Exploring threshold-tracking transcranial magnetic stimulation for cortical inhibition as a novel biomarker for γ-aminobutyric acid A α2,3 receptor signalling in humans

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I, Gintaute Samusyte, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Transcranial magnetic stimulation (TMS) is a non-invasive neurophysiological method used to investigate the human motor system. Variability of TMS measures remains one of the main concerns in the research field and a major limitation for their clinical application. Conventional TMS paradigms are largely based on the measurement of the motor evoked potential (MEP) size and are confounded by its large trial-to-trial variability. An alternative approach is to measure threshold, i.e. the stimulus intensity required to obtain a MEP of or above a certain size, and threshold-tracking TMS is emerging as a useful diagnostic test. However, little is known about its reliability and comparability with conventional methods.

In this thesis, threshold-tracking was for the first time directly compared with conventional TMS approaches in healthy volunteers. Estimation of resting motor threshold by threshold-tracking was validated against common probabilistic methods. The extent of changes in corticospinal excitability observed in individual recordings during a standard TMS session suggested that threshold-tracking may improve the reliability of paired-pulse TMS paradigms as it allows continuous monitoring and adjustment for these fluctuations.

This hypothesis was tested with one of the most widely-used paradigms – short-interval intracortical inhibition (SICI). Mean group SICI obtained at an interstimulus interval of 2.5 ms and a range of conditioning stimulus intensities by both conventional 'amplitude' method and threshold-tracking showed a close relationship suggesting that they reflect similar inhibitory mechanisms, while threshold-tracking had a potential for improved reproducibility and acquisition speed. Availability of a safe selective positive allosteric γ-aminobutyric acid A α2,3 receptor modulator AZD7325 allowed for the first time to test the hypothesis that SICI is mediated by this pathway and to directly compare the biomarker sensitivity of the two techniques in a randomised double-blind placebo-controlled cross-over study. This trial showed no modulatory effect of the drug on SICI at the exposure level used.

Impact Statement

Despite significant technological advances in the field of transcranial magnetic stimulation (TMS), variability of conventional TMS measures remains one of the main issues both in research and clinical practice. Therefore, alternative and more reliable ways to estimate motor excitability are being sought, and threshold measurements are gaining increasing interest.

In this work, threshold-tracking was for the first time compared to some of the most widely-used conventional single- and paired-pulse TMS measurements. The findings contribute towards a better understanding on how these two approaches relate, give insight into advantages and limitations of different techniques and provide reliability measurements that can be used for future experiment planning. The potential for improved reproducibility and acquisition speed with threshold-tracking suggests that this technique is a valuable addition to the array of biomarkers of motor excitability.

The technical part of this work led to implementation of new TMS-tailored features in QtracW software (© Institute of Neurology, University College London, London, UK). Through piloting and experimental work, optimal tracking parameters and analysis methods were defined and incorporated into recording and analysis scripts. This provides a basis for the development of standard TMS protocols for future research and clinical practice.

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List of Abbreviations

ACh	Acetylcholine
AE	Adverse event
ALS	Amyotrophic lateral sclerosis
ALT	Alanine transaminase
AMT	Active motor threshold
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AP	Anterior-to-posterior current flow direction
A-SICI	Conventional short-interval intracortical inhibition paradigm
AST	Aspartate transaminase
AT	A-SICI – T-SICI recording protocol sequence
AV	Audiovisual
BMI	Body Mass Index
BP	Blood pressure
CBI	Cerebellar inhibition
CC BY	Creative Commons Attribution Licence
CI	Confidence interval
C_{max}	Peak plasma concentration
cMEP	Conditioned motor evoked potential
CNS	Central nervous system
CR	Coefficient of repeatability
CS	Conditioning stimulus
CSE	Corticospinal excitability
CSF	Cerebrospinal fluid
cTS	Conditioned threshold
CV	Coefficient of variation
DA	Dopamine
DAQ	Data acquisition
DC	Direct current
ECG	Electrocardiogram
EEG	Electroencephalography
EMG	Electromyography
FDA	Food and Drug Administration
FDIO	First dorsal interosseous muscle
FFT	Fast Fourier Transform
GABA	γ-aminobutyric acid
Glu	Glutamate
HR	Heart rate
ICC	Intraclass correlation coefficient

Continued List of Abbreviations

ICF	Intracortical facilitation
IFCN	International Federation of Clinical Neurophysiology
IHI	Interhemispheric inhibition
IQR	Interquartile range
ISI	Interstimulus interval
JRO	Joint Research Office
LOA	Limits of agreement
LICI	Long-interval intracortical inhibition
LLT	Lowest level term
LM	Lateromedial induced current flow direction
KA	Kinematic analysis
(M)CD	(Mean) consecutive difference
MedDRA	Medical Dictionary for Regulatory Activities
MEP	Motor evoked potential
MSA	Mean square amplitude
MSO	Maximum stimulator output
MVC	Maximum voluntary contraction
MT	Motor threshold
NE	Norepinephrine
NO	Nitrous oxide
PA	Posterior-to-anterior current flow direction
PEST	Parameter Estimation by Sequential Testing
PET	Positron Emission Tomography
PC	Personal Computer
rmANOVA	Repeated measures Analysis of Variance
RF	Relative frequency method for motor threshold estimation
RMT	Resting motor threshold
rTMS	Repetitive transcranial magnetic stimulation
SAI	Short-latency afferent inhibition
SD	Standard deviation
SDC	Smallest detectable change
SDMT	Symbol Digit Modalities Test
SICF	Short-interval intracortical facilitation
SICI	Short-interval intracortical inhibition
SPECT	Single Photon Emission Computed Tomography
SPV	Saccadic peak velocity
SS	Sum of squares
TA	T-SICI – A-SICI recording protocol sequence
t_{max}	Time to peak plasma concentration
TMS	Transcranial magnetic stimulation

Continued List of Abbreviations

TS	Test stimulus
T-SICI	Short-interval intracortical inhibition obtained by threshold-tracking
TST	Triple stimulation tehcnique
TT	Threshold-tracking
VAS	Visual Analogue Scale

Chapter 1 - Introduction

Transcranial magnetic stimulation (TMS) is a neurophysiological technique that allows non-invasive investigation of the physiology and pathology of the human motor system. Single-pulse TMS paradigms can be used to assess the integrity of corticospinal tracts and provide measures of corticospinal excitability (CSE), while paired-pulse TMS protocols provide insight into various inhibitory or excitatory microcircuits in the human motor cortex (Rossini et al., 2015).

Conventional TMS paradigms employ a *constant stimulus* approach and rely on the motor response size as an outcome measure. Notoriously large trial-to-trial variability of motor evoked potentials (MEPs) neccesitates averaging of multiple responses which often leads to time-consuming recordings. As fixed stimulation intensities are used, slow changes in CSE which are likely to occur during prolonged protocols may remain undetected and unaccounted for, thus biasing the measurement reliability.

By contrast, a *constant response* approach is used in threshold-tracking in which the intensity of stimulation is altered to maintain a motor response of a pre-defined target size (Fisher et al., 2002). This technique allows continuous monitoring and online adjustment for changes in CSE and could potentially provide a more reliable outcome measure.

This thesis explores the comparability of threshold-tracking and conventional TMS paradigms with regards to their reliability and biomarker utility.

1.1 Basic principles of TMS

The principle of magnetic stimulation is based on electromagnetic induction: the electrical current in the coil creates a magnetic field, and changes in this field induce a current in the nearby conductors (Wassermann et al., 2008). First reports on the effects of alternating magnetic field on humans date back to the late 19th - early 20th centuries, but the true breakthrough in the field was prompted by the development of the first practical magnetic stimulator at the University of Sheffield in 1980s which enabled non-invasive and, most importantly, painless stimulation of the human nervous system (Figure 1.1). Availability of commercial magnetic stimulators led to a rapid expansion of the research in the field, and the first guidelines of the International Federation of Clinical Neurophysiology (IFCN) for the use of TMS in a routine clinical setting were published in mid-1990s (Rossini et al., 1994). In the last 30 years, TMS has been widely applied not only to investigate, but also to modulate cognition, behaviour, and disease, and is finding its way into clinical practice as a therapeutic intervention (Ziemann, 2017).

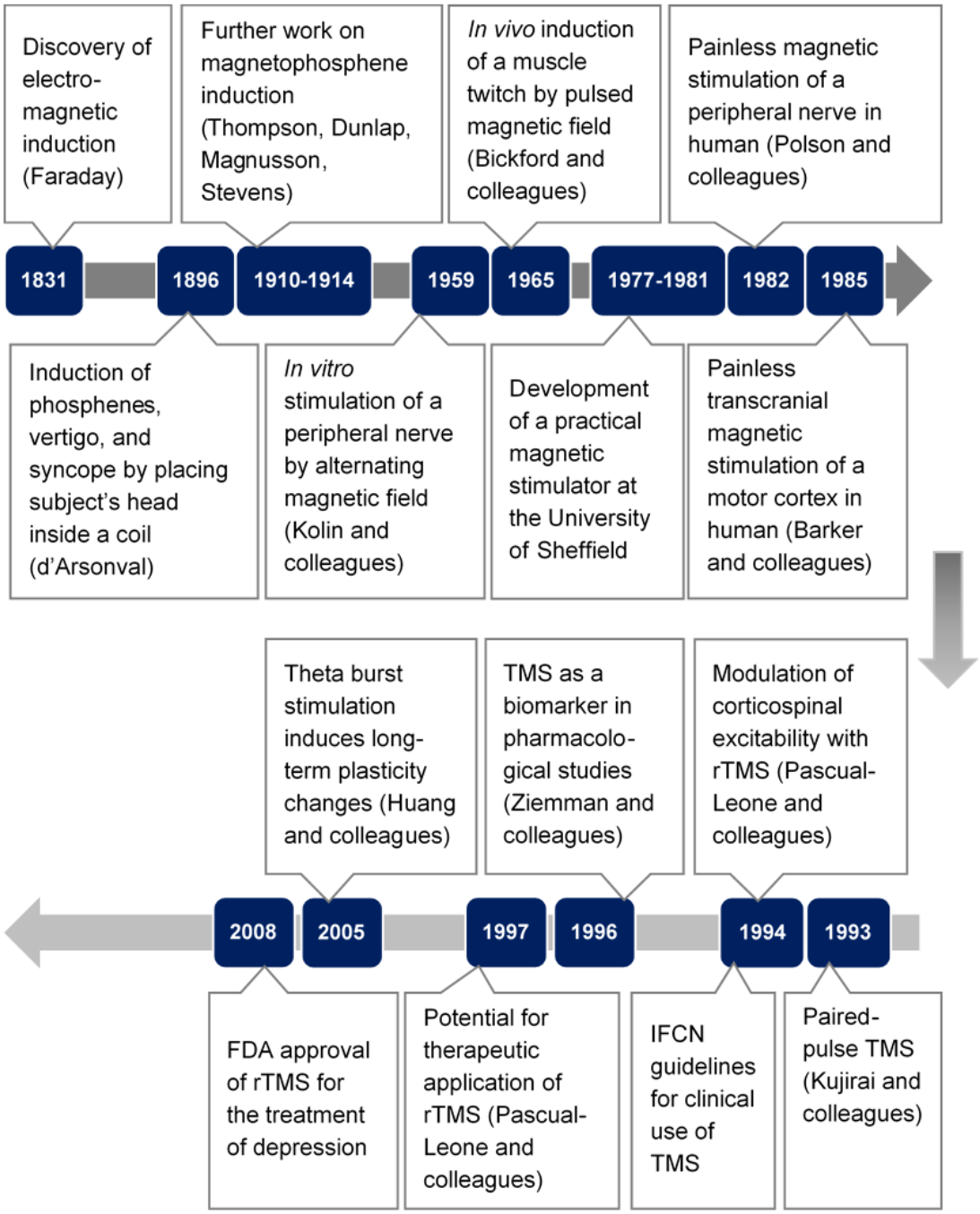


Figure 1.1. Brief history of magnetic stimulation. *Adapted from Barker, 1991; Geddes, 1991; Rossini et al., 1994; Ziemann, 2017 (references of the original work indicated in the figure are listed in the above-mentioned papers). FDA – Food and Drug Administration; IFCN – International Federation of Clinical Neurophysiology; rTMS – repetitive transcranial magnetic stimulation.*

How does TMS work? The basic principles of magnetic stimulation were outlined by Barker and colleagues (Barker, 1991). A magnetically-induced electric field passes through any volume (air, skin, skull, etc.). In conductive volumes, this electric field induces currents which in turn stimulate the axons in peripheral nerves or brain structures. Importantly, the magnetically-induced electric field attenuates less rapidly

with distance than the fields induced by electrical stimulation via surface electrodes. This allows stimulation of deeper structures with an electric field of relatively lower intensity at the surface, thus preventing activation of skin nociceptors and causing minimal, if any, discomfort to the tested subject.

The depth and focality of the magnetically-induced electric field largely depend on the shape of the stimulating coil. Modelling showed that with commonly used circular and figure-of-eight coils, the half-maximum electrical field in the brain can be achieved in up to 3.5 cm depth, but the spread of this field is several times smaller for figure-of-eight coils allowing a more precise targeting of brain areas (Deng et al., 2013).

Direction of the induced current is an important determinant of the efficacy of magnetic stimulation. It was noted early on that the latency and amplitude of magnetically-evoked motor responses varied depending on the orientation of the magnetic coil and that the largest responses were obtained with the figure-of-eight coil handle positioned at 45° angle to the mid-sagittal line so that induced current flowed posterior-to-anterior (PA) across the central sulcus (Rösler et al., 1989, Mills et al., 1992). Single motor unit recordings suggested that magnetic activation of the corticospinal tract was consistent with the 'D and I wave' hypothesis and that the recruitment of these waves depended on the direction of the magnetically-induced current (Day et al., 1989). This was later supported by epidural recordings of magnetically-evoked descending volleys over the cervical spine in humans (Nakamura et al., 1996, Di Lazzaro et al., 1998a).

The generally accepted 'D and I wave' hypothesis proposes that the multiple components observed in the descending volley recorded over the corticospinal tract following electrical or magnetic stimulation reflect different sites of activation of the pyramidal cells (Patton and Amassian, 1954, Day et al., 1989): the earliest D (direct) wave results from direct stimulation of corticospinal tract axons, while subsequent I (indirect) waves are thought to be generated trans-sinaptically in the cortex (Figure 1.2). PA magnetic stimulation, which has the lowest threshold for eliciting MEPs, recruits I1 wave first, then late I waves, and only at high stimulus intensities D wave may be recruited (Di Lazzaro et al., 2012).

Although the precise site of I wave origin is unknown, it is hypothesised that I1 wave originates in cortical layers II and III through activation of pyramidal cells with monosynaptic connections to layer V pyramidal neurons, while late I waves reflect recurrent activity in the microcircuit made up of layer II-III and V pyramidal cells and GABAergic interneurons (as reviewed in Di Lazzaro et al., 2012, Di Lazzaro and Ziemann, 2013). The I waves evoked by anterior-to-posterior (AP) magnetic stimulation

may arise in the premotor cortex neurons projecting onto primary motor cortex (as discussed in Di Lazzaro and Ziemann, 2013).

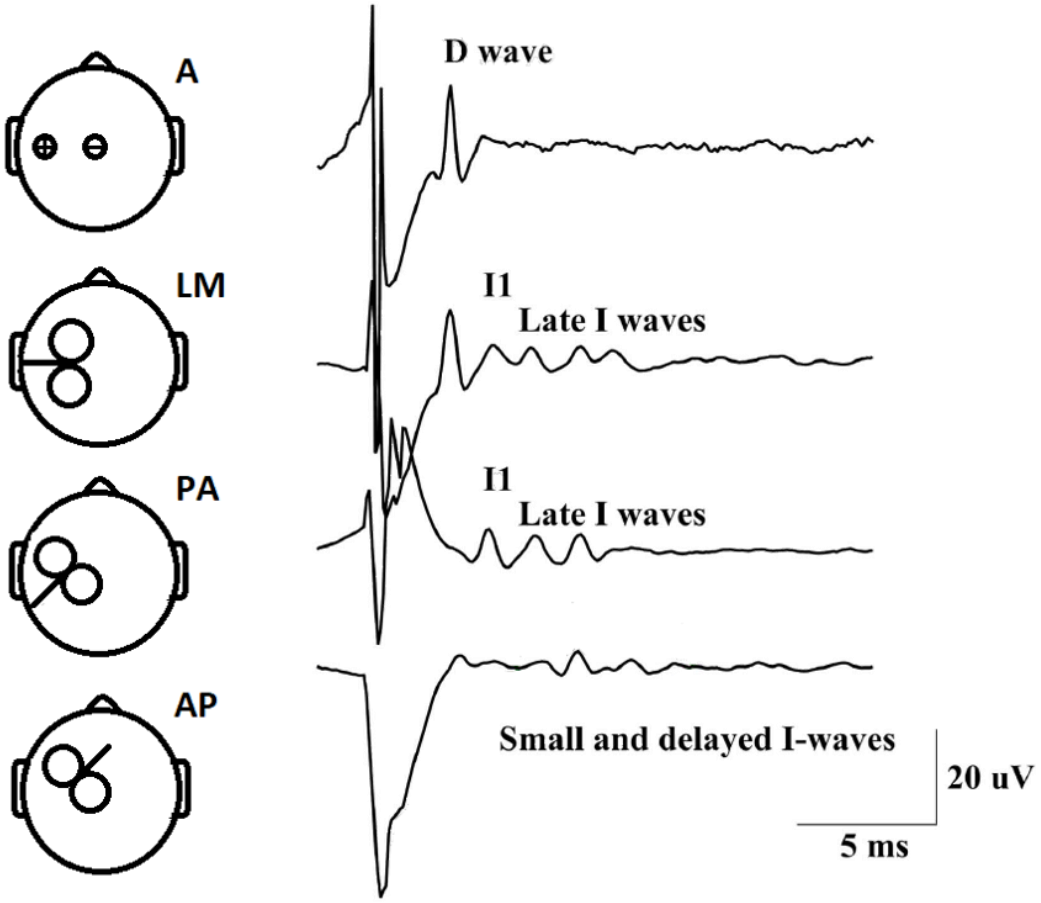


Figure 1.2. D and I wave recruitment depends on the induced current direction. *On the left, schematic illustration of transcranial brain stimulation method (from top to bottom): electrical anodal (A); magnetic inducing lateromedial current flow (LM); magnetic inducing posterior-to-anterior current flow (PA); magnetic inducing anterior-to-posterior current flow (AP). On the right, corresponding descending volleys recorded epidurally at the high cervical spine. Electrical anodal stimulation evokes D wave; LM magnetic stimulation evokes D wave followed by I1 wave and late I waves at high stimulus intensity; PA magnetic stimulation evokes I1 wave followed by late I waves and D wave at high stimulus intensities; AP magnetic stimulation evokes small and delayed volleys. Modified from (Di Lazzaro and Ziemann, 2013) under CC BY 3.0 licence.*

1.2 Measures of corticospinal excitability

The relationship between magnetic stimulus intensity and motor evoked potential size is S-shaped and can be modulated by factors altering CSE (e.g. voluntary activation of the target muscle; Devanne et al., 1997). Different parts of this stimulus-response curve

provide different information about CSE (Figure 1.3). The bottom part of the curve reflects the lowest-threshold corticospinal motoneurons and corresponds to the motor threshold, the mid-portion indicates the gain in the corticospinal pathway with increasing stimulus, while the plateau at the top of the curve reflects the maximum corticospinal response (Groppa et al., 2012). The latter is mainly used in the routine clinical setting to measure the central motor conduction time to assess the function of the fastest-conducting pyramidal tract fibers (Groppa et al., 2012, Rossini et al., 2015). Although a complete stimulus-response curve would provide more insight into the CSE-modulatory effects of an intervention (Devanne et al., 1997), this approach is often not feasible due to technical or time constraints. Thus, motor threshold and MEP amplitude in the linear portion of the sigmoid curve are commonly used as biomarkers of CSE (Groppa et al., 2012, Rossini et al., 2015).

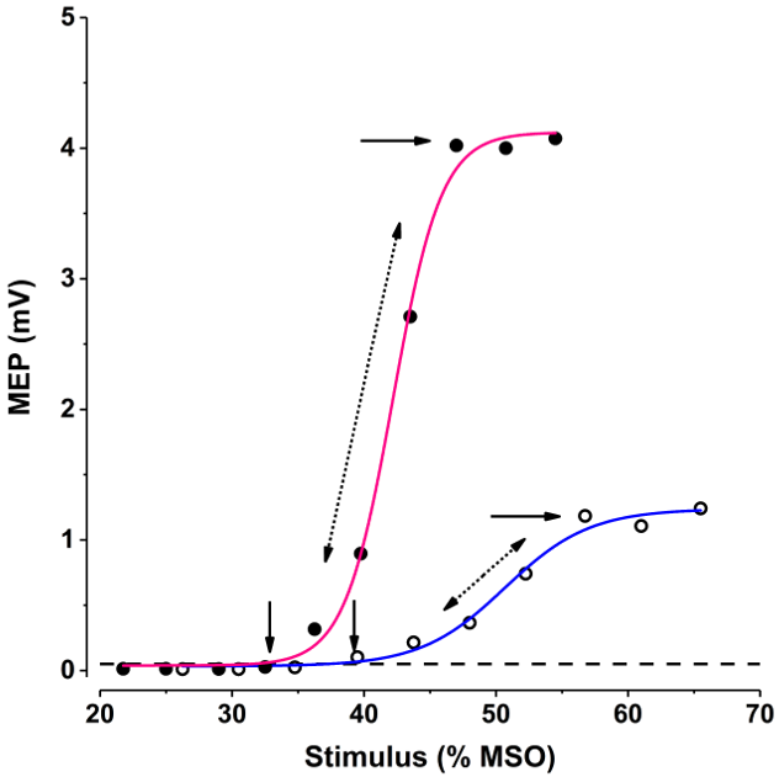


Figure 1.3. Magnetic stimulus-response relationship. *Representative stimulus-response curves from two subjects (closed and open circles). Recordings obtained in our lab using stimulus intensities of 60-150% of individual resting motor threshold (at 10% steps). Each data point is an average of 12 responses obtained at rest and plotted against the raw stimulus intensity in % of maximum stimulator output (% MSO), the coloured lines represent Boltzmann fit. The horizontal dashed line indicates the 0.05 mV cut-off value for the conventional definition of resting motor threshold. There are substantial interindividual differences in raw curve parameters, i.e. motor thresholds (vertical arrows), slopes (dotted arrows) and peak response amplitudes (horizontal arrows).*

1.2.1 Resting motor threshold

Resting motor threshold (RMT) is a baseline characteristic of CSE commonly used to adjust stimulation parameters for other TMS protocols (Rossini et al., 2015). Conventionally, motor threshold is defined as the stimulation intensity required to obtain a reliable MEP in 50% of consecutive trials and is expressed in percent of maximum stimulator output (% MSO; Rossini et al., 1994, Rothwell et al., 1999, Groppa et al., 2012, Rossini et al., 2015). The definition of a 'reliable MEP' has slightly changed over the years, from initially proposed peak-to-peak amplitude of ≥0.1 mV (Rossini et al., 1994) to currently recommended and most widely-used cut-off value of ≥0.05 mV (Rossini et al., 2015). Two approaches for RMT estimation are currently recommended by the IFCN in the most recent guidelines, i.e. relative frequency and adaptive methods (Rossini et al., 2015).

The relative frequency (RF) method was initially proposed by the IFCN committee in 1994 (Rossini et al., 1994), but a detailed procedure was described almost two decades later (Groppa et al., 2012). After finding the optimal scalp location (i.e. the so-called motor hotspot), the stimulation is started at a subthreshold intensity and is increased in steps of 5% MSO until TMS consistently evokes MEPs in each trial; the stimulus intensity is then decreased in 1% MSO steps until less than five out of ten positive (i.e. ≥0.05 mV) responses are obtained. The lowest stimulation intensity that elicits at least 5/10 positive responses is defined as the RMT. The most recent IFCN guidelines suggest using 10/20 positive response rule to increase the reproducibility of RMT estimates (Rossini et al., 2015). However, this proposition was based solely on mathematical simulations (Awiszus, 2012) under the assumption of a stable CSE, and no experimental data was provided to support this recommendation.

The main disadvantage of the RF method is the duration of the procedure which takes on average 57 stimuli if the algorithm prescribed in the 2012 IFCN guidelines and 5/10 positive trial rule are used (Silbert et al., 2013). Its duration could potentially be halved if stimulation is started at suprathreshold intensity used to locate the hotspot and then decreased in 2% MSO steps (Qi et al., 2011). Besides its inefficiency, the RF method has been criticised for its poor accuracy (Awiszus, 2003, Awiszus, 2012). Thus, an alternative adaptive threshold-hunting paradigm was introduced in 2003 (Awiszus, 2003) and a computer-based algorithm (MTAT 2.0) was made freely available by Awiszus and Borckardt (Awiszus and Borckardt, 2011). The best Parameter Estimation by Sequential Testing, or best PEST, procedure is based on a cumulative Gaussian distribution function which describes the probability of a positive response at a particular stimulus intensity (Awiszus, 2003). A maximum-likelihood function is used to predict the stimulus intensity which is expected to yield a 50% probability of eliciting a positive response and

is based on previous observations during the recording (Awiszus, 2003, Qi et al., 2011). Only 19 stimuli on average were required to achieve an improved accuracy over the RF method in mathematical simulations (Awiszus, 2003) and 12 stimuli were sufficient to obtain RMT estimates comparable to the RF method in experimental setting (Qi et al., 2011, Silbert et al., 2013). Use of a priori information and addition of Bayesian regression to the PEST algorithm allowed further reduction of the number of stimuli without introducing bias in RMT estimates (Qi et al., 2011).

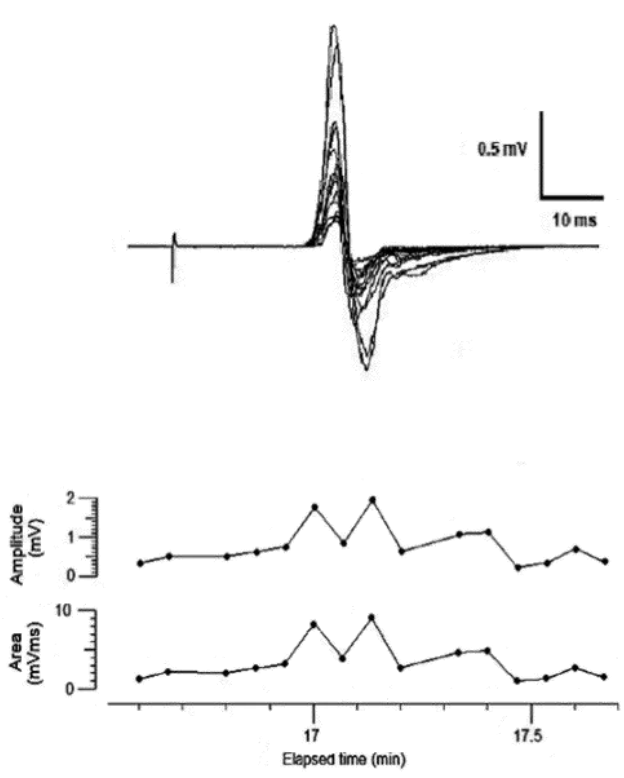
Both RF and best PEST methods are based on the probabilistic definition of threshold. Alternatively, threshold can be defined as the stimulation intensity required to maintain a response of a specific size (Bostock et al., 1998). This approach has been successfully used in peripheral nerve excitability testing (Bostock et al., 1998) and was pioneered in TMS studies by Bostock and colleagues (Awiszus et al., 1999, Fisher et al., 2002). In threshold-tracking paradigms, the stimulation intensity is continuously adjusted based on the size of the previous response (increased, if the response was below the target, decreased – if above, and unchanged – if on target). While probabilistic methods provide point estimates of RMT under the assumption that no systematic changes in CSE have occurred during the estimation procedure, threshold-tracking allows continuous recording of RMT and point estimates can be calculated for intervals of interest. However, this approach for RMT estimation has not been validated against conventional techniques in the experimental setting.

There is a substantial variability in RMT among healthy subjects (Wassermann, 2002), and individual coil- or skull-to-cortex distance is thought to be its major determinant (Herbsman et al., 2009, Danner et al., 2012). Pharmacological studies have shown that RMT is increased after administration of voltage-gated sodium channel blockers (as reviewed in Ziemann et al., 2015), thus it was proposed that intrinsic neuronal excitability determined by genetically defined sodium channel activity may also play a role (Wassermann, 2002). RMT is not a static property of the motor system: it fluctuates within an individual (Koski et al., 2005) and may be influenced by different technical and biological factors (Groppa et al., 2012) which will be described in more detail in the following section. Despite this, RMT is one of the most reliable TMS measurements (Beaulieu et al., 2017, Brown et al., 2017).

1.2.2 Motor evoked potentials

RMT is commonly used to adjust the stimulation parameters for other TMS protocols (single- or paired-pulse, repetitive), whereas MEP amplitude serves as a biomarker in the studies of human motor system physiology and pathology. Huge trial-to-trial variability is a well-known feature of MEPs (Wassermann, 2008). Several physiological

sources of the trial-to-trial variability of MEPs have been proposed (Figure 1.4), but to this day there is no consensus on which mechanisms (cortical or spinal) are largely responsible for it. MEP amplitude was found to correlate with electroencephalographic (EEG) parameters such as power and oscillatory phase of beta- (Keil et al., 2014) and alpha-band frequencies (Rossini et al., 1991, Zrenner et al., 2018). These observations led to the development of 'closed-loop' TMS paradigms, in which TMS trigger is synchronised with real-time brain oscillatory activity (Zrenner et al., 2016, Zrenner et al., 2018). For example, average MEP amplitude obtained during the negative phase of the mu-rhythm was almost 20-30% higher compared to that acquired during the positive phase, and similar differences in TMS-induced corticospinal plasticity were observed (Zrenner et al., 2018). However, the authors did not observe a reduction in the trial-to-trial variability of MEPs (personal communication).



Motor cortex and corticospinal tract:

- Intrinsic oscillations of pyramidal cell excitability [1]
- Multiple descending volleys (D and I waves) [2]
- Desynchronisation [3]

Spinal cord:

- Intrinsic oscillations of α-motoneuron excitability [4-6]
- Phase cancellation [3]
- Repetitive discharges of α-motoneurons [7, 8]

Peripheral nerve and muscle:

- Dispersion along peripheral nerve [9]

Figure 1.4. Physiological basis of MEP trial-to-trial variability. *On the left, raw traces of 15 consecutive MEPs obtained at a constant stimulus intensity (120% RMT) are superimposed. Both amplitude and area of motor responses varies in a similar fashion. On the right, mechanisms likely contributing to this variability are listed. [1] Adrian and Moruzzi, 1939, [2] Di Lazzaro et al., 1999, [3] Magistris et al., 1998, [4] Baranauskas and Nistri, 1995, [5] Schalow and Zach, 1996, [6] Gossard et al., 1994, [7] Day et al., 1987, [8] Hess et al., 1987a, [9] Roth and Magistris, 1989.*

Interaction between magnetically-evoked corticospinal volleys and α-motoneurons may play a major role in MEP variability. Collision techniques have been used to demonstrate the importance of desynchronisation of the descending volleys as well as repetitive firing of motor units in response to magnetic stimuli (Hess et al., 1987a, Magistris et al., 1998). The variability of MEPs obtained by triple stimulation technique (TST), in which transcranial magnetic stimulus is combined with electrical stimulation of peripheral nerves at distal and proximal sites in an attempt to overcome the desynchronisation of α-motoneuron discharges, was smaller (coefficient of variation (CV) 2.6% vs 8.1%) compared to magnetic stimulation alone at supramaximal intensities (Magistris et al., 1998). Rösler and colleagues found that at submaximal magnetic stimulus intensity the variability of conventional MEPs was larger than that of TST response by approximately one third and suggested that a third of the variability of MEP size is caused by the variations in synchronisation and the remaining two-thirds by the variation in the number of recruited α-motoneurons (Rösler et al., 2008). The authors reckoned that at least 10-15% of the whole α-motoneuron pool were subject to the trial-to-trial variability of MEPs. Given that no difference in the variability of conventional TMS and TST responses was seen between cortical and brainstem stimulation sites in the same study, it was proposed that the cortical level is not the major source of MEP variability.

In addition to intrinsic properties of the motor pathways determining the trial-to-trial variability of MEPs, various biological and technical factors have been found to modulate the MEP size and/or its variability (Table 1.1). It may be difficult to ensure a constant state of arousal, cognition, and relaxation in a subject, especially during lengthy recordings, but many factors, such as stimulation parameters, voluntary activation of the target muscle or coil positioning can be controlled for during the experiments. Kiers and colleagues (Kiers et al., 1993) demonstrated that stronger stimulation and, in particular, voluntary activation of the target muscle can reduce the trial-to-trial variability of MEPs rather considerably. However, this is of little practical use as many TMS protocols require a complete relaxation of the target muscle as well as submaximal stimulation intensity that evokes MEPs in the rising part of the stimulus-response curve (Di Lazzaro and Ziemann, 2013). Factors that affect the size of MEP may also alter the motor threshold. The effects of voluntary activation of the target muscle or magnetically-induced current direction on motor threshold are well-described (Hess et al., 1986, Kammer et al., 2001), but the importance of other factors remains less clear.

Precise coil positioning is considered as one of the most critical technical factors in TMS experiments. Smallest variability of MEPs is observed within 5 mm of the motor hotspot and increases with increasing distance from the initial target (Brasil-Neto et al., 1992). Use of navigation systems has been reported to reduce the displacement of the coil from

the optimal scalp location by up to 10 mm compared to non-navigated trials (Gugino et al., 2001, Julkunen et al., 2009), but the effects on MEP amplitude and its variability were mixed (Table 1.1). While the use of navigation systems may increase the probability of eliciting MEPs (Gugino et al., 2001), no difference in RMT was seen in studies comparing the two approaches despite up to 10 mm difference in the hotspot location (Julkunen et al., 2009, Jung et al., 2010). The variability in the size of MEPs is of less importance in the conventional threshold measurements. Use of RMT instead of MEP amplitude for motor mapping has been shown to be more accurate and reliable (Meincke et al., 2016), and it is likely that RMT is less sensitive to subtle variability in coil position occurring during the recording session.

Factors	**MEP size**	**MEP variability**
Biological and subject-related		
Voluntary activation		
Target muscle	↑ [1]	↓ [1, 2]
Contralateral homologous muscle	↑ [3, 4]	↓ [4]
Ipsilateral muscle of the same body region	↑ [3, 5]	
Contralateral muscle of other body region	↑ [4]	↓ [4]
Face and eye movements	↑ [6]	
Cognition and emotion		
Mental arithmetic		= [1]
Eyes open + mental arithmetic	↑ [7]	↓ [7]
Thinking about movement or muscle	↑ [8, 9]	
Emotional arousal	↑ [10]	
EEG activity and state of arousal		
Negative vs positive peak of mu-rhythm	↑ [11]	
Alpha power reduction	↑ [7]	
Beta power reduction	↑ [12]	
Beta oscillatory phase	↑ [12]	
REM sleep	↑ [13]	
Other		
Cardiac cycle	= [14]	= [14, 15]
Respiration		= [14]
Proximal vs distal upper limb muscles		↑ [16]
Continuous vibration of the target muscle	↑ [17]	

Table 1.1. *Continued on the next page*

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Factors	MEP size	MEP variability
Electrical pulse to the peripheral nerve 20-30 ms prior the magnetic pulse	↑ [18]	
Increase in proportion of recruited spinal motoneurons		↓ [16]
Technical and protocol-related		
Stimulation parameters		
Pulse duration	= [19]	= [19]
Waveform (biphasic vs monophasic)	↑ [20]	
Increase in stimulus intensity	↑ [1, 21]	↓ [1, 21]
Stimulation interval (10-15 s vs 4-5 s)	= [1], ↑ [22]	= [1]
Stimulation at short intervals (1-3 s)	↑ [23]	
Stimulation frequency ≤1 Hz	= [24]	
Stimulation frequency ≥3-5 Hz	↑ [24]	
Coil type and positioning		
Focal vs circular coil		↑ [1]
Suboptimal scalp position		↑ [16]
Clamped vs hand-held coil		= [15]
Sagittal and coronal vs standard coil placement	↓ [14]	
Posterior-to-anterior and lateromedial vs other direction of the current flow	↑ [25]	
Navigated vs non-navigated TMS	↑ [26, 27]	↓ [26]
Variability of actual coil output	= [21] ?	= [27, 21] ?

Table 1.1. Biological and technical factors that may affect MEP amplitude and its variability. *↑increase; ↓decrease; = no change; ? not reported. [1] Kiers et al., 1993, [2] Darling et al., 2006, [3] Hess et al., 1987a, [4] Stedman et al., 1998, [5] Hess et al., 1986, [6] Andersen et al., 1999, [7] Rossini et al., 1991, [8] Gandevia and Rothwell, 1987, [9] Izumi et al., 1995, [10] Hajcak et al., 2007, [11] Zrenner et al., 2018, [12] Keil et al., 2014, [13] Hess et al., 1987b, [14] Amassian et al., 1989, [15] Ellaway et al., 1998, [16] Brasil-Neto et al., 1992, [17] Claus et al., 1988, [18] Deletis et al., 1992, [19] Rothkegel et al., 2010, [20] Orth and Rothwell, 2004, [21] Jung et al., 2010, [22] Vaseghi et al., 2015, [23] Julkunen et al., 2012, [24] Pascual-Leone et al., 1994, [25] Mills et al., 1992, [26] Gugino et al., 2001, [27] Julkunen et al., 2009.*

The direction of the magnetically-induced current flow in the brain has a major impact on RMT (Kammer et al., 2001, Stephani et al., 2016) as well as MEP amplitude when same intensity stimuli are used (Amassian et al., 1989, Mills et al., 1992). However, for matched-size MEPs, the variability of similar degree was observed between different coil orientations (Ellaway et al., 1998). Electric field modelling suggests that small changes in coil orientation (≤10°) do not result in drastic changes of the electric field intensity (Janssen et al., 2015), but it is unclear whether such changes may have a significant effect on MEP amplitude (experimental studies exploring the effects of coil orientation on MEP amplitude used very crude increments). For RMT, no effect of the rotation of the coil handle from the optimal position within 15° in either direction has been demonstrated (Meincke et al., 2017). Changes in the tilt of the coil may also contribute to the variability of MEPs, but only rather large shifts (30° around the roll axis and 17° around the pitch axis) result in considerable reduction of MEP amplitude (Grey and van de Ruit, 2017).

1.3 Paired-pulse TMS

In the early 1990s, it was noted that the size of magnetically-evoked motor responses can be modified by applying a second electrical or magnetic stimulus to the CNS areas distant from the tested primary motor cortex (e.g. cerebellum (Ugawa et al., 1991), contralateral primary motor cortex (Ferbert et al., 1992)), providing neurophysiological evidence of modulatory effects of these structures on the primary motor cortex. Further work by Kujirai and colleagues showed that such modulatory effects could also be observed when the conditioning stimulus was applied to the ipsilateral motor cortex and likely reflected intracortical inhibitory and facilitatory circuits (Kujirai et al., 1993). These observations prompted development of other paired-pulse TMS protocols (Figure 1.5), while pharmacological studies employing CNS acting drugs provided further insight into the physiological basis of these phenomena (Ziemann, 2017).

1.3.1 Short-interval intracortical inhibition

Short-interval intracortical inhibition (SICI) is one of the most widely used paired-pulse TMS protocols (Rothwell et al., 2009). In their seminal experiment, Kujirai and colleagues (Kujirai et al., 1993) demonstrated that subthreshold magnetic conditioning stimulus (CS) resulted in the suppression of magnetically-evoked motor responses at short interstimulus intervals (1-6 ms; see Figure 1.6). Given that no inhibitory effect was observed on spinal H-reflex or on cortical test response evoked by an electrical anodal stimulus (which activates corticospinal axons directly), the authors suggested that the observed modulation at short ISIs likely occurred intracortically and possibly reflected GABAergic inhibitory activity (Kujirai et al., 1993). Single motor unit studies (Hanajima et

al., 1998) and epidural recordings of magnetically-evoked descending volleys (Di Lazzaro et al., 1998b) showed that subthreshold CS suppressed late I waves, but did not affect the early I1 wave, arguing against refractoriness of corticospinal tract as the mechanism of the observed MEP suppression (Di Lazzaro et al., 1998b). The duration of the late I wave suppression was compatible with GABA mediated inhibition described in animals, further supporting the hypothesis that SICI reflects trans-synaptic GABAergic inhibition in the motor cortex (as discussed in Hanajima et al., 1998). Moreover, pharmacological studies have repeatedly shown enhancement of SICI at ISI 2-3 ms with benzodiazepines which exert their effects through $GABA_A$ receptors (Ziemann et al., 2015; for more details and reference see section 1.4.2). Thus, SICI has been (and continues to be) widely used as a biomarker of $GABA_A$ mediated inhibition (Rothwell et al., 2009).

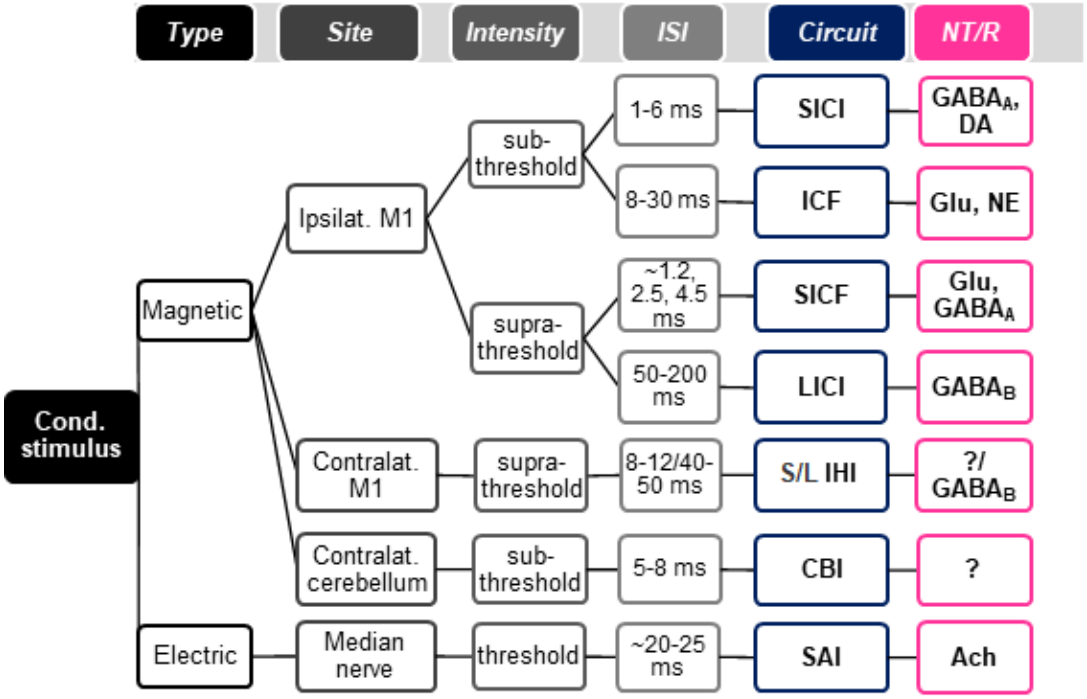

Figure 1.5. Summary of paired-pulse TMS protocols. *Modulatory effect of the conditioning stimulus on the MEP size (i.e. inhibition or facilitation) depends on its properties, such as type, application site, intensity, and temporal relationship to the test pulse (the latter is commonly set to suprathreshold intensity). Insight into physiological basis of these phenomena largely comes from pharmacological studies. M1 – primary motor cortex; ISI – interstimulus interval between conditioning and test pulses; NT/R – proposed neurotransmitter/receptor; SICI – short-interval intracortical inhibition; ICF – intracortical facilitation; SICF – short-interval intracortical facilitation; LICI – long-interval intracortical inhibition; S/L IHI – short-/long-interval interhemispheric inhibition; CBI – cerebellar inhibition; SAI – short-latency afferent inhibition; GABA - γ-aminobutyric acid, A and B represent receptor types; DA – dopamine; Ach – acetylcholine; NE – norepinephrine; Glu – glutamate; ? – not known. Adapted from Rossini et al., 2015.*

1.3.2 Methods to measure SICI

In conventional paradigms, *constant stimuli* are applied and multiple MEP amplitudes are measured and averaged to obtain a reliable estimate of SICI (Kujirai et al., 1993). Initially, the individual motor threshold (MT) is obtained either while the target muscle is at rest or during a weak tonic contraction[1]. Conditioning stimulus (CS) intensity is then set below the MT, while the test stimulus (TS) intensity is adjusted to evoke a MEP amplitude which lies approximately in the middle of the magnetic stimulus-response curve[2]. Multiple MEPs are then obtained and averaged for each condition and SICI is

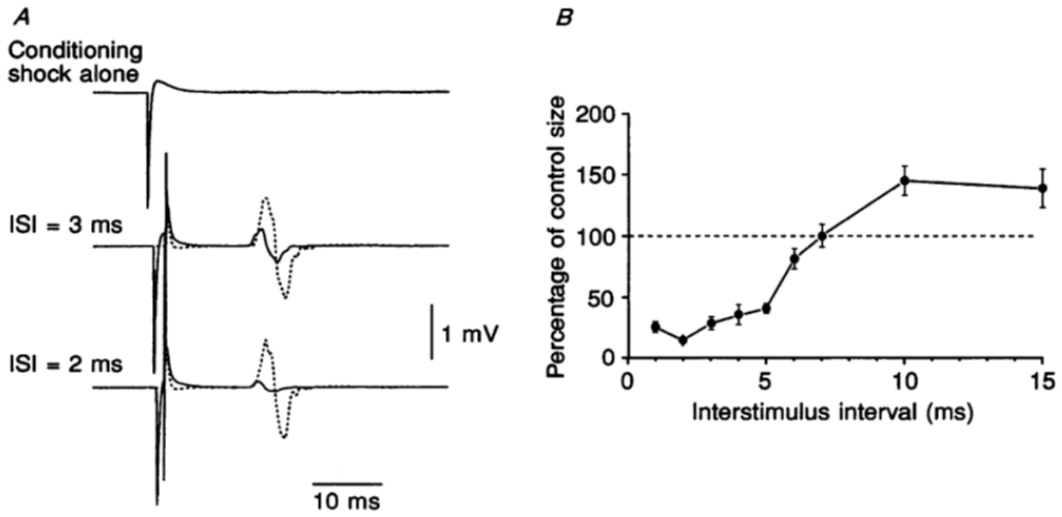

Figure 1.6. EMG responses to magnetic cortical stimulation in relaxed first dorsal interosseous are inhibited by a prior, subthreshold, magnetic conditioning stimulus. *A) shows examples of EMG data from a single subject. The first trace shows absence of any responses to the conditioning stimulus given alone. The lower two records have two superimposed traces, the response to the test stimulus given alone, and the response to the test stimulus when given 3 (middle traces) or 2 ms (lower traces) after a conditioning stimulus. The larger of the two traces (dotted line) is the response to the test stimulus alone. It is dramatically suppressed at these two interstimulus intervals (ISI). Note the shorter latency of the conditioned response at an ISI = 3 ms. In this and subsequent figures, each trace is the average of 10 sweeps. B) shows the mean ± SEM time course of suppression in 10 subjects. At each interstimulus interval, the size of the conditioned responses is expressed as a percentage of the size of the control response. In both A) and B), the conditioning and test stimuli were given through the same figure-of-eight coil oriented so that electric current in the junction region flowed from anterior to posterior over the lateral part of the motor cortex. Reproduced from Kujirai et al., 1993 with permission from the publisher (John Wiley and Sons, Inc., © 1993 The Physiological Society).*

[1] Active motor threshold (AMT) is obtained during a weak tonic contraction of the target muscle and a cut-off of 0.2 mV is usually used (Groppa et al., 2012).
[2] While CS is usually set as a fraction of MT, TS is often adjusted to evoke an average peak-to-peak MEP amplitude of 1 mV or set as a 110-120% of RMT (Rossini et al., 2015).

usually expressed as a ratio of conditioned MEP to unconditioned test MEP (the smaller the ratio, the stronger the inhibition, see Figure 1.6). Due to high trial-to-trial variability of MEPs, it is recommended to obtain at least 8-10 responses for each condition (Rossini et al., 2015). For this reason, recordings become time-consuming if multiple CS/TS intensities or ISIs are investigated. Another potential disadvantage of this approach is that it assumes that the CSE remains constant throughout the lengthy recording. However, it may change considerably due to biological or technical factors (e.g. subject's state of alertness, intrinsic fluctuations in MT, coil displacement), especially during long protocols (Groppa et al., 2012, Rossini et al., 2015). The CS intensity which was subthreshold at the beginning of the recording might become suprathreshold, if the CSE is enhanced, or become too low to elicit inhibition, if the CSE decreases. If the changes in MT are not continuously monitored, final SICI estimates are likely to be imprecise.

By contrast, *constant response* approach is used in threshold-tracking. This technique for SICI was pioneered by Bostock and colleagues (Awiszus et al., 1999, Fisher et al., 2002) and was adopted from peripheral nerve excitability studies (Bostock et al., 1998). The main principle of this technique is that instead of delivering fixed intensity stimuli and measuring the change in response, the stimulation intensity is dynamically adjusted to maintain the response at a certain target level (Figure 1.7). Therefore, if the CS suppresses the response to the TS, the intensity of the TS will be increased to counteract this effect. In this technique, MT is the control condition and SICI is usually expressed as a percentage increase over MT (the bigger the increase, the stronger the inhibition). The advantage of this paradigm is that it is less susceptible to the trial-to-trial variability of MEPs and allows to continuously monitor CSE and adjust for its changes, thus ensuring that relative CS intensities remain of a constant fraction of MT optimal for eliciting SICI. Moreover, as averaging of multiple responses is no longer required, the same amount of information could potentially be obtained much quicker.

Some phenomena were observed independently with both techniques, such as the ISIs for peak inhibition (Kujirai et al., 1993, Fisher et al., 2002, Roshan et al., 2003, Vucic et al., 2006), the effect of CS intensity (Kujirai et al., 1993, Vucic et al., 2009) and voluntary activation on SICI (Ridding et al., 1995c, Fisher et al., 2002), and overlap with short-interval intracortical facilitation (Ziemann et al., 1998, Awiszus et al., 1999, Fisher et al., 2002, Ilic et al., 2002). However, a head-to-head comparison of the two techniques has never been done before.

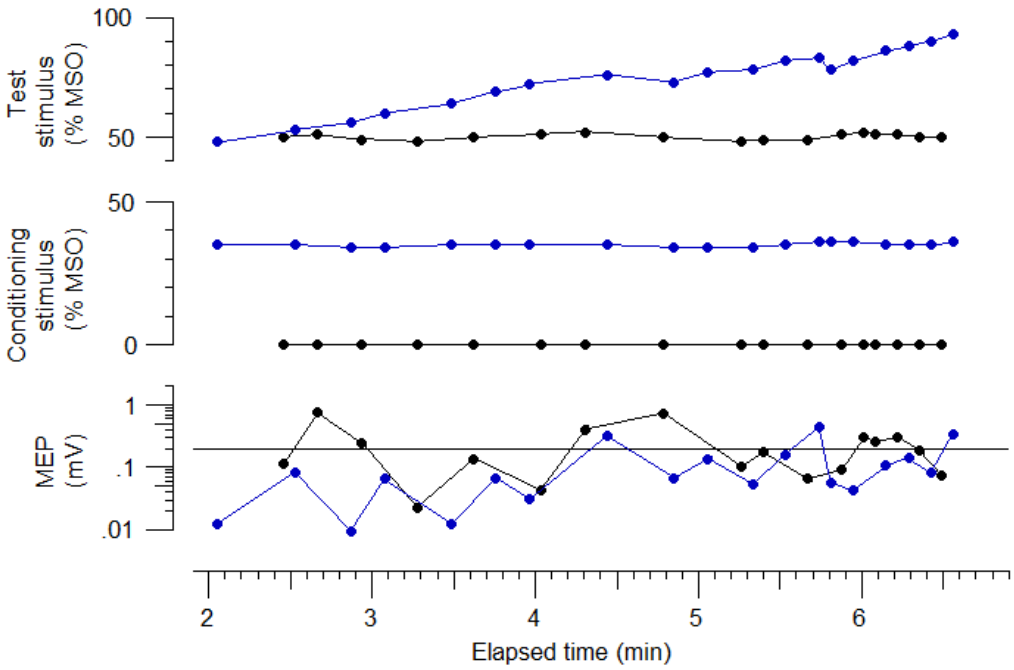

Figure 1.7. Threshold-tracking approach for SICI. *An example recording obtained in our lab is presented. Black circles represent control condition (i.e. motor threshold (MT) obtained by test stimulus alone), blue circles – paired-pulse condition (i.e. conditioning and test stimulus given together). In this paradigm, the test stimulus (top trace) is constantly adjusted to maintain a MEP at the target level (horizontal line in the bottom trace). An increase of conditioned threshold above the MT indicates SICI. Note that the conditioning stimulus intensity (middle trace) is not constant and is continuously adjusted in parallel to changes in MT to maintain it as a constant fraction of MT.*

1.3.3 Variability and reliability of SICI measurements in healthy volunteers

In the TMS literature, coefficient of variation (CV, expressed as a (percentage) ratio of the standard deviation to the mean) is often used to quantify and compare the variability of SICI measurements between subjects, operators, or sessions (Boroojerdi et al., 2000, Wassermann, 2002, Orth et al., 2003, Fleming et al., 2012, Ngomo et al., 2012). Despite differences in protocols (i.e. conditioning and test stimulus intensities, ISIs, number of MEPs averaged, TMS coil type), authors reported a substantial variability in SICI measurements, ranging between 45% and nearly 80% within a group of healthy individuals (Boroojerdi et al., 2000, Wassermann, 2002, Orth et al., 2003, Fleming et al., 2012, Ngomo et al., 2012). Although it was demonstrated that within-subject variability of SICI measurements can be significantly improved by averaging a larger number of MEPs, it remained relatively high at >30% (Boroojerdi et al., 2000, Orth et al., 2003). By contrast, the within-subject variability of RMT is only 4-5% (Koski et al., 2005).

