using a goniometer at 90° flexion prior to each experiment and maintained during contractions. Participants were instructed to grasp the handles on the side of the chair for support during maximal voluntary contractions (MVC) of the right knee extensors. Three MVCs of 3 s duration were performed prior to each trial, with 60 s between each contraction. The force trace

was displayed on a computer screen directly in front of participants in order to assist in providing maximal efforts (Baltzopoulos et al. 1991) and to provide the target force during submaximal contractions. The maximum force from the three MVCs was recorded in order to calculate the submaximal contraction values. EMG activity was recorded from the *rectus femoris*, using a bipolar setup, with surface electrodes (Ag/AgCl; Kendall H87PG/F, Covidien, Mansfield, MA, USA) placed 2 cm apart over the muscle belly, and a reference electrode placed on the patella. The placement of the EMG electrodes on the *rectus femoris* was based on Seniam guidelines. Specifically, the electrodes were placed at 50% on the line from the anterior spina iliaca superior to the superior part of the patella. The skin surface was shaved and cleaned prior to electrode placement, and marked with indelible ink to ensure consistent placement. Although the *vastus lateralis* has been studied when measuring responses to TMS during and following locomotor exercise (O'Leary et al. 2016; Sidhu et al. 2013b), this muscle is uni-articular and is involved in knee extension exclusively. Given that studies measuring responses to TMS in the knee extensors are most commonly conducted in response to activities involving locomotion (Brownstein et al. 2017; Thomas et al. 2017b; Weier et al. 2012), we believed that the *rectus femoris* was a more suitable muscle to study due to its biarticular make up and significant contribution to both hip flexion and knee extension, movements which are heavily involved in locomotion and activities of daily living. Signals were amplified: gain ×1,000 for EMG and ×300 for force (CED 1902; Cambridge Electronic Design, Cambridge, UK), band-pass filtered (EMG only: 20–2000 Hz), digitized (4 kHz; CED 1401, Cambridge Electronic Design) and analyzed offline.

Motor nerve stimulation

Peripheral stimulation of the right femoral nerve was administered using square wave pulses (200 μs) via a constant-current stimulator (DS7AH, Digitimer Ltd., Hertfordshire, UK) using self-adhesive surface electrodes (CF3200, Nidd Valley Medical Ltd., North Yorkshire, UK). The cathode was placed over the nerve, high in the femoral triangle in the position that elicited the greatest twitch amplitude (Q_{tw}) and compound muscle action potential (M-wave) in the *rectus femoris* (RF) at rest. The anode was placed halfway between the greater trochanter and iliac crest. Stimuli were delivered in 20 mA step-wise increments beginning at 20 mA until the maximum knee extensor twitch amplitude (Q_{tw}, N) and muscle compound action potential (M_{max}, mV) were elicited. The resulting intensity was then increased by 30% in order to ensure the stimulation intensity was supramaximal. The peak-to-peak amplitude of M_{max} was used as a measure of peripheral muscle excitability.

Transcranial magnetic stimulation

Single- and paired-pulse TMS were delivered over the motor cortex via a concave double cone coil using a BiStim unit and two Magstim 200^2 stimulators (The Magstim Company Ltd, Whitland, UK). The junction of the double cone coil was aligned tangentially to the sagittal plane, with its centre 1-2 cm to the left of the vertex, and was oriented to induce current in the posterior-to-anterior direction. The optimal coil placement was determined at the start of each trial as the position that elicited the largest MEP in the RF muscle during a light voluntary contraction (10% MVC). The optimal position was marked with indelible ink to ensure consistent placement throughout the trial. The stimulator intensity was based on an active motor threshold (AMT) established during a 10% MVC in all experiments apart from Experiment 2 (see below). In order to determine AMT, the stimulator intensity was increased in 5% steps beginning at 35% of stimulator output until a consistent MEP with peak-to-peak

amplitudes exceeding 200 μV were found, with an observable silent period. Thereafter, stimulus intensity was reduced in 1% steps until an MEP amplitude exceeding 200 μV was elicited in 3 out of 5 stimulations (Weier et al. 2012). For all experiments, the single-pulse and test-pulse intensity was set at 120% of AMT, as this intensity lies on the middle portion of the ascending part of the stimulus-response curve (Han et al. 2001), and is thus sensitive to changes in corticospinal excitability. 4-6 s were given between each pulse. During Experiments 1-3, the order in which SICI, ICF and/or CSE, and each stimulus variable was assessed was pseudo-randomised and counterbalanced using Latin square randomisation, while the order in which single- and paired-pulses were delivered was randomised using an online randomiser (www.randomizer.org).

Experimental procedures

Experiment 1 – Influence of conditioning stimulus intensity on SICI and ICF.

Twenty participants (aged: 25 ± 4 years; stature: 181.4 ± 6.6 cm; mass: 84.2 ± 13.3 kg) took part in this experiment. SICI and ICF were assessed using a subthreshold CS, followed by a suprathreshold test stimulus as described by Kujirai et al. (1993). Subthreshold CS intensities of 60, 70, 80 and 90% AMT were applied. Inter-stimulus intervals of 2 (Brownstein et al. 2018; Brownstein et al. 2017; Goodall et al. 2018) and 3 ms (O'Leary et al. 2016; Thomas et al. 2017b) for SICI and 10 (Di Lazzaro et al. 2006; Volz et al. 2012) and 15 ms (Chen et al. 1998; Orth et al. 2003) for ICF were examined at each CS intensity since these ISIs successfully elicited inhibition and facilitation in a number of previous studies. The order of conditions was pseudo-randomised and counterbalanced. During each experimental condition, a total of 24 pulses (12 single and 12 paired) were delivered in a randomised order in 4 sets of 6 during a submaximal contraction set at 10% of the MVC force (total of 96 single- and 96 paired-pulses

across all conditions). A short rest (30 s) was given in between each set of pulses to minimise the development of muscle fatigue. The CS intensity and ISI that elicited maximum SICI and ICF was used in Experiment 2.

Experiment 2 – Effect of different levels of muscle contraction on SICI and ICF.

Eighteen participants participated in Experiment 2 (25 ± 4 years; stature: 182.3 ± 6.1 cm; mass: 85.9 ± 13.4 kg), which aimed to assess the effects of four different contraction strengths (5, 10, 20 and 50% MVC) on SICI and ICF. Based on the results from Experiment 1, the CS and ISI were 70% AMT and 2 ms for SICI, and 60% AMT and 10 ms for ICF, respectively. During the 5% and 10% MVCs, AMT was defined, as above, the lowest stimulator intensity required to produce MEPs >200 μV in 3 out of 5 stimulations. During the 20% and 50% MVCs, AMT was defined as the minimum stimulator intensity that produced a discernible MEP which was 200 μV greater than the pre-stimulus EMG. This approach was employed due to background EMG activity being greater than 200 μV at contraction intensities of 20% and 50% MVC. At lower contraction strengths (5, 10 and 20% MVC), 24 pulses (twelve single and twelve paired) were randomly delivered in sets of six, with a short rest (30 s) given between sets. At 50% MVC, 16 pulses (eight single and eight paired) were randomly delivered in groups of four, with a longer rest interval (1 min) given between sets in order to minimise muscle fatigue (total of 44 single- and 44 paired-pulses across all conditions). The order of the 5, 10 and 20% MVC conditions were pseudo-randomised and counterbalanced, whilst the 50% MVC was always performed last because of the higher potential to induce muscle fatigue. The contraction strength that elicited maximum SICI and ICF was used in Experiment 3.

Experiment 3 – Effect of inter-stimulus interval on SICI and ICF.

Sixteen participants took part in Experiment 3 (aged: 24 ± 3 years; stature: 181.3 ± 6.5 cm; mass: 84.4 ± 10.2 kg). Using a CS of 70% AMT for SICI and 60% AMT for ICF and a contraction strength of 10% MVC based on the results from Experiments 1 and 2, this experiment assessed the influence of using different ISIs on SICI and ICF. For SICI, ISIs included 2, 3, 4 and 5 ms, while ICF ISIs included 10, 11, 12, 13, 14 and 15 ms. The order of conditions was pseudo-randomised and counterbalanced. At each ISI, 24 pulses (twelve single and twelve paired) were randomly delivered in four sets of six, with a short rest (30 s) given between sets (total of 60 single- and 60 paired-pulses across all conditions).

Experiment 4 – Assessment of the minimum number of measurements required to obtain an accurate estimation of CSE, SICI and ICF.

Experiment 4 was conducted on twenty subjects (aged: 24 ± 4 years; stature: 180.4 ± 7.1 cm; mass: 79.7 ± 12.8 kg). Based on the results from Experiments 1, 2 and 3, SICI was elicited with a CS of 70% AMT, contraction strength of 10% MVC, and an ISI of 2 ms. For ICF, the stimulus variables incorporated a CS of 60% AMT, contraction strength of 10% MVC, and an ISI of 10 ms. For SICI and ICF separately, 60 pulses (30 single and 30 paired) were delivered in a randomised order, with 30 single pulses delivered for assessment of CSE separate from the assessment of SICI and ICF (total of 90 single- and 60 paired-pulses across all conditions). All pulses were delivered in sets of 6, with a short rest between each set. The order of the conditions was pseudo-randomised and counterbalanced.