Subject-related factors	Trial-to-trial variability of MEPs	Increasing number of averaged MEPs from five to 20 significantly reduces the within-subject variability of SICI estimates (Boroojerdi et al., 2000)
	Age	Less SICI in young children compared to adults (Mall et al., 2004); reduced SICI in elderly (Peinemann et al., 2001); no effect of age on SICI with threshold-tracking technique (Shibuya et al., 2016a)
	Ethnicity	Lower SICI in Chinese compared to Caucasians when CS intensity adjusted to RMT (Yi et al., 2014)
	Sex	Reduced threshold-tracking SICI (averaged and peak at ISI 3.5 ms) in men (Shibuya et al., 2016a); fluctations in SICI during menstrual cycle in women (Smith et al., 1999, Smith et al., 2002, Hattemer et al., 2007)
	Voluntary activation	Reduced SICI during voluntary muscle contraction (Fisher et al., 2002, Roshan et al., 2003)
	Sleep	Enhanced SICI in slow-wave sleep (Salih et al., 2005, Avesani et al., 2008); increased SICI during the night with return to baseline in the morning during sleep deprivation (Manganotti et al., 2001); reduction in SICI after 24-hour sleep deprivation (Kreuzer et al., 2011)
	Interhemispheric asymmetry	Less SICI in the dominant hemisphere of right-handed subjects (Ilic et al., 2004)
Protocol-related factors	Interstimulus interval	Two distinct peaks at 1 ms and 2.5 ms (Fisher et al., 2002, Roshan et al., 2003); potential contamination with short-interval intracortical facilitation at ISI of 2-3 ms if high intensity CS is used (Fisher et al., 2002, Peurala et al., 2008)
	Conditioning and test stimulus intensities	U-shaped relationship between CS intentsity and SICI (Kujirai et al., 1993, Chen et al., 1998, Ilic et al., 2002, Vucic et al., 2009); optimal TS intensity for eliciting SICI is 110-120% RMT (Garry and Thomson, 2009); reduced SICI when TS intensity is set to evoke MEP of 0.2 mV (Sanger et al., 2001, Roshan et al., 2003)
	Coil type and orientation	Stronger SICI with anterior-to-posterior direction of stimulation (Hanajima et al., 2008); no difference between circular and figure-of-eight coils in conventional SICI (Badawy et al., 2011, Fleming et al., 2012), but less SICI with figure-of-eight coil with threshold-tracking (Van den Bos et al., 2018)

Table 1.2. Factors contributing to the variability of SICI.

The variability of SICI obtained by threshold-tracking has not been systematically studied. Raw data from four healthy volunteers who underwent repeated testing on at least two separate occasions suggests that a substantial variability in SICI between subjects and, to a lesser degree, within subjects is likely (Vucic et al., 2006). Factors that may contribute to the variability of SICI are summarised in Table 1.2.

Coefficient of variation shows the extent of variability in relation to the sample or population mean, but it does not provide any information on the size of the measurement error or the degree of agreement between repeated measurements, both of which are important in planning interventional trials or in the diagnostic decision making in individual patients.

Several methods have been proposed to quantify the reliability of measurements. Relative reliability, or reproducibility, indicates the degree to which subjects maintain their position within a group over repeated measurements (Bruton et al., 2000, Streiner et al., 2008). Simply, it is a ratio of true variance (i.e. between-subject variance) to the total variance (a sum of between- and within-subject variances) and is quantified using intraclass correlation coefficient (ICC; Fleiss, 1999, Streiner et al., 2008). Reproducibility of a test has important implications in interventional studies. Fleiss suggested that using an unreliable outcome measure increases the sample size required to detect a significant treatment effect by 1/ICC (Fleiss, 1999) and the effect of reproducibility on statistical power has been demonstrated in a recent study (Brown et al., 2017).

ICC is a dimensionless estimate that indicates how well a test can differentiate between the rank order of individuals with test repetition (i.e. the individuals with smallest or largest SICI value remain at the bottom and the top of a cohort; Streiner et al., 2008, Schambra et al., 2015), but it does not provide information on the absolute differences between repeated measurements (Rankin and Stokes, 1998). However, in clinical practice absolute reliability, i.e. the agreement between repeated measurements in an individual, is more important for determining the suitability of a diagnostic test for individual decision making. This can be assessed using Bland-Altman plots (Bland and Altman, 1986) or coefficient of repeatability (CR) - a value below which the differences of future measurements within a subject will lie with 95% probability (Bartlett and Frost, 2008). CR is derived from the standard error of measurement and is also referred to as the smallest detectable change (SDC), which indicates a true change in a test score beyond the measurement noise (de Vet et al., 2006, Schambra et al., 2015).

Several authors have previously reported good reproducibility of SICI measurements obtained by conventional technique with ICCs above 0.75 (Maeda et al., 2002, Fleming et al., 2012, Ngomo et al., 2012, Du et al., 2014, Schambra et al., 2015), while CRs of

varied from 17% to 147% of the test MEP (Fleming et al., 2012, Ngomo et al., 2012, Schambra et al., 2015). Limited data on threshold-tracking SICI showed that peak threshold change at ISIs of 2-3 ms could differ by more than two times in a healthy subject (Vucic et al., 2006), but no formal measures of reliability were available for this technique at the experimental planning stage for this thesis.

1.3.4 SICI as a diagnostic tool

Impairment of SICI has been reported across a wide range of neurological disorders such as stroke (Schambra et al., 2015, Huynh et al., 2016), Parkinson‘s disease (Ridding et al., 1995a), focal, generalised, and psychogenic dystonia (Edwards et al., 2003, Espay et al., 2006, Hanajima et al., 2008), Tourette syndrome (Orth et al., 2005), epilepsy (Manganotti et al., 2000, Hanajima et al., 2008, Silbert et al., 2015) and amyotrophic lateral sclerosis (Ziemann et al., 1997, Vucic et al., 2008, Menon et al., 2015a). This suggests that SICI is a non-specific measure that reflects an imbalance between inhibitory and facilitatory circuits in the brain rather than a disease-specific pathophysiological mechanism. There is a general concern that conventional SICI measures have limited diagnostic utility due to large variability between patients and overlap with normal subjects (Berardelli et al., 2008, Chen et al., 2008).

However, threshold-tracking SICI is emerging as a potentially useful diagnostic test in amyotrophic lateral sclerosis (ALS; Vucic and Kiernan, 2008, Vucic et al., 2008, Vucic et al., 2011, Menon et al., 2015a). Recent data shows that decrease in averaged SICI[3] or inexcitable motor cortex has 73% sensitivity and 81% specificity in distinguishing ALS from mimic disorders (Menon et al., 2015a). The diagnostic utility of this test was similar in both bulbar and limb onset groups. Among 209 patients who were eventually diagnosed with ALS, an extra 34% of patients could have been diagnosed as probable or definite ALS at the initial assessment (nearly 16 months earlier) if abnormal threshold-tracking TMS was used as a criterion of upper motoneuron involvement. Moreover, the same research group demonstrated that riluzole therapy led to a significant increase in averaged SICI at 4 and 8 weeks after its initiation (Geevasinga et al., 2016). These findings advocate the use of threshold-tracking TMS not only in a clinical setting, but also as a potential biomarker in the development of new therapies.

[3] SICI is measured at a single CS intensity (70% RMT) and a number of ISIs. SICI estimates are then averaged across the ISIs 1-7 ms. Values below 5.5% RMT have 70% sensitivity and 71% specificity in distinguishing ALS from mimic disorders (Vucic et al., 2011).

1.4 GABAergic signalling in the human brain

GABA (γ-aminobutyric acid) is the main inhibitory neurotransmitter in the human central nervous system. It exerts its effects through a variety of receptors that belong to the superfamilies of ligand-gated ion channels ($GABA_A$) and G-protein coupled receptors ($GABA_B$; Chebib and Johnston, 1999). $GABA_A$ receptors are heteropentamers with a central chloride channel that are composed of 19 known types of subunits (Rudolph and Knoflach, 2011). Different combinations of these subunits determine the physiology and pharmacology of the receptor (Johnston, 1996, Rudolph and Knoflach, 2011). For example, the binding site of benzodiazepines is formed by α and γ subunits, and the sensitivity to benzodiazepines and their pharmacodynamic effects are thought to be determined by the subtype of α subunit (Table 1.3).

Subunit type	**Frequency* and main expression sites**	**Subcellular site**	**Pharmacodynamic effects**
α1	Up to 60%: cerebral cortex (layers I-VI), subcortical nuclei, brainstem, and cerebellum	Synaptic and extrasynaptic	Sedation, anterograde amnesia, anticonvulsant activity, addiction
α2	Up to 20%: cerebral cortex (layers I-IV), subcortical nuclei, spinal cord	Mainly synaptic	Anxiolysis, myorelaxation
α3	Up to 15%: cerebral cortex (layers I-IV), subcortical nuclei, spinal cord	Mainly synaptic	Myorelaxation, sensorimotor gating, anxiolysis at high receptor occupancy
α5	Less than 5%: predominantly in hippocampus, some in deep cortical layers, subcortical nuclei, spinal cord	Extrasynaptic in hippocampus and cerebral cortex	Myorelaxation, sensorimotor gating, cognitive impairment

Table 1.3. Properties of benzodiazepine-sensitive $GABA_A$ receptors. *Receptor sensitivity to benzodiazepines and their pharmacodynamic effects are determined by α subunit. α4 and α6 containing $GABA_A$ receptors are benzodiazepine-insensitive. * Percent of all $GABA_A$ receptor subtypes. Adapted from Möhler et al., 2002, Fritschy and Brünig, 2003, Rudolph and Knoflach, 2011, Rudolph and Möhler, 2014.*

For decades, targeting of these receptors has been employed for the treatment of various CNS and psychiatric conditions, such as epilepsy, insomnia, anxiety, and movement disorders. However, the use of classical $GABA_A$ receptor modulating drugs, e.g.

benzodiazepines, is often limited by their side effect profile. A meta-analysis of the effects of long-term benzodiazepine use on cognition showed moderate-to-large negative effect sizes across all cognitive areas studied, including sensory processing, motor performance, attention, and memory (Barker et al., 2004). Addictive potential of these drugs has been one of the major safety concerns (Woods et al., 1992).

Better understanding of $GABA_A$ receptor pharmacology led to a search for more selective modulators. While drugs with preferential affinity to α1 subunit containing receptors have been used in clinical practice as hypnotics for several decades (e.g. zolpidem), none of the selective anxiolytic drugs targeting $GABA_A$ α2/α3/α5 receptor subtypes has been approved for clinical use so far (Rudolph and Knoflach, 2011, Rudolph and Möhler, 2014).

1.4.1 AZD7325

AZD7325 (4-amino-8-(2-fluoro-6-methoxy-phenyl)-N-propyl-cinnoline-3-carboxamide) is an experimental selective partial $GABA_A$ α2,3 receptor positive allosteric modulator intended for use as a non-sedating anxiolytic drug (Alhambra et al., 2011, Zhou et al., 2012). *In vitro,* it showed no functional activity at the α1 subunit and a twice higher relative efficacy at α2,3 subunits compared to α5 subunit (Alhambra et al., 2011, Chen et al., 2014). A positron emission tomography (PET) study in healthy volunteers showed a dose-dependent saturable displacement of $GABA_A$ receptor radioligand [^{11}C]flumazenil with no clear sedative or cognitive adverse effects at high receptor occupancy by AZD7325 (Jucaite et al., 2017). Although the effects of single 2 mg and 10 mg doses of AZD7325 on a validated battery of CNS tests in healthy volunteers did not reach statistical significance when compared to placebo, the drug showed saccadic peak velocity dominant effect profile when compared to lorazepam (Chen et al., 2014), suggestive of pharmacological selectivity to $GABA_A$ α2,3 receptor pathways[4] (Chen et al., 2012). In addition, the cognitive effects of AZD7325 did not differ from placebo, there were fewer adverse events (including somnolence and dizziness) when compared to lorazepam, and no clinically important abnormalities on vital signs, ECG, and laboratory findings were reported (Chen et al., 2014).

[4] Although AZD7325 did not have a significant effect on the saccadic peak velocity (SPV) when compared to placebo (in contrast to an active comparator lorazepam), the change in SPV relative to the change in other pharmacodynamic parameters such as body sway and alertness measured by visual analogue scale was more prominent with AZD7325 when compared to lorazepam (Chen et al., 2014). Such SPV-dominant effect profile has been observed with other selective $GABA_A$ α2,3 receptor positive allosteric modulators and is a potential biomarker of $GABA_A$ α2,3 receptor signalling (Chen et al., 2012).

1.4.2 Role of TMS in the assessment of GABAergic signalling

Two paired-pulse TMS paradigms are thought to reflect the GABAergic signalling in the human motor cortex: short-interval intracortical inhibition (SICI) and long-interval intracortical inhibition (LICI). While SICI is observed at short ISIs and subthreshold CS, LICI is seen when both conditioning and test stimuli are suprathreshold and delivered 50-200 ms apart (Valls-Solé et al., 1992, Wassermann et al., 1996, Sanger et al., 2001). LICI, but not SICI, was enhanced by $GABA_B$ receptor agonist baclofen (McDonnell et al., 2006), while increase in SICI at ISIs 2-3 ms was observed after a single dose of classical benzodiazepines (Table 1.4). Thus, LICI has been suggested as a biomarker of $GABA_B$, while SICI – $GABA_A$ mediated pathways (Paulus et al., 2008, Ziemann, 2013).

Findings of the previous studies exploring the effects of single doses of classical benzodiazepines on SICI in healthy volunteers are fairly consistent despite methodological differences (Table 1.4). All but three of the identified studies reported an enhancement of SICI at ISIs 2-4 ms at the time of peak plasma concentration (t_{max}). This effect could last for up to five hours (Ziemann et al., 1996a), but was no longer seen at six hours post-dose and beyond (Ziemann et al., 1996b, Di Lazzaro et al., 2005, Di Lazzaro et al., 2007). The lack of effect in the remaining studies could potentially be explained by insufficient dosing (Mohammadi et al., 2006), measurement of SICI during voluntary contraction (Inghilleri et al., 1996) or prominent baseline SICI which may have precluded detection of enhanced SICI due to a 'floor' effect (Boroojerdi et al., 2001). In contrast, SICI-enhancing effect of selective $GABA_A$ α1 agonist zolpidem was found in one small study (Mohammadi et al., 2006), but this was not confirmed by other groups (Di Lazzaro et al., 2006, Di Lazzaro et al., 2007, Teo et al., 2009). Given that $GABA_A$ α5 receptors are much less densely expressed in the cortex (Fritschy and Brünig, 2003) and thus less likely to contribute to SICI, it has been proposed that SICI is mediated by $GABA_A$ α2,3 signalling (Di Lazzaro et al., 2006, Ziemann, 2013). A recent study employing a selective $GABA_A$ α5 antagonist S44819 showed no effect on SICI recruitment curves supporting the hypothesis that SICI reflects synaptic inhibition (Darmani et al., 2016).

Study	Sample size	SICI protocol	Treatment arms	Time post-dose	Effect on SICI*
Ziemann et al., 1996a	11	$TS_{0.5\text{-}1.5mV}$, CS=AMT-5% MSO, ISI 1-5 ms	Lorazepam 2.5 mg po	2, 5, 24, 48 h	↑ 20% test MEP at ISI 4ms at 2-5 h [t_0 ~60% test MEP]
Inghilleri et al., 1996	5	TS=125% AMT, CS=80% AMT, ISI 3, 5 ms during 10% MVC	Diazepam 0.17 mg/kg iv Thiopental 2 mg/kg iv Baclofen 0.6 mg/kg iv	5 min 5, 10, 30 min 15, 30, 60 min	no effect [t_0 51.5% test MEP] not measured not measured
Boroojerdi et al., 2001	9	TS_{1mV}, CS=80% AMT, ISI 2, 3 ms	Placebo po Amphetamine 10 mg po Lorazepam 0.038 mg/kg po Lamotrigine 200 mg po	2.5 h	no effect no effect no effect [t_0 ~35% test MEP] no effect
Ilic et al., 2002	8	Interaction between S1/S2, ISI 1.5 ms	Diazepam 20 mg po	2 h	↑ 30% baseline SICI
Di Lazzaro et al., 2005	10	TS_{1mV}, CS=AMT-5% MSO, ISI 2, 3 ms	Placebo po Lorazepam 2.5 mg po Quetiapine 25 mg po	2, 6, 24 h	no effect ↑ 26% test MEP at 2 h [t_0 52.5% test MEP] no effect
Mohammadi et al., 2006	6	TS=120% RMT, CS=70% RMT, ISI 3 ms	Diazepam 5 mg po Zolpidem 10 mg po	30 min 60 min	no effect [t_0 61% test MEP] ↑ 18% test MEP [t_0 63% test MEP]

Table 1.4. *Continued on the next page*

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Di Lazzaro et al., 2006	7	TS_{1mV}, CS=AMT-5% MSO, ISI 2, 3 ms	Diazepam 20 mg po Lorazepam 2.5 mg po Zolpidem 10 mg po	1.5, 6, 24 h 2, 6, 24 h 2, 6, 24 h	↑ 12% test MEP at 1.5 h [t_0~ 40% test MEP] ↑ 26% test MEP at 2 h [t_0~55% test MEP] no effect [t_0~40% test MEP]
Di Lazzaro et al., 2007	10	TS_{1mV}, CS=AMT-5% MSO, ISI 2, 3 ms	Diazepam 20 mg po Lorazepam 2.5 mg po Zolpidem 10 mg po	1.5, 6, 24 h 2, 6, 24 h 2, 6, 24 h	↑ 12% test MEP at 2 h [t_0 52% test MEP] ↑ 10% test MEP at 2 h [t_0 52% test MEP] no effect [t_0 50% test MEP]
Müller-Dahlhaus et al., 2008	8	TS_{1mV}, CS=90% AMT then reduced until SICI~ 50% test MEP, ISI 3 ms	Placebo po Diazepam 20 mg po Baclofen 50 mg po	90 min 90 min 90 min	no effect ↑ 18% test MEP [t_0 ~47% test MEP] no effect
Teo et al., 2009	7	TS_{1mV}, CS=80% AMT and 100% AMT, ISI 3 ms	Lorazepam 2.5 mg po Zolpidem 10 mg po	2 h 2 h	↑ 27% test MEP [t_0 ~82% test MEP] no effect [t_0 ~72% test MEP]

Table 1.4. Summary of studies investigating the effects of benzodiazepines on SICI in healthy volunteers. *Despite slight methodological differences in SICI protocol, most studies reported enhanced inhibition after a single oral dose of a classical benzodiazepine, while no such effect was observed with $GABA_A$ α1 agonist zolpidem, other CNS acting drugs or placebo. * Baseline SICI (t_0) is indicated in square brackets; TS_{1mV}/$TS_{0.5\text{-}1.5mV}$ – test stimulus intensity adjusted to elicit a motor evoked potential (MEP) of 1 mV/0.5-1.5 mV; CS – conditioning stimulus intensity; RMT – resting motor threshold; AMT – active motor threshold; MSO – maximum stimulator output; ISI – interstimulus interval; po – oral administration; iv – intravenous administration; MVC – maximum voluntary contraction; SICI – short interval intracortical inhibition.*

1.5 Rationale for the experimental work and thesis outline

Threshold-tracking TMS is emerging as a useful diagnostic test, but little is known about its reliability and comparability with conventional techniques. Most conventional TMS measurements are highly variable and have poor reliability (Beaulieu et al., 2017) which not only limits their clinical application but may also confound the outcome in experimental studies. Theoretically, threshold-tracking should be advantageous over conventional paradigms as it is less susceptible to the trial-to-trial variability of MEPs and allows continuous monitoring of changes in CSE. Online adjustment for such changes could potentially improve the reliability of obtained measurements, especially in paired-pulse paradigms where this may affect the efficacy of conditioning stimulus at eliciting inhibition or facilitation (Rossini et al., 2015).

While similar phenomena have been described with both conventional and threshold-tracking techniques (see section 1.3.2), a head-to-head comparison of these methods has never been made. In this thesis, threshold-tracking is for the first time directly compared with conventional TMS approaches in both observational and interventional experiments in healthy volunteers. In addition, the availability of a safe selective $GABA_A$ α2,3 receptor positive allosteric modulator AZD7325 allows for the first time to test the hypothesis that SICI is mediated via this pathway.

Chapter 2 summarises the common methods used throughout the experimental work, including the description of experimental setup, hardware and software, stimulation techniques and data analysis.

In Chapter 3, threshold-tracking estimation of resting motor threshold at the conventional cut-off value of 0.05 mV is compared to the well-established methods (i.e. relative frequency and best PEST).

Chapter 4 explores the extent of changes in corticospinal excitability parameters and their relationship with coil positioning during a standard 20-minute recording session.

In Chapter 5, SICI recruitment curve obtained by threshold-tracking is compared to conventional ‘amplitude’ estimates and test-retest reliability of both techniques is assessed.

In Chapter 6, the hypothesis of SICI modulation via $GABA_A$ α2,3 receptor pathway is tested in a phase I randomised double-blind placebo-controlled three-way cross-over clinical trial using both conventional and threshold-tracking techniques.

In Chapter 7, the key findings are summarised and potential areas of application and directions for further development of threshold-tracking paradigms are discussed.

Chapter 2 - General methods

In this chapter, general methods used throughout the experimental work are presented. This includes description of the experimental setup, equipment, and software used to obtain TMS measurements, features of conventional and threshold-tracking protocols as well as statistical analysis of the data. Experiment-specific information will be presented in the relevant chapters.

2.1 Subject recruitment

All participants involved in the experiments were healthy adult volunteers. Subjects were recruited via word of mouth, advertisement, and a newsletter to University College London students. Observational studies were approved by local research ethics committee, and permissions from national bodies were obtained for the interventional study. Experiments were carried out in accordance with the Declaration of Helsinki and written informed consent was obtained from participants prior to investigations. Subjects with known neurological disorder potentially interfering with the studies, contraindications for TMS or taking CNS acting medication were excluded from further testing (more stringent selection criteria were applied for the interventional study, for details see section 6.1.3 of Chapter 6).

2.2 Experimental setup for TMS

All experiments were carried out in the same dedicated room in the department of Clinical Neurophysiology at the National Hospital for Neurology and Neurosurgery. Schematic illustration of hardware setup is presented in Figure 2.1. Ag/AgCl stick-on electrodes (Kendall 5500 Diagnostic Tab Electrodes, Covidien, Dublin, Ireland) were used to record surface electromyography (EMG). The EMG signal was amplified (x600 gain) and filtered (10-3000 Hz) using a Nicolet EA-2 amplifier (Nicolet Biomedical Inc., Madison, WI, USA) and sampled at 10 kHz using a NI PCI-6221 data acquisition (DAQ) card (National Instruments, Austin, TX, USA) and a shielded connector block NI BNC-2110 (National Instruments, Austin, TX, USA) running on a Viking Select EMG Unit (Nicolet Biomedical Inc., Madison, WI, USA). Signal from the preamplifier was passed via a HumBug 50/60 Hz Noise Eliminator device (Quest Scientific Instruments Inc., North Vancouver, BC, Canada). Custom-made QMSound software (Prof High Bostock, UCL Institute of Neurology, London, UK) run on a separate computer provided audiovisual feedback of the surface EMG to the subjects to help maintaining relaxation of the hand.

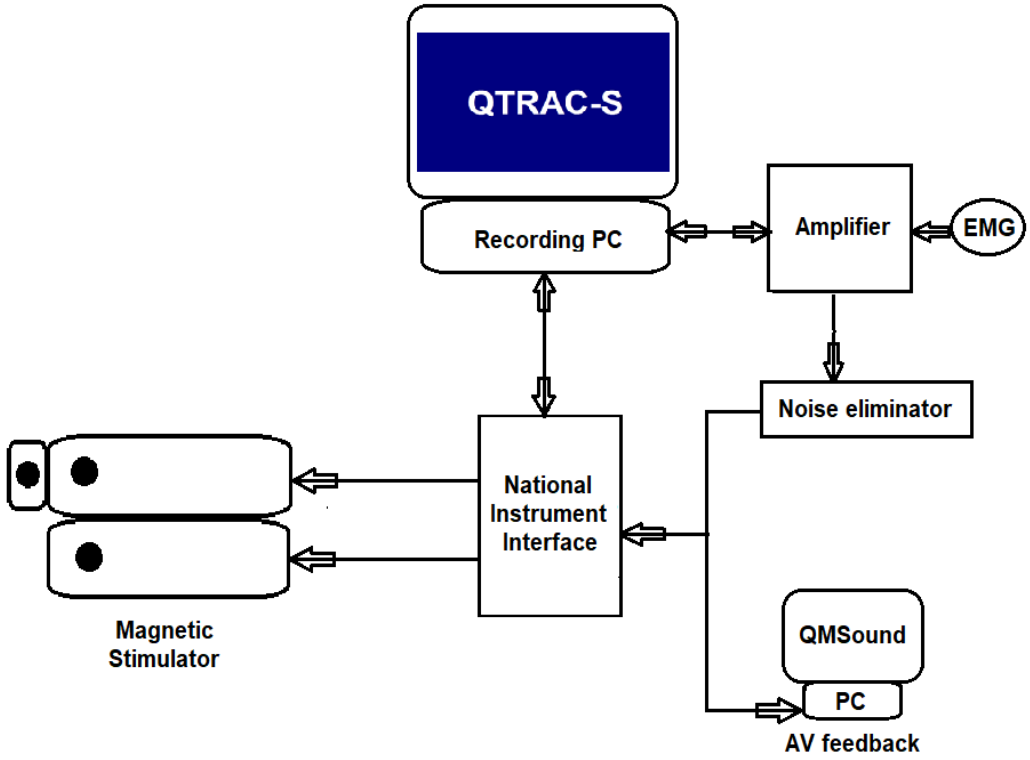

***Figure 2.1*. Schematic illustration of the hardware setup.** *Magnetic stimulation (intensity and triggering) is controlled by QtracS software. National Instrument Interface consists of a data acquisition card and a shielded connector block. PC – personal computer, AV – audiovisual feedback; EMG – surface electromyography. For details of the equipment, see text. A more detailed description of connections is presented in Appendix A*

TMS was carried out using two Magstim 200[2] stimulator units connected via BiStim module to a figure-of-eight D70[2] coil with 105 mm outer diameter of each wing (Magstim, Whitland, UK). For single-pulse TMS experiments, the coil remained connected to the BiStim module with the intensity of one of the units set to 0% MSO. This was done to maintain the strength of a magnetic field comparable between single- and paired-pulse TMS experiments as the magnetic stimulus is attenuated by approximately 20% in passing through the BiStim module (Magstim, 2016).

Magnetic stimulation delivery and data acquisition were controlled by QTRACW Version 3.0 software (© Institute of Neurology, University College London, London, UK, distributed by Digitimer Ltd. at www.digitimer.com) using bespoke parameter files. This is a flexible, multichannel data acquisition programme with averaging and threshold-tracking features, consisting of two parts: for stimulation (QtracS) and for data plotting and offline analysis (QtracP; Digitimer, 2018). Stimulation and data analysis scripts were written with the aid of Professor Hugh Bostock who, in the process of this work,

implemented new software features, such as online gating, conventional RMT estimation protocols, and automated TMS-specific analysis.

2.3 TMS procedure

For the duration of the experiments, participants were comfortably seated in an armchair with their head supported by an adjustable head-rest and hands resting on a cushion placed on their lap. Subjects were instructed to stay relaxed, but alert, to concentrate on the computer screen in front of them (where visual feedback from surface EMG was displayed) and not to move or talk unless there was a problem. A tight-fitting nylon swimming hat was placed on their head to prevent slippage of the coil from the scalp. Surface EMG was recorded from the relaxed first dorsal interosseous (FDIO) muscle with stick-on electrodes placed in a belly-tendon (of the index finger) montage and the ground electrode placed on the dorsum of the hand.

The coil was hand-held (except Experiment 2 in which the coil was clamped) over the hemisphere contralateral to the target muscle with the handle pointing postero-laterally at a 45° angle to the mid-sagittal line to induce posterior-to-anterior (PA) flow of the current in the motor cortex. Magnetic stimuli were delivered at regular 4.1 s (Experiments 3 and 4) or irregular 4.6 ± 0.5 s intervals (Experiments 1 and 2). To identify the optimal scalp location for stimulation (i.e. the motor hotspot), the coil was initially placed over the stimulated hemisphere approximately 5 cm lateral to the vertex (Thickbroom et al., 1996, Terao et al., 1998) and the stimulation intensity was set at 45% MSO. After delivering several magnetic pulses, the stimulation intensity was, if necessary, adjusted in 5% MSO steps to elicit a MEP of 0.3-0.8 mV peak-to-peak amplitude. The coil was then shifted in 0.5-1 cm steps anteriorly, posteriorly, laterally, and medially to identify the scalp location where magnetic stimuli consistently elicited the largest amplitude MEPs (Groppa et al., 2012). Once the hotspot was identified, the position of the coil was marked on the swimming hat to aid the investigator in maintaining a stable coil position. An automated stimulation protocol was then started, allowing a single operator to carry out the whole recording without having to reposition the TMS coil and manually control the stimulator.

2.4 Principles of threshold-tracking

In this paradigm, threshold is defined as the stimulation intensity required to maintain a response of a specific size (Bostock et al., 1998). During threshold-tracking, the stimulus intensity is dynamically adjusted based on a single previous response size: if it was below the target, the next stimulus intensity is increased; if the response was above the target, the next stimulus intensity is decreased; and if the stimulus was on the target, the stimulation intensity remains unchanged (Figure 1.7). The commonly used target size for

TMS is peak-to-peak MEP amplitude of 0.2 mV as it lies approximately in the middle of the linear range (between 0.02 and 2 mV) of the S-shaped relationship between logarithmically transformed MEP amplitudes and magnetic stimulus intensity (Fisher et al., 2002, Vucic et al., 2006). Responses within ± 20% range from the target are usually considered 'on target' to account for the trial-to-trial variability of MEPs and speed up the tracking (Vucic et al., 2006).

Threshold-tracking procedure in QtracS is not fixed and the following parameters can be customised:

1) target size (*QtracS command WH)* – should be set in the range where the relationship between stimulus and the log-response is linear; targets of 0.05, 0.2, and 1 mV were used in this work;

2) acceptable error size *(QtracS command TE)* – indicates the range around the target (in % of target size) within which the responses are considered 'on target'; ± 20% error was allowed in this work;

3) tracking step *(QtracS command TT)* – maximum step size (in % MSO) by which the stimulation intensity will be changed during tracking;

4) tracking mode *(QtracS command TM)* – in *fixed* mode (TM1), the stimulus intensity is altered by a constant pre-defined tracking step irrespective of how much the previous response deviated from the target; in *proportional* mode (TM0), the stimulation intensity is changed proportionally to the percentage error in the logarithm of the previous response, but not more than the predefined maximum tracking step: if the error is ≤-100% or ≥100%, the stimulation intensity will be changed by the maximum tracking step; if the error is between -100% and 100%, the change in stimulus intensity is [Error * Maximum tracking step], rounded to the nearest unit (% MSO);

5) tracking termination – *manual*, determined by the operator by pressing <Esc> button, or *automated*, when tracking is stopped after obtaining a predefined number of valid threshold estimates which are counted as hits or crosses of the target by MEPs (*QtracS command !CYCLES*; one cycle = one hit or cross).

The advantage of the threshold-tracking procedure is that it does not require averaging of multiple responses and allows continuous monitoring of CSE. However, the response to the changes in excitability will be delayed as it might take several steps for the procedure to reach the new threshold. Increasing the maximum tracking step allows a quicker detection of threshold changes, but this also results in 'noisier' tracking when the

excitability is relatively stable. Therefore, the above listed parameters were adjusted to the needs of each experiment and will be specified in the relevant chapters. The stimulation and analysis scripts used throughout this work are available upon request.

2.5 Resting motor threshold determination

In Experiment 1, RMT estimation by threshold-tracking was compared to conventional methods currently recommended by the IFCN (Rossini et al., 2015). In Experiments 2-4, threshold-tracking was used to determine RMT.

2.5.1 Relative frequency (RF) method

A modification of the Rossini-Rothwell method (Rossini et al., 1994, Rothwell et al., 1999) was implemented in QtracS, allowing to choose the initial stimulus intensity, the size of the step by which the stimulus intensity is changed, and the maximum number of trials at each step (n). In this protocol, the stimulus intensity is initially altered in 5% MSO steps until a change of a response from negative to positive, or vice versa, is observed. The stimulus intensity is then changed in the opposite direction by a predefined step and a maximum of n stimuli are given at that intensity. If more than n/2 positive responses are obtained, the stimulus intensity is decreased by one step. If more than n/2 responses are negative, the stimulus is increased by one step. The lowest stimulus intensity with >50% of positive responses out of n consecutive trials is considered the upper bound of RMT, the highest stimulus intensity with >50% of negative responses – the lower bound of RMT. The procedure is stopped when the difference between the upper and the lower bound is equal to the step size or there is an equal number of positive and negative responses in n consecutive trials. In the latter scenario, the last stimulus intensity is defined as RMT. Otherwise, the mean of the upper and lower bound estimates is defined as RMT (when the step size is ≥2% MSO).

To best adhere to the most recent IFCN procedure (as described in section 1.2.1), the step size was set to 1% MSO, and the maximum number of trials per step was 20. After finding the motor hotspot, subthreshold stimulation was started at 30% MSO and was increased in 5% MSO steps until the first positive response was obtained. The stimulus intensity was then decreased by 1% MSO. From this point, two scenarios were possible: i) if more than 10 responses (>50% or 11 out of 20) were positive, the stimulus was further decreased in 1% MSO steps until at least 10 out of 20 responses were negative (Figure 2.2); ii) if more than 10 responses were negative, the stimulus was increased in

1% MSO steps until at least 10 out of 20 responses were positive[5]. If procedure was stopped when 10 positive and 10 negative responses out of 20 consecutive trials were obtained, the last stimulus intensity was taken as RMT. Otherwise, the lowest stimulus intensity with >50% of positive trials was defined as RMT in the downward-step scenario or the highest stimulus intensity with >50% of negative trials plus 1% MSO was defined as RMT in the upward-step scenario.

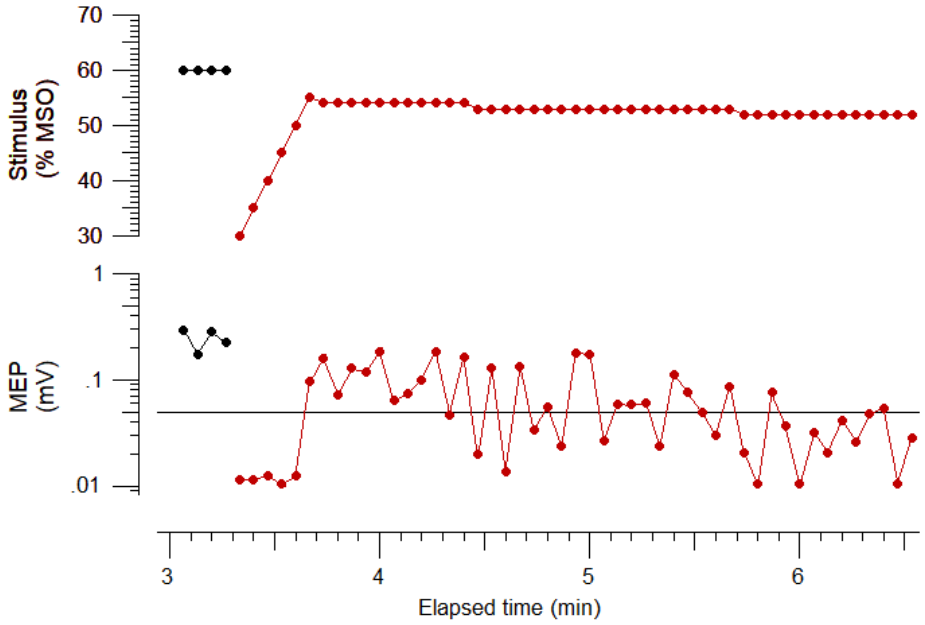

Figure 2.2. RMT estimation by relative frequency procedure. *An example recording obtained in our lab is presented. After finding the hotspot (black dots), a fully automated relative frequency procedure using 10/20 positive response rule was started (red dots). It was stopped when 11 negative responses (out of a maximum of 20) were obtained (horizontal line in the bottom trace indicates the conventional positive response cut-off of 0.05 mV). RMT was determined as the last stimulus intensity + 1% MSO.*

2.5.2 Best Parameter Estimation by Sequential Testing (PEST) method

This adaptive method for threshold estimation was first described by Awiszus (Awiszus, 2003). Although a computer-based algorithm (MTAT 2.0) was made freely available by Awiszus and Borckardt (Awiszus and Borckardt, 2011), the practical shortcoming of this software is that it runs separately from the data acquisition software. Thus, it requires a manual input of the outcome of each trial as well as manual adjustment of the next stimulus intensity.

[5] The stimulus intensity was changed once 11 positive or negative responses were obtained; therefore, not all intensity steps required delivery of 20 stimuli.

To automate the procedure (described in section 1.2.1), the best PEST algorithm was incorporated into QtracS software allowing to predefine the a priori limits of threshold, starting intensity, the maximum allowed stimulus intensity step size from trial to trial (default value 100% MSO), the expected relative spread of threshold (default value is 7% based on Awiszus data (Awiszus, 2003)), and the confidence limits as a stopping rule (default value 90%, i.e. the confidence limit range is <10% of the estimated threshold (Awiszus, 2011)). To replicate the best PEST procedure without a priori assumptions about RMT from MTAT 2.0 software (Awiszus and Borckardt, 2011), the initial stimulation intensity was set to 37% MSO and the default QtracS values of maximum allowed stimulus step size, expected threshold spread, and stopping rules were used. The last used stimulus intensity was defined as RMT (Figure 2.3).

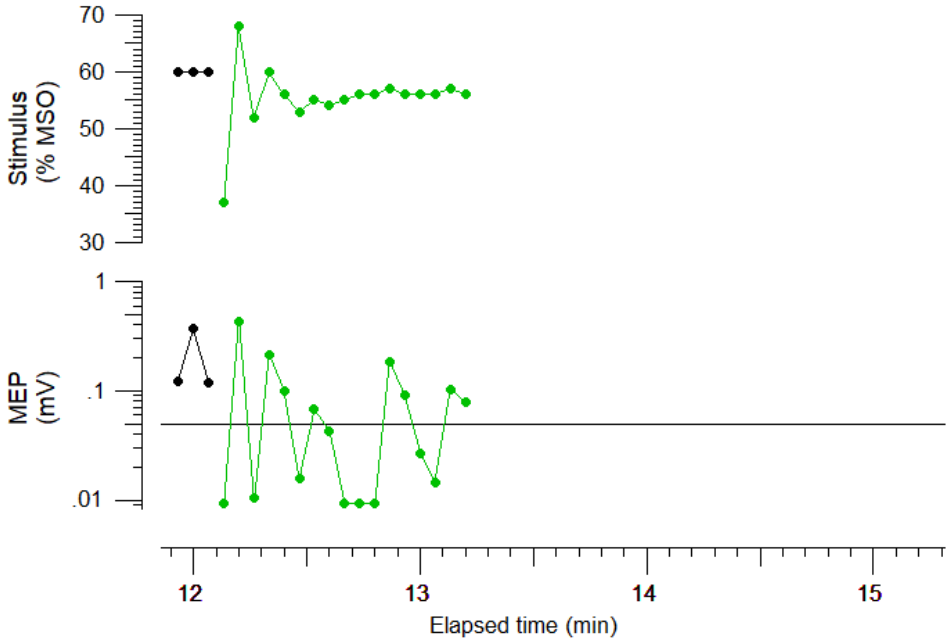

Figure 2.3. RMT estimation by best PEST procedure. *An example recording from the same subject as in Figure 2.2 is presented. After finding the hotspot (black dots), a fully automated best PEST procedure was started (green dots). It was stopped when 90% confidence limits were reached. Note that this method is much faster than the relative frequency method. The last stimulus intensity was determined as RMT. Horizontal line in the bottom trace indicates the conventional positive response cut-off of 0.05 mV.*

2.5.3 Threshold-tracking method

The commonly used RMT definition in threshold-tracking paradigms is the stimulation intensity that is required to maintain the peak-to-peak MEP amplitude of 0.2 mV (Fisher et al., 2002, Vucic et al., 2006) and is higher than the conventionally used cut-off value of 0.05 mV. As mentioned above, the tracking target could be set at any size as long as

it is in the linear part of the stimulus – log-transformed response curve. Previous observations demonstrated that 0.05 mV falls within this range (Fisher et al., 2002); therefore, principles of threshold-tracking could in theory be applied at this target level. Pilot recordings showed that threshold-tracking is feasible with the target set at 0.05 mV. While stopping criteria based on the frequency of positive responses or the accuracy of the estimate are used in RF and PEST methods (Silbert et al., 2013, Rossini et al., 2015), RMT could in theory be measured indefinitely with threshold-tracking, and currently there is no established stopping rule. It could either be terminated manually when the operator thinks that the tracking is stable (as in Vucic et al., 2006), or automatically by using a pre-determined number of stimuli that are considered as valid threshold estimates (as in Fisher et al., 2002).

To allow direct comparison with RF and best PEST methods, the target for tracking was set to 0.05 mV ± 20% (0.04-0.06 mV)[6]. The stimulation was started at the suprathreshold intensity used for identifying the hotspot, and tracking steps were fixed at 1% MSO (Figure 2.4). Tracking was automatically stopped when the MEP hit and/or crossed the target 30 times (one hit or crossing = one cycle; the cycle count represents the count of valid threshold estimates). The recording was marked every three cycles allowing to easily choose different durations of the recording for offline analysis[7].

Threshold-tracking analysis was performed offline using QtracP software. Stimulus intensities (y axis) were plotted against logarithmically transformed MEP amplitudes (x axis) and a linear least squares model was fitted, excluding MEP values outside 0.02-2 mV range (i.e. outside the linear part of the stimulus – log-transformed response curve). The y value at the intercept of a linear regression line with the target (0.05 mV) was defined as the threshold (Figure 2.4 B).

[6] The lower limit of the tracking target (0.04 mV peak-to-peak) was well above the noise level in our setup (0.02 mV peak-to-peak; see section 2.7 for details).

[7] The minimal duration and intervals of three cycles were chosen to allow obtaining a sufficient number of data points for the analysis method used in this experiment. With the target set at 0.05 mV, it is more likely that oscillations around the target rather than hits will be observed during the tracking procedure. In such scenario, three cycles would result in a minimum of four data points: two above and two below the target. If both below-the-target responses are of smaller than 0.02 mV amplitude, they will be excluded from the analysis, leaving two above-the-target responses (which is the minimum number of points required to fit a straight line).

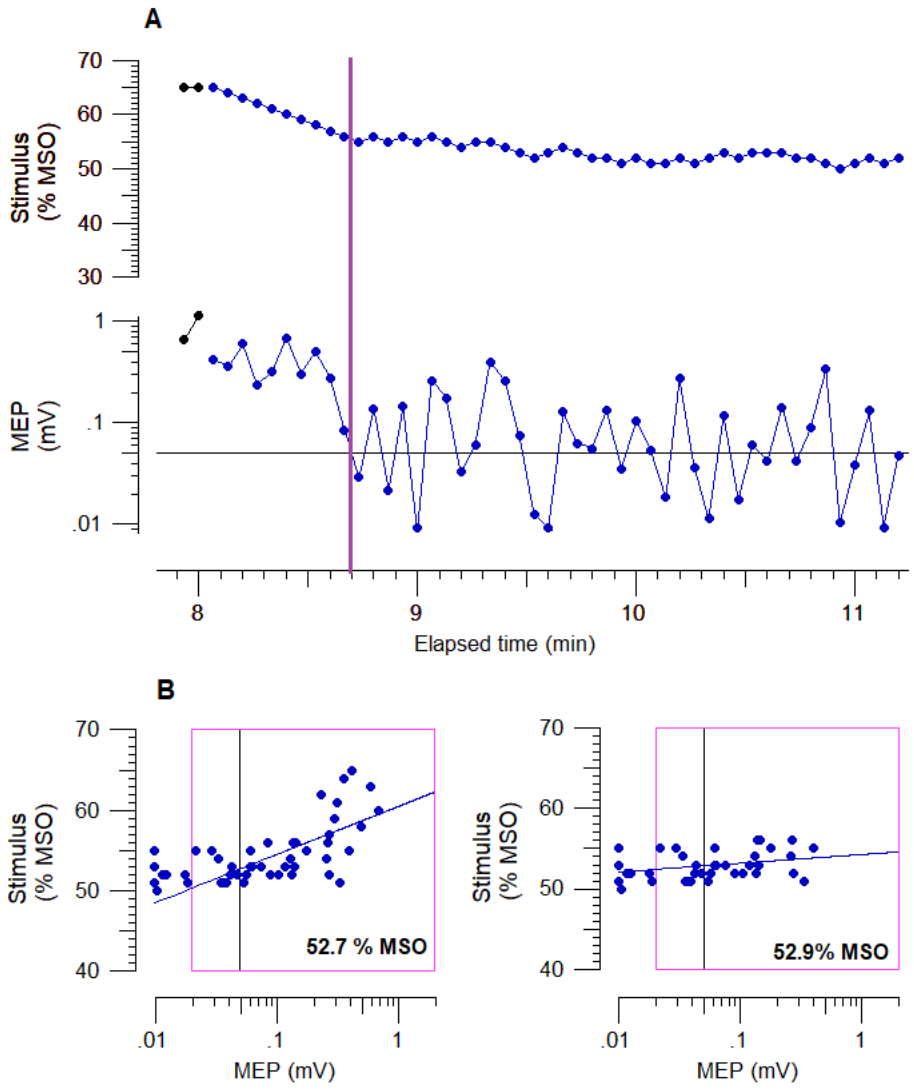

Figure 2.4. RMT estimation by threshold-tracking. *An example recording from the same subject as in Figure 2.2 and Figure 2.3 is presented. A) After finding the hotspot (black dots), threshold-tracking procedure was started (blue dots). It was stopped when the MEP hit or crossed the target line 30 times (horizontal line in the bottom trace set at 0.05 mV). Note that tracking was started at suprathreshold intensity used to locate the motor hotspot; therefore, it took some time to track down to the RMT level (marked by a vertical purple line in the top trace). B) RMT estimate was calculated by fitting a linear regression and finding its intercept with the target (vertical black line). The data points outside the magenta box were excluded from the analysis as they fall outside the linear range (0.02-2 mV) of the stimulus – log-transformed MEP curve. In the left graph, data from the start of the tracking is used, while on the right the data points obtained during the initial part of tracking (as delineated by vertical purple line in A) are excluded from the analysis. Note that this does not have a significant effect on the RMT estimate.*

2.6 Measurements of short-interval intracortical inhibition

In this work, SICI measurements were obtained using both conventional ('amplitude', A-SICI) and threshold-tracking (T-SICI) techniques. An interstimulus interval (ISI) of 2.5 ms was chosen as SICI at this interval is thought to reflect $GABA_A$ α2,3 receptor mediated inhibition in the motor cortex (Ziemann et al., 2015) and can serve as a biomarker of the effect of $GABA_A$ receptor modulating drugs. As the relationship between SICI and CS intensity is non-linear and varies between individuals (Chen et al., 1998, Rossini et al., 2015), a range of CS intensities, i.e. 50%, 60%, 70%, and 80% of RMT, was used to explore whether SICI recruitment curve may provide a more reliable measure than SICI estimates at a single CS intensity. Stimulation protocols were fully automated and required no manual adjustments of stimulus intensities.

2.6.1 Conventional SICI measurements (*constant stimulus* approach)

The conventional SICI protocol was modified from Kujirai et al. (Kujirai et al., 1993). Initially, RMT was determined by threshold-tracking[8]. Tracking was deemed stable when the MEP hit and/or oscillated around the target six times and was stopped automatically. The stimulation intensity that would have been used subsequently if tracking was continued was defined as RMT and used to adjust CS intensities. The test stimulus intensity was set to evoke MEPs of peak-to-peak amplitude of approximately 1 mV (TS_{1mV}). It was defined by threshold-tracking with the target set at 1 mV ± 20% and stopped automatically when stable tracking was achieved[9]. Stimulus intensity that would have been used subsequently if tracking was continued was defined as TS_{1mV}. Conditioning and test stimulus intensities were maintained constant and 15 MEPs were recorded for each condition in a pseudorandom order (Figure 2.5 D).

A-SICI analysis was performed offline using QtracP software and is summarised in Figure 2.5. The peak-to-peak MEP amplitudes were averaged for each condition. Mean conditioned MEP as a percent of mean test MEP was used as a conventional paired-pulse measure (Kujirai et al., 1993), with values below 100% reflecting inhibition and above 100% - facilitation.

[8] Tracking was started at suprathreshold intensity used to localise the motor hotspot. The tracking target, mode and maximum step size differed slightly between the experiments and will be specified in the relevant chapters.

[9] Tracking parameters such as starting intensity, tracking mode, maximum tracking step and stopping rule differed slightly between the experiments and will be specified in the relevant chapters.

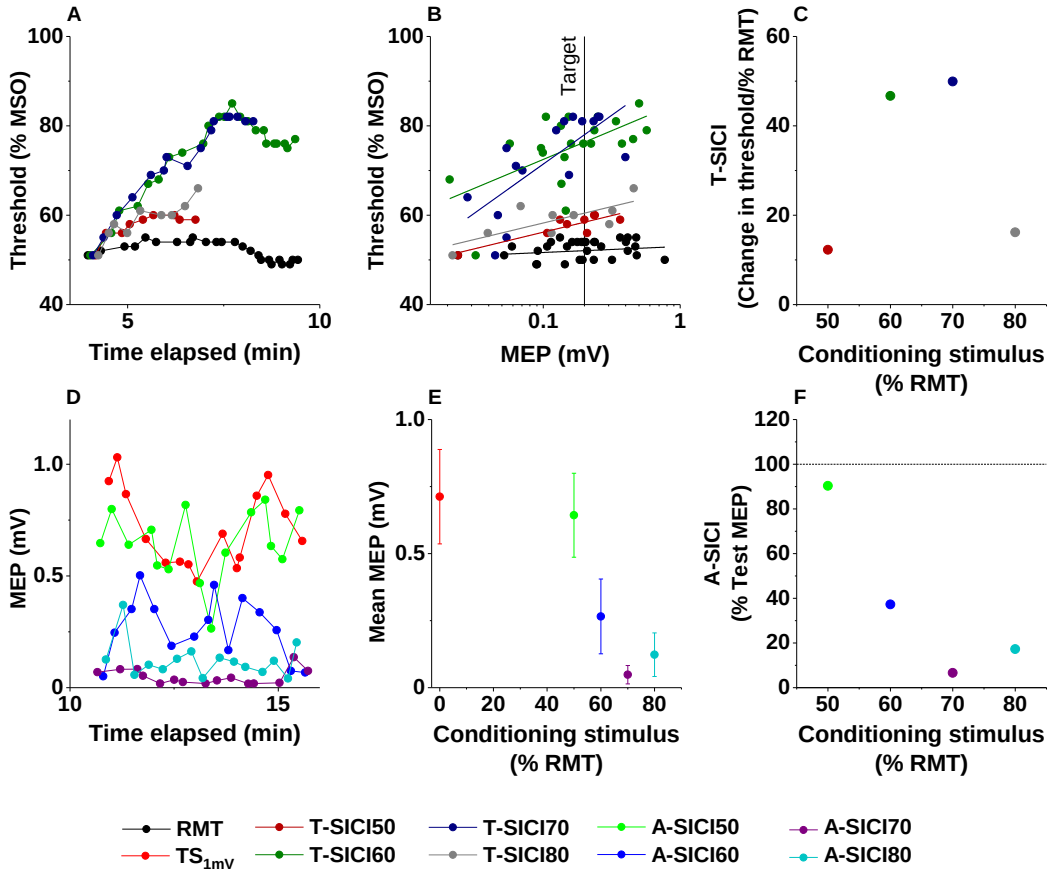

Figure 2.5. Constant response (T-SICI) and constant stimulus (A-SICI) protocols for SICI. *Data from a single recording is presented. A-C) For T-SICI (top row), target response was set to 0.2 mV and threshold-tracking was started at RMT intensity for each SICI condition. Test stimulus intensity was adjusted in 1-5% MSO steps proportionally to the error of the previous response. Tracking was stopped after motor responses hit and/or oscillated around the target six times (A). Linear regression was used to determine an estimate of the threshold for each condition by taking the y value at the intercept with the target (0.2 mV; B). SICI is reflected by the increase in stimulation intensity required to maintain the target response in the presence of conditioning stimulus and was calculated using formula T-SICI = (Conditioned threshold – RMT)/RMT*100% (C). D-E) For A-SICI (bottom row), test stimulus intensity was set to produce a response of 1 mV. Fifteen MEPs were recorded for each condition in a pseudorandomised order (D) and averaged (E); error bars represent standard deviation. SICI is reflected by the decrease in the mean amplitude of conditioned MEP compared to the test MEP and was calculated using formula A-SICI = Conditioned MEP/Test MEP*100% (F).*

2.6.2 Threshold-tracking SICI measurements (*constant response* approach)

The threshold-tracking SICI protocol was modified from Vucic et al. (Vucic et al., 2006). RMT was defined as the stimulus intensity required to maintain peak-to-peak MEPs of 0.2 mV ± 20% ($RMT_{0.2mV}$). It was then tracked throughout the protocol and CS intensities

were adjusted depending on the fluctuations in $RMT_{0.2mV}$ to maintain them as a constant fraction of $RMT_{0.2mV}$. Paired and control ($RMT_{0.2mV}$) stimuli were delivered in a pseudorandom order. Tracking was started at $RMT_{0.2mV}$ intensity and proportional tracking mode was used. Test stimulus intensities were adjusted to maintain the target response of 0.2 mV ± 20% when preceded by CS. Tracking for each SICI condition was stopped when the conditioned MEPs hit and/or oscillated around the target six times (Figure 2.5 A).