Experiment 5 – Within-day and between-day reliability of CSE, SICI and ICF

Twenty participants took part in Experiment 5 (aged: 24 ± 4 years; stature: 183 ± 6 cm; mass: 81 ± 10 kg), which assessed the within-day and between-day reliability of CSE, SICI and ICF

using the optimal stimulus variables obtained from the 4 previous experiments (CS of 70% AMT, ISI of 2 ms, and contraction strength of 10% MVC for SICI, CS of 60% AMT, ISI of 10 ms, and contraction strength of 10% MVC for ICF). Based on the results of Experiment 4, 20 conditioned and 20 unconditioned pulses were delivered in sets of 6 to determine SICI and ICF separately, with 20 single pulses delivered in sets of 5 for CSE separate from the assessment of SICI and ICF (total of 60 single-pulses and 40 paired-pulses across all conditions). For within-day reliability, participants visited the laboratory on two occasions in the morning and afternoon, separated by 4 h (e.g. 0900 and 1300). For between-day reliability, participants visited the laboratory on one further occasion at the same time of day as their previous morning session. In order to account for any within- or between-day fluctuations in peripheral muscle excitability, femoral nerve stimulation was administered at the beginning of each visit in order to assess M_{max}. In order to ensure consistent placement of electrodes during each visit in Experiment 5, electrodes were marked with indelible ink during each trial.

Data analysis

The peak-to-peak amplitude of the EMG responses to motor nerve stimuli and TMS were analysed offline. The root mean square EMG amplitude (RMS_{EMG}) and average force were calculated in the 80 ms prior to each TMS stimulus to ensure a similar level of background muscle activity during each stimulation, and excluded if pre-stimulation force was > 5% above or below the average force calculated from all stimulations in the set (< 1% excluded). To quantify SICI and ICF, the ratio of the average conditioned paired-pulse MEP amplitude was expressed relative to the average unconditioned MEP amplitude at 120% AMT. A ratio < 100% indicates inhibition, and a ratio > 100% indicates facilitation. Throughout the study, the stimulus variables which elicited the greatest degree of inhibition and facilitation and/or

produced inhibition and facilitation in the highest number of participants were used in the subsequent experiments of the study. While the average degree of inhibition and facilitation was prioritised as the most important factor in determining which stimulus variable was used in subsequent experiments of the study, the number of participants that exhibited inhibition and facilitation at each configuration was considered if the configuration which produced the highest average degree of inhibition or facilitation produced inhibition or facilitation in a substantially fewer number of participants (≤10%) than other configurations. In Experiment 4, the average MEP for CSE was calculated for subsets of consecutive stimuli as follows:

$$\overline{MEP_n} = \frac{MEP_1 + \ldots + MEP_n}{n}$$

where n = 2 to 30 consecutive MEPs for CSE(Cuypers et al. 2014). This procedure was also conducted for subsets of consecutive pairs of conditioned/unconditioned MEPs for SICI and ICF. For this experiment, the average of 30 consecutive measurements was considered as the true value for CSE, SICI and ICF. A 95% confidence interval (CI) was then calculated using all 30 measurements for each participant. Based on the CSE, SICI and ICF n value and the CI, it was determined whether the value for subsets of stimuli were included in the CI, yielding a binary variable (0 = not included in the CI, 1 = included in the CI). Subsequently, the number of consecutive measurements required as a probability of falling within the 95% CI was determined (Cuypers et al. 2014). In Experiment 5, CSE was assessed by averaging single MEP amplitudes across 20 pulses and normalizing the value relative to the M_{max}. Additionally, to investigate the influence of the number of measurements taken for the within- and between-day reliability of CSE, SICI and ICF, subsets of 5, 10, 12 and 15 stimuli (for CSE) or pairs of conditioned/unconditioned stimuli (for SICI and ICF) were calculated.

Statistical analysis

All data are presented as mean ± SD. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS, v22.0). Normality of the data was assessed using the Shapiro-Wilks test. If the assumption of normality was violated, appropriate transformations were performed, with common logarithm used for strongly positively skewed ICF and SICI data in Experiments 1 and 2, respectively, and reciprocal transformation used for extremely positively skewed ICF data in Experiment 2 (Bulmer 1979). For repeated measures ANOVA, sphericity was assessed using Mauchly's test. The Greenhouse-Geisser correction was used to compensate for non-spherical data. In the event of a significant main effect, *post hoc* pairwise comparison with Bonferroni corrections for multiple comparisons was applied. Statistical significance was accepted at $P < 0.05$. For Experiment 1, the effect of CS intensity (60, 70, 80, 90%) and ISI (2, 3, 10, 15 ms) on SICI and ICF was tested using a two-way repeated measures ANOVA. For Experiment 2, the effect of contraction strength (5, 10, 20, 50% MVC) on SICI and ICF was assessed using a one-way repeated measures ANOVA. For Experiment 3, a one-way repeated measures ANOVA was used to assess the effect of the ISI (2, 3, 4, 5 ms for SICI and 10, 11, 12, 13, 14, 15 ms for ICF) on SICI and ICF.

For Experiment 4, a linear regression was performed on the data of each participant to assess for change (slopes) in CSE, SICI or ICF over time. If the slope of the regression was statistically significant ($P < 0.05$), which would indicate a trend for scores to increase or decrease over time, the data from the corresponding participant was removed from the analysis of the specific condition. Although participants were given a rest period between each set throughout the experiment in order to prevent muscle fatigue, this analysis was performed in order to ensure the results were not confounded by fatigue-induced alterations in CSE, SICI or ICF. After excluding 4 participants from the CSE analysis, 2 participants from the SICI analysis, and 1

participant from the ICF analysis, 16 (CSE), 18 (SICI) and 19 (ICF) participants were included in the final analysis.

For Experiment 5, a one-way repeated measures ANOVA was performed on all neuromuscular and TMS variables to assess for any within- or between-day differences using 20, 15, 12, 10 and 5 responses. Relative reliability of all neuromuscular and TMS measures was assessed using intraclass correlation coefficient ($ICC_{3,1}$), while absolute reliability was assessed using typical error (TE) expressed in raw units (Hopkins 2000), and variability assessed through coefficient of variation (CV) determined using the formula: standard deviation/mean × 100. As per the guidelines recommended by Koo and Li (2016), ICCs between 0.5 and 0.75 were considered moderately reliable, values between 0.75 and 0.9 were considered of good reliability, and values above 0.9 considered of excellent reliability.

RESULTS

Experiment 1 − Influence of conditioning stimulus intensity on SICI and ICF.

Figure 2A and B, respectively, display the ratios of the conditioned to unconditioned pulses for SICI and ICF at different CS intensities and ISIs. A two-way ANOVA comparing SICI and different CS intensities and ISIs showed no main effect for CS ($F_{1.77,33.65} = 3.191$, $P = 0.059$), ISI ($F_{1,19} = 2.111$, $P = 0.163$) or CS*ISI ($F_{1.81,34.29} = 2.879$, $P = 0.075$). Similarly, for ICF, there was no main effect for CS ($F_{1.96,37.14} = 1.011$, $P = 0.372$), ISI ($F_{1,19} = 0.416$, $P = 0.572$) or CS*ISI ($F_{2.55,48.37} = 0.848$, $P = 0.473$). Although there were no statistically significant differences between stimulus variables, a CS of 70% with an ISI of 2 ms elicited the greatest degree of inhibition on average (67 ± 17% of unconditioned MEP), with 19 out of 20 participants displaying a conditioned/unconditioned MEP ratio < 100%. For ICF, although a

CS intensity of 80% AMT with an ISI of 10 ms produced the highest level of ICF on average (132 ± 40% of unconditioned MEP), only 16 out of 20 participants displayed a conditioned/unconditioned MEP ratio > 100%. In contrast, a CS of 60% AMT with an ISI of 10 ms induced facilitation (125 ± 20% of unconditioned MEP) in 18 out of 20 participants. Consequently, stimulus variables consisting of a 70% CS AMT with an ISI of 2 ms for SICI, and a CS of 60% AMT with an ISI of 10 ms for ICF, were applied in the subsequent parts of the study.

Experiment 2 − Effect of different levels of muscle contraction on SICI and ICF.

Figure 3 displays the ratios of the conditioned to unconditioned MEP at different contraction strengths. A main effect for contraction strength on SICI was observed ($F_{2.196,37.325} = 21.604$, $P < 0.001$). *Post hoc* analysis showed that there was more inhibition of the conditioned MEP at 5% MVC compared with 20% MVC ($P = 0.021$) and 50% MVC ($P < 0.001$). Similarly, there was more inhibition at 10% MVC compared with 20% MVC ($P = 0.037$) and 50% MVC ($P < 0.001$), with no differences between 5% and 10% MVC ($P = 1.000$), and more inhibition at 20% than 50% MVC ($P = 0.005$). For ICF, there was a main effect for contraction strength ($F_{3,51} = 4.741$, $P = 0.005$), with *post hoc* analysis showing more facilitation of the conditioned MEP at 10% MVC compared with 50% MVC ($P = 0.012$), and more facilitation at 20% than 50% MVC ($P = 0.006$), with no other differences ($P > 0.05$). A contraction strength of 10% MVC was chosen for further analysis during SICI and ICF measurements.

Experiment 3 – Effect of inter-stimulus interval on SICI and ICF.

Figure 4 displays the ratios of the conditioned to unconditioned MEP at different ISIs. A one-way ANOVA displayed a main effect for SICI ($F_{1.80,25.22} = 17.675$, $P < 0.001$). *Post hoc*

analysis revealed that a 2 ms ISI resulted in more inhibition of the conditioned MEP than 4 ms ($P = 0.001$) and 5 ms ($P < 0.001$), with no difference between 2 and 3 ms ($P = 0.092$). An ISI of 3 ms induced more inhibition than 5 ms ($P = 0.023$) with no difference between 3 and 4 ms ($P = 0.286$). No difference was found between inhibition at 4 and 5 ms ($P = 0.063$; Cohen's *d* effect size = 0.85). For ICF, there was a main effect for ISI ($F_{2.87,40.17} = 4.355$, $P = 0.011$), however, *post hoc* comparison revealed no differences between facilitation of the conditioned MEP at any ISI ($P > 0.05$). Although differences between SICI at ISIs of 2 and 3 ms were not observed, an ISI of 2 ms induced the greatest mean inhibition (59 ± 21% vs. 75 ± 31% of unconditioned MEP for 2 and 3 ms, respectively), and induced inhibition in more participants (16 at 2 ms vs. 14 at 3 ms). Similarly, the highest degree of facilitation on average was induced at 10 ms (120 ± 9% of unconditioned MEP), with the highest number of participants facilitated (13). As such, an ISI of 2 ms for SICI and 10 ms for ICF were used for the subsequent parts of the study.

Experiment 4 − Assessment of the minimum number of measurements required to obtain an accurate estimation of CSE, SICI and ICF.

The probability that MEP_n, $SICI_n$ and ICF_n fell within the 95% CI based on 30 TMS pulses or pairs of conditioned/unconditioned pulses increased with successive stimulations (Figure 5). At least 21, 18 and 17 stimuli were required for CSE, SICI and ICF, respectively, to reach a 100% probability that the average MEP fell within the 95% CI for all participants (Figure 6).

Supplementary experiment – Comparison of number of measures used in Experiments 1-3 with optimal number derived from Experiment 4.

The results from Experiment 4 displayed that the minimum number of measurements required to obtain an accurate estimate of SICI and ICF was 18 and 17, respectively. However, in Experiments 1-3, 12 measurements were used to determine the optimal combination of stimulus variables used to measure SICI and ICF. In order to determine whether using a suboptimal number of measurements in Experiments 1-3 could have had any bearing on the results, the level of uncertainty (assessed using 95% CIs) associated with using 12 and 17 (for ICF) and 18 measurements (for SICI) was determined using random sampling without replacement. This procedure involved taking 12 and 17 (for ICF), and 12 and 18 (for SICI) random conditioned/unconditioned MEP ratios (without replacement) derived from the 30 measurements taken in Experiment 4, and calculating the mean and 95% CIs from each sample. One thousand replicates of 12, 17 and 18 random samples were generated, with the average of the thousand means and upper and lower bound CIs calculated. The width of the 95% CIs were compared between 12 measurements and 17 (for ICF) and 18 measurements (for SICI).

The distribution of mean values derived from 1000 resamples of 12 and 18 measures (for SICI) and 17 measures (for ICF) are displayed in Figure 7. Differences in mean and 95% CIs between the number of measures used in Experiments 1-3 and the optimal number derived from Experiment 4 were negligible. For SICI, using 12 measurements produced a mean inhibition of the conditioned MEP of 71%, with 95% CIs spanning 67-75%, while using 18 measurements produced a mean inhibition of the conditioned MEP of 70%, with 95% CIs spanning 67-74%. For ICF, using 12 measures produced a mean facilitation of the conditioned MEP of 125%, with 95% CIs spanning 115-134%, while using 17 measures produced a mean facilitation of the conditioned MEP of 124%, with 95% CIs spanning 116-132%.

Experiment 5 − Within-day and between-day reliability of single- and paired-pulse TMS

Neuromuscular measures

There were no within- or between-day differences in MVC (within-day AM visit: 653.7 ± 151.7 N; within-day PM visit: 663.5 ± 150.2 N; between-day visit: 657.9 ± 153.1 N), M_{max} (within-day AM visit: 5.0 ± 1.7 mV; within-day PM visit: 5.2 ± 1.5 mV; between-day visit: 5.0 ± 1.5 mV), pre-stimulation force or EMG_{RMS} ($P > 0.05$). Both MVC and M_{max} demonstrated excellent within- and between-day reliability (ICC ≥ 0.90). TE and CV values for M_{max} were 0.7 mV and 8.6% for within-day measurements, and 0.7 mV and 8.3% for between day measurements, respectively. For MVC, TE and CV values were 26.3 N and 3.3% for within-day measurements, and 27.4 N and 3.0% for between-day measurements, respectively.

Transcranial magnetic stimulation measures

The within- and between-day reliability of CSE, SICI and ICF can be viewed in Table 1, while individual within- and between-day data points for single- and paired-pulse variables are displayed in Figure 8. There were no within- or between-day differences for any of the TMS measures using 5, 10, 12, 15 or 20 measurements (AMT, CSE, SICI or ICF) ($P > 0.05$). Based on 20 MEPs (CSE) or pairs of conditioned/unconditioned MEPs (SICI and ICF), within-day measures of SICI and ICF were good (ICC ≥ 0.77), while within-day measures of CSE and AMT were excellent (ICC ≥ 0.91). Between-day reliability analysis showed moderate reliability for ICF and SICI (ICC ≥ 0.61). Measures of CSE displayed good reliability (ICC = 0.87), while AMT demonstrated excellent reliability (ICC = 0.99). When comparing the reliability of CSE, SICI and ICF when taking 5, 10, 12, 15 and 20 measures, the ICCs were higher and the CVs lower the more measurements were taken (Table 1). For CSE, ICC values were excellent when using 10 or more stimuli for within-day measurements (≥ 0.90), and were good when using 5 or more stimuli for between-day measurements (≥ 0.87). For within-day

456 measurements of SICI, reliability was good when using 5 or more measurements (≥ 0.78),
457 while a minimum of 10 measurements were required to obtain moderate reliability between-
458 days (≥ 0.59). For ICF, a minimum of 15 measurements were required to obtain moderate
459 reliability both within- (ICC ≥ 0.71) and between-days (ICC ≥ 0.70).

460

461 **DISCUSSION**

462 The aims of the present study were: 1) to establish the optimal combination of stimulus
463 variables when measuring SICI and ICF in the *rectus femoris*, 2) to determine the minimum
464 number of stimuli required to obtain an accurate estimation of CSE, SICI and ICF and, 3) to
465 assess the within- and between-day reliability of CSE, SICI and ICF once the optimal
466 combination of stimulus variables, and number of pulses, had been established. The study
467 demonstrates that a number of stimulus variables can be used to induce inhibition and
468 facilitation in the evoked responses from *rectus femoris*. For SICI, a CS intensity of 70% AMT,
469 and ISI of 2 ms, with a contraction strength of 5 or 10% MVC induced the highest degree of
470 inhibition, suggesting that these stimulus variables are favourable when assessing SICI in the
471 *rectus femoris*. Intracortical facilitation was induced using most combinations of stimulus
472 variables, with large inter-subject variability evident across configurations. For accurate
473 estimates of CSE, SICI and ICF, the results indicate that 21, 18 and 17 evoked responses are
474 required, respectively. Finally, the study demonstrates that CSE, SICI and ICF can be measured
475 reliably both within- and between-days when assessing responses in the *rectus femoris*. Given
476 the role of the knee extensors in locomotion and activities of daily living, an increasing number
477 of studies are applying single- and paired-pulse TMS in the knee extensors in response to
478 various acute and chronic interventions (Thomas et al. 2017a; Weier et al. 2012). As such, the
479 results of the study could inform future investigations of this nature, and provide a standardised

approach to the stimulus variables used when taking TMS measures in the active knee extensors in order to facilitate comparisons between studies.

Effect of conditioning stimulus intensity on SICI and ICF. While there was no statistically significant effect of CS intensity on SICI, a CS of 70% AMT induced the highest level of inhibition on average, with 19 out of 20 participants exhibiting inhibition at this intensity with an ISI of 2 ms. Contrasting results exist throughout the literature concerning the influence of CS on SICI, with a range of CS intensities suggested as producing optimal SICI in muscles of both the upper and lower limb. For example, in the active knee extensors, studies have reported that a CS of 90% AMT elicits the greatest degree of SICI (O'Leary et al. 2015; Sidhu et al. 2013b), corroborating the findings of Ridding et al. (1995) in the upper limb muscles. Our findings are in agreement with those of Ortu et al. (2008), who similarly reported that a CS of 70% elicited optimal SICI during a 10% MVC in the first dorsal interosseous muscle. While it is unclear why SICI was reduced at CS intensities above 70% AMT, it is possible that higher CS intensities lead to the concurrent recruitment of both inhibitory and facilitatory interneurons, thereby reducing the magnitude of inhibition even at short ISIs. Indeed, previous work has shown that during a light, voluntary contraction (10% MVC), superimposed recruitment of intracortical facilitatory circuits during paired-pulse TMS at short intervals (1-5 ms) reduces the degree of SICI at specific CS intensities, due to concurrent activation of both inhibitory and facilitatory interneurons (Ortu et al. 2008). This facilitatory input, termed short-interval intracortical facilitation (SICF), overlaps in time with SICI, and can be assessed using a CS and test stimulus intensity which are both near AMT (Ziemann et al. 1998). By assessing both SICI and SICF during a 10% MVC, Ortu et al. (2008) found that a CS of 70% induced optimal SICI in the FDI because this intensity was not strong enough to simultaneously activate intracortical interneurons which mediate SICF. While previous work investigating SICF has

shown that facilitation occurs at discrete ISIs (1.1-1.5, 2.3-2.9 and 4.1-4.4 ms) (Hanajima et al. 2002; Ortu et al. 2008; Ziemann et al. 1998), these studies have been conducted exclusively in the upper limb muscles. As such, it is possible that differences in cortical circuitry between upper and lower limbs (Chen et al. 1998) could influence the interaction between SICI and SICF, providing a potential mechanistic explanation as to why a CS of 70% AMT induced the greatest degree of inhibition in our study. However, as SICF was not measured in the present study, this interpretation should be viewed with caution. While it is unclear why discrepancies exist in the optimal CS intensity found between studies, methodological differences such as differences in the test-pulse intensity, contraction strength, ISI and the muscle being investigated could all contribute to the observed disparities between studies. Therefore, caution should be aired when attempting to extrapolate the optimal CS intensity for SICI identified in the present study when used in combination with other paired-pulse TMS variables.