T-SICI analysis was performed offline using QtracP software. Stimulus intensities (y axis) were plotted against logarithmically transformed MEP amplitudes (x axis) and a linear least squares model was fitted, excluding MEP values outside the 0.02-2 mV range. The y value at the intercept of a linear regression line with the target (0.2 mV) was defined as the threshold. T-SICI was expressed as [(conditioned threshold – $RMT_{0.2mV}$)/$RMT_{0.2mV}$ *100%] (Vucic et al., 2006), with positive and negative values indicating inhibition and facilitation, respectively (Figure 2.5 B-C).

2.7 Control of pre-stimulus activation of the target muscle

In this work, all recordings were obtained from the relaxed target muscle. It is well known that voluntary activation of the target muscle has a facilitatory effect on MEP amplitude (as discussed in section 221.2.2). Brief periods of unintentional activation of the target muscle are not uncommon even in subjects who can otherwise maintain the relaxation well and this may affect the measurements (Darling et al., 2006). Voluntary activation is also known to decrease SICI (Fisher et al., 2002, Roshan et al., 2003). This may not be an issue in conventional SICI measurements, as traces contaminated with pre-stimulus EMG activity can be discarded offline. However, this becomes problematic if threshold-tracking is used, as stimulus intensity is altered with each trace.

For this reason, an online-gating function was implemented in QtracS. Raster options allow customising the interval before the magnetic stimulus and the size of the peaks in EMG activity which are then used for automated online gating. When any peak higher than the pre-defined gating threshold is detected inside the chosen pre-stimulus time window, the trace is discarded (i.e. not saved) and the stimulation parameters remain unchanged in the subsequent trace.

The minimum threshold for online gating was determined from the pilot recordings of a stimulus-response curve in 12 healthy volunteers. Traces obtained at stimulation intensity of 60-70% of individual RMT were used to assess the noise level of the background EMG signal. Each trace was reviewed offline, and those with pre-stimulus activation of the target muscle or a discernable MEP response were discarded from the

analysis. The mean peak-to-peak amplitude of the EMG signal measured within the MEP detection window was 0.012 ± 0.002 mV. We considered EMG activity up to 0.02 mV (i.e. approximately three standard deviations from the mean peak-to-peak noise level) non-distinguishable from the background noise. Given that gating function in QtracS is based on amplitude measurements from baseline to peak, the gating threshold of >0.01 mV (baseline to negative peak) was used in this work.

2.8 Statistical analysis

This is a general overview of statistical methods; a more detailed description will be presented in the relevant chapters.

Statistical analysis was performed using IBM SPSS Statistics Version 22.0 and 24.00 (IBM Corp., Armonk, NY, USA). Data was checked for normality using Shapiro-Wilk test. For normally distributed data (Shapiro-Wilk test, $p>0.05$) parametric tests were used for comparisons between groups and repeated measurements. For repeated measures analysis of variance (rmANOVA), the assumption of sphericity was tested; if violated (Mauchley's test of sphericity, $p>0.05$), Greenhaus-Geisser corrections were applied. Post hoc pairwise comparisons with Bonferroni adjustment were performed if significant main effects were identified. Non-parametric tests were used for non-normally distributed data. Data is presented as mean ± standard deviation (SD) when normally distributed or as median and interquartile range (IQR) of the 25^{th} and 75^{th} percentile, if non-normally distributed. The results were considered statistically significant if $p<0.05$.

Methods used for reliability analysis are summarised in Table 2.1. Coefficient of variation (CV) was calculated as SD/mean*100% and was used to compare the relative variability of measurements obtained by different techniques. Between-subject CV was calculated for each session (within-session SD/mean*100%), while within-subject CV was calculated for each subject (across-session SD/mean*100%).

Intraclass correlation coefficient (ICC) was used to assess reproducibility of TMS measurements. Two-way random model, absolute agreement type, single measures [ICC(2,1)] were used (Rankin and Stokes, 1998) with the following categories of reliability: excellent – ICC >0.75; intermediate-to-good – 0.4≤ ICC ≤0.75; poor – ICC <0.4 (Fleiss, 1999). Cohen's kappa was used to assess the agreement between the CS intensity at which peak inhibition was observed within and between the experimental days.

Parameter	Interpretation	Scale
Coefficient of variation (CV)	Variability: extent of the dispersion in relation to the sample mean.	Dimensionless
Intraclass correlation coefficient (ICC)	Reproducibility: the degree to which subjects maintain their position within a group over repeated measurements; the extent to which the variability in measurements is due to inherent variability rather than measurement errors.	Dimensionless
Standard error of measurement (SEM_{eas})	Measurement error: derived from within-subject standard deviation, defines the accuracy of a measurement irrespective of between-subject variability.	Same as measurement
Coefficient of repeatability (CR)	Repeatability/agreement: a value below which the differences of future measurements *within a subject* will lie with 95% probability.	Same as measurement
Bland-Altman bias	Systematic error between repeated measurements.	Same as measurement
Bland-Altman 95% limits of agreement	Repeatability/agreement: a range in which 95% of future differences between repeated measurements *in a population* are expected to lie.	Same as measurement

Table 2.1. Reliability measures.

Coefficient of repeatability (CR) was calculated using formula:

$$CR = 1.96*SD_{WS}*\sqrt{2} = 2.77*SD_{WS}$$ (Hopkins, 2000, Bartlett and Frost, 2008)

The within-subject standard deviation (SD_{WS}) was obtained by taking a square root of within-subject variance partitioned by fitting one-way ANOVA model with Subject as a factor (Bartlett and Frost, 2008). SD_{WS} reflects the standard error of measurement (SEM_{eas}), which defines the accuracy of a measure (i.e. size of measurement error) irrespective of between-subject variability (Schambra et al., 2015). CR is equivalent to the smallest detectable change (SDC), which indicates a true change in a test score beyond the measurement noise, e.g. due to intervention or disease progression (de Vet et al., 2006, Schambra et al., 2015). SDC can also be calculated for the group ($SDC_{group}= SDC/\sqrt{n}$, where n represents the sample size (Schambra et al., 2015)) and may aid in planning interventional studies.

Bland-Altman plots were constructed to assess the repeatability of TMS measurements (Bland and Altman, 1986). Differences between two measurements (y axis) were plotted against the means of these measurements (x axis). The mean and standard deviation were calculated for these differences and three lines were added to the plot: 1) mean difference (bias), indicating a systematic error between the two repeated measurements if significantly different from zero; 2) 95% limits of agreement (bias ± 2*SD), indicating a range in which 95% of future differences between repeated measurements are expected to lie. 95% confidence intervals were also calculated for bias and limits of agreement.

Chapter 3 - Comparison of threshold-tracking and conventional methods for resting motor threshold estimation (Experiment 1)

Resting motor threshold (RMT) is a baseline characteristic of corticospinal excitability (CSE) commonly used to adjust stimulation parameters for other TMS protocols (Rossini et al., 2015). It is also one of the most reliable TMS measurements (Beaulieu et al., 2017, Brown et al., 2017). The most recent IFCN guidelines recommend two approaches for RMT estimation – relative frequency and adaptive methods (Rossini et al., 2015), with the latter being preferable due to a smaller number of stimuli required (Groppa et al., 2012, Rossini et al., 2015).

The main limitation of the conventional approaches to RMT determination is that they provide point estimates only and do not allow continuous monitoring of the CSE. This could be done by using threshold-tracking. However, the target MEP size for RMT determination in standard threshold-tracking paradigms is higher than the conventional cut-off value used in probabilistic methods (0.2 vs 0.05 mV). Our pilot data showed that threshold-tracking is feasible with the target set at 0.05 mV.

The aim of this experiment was to compare threshold-tracking and its reliability to the well-established relative frequency and best PEST methods for RMT estimation and to determine the optimal duration for tracking that allows to obtain a reliable RMT estimate.

3.1 Methods

3.1.1 Subjects

Twenty-four healthy volunteers (11 men; median age 22 years, age range 18-55 years; all self-reported right-handed) with no known neurological disorder or contraindications for TMS (as determined by safety questionnaire modified from Rossi et al., 2011) and not on any regular CNS acting prescription medication participated in the experiment.

3.1.2 Experimental setup

The experimental setup and TMS procedure were as described in sections 2.2 and 2.3 of Chapter 2. Surface EMG was recorded from a relaxed FDIO muscle of the self-reported dominant hand. Magnetic stimuli were delivered via a figure-of-eight coil hand-held over the contralateral hemisphere so that they induced posterior-to-anterior current flow in the motor cortex. Stimuli were delivered at 4.6 ± 0.5 s intervals (1 s jitter added to prevent anticipation).

Audiovisual feedback was provided for the subjects to help maintain relaxation of the target muscle. In addition, online gating of pre-stimulus activation was used during the recordings: traces with EMG activity occurring 500 ms before the magnetic stimulus and with a negative peak exceeding 0.025 mV were automatically discarded and the same stimulus intensity was used subsequently to replace the discarded trace.

3.1.3 Experiment design

Three RMT estimation methods were compared head-to-head in this experiment: relative frequency (RMT_{RF}) as described in section 2.5.1, best PEST (RMT_{PEST}) as described in section 2.5.2, and threshold-tacking (RMT_{TT}) as described in section 2.6.2 of Chapter 2. The cut-off value for RMT estimation was conventionally set at 0.05 mV for all methods (Rossini et al., 1994, Rothwell et al., 1999). To establish the optimal duration of threshold-tracking required to obtain a reliable RMT estimate, different durations of the recording were analysed: from the beginning of tracking to 3, 6, 9, ..., 30 cycle marks (a total of ten different durations).

	1 ↓	**2 ↓**	**3 ↓**
4 →	**RF**	**PEST**	**TT**
5 →	**TT**	**RF**	**PEST**
6 →	**PEST**	**TT**	**RF**

Table 3.1. Latin square design. *Each method appears once in each row and each column, resulting in six possible combinations.*

To avoid the operator bias, all three RMT estimation methods were fully automated. All the recordings were obtained by a single operator. To control for period effects, the order of the methods in the recording was balanced based on Latin Square design (Table 3.1). Participants were pseudorandomly assigned to one of the six blocks to maintain an equal number of subjects and a similar proportion of males and females in each block.

Each recording consisted of all three RMT estimation methods. Between each of the methods there was a short pause allowing the subjects to re-adjust their position, if needed, and the motor hotspot was identified anew before the start of each method. To assess the test-retest reliability, two recordings were obtained on the same experimental day with a short break between them to change the magnetic coil to prevent overheating during the second session. The surface EMG electrodes were kept in the same position throughout the experiment.

3.1.4 Statistical analysis

For normally distributed data (Shapiro-Wilk test, $p>0.05$), parametric tests (rmANOVA, one-way ANOVA, Student's t-test, paired-sample t-test) were used to test for differences between groups, methods, and repeated measurements. Otherwise, non-parametric tests (Friedman's test, Wilcoxon Signed Rank test) were applied. Test-retest reliability of RMT estimates was assessed as described in section 2.8 of Chapter 2.

Data is presented as mean ± standard deviation (SD) when normally distributed or as median and interquartile range (IQR) of the 25^{th} and 75^{th} percentile, if non-normally distributed.

3.2 Results

3.2.1 Comparability of RMT estimates

Mean group RMT estimates and the duration of the procedure for each method are summarised in Figure 3.1. To compare the RMT estimates between the methods, two-way rmANOVA was used with two within-subject factors: Time (2 levels: run 1 and 2) and Method (12 levels: RF, PEST, and ten TT protocols of different duration). A significant main effect of Method was observed ($F_{2.7,61}=9.57$, $p<0.001$) with post hoc comparisons showing that RMT_{TT} estimate after three cycles was approximately 2-3% MSO higher than RMT obtained by RF, PEST and TT at 9-30 cycles ($p\leq0.045$). There were no significant differences in RMT estimates between RF, PEST, and TT durations of 6-30 cycles ($p>0.082$). No main effect of Time ($F_{1,23}=1.65$, $p=0.212$) or Time and Method interaction ($F_{2.7,62.5}= 2.52$, $p=0.071$) were observed.

While RMT estimates were similar between the different methods, the duration of the procedure varied greatly (Figure 3.1 B). rmANOVA with Time and Method as the within-subject factors showed a significant main effect of Method on the duration of the procedure ($F_{1,23}=6.17$, $p=0.021$). Post hoc comparisons showed that only RF and TT at 30 cycles as well as PEST and TT at 12 cycles had a similar duration ($p=0.212$ and $p=0.110$, respectively), while other methods differed significantly between each other ($p\leq0.027$). Overall, approximately three more stimuli were required to obtain the estimates on the second run (rmANOVA, $F_{1,23}=6.17$, $p=0.021$), but no interaction between Method and Time was found (rmANOVA, $F_{1.2,26.6}=0.219$, $p=0.679$).

RMT estimates averaged across two runs were used to explore differences between males and females as well as protocol types. rmANOVA with Method as a within-subject factor (12 levels) and Sex (2 levels) and Protocol (6 levels) as between-subject factors

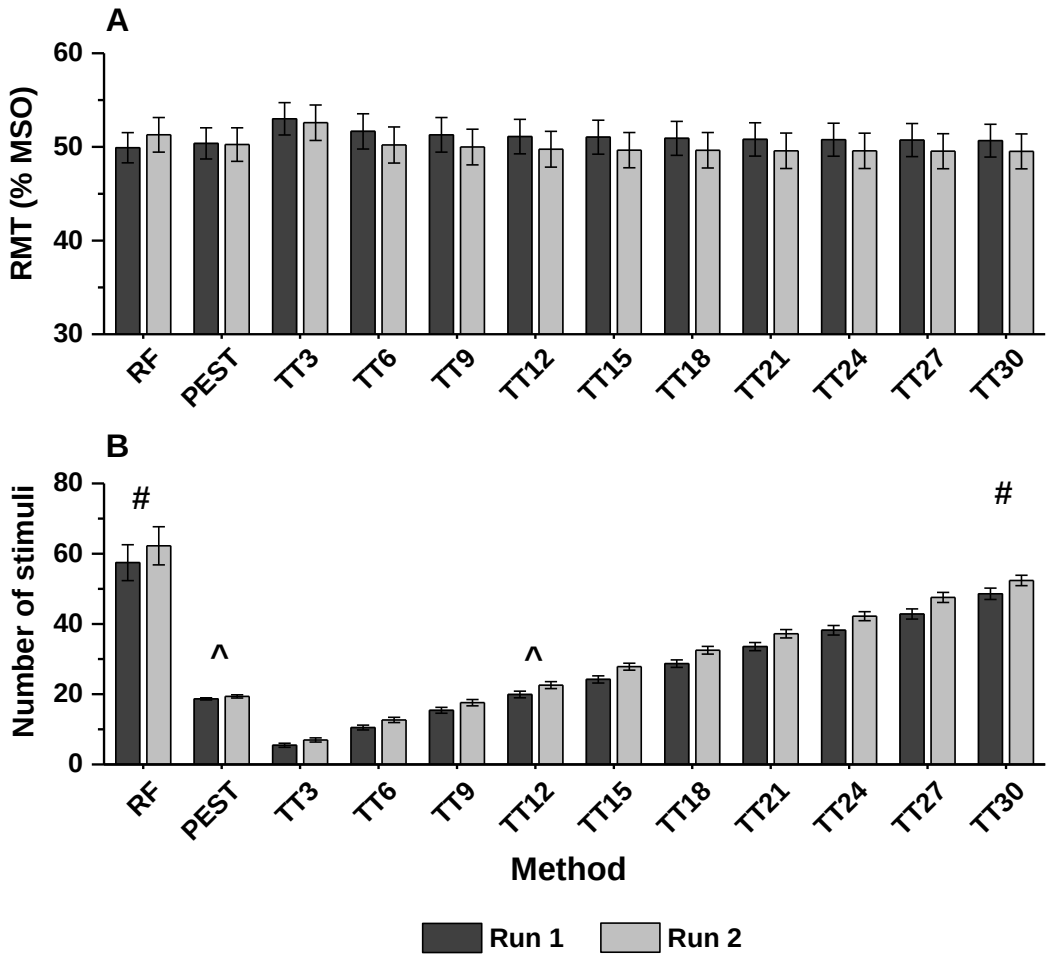

Figure 3.1. RMT estimates (A) and duration of the procedure (B). *While the mean group RMT estimates were similar, the number of stimuli required to obtain them differed greatly between the methods. * RMT_{TT3} was approximately 2-3% MSO higher than most of other estimates (p≤0.045). # and ^ mark the methods of similar duration. Columns represent group means, error bars – standard error of the mean. RF – relative frequency method, TT – threshold-tracking (numbers indicate the duration of tracking from the start to the respective cycle count mark).*

showed no main effect of Sex ($F_{1,12}$<0.001, p=0.990) or interaction between Method and Sex ($F_{1.9,22.3}$=0.82, p=0.444). A main effect of Protocol type was observed ($F_{5,12}$=4.64, p=0.014), which can be explained by the allocation of a small number of subjects (n=4) to each protocol type without a prior knowledge of their RMT. No interaction between Method and Protocol type was seen ($F_{9.3,22.3}$=1.26, p=0.311). The duration of the procedure did not differ between the sexes ($F_{1,12}$=4.30, p=0.060) or protocol types ($F_{5,12}$=1.70, p=0.208).

Across 48 recordings, the median absolute difference in RMT was low between the methods: 1.4 (IQR 2.7) % MSO between RF and TT at six cycles, 2 (IQR 2) % MSO

between RF and best PEST, and 2.2 (IQR 1.8) % MSO between best PEST and TT at six cycles (Friedman's test, p=0.918) with up to 10.5%, 10%, and 7.5% MSO differences in individual recordings, respectively.

In this study, the automated RF procedure deviated from the IFCN standard in 21 out of 48 recordings, but no difference in RMT_{RF} estimates was seen between these groups (Student's t-test, t=-0.15, df=46, p=0.884). In the IFCN procedure, the stimulus intensity is altered in a downward step fashion and estimation is stopped when 6/10 (or 11/20) responses are negative (section 1.2.1). Meanwhile in the QtracS RF protocol, stimulus intensity could be altered either in a downward (38 out of 48 recordings) or an upward (10 out of 48 recordings) manner (as described in section 2.5.1). In 18 subjects, the RMT estimation had the same direction (16 – downward, 2 – upward) in both sessions. There was no effect of the direction of the recordings (same upward/downward or opposite between runs) on RMT_{RF} estimates (rmANOVA, $F_{2,21}$=0.33, p=0.726) or inter-session difference in them (one-way ANOVA, $F_{2,23}$=0.24, p=0.790). Also, the RF frequency procedure may had been stopped either at 10 positive and 10 negative responses out of 20 consecutive trials or when 11 out of 20 consecutive trials were negative or positive (depending on the direction of the stepwise procedure). Thus, RMT_{RF} estimate determination depended on the stopping criterion. If the stopping rule was different between the sessions, this may have potentially introduced bias to the reliability of RMT_{RF} estimates in this experiment. In 13 subjects, the stopping criterion was the same in both runs, and the inter-session difference in RMT_{RF} estimates in this group did not differ from those in which the stopping criterion differed between the runs (one-way ANOVA, $F_{1,23}$=0.04, p=0.845).

3.2.2 Test-retest reliability of RMT estimates

There was no difference in mean group RMT estimates between the runs for any of the methods. Individual absolute differences in RMT between the runs are presented in Figure 3.2. Although within the group, the range of inter-session differences in RMT was smallest with best PEST method (up to 7% MSO or up to 15% of the initial RMT estimate), it did not prove to be significantly superior to other methods in absolute or relative terms (Friedman's test, p=0.973 and p=0.949, respectively). All methods showed excellent reproducibility with no significant differences between them (Figure 3.3 A) and a similar standard error of measurement of about 3% MSO (Figure 3.3 B). Coefficients of repeatability (CRs) differed slightly between the methods with nominal values ranging from 7 to 9% MSO (Figure 3.3 B). This reliability parameter indicates the range within which the difference in repeated measurements in an individual would not be considered a significant change. For example, if the best PEST method was used to assess the

disease progression or a treatment effect on RMT in an individual with a baseline estimate of 50% MSO, the subsequent measurements between 43% and 57% MSO would reflect the natural variability, but not a significant change due to a disease or a treatment (for RMT_{RF} this range would be between 42% and 58% MSO, for RMT_{TT} at six cycles – between 41% and 59% MSO).

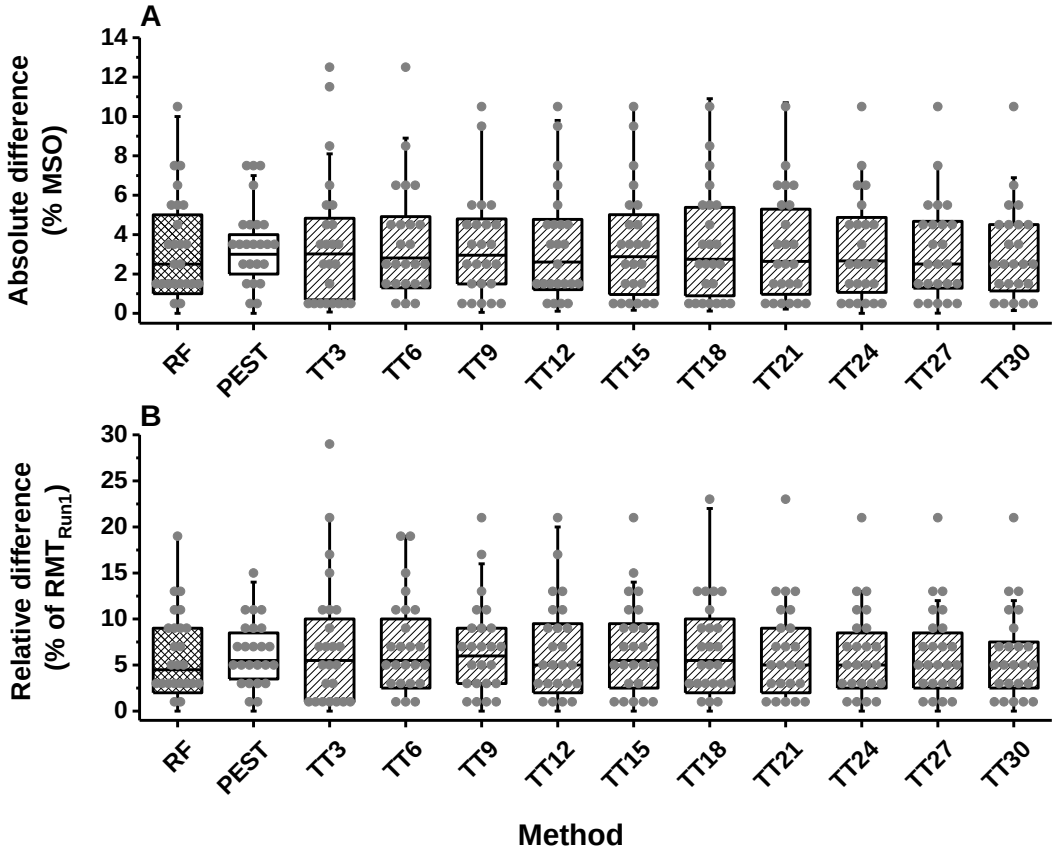

Figure 3.2. Intersession differences in RMT estimates. *Although the range of absolute (A) and relative (B) differences in RMT estimates between the runs varied depending on the method, none of them proved to be superior on a group level (Friedman's test, p≥0.949). Absolute differences were calculated as [RMT_{Run1} – RMT_{Run2}] (A), relative differences - as [(RMT_{Run1} – RMT_{Run2})/RMT_{Run1}*100%] (B). Grey dots represent intersession differences for each subject; data bin size is 1% MSO in A) and 2% RMT_{Run1} in B); boxplots – median and interquartile range; whiskers – 1.5 boxplot length. RF – relative frequency method, TT – threshold tracking (numbers indicate the duration of tracking from the start to the respective cycle count mark).*

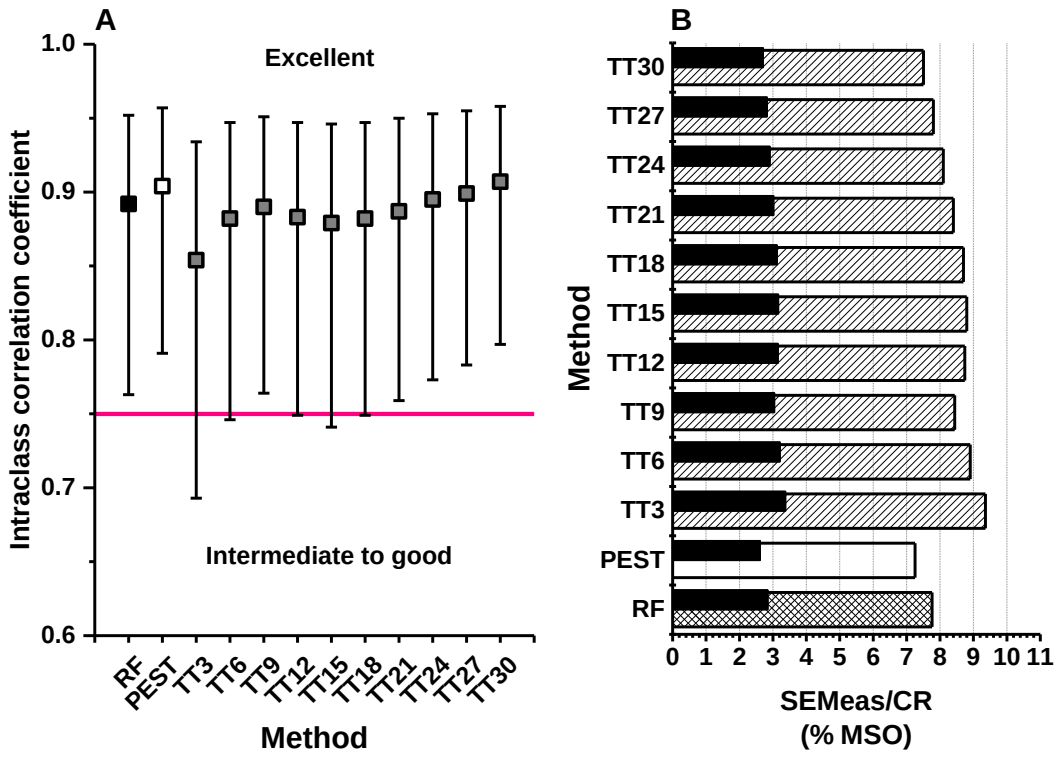

Figure 3.3. Reliability of RMT estimates. *A) All RMT estimation methods showed excellent reproducibility (ICC(2,1) values presented; error bars indicate 95% confidence interval). B) Standard errors of measurement (SEM_{eas}) were similar across the methods, ranging from 2.6 to 3.4 % MSO (black bars). Coefficient of repeatability (CR) – a value below which the differences of future measurements within a subject will lie with 95% probability – ranged from 7.3 to 9.4% MSO, depending on the method (patterned bars). RF – relative frequency method, TT – threshold tracking (numbers indicate the duration of tracking from the start to the respective cycle count mark).*

3.2.3 Optimal duration of threshold-tracking for RMT estimation

On a group level, RMT estimate obtained by threshold-tracking for three cycles (RMT_{TT3}) was significantly higher compared to RMT at different tracking durations or methods (Figure 3.1 A). Across 48 recordings, the mean difference between RMT_{TT3} and RMT_{TT6} estimates was 1.9 ± 3.5% MSO. However, the absolute difference exceeded 2% MSO in a quarter of the recordings and could be as high as 14-18% MSO. In comparison, the mean difference between RMT_{TT6} and RMT_{TT9} estimates was only 0.3 ± 0.8% MSO and did not exceed an absolute nominal difference of 2% MSO in any single recording. This shows that in some recordings threshold-tracking for three cycles may be insufficient to reach the intensity representative of the individual's RMT. As RMT is used to adjust other stimulation parameters for paired-pulse or repetitive TMS, such discrepancies would significantly bias the results or may even pose safety risks if used to set the stimulation intensity for repetitive TMS.

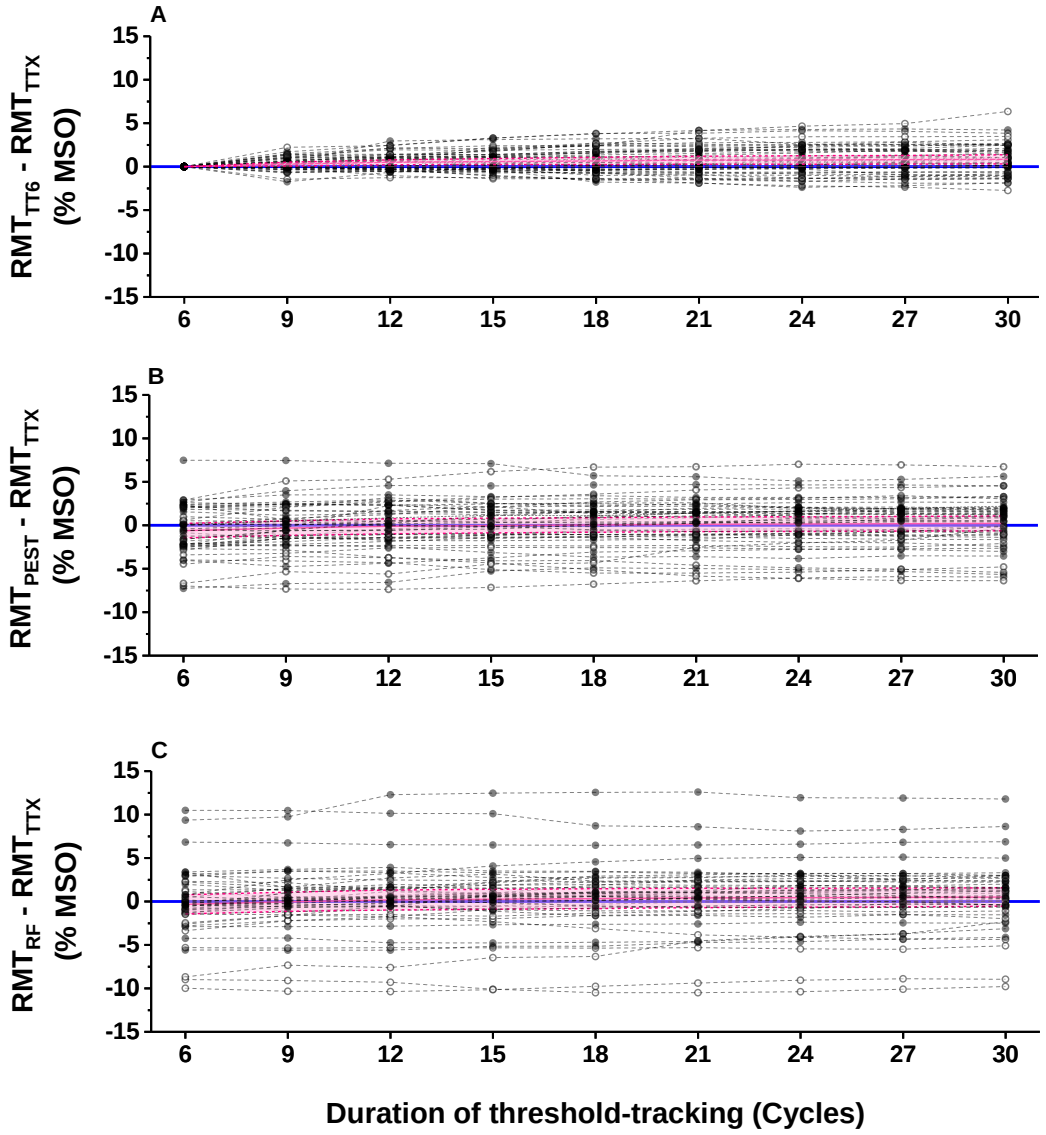

Figure 3.4. Optimal duration of threshold-tracking for RMT determination*. RMT estimates at different tracking durations were compared to RMT estimates obtained by RF, PEST, and TT at six cycles. Each data point indicates a difference between a control RMT estimate (RF, PEST, and TT at six cycles) and an estimate at different tracking durations (indicated on x axis). Data from two sessions (48 recordings) is presented. Open circles represent data from Run 1, filled circles – Run 2; dashed lines connect data points from the same recording; pink lines and shaded area represent mean and 95% confidence limits. RF – relative frequency method; TT6 – threshold-tracking for six cycles; TTX – threshold-tracking duration where X corresponds to number of cycles on x axis. The range of differences between RMT_{TT6} and other RMT_{TTX} estimates gradually increased as the length of tracking increased (A), but the mean difference never exceeded 1% MSO. The range of differences between RMT_{RF}/RMT_{PEST} and RMT_{TTX} estimates was similar across all tracking durations (B and C). This suggests that although the duration of tracking may have some impact on RMT_{TT} estimates, a longer tracking procedure does not appear to be advantageous when the same estimates are compared to other techniques.*

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Mean group RMT_{TT} estimates at other tracking durations did not differ between themselves or when compared to other methods. Up to 6% MSO difference between RMT_{TT6} and RMT_{TT} at other tracking durations was observed in individual recordings (Figure 3.4 A), but in general long tracking procedures did not appear to be advantageous when compared to RF or best PEST methods (Figure 3.4 B and C). Although long tracking duration (for ≥27 cycles) resulted in a marginally better repeatability (Figure 3.3 B), the overall reliability profile was similar between RMT_{TT} estimates obtained at various protocol durations.

Given that the RMT_{TT} estimates obtained at various tracking durations and their reliability are essentially similar, the duration of the procedure may be the main decisive factor in choosing the method. RMT_{TT6} required on average 12 ± 4 stimuli and was significantly shorter than PEST (19 ± 2 stimuli), RF (60 ± 26 stimuli), and longer tracking durations (from 17 ± 4 to 50 ± 8 stimuli; Figure 3.1 B). Bland-Altman plot for inter-session agreement of RMT_{TT6} showed slightly, but not significantly, broader limits of agreement than those of RMT_{RF} and RMT_{PEST} (Figure 3.5). However, the agreement between RMT_{TT6} and other methods was similar to the inter-session agreement of those methods (Figure 3.5).

3.2.4 RMT fluctuations

In the literature on RMT estimation, the term 'accuracy' is used to describe how well the method represents individual's 'true RMT', and safety implications[10] of this 'accuracy' are often used as one of the main arguments for the choice of the method (Awiszus, 2003, Tranulis et al., 2006, Awiszus, 2011, Qi et al., 2011, Silbert et al., 2013). This is done under the assumption that the 'true RMT' is a stable parameter of CSE. However, many physiological factors are known to influence CSE (Rossini et al., 2015), and very slow underlying fluctuations of cyclic pattern in the MEP size have been observed (Wassermann, 2008). Probabilistic methods (RF and PEST) enable RMT sampling at certain intervals only, while threshold-tracking allows uninterrupted monitoring of RMT and obtaining an estimate for an interval of interest. In this study, the RMT appeared to remain relatively stable throughout the whole threshold-tracking procedure in some recordings; in others, clear shifts were noted (Figure 3.6).

[10] This may be particularly important in repetitive TMS (rTMS) protocols in which stimulation intensity is adjusted to the individual's RMT. Induction of seizures is one of the main safety concerns, and a maximum safe duration of single trains of rTMS in relation to the stimulus intensity has been proposed (Rossi et al., 2009). The stronger the stimulus in relation to the individual's RMT, the shorter the trains of pulses are considered safe at higher stimulation frequencies. Therefore, overestimation of RMT could potentially lead to an increased risk of seizure induction.

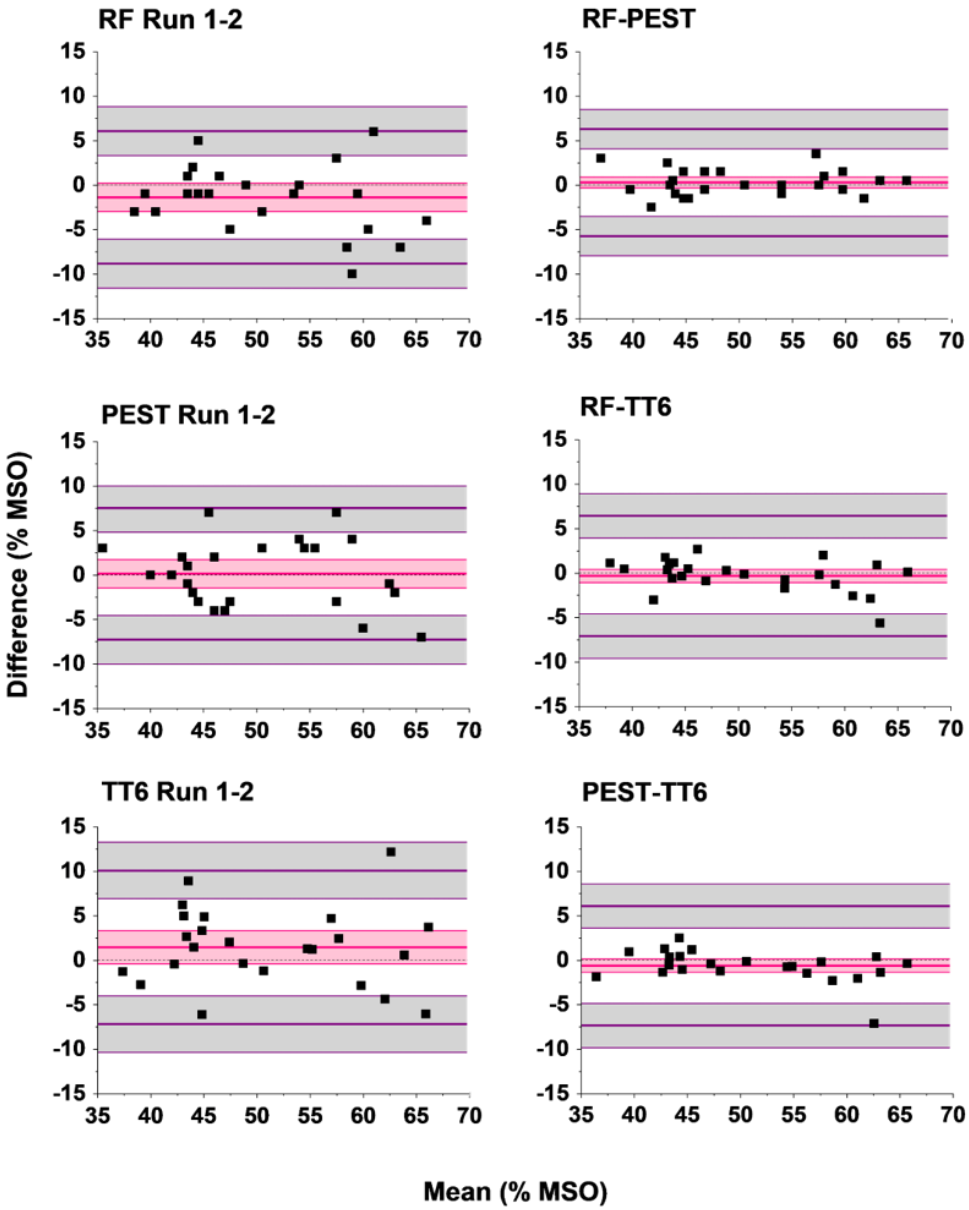

Figure 3.5. Agreement between repeated measurements and techniques. *Bland-Altman plots where constructed. For inter-session agreement (left column), the difference in RMT estimates between the runs (Run1 – Run2, y axis) was plotted against their mean (x axis). The mean and standard deviation of these differences were calculated and used to determine bias and limits of agreement (LOA). For comparison between the methods (right column), RMT estimates averaged across two runs were used. Differences between the methods (calculated as indicated in the graph title, y axis) were plotted against their mean (x axis). LOA were calculated after adjusting standard deviation for repeated measurements (Bland and Altman, 1986). Each data point represents an individual subject. Pink lines and shaded area indicate the mean difference (bias) and its 95% confidence interval; purple lines and grey shaded area – upper and lower 95% LOA and their 95% confidence limits; dashed black line – line of identity. There was no significant bias between the sessions or between the methods. The LOA, indicating the range within which 95% of differences between repeated measurements or methods are expected to lie within the population, appear to be tighter (although not significantly) between the methods than between sessions within the same method.*

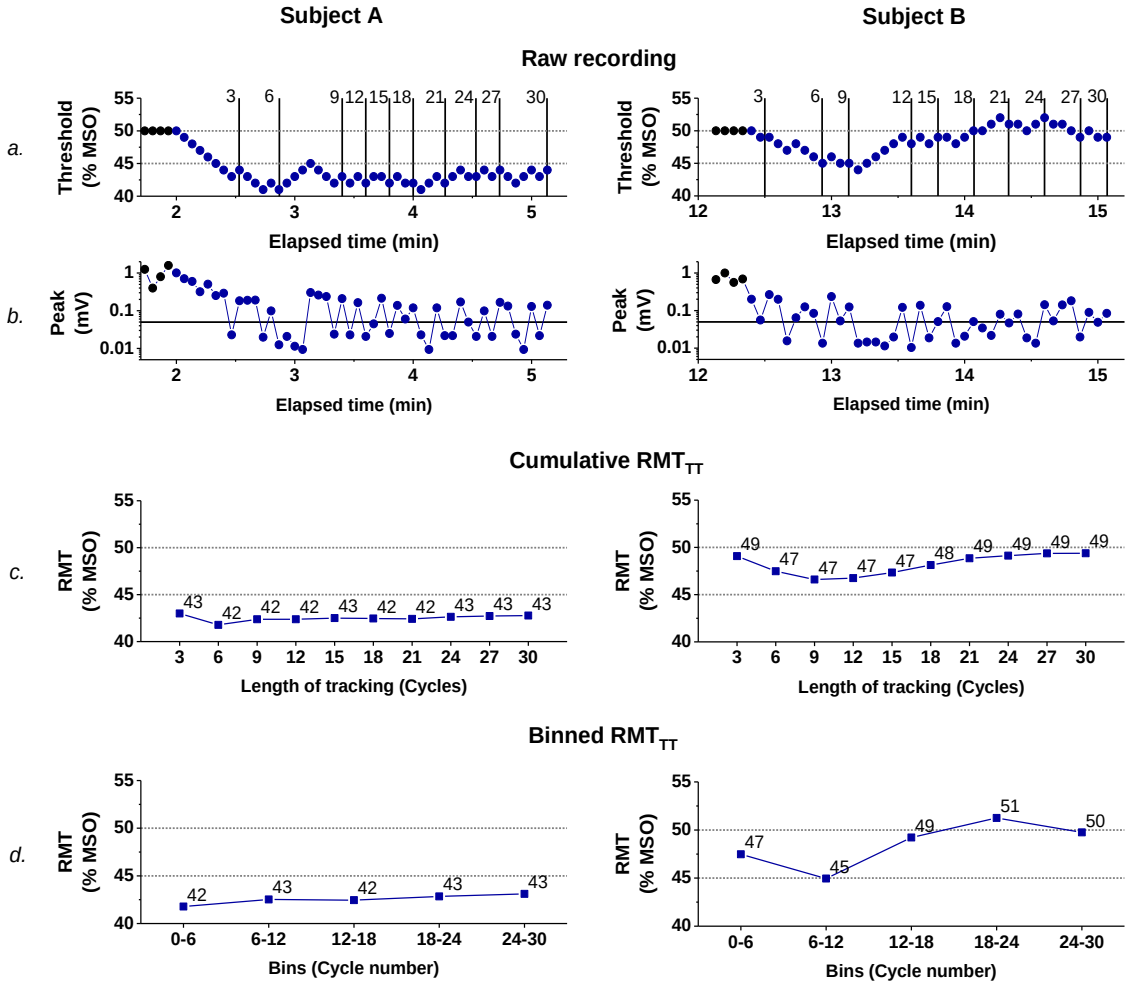

Figure 3.6. Continuous monitoring of RMT with threshold-tracking. *Representative raw recordings and RMT$_{TT}$ estimates of two subjects. Circles represent raw data points (black – the hotspot search; blue – RMT determination); squares – RMT estimates. Vertical lines linked with numbers indicate tracking length in cycles (row a), horizontal solid line – target MEP size (row b). Cumulative RMT$_{TT}$ (row c) indicates RMT estimates at increasing lengths of the protocol (i.e. each estimate includes all data from the beginning of tracking to the relevant cycle mark, and thus is related to all previous estimates), while binned RMT$_{TT}$ (row d) represents RMT estimates obtained at discrete parts of the protocol (the whole recording was divided into five bins of six cycles; RMT estimate for each bin is independent from other estimates). While in subject A the RMT appears to be rather stable throughout the whole three minutes of recording, clear shifts are seen in subject B (data point labels in rows c) and d) represent RMT estimates rounded to the nearest integer). Note that the range within which the MEP amplitudes oscillate around the target (row b) remains similar throughout the whole recording.*

When RMT$_{TT}$ estimates were calculated for every independent six-cycle interval, gradual rather than abrupt changes in RMT were seen over the course of the recording (Figure 3.7).

Within the recordings, the arithmetic mean of binned RMT_{TT} estimates was essentially equivalent to the cumulative RMT_{TT30} estimate (mean difference -0.02% MSO, range -0.85-0.85% MSO; paired-sample t-test, t=-0.53, df=47, p=0.599). This may explain the differences in RMT_{TT} estimates at different tracking durations: any fluctuations in RMT that occurred throughout the recording will be 'averaged out' in RMT_{TT30}, while RMT_{TT6} provides a more instantaneous estimate of the motor threshold. The differences between the minimum and the maximum binned RMT_{TT} within the recording were comparable to the absolute differences in RMT_{TT6} between the runs (Wilcoxon Signed Rank test, p=0.668).

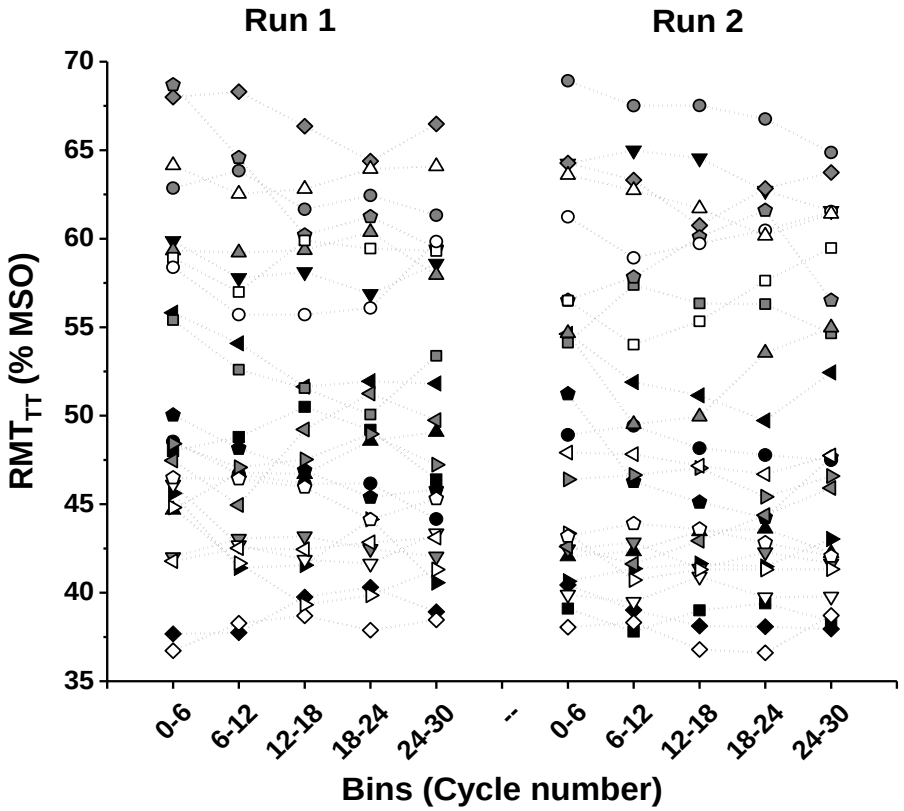

Figure 3.7. Within-session variation of RMT estimates. *The mean duration of the threshold-tracking procedure was 3.8 ± 0.6 min (range 2.9-5.3 min). Each recording was divided into independent six-cycle bins, and RMT_{TT} estimates were calculated for each bin (note that the actual elapsed tracking time varies between the bins). Different symbols represent individual subjects. In 48 recordings, the median within-run coefficient of variation (within-run standard deviation/mean*100%) of RMT_{TT} was 2.3 (IQR 2) % (range 1-5%). The averaged binned RMT_{TT} estimates were essentially equal to the cumulative RMT_{TT30} (paired-sample t-test, p=0.599). The group median absolute range between the minimum and the maximum binned RMT_{TT} estimates within a recording was 3 (IQR 2.5) % MSO but could be as high as 9% MSO in some recordings. In 40 recordings, the binned RMT_{TT} estimates varied within 10% of the overall RMT_{TT30} and were up to 15% of RMT_{TT30} in the rest. In most of the recordings gradual rather than abrupt changes in RMT_{TT} estimates were seen. This may suggest slow underlying changes in corticospinal excitability.*

3.3 Discussion

In summary, there was no difference in mean group RMT estimates obtained by the three methods used in this study, but the duration of the procedure differed considerably. All the methods had similar test-retest reliability profile. Minimal duration for threshold-tracking procedure allowing to obtain a reliable RMT estimate was determined, and it was significantly shorter compared to other methods. Besides potential for improved speed, the threshold-tracking method allows uninterrupted monitoring of corticospinal excitability and may open new avenues in TMS research.

3.3.1 Comparability of RMT estimates

Direct comparison of different RMT estimation methods has been reported by several groups (Table 3.2). Despite methodological variations, the findings of these studies were consistent: there was no difference in mean group RMT estimates between the methods, but the number of stimuli required to obtain them varied greatly. Based on these observations, IFCN proposed that any method can be used for RMT determination, but the adaptive methods may be preferable due to a smaller number of stimuli required (Groppa et al., 2012, Rossini et al., 2015).

The findings of this study are in line with previous reports: no difference in RMT estimates obtained by RF and best PEST methods were observed, and the PEST procedure was significantly quicker. The 10/20 positive response rule was used in this study for RF method as per latest IFCN recommendations (Rossini et al., 2015). One would expect that it would further prolong the recording, but interestingly, the number of stimuli required to obtain RMT_{RF} estimate was similar to the one reported by Silbert et al. where 5/10 positive trial rule was applied (Silbert et al., 2013). This may be due to several differences between the RF algorithm in QtracS (see section 2.5.1) and the standard IFCN procedure (Groppa et al., 2012, Rossini et al., 2015) used in the above-mentioned study. In particular, the condition to begin decreasing stimulus intensity and stopping of the procedure when an equal number of negative and positive responses is obtained could have potentially shortened the RF procedure in this experiment. The duration of the best PEST method was the same as initially reported by Awiszus (Awiszus, 2003), but longer than observed by other groups (Qi et al., 2011, Silbert et al., 2013). This is likely due to different stopping rules used across the studies. Although modifications to the PEST algorithm that may reduce the number of stimuli have been criticised as mathematically ‘unsafe’ (Awiszus, 2011), they seem to arrive at the same estimate under experimental conditions (Qi et al., 2011).