Another important finding from Experiment 1 was the substantial inter-subject variability in the optimal CS intensity used when measuring SICI and ICF. Although a CS of 70% AMT with a 2 ms ISI produced the highest level of SICI on average, only 7 out of 20 (35%) participants exhibited optimal SICI using these stimulus variables. Previous work has displayed comparable inter-subject variability in SICI when assessing individual responses to different CS intensities in the upper limb (Orth et al. 2003; Ortu et al. 2008). Similarly, a high degree of inter-subject variability was found in ICF, with negligible differences in the mean level of facilitation using different stimulus variables. While a CS intensity of 80% AMT produced the highest level of ICF on average, corroborating the findings of previous work (Hunter et al. 2016), only 16 out of 20 participants displayed facilitation at this intensity, with a high degree of inter-subject variability found in the level of facilitation induced at this intensity. Although a CS of 60% AMT did not produce the highest level of ICF on average, the inter-subject

variability in facilitation at this intensity was low, with ICF elicited in the highest number of subjects when used in combination with an ISI of 10 ms, with 18 out of 20 participants displaying some degree of facilitation, albeit a smaller magnitude. Furthermore, that ICF was induced using this CS intensity in combinations with different contraction strengths and inter-stimulus intervals in Experiments 2 and 3 suggests that, while this intensity might not elicit maximal levels of facilitation, it consistently induces ICF in the vast majority of participants. While these results suggest a high degree of inter-subject variability in the optimal CS intensity to elicit inhibition and facilitation, the differences noted between subjects could be a consequence of the variability inherent in measures of SICI and ICF. Alternatively, it is possible that differences in the electrophysiological properties of inhibitory and facilitatory interneurons between-subjects might have contributed to the inter-subject variability (Orth et al. 2003).

Effect of contraction strength on SICI and ICF. Although it is well established that the magnitude of SICI is reduced during voluntary contraction (Kujirai et al. 1993; Ridding et al. 1995), it is recommended that assessments of corticospinal and intracortical activity should be conducted with the muscle in an active state when assessing responses in relation to locomotor activity (Gruet et al. 2013; Kalmar 2018), as this is thought to be more reflective of motor cortical behaviour during locomotion (Sidhu et al. 2013a). Given the key role of this muscle group in locomotion and athletic activity, the majority of studies using single- and paired-pulse TMS in the knee extensors relate to locomotor activities, such as fatiguing exercise (Brownstein et al. 2017; Thomas et al. 2017a), neural adaptations to strength training (Thomas et al. 2017b; Weier et al. 2012), and the assessment of movement disorders (Cantello 2002). As such, we considered that because of the muscle group under investigation, it was more appropriate to assess responses to TMS with the muscle in an active state, and to examine the

555 effects of varying contraction intensities on SICI and ICF. The results displayed that SICI was
556 elicited at contraction strengths of 5%, 10% and 20% MVC, but was progressively reduced
557 with higher contraction strengths (Figure 3). Although a contraction strength of 5 and 10%
558 MVC induced a similar degree of SICI on average (60 ± 19% and 62 ± 20% of unconditioned
559 MEP for 5 and 10% MVC, respectively), we chose to apply a contraction strength of 10%
560 MVC because we believed that using this contraction strength is more representative of the
561 recruitment of neural pathways involved in locomotion (where single- and paired-pulse TMS
562 paradigms are regularly applied when assessing responses in the knee extensors) when
563 compared with a 5% MVC.

564

565 Previous work has similarly displayed a progressive reduction in SICI with stronger contraction
566 strengths (Ortu et al. 2008; Zoghi and Nordstrom 2007). The release of inhibition during
567 contraction has been attributed to modulation of corticospinal neurons by GABAergic circuits
568 (Zoghi and Nordstrom 2007), and concomitant superimposition of facilitation during voluntary
569 contraction (Ortu et al. 2008). From a functional perspective, it has been suggested that the
570 reduction in SICI during voluntary contraction represents a transient compensatory down-
571 regulation of inhibitory processes, such that there is a gradual reduction in SICI with increasing
572 contraction strengths in order to preserve cortical output to the target muscle (Maruyama et al.
573 2006; Vucic et al. 2011).

574

575 Intracortical facilitation was also induced at contraction strengths of 5%, 10% and 20% MVC,
576 with no ICF at 50% MVC. Limited evidence exists on the effect on contraction strength on
577 ICF; however, contrasting evidence has suggested during voluntary contraction, ICF is reduced
578 compared with rest (Hanajima et al. 2002; Kujirai et al. 1993; Ridding et al. 1995), with others

reporting an increase in glutamate mediated SICF during contraction compared with rest (Ortu et al. 2008). Furthermore, it is unclear why ICF was abolished at 50% MVC. Ortu *et al.* (2008) suggested that at high contraction intensities, a 'busy line' phenomenon might occur, whereby there is too much activity within glumatergic circuits for facilitation to be observed. Alternatively, given that the largest MEPs are commonly evoked during a 50% MVC in the knee extensors (Goodall et al. 2014), it is possible that a ceiling effect exists in MEP amplitude, whereby no increase in the conditioned MEP amplitude can be observed.

While previous authors have advocated taking measures of SICI and ICF with the muscle in an active state in order to better reflect motor cortical behaviour compared with taking measures at rest (Gruet et al. 2013; Kalmar 2018), the limitations associated with taking measurements of paired-pulse TMS in relation to locomotor activities should be acknowledged. Specifically, because SICI and ICF are abolished at higher contraction intensities, the capacity to capture these measures at higher contraction intensities consistent with those used during and following high-intensity locomotor exercise, to which they are commonly applied (O'Leary et al. 2016; Thomas et al. 2017b; Weier et al. 2012), is precluded. These limitations were highlighted in a recent review by Kalmar (2018), who suggested that in an ideal scenario, we would take measures of corticospinal excitability, and in this case SICI and ICF, across a range of time points and contraction intensities that reflect the planning or execution phases of motor output that we consider most pertinent to the questions we pose. However, due to the constraints associated with taking such measures, this is of course not possible. Consequently, we are required to sacrifice some degree of ecological validity in order to ensure measures are taken in a controlled and reproducible environment. As a compromise, taking measures under conditions which more closely replicate the 'real-life' motor task has been advocated (Kalmar 2018). Despite their limitations, measuring SICI and ICF during light voluntary contractions

has previously been shown be responsive to changes in intracortical excitability following locomotor exercise interventions such as fatiguing exercise, acute and chronic strength training interventions involving high force contractions. Taking these considerations into account, we believe that measuring SICI and ICF during a low intensity voluntary contraction offers a reasonable compromise when attempting to assess changes in response to muscular exercise.

Effect of inter-stimulus interval on SICI and ICF. The level of SICI was influenced by the ISI, with significant inhibition at 2 and 3 ms and no inhibition at 4 and 5 ms. Previous work has found that SICI is most prominent at 1 ms and 2.5 ms ISIs, with inhibition at 1 ms attributed to the refractory period of the interneurons activated by the preceding CS, and inhibition at 2.5 ms mediated by $GABA_A$ interneurons (Fisher et al. 2002; Hanajima et al. 2003). It is now generally accepted that all SICI occurring at 2-5 ms is a consequence of the activity of GABAergic inhibitory interneurons acting via $GABA_A$ receptors (Vucic et al. 2011). While no statistically significant difference in SICI was found between 2 and 3 ms, a 2 ms ISI induced the most inhibition on average, and the highest level of MEP suppression in 12 out of 16 participants. These results are in contrast to Hanajima *et al* (2003), who found no suppression of late indirect waves (I-waves; descending volleys produced by indirect activation on pyramidal tract neurons), which are normally susceptible to inhibition, in the active first dorsal interosseous at an ISI of 2 ms, while 3-5 ms produced substantial inhibition. Moreover, previous studies investigating responses in the upper-limb have successfully induced SICI at ISIs of 4 and 5 ms (Beck et al. 2007; Kujirai et al. 1993; Ortu et al. 2008). While it is unclear why these discrepancies exist, the disparity between the studies highlight that the optimal stimulus variables for inducing SICI in one muscle group cannot necessarily be generalised across all muscle groups.