Study and sample size	Method	Number of stimuli	Difference in RMT estimates between methods
Mishory et al., 2004 (n=1)	Modified Mills-Nithi (2% MSO steps; 3/6 trials)	~55*	Not reported
	Best PEST	~16*	
Tranulis et al., 2006 (n=10)	Modified relative frequency method (2% MSO steps, 5/10 trials)	75 ± 18	Up to 16.6% MSO (≤5% MSO in approximately half of the method comparison pairs)
	Mills-Nithi method (1% MSO steps, 5/10 trials)	157 ± 41	
	Supervised parametric method	173 ± 36	
Qi et al., 2011 (n=10)	Modified relative frequency method (2% MSO steps, 5/10 trials)	29.9 ± 11.6	<5% MSO (except one method comparison pair with 6% MSO difference)
	Best PEST	12.2 ± 5.5	
	Bayesian PEST with common prior	6.6 ± 2.6	
	Bayesian PEST with subject prior	2.7 ± 0.5	
Silbert et al., 2013 (n=10)	Relative frequency method (1% MSO steps, 5/10 trials)	56.8 (4.3)	≤5% MSO (median absolute difference 2.3% MSO)
	Best PEST	12 (0)	
Ah Sen et al., 2017 (n=15)	Modified relative frequency method (1% MSO steps, 5/10 trials)	35.1 ± 8.21	Up to 14% MSO (≤5% MSO in 13 out 15 subjects)
	Best PEST	20†	

Table 3.2. Comparison of RMT estimation methods. *No significant difference in mean group RMT estimates were found between various methods in any of the studies (for surface EMG recordings). Modified relative frequency method refers to a relative frequency procedure other than described in the recent IFCN guidelines (Groppa et al., 2012, Rossini et al., 2015). Number of stimuli is presented as mean ± standard deviation or mean (standard error), except marked with *, for which approximate means were obtained from the reported graphs. † procedure stopped after 20 stimuli in all subjects.*

The validity of threshold-tracking technique for RMT estimation has not been previously investigated. In contrast to the conventional cut-off value of 0.05 mV used for the probabilistic definition of RMT, the target size of 0.2 mV is commonly used in threshold-tracking and the RMT estimation is based on the linearity of the relationship between stimulus intensity and log-transformed response size (Fisher et al., 2002, Vucic et al.,

2006). This study showed that threshold-tracking can be successfully used with the target set at 0.05 mV, and the RMT estimates obtained by this technique were comparable to the well-established methods. Moreover, the minimal duration of tracking needed to obtain a reliable RMT estimate was significantly shorter than other procedures. It required on average seven stimuli less than the best PEST technique and was five times quicker than the RF method.

In this study, the absolute differences in RMT estimates between the methods were comparable to previously reported (see Table 3.2), and the absolute difference between RMT_{TT6} and RF or best PEST estimates was the same as the difference between RMT_{RF} and RMT_{PEST}.

3.3.2 Reliability of RMT estimates

RMT is one of the most reliable measures of CSE with low within-subject variability (Wassermann, 2002, Koski et al., 2005, Ngomo et al., 2012) and good reproducibility (Fleming et al., 2012, Ngomo et al., 2012, Liu and Au-Yeung, 2014, Beaulieu et al., 2017). In most of the previous test-retest reliability studies, RF method with 5/10 positive trial rule was used, and the reported intraclass correlation coefficients (ICCs) ranged from 0.61 to 0.98 (Fleming et al., 2012, Ngomo et al., 2012, Liu and Au-Yeung, 2014, Schambra et al., 2015, Brown et al., 2017).

It is important to note that ICC is a dimensionless measure of relative reliability, indicating the degree to which subjects maintain their position within a group over repeated measurements (Bruton et al., 2000, Streiner et al., 2008). It is highly dependent on the heterogeneity of the sample it was obtained from (the more different the subjects within the sample, the higher the ICC), thus direct comparison between the studies cannot be easily made (Streiner et al., 2008, Beaulieu et al., 2017). Measures of absolute reliability, such as standard error of measurement (SEM_{eas}) or coefficient of repeatability (CR, also referred to as smallest/minimal detectable change (Schambra et al., 2015, Beaulieu et al., 2017)), may be more informative, especially when reliability of a test in a clinical setting is assessed. CR is derived from SEM_{eas} and indicates a range (± CR) within which the differences between repeated measurements will reflect measurement noise (de Vet et al., 2011).

The repeatability of RMT has been summarised in a recent systematic review (Beaulieu et al., 2017). For RMT measured from the intrinsic hand muscles, the CRs varied between 3.58% and 9% MSO across five studies (Beaulieu et al., 2017). RF method was applied in three of them, with lower CRs reported by groups employing navigation

systems (CRs 3.58-5% MSO (Ngomo et al., 2012, Schambra et al., 2015) compared to CR of 7.91% MSO for non-navigated TMS (Liu and Au-Yeung, 2014)).

Reliability data for the best PEST paradigm is limited. In one study, RMT_{PEST} was measured by five operators on a single subject with same-day differences in estimates of up to about 25% MSO (Mishory et al., 2004). Silbert and colleagues (Silbert et al., 2013) carried out four serial RMT_{PEST} measurements at four-minute intervals with individual inter-session differences of up to 4% MSO. However, none of these studies reported formal reliability measures (i.e. ICCs or CRs).

The findings of this study are in line with the previous reports. All three methods showed excellent reproducibility with no significant differences between them. The repeatability of RMT_{RF} (CR 7.8% MSO) was comparable to that reported by Liu et al. (Liu and Au-Yeung, 2014) where 5/10 positive trial rule was applied, and no navigation system was used, but was poorer than in the studies with navigated TMS (Ngomo et al., 2012, Schambra et al., 2015). The repeatability of best PEST was only marginally better (CR 7.3% MSO), while for threshold-tracking it depended on the duration of the procedure: it was best for the longest recordings (27-30 cycles; CRs 7.5-7.8% MSO) and ranged between 8 and 9.4% MSO for other durations.

3.3.3 Optimal duration of threshold-tracking for reliable RMT estimation

The RF procedure is stopped based on the proportion of negative responses, and a minimal number of stimuli has been proposed for the best PEST algorithm to make the procedure 'safe' (Awiszus, 2011). With threshold-tracking, RMT could theoretically be monitored indefinitely, and currently there is no clear guidance on the optimal duration of the procedure allowing to obtain reliable estimates. Tracking is considered successful when the responses consistently hit and/or oscillate around the target. Various stopping rules ranging from two to six hits and/or crosses of the target line have been used in the previous threshold-tracking experiments in a context of paired-pulse TMS (Awiszus et al., 1999, Fisher et al., 2002, Vucic et al., 2006), but the optimal duration for reliable estimates of RMT has not been systematically investigated.

In this study, no difference was observed in the mean group RMT_{TT} estimates between different threshold-tracking durations, except for the shortest duration of three cycles. The minimum duration of the procedure required to obtain RMT_{TT} estimates comparable to RF and best PEST methods was six cycles (i.e. six valid threshold estimates), which on average took less than one minute to complete. The inter-session differences in RMT_{TT} estimates were similar across all tracking durations as well as methods (Figure 3.2). All estimates had excellent reproducibility (Figure 3.3 A), and Bland-Altman analysis

showed no significant difference in the inter-session agreement between RMT_{TT6} and RMT_{RF} or RMT_{PEST} estimates (Figure 3.5). Nevertheless, only the longest threshold-tracking protocols (27-30 cycles) resulted in repeatability similar to the best PEST or RF methods. Shorter tracking durations had marginally higher CRs (Figure 3.3 B).

There are two possible explanations for these findings. One is related to the analysis of the threshold-tracking data, which relies on the stimulus – log-transformed response relationship. Longer tracking protocols produce more data points which compensate for the high variability of MEP amplitudes, thus making the RMT_{TT} estimates more reliable. This would be the most plausible explanation if RMT remained stable throughout the whole recording. However, observations of the raw threshold-tracking recordings suggest that RMT may change considerably during the lengthy procedure (Figure 3.6, subject B). Furthermore, when individual recordings were split into independent six-cycle bins and RMT_{TT} estimates were calculated for each interval (Figure 3.7), gradual shifts in threshold were observed in nearly every recording within a median range of 3% MSO (up to a maximum of 9% MSO). This study was not designed to identify the cause of these shifts. They could be related to technical or biological factors, such as subtle changes in the coil position or subject's level of alertness. Very slow fluctuations in MEP amplitude with a cyclic pattern have been reported (Wassermann, 2008), and slow changes in RMT can be seen in published raw threshold-tracking recordings from paired-pulse TMS experiments (Vucic et al., 2006). It may be possible that the gradual changes in RMT_{TT} observed in this study reflect the aforementioned cyclic fluctuations in CSE, but further experiments are needed to elucidate their source.

The observed drifts in RMT_{TT} may explain why shorter tracking protocols resulted in marginally poorer repeatability. They likely reflect a more instantaneous state of CSE, while estimates obtained from a long recording will 'average out' any fluctuations in motor threshold that occurred during it. Thus, the duration of the tracking procedure may be chosen depending on the purpose the RMT estimates will be used for.

3.3.4 The choice of method for RMT estimation

This study demonstrated no difference in mean group RMT obtained by three estimation methods. Thus, other factors, such as the duration of the procedure, test-retest reliability, and the availability of a specific software are decisive in choosing the method for RMT.

The RF procedure using 10/20 positive response rule was one of the longest. It lasted nearly four minutes on average, but its duration in this experiment was rather unpredictable and could take anywhere between two to ten minutes to complete. Such a long procedure would not be desirable neither in clinical, nor research setting.

Furthermore, it did not show an improved reliability compared to the commonly used 5/10 positive response rule (Liu and Au-Yeung, 2014) or other RMT estimation methods used in this study. The choice of RF method could only be justified if no software required for adaptive methods is available.

The choice between the best PEST and threshold-tracking methods is less obvious. RMT estimates could be obtained quicker with threshold-tracking compared to the best PEST, but the latter method showed a marginally better test-retest reliability. Thus ultimately, the choice of the method should be based on the intended use of the RMT estimates. For example, if RMT was used as an independent outcome measure of a treatment effect or disease progression, especially on an individual rather than a group level, a method with the best reliability would be preferred (i.e. best PEST)[11]. Meanwhile, threshold-tracking would be advantageous where time constraints are more important. Moreover, probabilistic methods can only provide point estimates of the RMT, while threshold-tracking allows uninterrupted online monitoring of the changes in CSE.

The accuracy of an estimate in reflecting the 'true RMT' of an individual has often been used as the main argument for the choice of a 'safe' RMT estimation method (Awiszus, 2003, Awiszus, 2011, Qi et al., 2011). However, such safety has only been defined mathematically under the assumption that the 'true RMT' is a stable parameter of CSE (Awiszus, 2003, Tranulis et al., 2006, Awiszus, 2011, Qi et al., 2011, Silbert et al., 2013). Meanwhile, threshold-tracking findings suggest that considerable changes in RMT may occur during a five-minute recording, in some instances exceeding 10% of the individual's average RMT (Figure 3.6 and Figure 3.7). This may have important implications in TMS protocols in which stimulation intensities are set based on individual's RMT. It is a common approach to obtain a single RMT estimate at the start of an experiment and then use it to set other TMS parameters for the remainder of the session irrespective of its duration. However, if RMT changes significantly due to technical or biological factors, the pre-set conditioning stimulus intensities may become suboptimal in eliciting inhibitory or facilitatory effects in paired-pulse protocols (Groppa et al., 2012), potentially contributing to the variability of such TMS measurements. Similarly, this may result in insufficient dosing or a potential safety hazard in repetitive TMS protocols (Rossi et al., 2009). Thus, TT may be valuable in those circumstances where controlling for changes in CSE is crucial; it could be used both for continuous monitoring of CSE or as a quick way to obtain RMT estimates between different portions

[11] Coefficient of repeatability, or smallest detectable change, calculated for the group can be useful in estimating the sample size for interventional studies (section 2.8). For instance, to detect a significant change in RMT of 2% MSO due to intervention, an approximate sample size of 12 subjects would be required if PEST method was used, while larger samples would be needed using RF and TT methods (16 and 20 subjects, respectively).

of the stimulation session. Furthermore, threshold-tracking may be useful in demonstrating the immediate effects of various interventions used to modulate the CSE, such as repetitive TMS, transcranial direct current stimulation or administration of fast-acting CNS active drugs.

3.3.5 Limitations

This study had several potential limitations. Firstly, the RF algorithm available in QtracS is based on the initial descriptions by Rossini (Rossini et al., 1994) and Rothwell (Rothwell et al., 1999) and has some differences from the most recent protocol proposed by the IFCN (Groppa et al., 2012, Rossini et al., 2015). Despite the use of currently recommended parameters such as starting intensity, stimulation step size, or the maximum number of stimuli per step, the procedure deviated from the IFCN standard in 21 out of 48 recordings. However, none of these deviations (i.e. the direction of the stimulus steps or stopping rule) seemed to have an impact on the RMT_{RF} estimates or their inter-session differences. It is important to note that the current IFCN RF procedure has been proposed to standardise the method, but its superiority against other modifications has not been experimentally validated (Groppa et al., 2012). A variety of RF procedure modifications have been used in other studies, yielding similar outcome when compared to other RMT estimation methods (see Table 3.2). To our knowledge, this is the first study to report the reliability data of the RF procedure using 10/20 positive trial rule. On mathematical grounds, it has been argued that 5/10 positive trials are not sufficient to ensure the reliability of the RF procedure (Awiszus, 2012). Although a direct comparison was not carried out in this study, its findings suggest that increasing the number of stimuli does not necessarily improve the reliability of RMT estimates under 'real life' conditions.

The reliability of the RMT estimates in this study was comparable to the previous work in which no navigation system was used (Kimiskidis et al., 2004, Liu and Au-Yeung, 2014), but was poorer than in the navigated TMS experiments (Livingston and Ingersoll, 2008, Ngomo et al., 2012, Schambra et al., 2015). It is conceivable that the more precise TMS coil (re)positioning with the help of navigation system may improve the reliability of RMT measurements. However, several studies that compared navigated versus non-navigated TMS procedures showed no definite benefit of the use of navigation system for RMT estimation or its reliability (Danner et al., 2008, Julkunen et al., 2009, Jung et al., 2010, Fleming et al., 2012).

3.4 Conclusions

This study validates threshold-tracking for RMT estimation using a cut-off value of 0.05 mV. A minimal duration of tracking required to obtain estimates comparable to the well-established relative frequency and best PEST methods has been determined. All estimation methods were fully automated to allow for a single operator to carry out the recordings and to prevent operator bias with repeated measurements. The hotspot was identified anew before each method with the aim to reproduce the circumstances of a routine experimental or clinical setting. The 10/20 positive trial rule used for the RF method did not result in a better reliability when compared to the reports of other studies in which 5/10 positive trials were used. Threshold-tracking offered an improved speed of RMT determination compared to the other methods without fundamentally compromising the reliability of repeated measurements. While probabilistic methods provide point estimates only, threshold-tracking allows uninterrupted monitoring of RMT. It revealed that considerable shifts in RMT may occur even during relatively short recordings, which may in turn have implications for other TMS protocols in which stimulation intensities are set based on individual motor threshold. Continuous monitoring and online adjustment for such fluctuations in corticospinal excitability may potentially improve the reliability of paired-pulse TMS measurements or the safety and efficacy of repetitive TMS. Furthermore, threshold-tracking could potentially be used to demonstrate the instantaneous effects of various interventions that modulate the corticospinal excitability, thus opening new avenues for TMS research.

Chapter 4 - Fluctuations in corticospinal excitability (Experiment 2)

Resting motor threshold (RMT) and motor evoked potential (MEP) amplitude are used as the baseline characteristics of corticospinal excitability (CSE). While RMT is one of the most reliable TMS measurements (Beaulieu et al., 2017, Brown et al., 2017), huge trial-to-trial variability is a well-known feature of MEPs (Wassermann, 2008). Thus, averaging of multiple responses is required to obtain a more accurate estimate of CSE (Rossini et al., 2015).

Various technical and biological factors that contribute to the variability of MEP size and RMT were discussed in Chapter 1. In previous studies (see section 1.2.2), the impact of these factors was investigated in a systematic way. However, during a standard TMS recording, they are likely to occur in an unpredictable manner. For example, brief periods of unintentional activation of the target muscle are not uncommon even in subjects who can otherwise maintain the relaxation well and this may affect the measurements (Darling et al., 2006). Eye movements or opening/closing, movements of the face or limbs are rarely monitored and may contribute to the variability of MEPs within the recording session. There may also be systematic factors that affect the CSE. Subjects may become drowsy as the recording progresses or, on the contrary, may start feeling uncomfortable and tense. This may alter the level of CSE so that preset stimulus intensities become suboptimal in the middle of the recording. Operator fatigue may occur during lengthy recordings resulting in shifts of the hand-held coil position that may remain unnoticed. Alternatively, head movements of the subject are problematic if the coil is clamped. Navigation systems could improve the stability of the coil placement, but they are expensive and require additional time for setting up; thus, their use may be impractical in certain circumstances.

Factors that contribute to the variability of RMT and MEP amplitude may be critical in paired-pulse TMS protocols. For example, considerable changes in CSE during the recording session may result in the pre-set test and/or conditioning stimulus intensities becoming suboptimal for eliciting intracortical inhibition or facilitation, thus contributing the variability of the paired-pulse outcomes (Groppa et al., 2012, Rossini et al., 2015). Threshold-tracking would allow to observe such changes in CSE and adjust the stimulation intensities accordingly.

The aim of this experiment was to determine the extent of variability of the baseline TMS measurements (i.e. MEP amplitude and RMT) during a 20-minute recording in the typical experimental setting of our lab and to assess whether it can be attributed to the unintentional shifts in coil position occurring during the recording.

4.1 Methods

4.1.1 Subjects

Fourteen healthy volunteers (9 men; median age 26 years, age range 22-52 years; 11 right-handed) with no known neurological disorder or contraindications for TMS and not on any regular medication took part in the experiment.

4.1.2 Experimental setup

The experimental setup and TMS procedure were largely as described in sections 2.2 and 2.3 of Chapter 2. Surface EMG was recorded from a relaxed FDIO muscle of the self-reported dominant hand. Magnetic stimuli were delivered via the figure-of-eight coil to the contralateral hemisphere so that posterior-to-anterior current flow in the motor cortex was induced. Stimuli were delivered at 4.6 ± 0.5 s intervals (1 s jitter added to prevent anticipation). After identifying the motor hotspot, the coil position was fixed using a clamp and the subjects were instructed to keep their head as still as possible throughout the recording. The position of the subject's head in relation to the coil was further supported by the operator. Audiovisual feedback was provided for the subjects to help maintain relaxation of the target muscle.

A frameless optical navigation system consisting of a standard Polaris Vicra Optical Tracking System and a TMS Manager 2.0 software (Northern Digital Inc., Toronto, Canada) were used to monitor the coil position with reference to the subject's head[12]. The co-ordinates of the coil in respect to the subject's head at the start of data collection were set as the target reference and were subsequently registered with each TMS pulse. Distance, rotation (about the axis perpendicular to the subject-facing hotspot of the coil) and plane (tangential angle of the coil with respect to the skull) of the coil relative to the initial target reference were calculated by the navigation software for each TMS trace[13]. A visual feedback was provided on a screen in front of the subject and the operator by means of three circles (for distance, rotation, and plane) that changed colour in the

[12] Navigation system was set up prior to the TMS session. A head-strap with rigid bodies was placed on subject's head and head registration was carried out using a digitising probe to point the landmarks on the subject's head (nasion, inion, left and right tragus, vertex). For coil registration, rigid bodies were attached to the coil and a digitising probe was used to point the outline of the coil (the right and left outer wings and subject-facing hotspot of the coil).

[13] To assess for the navigation system error, one stimulation session was carried out with the magnetic coil securely placed on a desk. The mean error of the navigation system was 0.23 ± 0.08 mm for distance and 1.62 ± 0.17° for plane and rotation. The median trial-to-trial variability of the navigation system error was 0.003 (IQR 0.15) mm for distance, 0.006 (IQR 0.32)° for plane, and 0.003 (IQR 0.32)° for rotation.

spectrum from green to red as the displacement of the coil from the initial target increased.

The experimental setup required two operators: one to carry out the TMS recordings and another to monitor the navigation system to ensure that TMS and position data is aligned.

4.1.3 Stimulation protocols and data analysis

After finding the hotspot, one of the two stimulation protocols was initiated:

1) Constant stimulation at 120% $RMT_{0.2mV}$ intensity. This stimulation intensity was chosen as it is commonly used to obtain control MEPs in conventional paired-pulse TMS protocols (Rossini et al., 2015). Initially, RMT was determined by threshold-tracking with the target MEP set at 0.2 mV ± 20% ($RMT_{0.2mV}$). Proportional tracking mode with a maximum step of 2% MSO was used. Once stable $RMT_{0.2mV}$ tracking was reached, the coil position was registered by the navigation system as the target reference. Stimulation intensity was then automatically set to 120% $RMT_{0.2mV}$ and remained fixed at this level for the rest of the session. Peak-to-peak amplitude and area of MEPs were measured.

2) Continuous $RMT_{0.2mV}$ tracking. The tracking was carried out on two alternating channels producing two independent $RMT_{0.2mV}$ samples. The stimulation interval was approximately 9.2 s on each channel. The purpose for tracking on two channels was to establish whether any changes in threshold occurring during the recording are incidental due to a random variability of MEP amplitude or whether they reflect underlying fluctuations in CSE. If the latter were true, progressive changes in threshold would occur over a series of pulses and should be seen on both channels concomitantly. Tracking was started at suprathreshold intensity used to localise the hotspot. Proportional tracking mode with a maximum step of 2 % MSO was used throughout. The reference coil position was registered by the navigation system at the start of tracking.

Both types of recordings ($RMT_{0.2mV}$ and MEP) were continued until the capacity of the position data storage of the navigation system was reached (co-ordinates of 256 stimuli) or until the TMS coil overheated. The online gating of pre-stimulus activation of the target muscle was not used in this experiment for several reasons. Firstly, the navigation software registered coil coordinates with each magnetic pulse, while gated-out traces were not saved by QtracS. Therefore, online gating would have increased the chance of mis-aligning the TMS and coil position data. Secondly, gating would result in irregular intervals between recorded responses, especially if several in a row traces were

discarded, and this might bias the calculations of trial-to-trial variability of MEPs or the threshold-tracking resolution on two independent channels.

A total of 20 recordings were obtained for this experiment (ten $RMT_{0.2mV}$ and ten MEP). Six subjects underwent both $RMT_{0.2mV}$ and MEP recordings (done on separate experimental days).

$RMT_{0.2mV}$ estimate was determined offline in QtracP as described in section 2.5.3 (except that a target of 0.2 mV instead of 0.05 mV was used). MEP area was calculated offline by integrating and rectifying response 15-55 ms after the TMS stimulus. Absolute consecutive difference (CD) between adjacent MEPs was calculated for both amplitude and area, and a mean consecutive difference (MCD) was used to quantify the trial-to-trial variability of MEPs in a recording (Kiers et al., 1993, Rösler et al., 2008). MCD of amplitude and area were normalised to the individual's mean MEP size and area, respectively, to allow a direct comparison between the variability of the two measurements as well as subjects. Coefficient of variation (standard deviation/mean) was also calculated for both MEP amplitude and area. Raw traces were reviewed offline and those with pre-stimulus activation of the target muscle 200 ms prior to the magnetic stimulus were marked.

4.1.4 Statistical analysis

For normally distributed data (Shapiro-Wilk test, $p>0.05$) parametric tests (paired-sample t-test, Pearson's correlation coefficient) were used for comparisons between repeated measurements and associations between TMS and navigation system data. Non-parametric tests (Mann-Whitney U-test, Wilcoxon signed rank test, Spearman's correlation coefficient) were used for non-normally distributed data. Cross-correlation analysis was carried out to determine the association between various TMS parameters and coil position in time series. The effect of pre-stimulus activation of the target muscle on MEP amplitude and trial-to-trial variability was assessed by non-parametric Mann-Whitney U-test. For this purpose, traces with and without pre-stimulus activation within a recording were considered to be independent observations.

To assess for any underlying frequencies in CSE, Fast Fourier Transform (FFT) analysis of CSE parameters was carried out using OriginPro software version 9.40.00 (OriginLab, Northampton, MA, USA). To correct for direct current (DC) component of the signal, the overall average estimate of the recording was subtracted from each data point. The following parameters were used: sampling interval of 4.6 s for MEPs and 9.2 s for $RMT_{0.2mV}$, rectangle window with correction for amplitude; power of the frequency band

was normalised to the mean square amplitude (MSA). The analysis covered a frequency range of up to 0.1087 Hz for MEPs and up to 0.0543 Hz for RMTs.

Data is presented as mean ± standard deviation (SD) when normally distributed or as median and interquartile range (IQR) of the 25th and 75th percentile, if non-normally distributed.

4.2 Results

4.2.1 Motor evoked potentials

The recording session lasted around twenty minutes, and 256 MEPs were obtained for all but two subjects with the highest $RMT_{0.2mV}$, for whom the recording session was terminated early due to coil overheating (210 and 218 MEPs were obtained, respectively).

4.2.1.1 Amplitude vs area

The main mean group TMS parameters are summarised in Table 4.1. No significant difference was observed between the trial-to-trial variability of MEP amplitude and area (normalised $MCD_{amplitude}$ vs normalised MCD_{area}, paired-sample t-test, t=-1.36, df=9, p=0.207; $CV_{amplitude}$ vs CV_{area}, paired-sample t-test, t=-1.89, df=9, p=0.091). Thus, MEP amplitude was used for further analysis.

Parameter	**Mean ± SD**	**Parameter**	**Mean ± SD**
$RMT_{0.2mV}$ (% MSO)	50.4 ± 6.9	TS (% MSO)	60.5 ± 8.3
$MEP_{amplitude}$ (mV)	1.248 ± 0.72	MEP_{area} (mVms)	4.904 ± 2.40
$MCD_{amplitude}$ (mV)	0.554 ± 0.22	MCD_{area} (mVms)	2.340 ± 0.98
$CV_{amplitude}$	0.55 ± 0.16*	CV_{area}	0.58 ± 0.17†
$nMCD_{amplitude}$	0.49 ± 0.16*	$nMCD_{area}$	0.51 ± 0.17†

Table 4.1. Summary statistics of TMS parameters. *SD – standard deviation; $RMT_{0.2mV}$ – resting motor threshold (target 0.2 mV); TS – test stimulus intensity used to obtain MEPs; MEP – motor evoked potential; CV – coefficient of variation; MCD – mean consecutive difference; nMCD – mean consecutive difference normalised to the subject's mean MEP amplitude/area. * paired-sample t-test, t=5.71, df=9, p<0.001, † paired-sample t-test, t=6.52, df=9, p<0.001.*

4.2.1.2 Pre-stimulus activation of the target muscle

During the recordings, subjects were instructed to maintain their target muscle relaxed. However, traces with pre-stimulus activation were found in all recordings with a frequency varying from 1.2% to 28.4% of the total number of traces recorded. In seven out of ten recordings, less than 10% of traces were contaminated by pre-stimulus activation (formal statistical tests were not carried out due to a very small number of traces with pre-stimulus activation). In the remaining three recordings, no significant difference in median MEP amplitude or CD was seen between traces with and without pre-stimulus activation (Mann-Whitney U-test, $p \geq 0.285$ and $p \geq 0.129$, respectively).

4.2.1.3 MEP variability and coil positioning

The coil displacement from the initial target was very small in most recordings (see Table 4.2). The distance from the initial target exceeded 5 mm in three recordings, tilt from the initial plane exceeded 5° in one recording, whilst the rotation from the initial coil handle position was less than 5° in all recordings. The relationship between MEP and coil positioning was analysed on a trial-to-trial basis. Significant positive correlations of raw MEP amplitude with at least one of the coil displacement indices was seen in half of the recordings, but they were mostly weak (Spearman's $r=0.136\text{-}0.282$, $p \leq 0.032$), except for one recording with the largest coil displacement showing moderate correlations (Recording No. 9, Table 4.2). Correlating MEP amplitudes with a combined coil displacement measure (sum of displacement in three dimensions) did not reveal further associations. Raw consecutive differences of MEP amplitude and coil displacement indices were calculated to assess whether MEP variability can be explained by small trial-to-trial movements of the coil. Significant correlations with the change in coil distance from the target were found in three recordings, but they were weak (Pearson's/Spearman's $r=-0.160\text{-}0.206$, $p \leq 0.013$) and would be negligible in practical terms (Figure 4.1).

Positive correlations between the time elapsed from the start of the session and the corresponding coil displacement were seen in all recordings and were largely of moderate to very strong degree (Spearman's $r=0.178\text{-}0.998$, $p \leq 0.004$). This suggests a gradual shift of the coil from the initial target as the recording progressed. Meanwhile, significant correlations between the elapsed time and MEP amplitude were seen in two recordings only: weak negative (Spearman's $r=-0.149$, $p=0.017$) in a recording with essentially stable coil positioning throughout and moderate positive (Spearman's $r=0.384$, $p<0.001$) in a recording with the largest coil displacement occurring in the second half of the session.

Rec. No.	Coil displacement			Correlation of MEP amplitude and coil displacement*			
	Distance, mm	Plane, °	Rotation, °		Distance	Plane	Rotation
1	0.81 (0.56)	1.66 (0.84)	1.72 (0.91)	r	0.113	-0.029	0.069
	1.91	2.02	2.12	p	0.072	0.650	0.274
2	1.45 (0.65)	1.71 (0.43)	1.77 (0.37)	r	**0.271**	**0.227**	0.123
	2.55	2.34	2.40	p	**<0.001**	**0.001**	0.087
3	2.09 (0.54)	2.08 (0.37)	3.39 (1.86)	r	**0.156**	**0.270**	**0.136**
	3.10	2.47	4.83	p	**0.014**	**<0.001**	**0.032**
4	0.98 (0.60)	1.36 (0.30)	2.34 (1.09)	r	0.061	-0.015	-0.156
	3.98	1.87	3.49	p	0.447	0.853	0.051
5	0.75 (0.32)	1.69 (0.31)	1.66 (0.26)	r	**0.185**	0.064	0.144
	1.34	2.15	2.04	p	**0.012**	0.393	0.127
6	2.81 (1.65)	1.61 (0.57)	2.06 (0.68)	r	0.107	**0.160**	0.073
	5.01	2.69	2.77	p	0.092	**0.012**	0.250
7	3.10 (3.56)	3.00 (1.51)	2.48 (1.03)	r	0.096	0.075	0.081
	5.23	4.24	3.33	p	0.132	0.241	0.207
8	1.15 (0.54)	2.92 (1.74)	3.19 (1.21)	r	0.059	-0.059	-0.051
	2.87	4.33	4.84	p	0.370	0.371	0.438
9	1.98 (3.83)	3.04 (2.47)	3.05 (2.09)	r	**0.390**	**0.427**	**0.282**
	7.24	6.28	4.72	p	**<0.001**	**<0.001**	**<0.001**
10	0.53 (0.22)	1.64 (0.26)	1.69 (0.28)	r	-0.068	-0.056	-0.082
	1.60	2.04	2.32	p	0.288	0.382	0.200

Table 4.2. Coil displacement and its association with MEP amplitude*. On the left side of the table, the summary statistics of coil displacement is presented for each recording (median (interquartile range) in the top row and maximum in the bottom row of each cell). On the right side of the table, correlations of coil displacement with MEP amplitude are presented (* traces with pre-stimulus activation removed from the analysis). Raw MEP and navigation data was used for analysis. r - Spearman's correlation coefficient; significant (p<0.05) correlations are in bold.*

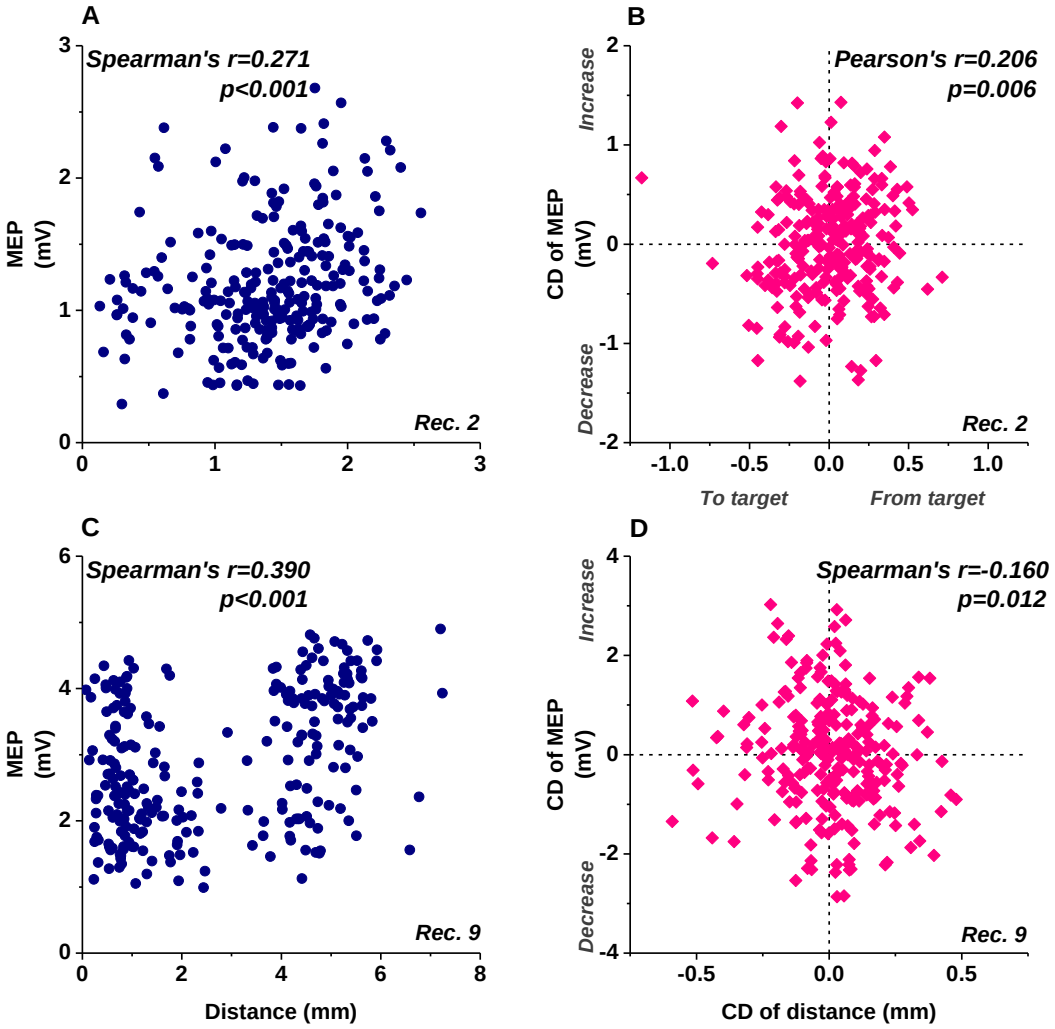

Figure 4.1. Relationship between coil displacement and trial-to-trial variability of MEPs. *Scatterplots of two recordings with significant correlations are presented (traces with pre-stimulus activation of the target muscle removed from the analysis). To assess the relationship between coil movements and MEP amplitude changes on a trial-to-trial basis, the consecutive differences (CD) were calculated for both parameters; negative CD of MEP values indicate decrease in amplitude compared to previous trial; negative CD of distance values – coil movement closer to the target compared to the previous trial. Correlation analysis suggests that MEP amplitude increased with increasing coil distance from the initial target in both recordings (A and C). Although statistical significance was reached on a trial-to-trial change basis (B and D), only a very small part of trial-to-trial variability of MEPs could be explained by coil movements and was negligible in practical terms. Note that the axes are scaled to individual parameters.*

4.2.1.4 Fluctuations in MEP amplitude

Averaging of several responses is used to overcome the trial-to-trial variability of MEPs (Rossini et al., 2015). To assess the within-session reliability of MEP estimates, every

15 responses were averaged resulting in 14-17 estimates per recording. The within-session variability (CV) of these estimates was 25 ± 5%. They showed excellent reproducibility (ICC(2,1) 0.862, 95% confidence intervals 0.721-0.964), but rather poor agreement (CR 0.92 mV). To assess for any slow underlying changes in MEP amplitudes or their trial-to-trial variability, the data was smoothed by calculating one-sided rolling averages for MEP amplitude (MEP_{avg}) and the absolute consecutive difference. Fifteen responses were used as this is the number of control MEPs that are usually averaged in paired-pulse TMS protocols in our lab. Similarly, rolling averages using 15 points were also calculated for the coil displacement data (Figure 4.2).

During the course of the recording, the coil displacement from the initial target was gradually increasing, but no systematic change in MEP_{avg} occurred on a group level (Figure 4.3). There was no difference in the initial MEP_{avg} (the first 15 responses) and the overall average MEP (all responses) amplitudes or their trial-to-trial variability expressed as MCD (1.15 ± 0.73 vs 1.25 ± 0.72 mV, paired-sample t-test, t=-0.95, df=9, p=0.365 and 0.57 ± 0.34 vs 0.55 ± 0.22 mV, paired-samples t-test, t=0.30, df=9, p=0.768, respectively). However, individual recordings showed slow cyclic fluctuations in averaged MEP amplitude which appeared to be independent from the changes in coil position (Figure 4.3). The mean group intra-individual difference between the smallest and the largest MEP_{avg} was 1.23 ± 0.6 mV (or 103 ± 21% of the overall MEP_{avg}), and the MEP_{avg} amplitude fluctuated between 62 ± 19% to 177 ± 28% of the subject's initial MEP_{avg} during the course of the recording. In all subjects, the deviation of MEP_{avg} from the initial average was statistically significant (as indicated by non-overlapping 95% confidence intervals on visual inspection of plotted data) at least once during the 20-minute recording for intervals of tens of seconds to several minutes.

Figure 4.2. Representative recordings of MEPs (see the next page). *Data of three subjects is presented: A) – raw MEP amplitude; B) – absolute consecutive difference (CD); C) – smoothed MEP amplitude and mean consecutive difference (prior rolling average of 15 responses); D) – smoothed coil displacement data (prior rolling average of 15 data points). Black triangles in A) and B) indicate traces with pre-stimulus activation; grey shaded areas in C) – 95% confidence interval for averaged MEP amplitude. In C), rolling averages were calculated using all consecutive MEPs (thick dark blue line) and CDs (thick pink line) as well as without traces contaminated by pre-stimulus activation (thin light blue line and thin brown line, respectively). The solid horizontal line indicates the average amplitude of the first 15 MEPs, dashed lines – its 95% confidence interval. Note that pre-stimulus activation does not have a significant impact on average MEP size and its fluctuations, even in a subject with relatively high proportion of traces contaminated by pre-stimulus activation (Rec. 4). Also, these fluctuations are not related to the changes in coil position, except for Rec. 9, in which increase in average MEP amplitude towards the end of the recording could be attributed to increasing distance of the coil from the initial target.*

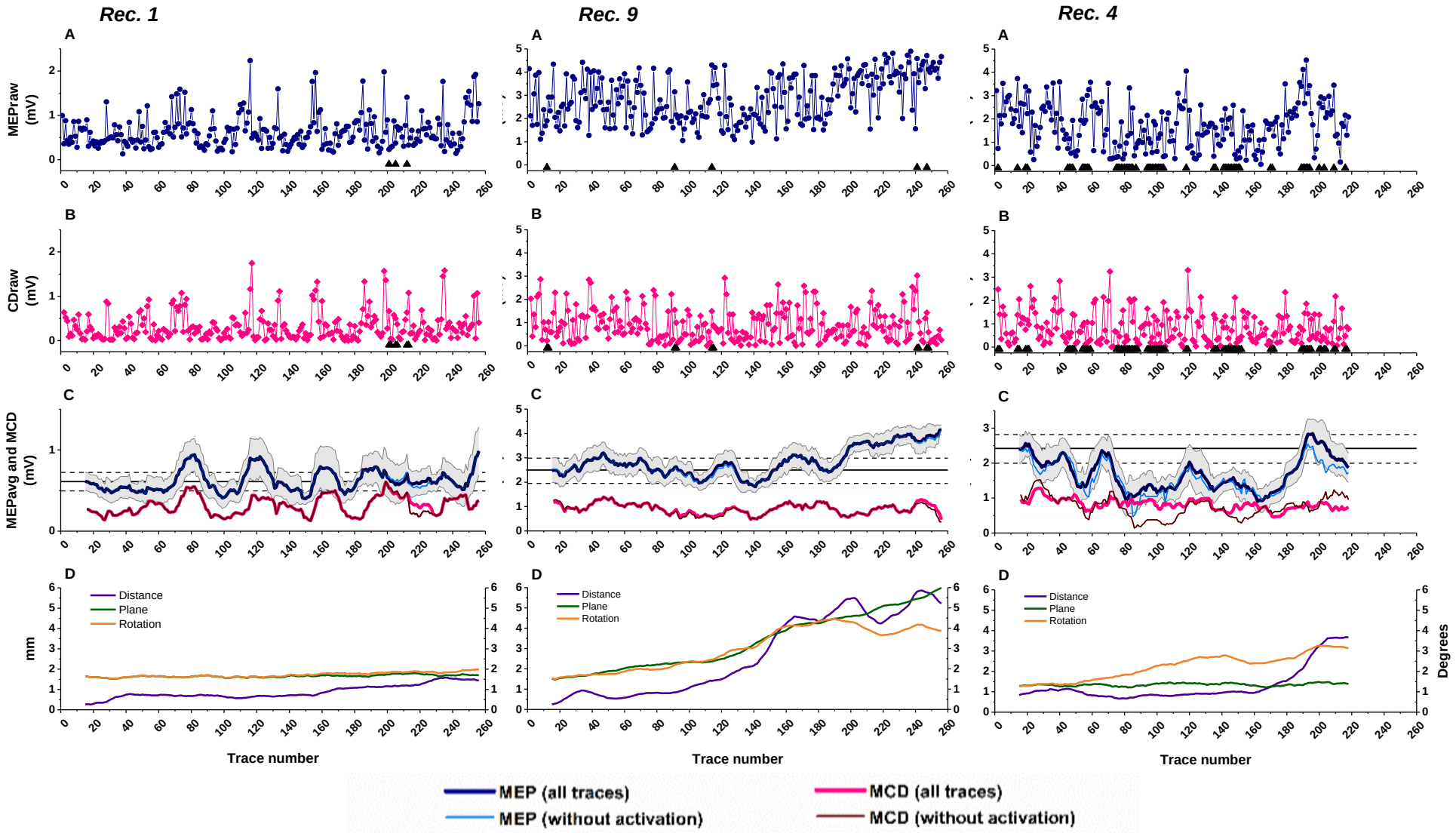

Figure 4.2. *For legend see the previous page*

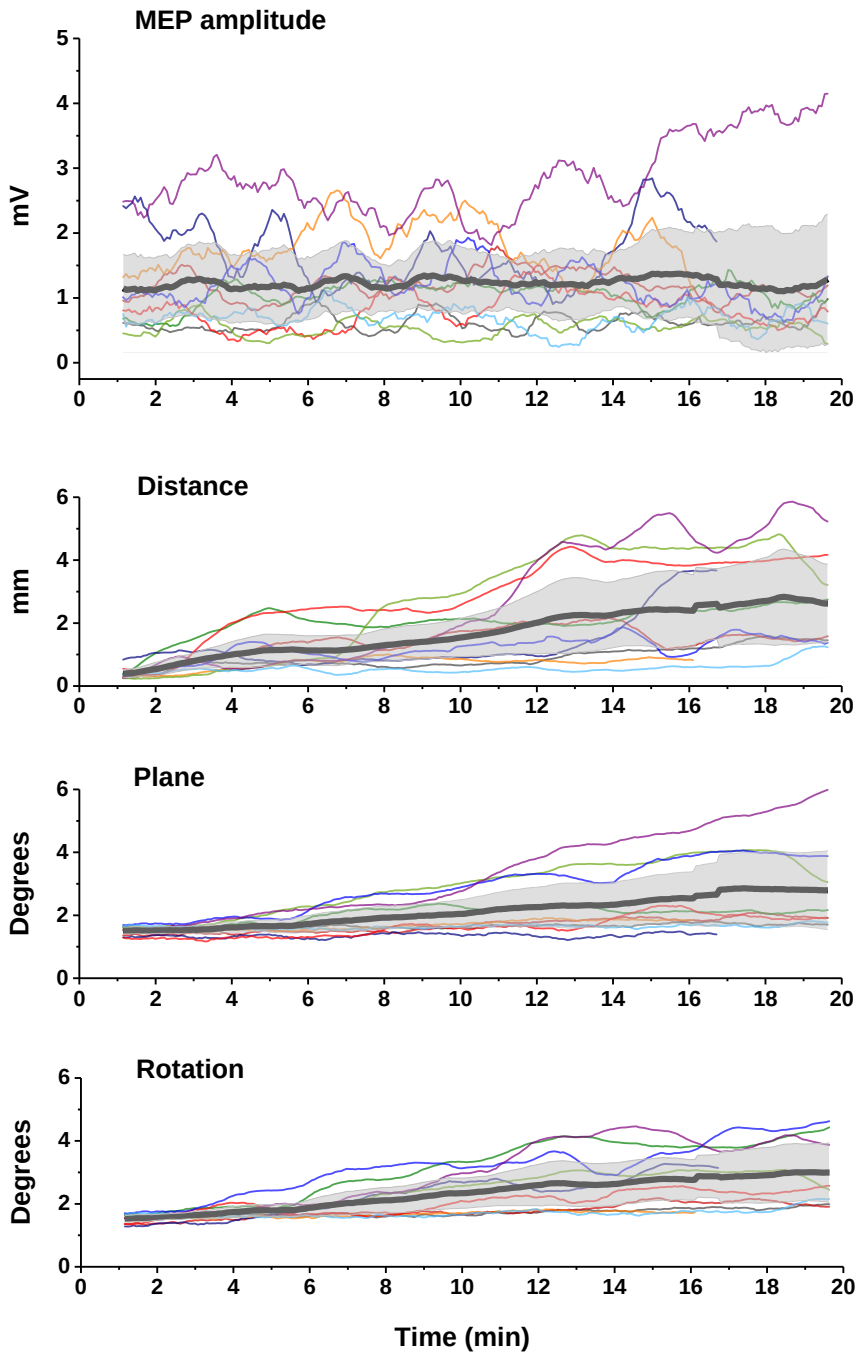

Figure 4.3. No systematic change in average MEP amplitude was observed during a 20-minute recording on a group level despite a gradual increase in coil displacement. *The thin lines represent smoothed data (a rolling average of 15 preceding trials), different colours indicate individual subjects. Thick black lines indicate the group mean, grey shaded areas – 95% confidence intervals. On the group level, a systematic change in coil position was observed in all three dimensions. Although the increase in coil displacement was statistically significant (average of first vs last 15 traces, Wilcoxon signed rank test, p=0.005), it was likely negligible in practical terms (mean change in distance 2.2 ± 1.4 mm, plane 1 ±1.4°, and rotation 1.4 ± 1°). No systematic changes in the average MEP amplitude were seen on a group level. However, slow cyclic fluctuations in average MEP amplitude were observed in individual recordings which appeared to be independent of the changes in coil position.*

To assess the underlying frequencies of the observed fluctuations, FFT analysis of smoothed MEP data was carried out. The power spectrum of individual recordings revealed two to five distinct peaks in extremely slow frequency range (0.001-0.01 Hz or a period of approximately 2-17 min/cycle; Figure 4.4). In seven recordings, the highest power was seen in the 0.001 Hz frequency band, which is equivalent to a cycle duration of roughly 17 minutes. Given that the whole recording lasted around 20 minutes, it is not possible to determine whether this represents periodic fluctuations in CSE or merely reflects the slow drifts occurring during the session. Superimposed on these very slow drifts, faster oscillations were observed. Across ten recordings, three additional frequency bands could be distinguished (Figure 4.8): ~0.0035 Hz (4.6 min/cycle), ~0.0055 Hz (3.1 min/cycle), and ~0.009Hz (1.9 min/cycle). FFT analysis of raw MEP data failed to reveal any underlying frequency.

In a group, there was a very strong positive linear correlation between subject's overall CV and nMCD (Pearson's $r=0.978$, $p<0.001$), but none of these variability parameters correlated with the overall average MEP amplitude (Pearson's $r=-0.601$, $p=0.066$ and $r=-0.530$, $p=0.115$, respectively). Within a subject, the MEP variability was not constant throughout the recording and fluctuated in a similar fashion as MEP amplitude (Figure 4.2). To assess whether fluctuations in MEP amplitude and variability occurred simultaneously, cross-correlation analysis was carried out with smoothed data using first order differencing to correct for autocorrelation of time series and a maximum of 15 lags. Cross-correlation peaks at 0 lag were assessed for measures of dispersion (standard deviation (SD) and coefficient of variation (CV)) and at -1 – 1 lag for measures of trial-to-trial variability (raw and normalised MCD). An increase in MEP_{avg} amplitude was associated with an absolute increase in both dispersion (SD; $r=0.171$-0.687, $p<0.05$) and trial-to-trial variability of MEPs (MCD; $r=0.219$-0.602, $p<0.05$) in all subjects, and there was a positive cross-correlation between the two variability parameters (SD vs MCD, $r=0.289$-0.613, $p<0.05$; CV vs nMCD, $r=0.233$-0.620, $p<0.05$). However, the relationship between the MEP_{avg} amplitude and relative variability of MEPs (i.e. CV and nMCD) differed between subjects.

***Figure 4.4. Fluctuations in average MEP amplitude (see the next page)**. In the left column, smoothed MEP amplitudes of individual recordings are presented. Blue lines indicate one-sided rolling averages of 15 MEPs; grey shaded area – 95% confidence intervals; black horizontal lines – subject's overall average MEP amplitude. In the right column, the corresponding frequency analysis data using smoothed MEP amplitudes is presented. To allow comparison between subjects, normalised data is plotted on the y axes: smoothed MEP amplitude expressed as percent of the subject's overall average MEP amplitude (left column); mean square amplitude of the power spectrum expressed as percent of the subject's largest power spectrum peak (right column).*

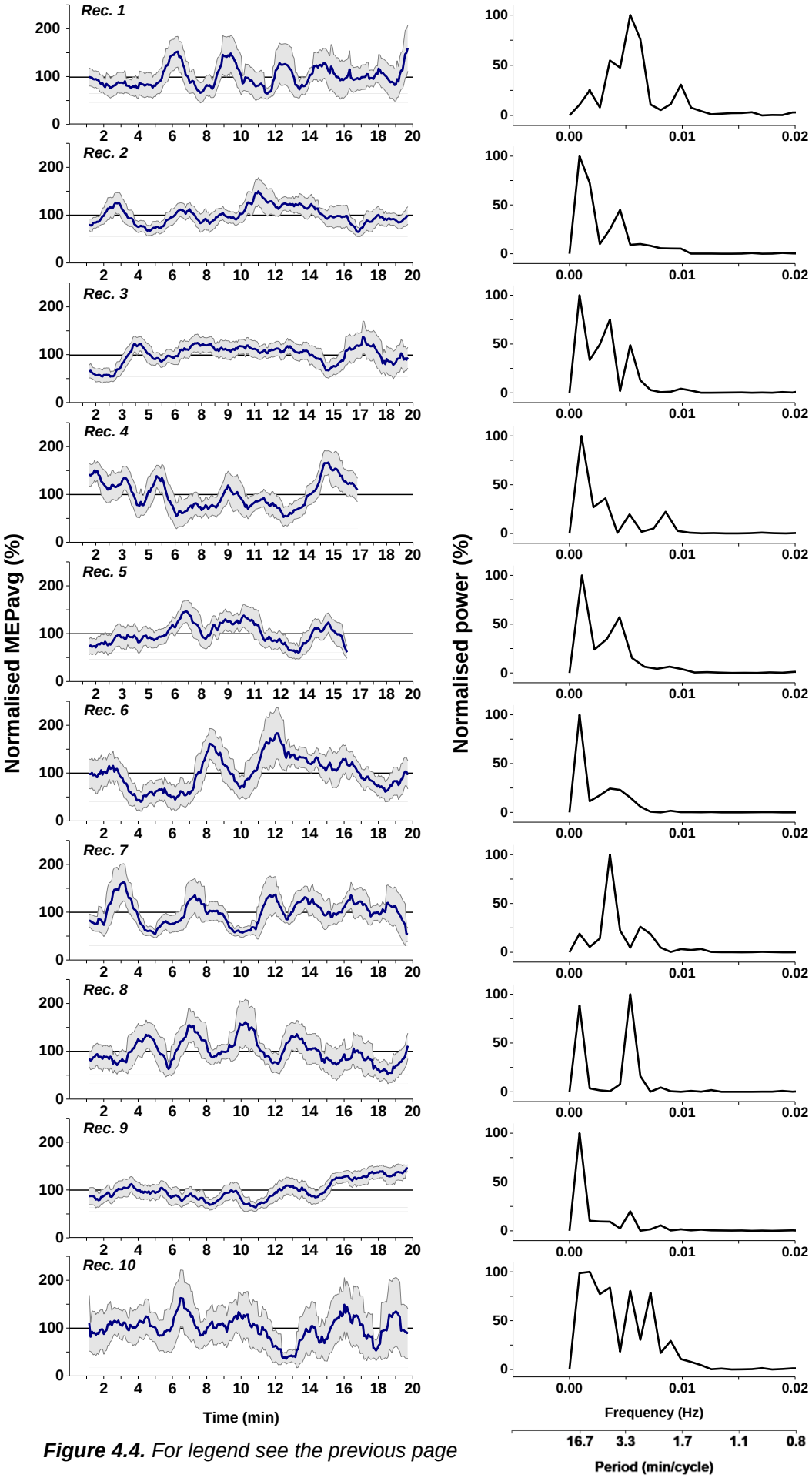

Figure 4.4. *For legend see the previous page*

No significant cross-correlations were found between MEP amplitude and CV in four subjects, and between MEP amplitude and nMCD in one subject. Higher MEP amplitudes were associated with lower relative dispersion (CV) in four subjects (cross-correlation r=-0.346 - -0.170, p<0.05), and the opposite was seen in two subjects (cross-correlation r=0.307-0.401, p<0.05). Meanwhile, relative trial-to-trial variability (nMCD) decreased as the MEP amplitude increased in six subjects (cross-correlation r=-0.496 - -0.246, p<0.05), and increase was observed in three subjects (cross-correlation r=0.460-0.535, p<0.05).