. Although no significant differences between the level of ICF were found between different ISIs in the present study, we maintained an ISI of 10 ms when assessing ICF in Experiments 4 and 5, because this ISI induced the highest level of facilitation on average and in the greatest number of participants (14 out of 16) in comparison with other stimulus variables. However, even when using these stimulus variables, substantial inter-subject variability existed in the level of facilitation induced (average conditioned/unconditioned MEP ratio: 120 ± 10%, range: 98 to 169%). Furthermore, a high degree of inter-subject variability existed in the ISI which induced the highest level of ICF, with only 4 of 16 participants displaying the highest conditioned/unconditioned MEP ratio at this ISI. The erratic nature of ICF in the present study is in line with previous studies attempting to elicit ICF in the knee extensors (Brownstein et al. 2018; O'Leary et al. 2015). For example, a recent study from our laboratory attempting to compare intracortical and corticospinal responses between isometric squat and knee extension exercise found that only a limited number of participants exhibited facilitation in the *vastus lateralis* during both exercise modalities (Brownstein et al. 2018), and the measure was consequently omitted from the analysis due to the small number of valid cases. Similarly, O'Leary et al (2015) displayed an average ratio of conditioned/unconditioned MEP amplitude below 1.0 in a cohort of 16 participants when assessing the reliability of ICF. While ICF is thought to reflect the excitability of glutamate mediated N-methyl-D-aspartate excitatory interneurons, the lack of facilitation suggests that using a subthreshold CS with an ISI of 10-15 ms fails to activate these interneurons in some participants. Consequently, future studies should exercise caution when attempting to measure and interpret ICF when assessing responses in the knee extensors. A prudent approach when assessing ICF could be to exclude participants who do not exhibit a conditioned/unconditioned MEP ratio > 1.0 from the analysis, and to only proceed with the analysis if a sufficient number of participants exhibit facilitation.

Assessment of the minimum number of measurements required to obtain an accurate estimation of CSE, SICI and ICF. The number of measurements required to obtain an accurate estimate of CSE, SICI and ICF, i.e. the number of measurements required to fall within the 95% CI, was 21, 18 and 17, respectively. Responses to single- and paired-pulse TMS are inherently variable, with a high degree of pulse-to-pulse fluctuation in the MEP amplitude. As such, it is important to understand the optimal number of pulses required to obtain a 'true' estimate of CSE, SICI and ICF in order to maximise the reliability of these measurements. A number of recent studies have similarly assessed the minimum number of pulses required to obtain an accurate estimate of CSE; Bashir et al. (2017) and Cuypers et al. (2014) reported that a minimum of 30 stimuli were required, while Chang et al. (2016) reported that at least 20 and 25 pulses were required to obtain an accurate estimate of SICI and ICF, respectively. However, all of these studies measured responses in the resting first dorsal interosseous, while the present study was conducted in the active knee extensors. Given that it has previously been shown the variability of MEPs are reduced when measurements are taken during muscle contraction (Darling et al. 2006), this likely explains the lower number of pulses required to fall within the 95% CI in comparison with previous work (Bashir et al. 2017; Chang et al. 2016; Cuypers et al. 2014). In the majority of studies assessing responses in the knee extensor musculature, 10-15 measurements are arbitrarily applied when assessing CSE, SICI and/or ICF (O'Leary et al. 2016; Thomas et al. 2017b; Weier et al. 2012). Based on the results from the present study, using 10-15 pulses would reduce the probability of the value for averaged consecutive measurements falling within the 95% CI based on 30 stimuli for CSE (0.60-0.75), SICI (0.65-0.90) and ICF (0.80-0.90). As such, the degree of error in the estimate of CSE, SICI and ICF is reduced considerably when using the number of stimuli commonly employed when measuring responses in the knee extensors (O'Leary et al. 2016; Thomas et al. 2017b; Weier et

al. 2012). Thus, the information provided from this study on the optimal number of pulses required during single- and paired-pulse TMS measurement provides important practical information when assessing responses in the active knee extensors.

Within-day and between-day reliability of single- and paired-pulse TMS. Using the optimal number of measurements established in the previous experiment, reliability analyses revealed that CSE, SICI and ICF can be measured with moderate-to-excellent relative reliability both within- and between-days. Corticospinal excitability was highly reproducible both within- and between-days, corroborating findings from previous studies in the active *rectus femoris* (Temesi et al. 2017). The level of within- and between-day reliability of CSE was slightly higher than reported by O'Leary et al. (2015) (ICC = 0.85 and 0.82, respectively). However, their study investigated responses in the *vastus lateralis*, and was based on averaged responses from 10 measurements rather than the 20 used in the present study, possibly contributing to the differences in ICCs. Despite the high reproducibility of CSE in the present study, there was also a higher degree of variability for within- and between-day measurements when compared with SICI and ICF measurements, which should be taken into account when taking multiple measures of CSE throughout an intervention. Based on 20 measurements, both SICI and ICF displayed good reliability within-day, and moderate reliability between-days, similar to previous findings in the *vastus lateralis* (O'Leary et al. 2015). Furthermore, the excellent reliability of MVC and M_{max} suggest that the variability in CSE, SICI or ICF was not a result of changes in contraction strength or neuromuscular transmission.

While Experiment 4 identified the optimal number of measurements as 21, 18 and 17 when assessing CSE, SICI and ICF, respectively, many studies require responses to single- and

paired-pulse TMS to be captured in a more timely fashion. For example, several studies have measured CSE and SICI during and following exercise interventions in order to assess fatigue-induced alterations in corticospinal or intracortical activity (Brownstein et al. 2017; Sidhu et al. 2013b; Thomas et al. 2017a). As such, it is often impractical to employ a prolonged testing battery during which intervention-induced changes in CNS activity could dissipate, and using a lower number of stimuli might be more appropriate in order to reduce the time required for assessment. In these circumstances, it is important to understand the reliability and sensitivity of single- and paired-pulse TMS in detecting changes when a suboptimal number of stimuli have been used. In general, using a higher number of measurements resulted in greater relative and absolute reliability and lower variability, particularly for between-day measurements. Despite this, the reliability and variability for measurements of CSE and SICI were not markedly impaired between 20 and 5 measurements when assessed within-day. In contrast, SICI and ICF displayed a substantial drop in between-day reliability and increase in variability when taking under 15 measurements. Based on these results, we suggest that taking 20 measurements of CSE, SICI and ICF will improve the accuracy and reliability of results both within- and between-days.

Limitations. While the present study provides important methodological information which can be used to guide future investigations employing single- and paired-pulse TMS in the knee extensors, the study is not without its limitations. Specifically, in Experiments 1-3, 12 measurements were used to assess the effect of each combination of stimulus variables on SICI and ICF. However, in Experiment 4, it was determined that 18 and 17 measurements were required to ensure 100% probability of falling within the 95% CI based on 30 measurements for SICI and ICF, respectively. Consequently, the number of stimuli used in Experiments 1-3 was below the minimum required to ensure the SICI or ICF value fell within the 95% CI for

all participants. However, had the sequence of the experiments been such that Experiment 4 was conducted before Experiment 1, the optimal configuration used to assess SICI and ICF would not yet have been determined. As such, it is possible that performing the experiments in this sequence would have resulted in using a different set of stimulus variables for measurements of SICI and ICF then would subsequently be determined in the next three experiments. In turn, using different stimulus variables could have influenced the variability in responses to paired-pulse TMS if a different population of inhibitory or facilitatory interneurons were activated, potentially invalidating the results of the experiment. To account for this limitation, we performed statistical resampling in order to establish the uncertainty (measured through 95% CIs) associated with using 12 measurements (i.e. the number used in Experiments 1-3) to quantify the level of SICI and ICF, compared with the level of uncertainty associated with using the 'optimal' number of measurements derived from Experiment 4, i.e. 18 for SICI and 17 for ICF. The results displayed that differences between the mean values and 95% CIs derived from using 12 measurements compared with the 'optimal' number were negligible. Specifically, 95% CIs were 1 and 3% wider when using 12 measurements compared with using 18 and 17 for SICI and ICF, respectively, suggesting that it is unlikely that using a suboptimal number of measurements in Experiments 1-3 had bearing on the results of the study.

CONCLUSION

The present study demonstrates that a number of stimulus variables can be used to assess short-interval intracortical inhibition and intracortical facilitation in the active *rectus femoris*. For measurements of short-interval intracortical inhibition, a conditioning stimulus of 70% active motor threshold with an inter-stimulus interval of 2 ms during a contraction (5 or 10% maximum voluntary contraction) was the optimal combination of stimulus variables to elicit

maximum inhibition. For intracortical facilitation, there appeared to be no optimal combination of stimulus variables to maximise facilitation, with low levels of facilitation induced using most stimulus variables, and large inter-subject variability evident across all combinations of stimulus variables. A minimum of 21, 18 and 17 measurements were required to obtain an accurate estimate of corticospinal excitability, short-interval intracortical inhibition and intracortical inhibition, respectively. Furthermore, using these stimulus variables and number of stimuli, the study demonstrated that corticospinal excitability, short-interval intracortical inhibition and intracortical inhibition can be measured reliability both within- and between-days in the active *rectus femoris*. The results of this study can be used to guide future investigations employing single- and paired-pulse transcranial magnetic stimulation in the active *rectus femoris*, and reduce the heterogeneity which currently exists between studies.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

CB, KT, GH and SG contributed to the conception/design of the work and contributed to the interpretation and analysis of the data. CB, PA and JS acquired the data for the study. All authors have drafted/revised the intellectual content and approved the final version. All listed authors qualify for authorship, and all those who qualify for authorship are listed.

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Table and Figure Legends

Table 1. Intraclass correlation coefficients, typical error expressed in raw units, and coefficient of variation for within- and between-day measures of single- and paired-pulse transcranial magnetic stimulation (n = 20).

Figure 1. Flow chart displaying study design. Experiments 1-3 aimed to determine the optimal stimulus variables used to measure short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) in the rectus femoris by investigating the effects of conditioning stimulus (CS) intensity, contraction strength and inter-stimulus interval (ISI), respectively, on the level of inhibition and facilitation. Experiment 4 assessed the minimum number of measurements required to obtain an accurate estimate of corticospinal excitability (CSE), SICI and ICF using the optimal stimulus variables determined from Experiments 1-3. Using the optimal stimulus variables and number of measurements obtained from Experiments 1-4, Experiment 5 assessed the within- and between-day reliability of CSE, SICI and ICF.