4.2.2 Resting motor threshold

No bias was observed between any of the mean group $RMT_{0.2mV}$ estimates simultaneously obtained as two independent samples (Table 4.3). Inspection of the raw recordings showed that thresholds measured on two channels were closely following each other for the greater part of the session (Figure 4.5). Given the lag between the values on two channels due to alternating stimulation, raw threshold values on channel 1 were correlated with a mean of two adjacent threshold values on channel 2. Significant correlations between the channels were seen in all recordings (Pearson's r=0.455-0.724, p<0.001), except for one (Pearson's r=0.124, p=0.170). Absence of correlation in this recording despite essentially overlapping raw thresholds (Rec. 2 in Figure 4.5) can be explained by a relatively high tracking noise in relation to the extent of slow $RMT_{0.2mV}$ changes. Overall, the strength of correlation between the two channels was inversely related to the ratio between the maximum tracking step (i.e. 2% MSO) and the extent of slow changes in $RMT_{0.2mV}$ (i.e. ΔRMTabs; Pearson's r=-0.882, p=0.001).

Parameter	**Channel 1**	**Channel 2**	**Paired-sample t-test**
$RMT_{initial}$, % MSO	47.2 ± 8.4	47.0 ± 8.1	t=0.68, df=9, p=0.516
$RMT_{overall}$, % MSO	46.6 ± 7.9	46.6 ± 7.8	t=-0.08, df=9, p=0.938
RMT_{min}, % $RMT_{overall}$	95.0 ± 2	94.2 ± 1	t=1.38, df=9, p=0.202
RMT_{max}, % $RMT_{overall}$	104.7 ± 3	105.5 ± 2	t=-1.53, df=9, p=0.162
ΔRMT_{abs}, % MSO	4.6 ± 2.6	5.3 ± 2.1	t=-1.43, df=9, p=0.187
ΔRMT_{rel}, % $RMT_{overall}$	9.6 ± 4	11.2 ± 3	t=-1.55, df=9, p=0.156

Table 4.3. Summary statistics of RMT recordings. *RMT was determined by threshold-tracking with the target size set at 0.2 mV; initial RMT estimate was calculated using the first six valid threshold estimates on each channel, overall RMT estimate – using all the traces in the recording.* RMT_{min} *and* RMT_{max} *– minimum and maximum smoothed RMT values in the recording (after achieving stable tracking on both channels);* ΔRMT_{abs} *and* ΔRMT_{rel} *– absolute and relative intra-individual difference between the maximum and minimum RMT estimates within a recording. No significant difference in mean group estimates was observed between the estimates obtained on two independent channels.*

4.2.2.1 Effect of pre-stimulus activation of the target muscle

The frequency of traces with pre-stimulus activation ranged from 0 to 32.4% across ten subjects and exceeded 10% in three recordings. However, the assessment of the importance of pre-stimulus activation of the target muscle for $RMT_{0.2mV}$ tracking is not straightforward due to the constantly changing stimulation intensity. The effect of the 'unintentional' activation of the target muscle on MEP amplitudes was negligible in this experiment. In threshold-tracking, sporadic pre-stimulus activation could potentially contribute to the measurement noise. However, longer periods of sustained activation would be required to observe the resulting decrease in RMT due to time needed for threshold-tracking to 'catch-up' with changes in CSE. No such pattern was seen in any of the recordings.

4.2.2.2 Changes in RMT and coil positioning

As in MEP experiment, the coil displacement from the initial target was largely negligible in most recordings. The distance from the initial target exceeded 5 mm in two recordings (up to 5.32 mm and 7.12 mm, respectively), the rotation from the initial coil handle position exceeded 5° in one recording (up to 7.88°), whilst the tilt from the initial plane remained under 4° in all recordings.

During the course of the recording, the coil displacement from the initial target was gradually increasing, although this shift was minimal in most recordings. No systematic change in $RMT_{0.2mV}$ occurred on a group level (Figure 4.6). There was no difference in the initial $RMT_{0.2mV}$ estimate (after the first six valid threshold estimates) and the overall $RMT_{0.2mV}$ estimate (the whole recording; rmANOVA with Channel and Duration as within-subject factors, $F_{1,9}=0.54$, $p=0.482$).

In individual recordings, slow changes (drifts and/or fluctuations) in $RMT_{0.2mV}$ where observed. To determine whether these changes were related to shifts in coil positioning, cross-correlations were calculated[14]. If the coil shift from the initial target is significant enough to result in changes in CSE, a delayed response would be seen in threshold as several steps may be required for the tracking algorithm to adapt to this change. $RMT_{0.2mV}$ recordings from channels 1 and 2 were analysed separately, the lag in cross-correlation analysis was limited to ± 15 traces per channel (approximately 2.3 minutes).

[14] Meaningless correlations may exist between two independent time series which are autocorrelated (Dean and Dunsmuir, 2016). Autocorrelation plots for $RMT_{0.2mV}$ and coil displacement variables were assessed for each recording. With only a few exceptions, all variables showed significant autocorrelations. To correct for this, first order differencing was used while calculating cross-correlations.

Given that the coil shifts were minimal in most recordings, cross-correlation peaks, if any, were expected to be seen with a lag of 2-3 traces.

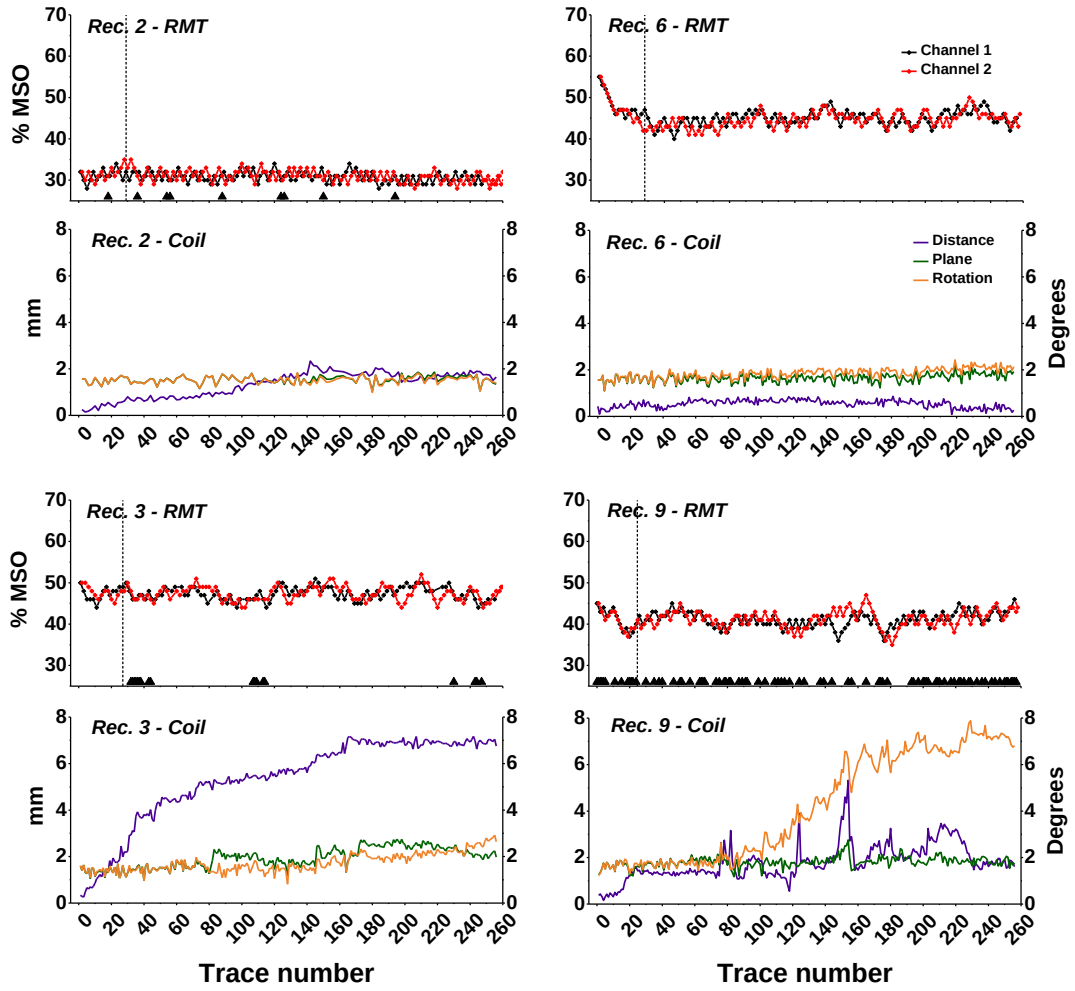

Figure 4.5. Thresholds tracked on two independent channels followed each other closely. *Raw $RMT_{0.2mV}$ (black and red lines) and coil displacement data (purple, green, and orange lines) of four representative recordings is presented. Black triangles indicate traces with pre-stimulus activation, vertical dotted lines – time of the recording when stable tracking was achieved on both channels (i.e. at least six valid threshold estimates observed). In the coil displacement graphs, the left-handed y axis indicates coil displacement from the initial target, the right-handed y axis – coil tilt and rotation from the initial target. The $RMT_{0.2mV}$ recordings on channels 1 and 2 followed each other closely throughout the greater part of the sessions, and slower changes in opposite directions were rarely seen (e.g. Rec. 9). Slow fluctuations in $RMT_{0.2mV}$ could be seen over the course of most recordings, irrespective of whether the coil position was stable during the recording (Rec. 8) or clear coil shifts were seen (Rec. 3 and 9). Changes in $RMT_{0.2mV}$ did not appear to be time-locked to the changes in coil position or associated with pre-stimulus activation of the target muscle.*

Statistically significant positive and negative cross-correlation peaks were seen in all recordings with at least one coil positioning variable across the whole range of both positive (coil shift precedes change in RMT) and negative (change in RMT precedes coil shift) lags. However, they were all weak (r= -0.276-0.376, p<0.05) and the lags at which they were seen on channels 1 and 2 in the same recording were highly inconsistent. Thus, the observed changes in $RMT_{0.2mV}$ were highly unlikely to result from coil displacement.

In some recordings, $RMT_{0.2mV}$ seemed to fluctuate in a cyclic manner. FFT analysis of smoothed $RMT_{0.2mV}$[15] showed two to six peaks in the infra-slow frequency range (Figure 4.7). In most recordings, the highest power was seen in the 0.001 Hz frequency band (~17 min/cycle), reflecting slow drifts in threshold occurring during the session. Across twenty $RMT_{0.2mV}$ recordings (combined from channels 1 and 2), peaks at 0.0035-0.0045 Hz (~3.7-4.8 min/cycle) and 0.007 Hz (~2.4 min/cycle) were more common (Figure 4.8). Despite good correlation between the thresholds obtained on two independent channels, the FFT peaks were not entirely congruent between them. This likely reflects the fact that although threshold-tracking is less sensitive to the trial-to-trial variability of MEPs, it still introduces some degree of measurement error.

Figure 4.6. No systematic change in RMT was observed during a 20-minute recording on a group level despite a gradual increase in coil displacement (see the next page). *The thin lines represent raw data (RMT data from channel 1; different colours indicate individual subjects). Thick black lines indicate the group mean, grey shaded areas – 95% confidence intervals; vertical dotted line – time of the recording when stable tracking was achieved in all subjects (i.e. at least six valid threshold estimates observed). On the group level, a significant change in coil position was observed in distance and rotation from the initial target (Wilcoxon signed rank test, p=0.017 and p=0.013, respectively), but it was likely negligible in practical terms (median change in distance 0.9 (IQR 1.1) mm, rotation 0.4 (IQR1.2) degree). No systematic changes in $RMT_{0.2mV}$ were seen on a group level.*

[15] To eliminate the noise related to tracking, smoothing of $RMT_{0.2mV}$ recordings was carried out using a one-sided prior rolling average of ten stimuli. This number was chosen based on the data from previous $RMT_{0.05mV}$ experiment, which deemed tracking until six valid threshold estimates are obtained to be sufficient to obtain a reliable RMT estimate. Once stable tracking was achieved (after the first six valid threshold estimates), each subsequent six cycle interval required approximately ten stimuli. Our observations showed that once tracking is stable, the estimates obtained by regression analysis and by simple arithmetic average differ minimally, by decimal points. For practical reasons, data was smoothed using a rolling average.

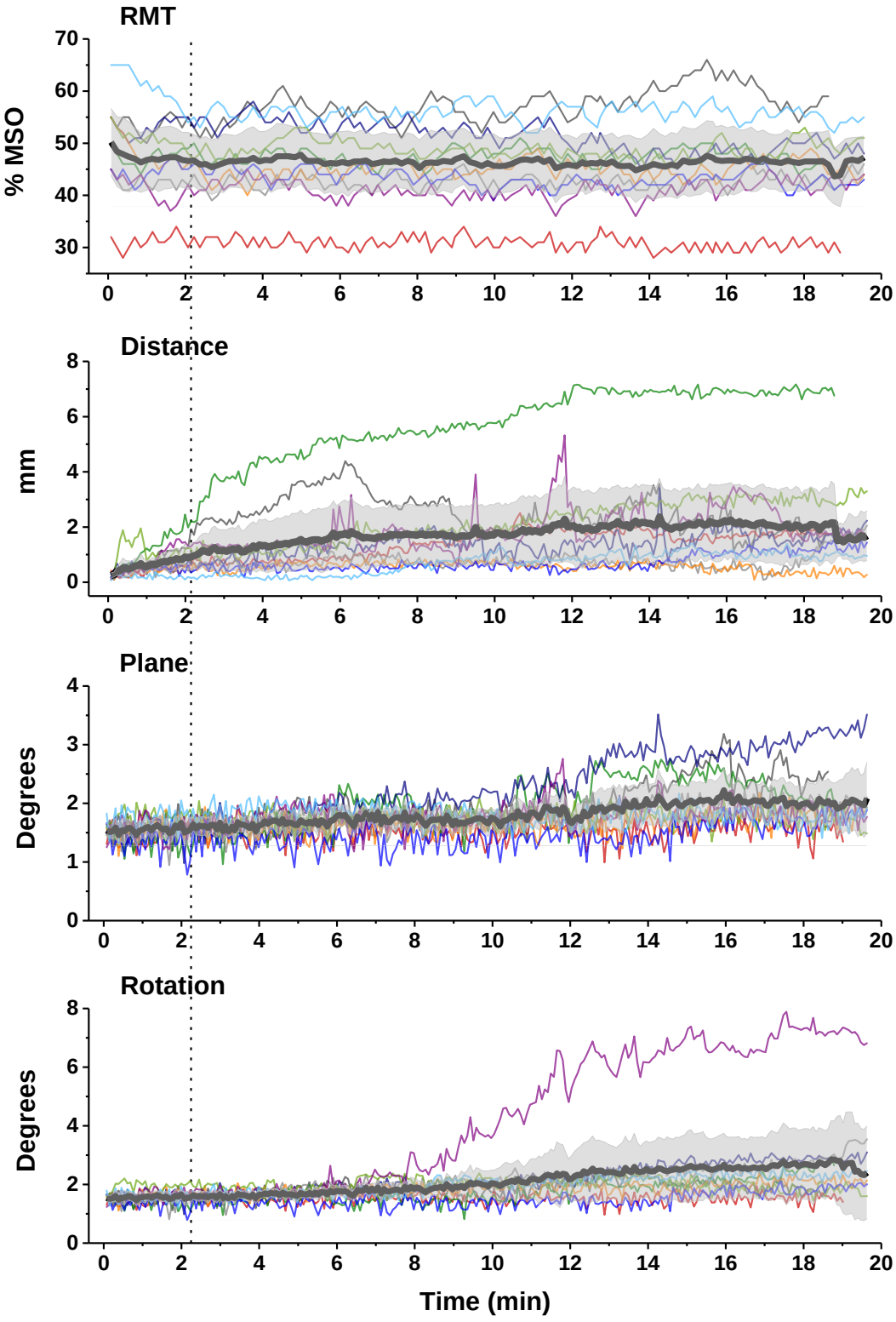

Figure 4.6. *For legend see the previous page*

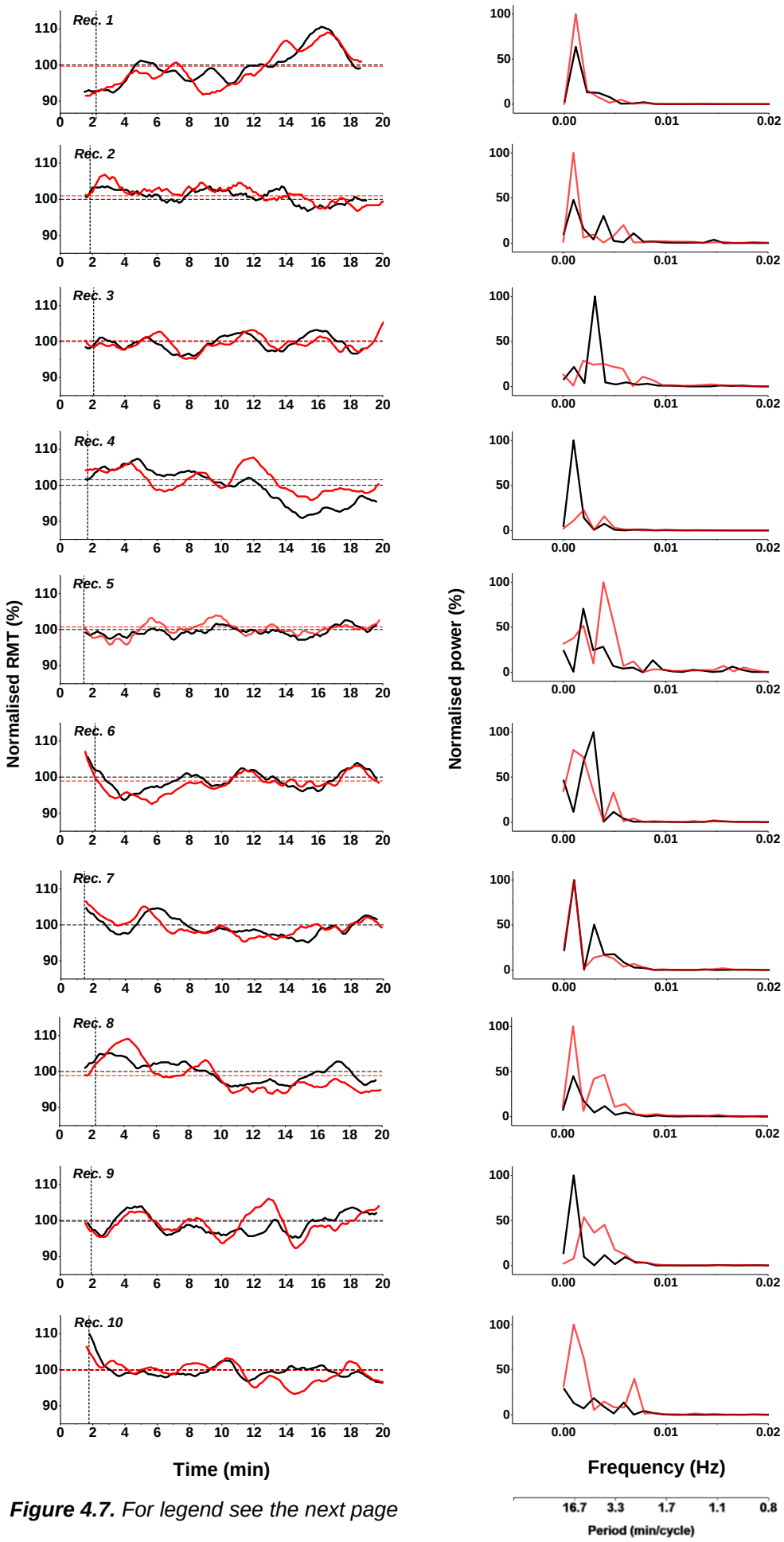

Figure 4.7. *For legend see the next page*

4.3 Discussion

In summary, there was no significant difference in the trial-to-trial variability between the MEP amplitude and area. In a group, both average MEP amplitude and $RMT_{0.2mV}$ remained stable throughout the 20-minute recording despite gradual increase in coil displacement from the initial target. However, slow cyclic fluctuations were observed in individual recordings. They were more prominent for MEPs than $RMT_{0.2mV}$ and were unrelated to the changes in coil position.

4.3.1 Coil positioning and corticospinal excitability parameters

In this experiment, there was no association between MEP size and coil positioning. In a group, the mean MEP_{avg} amplitude remained constant throughout the recording despite the gradual increase in coil displacement. This suggests that, although statistically significant, the magnitude of the mean group coil displacement at the end of the session (increase by ~2 mm in distance, 1° in tilt, and ~1.5° in rotation compared to the first minute of the recording) was negligible in practical terms. Indeed, more prominent shifts of the coil from the optimal target were reported in the previous studies which demonstrated a significant effect of coil position on MEP size (Gugino et al., 2001, Julkunen et al., 2009, Grey and van de Ruit, 2017).

Significant correlations between raw MEP amplitude and coil displacement parameters were found in half of individual recordings, however, they were mainly weak. The somewhat unexpected finding was that these correlations were positive, i.e. MEP amplitude increased as the coil shifted away from the initial target. There are several possible explanations for this: either the motor hotspot determined at the beginning of the recording was suboptimal, or both changes in MEP amplitude and coil displacement were caused by other factors.

***Figure 4.7. Fluctuations in threshold (see the previous page)**. In the left column, smoothed $RMT_{0.2mV}$ of individual recordings is presented. Black (channel 1) and red (channel 2) solid lines indicate one-sided rolling averages of ten stimuli; horizontal dashed lines – subject's overall $RMT_{0.2mV}$ estimate, dotted vertical line - time of the recording when stable tracking was achieved on both channels (i.e. at least six valid threshold estimates obtained). In the right column, the corresponding frequency analysis data using smoothed $RMT_{0.2mV}$ is presented. To allow comparison between subjects, normalised data is plotted on the y axes: smoothed $RMT_{0.2mV}$ expressed as percent of the subject's overall $RMT_{0.2mV}$ estimate on channel 1 (left column); mean square amplitude of the power spectrum expressed as percent of the subject's largest power spectrum peak across both channels (right column). The frequency peaks were congruent between channels 1 and 2 across the whole range in two subjects only (Rec. 5 and Rec. 8).*

The coil was clamped during the experiment, therefore, any changes in coil position in fact reflect the changes in subject's head position. Staying completely still for twenty minutes would not be an easy task for most individuals, and head movements are more likely to occur if the subject starts feeling drowsy or uncomfortable. Thus, trying to keep the head in the same position or prevent further shift using the visual feedback from the navigation system may require additional effort from the subject which in turn may increase the CSE excitability and MEP amplitude.

Very similar observations were made in the RMT experiment. Across the subjects, the motor threshold remained stable throughout the course of the recording despite the gradual increase in the distance and rotation of the coil from the initial target, but the coil displacement in this part of the experiment was even smaller than in the MEP sessions. Cross-correlation analysis of individual recordings did not reveal any meaningful associations between the coil position and $RMT_{0.2mV}$, even in the recordings with the largest coil shifts. This is consistent with the previous reports which showed no significant change in RMT with coil distance of approximately 10 mm (Julkunen et al., 2009) and rotation up to 15° (Meincke et al., 2017) from the optimal target.

4.3.2 Trial-to-trial variability of MEPs

Variability of MEPs is commonly quantified using coefficient of variation (CV; Kiers et al., 1993, Ellaway et al., 1998, Stedman et al., 1998, Rösler et al., 2008, Julkunen et al., 2009, Jung et al., 2010), and only a few studies also report mean consecutive difference (MCD; Kiers et al., 1993, Rösler et al., 2008). CV, expressed as the ratio of the standard deviation to the mean, determines the relative dispersion of the MEP size across multiple measurements. Meanwhile MCD, which is the mean absolute difference between successive measurements, provides information on the magnitude of its change from trial to trial (to allow comparison between the subjects, MCD normalised to individual's average MEP amplitude is used (Rösler et al., 2008)). The comparison between the two parameters may provide useful information on the variations in MEP amplitude. Combination of low nMCD and high CV would suggest that MEP amplitude is more stable at short intervals compared to its changes over time. In such instance, it would be more rational to record control and conditioned MEPs sequentially instead of randomising their order in paired-pulse TMS protocols.

The MEP variability in this experiment falls within the range of previously reported (Kiers et al., 1993, Ellaway et al., 1998, Rösler et al., 2008, Julkunen et al., 2009, Jung et al., 2010). There was a very strong positive association between CV and nMCD in a group. CV was significantly higher than nMCD in this sample, but the actual difference between the two measures was relatively small. This suggests that although MEP amplitudes

varied more over the course of the recording than between successive stimuli, the major part of the dispersion of MEP size was due to its trial-to-trial variability. This is further supported by positive cross-correlations between the two variability parameters in time series analysis. These observations suggest that randomisation is appropriate in paired-pulse TMS protocols.

In this study, trial-to-trial variability of MEPs could not be explained by small shifts in coil position from stimulus to stimulus or unintentional activation of the target muscle – the two factors that are probably easiest to control for in TMS experiments. This is consistent with the previous reports. Ellaway et al. (Ellaway et al., 1998) found that clamping the coil did not reduce the variability of MEPs compared to hand-held coil sessions, and substantial variability of MEP amplitudes remains despite markedly improved spatial accuracy of stimulation using navigation systems (Julkunen et al., 2009).

Given the contribution of the desynchronisation of corticospinal volleys to the variability of MEPs (as discussed in section 1.2.2 of Chapter 1), one would expect that MEP area instead of amplitude would provide a more robust measure. In this study, the variability of MEP area was slightly, but not significantly, higher than that of amplitude. This is consistent with the previous observations (Kiers et al., 1993, Magistris et al., 1998, McDonnell et al., 2004) and could be explained by the repetitive discharges of α-motoneurons in response to magnetic stimuli which would largely contribute to the area, but not peak-to-peak amplitude measurements (Hess et al., 1987a, Magistris et al., 1998).

A technical factor that could potentially contribute to MEP variability, but has not been adequately studied, is fluctuations in the actual coil output. For example, Magstim 200[2] operating manual states that the accuracy of the stimulator output is ±1% of the voltage at a particular stimulus intensity level (Magstim, 2015). This would be equivalent to ±0.5% MSO range at 50% MSO intensity level if the coil is connected to the BiStim module as in this experiment. Such fluctuations in the actual coil output may contribute to the trial-to-trial variability of MEPs, especially in subjects with a very steep stimulus-response slope, and the degree to which this technical variability may affect TMS measurements remains to be elucidated.

4.3.3 Fluctuations in corticospinal excitability

While in a group the CSE parameters remained stable despite increasing coil displacement, slow changes in MEP_{avg} amplitude and $RMT_{0.2mV}$ were observed in individual recordings. During a 20-minute recording, the average MEP amplitude fluctuated within a range of approximately 100% of the initial MEP_{avg}, while changes in

$RMT_{0.2mV}$ were less prominent constituting roughly 10% of the initial estimate. Importantly, these changes in CSE parameters were unrelated to the shifts in coil position and often had a cyclic appearance (Figure 4.4 and Figure 4.7). The reports on the periodicity of MEPs are very scarce and mainly limited to single example recordings (Kiers et al., 1993, Wassermann, 2008). No reports are available on periodic fluctuations in RMT (traditional estimation methods would prevent detection of such changes), but they can be seen in published raw recordings from threshold-tracking experiments (Vucic et al., 2006). Kiers and colleagues did not identify any underlying dominant frequencies in the recordings of 300 consecutive MEPs (Kiers et al., 1993), but they used raw data which is likely to introduce noise into the FFT analysis due to large trial-to-trial variability of MEPs (in the example recording in Figure 6 of their paper, trial-to-trial variation of MEPs appears to be superimposed on slow fluctuations of its average size). For this reason, smoothed data was used for the frequency analysis in this experiment.

Extremely slow fluctuations of MEP_{avg} and $RMT_{0.2mV}$ in the 0.001-0.01 Hz range were observed in this experiment. In most recordings, the highest power frequency peaks were seen in the 0.001 Hz band (~17 min/cycle), but the recordings were way too short to determine whether this represents true cyclic changes in CSE. Across all recordings, several additional peaks, often of much lower power, clustered at 0.0035-0.0055 Hz (or roughly 4 min/cycle) and 0.007-0.009 Hz (~2 min/cycle) frequency bands. In most

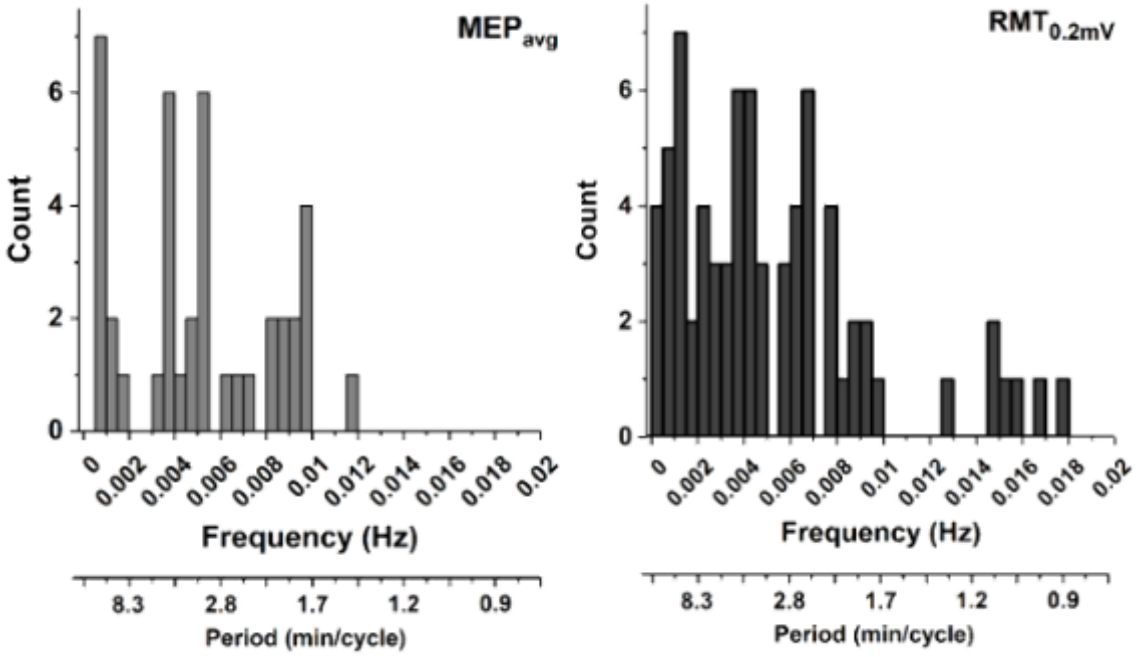

Figure 4.8. The distribution of the frequency power spectrum peaks across MEP_{avg} and $RMT_{0.2mV}$ recordings. *The histogram bin size 0.0005 Hz. For $RMT_{0.2mV}$, combined data from channels 1 and 2 is presented.*

recordings, the CSE fluctuations had rather irregular periods (which is reflected by multiple FFT peaks of similar power), but there were some with very regular periodicity (e.g. Rec. 1, 7, and 8 in Figure 4.4, Rec. 3 in Figure 4.7).

What could these fluctuations in CSE represent? Do they reflect intrinsic changes in the excitability of the motor system itself or more extensive brain networks or are they secondary due to other physiological processes? This experiment was not designed to answer any of the questions, and only speculations can be made based on the literature. It is unlikely that they represent plasticity changes induced by the stimulation itself, as on a group level both MEP_{avg} and $RMT_{0.2mV}$ were stable throughout the recording. Infra-slow fluctuations (<0.1 Hz) have been observed in various neurophysiological, neuroimaging as well as behavioural studies and were proposed to reflect quasi-periodic fluctuations in the excitability of brain networks that determine brain-state dynamics (Palva and Palva, 2012). However, frequencies below 0.01 Hz were rarely reported in this context.

Contribution of cardiac and cerebrovascular factors may also be considered. Very low frequency (0.003-0.03 Hz) fluctuations in R-R interval variability have been observed and linked to the parasympathetic outflow (Taylor et al., 1998, Tripathi, 2011). Spontaneous oscillations in cerebral haemodynamics of 0.1 Hz and 0.04 Hz have been reported (Obrig et al., 2000), and even slower fluctuations in microcirculation at around 0.01 Hz and 0.005-0.0095 Hz were found to be mediated by endothelial factors (NO-dependent and NO-independent, respectively; Kvandal et al., 2006). It is well known that cerebrospinal fluid (CSF) dynamics is influenced by cerebral blood flow, and while CSF flow was found to be primarily affected by the heart rate, very slow frequencies were more prevalent in fluctuations of the venous sinus size (0.008-0.05 Hz; Strik et al., 2002) and the subarachnoid space width (0.005-0.0095 Hz; Gruszecki et al., 2018). CSF distribution was found to be of major importance in the location of peak electric fields induced by a magnetic pulse (Bijsterbosch et al., 2012). Thus, fluctuations in CSF thickness could potentially modulate the electric field strength in the grey matter. Changes in subject's state may also have contributed to the observed fluctuations, but this is less likely given that they were required to concentrate on the visual feedback from the navigation system. Finally, external source of the observed fluctuations, such as stimulator output, cannot be entirely ruled out.

It is important to note that the frequency analysis in this experiment has certain limitations. Firstly, the range of frequencies that could be detected (up to 0.11 Hz for MEPs and up to 0.05 Hz for RMTs) was limited by the rate of magnetic stimulation. Secondly, rolling average was used to smooth the data; this acts as a filter for frequencies faster than 1/t, where t is the period over which the data was averaged (Owens, 1978; >0.014 Hz for MEPs and >0.011 Hz for RMTs in this study). Sale and

colleagues found a stronger autocorrelation of subsequent MEPs obtained at 1 Hz stimulation rate compared to stimulation at 5 or 20 second intervals (Sale et al., 2016). Although higher stimulation rates would potentially allow detection of faster fluctuations, in practice this approach is limited by the induction of plasticity changes (Pascual-Leone et al., 1994). The main aim of this study was to determine the variability of the estimates that are used for paired-pulse TMS protocols in our lab (i.e. average amplitude of 15 MEPs and $RMT_{0.2mV}$ obtained from six valid threshold estimates), therefore impact of different data smoothing approaches was not explored, and only tentative FFT analysis was used to roughly approximate the periodicity of the visually observed fluctuations of CSE.

An interesting observation in this experiment is that the MEP variability was not static and fluctuated in a similar fashion to the MEP size. In all subjects, MEP_{avg} amplitude was positively cross-correlated with absolute variability of MEPs (i.e. SD and MCD), but the relationship between MEP amplitude and its relative variability (CV and nMCD) in the time series analysis differed between subjects. In about half of them, the MEP_{avg} amplitude fluctuations were relatively more prominent than fluctuations in its variability, while in the rest no significant relationship was found, or the opposite was observed. The cause and significance of these findings are unclear, but they probably reflect the large inter-individual variability of the response to magnetic stimulus which is common across the whole range of TMS measurements.

4.3.4 Conclusions

Slow cyclic fluctuations in individual's corticospinal excitability occurring over a 20-minute recording session is the key finding of this experiment. They were not related to changes in coil position which were minimal when the coil was clamped, and subjects were provided with a visual feedback from the navigation system to help maintain their head position.

What are the practical implications of this finding? On a group level, they are unlikely to have a significant impact, as no systematic changes in either MEP_{avg} or $RMT_{0.2mV}$ occurred during the 20-minute recording. However, in an individual they are likely to contribute to the measurement noise and high intra-individual variability of TMS parameters limiting their use for individual decision making. It also suggests that caution should be taken while classifying subjects into responders and non-responders in interventional trials based on individual change in RMT or MEP amplitude. Currently there is no consensus on the number of MEPs that should be recorded to obtain a reliable CSE estimate. The IFCN guidelines recommend obtaining at least 8-10 MEPs (Rossini et al., 2015). However, several reports suggest that as much as 20-30 MEPs are required

to achieve a ‘steady state’ (Schmidt et al., 2009) and obtain an accurate and reliable estimate of CSE (Chang et al., 2016, Bashir et al., 2017). Slow fluctuations of CSE may also be problematic in paired-pulse TMS protocols, particularly if multiple conditions are explored resulting in prolonged recordings. Findings of this study underscore the importance of simultaneous recording and pseudorandomisation of the control and conditioned responses. Furthermore, conditioning stimulus intensities are commonly set as a fraction of RMT, which was also found to fluctuate considerably in this experiment. Conventional paired-pulse TMS paradigms use constant stimulation intensities, thus the pre-set conditioning stimulus intensity may become suboptimal for eliciting inhibition or facilitation during a prolonged recording and thus contribute to the variability of paired-pulse TMS outcomes. Threshold-tracking paradigms allow continuous monitoring of RMT and online adjustments for its changes. This could potentially improve the reliability of paired-pulse TMS parameters.

Chapter 5 - Comparability of conventional and threshold-tracking techniques for short-interval intracortical inhibition (Experiment 3)[16]

Conventional paradigms for short-interval intracortical inhibition employ a constant stimulus approach in which stimulation intensities are maintained fixed throughout the recording and inhibition is quantified by a relative reduction in motor response amplitude (Kujirai et al., 1993). By contrast, stimulation intensity is dynamically adjusted in threshold-tracking and inhibition is measured as a relative increase in threshold required to maintain a constant response (Fisher et al., 2002, Vucic et al., 2006).

The main advantage of the conventional technique is that the vast majority of knowledge on the physiology and pharmacology of SICI as well as its impairment in pathological conditions has been obtained with this method. However, conventional SICI measurements have poor test-retest reliability (Beaulieu et al., 2017) which is an important factor limiting their use, particularly for individual decision making in clinical practice.

Threshold-tracking TMS is emerging as a useful diagnostic test (Menon et al., 2015a). However, its reliability has not been formally tested[17]. Although similar phenomena have been described with both methods (as discussed in section 1.3.2), conventional and threshold-tracking SICI measurements as well as their reliability have not been directly compared.

This experiment tests the hypothesis that threshold-tracking paradigms which are less susceptible to trial-to-trial variability of MEPs and can adapt to the naturally occuring fluctuations in corticospinal excitability can reduce the variability of SICI and would therefore be a more reliable test for both clinical practice and research.

5.1 Methods

5.1.1 Subjects

Twelve healthy volunteers (6 men; median age 30 years, age range 23-52 years) with no known neurological disorder or contraindications for TMS and not taking any CNS acting medication completed the full set of recordings. Four subjects were excluded due

[16] This chapter is based on a previously published work (Samusyte et al., 2018).

[17] At the time of the experiment, no such data was available in the literature.

to inability to maintain relaxation of the target muscle (n=1) and incomplete recordings resulting from overheating of the stimulation coil (n=3).

5.1.2 Experimental setup

The experimental setup and TMS procedure were largely as described in sections 2.2 and 2.3 of Chapter 2. Surface EMG was recorded from a relaxed right FDIO muscle. Magnetic stimuli were delivered via a figure-of-eight coil hand-held over the left hemisphere so that they induced posterior-to-anterior current flow in the motor cortex. Stimuli were delivered at 4.1 s intervals. Visual feedback from surface EMG was provided for the subjects on a screen in front of them to help maintain the relaxation of the target muscle.

5.1.3 Stimulation protocols and experiment design

SICI at an ISI of 2.5 ms and CS intensities of 50, 60%, 70%, and 80% of RMT was measured using both conventional (A-SICI) and threshold-tracking (T-SICI) methods which were described in section 2.6 of Chapter 2.

Tracking parameters used in this experiment are summarised in Table 5.1. To ensure comparable CS intensities between the techniques, resting motor threshold was defined by threshold-tracking with the target set at 0.2 mV ± 20% ($RMT_{0.2mV}$) for both paradigms. For A-SICI, the test stimulus intensity was set to evoke a MEP of a peak-to-peak amplitude of approximately 1 mV (TS_{1mV}) and was defined by threshold-tracking. CS and TS intensities were then maintained fixed and 15 MEPs were obtained for each condition in a pseudorandom order.

For T-SICI, $RMT_{0.2mV}$ served as a control condition as was tracked throughout. CS intensities were continuously adjusted depending on the change in $RMT_{0.2mV}$ so that they remained as a constant fraction of $RMT_{0.2mV}$. Threshold-tracking was used to determine the test stimulus intensities required to maintain a target response of 0.2 mV when preceded by CS (further referred to as conditioned thresholds). Single and paired stimuli for each condition were delivered pseudorandomly and tracking was stopped automatically when the stopping rule was met.

Tracking parameter	$RMT_{0.2mV}$	TS	T-SICI
Target level	0.2 mV	1 mV	0.2 mV
Tracking mode	Proportional		
Maximum tracking step	2% MSO	5% MSO	5% MSO
Starting intensity	Suprathreshold used for hotspot	$RMT_{0.2mV}$	$RMT_{0.2mV}$
Stopping rule	After six valid threshold estimates		

Table 5.1. Threshold-tracking parameters used in Experiment 3. *Larger tracking step was chosen for TS_{1mV} and T-SICI to allow faster estimation of these parameters. One valid threshold estimate = one hit or cross of the target line by MEP.*

The automated stimulation protocol included both A-SICI and T-SICI which were recorded over the same motor hotspot defined at the start of the session (Figure 5.1). To control for a period effect, two stimulation protocols were employed: 1) T-SICI, followed by A-SICI (TA), and 2) A-SICI, followed by T-SICI (AT). Subjects were pseudorandomly assigned to one of these protocols for the duration of the experiment. To assess the test-retest reliability, SICI measurements were made on two experimental days separated by at least one week (interday reliability) during a similar time of day. On each day, recordings were made twice (using the same sequence) with a short 10-minute break (intraday reliability). Between the same-day sessions, the TMS coil was replaced to prevent overheating, while position of the surface EMG electrodes remained unchanged. Hotspot was identified anew before each recording. All measurements were carried out by a single operator. SICI analysis were carried out offline as described in sections 2.6.1 and 2.6.2.

5.1.4 Statistical analysis

For normally distributed data (Shapiro-Wilk test, $p>0.05$), parametric tests were used for comparisons between groups (Student's t-test) and repeated measurements (paired-sample t-test). To assess whether SICI was different from control condition, one sample t-test was used. Non-parametric tests (Wilcoxon signed rank test, Mann-Whitney U-test, Friedman's test) were used for non-normally distributed data.

Repeated measures analysis of variance (rmANOVA) was used: i) to compare CSE parameters between the sessions (4 levels) and ii) to explore the effect of CS intensity on SICI (4 levels). Pearson's correlation coefficient (r) was used to determine association between A-SICI and T-SICI. The relationship between measurements was considered very weak if $r <0.2$, weak if $0.2< r <0.39$, moderate if $0.4< r <0.59$, strong if $0.6< r <0.79$, and very strong if $r \geq 0.8$ (Evans, 1996).

Between-subject coefficient of variation (CV) was calculated for each session (within-session standard deviation/mean*100%), while within-subject CV was calculated for each subject (across-session standard deviation/mean*100%). Test-retest reliability of TMS parameters was assessed as described in section 2.8.

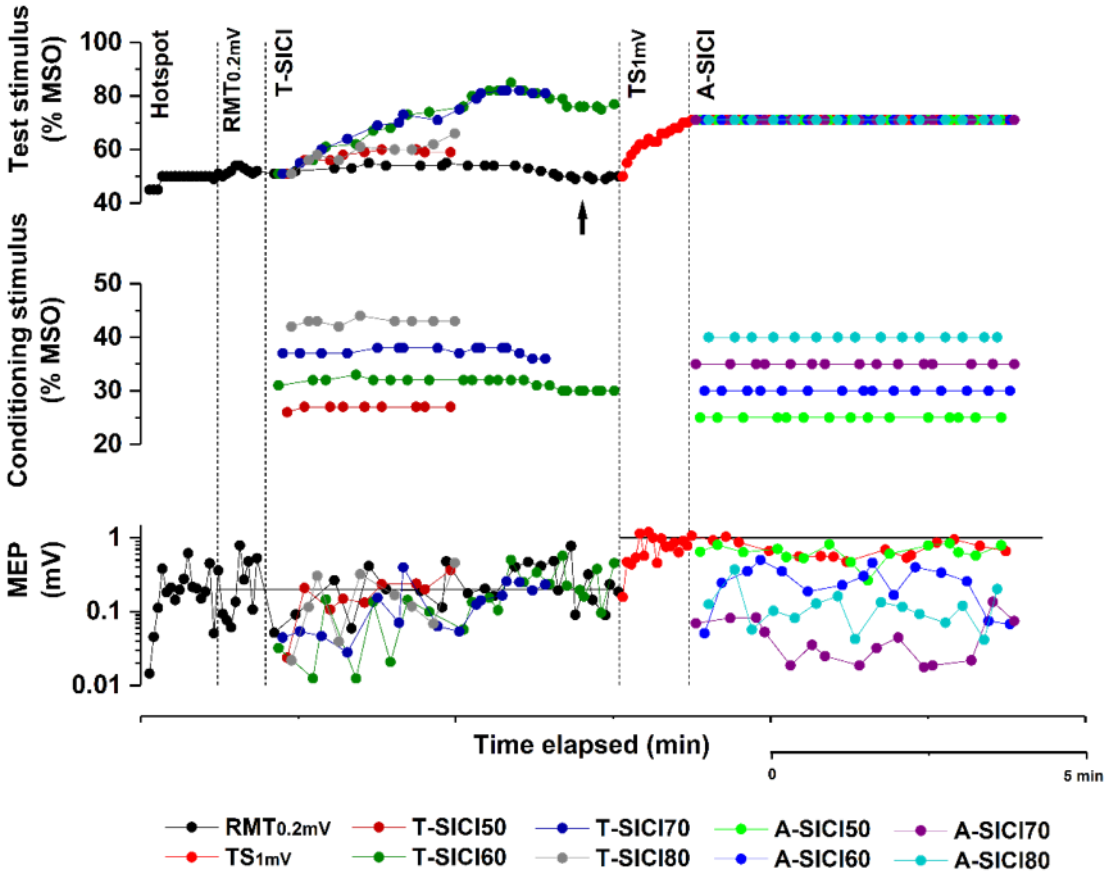

Figure 5.1. Illustration of the automated stimulation protocol in a single subject. *After finding the hotspot, the following parameters were recorded (separated by vertical dotted lines): resting motor threshold ($RMT_{0.2mV}$); SICI using constant response (T-SICI) and constant stimulus (A-SICI) techniques at conditioning stimulus intensities of 50-80% $RMT_{0.2mV}$. The target response of 1 mV (TS_{1mV}) was determined by tracking to this target and this value was then used for the entire A-SICI protocol. Test stimulus intensity (top), conditioning stimulus intensity (middle), and MEP amplitude (bottom) were recorded throughout the protocol for each condition (indicated by different colours). All stimulation intensities were adjusted automatically by the QtracS software, thus enabling a single operator to carry out the whole recording without having to reposition the TMS coil or to manually adjust the intensity of the stimuli. Horizontal solid lines (bottom graph) represent target MEP size: 0.2 mV for $RMT_{0.2mV}$ and T-SICI, 1 mV for TS_{1mV} and A-SICI. Note that $RMT_{0.2mV}$ drifts in T-SICI part (indicated by an arrow) and conditioning stimulus intensities are adjusted accordingly, whereas similar compensations are not possible in A-SICI part. When A-SICI was preceded by T-SICI, $RMT_{0.2mV}$ from T-SICI part was used to set the conditioning stimulus intensities for A-SICI part. Figure reproduced from Samusyte et al., 2018.*

Data is presented as mean ± standard deviation (SD) or as median and interquartile range of the 25th and 75th percentile (IQR), if not normally distributed.

5.2 Results

5.2.1 Parameters of corticospinal excitability and their reliability

The interval between the two experimental days ranged from 1 to 14 weeks (median interval of 2 (IQR 2) weeks). Across four sessions, mean $RMT_{0.2mV}$ was 48.6 ± 6.7 % MSO. The mean TS_{1mV} intensity was 57.6 ± 9.4% MSO (or 118.4 ± 10.6% $RMT_{0.2mV}$) and produced a control test MEP of 1.16 ± 0.25 mV. No significant difference was observed between the sessions in the mean group $RMT_{0.2mV}$ and TS_{1mV} (rmANOVA, $F_{1.7,18.2}$=1.38, p=0.274 and $F_{3,33}$=0.51, p=0.681, respectively). No difference in these measurements was observed between the sexes (Student's t-test, t=-0.30-0.14, df=10, p≥0.769) or between the TA and AT sequences of the recording protocol (Student's t-test, t=-0.17-0.51, df=10, p≥0.624).

The variability and reliability of CSE parameters are summarised in Table 5.2.

Parameter		**$RMT_{0.2mV}$**	**TS_{1mV}**	**Test MEP**
Variability (CV)	Between-subject	15 ± 2%	17 ± 4%	35 ± 6%
	Within-subject	6 ± 3%	7 ± 4%	27 ± 12%
Reproducibility (ICC)	Intraday	0.935 (0.793-0.981)	0.915 (0.715-0.975)	0.154 (-0.142-0.555)
	Interday	0.678 (0.226-0.894)	0.725 (0.288-0.912)	0.254 (-0.267-0.693)
	Interday averaged	0.811 (0.487-0.941)	0.750 (0.322-0.922)	0.112 (-0.492-0.631)
Repeatability (CR/SDC)	Intraday	5.5% MSO	10% MSO	1.21 mV
	Interday	11% MSO	14% MSO	0.98 mV
	Interday averaged	8.5% MSO	15% MSO	0.87 mV

Table 5.2. Variability and reliability of corticospinal excitability parameters. *CV – coefficient of variation (calculated across four sessions); ICC – intraclass correlation coefficient (ICC(2,1) model values are presented; 95% confidence intervals are indicated in brackets); CR/SDC – coefficient of repeatability/smallest detectable change. Data from Run 1 and Run 2 were used to assess intraday reliability, Run1 and Run 3 – interday reliability; average of two subsequent measurements made on the same day was used to calculate interday averaged reliability. Test MEP represents mean amplitude of 15 responses obtained at TS_{1mV} intensity,*

5.2.2 SICI recruitment curve

All SICI measurements averaged across four sessions were normally distributed (Shapiro-Wilk test, p>0.05), except for T-SICI at CS 60% $RMT_{0.2mV}$ where one subject consistently showed strong inhibition above 40% $RMT_{0.2mV}$ (Figure 5.7). No significant difference was observed between the sessions in mean group A-SICI and T-SICI (Figure 5.2), neither at individual CS intensities, nor peak or combined slope measurements (rmANOVA, F=0.21-0.99, p≥0.410, Friedman's test, p≥0.849).

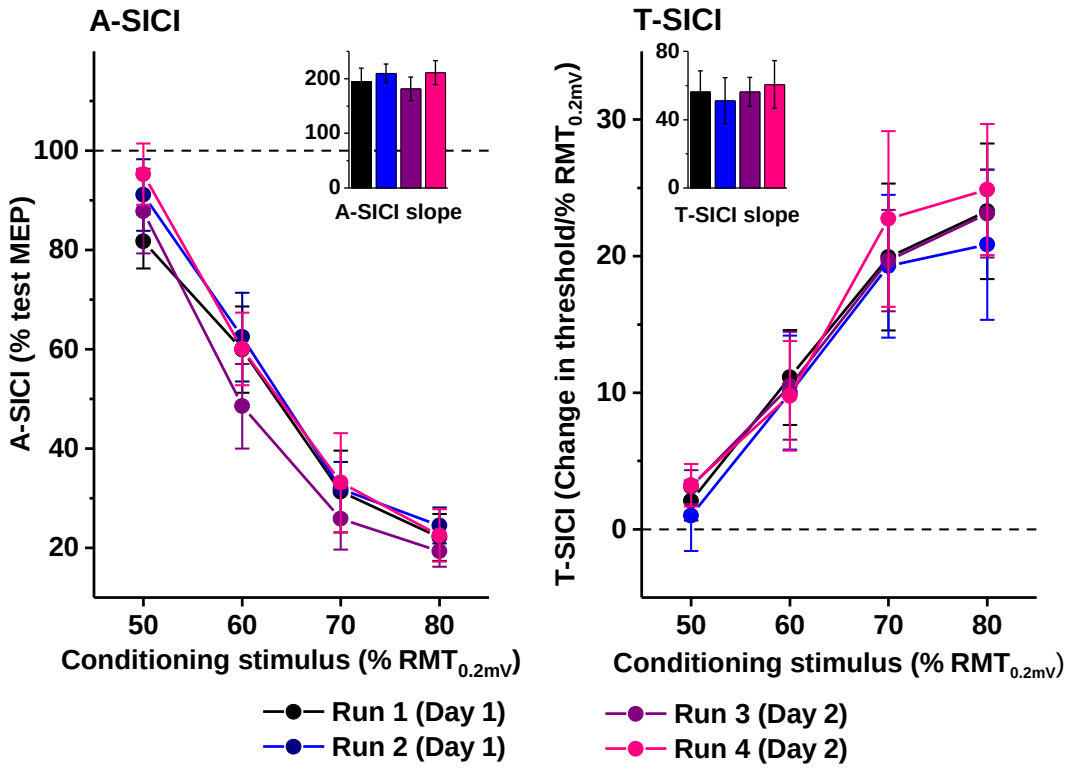

Figure 5.2. SICI recruitment curve. *No significant difference was observed between the sessions in both mean group A-SICI and T-SICI, neither at any single conditioning stimulus intensity, nor combined slope (inset) measurements (rmANOVA, p≥0.410; Friedman's test, p≥0.849). Dotted lines indicate the test condition (100% test MEP for A-SICI, 0% $RMT_{0.2mV}$ for T-SICI), error bars represent standard error of the mean. Figure reproduced from Samusyte et al., 2018.*

A main effect of CS intensity on inhibition was observed with both techniques (A-SICI rmANOVA $F_{3,33}$=76.25, p<0.001; T-SICI rmANOVA $F_{1.6,15.5}$=19.39, p<0.001, one outlier[18] with strong T-SICI at CS 60% RMT removed). Post hoc pairwise comparisons showed

[18] To identify outliers, boxplots were constructed for SICI measurements averaged across four sessions. Averaged but not single-session values were chosen due to considerable within-subject variability. For the purpose of this study, subjects who had extreme data points with values greater than 3 box-lengths from the edge of the box were considered as significant outliers.

that with both methods SICI differed between all CS intensity levels ($p \leq 0.004$), except for CS 70% and 80% $RMT_{0.2mV}$ ($p \geq 0.069$).

In approximately two thirds of the recordings, peak inhibition was observed at CS 80% $RMT_{0.2mV}$ (28 and 32 out of 48 recordings for A-SICI and T-SICI, respectively) and only rarely at CS 60% $RMT_{0.2mV}$ (in 3 out 48 recordings with each technique). The CS intensity level which resulted in peak inhibition was the same in 33 out of 48 recordings showing a fair agreement between the techniques within the same recording session (Cohen's kappa 0.389, $p=0.001$). However, the CS intensity level at which peak SICI was observed had poor agreement between the sessions with either of the methods, irrespective of whether the recordings were obtained on the same or different experimental days (Cohen's kappa 0.068-0.304, $p \geq 0.074$).

There was no significant difference in average SICI measurements between men and women (Student's t-test, t=-0.68-0.42, df=10, $p \geq 0.657$; Mann-Whitney U-test, $p \geq 0.818$) or TA and AT protocol sequences (Student's t-test, t=-1.76-0.98, df=10, $p \geq 0.109$; Mann-Whitney U-test, $p=0.394$).

5.2.3 Comparability of the two techniques

To assess correlations across all the sessions, SICI data was pooled. Because an ordinary correlation coefficient is not appropriate for repeated measurements (Bland and Altman, 1994), 'between-subjects' and 'within-subject' correlations were calculated. The 'between-subjects' correlation indicates whether individuals who have a strong inhibition measured by conventional method (A-SICI) also show a strong inhibition if threshold-tracking is used (T-SICI). For this purpose, SICI measurements were averaged across sessions for each individual and correlated. As all individuals had an equal number of observations, no weighting was required (Bland and Altman, 1995a). There were significant negative correlations between the two techniques at peak SICI and SICI slope (Pearson's $r=-0.847$, $p<0.001$ and $r=-0.665$, $p=0.018$, respectively) and all but 60% $RMT_{0.2mV}$ CS intensities (Figure 5.3). On a group level, the relationship between mean SICI recruitment curves obtained by two different techniques was linear (Figure 5.4 A), but there was a considerable inter-individual variability (Figure 5.4 C). Across all subjects, a strong non-linear relationship between A-SICI and T-SICI slopes was seen (Figure 5.4 B), which could be explained by the 'floor' effect observed in the conventional method at strong inhibition levels.

The 'within-subject' correlations show whether an increase in A-SICI was associated with an increase in T-SICI within the same individual. For this purpose, the procedure

proposed by Bland and Altman (Bland and Altman, 1995b) was used[19]. Weak negative 'within subject' correlations were observed between A-SICI and T-SICI measurements at CS 70% $RMT_{0.2mV}$ (r= -0.371, p=0.024) and CS 60% $RMT_{0.2mV}$ (r= -0.323, p= 0.051).

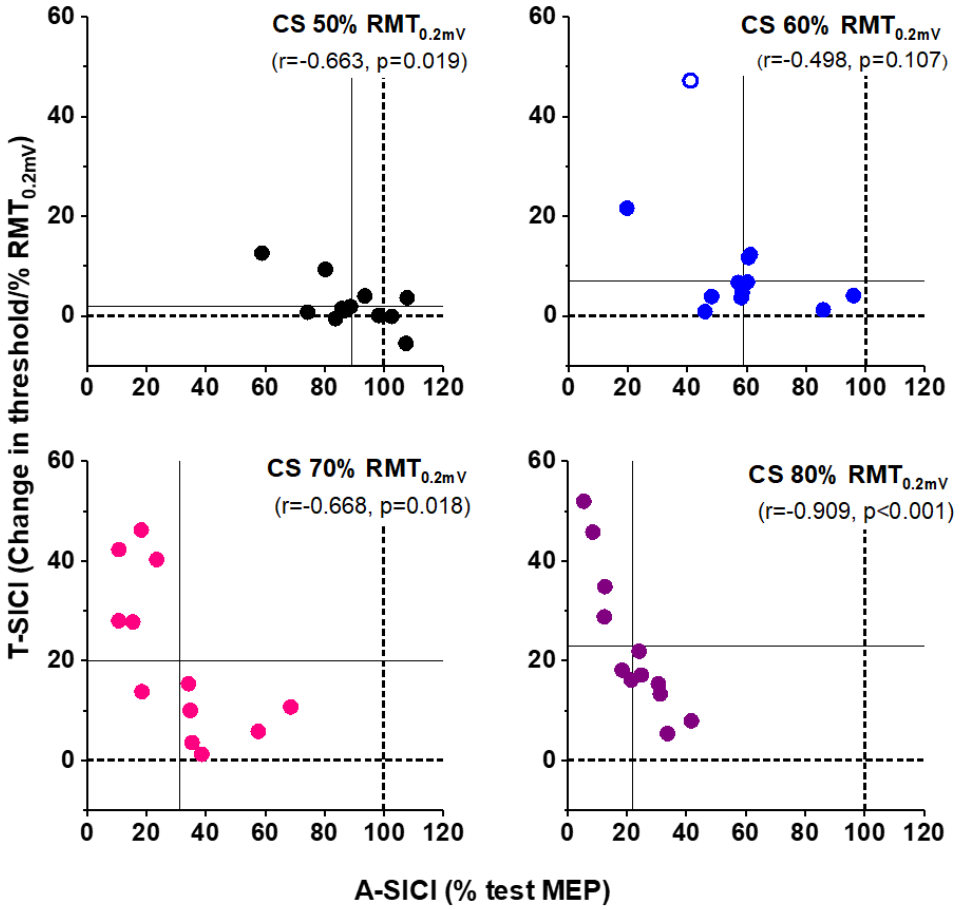

Figure 5.3. Comparability of A-SICI and T-SICI at individual conditioning stimulus intensities. *The scatter plots demonstrate the relationship between SICI obtained by conventional technique (x axis) and threshold-tracking (y axis). Circles indicate SICI averaged across four sessions for each subject, open circle indicates an outlier. Different colours indicate conditioning stimulus (CS) intensities; dotted lines represent the test conditions (100% test MEP for A-SICI, 0% $RMT_{0.2mV}$ for T-SICI), solid lines – group means. Pearson's correlation coefficients were calculated for average SICI. SICI conditions at CS intensity of 50, 70, and 80% $RMT_{0.2mV}$ showed significant strong negative linear correlations. At CS 60% $RMT_{0.2mV}$, the correlation improved when an outlier (open circle) was removed from the analysis (r=-0.581, p=0.061). Figure reproduced from Samusyte et al., 2018.*

[19] To partition the variability in parameters due to different sources, analysis of covariance (ANCOVA) was performed with T-SICI as a dependent variable, A-SICI as an independent variable and Subject as a fixed factor. The sum of squares (SS) for A-SICI and residual sum of squares were used to calculate the coefficient of determination (r^2) using formula [$SS_{A\text{-}SICI}/(SS_{A\text{-}SICI} + SS_{residual})$], and the correlation coefficient (r) was obtained by taking a square root of this proportion. The sign of this correlation coefficient was taken from the regression slope for A-SICI (coefficient B in Parameter estimates table) as well as the p value.

There was no significant 'within-subject' relationship between other SICI parameters obtained by the two techniques. This suggests that within an individual, A-SICI and T-SICI measures obtained at the same CS intensity may represent interaction of different neuronal pools.

Both conditioning and test stimulus intensities are important in determining the size of SICI (Ilic et al., 2002, Garry and Thomson, 2009, Vucic et al., 2009). Raw CS intensities (in % MSO) used in this experiment did not differ between the two techniques at any of the SICI conditions (paired-sample t-test, t=-1.64 - -1.03, df=11, p≥0.129), while this was not the case for test stimulus. Conditioned thresholds reached with threshold-tracking at CS 50-60% $RMT_{0.2mV}$ were significantly lower than the TS_{1mV} intensity used for A-SICI (absolute difference of 7 ± 4.7% MSO and 3 ± 2.9% MSO, respectively; paired sample t-test, t=3.40-5.08, df=11, p≤0.006). Thresholds at CS 70-80% $RMT_{0.2mV}$ were 2 ± 5.5% and 3 ± 9.4% MSO higher than TS_{1mV} intensity used in the conventional technique, but this was not significant (paired sample t-test, t=-1.24 - -1.17, df=11, p≥0.242).

Figure 5.4. Relationship between A-SICI and T-SICI recruitment curves (see the next page). *Scatter plots demonstrate the relationship between mean SICI recruitment curves obtained by conventional technique (x axis) and threshold-tracking (y axis). Dotted lines indicate control conditions (100% test MEP for A-SICI, 0% $RMT_{0.2mV}$ for T-SICI). A) shows a strong linear relationship between the mean group SICI obtained by the two techniques. Group means were obtained by averaging SICI at matching conditioning stimulus levels (indicated by colours and labels: black/CS50 - CS 50% $RMT_{0.2mV}$; blue/CS60 - CS 60% $RMT_{0.2mV}$; pink/CS70 - CS 70% $RMT_{0.2mV}$; purple/CS80 - CS 80% $RMT_{0.2mV}$; dashed line – linear fit, error bars – standard error of the mean). B) shows a non-linear relationship between individual SICI means averaged across four sessions for each subject. Individual SICI recruitment curves from C) were superimposed (symbols represent different subjects and correspond to symbols in C); colours – conditioning stimulus levels as in A); dashed line - the best fitting curve with two parameters satisfying the boundary conditions y=0 when x=100 and y=infinity when x=0 [y=a(100-x)+b(100-x)/x]). Fit further improved when an outlier (open symbol) was removed (y=9.94-0.127x+276/x, R^2=0.78). C) depicts the relationship between A-SICI and T-SICI varied among individuals over the same range of conditioning stimulus levels. Data points indicate SICI averaged across four sessions (symbols correspond to subject's symbol in B); colours and labels indicate conditioning stimulus levels as in A) and B); thick dotted line – fitted curve y=a(100-x)+b(100-x)/x). In almost half of the subjects, the relationship was near linear (e.g. a, d, l), while a 'floor effect' with conventional method was observed in others (e.g. b, e). In some subjects, no apparent correlation between A-SICI and T-SICI recruitment curves was seen (e.g. i). Figure reproduced from Samusyte et al., 2018.*

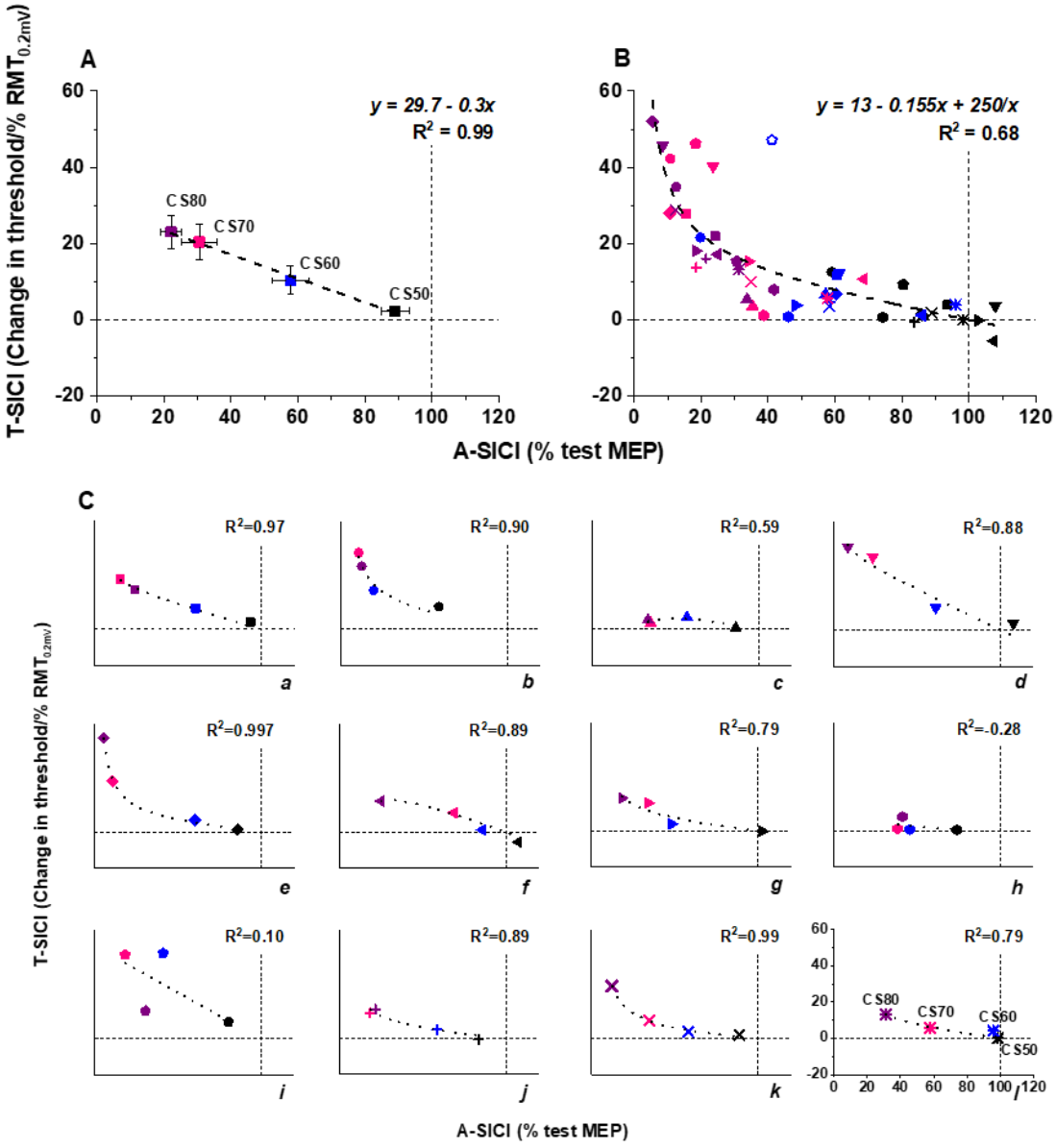

Figure 5.4. *For legend see the previous page*

5.2.4 Variability of SICI parameters

Coefficient of variation (CV) is a dimensionless measure of relative dispersion and can be used to compare the variability of parameters which are measured on different scales. Overall, the CVs of the commonly used SICI measures (i.e. % ratio of conditioned MEP to test MEP for A-SICI and % change in threshold for T-SICI) were high both between and within subjects (Table 5.3). However, certain drawbacks of this measure should be kept in mind. Firstly, it is only suitable to express the variability of data measured on a ratio scale. Therefore, CV is invalid if the mean is negative or equal to zero. Secondly, means that are close to zero result in large CVs and potentially overestimate the variability of the data. These limitations are important to be kept in mind while calculating and interpreting CVs for SICI, particularly if measures are obtained by threshold-tracking.

An illustrative example is presented in Figure 5.5.

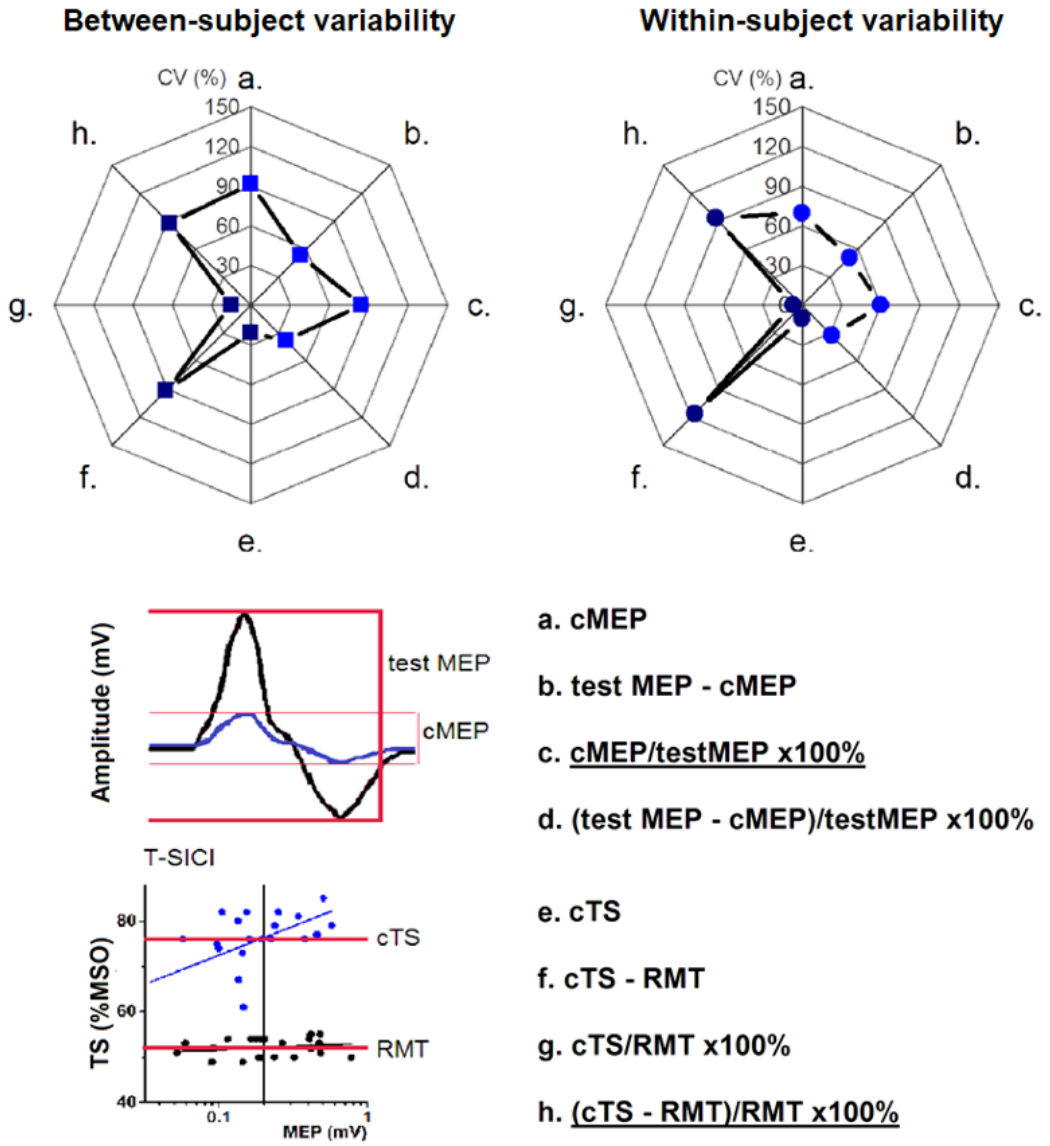

Figure 5.5. Variability of SICI depends on the expression method. *Between- and within-subject coefficients of variation of SICI at CS70% RMT$_{0.2mV}$ are presented in the top graphs (A-SICI in light blue, T-SICI in dark blue). In A-SICI, inhibition is reflected by reduction in MEP amplitude, while in T-SICI – by increase in threshold. This can be expressed in several different ways: using 1) raw values (a and e); 2) raw change from control condition (b and f); 3) percentage of control condition (c and g); and 4) percentage change from control condition (d and h). The commonly used ways of expressing A-SICI and T-SICI are underlined. When SICI is expressed in relation to control condition, the dispersion of the data (i.e. standard deviation) remains the same irrespective of whether it is calculated as percentage (c and g) or percentage change (d and h). However, this affects the mean of the data, biasing it towards higher (d and g) or lower (c and h) values and thus resulting in very different coefficients of variation. This is especially true for T-SICI, where some measurements can have negative values, bringing group mean values closer to zero.*

CS (%RMT)	Between-subject coefficient of variation (%)							
	A-SICI				T-SICI			
	Raw	Relative			Raw	Relative		
	cMEP	*MEP change*	*% ratio*	*% change*	*cTS*	*TS change*	*% ratio*	*% change*
50	37	99	23	103	17	227	5	241
60	72	84	50	75	22	118	11	108
70	106	47	91	42	23	101	16	94
80	92	34	74	21	18	75	14	74
CS (%RMT)	**Within-subject coefficient of variation (%)**							
	A-SICI				**T-SICI**			
	Raw	Relative			Raw	Relative		
	cMEP	*MEP change*	*% ratio*	*% change*	*cTS*	*TS change*	*% ratio*	*% change*
50	37	184	22	25	8	96	4	142
60	60	65*	45	95	7	175	4	152
70	70	51	60	32	10	116	7	93
80	56	35	48	14	8	44	6	43

Table 5.3. Between- and within-subject coefficients of variation were calculated for different ways of quantifying SICI*. A-SICI: cMEP = conditioned MEP amplitude (mV); MEP change = test MEP amplitude – conditioned MEP amplitude (mV); % ratio = conditioned MEP/test MEP*100%; % change = (test MEP-conditioned MEP)/test MEP*100%. T-SICI: cTS = conditioned threshold (% MSO); TS change = conditioned threshold – $RMT_{0.2mV}$ (% MSO); % ratio = conditioned threshold/$RMT_{0.2mV}$*100%; % change = (conditioned threshold – $RMT_{0.2mV}$)/$RMT_{0.2mV}$*100%. The commonly used SICI expression methods are underlined. *n=11 (an outlier with negative coefficient of variation excluded). CS – conditioning stimulus.*

5.2.5 Reproducibility of SICI measurements

Recordings from Day 1 (Runs 1 and 2) were used to assess the intraday reproducibility, and recordings from the first session of the experimental day (Runs 1 and 3) - interday reproducibility of SICI measurements.

Overall, SICI parameters obtained by threshold-tracking had adequate-to-excellent reproducibility, while most conventional measurements tended to have poorer ICCs (Figure 5.6). However, it is important to note that these differences did not reach statistical significance as the 95% confidence intervals for ICCs were wide and overlapping with both techniques.

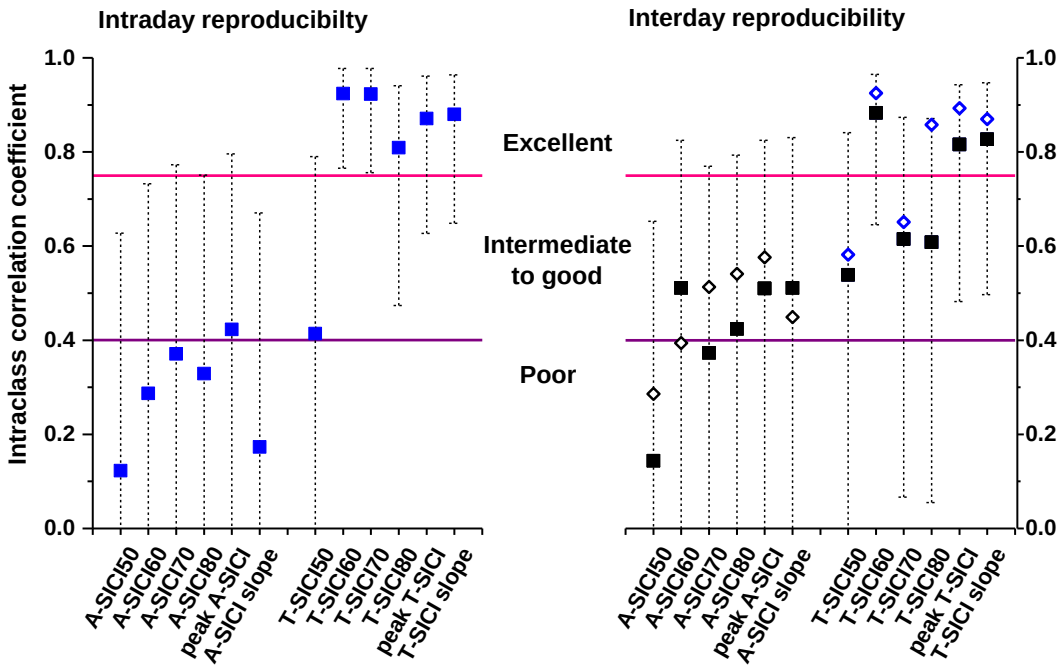

Figure 5.6. Reproducibility of SICI. *Recordings from Day 1 (runs 1 and 2) were used to assess the intraday reproducibility of SICI estimates and results of the first session on Days 1 and 2 (i.e. runs 1 and 3) – for interday reproducibility (filled squares). A-SICI had poor intra- and poor-to-adequate interday reproducibility, while T-SICI showed adequate-to-excellent intra- and interday reproducibility. Single measures [ICC (2,1)] model data is presented. Error bars represent 95% confidence intervals for intraclass correlation coefficients. Averaging two SICI estimates obtained on the same day (i.e. Runs 1 and 2 on Day 1, Runs 3 and 4 on Day 2) did not improve the interday reproducibility considerably (open diamonds), except maybe for T-SICI at CS 80% $RMT_{0.2mV}$. Figure reproduced from Samusyte et al., 2018.*

It is noteworthy that the sample heterogeneity should be considered while interpreting ICCs (Bartlett and Frost, 2008, Schambra et al., 2015, Brown et al., 2017). For example, the ICCs of T-SICI at CS 60% $RMT_{0.2mV}$ were among the highest when data of all subjects was used for calculation (Figure 5.6). However, when the measurement of one outlier with strong inhibition (more than 3 IQRs outside the boxplot) was removed from the analysis, the intraday ICC dropped from 0.924 to 0.776 and interday ICC – from 0.883 to 0.501, but the measurement error was not affected (Table 5.4). This illustrates the counter-intuitive aspects of the ICC that increase in this parameter does not necessarily mean an improved measurement error.

5.2.6 Repeatability of SICI measurements

Although there were no significant differences in mean group SICI estimates across experimental sessions, fluctuations over time were observed in individual subjects with up to 10-fold differences from the initial measurements (Figure 5.7). When these

estimates are used for decision making in an individual, it is important to know whether a change in the score is meaningful (e.g. due to treatment or disease progression) or whether it merely represents measurement noise.

ICC is a dimensionless estimate of relative reliability and does not provide information on the absolute differences between repeated measurements (Rankin and Stokes, 1998). This can be assessed using Bland-Altman plots (Bland and Altman, 1986) and coefficient of repeatability (CR) which is derived from the standard error of measurement (Table 5.4) and is equivalent to the smallest detectable change (SDC) indicating a true change in a test score beyond the measurement noise (de Vet et al., 2006, Schambra et al., 2015). SDC can be calculated not only for an individual, but also for a group (de Vet et al., 2011).

Overall, the agreement of repeated SICI measurements was poor (Table 5.4, Figure 5.8). For example, CR of 52% test MEP means that if initial measurement of A-SICI at CS 70% $RMT_{0.2mV}$ was 50% test MEP, a repeat measurement between 0 and 102% test MEP would not be considered a significant change. Similarly, if initial T-SICI at CS 70% $RMT_{0.2mV}$ was 20% $RMT_{0.2mV}$, a repeat measurement between 6 and 34% $RMT_{0.2mV}$ would reflect a measurement noise, not a true change. The intra- and interday repeatability was essentially similar for all parameters except for T-SICI at CS 70% $RMT_{0.2mV}$ (Table 5.4) and could not be considerably improved by averaging two same-day estimates (Samusyte et al., 2018).

Bland-Altman plots showed no significant bias between the repeated measurements obtained on the same day indicating that there was no systematic error between the runs. However, the limits of agreement were broad for all SICI conditions obtained with both techniques (Figure 5.8). Very similar outcome was observed for measurements obtained on separate experimental days. Although these findings could partially be explained by a small sample size (Rankin and Stokes, 1998), they also point towards a substantial variability of SICI measurements.

5.2.7 Protocol duration

The estimation of $RMT_{0.2mV}$ by threshold-tracking lasted less than 90 s on all occasions and required a median of 10 (IQR 2) stimuli. TS estimation was of similar duration (11 (IQR 4) stimuli). Threshold-tracking protocol for SICI was significantly shorter than the conventional amplitude method (3.8 (IQR 1.2) vs 5.8 (IQR 0.3) minutes; Wilcoxon signed rank test, $p<0.001$).

Parameter	Intraday			Interday		
	SEM_{eas}	**CR/SDC**	**SDC_{group}**	**SEM_{eas}**	**CR/SDC**	**SDC_{group}**
A-SICI50	21	58	16.7	23	62	17.9
A-SICI60	25	69	19.9	21	59	17.0
A-SICI70	19	52	15.0	20	55	15.9
A-SICI80	12	32	9.2	10	29	8.4
A-SICI slope	65	179	51.7	55	152	43.9
peak A-SICI	10	28	8.1	10	28	8.1
T-SICI50	5	15	4.3	3	9	2.6
T-SICI60	4 (4)*	10 (10)*	2.9	4 (4)*	12 (12)*	3.5
T-SICI70	5	14	4.0	10	27	7.8
T-SICI80	8	22	6.4	9	25	7.2
T-SICI slope	15	43	12.4	15	42	12.1
peak T-SICI	7	20	5.8	7	20	5.8

Table 5.4. Repeatability of SICI measurements. *SEM_{eas} – standard error of measurement, CR/SDC – coefficient of repeatability/smallest detectable change; SDC_{group} – smallest detectable change for a group calculated as SDC/ √n (de Vet et al., 2011), where n – sample size; values presented in the table were calculated for a sample of 12 subjects; * values in brackets were calculated after removing an outlier. All repeatability parameters are expressed on the same scale as the measurement (i.e. % test MEP for A-SICI, % $RMT_{0.2mV}$ for T-SICI).*

5.3 Discussion

In summary, SICI obtained by both conventional and threshold-tracking techniques showed good correlation on a group level. No difference in any of the mean group TMS parameters was observed between the recording sessions. While T-SICI protocol was shorter compared to A-SICI measurements and tended to have better reproducibility (particularly when recorded on the same day), the agreement of repeated measurements was poor with both techniques suggestive of limited use of SICI for individual decision making.

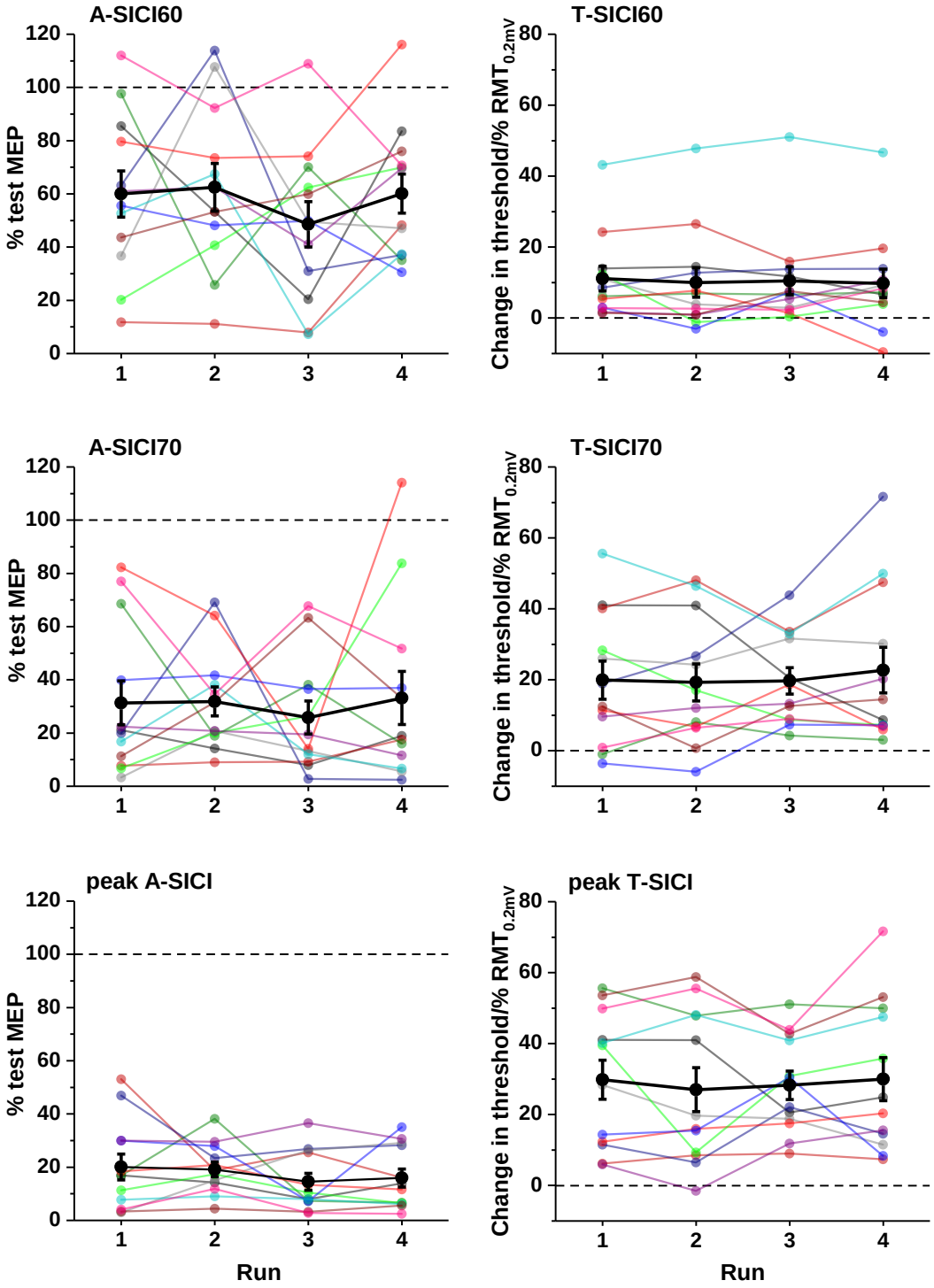

Figure 5.7. Variability of SICI over time. *There were no significant differences between the sessions in mean group SICI. Although some subjects had relatively stable measurements over time, most individuals showed fluctuations with up to 10-fold differences from the initial measurement. Large black circles indicate group means, error bars - standard error of the mean, small coloured circles – raw values of each subject. Figure reproduced from Samusyte et al., 2018.*

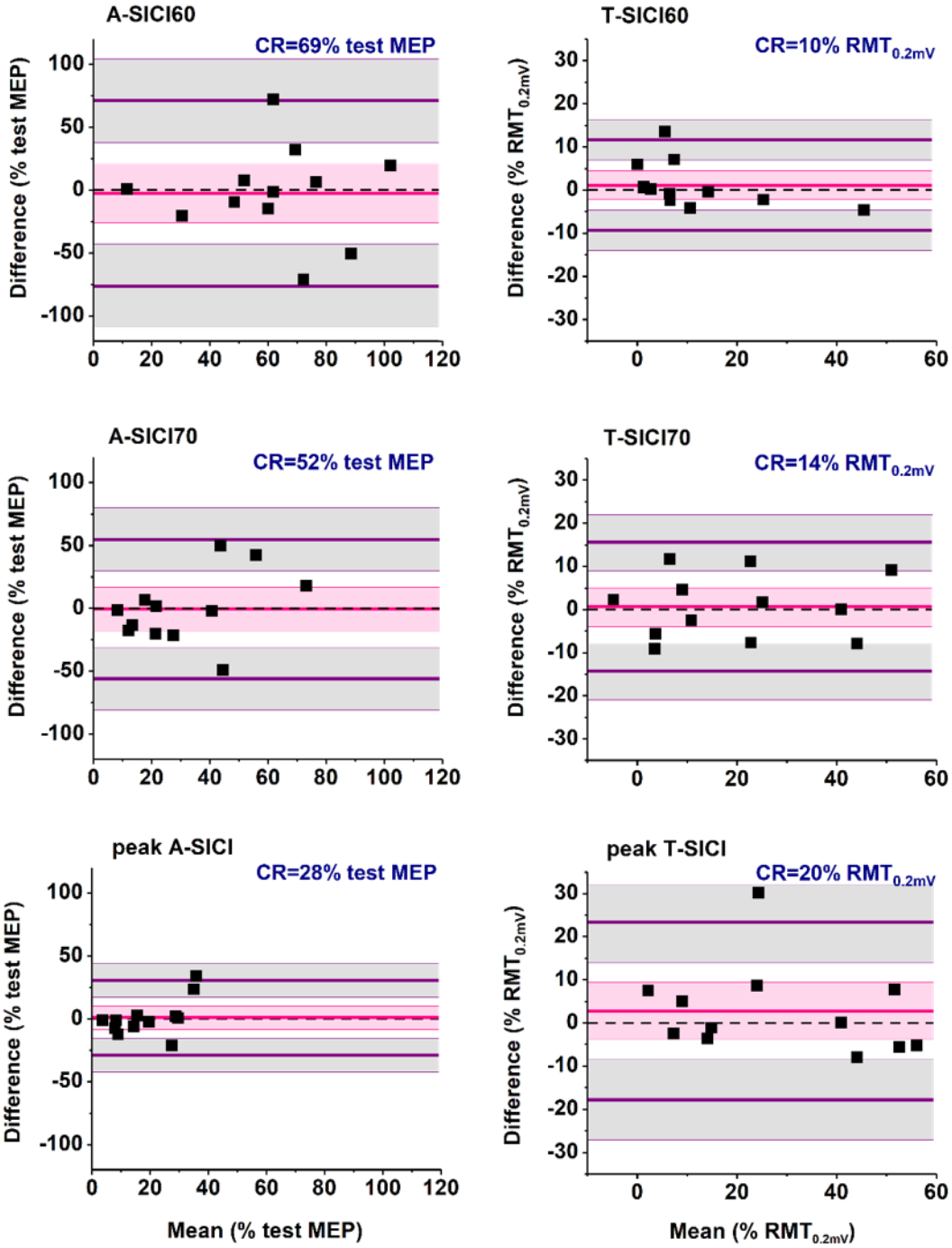

Figure 5.8. Intraday repeatability of SICI measurements. *None of the SICI measurements showed a significant bias between the recording sessions within the same experimental day (runs 1 and 2). However, broad 95% limits of agreement and large coefficients of repeatability indicate the considerable variability of these parameters within subjects. Difference between sessions was calculated as (Run 1 - Run 2). Dots represent data of individual subjects, bold pink line indicates mean difference (bias), bold purple lines - upper and lower 95% limits of agreement. Shaded areas represent 95% confidence intervals for bias (light pink) and 95% limits of agreement (grey). Black dotted line - line of identity. CR – coefficient of repeatability. Figure reproduced from Samusyte et al., 2018.*

5.3.1 Threshold-tracking for corticospinal excitability parameters

The variability and reliability of $RMT_{0.2mV}$ estimate obtained by threshold-tracking was comparable to that of estimates obtained using conventional cut-off value of 0.05 mV (as discussed in section 3.3.2 of Chapter 3).

Although test stimulus intensity for conventional SICI protocols is commonly set to evoke a MEP of 1 mV (Rossini et al., 2015), there is no consensus on a standard procedure and it is rarely described in detail when reporting SICI results. Using a standardised method is important for longitudinal experiments as well as disease or treatment monitoring to avoid operator bias. Threshold-tracking with the target set at 1 mV was used to define the control stimulus intensity for A-SICI in this experiment. This fully automated procedure was fast and produced a valid and reliable parameter for conventional SICI paradigm.

5.3.2 Variability of SICI

Most studies that assessed variability of conventional SICI measurements used single intensity conditioning stimuli (Boroojerdi et al., 2000, Wassermann, 2002, Orth et al., 2003, Fleming et al., 2012, Ngomo et al., 2012), while a range of conditioning stimuli was explored in this experiment. The variability of A-SICI was high and varied considerably across conditions. The between-subject variability was essentially comparable to the reported in the literature, while the within-subject variability was slightly higher (CV 45-48%). The variability (i.e. CVs) of T-SICI varied greatly depending on the expression method used, confounding the comparison between the techniques based on this measure.

5.3.3 Reproducibility of SICI measurements

In the literature, reproducibility of conventional SICI measurements varies greatly between studies ranging from poor to excellent (ICC 0.23-0.91; Maeda et al., 2002, Fleming et al., 2012, Ngomo et al., 2012, Du et al., 2014, Schambra et al., 2015). Although findings of this experiment fall into this broad range, the ICCs of A-SICI (≤0.511) were generally lower than in most of the above studies. If solely numerical values of ICCs were compared, one may suggest that certain aspects of the protocol for conventional SICI used in this experiment (such as unconventional definition of RMT, full automation, etc.) or (in)experience of the operator may contribute to the reduced reproducibility of the measurements. However, ICC values between studies should never be compared in isolation, as factors such as model, precision and the heterogeneity of the sample have

impact on the size of the estimates (Bartlett and Frost, 2008, Schambra et al., 2015, Brown et al., 2017).

In most of the previous studies, the ICC model was either not specified or a model with average measures [ICC(2,k), ICC(3,k)] was used, which is known to increase the ICC value (Streiner et al., 2008, Brown et al., 2017). However, this overestimates the reproducibility of the test in those circumstances where a single measurement is used as an outcome[20] (a common approach in TMS research and clinical practice). Thus, a model with single measures [ICC(2,1)] was used in this study. The precision of the ICC estimates (i.e. 95% confidence intervals) was also rarely presented. Most importantly, the reproducibility of a measurement highly depends on the heterogeneity of the sample or population it was obtained from (Bruton et al., 2000, Bartlett and Frost, 2008). That is, the more heterogeneous the sample is, the easier it is to make a distinction between the subjects. For instance, Schambra and colleagues (Schambra et al., 2015) found that reproducibility of SICI in chronic stroke patients was worse on the lesional side than in the unaffected hemisphere (ICC 0.33 and 0.64, respectively). The measurement errors were essentially equivalent in both hemispheres, but the inter-individual dispersion of SICI was smaller in the affected hemisphere, thus resulting in lower ICC. An example of reduced reproducibility of T-SICI at CS 60% $RMT_{0.2mV}$ after exclusion of an outlier in this experiment also illustrates this counter-intuitive aspect of ICC. When the ICCs of conventional SICI from previous studies were adjusted to the heterogeneity of the sample in this experiment (as proposed by (Streiner et al., 2008) [21]), the reproducibility of SICI measurements became much more comparable between the studies (adjusted ICC in Fleming et al., 2012 increased from 0.23 to 0.53 and decreased from 0.91 to 0.33 in Ngomo et al., 2012).

In this experiment, T-SICI had higher numerical ICCs values than A-SICI, especially when measured on the same day. However, these differences did not reach statistical significance due to large and overlapping 95% confidence intervals. This could be explained by a small sample size which was adequate to detect ICCs of >0.9 with 95% confidence interval width of 0.2[22], but about 100-300 subjects more would have been

[20] ICC estimates with both single [ICC(2,1)] and averaged measures [ICC(2,k)] are presented in Table 1 in (Samusyte et al., 2018), which illustrates the impact of the model type on the size of the estimates.

[21] $ICC_{adjusted} = (ICC*SD_{new}^2)/(ICC*SD_{new}^2 + (1-ICC)*SD^2)$, where ICC and SD (standard deviation) are obtained from the reported study and SD_{new} from the new study to which the reported result is being adjusted.

[22] Sample size required to obtain an ICC estimate with a pre-determined precision can be calculated using Bonett formula: $n = 1 + 8*1.96^2*[(1 - \rho)^2*(1 + (k - 1)\rho)^2]/k(k - 1)*w^2$, where n – sample size; ρ – chosen ICC level, w – chosen 95% confidence interval width, k – number of raters or repeated measurements.

required to achieve the same precision when ICC falls between 0.7 and 0.3 (the lower the ICC, the larger the sample size needed; Bonett, 2002). Conducting studies with such sample sizes would be impractical.

Parameters obtained from T-SICI recruitment curve (i.e. T-SICI slope and peak T-SICI) had better reproducibility (Figure 5.6) than individual SICI conditions between the experimental days. Thus, using a range of CS intensities may provide a more stable T-SICI measure over time.

No data of the reliability of threshold-tracking SICI was available in the literature until very recently. Matamala and colleagues assessed test-retest reliability of a standard threshold-tracking TMS protocol in which SICI at CS 70% $RMT_{0.2mV}$ is measured across a range of interstimulus intervals from 1 to 7 ms (Matamala et al., 2018). The reported ICC(2,k) estimates ranged from 0.39 to 0.95 for different parameters and were highest for averaged SICI. These findings are consistent with the observations of this experiment, despite methodological differences in SICI measurements.

Trend for better reproducibility suggests that T-SICI may be advantageous at detecting inter-individual differences and may be more suitable for discriminative purposes (e.g. disease staging; de Vet et al., 2006). However, reproducibility estimates of 'healthy-state' SICI may be inappropriate in conditions in which SICI is impaired (e.g. ALS), especially if patients are a very homogeneous group (i.e. all have markedly reduced/absent SICI). Hence, reproducibility of SICI measurements in patient groups should be determined independently.

5.3.4 Repeatability of SICI measurements

Measures of absolute reliability are more important in determining the diagnostic value of a test in clinical practice (e.g. for the assessment of treatment response or disease progression) where decisions are made on an individual basis. In this experiment, the agreement between repeated measurements was poor for both techniques (as indicated by high coefficients of repeatability), irrespective of whether the measurements were taken on the same day or at least one week apart. This is consistent with previous studies in which agreement between repeated individual SICI measurements varied from 17% to 147% test MEP with conventional technique (Fleming et al., 2012, Ngomo et al., 2012, Schambra et al., 2015) and 14-21% $RMT_{0.2mV}$ with threshold-tracking (Matamala et al., 2018). Averaging inhibition across the range of interstimulus intervals was found to somewhat improve repeatability of threshold-tracking estimates; however, it still remained relatively poor at ± 6.68% $RMT_{0.2mV}$ vs group mean of approximately 12% $RMT_{0.2mV}$ (Matamala et al., 2018). Although a small sample size could partially explain

poor agreement of SICI measurements in our experiment (Rankin and Stokes, 1998), overall the data is suggestive of a substantial biological variability.

5.3.5 Comparability of A-SICI and T-SICI

Shorter acquisition time and potential for better reproducibility of SICI measurements make threshold-tracking a more appealing method. But can these two techniques be used interchangeably?

In this experiment, subjects with strong inhibition measured by conventional method also showed strong inhibition when threshold-tracking was used (Figure 5.3). This was true for most individual SICI conditions, with strongest correlations seen when inhibition was at its peak (i.e. at CS 80% $RMT_{0.2mV}$ and peak SICI). On a group level, the relationship between mean group A-SICI and T-SICI recruitment curves was also strongly linear (Figure 5.4 A). However, individual plots of SICI recruitment curves (Figure 5.4 C) showed non-linear relationships with notably different slopes (or even lack of correlation) in some subjects suggesting that SICI estimates cannot be easily extrapolated between the techniques. The 'floor' effect seen in A-SICI is the most likely explanation for such non-linearity in some individuals, but it may also indicate that different subsets of neuronal pools are interrogated with different methods.

The effect of test stimulus intensity on SICI is well established (Sanger et al., 2001, Ilic et al., 2002, Roshan et al., 2003, Garry and Thomson, 2009). Whereas the TS_{1mV} intensity used in the conventional A-SICI was optimal for eliciting maximum inhibition (Garry and Thomson, 2009) and was constant for all SICI conditions, stimulation intensities in T-SICI varied depending on the CS intensity. If one assumes that the size of the recruited pool of cortical motoneurons is determined by the test stimulus intensity, then it is likely that different sets of these neurons are assessed by the two techniques at different CS levels (Figure 5.9).

This is a very simplified explanation which does not take into account other potentially important factors such as slope of individual stimulus-response function, overlap with short-interval intracortical facilitation, composition of the descending corticospinal volleys and their interaction at the spinal level. Nevertheless, the close relationship between A-SICI and T-SICI across a range of conditions suggests that the neurons explored by the two techniques have much in common. Future pharmacological interventions may provide further insight into the similarities and differences of the neuronal pathways interrogated by the two techniques.

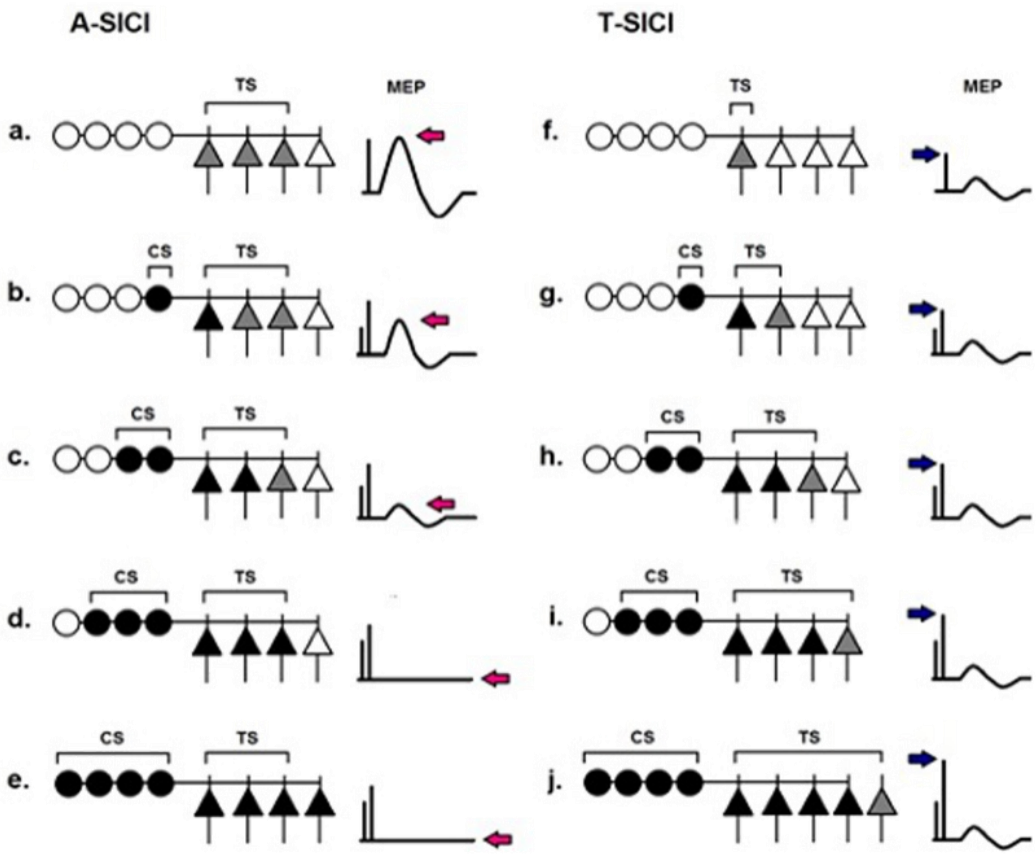

Figure 5.9. Schematic illustration of hypothetical neuronal pools assessed by conventional (A-SICI) and threshold-tracking (T-SICI) techniques. *This diagram is based on an assumption that the size of the neuron pool under investigation is defined by the stimulation intensity. Triangles represent upper (cortical) motoneuron pool, circles – inhibitory interneurons projecting onto motoneurons. In A-SICI, the size of the motoneuron pool (represented in a) by 3 of 4 neurons; grey triangles) that will be tested and will generate a control motor response is pre-determined by the test stimulus (TS) intensity. As the intensity of the conditioning stimulus increases (b to d), inhibitory (GABAergic) interneurons are progressively recruited (black circles), exerting increasingly stronger inhibitory effect on the upper motoneuron pool (black triangles). As a result, the conditioned MEP amplitude decreases (b, c) and is eventually abolished (d). Although even more inhibitory interneurons might be recruited by stronger conditioning stimuli (e), this cannot be further quantified (as the inhibited neurons are not activated by the test stimulus) thus producing a 'floor' effect. By contrast, in T-SICI, test stimulus intensity is adjusted to counteract the effect of the inhibitory interneurons so that a small response (represented by a single grey neuron) is always obtained (g to j). Although potentially different subsets of motoneurons are assessed at different conditioning stimulus levels, this allows the inhibitory potential of GABAergic interneuron pool to be fully evaluated. Arrows indicate change in MEP amplitude (pink) and test stimulus intensity (blue). Figure reproduced from Samusyte et al., 2018.*

5.3.6 The choice of method for SICI estimation

The main advantage of the conventional technique is that the majority of data on the physiology and pharmacology of SICI as well as its impairment in many pathological conditions has been obtained with this method. It is unclear to what extent these findings (e.g. enhancing effect of benzodiazepines) are 'transferable' to threshold-tracking SICI.

A-SICI might be a more appropriate method if one is interested in investigating effects of an intervention on a particular motoneuron subset. However, the 'floor' effect might prevent from fully quantifying SICI-enhancing effects, especially if a single SICI condition is used and the baseline inhibition is strong. This could be prevented by adjusting CS intensity to produce 50% inhibition at baseline (Müller-Dahlhaus et al., 2008). However, such approach would result in varying CS intensities in relation to individual motor thresholds and could introduce bias. Therefore, obtaining a SICI recruitment curve may be favourable.

T-SICI allows the inhibitory potential to be fully evaluated and might be better at detecting inter-individual differences within a group as well as outliers. It is potentially quicker to obtain, thus could be advantageous where time constraints play an important role. Assuming proportionate modulatory effects of an intervention on T-SICI and A-SICI, smaller sample sizes may be sufficient in cross-over experiments if threshold-tracking was used (Samusyte et al., 2018). However, it is yet to be determined whether the sensitivity to intervention of the two SICI methods is similar. A conceivable technical limitation of T-SICI is related to the power of the magnetic stimulator. In subjects with high RMT and strong inhibition, test stimulus intensities of >100% MSO may be required to demonstrate full inhibition, resulting in a 'ceiling' effect.