Figure 2. Effect of conditioning stimulus intensity relative to active motor threshold (AMT) and inter-stimulus interval (ISI) on short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) measured in the *rectus femoris* (n = 20) during a 10% MVC. Solid horizontal line represents threshold between inhibition (< 100%), and facilitation (> 100%). Values are mean ± SD.

Figure 3. Effect of contraction strength relative to maximal voluntary contraction (MVC) on short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) measured in the *rectus femoris* (n = 18). Solid horizontal line represents threshold between inhibition (< 100%), and facilitation (> 100%). Values are mean ± SD.

Figure 4. Effect of inter-stimulus interval (ISI) on short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) in the *rectus femoris* (n = 16) during a 10% MVC. Solid

horizontal line represents threshold between inhibition (< 100%), and facilitation (> 100%). Solid vertical line represents cut off between ISIs used to measure SICI (2-5 ms) and ICF (10-15 ms). Values are mean ± SD.

Figure 5. Corticospinal excitability (CSE, A), short-interval intracortical inhibition (SICI, B) and intracortical facilitation (ICF, C) during consecutive TMS stimuli from a representative participant measured during a 10% MVC. White dots represent the individual (raw) MEP (A) or ratio of conditioned to unconditioned MEPs (B and C), while black dots represent the average of consecutive MEPs or SICI and ICF ratios. Dashed lines represent the 95% confidence interval (CI), which is based on 30 stimuli. For this particular participant, 17, 16 and 17 consecutive stimuli for CSE, SICI and ICF, respectively, were sufficient to enter the 95% CI.

Figure 6. Probability that the motor evoked potential (MEP) during single-pulse measures of corticospinal excitability (CSE, A) or the ratio of conditioned to unconditioned MEP during measures of short-interval intracortical inhibition (SICI, B) and intracortical facilitation (ICF, C) for averaged consecutive stimuli and pairs of stimuli will fall within the 95% confidence interval (CI) based on 30 stimuli. 21, 18 and 17 measurements were required to a probability of 1 for inclusion in the 95% CI for CSE, SICI and ICF, respectively (CSE n = 16, SICI n = 18, ICF n = 19).

Figure 7. Histogram displaying distribution of mean values derived from 1000 resamples of 12 (solid line) and 18 measurements (dashed line) of SICI (A) and of 12 (solid line) and 17 measurements (dashed line) of ICF (B).

Figure 8. Individual data points for within- and between-day measures of corticospinal excitability (CSE, A), short-interval intracortical inhibition (SICI, B) and intracortical facilitation (ICF, C) measured during a 10% MVC. White dot represents between-day

measurements, while black dots represent within-day measurements. The dashed lines represent lines of agreement (n = 20).

Table 1. Intraclass correlation coefficients, typical error expressed in raw units (CSE: % of M_{max}, SICI and ICF: % of unconditioned MEP), and coefficient of variation (%) for within- and between-day measures of single- and paired-pulse transcranial magnetic stimulation (n = 20).

	20 measurements			15 measurements			12 measurements			10 measurements			5 measurements		
Within-day															
	ICC	TE	CV	ICC	TE	CV	ICC	TE	CV	ICC	TE	CV	ICC	TE	CV
CSE	0.91	6	17.9	0.90	6	20.3	0.87	6	22.8	0.90	6	21.3	0.87	6	24.8
SICI	0.84	9	10.9	0.84	9	11.3	0.78	11	12.7	0.78	11	12.9	0.80	11	12.1
ICF	0.77	15	6.9	0.71	13	7.1	0.80	10	7.3	0.36	17	9.6	0.30	30	14.2
Between-day															
CSE	0.87	5	18.3	0.84	5	18.0	0.77	5	17.0	0.78	6	19.6	0.77	7	20.2
SICI	0.74	11	10.6	0.70	10	13.1	0.68	11	13.3	0.59	12	14.3	0.23	17	21.1
ICF	0.61	15	8.2	0.70	13	7.8	0.78	15	7.8	0.67	17	8.0	0.11	30	15.1

ICC = intraclass correlation coefficient, TE = typical error, CV = coefficient of variation, CSE = corticospinal excitability, SICI = short-interval intracortical inhibition, ICF = intracortical facilitation

Figure 1

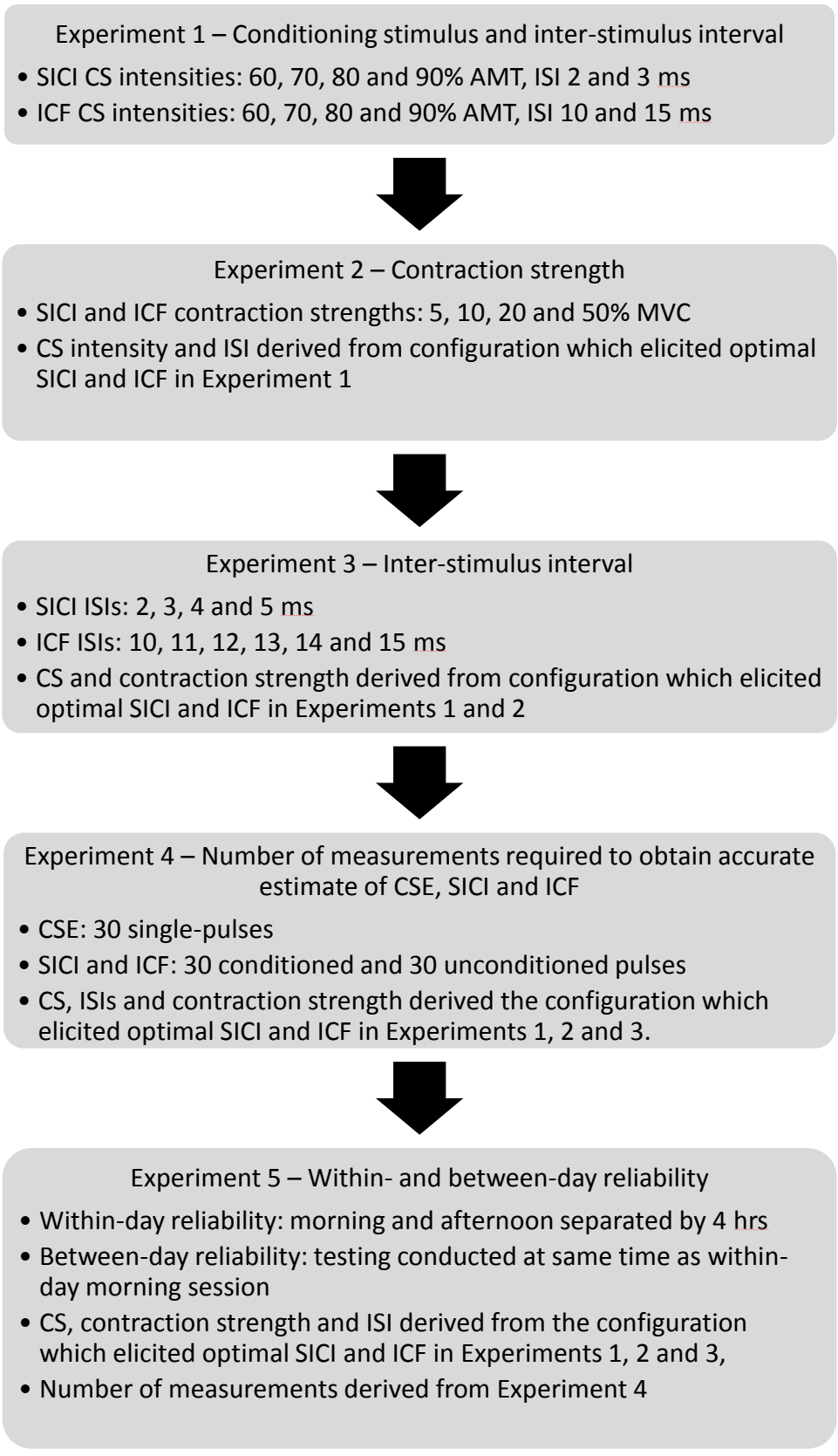

Experiment 1 – Conditioning stimulus and inter-stimulus interval
• SICI CS intensities: 60, 70, 80 and 90% AMT, ISI 2 and 3 ms
• ICF CS intensities: 60, 70, 80 and 90% AMT, ISI 10 and 15 ms
Experiment 2 – Contraction strength
• SICI and ICF contraction strengths: 5, 10, 20 and 50% MVC
• CS intensity and ISI derived from configuration which elicited optimal SICI and ICF in Experiment 1
Experiment 3 – Inter-stimulus interval
• SICI ISIs: 2, 3, 4 and 5 ms
• ICF ISIs: 10, 11, 12, 13, 14 and 15 ms
• CS and contraction strength derived from configuration which elicited optimal SICI and ICF in Experiments 1 and 2
Experiment 4 – Number of measurements required to obtain accurate estimate of CSE, SICI and ICF
• CSE: 30 single-pulses
• SICI and ICF: 30 conditioned and 30 unconditioned pulses
• CS, ISIs and contraction strength derived the configuration which elicited optimal SICI and ICF in Experiments 1, 2 and 3.
Experiment 5 – Within- and between-day reliability
• Within-day reliability: morning and afternoon separated by 4 hrs
• Between-day reliability: testing conducted at same time as within-day morning session
• CS, contraction strength and ISI derived from the configuration which elicited optimal SICI and ICF in Experiments 1, 2 and 3,
• Number of measurements derived from Experiment 4