5.3.7 Limitations

Exclusion rate due to coil overheating (three out of 16 recruited participants) was relatively high and biased towards subjects with high $RMT_{0.2mV}$. A single interstimulus interval was used in this experiment, therefore it remains unclear whether the relationship between A-SICI and T-SICI is similar across the whole range of interstimulus intervals. It should also be kept in mind that CS intensities for A-SICI were set based on $RMT_{0.2mV}$ which, according to limited reports, is equal to about 109% $RMT_{0.05mV}$ (Awiszus, 2005, Cirillo and Byblow, 2016). Thus, they may not be directly comparable to the studies where conventional RMT estimates were used.

5.3.8 Conclusions

Threshold-tracking technique provided fast and reliable estimates of test stimulus intensity for conventional SICI protocols. No systematic bias in SICI estimates was observed between the recording sessions with either technique. T-SICI was significantly quicker to obtain and showed good reproducibility on a group level. However, both techniques showed poor agreement of repeated measurements indicating limited utility for individual decision making in clinical practice. Although T-SICI and A-SICI estimates correlated across a range of conditioning stimuli, it remains unclear whether they represent the same neuronal populations. Head-to-head comparison in pharmacological studies and disease states are required to elucidate the comparability of the two techniques.

Chapter 6 - Exploring modulation of short-interval intracortical inhibition via $GABA_A$ α2,3 receptor pathway (Experiment 4)

Pharmacological studies have been crucial in better understanding the physiology underlying various TMS measures (Ziemann, 2017). As a result, some of these measures have become widely-accepted biomarkers for neurotransmitter, receptor, or ion channel function in the study of physiology, pathology, and pharmacology of CNS. For example, motor threshold is affected by voltage-gated sodium channel blockade, short-latency afferent inhibition is thought to reflect central cholinergic activity, whereas short- and long-interval intracortical inhibition are mediated by $GABA_A$ and $GABA_B$ receptor signalling, respectively (Paulus et al., 2008, Ziemann et al., 2015).

TMS measures could potentially be utilised in the new drug development research as biomarkers of target engagement or pathophysiology of the disease. Based on the observed effects of $GABA_A$ modulating drugs with well-described pharmacology, it has been proposed that SICI is mediated via $GABA_A$ α2,3 receptor pathway (Ziemann, 2013; for details see section 1.4.2). Although this hypothesis is yet to be supported by direct empirical evidence, SICI could in theory be used as a target-engagement biomarker in the development of selective $GABA_A$ α2,3 receptor positive allosteric modulators. While non-sedative anxiolysis has been the primary incentive for the development of such drugs (Rudolph and Möhler, 2014), the improved side-effect profile may be beneficial in other therapeutic areas.

For instance, muscle relaxing properties of classical benzodiazepines have been utilised for the symptomatic treatment of various disorders affecting the motor system such as dystonia, stiff person syndrome, and spasticity (Chang et al., 2013, Thenganatt and Jankovic, 2014, Bhatti and Gazali, 2015). As myorelaxation has been linked to α2, α3, and α5 subunit types (Table 1.3), selective modulation of $GABA_A$ receptors devoid of sedating and addictive CNS adverse effects could result in significant advance in the management of such conditions.

Dystonia is a group of neurological disorders that are characterised by sustained or intermittent muscle contractions causing abnormal movements and/or postures (Albanese et al., 2013). The pathophysiology of these conditions is thought to be linked to impairment in GABA signalling. Changes in the expression of $GABA_A$ receptors in the sensorimotor and premotor cortex as well as cerebellum have been observed in PET studies (Garibotto et al., 2011, Berman et al., 2018, Gallea et al., 2018), while loss of inhibition at cortical, brainstem, and spinal levels was demonstrated by a number of neurophysiological techniques, including paired-pulse TMS (Hallett, 2011). Reduction in SICI was found in primary dystonia patients (Ridding et al., 1995b, Hanajima et al., 2008)

with short-term normalisation following 1 Hz repetitive TMS (Siebner et al., 1999) and long-term normalisation with deep brain stimulation (Ruge et al., 2011). Importantly, increase in SICI was paralleled by clinical improvement in these studies. If selective $GABA_A$ α2,3 modulators proved to enhance SICI, the use of this measure in conjunction with clinical endpoints in clinical trials of dystonia could provide invaluable insight into the pathophysiology of these disorders.

The availability of a safe non-sedating $GABA_A$ α2,3 receptor positive allosteric modulator AZD7325 now allows for the first time to study the hypothesis that SICI is mediated by this pathway. If SICI is enhanced in healthy volunteers, the subsequent step would be to explore the utility of this medication as a novel treatment for dystonia.

6.1 Methods

A phase I single site, single dose, randomised, double-blind, placebo controlled, 3-way cross-over biomarker study investigating the effect of the GABA modulator AZD7325 on Short Interval intracortical Inhibition (SICI) in healthy volunteers (short title 'Effects of AZD7325 on SICI') was conducted in the department of Clinical Neurophysiology at the National Hospital for Neurology and Neurosurgery over the period from October 2014 to August 2015.

6.1.1 Preparation for the clinical trial and regulatory approvals

The study was funded by the Medical Research Council (MRC) in collaboration with AstraZeneca (Grant Reference MR/K015222/1). Given that AZD7325 is an unlicensed drug, the study was classified as a phase I clinical trial and was registered on the European Clinical Trials Database (EudraCT Number 2013-005472-17). University College London (UCL) acted as a Sponsor, and all study documentation was prepared in liaison with Sponsor's representative at the Joint Research Office (JRO) following stringent regulatory requirements for this type of studies (the timeline of the preparation for the clinical trial is presented in Figure 6.1).

Approvals to conduct the study were obtained from three different national and local bodies in July 2014: Medicines and Healthcare products Regulatory Agency, National Research Ethics Service, and local ethics committee of the University College London Hospital. Sponsor's permission to start the study was issued in September 2014 after finalising the local procedures for data management, serious adverse event reporting, and emergency unblinding.

6.1.2 Recruitment process

Study participants were recruited by advertisement, word of mouth and email to UCL students. All advertisement material was approved by ethics committee. After expressing their interest in the study, potential volunteers were sent a copy of the Participant's Information Sheet and a suitable time was arranged for them to attend the study site to discuss the trial in detail. Following this meeting, they were given as much time as needed (but not less than 24 hours) to consider participation in the trial. Written informed consent was obtained from all volunteers before carrying out any screening procedures.

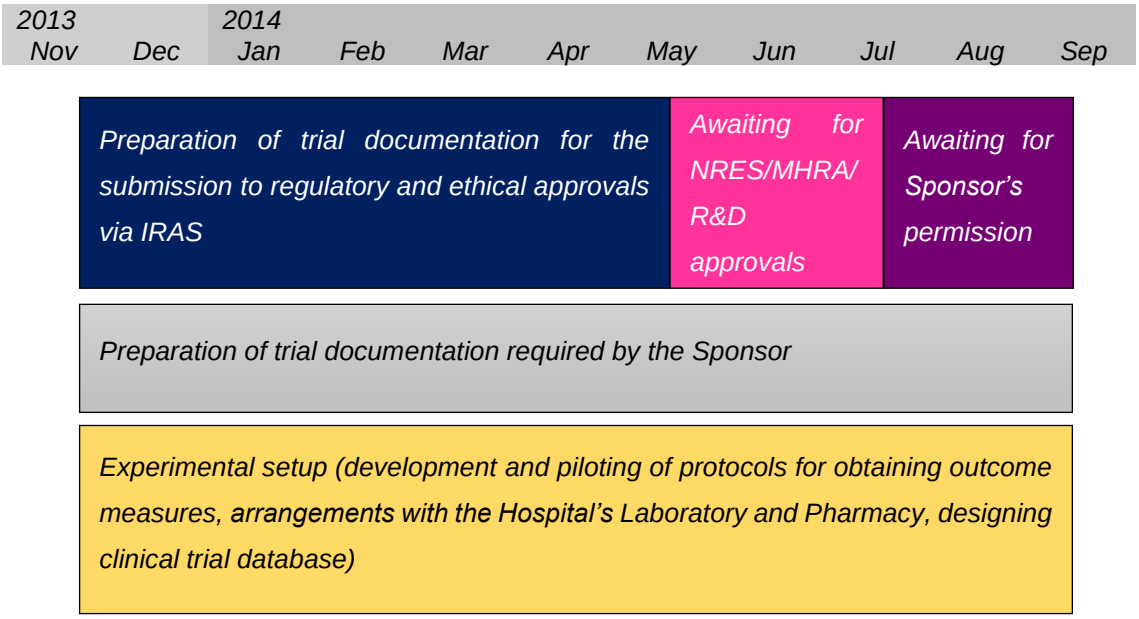

Figure 6.1. The timeline of the preparation for the clinical trial. *IRAS – Integrated Research Application System; MHRA – Medicines and Healthcare products Regulatory Agency; NRES – National Research Ethics Service; R&D – local ethics committee of the University College London Hospital.*

6.1.3 Eligibility determination

Participant's eligibility for the study was determined following the Screening visit, strictly adhering to the selection criteria summarised in Table 6.1.

Inclusion criteria	
1.	Male adults aged 18 to 55 years (extremes are included)
2.	A body weight resulting in a body mass index (BMI) of 18≤ BMI ≤30 kg/m^2 (extremes included) using the formula BMI = body-weight [in kg] / body-height [in m]^2
3.	Able and willing to sign the Informed Consent Form prior to screening evaluations
4.	History of good physical and mental health as determined by history taking and laboratory examinations, ECG, blood pressure and heart rate recordings as judged by the investigator
5.	Willing not to consume alcohol or to smoke or chew tobacco on days of assessments
6.	Subjects must be willing to avoid unprotected vaginal intercourse with women of child bearing potential or donating sperm until 12 weeks after drug administration. They also must be willing to use a condom when having sex with a pregnant woman until one week after drug administration.
Exclusion criteria	
1.	History of sensitivity/idiosyncrasy to AZD7325 or chemically related compounds or excipients which may be employed in the study or to any other drug used in the past
2.	Subject has taken systemically (oral or intravenous route) any potent or moderate CYP3A4 or CYP2C9 inhibitor 1 month prior to screening (topical or inhaled are permitted) such as: aprepitant, barbiturates, carbamazepine, clarithromycin, erythromycin, cyclosporine, diltiazem, efavirenz, fluconazole, HIV protease inhibitors, glucocorticoids, itraconazole, ketoconazole, nefazodone, nevirapine, phenytoin, pioglitazone, primidone, rifabutin, rifampicin, telithromycin, St. John's wort, verapamil
3.	Use of any prescription drug within two weeks prior to the first dosing, except for topical medication without systemic exposure
4.	Clinically relevant history or presence of any medical disorder, potentially interfering with this trial*
5.	Clinically relevant abnormal laboratory, ECG, HR or BP at screening as judged by the investigator
6.	History of or current abuse of drugs (including prescription medication) or alcohol or solvents
7.	Smoking in excess of 5 cigarettes per day or the equivalent within 28 days prior to the first study day
8.	Smoking or chewing of tobacco or consumption of alcohol 24 hours before and on the days of assessment
9.	Subject is a family member or in the employment line management of study personnel

Table 6.1. *Continued on the next page*

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10.	Subject has abnormal screening laboratory values: AST >1x upper limit of normal; ALT > 1x upper limit of normal; total bilirubin >1x upper limit of normal; serum creatinine >1x upper limit of normal
11.	Subject's partner is planning pregnancy within 3 months of last dosing
12.	Participation in an IMP intervention trial within the last month or more than four in the previous 12 months†
13.	Abnormal SICI response, KA analysis, SDMT, VAS outside 95% confidence interval of normal at screening visit
14.	Contraindications for TMS (a questionnaire modified from Rossi et al. (Rossi et al., 2011), see Appendix B.1)

Table 6.1. Eligibility criteria. *All eligibility criteria had to be met for the participant to be included in the trial. * Following the screening visit, a letter was sent to subject's general practitioner to request for a summary of their medical records which included past medical history and recent prescriptions. † As per regulatory requirements, all participants were registered on the Over-Volunteering Prevention System (TOPS) database held by Health Research Authority where record of previous participation in clinical trials was checked. ECG – electrocardiogram; HIV – human immunodeficiency virus; HR – heart rate; BP – blood pressure; AST - aspartate transaminase; ALT - alanine transaminase; IMP – investigational medicinal product; SICI – short interval intracortical inhibition; KA – kinematic analysis of circle drawing; SDMT – Symbol Digit Modalities Test; VAS – visual analogue scale for sedation; TMS – transcranial magnetic stimulation.*

6.1.4 Experiment design

Schematic illustration of the study design is presented in Figure 6.2. The 2 mg and 10 mg doses of AZD7325 were chosen based on PET study in healthy volunteers in which 50% receptor occupancy was observed at 2 mg dose, while doses above 5 mg resulted in high (80%) receptor occupancy (Jucaite et al., 2017). In addition, phase I clinical trials have shown that these doses resulted in fewer sedative and cognitive side effects compared to lorazepam (AstraZeneca, 2009, Chen et al., 2014). A washout period was determined based on the pharmacokinetic properties of the drug (>10 times the half-life of 12 hours (AstraZeneca, 2009)) to prevent carry-over effects.

A randomised double-blind design was chosen to prevent the bias related to subject's and/or investigator's knowledge of the treatment, while a cross-over design allowed to reduce the number of individuals exposed to the study medication. Randomisation was based on a balanced Latin square design to control for both treatment period and dose order effects. This resulted in six blocks to which eligible participants were allocated

randomly. To maintain blinding of the investigators, randomisation code was provided by Almac Clinical Services (Almac Group, Craigavon, United Kingdom) together with a substitution list to ensure balance in case of drop-outs. First Screening visit of the first subject denoted the start of the trial (15 October 2014), the last follow-up phone call to the last participant denoted the end of trial (24 August 2015).

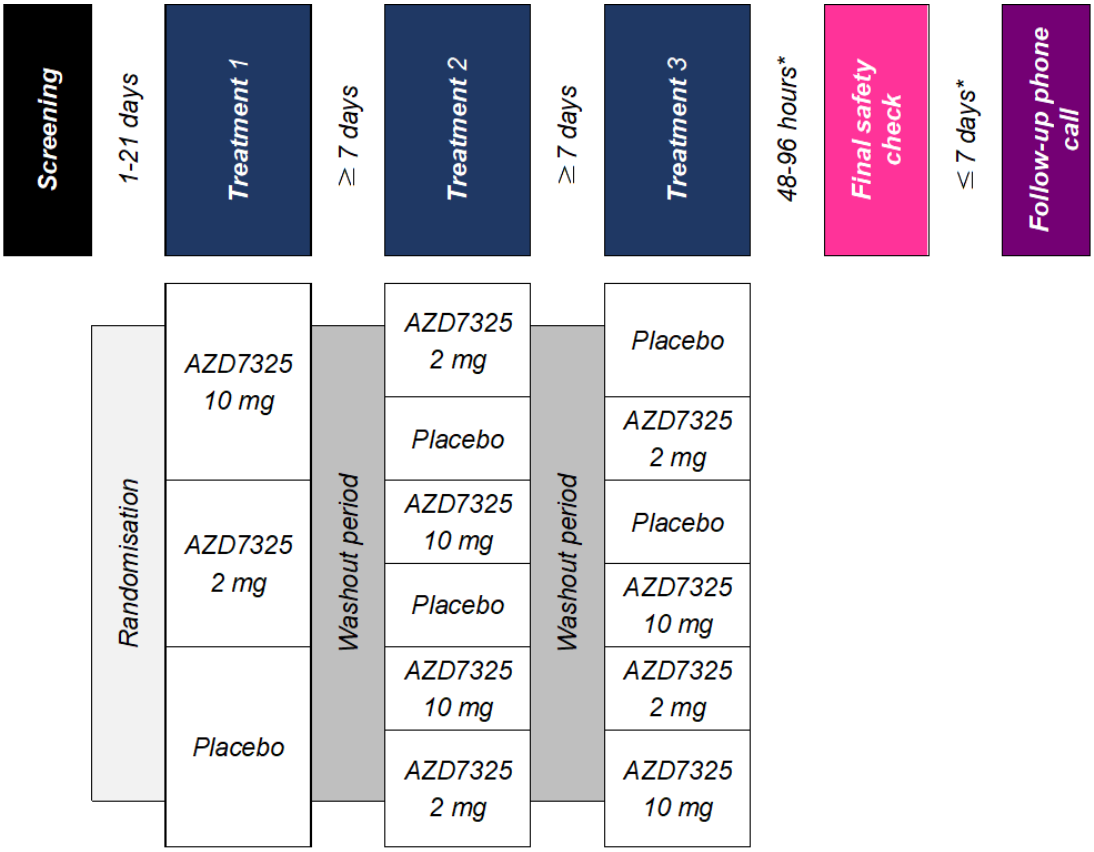

Figure 6.2. Schematic diagram of the study design. ** Time interval from the last Treatment visit.*

6.1.5 Pharmacodynamic assessments

Pharmacodynamic assessments were carried out once during the Screening visit and four times during the Treatment visits (before and 1, 2, and 8 hours after a single dose of the study medication) in the following order:

1) Sedation. Visual analogue scale ($VAS_{sedation}$) was anchored at 'No sedation' at the beginning of 100-mm long line and at 'Maximal imaginable sedation' at the end of it (Appendix B.2). Subjects were instructed to place a mark which represented the amount of sedation they experienced at the time of assessment. The distance between 'No sedation" and the mark measured in millimetres was recorded.

2) Psychomotor performance. The Symbol Digit Modalities Test (SDMT) was used to assess the psychomotor performance. It is a simple substitution task that was developed to screen for cerebral dysfunction in children and adults (Aaron, 1982). It assesses information processing speed, including attention, visual scanning, and motor performance (Lezak et al., 2004, Sheridan et al., 2006). A similar substitution test was found to be sensitive to the effects of benzodiazepines (Greenblatt et al., 1988). Briefly, subjects were presented with a key containing nine symbols matched with numbers from 1 to 9 (Appendix B.3). After a short practice, subjects were given 90 seconds to fill in as many blank boxes below the symbols as possible with the corresponding numbers. After completion, the number of correct substitutions was counted and recorded (maximum score of 110). SDMT score outside the normative range (Lezak et al., 2004) at Screening visit was considered as an exclusion criterion.

3) Motor control of the hand. Kinematic analysis of circle drawing (KA; Marquardt and Mai, 1994, Mergl et al., 1999) was used to assess the motor control of the hand. Digitising analysis of handwriting was found to be sensitive at detecting motor impairment in psychiatric (Tigges et al., 2000, Mavrogiorgou et al., 2001, Mergl et al., 2004, Mergl et al., 2007) and cognitive (Schröter et al., 2003) disorders or after alcohol (Phillips et al., 2009) and nicotine (Tucha and Lange, 2004) intake. It can be used to objectively quantify the motor dysfunction of the hand in movement disorders, such as Parkinson's disease (Eichhorn et al., 1996), writer's cramp (Zeuner et al., 2007), or drug-induced parkinsonism (Caligiuri et al., 2006) as well as to assess treatment effects (Baur et al., 2006, Tucha et al., 2006).

   During the procedure, participants were comfortably seated at a table. A white sheet of paper with a 2-cm horizontal grid was placed over the active area of the digitising tablet (WACOM Intuos®pro, Wacom Europe GmbH, Krefeld, Germany; sampling rate 200 Hz, spatial resolution 0.05 mm). Participants were given an inking digitising pen (WACOM Inking Pen, Wacom Europe GmbH, Krefeld, Germany) and instructed to draw superimposed circles at high but still comfortable speed using the preferred hand. The positional data of the tip of the pen and the pressure against the digitising tablet were analysed using CSWin software version 2012 (MedCom, Munich, Germany). The software handles the recording and analysis of the handwriting movements and includes procedures for filtering and smoothing the kinematic data (MedCom, 2013). After an initial practice trial, five 3-s long trials of circle drawing were recorded. First 500 ms of each trial representing the tuning-in phase were excluded from the analysis

(personal communication with Christian Marquardt). The following variables were calculated and a mean value of five trials was used as a single data point:

i. mean stroke frequency (Hz) - a measure of fluency and automaticity of drawing movements;

ii. mean axial pressure (N);

iii. coefficient of variation of mean peak velocity ($CV_{velocity}$ = standard deviation of the positive velocity peaks divided by the mean peak velocity) – an index of the regularity of movement kinematics during the task;

Values outside the normative range (Zeuner et al., 2007, MedCom, 2013) at Screening visit were considered as an exclusion criterion.

4) Transcranial magnetic stimulation. The experimental setup and TMS procedure was largely as described in sections 2.2 and 2.3 of Chapter 2. Surface EMG was recorded from the relaxed FDIO muscle of the self-reported dominant hand (i.e. the hand used for writing in KA). The coil was hand-held over the contralateral hemisphere with the handle pointing postero-laterally at a 45° angle to the mid-sagittal line to induce posterior-to-anterior flow of the current in the motor cortex. Stimuli were delivered at 4.1 second intervals. Online gating was used to discard traces with pre-stimulus activation of the target muscle (900 ms prior the stimulus, negative EMG activity peak >0.01 mV).

The following TMS parameters were obtained (Figure 6.3):

i. resting motor threshold using the conventional cut-off value of 0.05 mV, defined by threshold-tracking with the target set at 0.05 mV ± 20% ($RMT_{0.05mV}$)[23];

ii. test stimulus intensity required to elicit a MEP of peak-to-peak amplitude of approximately 1 mV, defined by threshold-tracking with the target set at 1 mV ± 20% (TS_{1mV})[24];

[23] Threshold-tracking was started at suprathreshold intensity used to find the motor hotspot. Fixed step tracking mode with a maximum step of 1% MSO was used. Tracking was automatically stopped when MEP hit and/or crossed the target six times. Stimulus intensity that would have been used subsequently for tracking was used to set the conditioning stimulus intensities for A-SICI.

[24] Several modifications were made to the protocol of TS_{1mV} estimation by threshold-tracking compared to Experiment 3. Firstly, tracking was started at suprathreshold intensity (180% $RMT_{0.05mV}$). Secondly, the tracking protocol consisted of two parts: 'fast' and 'stable'. In the initial part, proportional tracking mode with a maximum step of 4% MSO was used and was continued until six valid threshold estimates were obtained. Then tracking was continued in fixed step mode

iii. conventional 'amplitude' short-interval intracortical inhibition (A-SICI) at an interstimulus interval (ISI) of 2.5 ms, conditioning stimulus (CS) intensities of 50%, 60%, 70%, and 80% of $RMT_{0.05mV}$, and test stimulus intensity eliciting a MEP of peak-to-peak amplitude of approximately 1 mV (TS_{1mV}). Fifteen responses were recorded in a pseudorandomised order for each paired and test conditions. A-SICI was expressed as a percent ratio of mean conditioned MEP to mean test MEP amplitude [conditioned MEP/test MEP*100%], with values below 100% indicating inhibition;

iv. resting motor threshold as a control condition for threshold-tracking SICI protocol, defined by threshold-tracking with the target set at 0.2 mV ± 20% ($RMT_{0.2mV}$)[25];

v. threshold-tracking short interval intracortical inhibition (T-SICI) at an ISI of 2.5 ms, CS intensities of 50%, 60%, 70%, and 80% of $RMT_{0.2mV}$, and tracking target set at 0.2 mV ± 20%. Paired and control stimuli were delivered in a pseudorandomised order and tracking was deemed stable when the responses hit and/or crossed the target line six times[26]. T-SICI was expressed as a relative threshold change over $RMT_{0.2mV}$ [(conditioned threshold – $RMT_{0.2mV}$)/$RMT_{0.2mV}$*100%], with values >0% indicating inhibition.

Subject's inability to maintain relaxation of the target muscle, overheating of the coil prior the completion of the stimulation protocol, absent SICI or a 'floor effect' observed with conventional technique at Screening visit were considered an exclusion criterion.

Pharmacokinetic analysis was not done as pharmacokinetics of the drug had been established before and the dosing was carried out under supervision. In a previous phase I clinical trial exploring CNS pharmacodynamic effects of the 2 mg and 10 mg doses of AZD7325, the mean peak plasma concentration (C_{max}) of 14.2 ± 5.36 ng/ml and 67.4 ±

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and a maximum step of 1% MSO until four valid threshold estimates were obtained. Stimulus intensity that would have been used subsequently for tracking was used to set the conditioning stimulus intensities for A-SICI. Proportional tracking mode with large tracking step allowed fast tracking to approximate the threshold, while fixed mode with small tracking step was used to fine-tune the tracking.

[25] Threshold-tracking was started at $RMT_{0.05mV}$ intensity. Proportional tracking mode with a maximum step of 2% MSO was used. When tracking was deemed stable (i.e. after obtaining six valid threshold estimates), T-SICI protocol was started.

[26] Proportional tracking mode with a maximum step of 2% MSO was used throughout. Tracking was started at $RMT_{0.2mV}$ intensity for each paired condition.

33.5 ng/ml was reached at the median t_{max} of 1.75 (range 1-3.25) and 2 (range 0.5-3.25) hours, respectively, while the mean elimination half-time was 8.5-9 (range 5-15) hours (Chen et al., 2014). As SICI-enhancing effects of benzodiazepines were observed at t_{max}, 1 and 2-hour post-dose assessments were chosen for this study to capture the effects corresponding to the expected peak plasma concentration of AZD7325. The 8-hour post-dose assessment corresponded roughly to the mean elimination half-life and was chosen to assess whether pharmacodynamic effects of the study medication correlated with the expected pharmacokinetics of the drug.

6.1.6 Safety assessments

Safety assessments were carried out at every visit (Screening, at baseline on Treatment visits, Final safety check visit) and included:

1) Physical and neurological examination. This included measurement of height and weight as well as calculation of the body mass index (BMI) at Screening visit. BMI less than 18 kg/m^2 and more than 30 kg/m^2 was considered as an exclusion criterion.

2) Vital signs. This included oral temperature, heart rate, and blood pressure.

3) Laboratory tests. Blood samples were processed at the local Hospital lab and included full blood count, serum creatinine, aspartate transaminase (AST), alanine transaminase (ALT), and total bilirubin. A dipstick test was used for urinalysis (Multistix® 10 SG, Siemens, Munich, Germany) as per routine Hospital procedures.

4) Electrocardiogram (ECG). A standard 12-lead ECG was obtained using the ECG service of the Hospital's Outpatient Department.

5) Urine drug screen test. A validated dip stick test Drug-Screen-Cup II (Nal Von Minden GmbH, Moers, Germany) was used for urine drug screening. The immunoassay test included ten recreational and prescription drugs (Appendix B.4). A positive test for any of these drugs at Screening visit was considered an exclusion criterion.

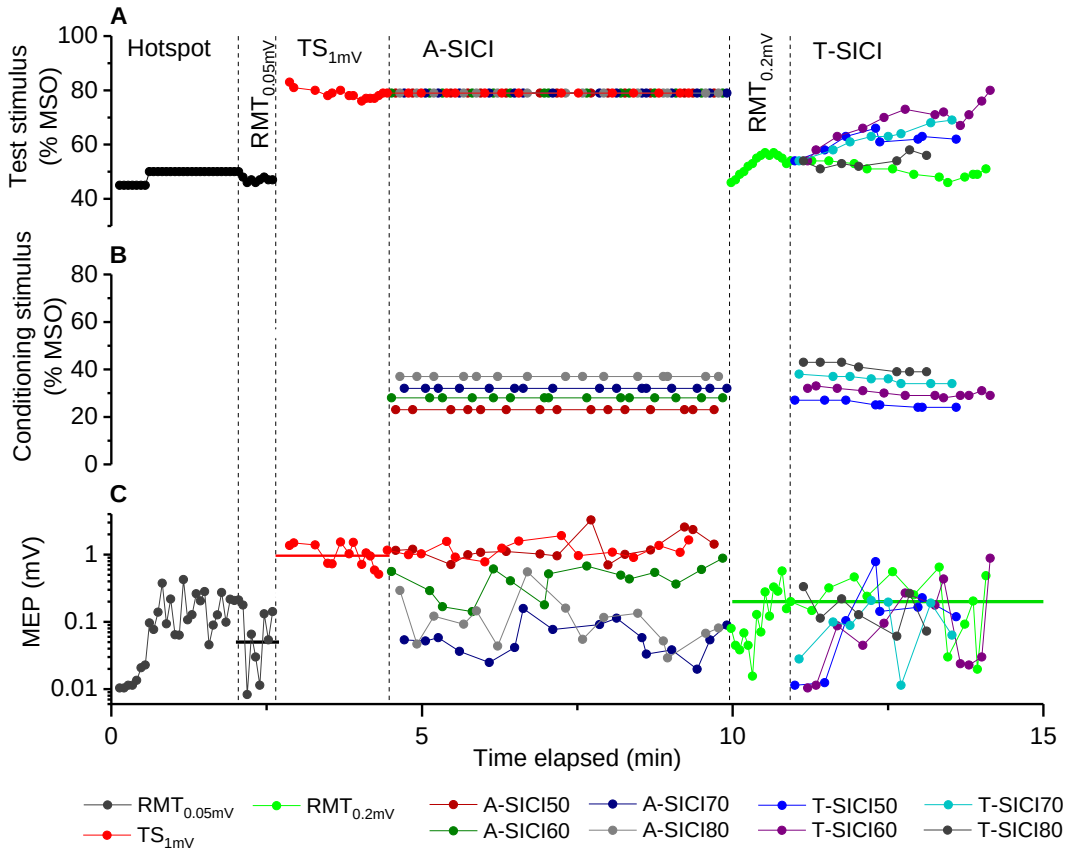


Figure 6.3. Representative illustration of the automated stimulation protocol in a single subject*. After finding the hotspot, conventional followed by threshold-tracking SICI was recorded. Vertical dotted lines indicate parts of the recording in which different TMS parameters were obtained. For conventional SICI (A-SICI), conditioning stimulus intensities of 50-80% $RMT_{0.05mV}$ and test stimulus required to elicit a peak-to-peak MEP of approximately 1mV (TS_{1mV}) were used. $RMT_{0.05mV}$ and TS_{1mV} were obtained by threshold-tracking. For threshold-tracking SICI (T-SICI), $RMT_{0.2mV}$ was used as a control condition and conditioning stimulus intensities were set to 50-80% of $RMT_{0.2mV}$. Test stimulus intensity (top), conditioning stimulus intensity (middle), and motor evoked potential (MEP) amplitude (bottom) were recorded throughout the protocol for each condition (indicated by different colours). All stimulation intensities were adjusted automatically by the QtracS software, thus enabling a single operator to carry out the whole recording without having to reposition the TMS coil or to manually adjust the intensity of the stimuli. Horizontal solid lines (bottom graph) represent target MEP size: 0.05 mV for $RMT_{0.05mV}$ (black), 1 mV for $TS_{1\ mV}$ (red), 0.2 mV for $RMT_{0.2mV}$ and T-SICI (green).*

6) <u>Adverse event questioning.</u> Adverse events (AEs) were assessed after the completion of assessments at Screening visit, at Treatment visits (at baseline and 1, 2, and 8 hours post-dose), Final Safety Check visit, and Telephone call. The AEs were recorded in verbatim terms in the study notes and Case Report Forms and were coded for the entry to the trial database in accordance with the Medical Dictionary for Regulatory Activities (MedDRA version 17.0). Lowest level terms

(LLTs) were used in the study database and the severity of AEs as well as their relationship to the study medication were assessed. Where possible, AEs were followed-up until resolution.

6.1.7 Dosing

The Treatment visits were scheduled in the morning. Subjects were advised to have a light breakfast prior to the visit and no food intake was allowed until the completion of 2-hour post-dose assessments. During each Treatment visit, a single oral dose of 2 mg or 10 mg of AZD7325 or 10 mg of microcrystalline cellulose (placebo) was administered following a completion of baseline safety and pharmacodynamic assessments (the active substance and placebo were presented in identical capsules). Each participant who completed the trial received all three doses.

6.1.8 Data management and statistical analysis

The primary and secondary outcome measures for this trial are summarised in Table 6.2. Sample size was calculated for the primary outcome variable. It was based on previous studies which showed an absolute increase in conventional SICI by more than 25% test MEP following a single dose of a benzodiazepine at the time of peak plasma concentration (Di Lazzaro et al., 2006, Teo et al., 2009) and a within-subject standard deviation of 16% test MEP from the previous reliability study[27] (Orth et al., 2003). For the primary outcome of A-SICI at the highest dose of AZD7325, a cross-over design study of nine participants was shown to have a power of 82% to detect an absolute change in A-SICI of 25% test MEP at a two-sided significance level of 0.05 (Schoenfeld, 2010). A sample size of 12 subjects was chosen to maintain a balanced randomisation, which would have allowed a detection of treatment effect of 20% test MEP at the same power and significance level. Furthermore, a sample of 12 subjects was shown to have a power of 80% to detect a statistically significant treatment effect when using Bonferroni correction for multiple comparisons for the dose proportionality analysis.

The safety and pharmacodynamic assessment data was recorded in paper Case Report Forms as well as a custom-made electronic trial database created in Microsoft Office Access 2003 database engine (Microsoft Corporation, Redmond, WA, US). The accuracy of the trial data records was periodically checked by an external trial monitor.

[27] Reliability data from Experiment 3 was not available at the time of submission of this clinical trial for regulatory approvals, therefore previously reported data was used. Sample size re-calculations with intraday within-subject standard deviation of A-SICI at CS 70% $RMT_{0.2mV}$ from Experiment 3 showed that a sample of 12 subjects would have a power of 83% to detect an absolute change in A-SICI of 25% test MEP at a two-sided significance level of 0.05. Therefore, no amendments to the study protocol were made.

After completion of the trial and the final monitoring visit, the database was locked, and data was exported for statistical analysis.

For normally distributed data (Shapiro-Wilk test, $p>0.05$) parametric tests were used for comparisons between groups and repeated measurements. Non-parametric tests (Wilcoxon signed rank test, Mann-Whitney U-test, Friedman's test) were used for non-normally distributed data. Repeated measures analysis of variance (rmANOVA) with Dose and Time as within-subject factors was carried out for both raw outcome variables and change from baseline to determine the effect of study medication as well as its time course. Analysis outcomes are summarised in Table 6.4. If significant main effects were identified, post hoc pairwise comparisons with Bonferroni adjustment were performed and are reported where relevant. Depending on data normality, Pearson's or Spearman's correlation coefficient was used to assess association between different measures.

Primary outcome	Change in conventional SICI at ISI of 2.5 ms and CS intensity of 70% $RMT_{0.05mV}$ at t_{max} (2 hours post-dose)
Secondary outcome	Change in conventional SICI at ISI of 2.5 ms and CS intensity of 70% $RMT_{0.05mV}$ at 1 and 8 hours post-dose
	Change in mean stroke frequency, mean axial pressure, and mean coefficient of variation of peak velocity in the KA of circle drawing
	Change in SDMT score
	Change in $VAS_{sedation}$
Exploratory outcome	Change in conventional SICI slope
	Change in threshold-tracking SICI slope

Table 6.2. Outcome variables. *SICI – short interval intracortical inhibition; CS - conditioning stimulus; KA - kinematic analysis; SDMT - Symbol Digit Modalities Test; VAS - visual analogue scale; $RMT_{0.05mV}$ - resting motor threshold (conventional cut-off value of 0.05 mV); t_{max} - time of peak plasma concentration.*

Reliability analysis for TMS parameters was carried out as described in section 2.8 of Chapter 2. Measurements obtained at Screening visit as well as baseline of three Treatment visits were used to assess their reliability during the trial.

Data is presented as mean ± standard deviation (SD) when normally distributed or as median and interquartile range (IQR) of the 25^{th} and 75^{th} percentile, if non-normally distributed.

6.2 Results

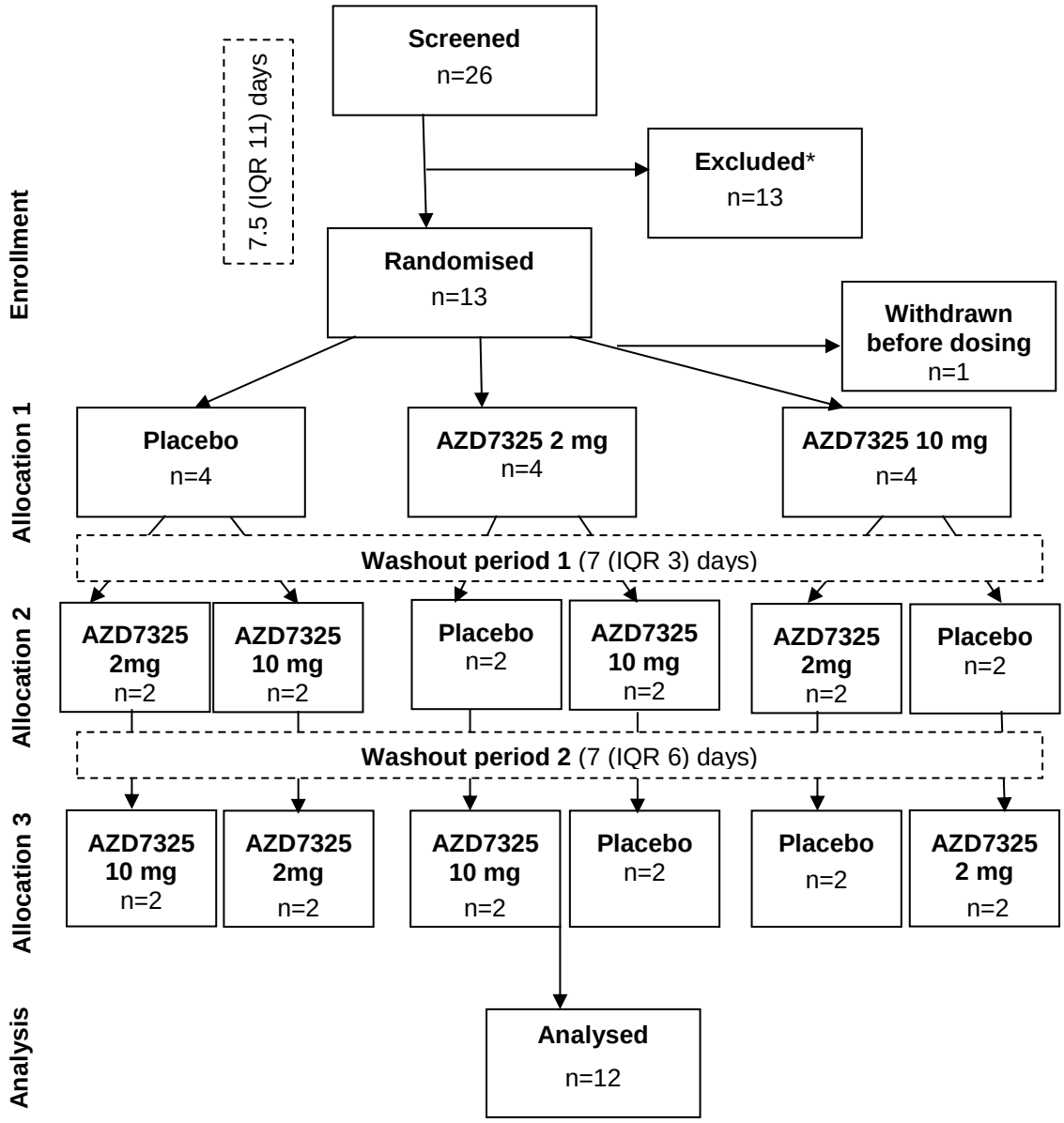
6.2.1 Subjects

Twenty-six subjects were screened, out of whom 13 were deemed eligible for the trial. One subject was withdrawn from the study prior the first dosing due to the use of prescription painkillers following back injury, and 12 participants (median age 23.5 (IQR 5), range 18-33 years, 2 self-reported left-handed) successfully completed the trial (Figure 6.4).

6.2.2 Reliability of TMS parameters

The mean group TMS parameters remained stable throughout the trial. The reliability data is presented in Appendix C . No difference between trial visits was observed in the parameters of corticospinal excitability (rmANOVA: $RMT_{0.05mV}$ $F_{3,33}$=0.45, p=0.721; $RMT_{0.2mV}$ $F_{3,33}$= 0.91, p=0.445; TS_{1mV} $F_{3,33}$=2.18, p=0.110*).* The motor thresholds ($RMT_{0.05mV}$ and $RMT_{0.2mV}$) as well as stimulus intensity required to evoke a peak-to-peak MEP of 1 mV (TS_{1mV}) were the most reliable measurements that showed excellent reproducibility and good agreement (Appendix C.2). $RMT_{0.2mV}$ was equal to 104 ± 6% $RMT_{0.05mV}$, while TS_{1mV} constituted a median of 122 (17) % $RMT_{0.05mV}$ and did not differ between trial visits (rmANOVA, $F_{3,33}$=0.75, p=0.529 and Friedman's test, p=0.107, respectively).

While no difference in individual SICI conditions or combined slope measurement was observed between the trial visits with either technique (Appendix C.1), the reliability of these parameters ranged from poor to intermediate-good (Appendix C.2). Although most of the threshold-tracking parameters had higher ICCs compared to the conventional technique, this was not statistically significant. The agreement between the repeated SICI measurements, as indicated by coefficients of repeatability (or smallest detectable change), was poor with both techniques. These findings are overall similar to those in Experiment 3.



***Reasons for exclusion:**

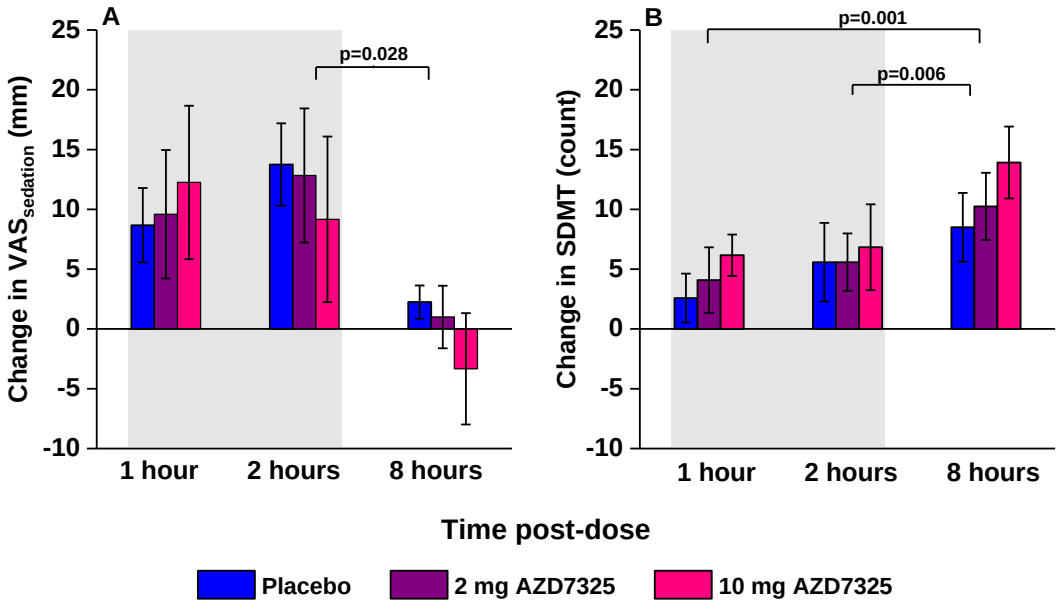
1) TMS related (n=7): absent SICI (n=1); 'floor' effect in conventional SICI (n=1); inability to relax for TMS (n=1); contraindications for TMS (n=4);
2) Abnormal laboratory/ECG findings (n=3): neutropenia (n=1); increased AST (n=1); I° atrioventricular block (n=1);
3) Kinematic analysis values outside 95% reference range (n=1);
4) History of allergy to a medication (n=1);
5) Withdrawn before randomisation (n=1).

Figure 6.4. CONSORT trial flow diagram. *The exclusion rate was higher than expected and was largely TMS-related. One subject was withdrawn from the study prior to the first dosing due to the use of prescription medication after the screening visit. None of the participants was withdrawn due to adverse events. The time intervals between the visits are presented as median (IQR) range.*

6.2.3 Effect of AZD7325 on sedation and psychomotor performance

There was no difference in sedation or psychomotor performance indices between the treatment arms at baseline (rmANOVA: $VAS_{sedation}$ $F_{1.3,14.7}$=3.58, p=0.069 and SDMT $F_{2,22}$=0.10, p=0.907). No significant correlation between raw sedation and SDMT scores was found at any timepoint (Pearson's r, p≥0.236) and no associations were observed in post-dose change of these parameters within a group (Pearson's r, ≥0.191).

Overall, participants reported little sedation at baseline. An increase was seen at 1 and 2 hours post-dose with return to baseline levels at 8 hours (Figure 6.5 A). rmANOVA with Dose and Time as within-subject factors revealed a significant main effect of Time, but no effect of Dose or Dose and Time interaction with both raw and change from baseline scores (Table 6.4). Post hoc comparisons of raw scores showed a trend towards a significant increase in sedation at 2 hours post-dose compared to baseline (p=0.059) and 8 hours timepoint (p=0.056), but this was not treatment-related.



	Placebo	2 mg AZD7325	10 mg AZD7325
Baseline $VAS_{sedation}$	11.9 ± 10.1	10.0 ± 8.1	18.1 ± 14.7
Baseline SDMT	85.6 ± 16.9	84.3 ± 22.5	80.8 ± 17.9

Figure 6.5. Effect of AZD7325 on sedation (A) and psychomotor performance (B). *Change from baseline calculated as [post-dose – baseline] is presented. Positive change indicates increase in sedation (A) and improvement in psychomotor performance (B). There was a main effect of Time on change in both variables (rmANOVA, p≤0.006; brackets indicate significant post hoc comparisons between timepoints), but no effect of Dose or Dose and Time interaction (rmANOVA, p≥0.572). Error bars represent standard error of the mean; shaded areas indicate time of maximum plasma concentration.*

The time course of changes in performance in SDMT suggests a learning effect (Figure 6.5 B). Indeed, improving performance was observed throughout the duration of the trial, with no significant change between Screening (62 ± 11.6) and the baseline of the first Treatment visit (65 ± 11.0), but marked increase in SDMT count between subsequent visits up to 98 ± 15.0 at baseline of the last Treatment visit. On Treatment days, rmANOVA showed a main effect of Time, but no effect of Dose or Time and Dose interaction (Table 6.4). Post hoc comparisons showed a significantly higher increase in SDMT count at the end of the Treatment visit compared to 1 and 2 hours timepoints ($p \leq 0.006$). Again, this improvement was not treatment-related.

6.2.4 Effect of AZD7325 on corticospinal excitability

There was no difference in CSE parameters at baseline between the treatment arms (rmANOVA $RMT_{0.05mV}$ $F_{2,22}=0.97$, $p=0.397$, $RMT_{0.2mV}$ $F_{2,22}=1.56$, $p=0.109$, TS_{1mV} $F_{2,22}=0.69$, $p=0.510$; Figure 6.6 A). Although a trend for a main effect of Dose on test MEP amplitude was seen at baseline (rmANOVA, $F_{2,22}=3.43$, $p=0.051$; Figure 6.6 B), post hoc comparisons did not reveal significant differences between the treatment arms ($p \geq 0.173$). rmANOVA with Dose and Time as within-subject factors showed no main effect of Dose or Dose and Time interaction with either raw or change from baseline scores (Table 6.4). Except for $RMT_{0.2mV}$, no main effect of Time was also observed. Post hoc comparisons revealed a significant increase in mean group $RMT_{0.2mV}$ at 2 hours post-dose compared to baseline ($p=0.024$), but this was not treatment-related.

Although no significant main effect of Dose was found on change in TS_{1mV} across three post-dose timepoints, the time course of changes in this parameter clearly differed between treatments on visual inspection: at expected t_{max}, i.e. 1 and 2 hours post-dose, an increase in TS_{1mV} was seen after AZD7325, while a decrease was noted after placebo. Indeed, rmANOVA at these timepoints showed a significant main effect of Dose ($F_{2,22}=4.11$, $p=0.030$), while post hoc comparisons revealed a significant mean increase in TS_{1mV} of approximately 7% MSO at t_{max} after 10 mg AZD7325 dose when compared to placebo ($p=0.007$). Importantly, there was no significant difference in change of average test MEP amplitude obtained using TS_{1mV} between the treatment arms (rmANOVA $F_{2,22}=1.71$, $p=0.205$).

This observation suggests that AZD7325 may alter the response to single-pulse stimulation. Increase in TS_{1mV} implies a reduction in CSE, which could be a result of a rightward shift of magnetic stimulus-response function and/or change in its slope. Given that no treatment-related change was seen in RMTs, change in slope rather than rightward shift of the curve is the more likely explanation. No correlation between changes in TS_{1mV} and sedation score was observed in any of the treatment arms

(Pearson's r, p≥0.083).

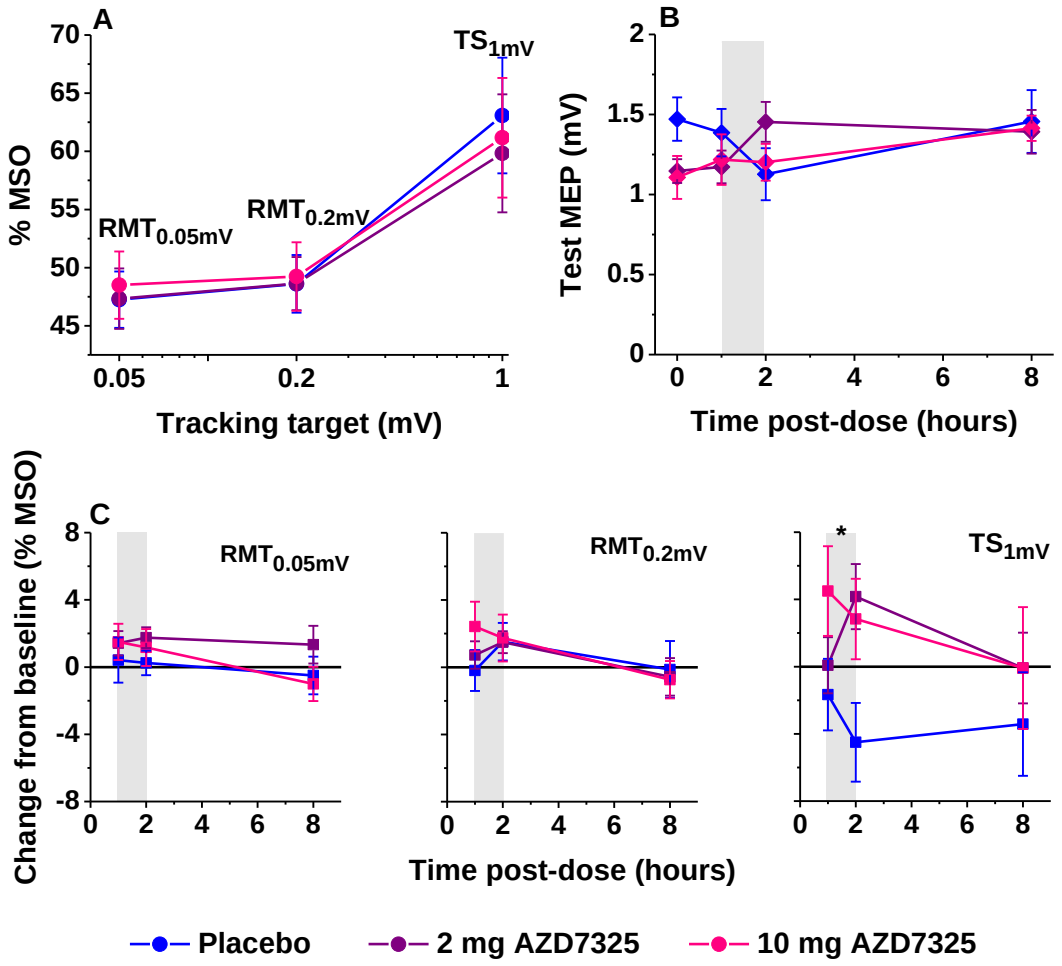


Figure 6.6. Effect of AZD7325 on corticospinal excitability. *The baseline corticospinal excitability was comparable between the treatment arms (A). Although conventional magnetic stimulus-response function was not recorded in the trial due to time constraints, some information on the input/output slope may be obtained from thresholds measured with different target MEP levels (i.e. 0.05, 0.2, and 1 mV). While no significant difference in the change of RMTs from baseline was observed between the treatment arms (rmANOVA, p≥0.475),* TS_{1mV} *changed in opposite directions after the intake of AZD7325 and placebo at the time of peak plasma concentration (i.e. 1 and 2 hours post-dose; indicated by shaded area in B and C). A main effect of Dose was observed (rmANOVA, p=0.030), and post hoc comparisons showed a significant difference between 10 mg dose and placebo (p=0.007, marked by asterisk). Baseline test MEP amplitude obtained at* TS_{1mV} *intensity was higher in the placebo arm (B). Despite a trend for main effect of Dose (rmANOVA, p=0.051), post hoc comparisons did not reveal significant differences between the treatment arms (p≥0.173). No main effect of Dose on change in test MEP from baseline was found at* t_{max} *(rmANOVA, p=0.205). This suggests that AZD7325 may decrease the corticospinal excitability by reducing the slope of the stimulus-response curve. Error bars represent standard error of the mean.*

6.2.5 Effect of AZD7325 on short-interval intracortical inhibition

6.2.5.1 Conventional SICI

Conventional SICI (A-SICI) measurements were defined as primary and secondary outcome measures in this trial, as they have been shown to be enhanced by $GABA_A$ modulating drugs in the past and no such studies have been carried out with threshold-tracking. There was no difference in baseline A-SICI measurements between the treatment arms in any A-SICI recruitment curve parameters, including combined A-SICI slope measurement and peak A-SICI (Figure 6.7., rmANOVA $F_{2,22}$=0.11-2.00, p≥0.159; Friedman's test, p≥0.368).