Figure 2

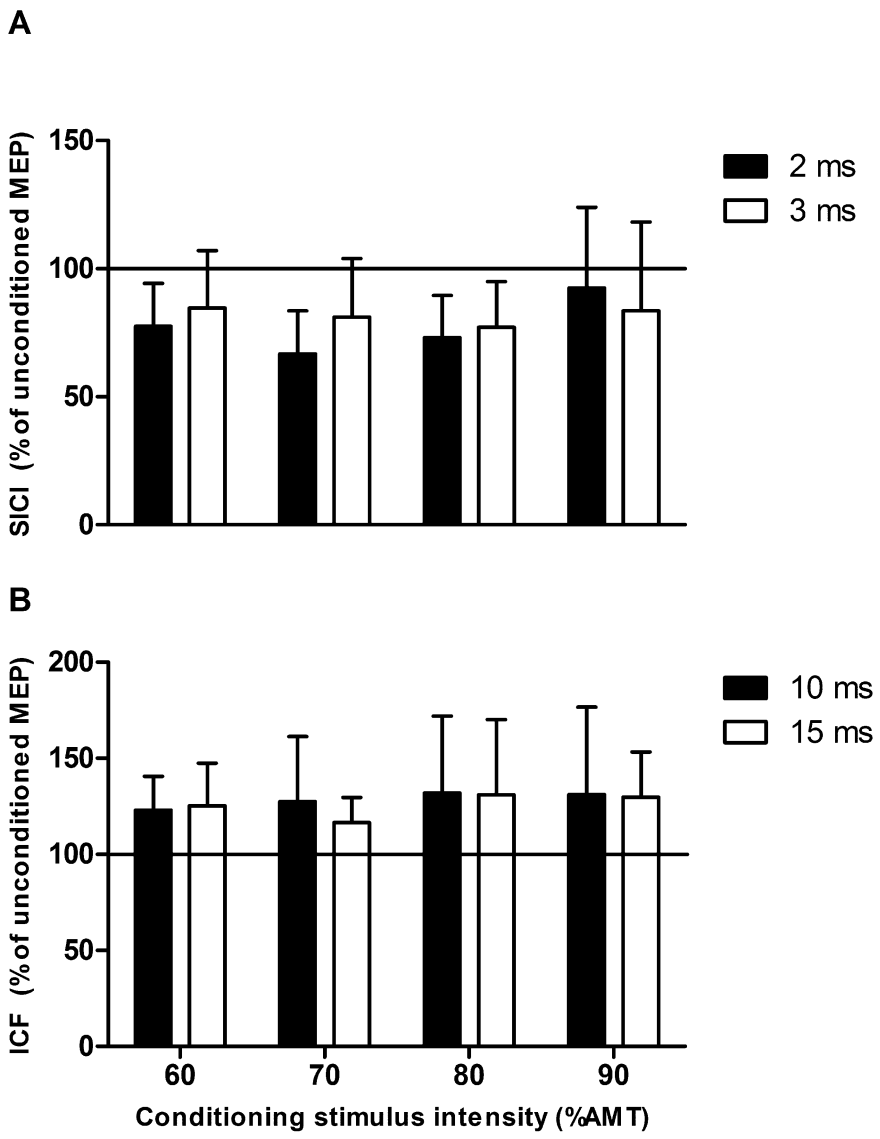

A
SICI (% of unconditioned MEP)
150
100
50
0
2 ms
3 ms
B
ICF (% of unconditioned MEP)
200
150
100
50
0
10 ms
15 ms
60
70
80
90
Conditioning stimulus intensity (%AMT)

Figure 3

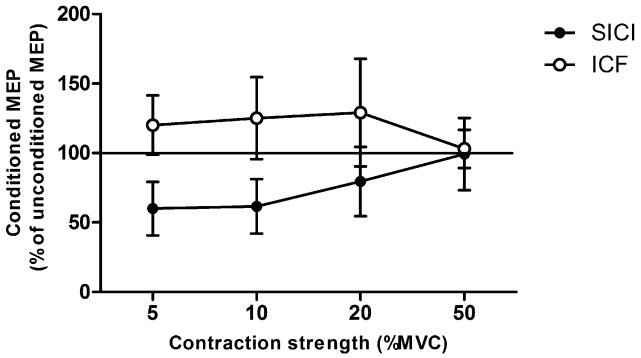

Conditioned MEP
(% of unconditioned MEP)
200
150
100
50
0
5
10
20
50
Contraction strength (%MVC)
SICI
ICF

Figure 4

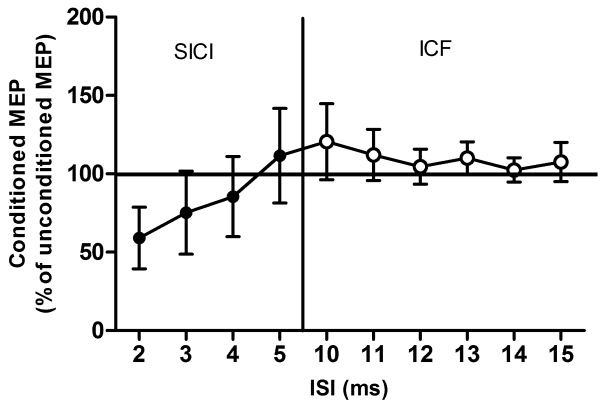

200
150
100
50
0
SICI
ICF
Conditioned MEP
(% of unconditioned MEP)
2
3
4
5
10
11
12
13
14
15
ISI (ms)

Figure 5

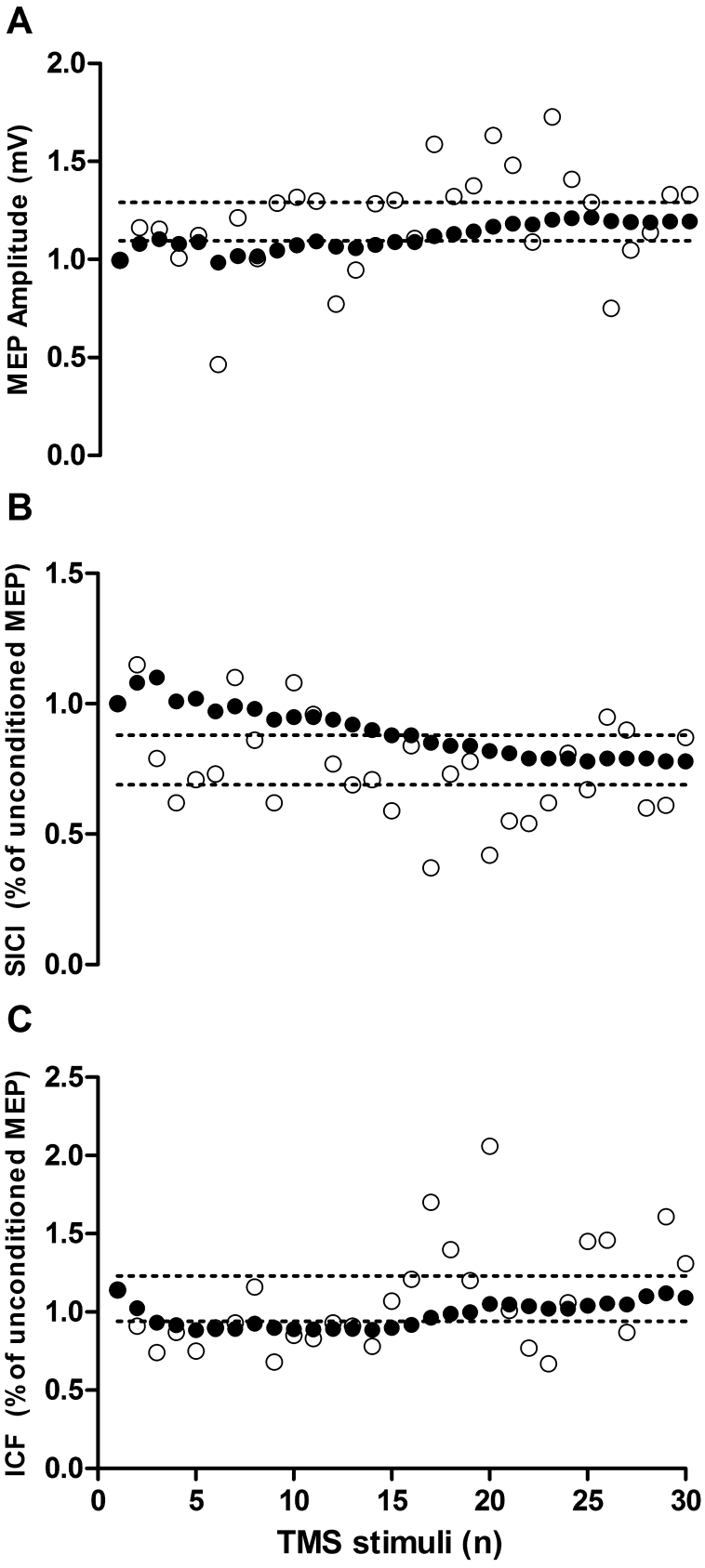

A
MEP Amplitude (mV)
2.0
1.5
1.0
0.5
0.0
B
SICI (% of unconditioned MEP)
1.5
1.0
0.5
0.0
C
ICF (% of unconditioned MEP)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
TMS stimuli (n)

Figure 6

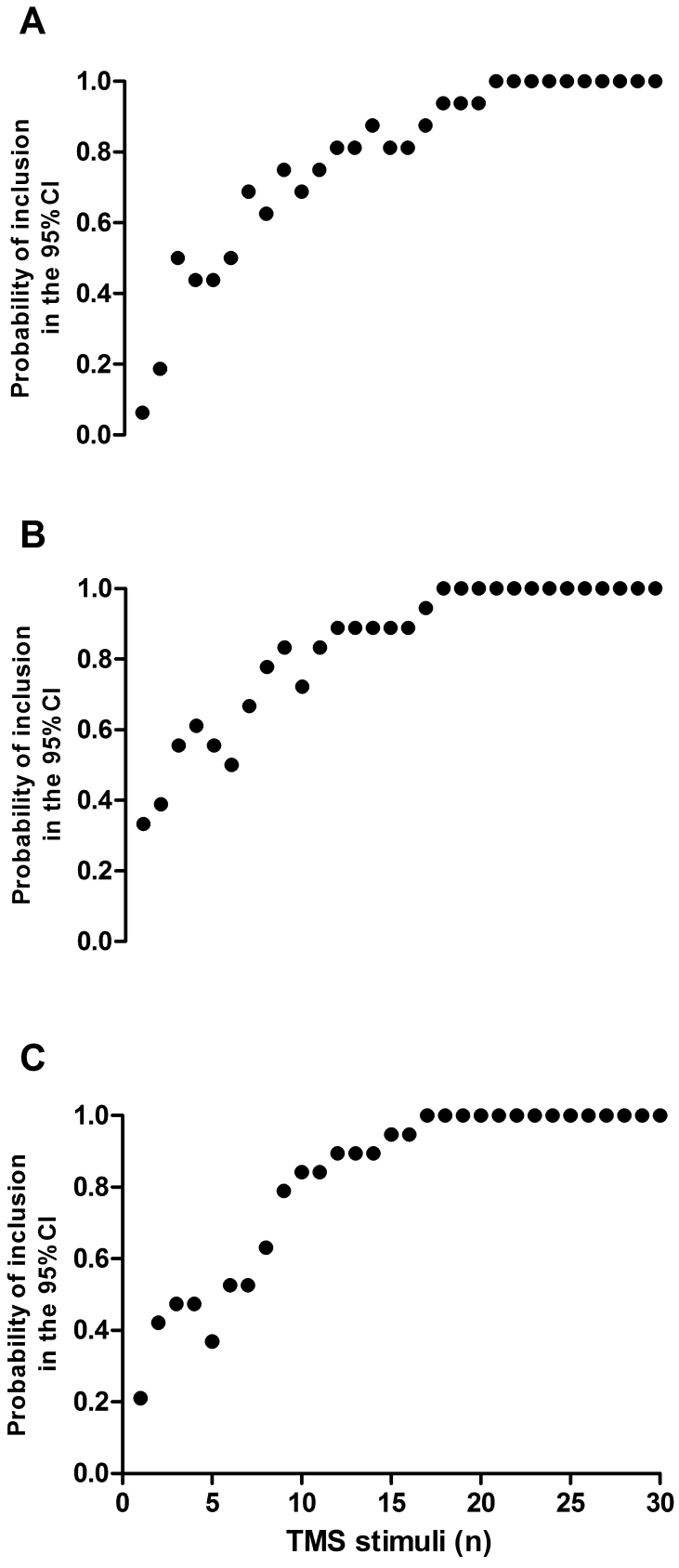

A
Probability of inclusion in the 95% CI
1.0
0.8
0.6
0.4
0.2
0.0
B
Probability of inclusion in the 95% CI
1.0
0.8
0.6
0.4
0.2
0.0
C
Probability of inclusion in the 95% CI
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
TMS stimuli (n)

Figure 7

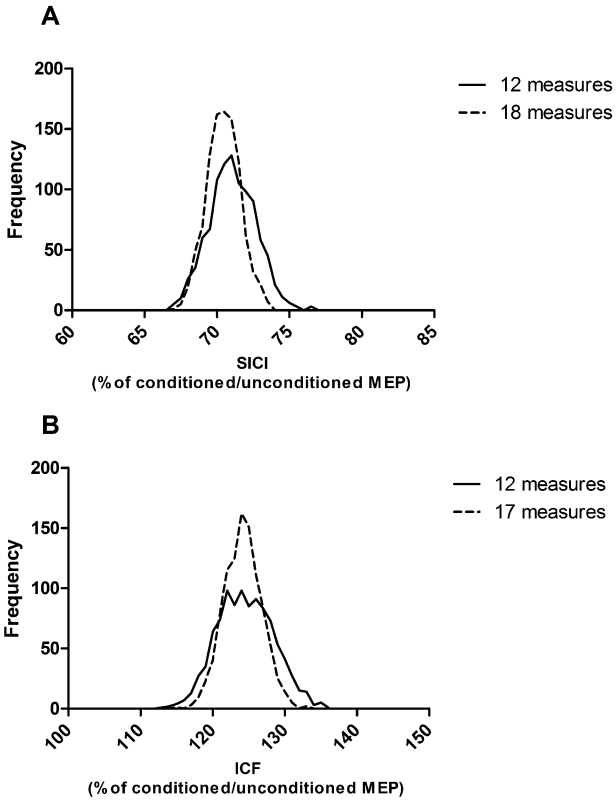
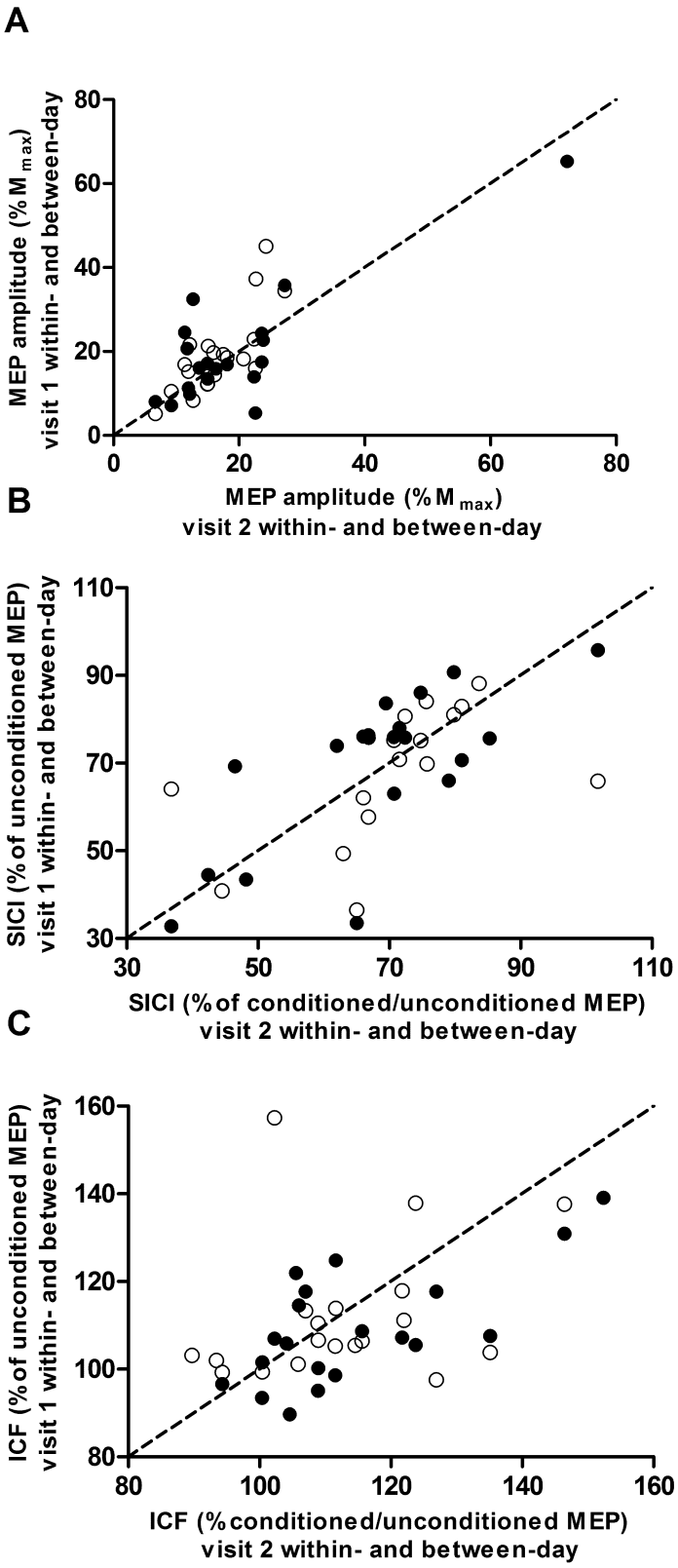

A
200
150
100
50
0
Frequency
60
65
70
75
80
85
SICI
(% of conditioned/unconditioned MEP)
12 measures
18 measures
B
200
150
100
50
0
Frequency
100
110
120
130
140
150
ICF
(% of conditioned/unconditioned MEP)
12 measures
17 measures

Figure 8


A
MEP amplitude (%M max)
visit 1 within- and between-day
0
20
40
60
80
MEP amplitude (%M max)
visit 2 within- and between-day
B
SICI (% of unconditioned MEP)
visit 1 within- and between-day
30
50
70
90
110
SICI (% of conditioned/unconditioned MEP)
visit 2 within- and between-day
C
ICF (% of unconditioned MEP)
visit 1 within- and between-day
80
100
120
140
160
ICF (% conditioned/unconditioned MEP)
visit 2 within- and between-day