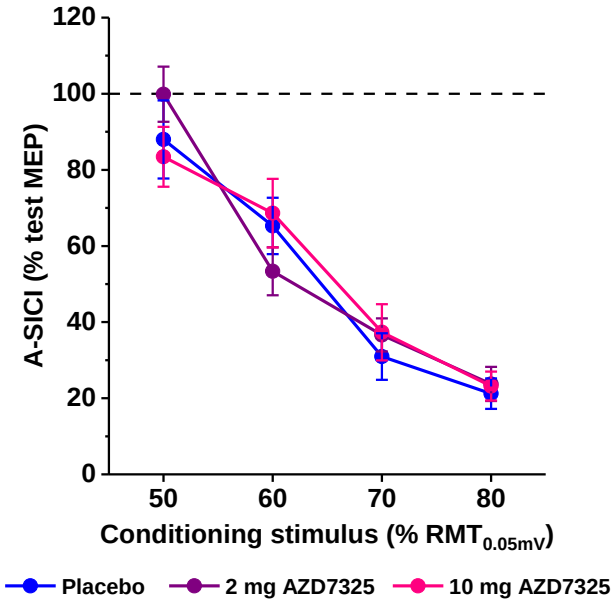


Figure 6.7. Baseline A-SICI recruitment curves. *No difference in A-SICI measurements between treatment arms was observed at baseline (rmANOVA, p≥0.159, Friedman's test p≥0.368). Error bars represent standard error of the mean; dashed line – control condition.*

A mean absolute increase of 5.9 ± 23.6 % test MEP was observed in A-SICI at CS 70% $RMT_{0.05mV}$ at 2 hours after the intake of 10 mg of AZD7325 (Figure 6.8 A), but this was not significant when compared to other treatment arms (rmANOVA, $F_{2,22}$=0.71, p=0.503). No main effect of Dose on change in A-SICI at CS 70% $RMT_{0.05mV}$ was found at 1 hour post-dose (rmANOVA $F_{2,22}$=0.75, p=0.485), but a trend was seen at 8 hours after the intake of study medication (rmANOVA, $F_{2,22}$=2.72, p=0.088). Post hoc comparisons did not show any difference between placebo and AZD7325 (p≥0.190), and the trend was no longer seen when an outlier with a 73% test MEP increase in inhibition after the 10 mg dose was removed from the analysis (rmANOVA, $F_{2,22}$=1.52, p=0.242). Sample size

calculation for this study was based on an expected change of 20-25% test MEP in the primary endpoint. However, the baseline inhibition at CS 70% $RMT_{0.05mV}$ was already relatively strong (around 35% test MEP across treatment arms), and a treatment effect of this magnitude may have not been possible to achieve due to the 'floor' effect. Being roughly in the middle of A-SICI recruitment curve, A-SICI at CS 60% $RMT_{0.05mV}$ may have been a more appropriate primary outcome measure in this study. Indeed, at this CS intensity level, a much larger mean absolute increase in inhibition (up to 17.1 ± 30.5% test MEP) was seen at t_{max} after 10 mg of AZD7325 and persisted for up to 8 hours post-dose (Figure 6.8 A). However, at neither of the timepoints a significant main effect of Dose was found (rmANOVA $F_{2,22}$=0.74-1.09, p≥0.346).

Given the large between-subject variability of baseline A-SICI, relative change from baseline was calculated for A-SICI at CS 60% and 70% $RMT_{0.05mV}$ as [(post-dose – baseline)/baseline*100%]. The largest relative increase in A-SICI of approximately 26% was seen at CS 70% $RMT_{0.05mV}$ at 8 hours after the intake of AZD7325 and was comparable between both doses of the study medication (Figure 6.8 B). A significant main effect of Dose was found (rmANOVA $F_{1.3,14.3}$=4.75, p=0.039), and post hoc comparisons showed that only the effect of 10 mg of AZD7325 tended to differ from placebo (p=0.082). This trend remained after an outlier with approximately 300% reduction in inhibition after placebo was removed from the analysis (rmANOVA $F_{2,22}$=3.40, p=0.054, post hoc p≥0.120).

rmANOVA with Dose and Time as within-subject factors was carried out for other A-SICI recruitment curve parameters, including combined slope measurement and peak inhibition, but no main effect of Dose, Time, or Dose and Time interaction was found (Table 6.4).

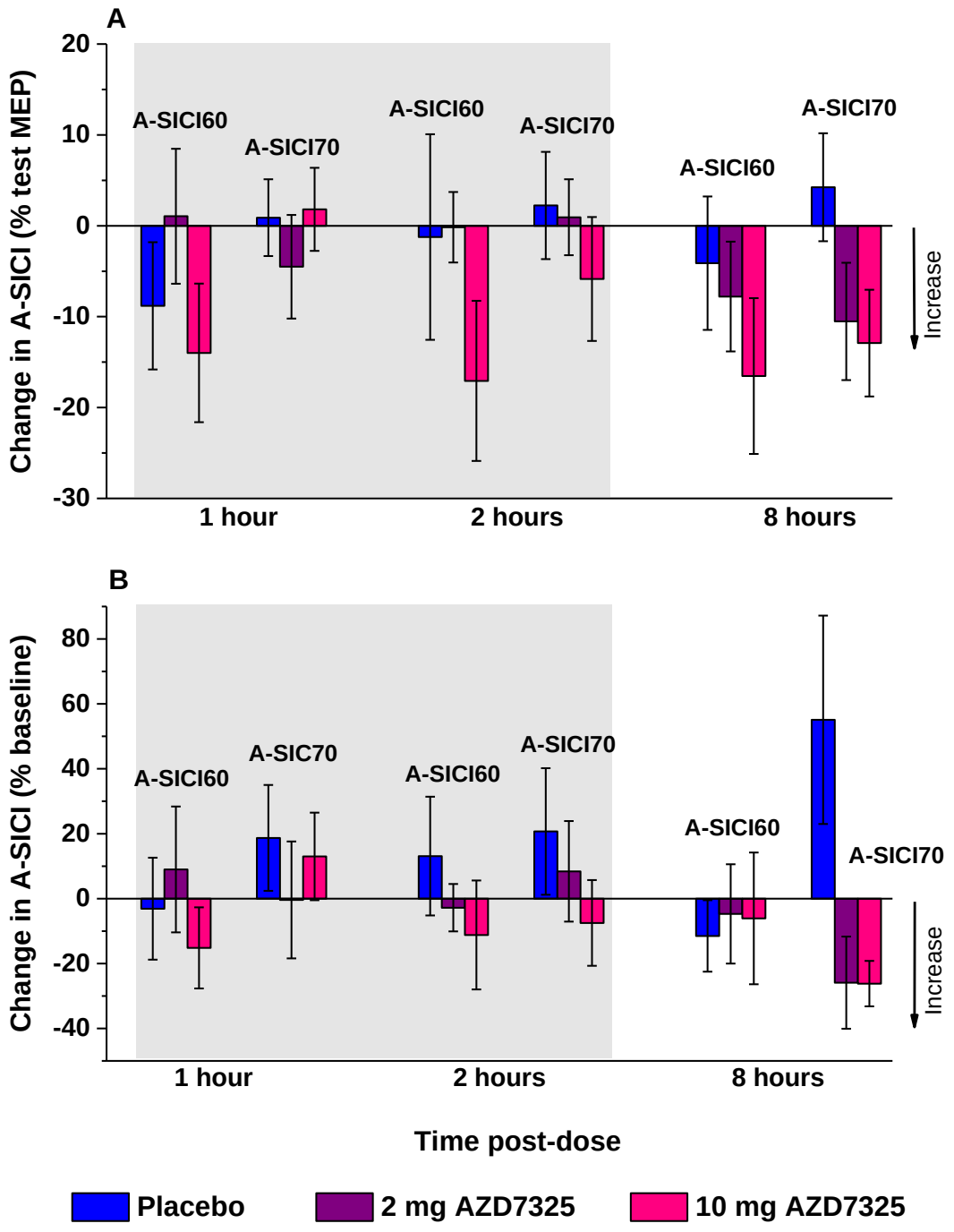


Figure 6.8. Effect of AZD7325 on A-SICI. *Absolute (A) and relative (B) change from baseline in A-SICI parameters is presented (negative values indicate increase in inhibition). The bars indicate group mean, error bars – standard error of the mean; shaded area – time of peak plasma concentration. Although increase in inhibition at both conditioning stimulus intensities was consistently observed at 1 and/or 2 hours after the intake 10 mg of AZD7325, it was not significant when compared to other treatment arms (rmANOVA, p≥0.346). A-SICI remained increased at 8 hours post-dose and the main effect of Dose was close to reaching statistical significance at CS 70% $RMT_{0.05mV}$ (rmANOVA, p=0.088). Largest relative increase in A-SICI was seen at CS 70% $RMT_{0.05mV}$ and 8 hours after the intake of both AZD7325 doses (rmANOVA, p=0.039), but only 10 mg dose tended to differ from placebo (post hoc p=0.082).*

6.2.5.2 Threshold-tracking SICI

T-SICI at CS 60% and 70% $RMT_{0.2mV}$ were non-normally distributed (Shapiro-Wilk test, $p<0.05$) with one subject consistently showing stronger inhibition at CS 60% and 70% $RMT_{0.2mV}$ compared to the rest of the group. Furthermore, in the same subject a 'ceiling effect' was observed at 1 hour following the 10 mg of AZD7325 dose at CS 50-70% $RMT_{0.2mV}$ (i.e. the target MEP level could not be achieved in the presence of CS despite test stimulus reaching 100% MSO). Therefore, this subject was excluded from further analysis of the threshold-tracking data.

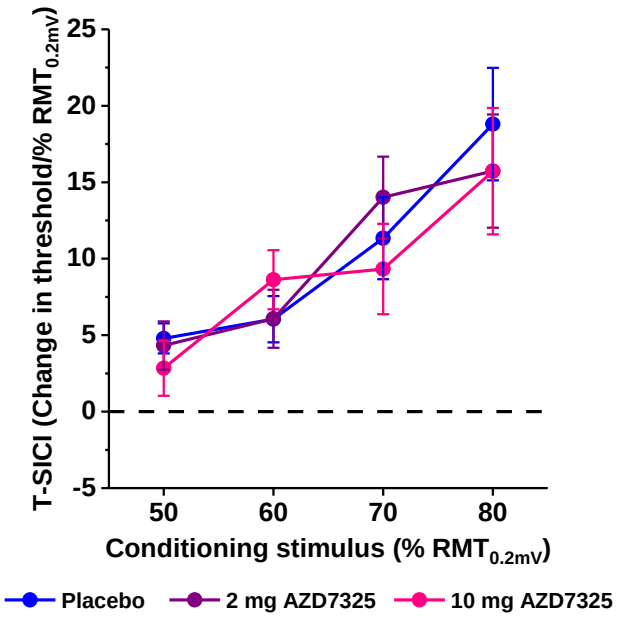


Figure 6.9. *Baseline T-SICI recruitment curves. No difference in T-SICI measurements between treatment arms was observed at baseline (rmANOVA (n=11), p≥0.264). Error bars represent standard error of the mean; dashed line – control condition.*

There was no significant difference in baseline threshold-tracking SICI (T-SICI) recruitment curves, T-SICI slope and peak T-SICI between the treatment arms (Figure 6.9; rmANOVA $F_{2,20}$= 0.19-1.43, p≥ 0.264). rmANOVA with Dose and Time as within-subject factors showed no main effect of these factors or their interaction (Table 6.4). Given the high between-subject variability of baseline T-SICI measurements, absolute and relative change from baseline was calculated for each post-dose timepoint[28].

A mean increase in T-SICI at CS 70% $RMT_{0.2mV}$ of up to 4.7 ± 15.2 % $RMT_{0.2mV}$ was

[28] For relative change calculation, baseline T-SICI values were expressed as [conditioned threshold/$RMT_{0.2mV}$*100%] instead of [change in threshold/$RMT_{0.2mV}$*100%]. This was done as in some subjects facilitation rather than inhibition was observed at baseline (i.e. negative T-SICI values if expressed conventionally), and normalising to negative values would have biased the group means.

observed at 1-2 hours after the intake of 10 mg of AZD7325 (Figure 6.10 A). However, an increase in inhibition of similar magnitude was observed at 1 hour after the intake of placebo, while reduction was observed at 2 hours after 2 mg of AZD7325. There was no significant main effect of Dose at any of these timepoints (rmANOVA, $F_{2,20}$=0.43-2.51, p≥0.107).

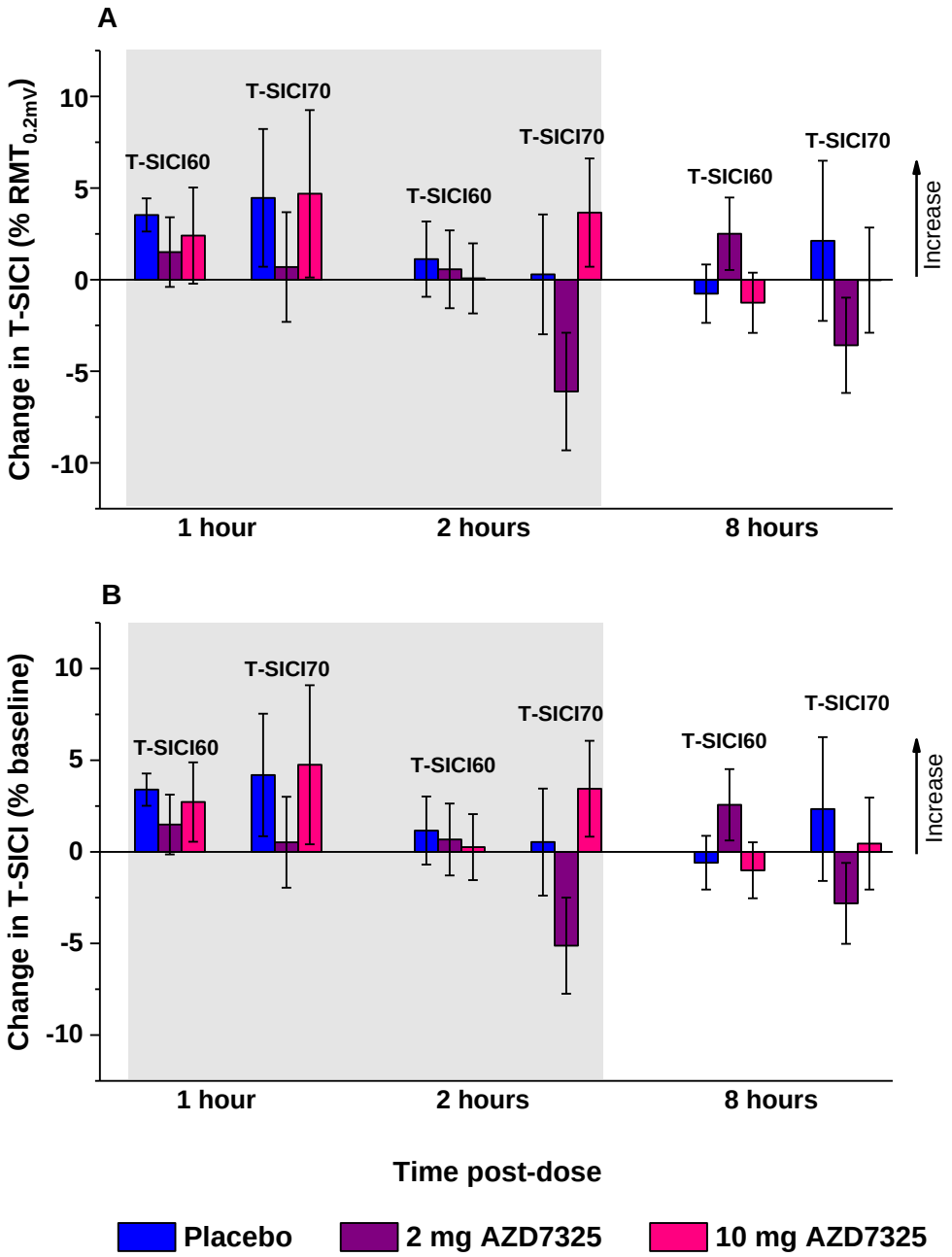


Figure 6.10. Effect of AZD7325 on T-SICI. *Absolute (A) and relative (B) change from baseline in T-SICI parameters is presented (n=11; positive values indicate increase in inhibition). The bars indicate group mean, error bars – standard error of the mean; shaded area – time of peak plasma concentration. Absolute and relative increase in T-SICI at CS 70% $RMT_{0.2mV}$ was observed at the of peak plasma concentration after the 10 mg of AZD7325, however, this was not significant when compared to other treatment arms (rmANOVA, p≥0.107).*

There was no correlation between mean baseline A-SICI and T-SICI neither at individual conditions (Pearson's r, p≥0.146) nor at slope or peak measurements (Pearson's r, p≥0.631). This could be explained by the fact that actual CS intensities (in % MSO) differed significantly between the techniques (paired-sample t-test, t=-9.16 - -6.68, df=11, p<0.001) as they were set based on different RMT estimates. Meanwhile, the mean baseline conditioned thresholds reached at CS 70-80% $RMT_{0.2mV}$ did not differ significantly from TS_{1mV} intensity used in A-SICI (paired-sample t-test, t=-1.54-0.41, df=32, p≥0.132), but were (or tended to be) lower for CS 50-60% $RMT_{0.2mV}$ conditions (paired-sample t-test, t=2.02-3.12, df=32, p=0.004-0.052). No significant within-group correlations were found between the maximum change in A-SICI and T-SICI at any timepoint after any of the doses[29] (Pearson's r, p≥0.355).

6.2.6 Statistical power considerations for SICI measurements

Sample size for this trial was calculated for primary outcome (A-SICI at CS70% $RMT_{0.05mV}$) based on previous reports on the variability of conventional SICI measurements and effect sizes observed with benzodiazepines. However, the effect of AZD7325 on SICI was smaller than expected, thus the absence of statistical significance could potentially be explained by inadequate sample size.

Parameter	Observed		SDC_{group}*	Cohen's d	Observed power	Required sample size
	Change*	Treatment effect*				
A-SICI70	-5.9	-8.08	± 9.2	-0.37	34%	34
A-SICI60	-17.7	-15.8	± 17.3	-0.45	37%	32
T-SICI70	3.7	3.4	± 6.8	0.27	13%	101

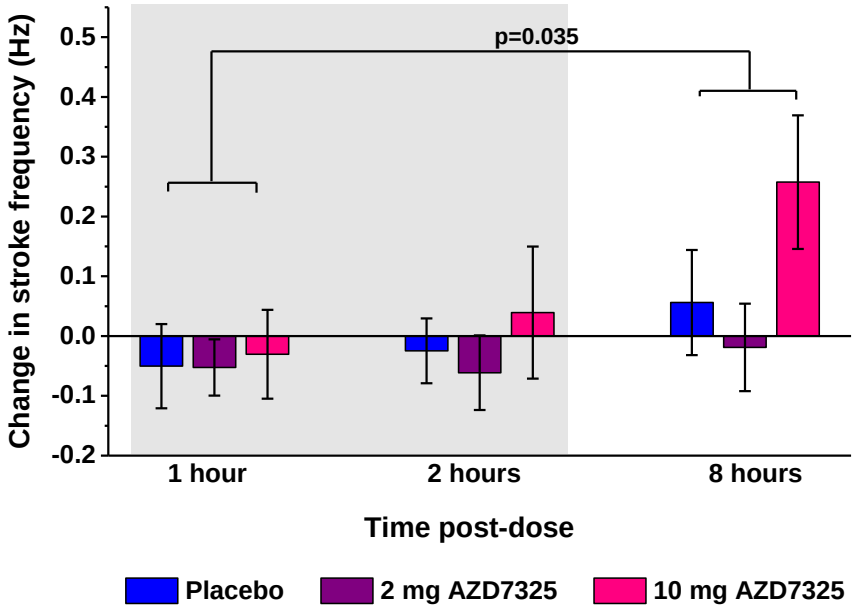
Table 6.3. Observed statistical power of the study. *Observed change represents the absolute change in SICI at 2 hours post-dose after 10 mg of AZD7325 compared to baseline, observed treatment effect – change compared to placebo; Cohen's d – standardised effect size (treatment effect/pooled standard deviation);* SDC_{group} *(smallest detectable change for sample) is based on observed reliability of SICI measurements in this study and indicates the measurement noise; observed power was calculated based on the observed treatment effect and within-subject variability in this sample; required sample size represents number of subjects needed to demonstrate statistical significance (p<0.05, not corrected for multiple comparisons) of the observed treatment effect with 80% power (Schoenfeld, 2010). * % test MEP for A-SICI,* $RMT_{0.2mV}$ *for T-SICI.*

[29] Given the lack of correlation between baseline A-SICI and T-SICI at individual conditions, only the relationship between maximum change in inhibition across all CS intensities was assessed.

Table 6.3 summarises the observed power of the study and sample size that would have been required to demonstrate statistically significant treatment effect on SICI measurements. Only the absolute change in A-SICI at CS 60% $RMT_{0.2mV}$ was greater than the measurement noise in this sample (i.e. greater than SDC_{group}), and roughly three times larger sample size would have been required to detect a significant treatment effect of 10 mg of AZD7325 at 80% power. The standardised effect size of AZD7325 was lower than previously reported for benzodiazepines (Ziemann et al., 2015).

6.2.7 Kinematic analysis of circle drawing

At baseline, a significant main effect of Dose on mean axial pressure was observed (rmANOVA, $F_{2,22}$=3.56, p=0.046), but post hoc comparisons revealed no significant difference between the treatment arms (p≥0.120), and no main effect of Dose was found



	Placebo	2 mg AZD7325	10 mg AZD7325
Baseline stroke frequency (Hz)	4.9 ± 0.39	5.0 ± 0.53	4.8 ± 0.52

Figure 6.11. Effect of AZD7325 on stroke frequency. *Change from baseline was calculated as [post-dose – baseline], positive values indicate increase in stroke frequency. There was a main effect of Time (rmANOVA, p=0.034; brackets indicate significant post hoc comparisons between timepoints), but no effect of Dose or Dose and Time interaction (rmANOVA, p≥0.172). At 8 hours post-dose, largest increase in stroke frequency was noted after intake of 10 mg of AZD7325, but this was not significant when compared to placebo (post hoc pairwise comparisons, p=0.512). Error bars represent standard error of the mean; shaded areas indicate time of maximum plasma concentration.*

on other kinematic analysis (KA) parameters between the treatment arms prior to the dosing (rmANOVA, $F_{2,22}$=2.06-2.38, p≥0.116).

There was no significant main effect of Dose and Time or Dose and Time interaction on KA parameters, except for stroke frequency (Table 6.4). A significant main effect of Time was observed on the change from baseline in this parameter, and post hoc comparisons revealed a significant increase at 8 hours post-dose compared to the first post-dose assessment (p=0.035; Figure 6.11). A trend for main effect of Dose was found at this timepoint (rmANOVA, $F_{2,22}$=3.16, p=0.062) with the largest increase in stroke frequency noted after 10 mg of AZD7325. However, this was not significant when compared to placebo (post hoc comparisons, p=0.512).

6.2.8 Correlation of pharmacodynamic outcomes

It is conceivable that the pharmacodynamic outcomes used in this study could be inter-dependent. For example, the level of sedation may have had an impact on the TMS parameters or psychomotor performance, while circle drawing variables could be related to the amount of SICI. The rmANOVA procedure is limited to time-invariant covariates only; therefore, within-subject correlations[30] were calculated as proposed by Bland and Altman (Bland and Altman, 1995b) from pooled data to screen for possible associations between the variables used in this study across all treatment arms and timepoints.

At baseline, there were no significant associations between $VAS_{sedation}$ and psychomotor performance (p=0.136) or circle drawing task parameters (p≥0.157). However, the change in sedation post-dose was negatively correlated with the change in SDMT score (r=-0.235, p=0.020) as well as stroke frequency in circle drawing task (r=-0.248, p=0.014).

No association between $VAS_{sedation}$ score and TMS parameters (including SICI) was observed within individuals at baseline (p≥0.146), except for conventional $RMT_{0.05mV}$ measure (r=0.512, p=0.009). Meanwhile, the change in $VAS_{sedation}$ score post-dose correlated only with the change in conventional A-SICI at CS 70% $RMT_{0.05mV}$ (r=0.227, p=0.025; more increase in sedation was associated with less increase in inhibition in an individual), but not with change in any other TMS parameter (p≥0.100).

[30] Ordinary correlation coefficients are not suitable for repeated measurements (Bland and Altman, 1994), but pooled data can be used to determine 'between subjects' and 'within subject' correlations. 'Between subjects' correlations define the association between two variables across the subjects, while the 'within subject' correlations describe the association between variables across repeated measurements within the individuals, and thus are more relevant for the purpose of this study.

At baseline, there were no significant within-subject correlations between CSE parameters (RMTs and TS_{1mV}) and SICI obtained with either of the techniques ($p \geq 0.065$). Post-dose, more increase in $RMT_{0.05mV}$ was associated with more increase in A-SICI at CS 60% $RMT_{0.05mV}$ ($r=-0.302$, $p=0.003$)**,** while more increase in TS_{1mV} - with less increase in A-SICI at CS 70% $RMT_{0.05mV}$ ($r=0.295$, $p=0.003$)**.** Change in T-SICI at CS 60-70% $RMT_{0.2mV}$ was not associated with changes in $RMT_{0.2mV}$ ($p \geq 0.690$) or TS_{1mV} ($p \geq 0.088$).

At baseline, no significant within-subject correlations were observed between SICI and KA variables ($p \geq 0.058$). Post-dose, more increase in A-SICI at CS 60% $RMT_{0.05mV}$ was associated with larger increase in stoke frequency in the circle drawing task ($r=-0.207$, $p=0.041$), while more increase in T-SICI at CS 70% $RMT_{0.2mV}$ – with more increase in CV of peak velocity ($r=0.248$, $p=0.019$). There were no significant within-subject correlations between change in SICI measurements obtained by the two techniques ($p \geq 0.545$).

6.2.9 Adverse events

All recorded adverse events (AEs) are summarised in Appendix D . There were no serious adverse events and none of the subjects was withdrawn from the trial due to AEs. Throughout the duration of the trial, each subject reported at least three AEs that were judged as treatment-related. There were more AEs in the 10 mg of AZD7325 arm than with other treatments (49 in 10 mg AZD7325, 19 in 2 mg AZD7325, 20 in the placebo arm) and the majority of them were reported at 1 hour post-dose (37 with 10 mg AZD7215, 11 with 2 mg AZD7325, 11 with placebo). Most AEs (84%) were mild, and there was one instance of severe somnolence following the intake of placebo. All AEs resolved without sequelae.

As expected, most treatment-related AEs were linked to the nervous system (57%) with somnolence/sedation being the most frequent (31% of all treatment-related AEs). Six subjects reported it after each dosing, and overall there was no significant difference in its frequency between the treatment arms (Fisher's Exact test, $\chi^2=0.95$, $p=0.887$). Dizziness was the second most frequent AE (9%), reported by five subjects following 10 mg AZD7325 dose, two subjects in the placebo and one in 2 mg AZD7325 arms. Feeling drunk (three subjects), hypoaesthesia or paraesthesia (two subjects each), and euphoric mood (three subjects) were observed only after the intake of 10 mg AZD7325.

At the end of the trial, subjects were asked whether they could identify the treatment sequence. Interestingly, all 12 subjects correctly indicated the visit during which they received 10 mg AZD7325, and the correct sequence of all three treatments was reported by half of the subjects suggestive of no subjectively noticeable difference between placebo and 2 mg dose of AZD7325.

Parameter	Factor	rmANOVA					
		Raw			Change from baseline		
		df	F	p	df	F	p
$VAS_{sedation}$	DOSE	2,22	2.36	0.118	1.4,15.1	0.13	0.802
	TIME	**3,33**	**6.94**	**0.001**	**2,22**	**6.58**	**0.006**
	DOSE x TIME	6,66	0.56	0.764	4,44	0.74	0.572
SDMT	DOSE	2,22	0.09	0.918	2,22	0.37	0.694
	TIME	**3,33**	**25.36**	**<0.001**	**2,22**	**14.07**	**<0.001**
	DOSE x TIME	6,66	0.49	0.815	4,44	0.72	0.585
$RMT_{0.05mV}$	DOSE	2,22	3.34	0.054	2,22	0.77	0.475
	TIME	3,33	2.19	0.108	2,22	2.05	0.152
	DOSE x TIME	6,66	0.81	0.564	4,44	0.84	0.510
$RMT_{0.2mV}$	DOSE	2,22	0.71	0.505	2,22	0.14	0.872
	TIME	**3,33**	**3.45**	**0.028**	**2,22**	**3.74**	**0.040**
	DOSE x TIME	6,66	0.68	0.667	4,44	1.00	0.417
TS_{1mV}	DOSE	1.4,15.1	1.19	0.312	2,22	2.25	0.129
	TIME	3,33	0.80	0.502	2,22	1.27	0.299
	DOSE x TIME	6,66	2.09	0.067	4,44	1.98	0.115
Test MEP	DOSE	2,22	0.73	0.494	2,22	1.51	0.244
	TIME	3,33	0.84	0.481	2,22	1.45	0.257
	DOSE x TIME	6,66	2.06	.070	4,44	0.52	0.724
A-SICI50	DOSE	2,22	1.62	0.220	2,22	0.75	0.486
	TIME	3,33	1.02	0.398	2,22	0.50	0.611
	DOSE x TIME	6,66	0.56	0.758	4,44	0.49	0.744
A-SICI60	DOSE	2,22	2.05	0.153	2,22	1.73	0.201
	TIME	3,33	1.48	0.238	2,22	0.22	0.802
	DOSE x TIME	3.2,35.2	0.59	0.638	4,44	0.32	0.861
A-SICI70	DOSE	1.1,11.7	0.01	0.940	2,22	1.48	0.249
	TIME	3,33	1.27	0.301	2,22	1.64	0.217
	DOSE x TIME	6,66	1.65	0.148	2.2,24.4	1.69	0.203
A-SICI80	DOSE	2,22	0.40	0.676	2,22	0.24	0.790
	TIME	3,33	0.38	0.768	2,22	0.55	0.587
	DOSE x TIME	6,66	0.44	0.851	4,44	0.52	0.719
A-SICI slope	DOSE	2,22	0.30	0.744	2,22	0.62	0.547
	TIME	3,33	1.62	0.204	2,22	0.56	0.578
	DOSE x TIME	6,66	0.55	0.772	4,44	0.53	0.718
Peak A-SICI	DOSE	2,22	1.03	0.375	2,22	0.10	0.903
	TIME	3,33	1.37	0.268	2,22	2.04	0.154
	DOSE x TIME	6,66	0.35	0.909	4,44	0.58	0.680

Table 6.4. *Continued on the next page*

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Parameter	Factor	rmANOVA					
		Raw			Change from baseline		
		df	F	p	df	F	p
T-SICI50*	DOSE	2,20	0.04	0.960	2,20	1.76	0.197
	TIME	3,30	0.31	0.818	2,20	0.34	0.719
	DOSE x TIME	6,60	0.97	0.456	4,40	0.70	0.597
T-SICI60*	DOSE	2,20	1.51	0.245	2,20	0.19	0.829
	TIME	3,30	1.60	0.209	2,20	1.64	0.219
	DOSE x TIME	2.9,28.6	0.79	0.505	1.9,19.2	0.97	0.394
T-SICI70*	DOSE	1.3,12.6	0.48	0.542	2,20	1.63	0.221
	TIME	3,30	1.59	0.211	1.3,13.0	2.61	0.125
	DOSE x TIME	2.9,28.7	0.83	0.482	4,40	0.55	0.703
T-SICI80*	DOSE	2,20	0.27	0.763	2,20	0.11	0.895
	TIME	3,30	0.06	0.980	2,20	0.07	0.934
	DOSE x TIME	2.7,27.4	0.68	0.560	4,40	1.15	0.349
T-SICI slope*	DOSE	2,20	0.14	0.872	2,20	0.26	0.773
	TIME	3,30	0.99	0.411	2,20	2.05	0.155
	DOSE x TIME	6,60	0.39	0.882	2.2,22.0	0.47	0.650
Peak T-SICI*	DOSE	2,20	0.26	0.774	2,20	0.04	0.960
	TIME	3,30	1.19	0.331	2,20	1.65	0.218
	DOSE x TIME	3.1,30.5	0.77	0.520	4,40	1.19	0.331
KA frequency	DOSE	2,22	0.74	0.489	2,22	1.40	0.268
	TIME	3,33	2.65	0.065	2,22	**3.98**	**0.034**
	DOSE x TIME	6,66	1.59	0.164	4,44	1.68	0.172
KA mean axial pressure	DOSE	1.3,14.4	4.75	0.038	2,22	0.30	0.743
	TIME	1.4,15.6	1.53	0.244	1.3,14.5	2.51	0.129
	DOSE x TIME	3.3,36.3	0.26	0.873	4,44	0.19	0.940
KA CV of peak velocity	DOSE	2,22	0.91	0.417	2,22	1.93	0.169
	TIME	3,33	2.29	0.096	2,22	0.72	0.497
	DOSE x TIME	6,66	0.84	0.541	2.4,25.9	0.32	0.764

Table 6.4. Repeated measures ANOVA summary table. *Raw scores and change from baseline were analysed for each variable with two within-subject factors DOSE (3 levels) and TIME (4 levels for raw scores, 3 levels for change from baseline). Significant main effects (p<0.05) are marked in bold. A-SICI and T-SICI – short-interval intracortical inhibition obtained by conventional and threshold-tracking methods, respectively, number indicates conditioning stimulus intensity level; KA – kinematic analysis of circle drawing; df – degree of freedom. * n=11.*

6.3 Discussion

In summary, this clinical trial failed to reach the primary endpoint. This means that at the chosen exposure levels, SICI is not affected by $GABA_A$ α2,3 signalling. The observed increase in TS_{1mV} intensity suggests that AZD7325 may have a direct depressive effect on corticospinal excitability. Although an increase in some of the conventional and threshold-tracking SICI outcomes was observed at t_{max} following 10 mg of AZD7325, it did not differ significantly from placebo. Unexpectedly, the largest relative increase in SICI was observed at 8 hours post-dose with the conventional technique (but not threshold-tracking) and was close to reaching statistical significance after 10 mg of AZD7325. In contrast, previously reported SICI-enhancing effects of benzodiazepines were observed at t_{max} (Table 1.4). AZD7325 was non-sedating, did not impair psychomotor performance and did not affect handwriting parameters.

6.3.1 Effect of $GABA_A$ α2,3 receptor modulation on corticospinal excitability

Neither of AZD7325 doses had an effect on motor thresholds, but a significant increase in TS_{1mv} intensity was seen at t_{max} (i.e. 1-2 hours) after the intake of 10 mg of AZD7325 when compared to placebo. These findings are consistent with the effects of other positive $GABA_A$ receptor modulators which suppressed MEP amplitude, but did not affect motor thresholds (Ziemann, 2004, Ziemann, 2013; also see reference in Table 1.4). Recordings of the TMS-evoked descending epidural volleys showed a significant suppression of late I-waves with concomitant reduction in MEP amplitude following a single dose of lorazepam (Di Lazzaro et al., 2000). More than 50% reduction of control MEP amplitude in the 1 mV range was observed after the intake of 20 mg diazepam in a plasticity study, and the increase in the test stimulus intensity required to maintain the pre-dose MEP size was 2% MSO higher compared to placebo (Heidegger et al., 2010). Studies employing magnetic stimulus-response functions showed that benzodiazepines induce the most prominent suppression of MEP amplitude at the top part of the curve (Boroojerdi et al., 2001, Kimiskidis et al., 2006) without significant effect on its slope (Kimiskidis et al., 2006). Unfortunately, obtaining a detailed stimulus-response function was not feasible in this study due to time constraints. Therefore, it is not possible to determine whether the effect of AZD7325 on corticospinal excitability has a similar pattern to that of benzodiazepines.

6.3.2 Effect of $GABA_A$ α2,3 receptor modulation on SICI

Availability of a safe selective $GABA_A$ α2,3 positive allosteric modulator AZD7325 allowed for the first time to directly assess the hypothesis that SICI is modulated via this pathway. Although a consistent increase in conventional SICI was observed after the

intake of 10 mg of AZD7325, this change was not significant when compared to placebo. At the chosen exposure level, this clinical trial failed to prove the hypothesis of $GABA_A$ α2,3 modulation of SICI. Nevertheless, several factors should be considered while interpreting these findings.

While the within-subject variability of SICI measurements in this study was similar to the previously reported (Orth et al., 2003, Samusyte et al., 2018), the observed change in SICI was smaller than the anticipated treatment effect used for sample size calculation. Thus, the study of 12 subjects was underpowered to demonstrate statistically significant treatment effect of the observed magnitude (Table 6.3). Another important consideration is the overall lack of efficacy of the study medication. The doses of AZD7325 used in this trial have previously failed to exert a significant effect on various CNS pharmacodynamic outcomes when compared to placebo in contrast to robust effects of an active comparator lorazepam (Chen et al., 2014). Similar findings were reported in another study in which intake of 2 mg of lorazepam resulted in significant increase in plasma prolactin levels[31], while 10 mg of AZD7325 showed only a trend with a four-fold smaller effect size (Te Beek et al., 2015). Furthermore, lack of efficacy of doses up to 15 mg twice a day was also observed in patients with generalised anxiety disorder (AstraZeneca, 2010a, AstraZeneca, 2010b). This suggests that despite achieving high $GABA_A$ receptor occupancy (Jucaite et al., 2017), AZD7325 may have a low pharmacodynamic potency.

On the other hand, the sensitivity of SICI as a biomarker for $GABA_A$ receptor modulation may be low. The doses of classical benzodiazepines used in most previous studies were relatively high, especially for diazepam, exceeding the recommended starting daily doses for the treatment of muscle spasms, anxiety, and even insomnia (Joint Formulary Committee, 2018). Memory impairing effects of diazepam can be seen even at low doses (0.1 mg/kg; Ghoneim et al., 1984), and dose proportionality of the effects of benzodiazepines on psychomotor performance has been well-described (Wittenborn, 1979). Meanwhile, dose relationship for SICI has not been systematically investigated, and it is not clear whether any effect would be evident at lower doses which are commonly prescribed at the initiation of benzodiazepine therapy to minimise the risk of side effects.

Interestingly, the increase in A-SICI at CS 60% $RMT_{0.05mV}$ was of similar magnitude at t_{max} as well as 8 hours post-dose, while the largest change in A-SICI at CS 70% $RMT_{0.05mV}$

[31] Increased prolactin level is a biomarker of reduced dopaminergic activity which can be modulated via $GABA_A$ receptors (α3-mediated inhibition and α1-mediated disinhibition; Tan et al., 2010, Rudolph and Knoflach, 2011, Te Beek et al., 2015).

was observed at 8 hours post-dose and a trend for a significant main effect of Dose was found. Furthermore, the relative increase in A-SICI at CS 70% $RMT_{0.05mV}$ at 8 hours after the intake of 10 mg of AZD7325 tended to be significant when compared to placebo. This is an unexpected and likely spurious observation, as SICI-enhancing effect of benzodiazepines with longer elimination half-lives than AZD7325 (Greenblatt et al., 1989, Chen et al., 2014) was commonly seen at t_{max}, but was no longer present at 6 hours post-dose (Di Lazzaro et al., 2005, Di Lazzaro et al., 2007). A similar time-course of lorazepam effects on other CNS biomarkers has been reported (Chen et al., 2014). Nevertheless, it cannot be entirely ruled out that modulation of SICI via α2,3 pathway may differ from the non-selective $GABA_A$ receptor modulation.

6.3.3 Effect of $GABA_A$ α2,3 modulation on threshold-tracking SICI

This is the first pharmacological study employing both conventional and threshold-tracking techniques for SICI. It has been shown that mean group SICI measurements obtained by the two methods have a strong linear relationship across a range of conditions when comparable conditioning stimulus intensities (in % MSO) are used (Samusyte et al., 2018). Thus, one would expect that SICI-enhancing effects of a drug would be captured by both techniques, and threshold-tracking may even be superior at demonstrating this effect as it is not limited by the 'floor' effect and potentially has a better reproducibility.

No effect of AZD7325 on T-SICI was found in this study. The highest numerical increase in inhibition was seen at CS 70% $RMT_{0.2mV}$ at t_{max} after 10 mg dose, but it was not significant when compared to placebo. A return to baseline of T-SICI was observed at 8 hours post-dose, while conventional SICI remained increased.

Has this clinical trial provided further insight into the comparability of conventional and threshold-tracking techniques for SICI? Although several observations point towards potential differences between the techniques (i.e. smaller standardised effect size of AZD7325 on T-SICI, CS intensity levels at which the largest change was observed and the discrepancy in the time-course of these changes), no conclusion can be confidently drawn as no statistically significant modulation of SICI was observed with either of the methods after the intake of AZD7325. Therefore, this study does not provide data to indicate that A-SICI and T-SICI are mechanistically different.

6.3.4 Effects of $GABA_A$ α2,3 modulation on other pharmacodynamic outcomes

No significant increase in sedation or impairment of psychomotor performance was found in this study following intake of AZD7325 when compared to placebo. This is consistent

with previous reports from phase I clinical trials (Chen et al., 2014, Jucaite et al., 2017) and provides further evidence for a potentially improved side effect profile of AZD7325.

In this study, a main effect of time of assessment on sedation and SDMT scores was observed and was unrelated to the received treatment. Sedation tended to increase at 2 hours post-dose and return to baseline at the end of the experimental day. It is unclear whether this was related to underlying circadian fluctuations in alertness or to the experimental conditions (e.g. quiet environment of the lab, repetitiveness of the tasks, etc.). Level of alertness or sedation may affect other CNS pharmacodynamic outcome measures, including TMS parameters. In this study, there was a negative within-subject association between the change in $VAS_{sedation}$ score and the change in SDMT count as well as stroke frequency in circle drawing task. Of all TMS measurements, only the change in A-SICI at CS 70% $RMT_{0.05mV}$ was associated with the change in sedation within a subject. However, all these correlations were weak suggesting that the variation in sedation could explain only a small portion of change in other outcome measures.

The performance of participants in SDMT improved both during the course of the trial and the experimental days. This indicates a learning effect which may have limited the utility of this test to detect treatment-related changes. Use of alternate forms of this test can prevent practice-dependent changes in its score (Benedict et al., 2012, Pereira et al., 2015), and thus would be more appropriate for future clinical trials.

AZD7325 did not affect motor control of the hand. Although kinematic analysis of handwriting was found to be helpful in the assessment of treatment effect in pathological conditions (Tucha et al., 2006, Mergl et al., 2007), very little is known about the effects of pharmacological interventions in healthy volunteers (Tucha and Lange, 2004) and no such data is available for benzodiazepines. In this clinical trial, the change from baseline in some SICI measurements was weakly associated with the change in kinematic analysis parameters within subjects. Post-intervention normalisation of SICI with concomitant improvement in kinematic analysis of handwriting parameters has been reported in writer's cramp patients (Siebner et al., 1999). Therefore, this simple and quick test could potentially be utilised to explore whether pharmacological modulation of TMS biomarkers translates into any detectable change in motor performance.

6.3.5 Safety profile of AZD7325

The adverse event profile of AZD7325 observed in this study is in keeping with the previous findings (AstraZeneca, 2009, Chen et al., 2014). The outcome of both quantitative (VAS) and qualitative (AE questioning) assessments was consistent showing no significant increase in sedation after AZD7325 when compared to placebo.

However, other CNS-related AEs, such as dizziness, euphoric mood, feeling drunk or paraesthesia, were more common or reported only after 10 mg of AZD7325. Furthermore, all subjects retrospectively identified the sequence number of this dose correctly. This suggests that the effect of the higher dose of the study medication may have differed from the lower dose and placebo resulting in subjective perception of the drug action, but the objective pharmacodynamic outcomes used in this study were not sensitive (or not appropriate) to capture it. It also shows that achieving complete blinding of both the subject and the investigator in placebo-controlled cross-over studies employing CNS acting drugs can be problematic despite meeting strict procedural requirements.

6.3.6 Strengths and limitations of the study

This study was a phase I randomised double-blind placebo-controlled cross-over clinical trial meeting the 'gold standard' methodological requirements for interventional studies. Two doses of the study medication were used allowing assessment of dose proportionality on SICI response. SICI recruitment curve rather than a single condition was obtained in order to increase the yield of detection of modulatory effects of the study medication. In addition to neurophysiological variables, functional outcomes were assessed. A fully automated magnetic stimulation protocol for SICI eliminated the operator bias arising from manual adjustments of stimulation intensities and ensured consistency of the neurophysiological assessments across the treatment visits and timepoints.

However, the interpretation of the study results is hindered by two major limitations: an absence of an active comparator and the arguable potency of the study medication. An active comparator was not included in this study and we therefore do not know whether our setup had a sufficient essay sensitivity. An additional benzodiazepine treatment arm would have added more certainty to the interpretation of conventional SICI findings (i.e. is the enhancement after 10 mg dose a spurious effect or could the lack of statistical significance be explained by an inadequate sample size and/or weak potency of the used doses of AZD7325?). It would also have provided more insight into the comparability of conventional and threshold-tracking techniques as well as their sensitivity to detect $GABA_A$ modulatory effects. Obtaining a detailed magnetic stimulus-response function would have allowed to better characterise the effects of AZD7325 on corticospinal excitability.

The AZD7325 dose choice was based primarily on the receptor occupancy data and side effect profile. The reported receptor occupancy in the occipital cortex was high (Jucaite et al., 2017), but it is unclear whether the receptor binding of AZD7325 in the primary

motor cortex would be comparable. Although the trial assessments were timed to capture the drug effects at the expected t_{max} and elimination half-life, drug plasma concentration measurements would have aided in determining the inter-individual differences in the pharmacokinetics of the drug which may have contributed to the variable effects on pharmacodynamic outcomes.

6.4 Conclusion

AZD7325 was non-sedating and did not impair psychomotor performance or motor control of the hand. Depression of corticospinal excitability as indicated by increased TS_{1mV} intensity was the only significant effect of AZD7325 observed in this study and is consistent with the mode of action of other $GABA_A$ receptor modulators (Ziemann, 2004, Ziemann, 2013). However, the enhancement of SICI with AZD7325 did not reach statistical significance, thus failing to provide direct evidence for the hypothesis of SICI modulation via $GABA_A$ α2,3 receptor pathway. Given that AZD7325 may have a generally weak pharmacodynamic potency at 2 mg and 10 mg doses (Chen et al., 2014, Te Beek et al., 2015), larger studies employing a more potent drug or higher doses of AZD7325 and an active comparator are required to further explore the hypothesis of $GABA_A$ α2,3 modulation of SICI and comparability of the conventional and threshold-tracking techniques.

Chapter 7 - General discussion

This work explored the reliability of threshold-tracking technique for the estimation of some of the most widely used single- and paired-pulse TMS parameters, namely resting motor threshold (RMT) and short-interval intracortical inhibition (SICI). For the first time, measures obtained by threshold-tracking were compared head-to-head to the conventional estimates, and the hypothesis of SICI modulation by $GABA_A$ α2,3 receptor signalling was directly tested in a pharmacological study. A summary of the main findings is presented in Figure 7.1. While the results of each experiment were discussed in detail in the relevant chapters, this chapter focuses on the general advantages and limitations of threshold-tracking, potential areas of application and directions for further development of threshold-tracking paradigms.

Experiment 1: Threshold-tracking not only provides quick and reliable point estimates of $RMT_{0.05mV}$ which are comparable to conventional methods, but also allows uninterrupted monitoring of its change

Experiment 2: Very slow fluctuations in average MEP amplitude and $RMT_{0.2mV}$ with a period of approximately two to five minutes were seen in individual recordings that could not be explained by the change in coil position during the recording

Experiment 3: Mean group SICI estimates obtained by conventional and threshold-tracking techniques showed a strong linear relationship across a range of conditioning stimulus intensities; threshold-tracking procedure was quicker and showed a trend towards improved reproducibility

Experiment 4: Significant enhancement of SICI by selective $GABA_A$ α2,3 receptor modulation after a single oral dose of AZD7325 could not be demonstrated with either of the techniques at the exposure to the drug level used in the present setting

Figure 7.1. Key findings.

7.1 Response size vs threshold

For decades, MEP amplitude has been be the most widely used marker of corticospinal excitability (Rossini et al., 2015). However, its reliability is hindered by huge variability. Two aspects of this variability can be distinguished: i) the trial-to-trial variability of MEPs which requires averaging of multiple responses to obtain a representative estimate and ii) the stability of these estimates over time.

Numerous factors have been found to have an impact on MEP variability (summarised in Table 1.1 in Chapter 1) and various tactics have been attempted to minimise it. This ranges from simple measures such as stabilising/fixing the position of the stimulation coil and/or subject's head, controlling for pre-stimulus activation of the target muscle and averaging a larger number of responses for MEP size estimate, to sophisticated methods such as use of navigation systems in combination with individual brain images for more precise coil (re)positioning (Gugino et al., 2001, Julkunen et al., 2009), 'closed-loop' stimulation to synchronise the TMS pulse with the ongoing oscillatory EEG activity in the motor cortex (Zrenner et al., 2018), or triple stimulation technique to overcome the desynchronisation of the α-motoneuron discharges (Rösler et al., 2008). However, even these advanced methods show limited utility in eliminating the variability of MEPs, especially taken into consideration their cost, additional time requirements or discomfort to the subjects.

The variability of MEP amplitude and area in Experiment 2 (Chapter 4) was consistent with previous reports (Kiers et al., 1993, Ellaway et al., 1998, Rösler et al., 2008). Both variability measures indicating trial-to-trial variability (nMCD) and dispersion over time (CV) constituted approximately half of the MEP amplitude elicited at a constant stimulus of 120% $RMT_{0.2mV}$. This was not related to shifts in coil position or unintended pre-stimulus activation of the target muscle during the session.

Interestingly, the within-session variability and agreement of MEP estimates obtained by averaging every 15 responses in Experiment 2 (section 4.2.1.4) was very similar to the within-subject variability and between-session agreement of the test MEP estimate in Experiment 3 (Table 5.2), even though in the latter they were obtained at intervals of tens of minutes or days with the coil being repositioned each time. This strongly suggests that the role of technical factors (specifically coil positioning) as a confounder of the reliability of MEP estimates may be rather limited in comparison to the inherent biological variability.

The extremely slow underlying fluctuations in MEP estimate is another important observation of this work (Chapter 4). They were seen in individual recordings, appeared to be cyclic with a period of two to five minutes and could not be explained by shifts in

coil position or pre-stimulus activation of the target muscle. They were prominent with the difference between the session's extreme estimates of more than 100% of the initial estimate. It is unlikely that they represent magnetic-stimulus induced plasticity changes as MEP estimates averaged across subjects remained stable throughout the session (Figure 4.3). Even though the cause of these slow fluctuations is not understood, it is likely to have practical consequences. It cautions against the use of MEP amplitude estimates for individual decision making (e.g. for classifying individuals into responders and non-responders in interventional studies).

An alternative measure of CSE is threshold, i.e. stimulus intensity required to obtain a response of (or above) a certain size. In the TMS field, this concept traditionally relates to the most excitable structures of the motor system which generate the smallest response that can be reliably measured (i.e. resting and active motor threshold). However, the same principle could be applied for any response size. Construction of TMS input-output curves by obtaining thresholds rather than MEP amplitudes has been proposed in the past (Awiszus, 2005, Julkunen et al., 2011) and threshold approach has been used for paired-pulse TMS paradigms (Fisher et al., 2002, Vucic et al., 2006, Cirillo and Byblow, 2016).

The main appeal of this approach is that threshold estimates are much less affected by the trial-to-trial variability of MEPs and could potentially improve the reliability of TMS measurements. For example, the variability and reliability of stimulus intensity required to evoke a MEP amplitude of approximately 1 mV (TS_{1mV}) in Experiment 3 was considerably better than that of the MEP estimate obtained at this intensity (Table 5.2). Similarly, conditioned thresholds were less variable than conditioned MEPs in SICI paradigm (Table 5.3).

Nevertheless, motor thresholds were also subject to extremely slow underlying changes (Experiment 2). There was no difference in the initial and overall estimates, but during the 20-minute session relative fluctuations of $RMT_{0.2mV}$ ranged within 10% of these estimates (Table 4.3). Although less clearly defined compared to MEP estimates, the cyclic pattern with a similar period of two to five minutes was observed in most RMT recordings (Figure 4.7). Simultaneous recordings of RMT and MEP or tracking at different target levels would allow to further elucidate whether these fluctuations in CSE parameters have a common source.

7.2 Probabilistic threshold estimation vs threshold-tracking

The conventional approach to threshold measurements is probabilistic (as discussed in section 1.2.1 of Chapter 1) given the observations that at liminal stimulus intensity the

response amplitudes vary from none to ‘giant’ (Rossini et al., 1994). The size of the response is used only to classify it as negative or positive (i.e. below or above the cut-off value) and the information encoded in the MEP amplitude is otherwise discarded. A single point estimate of threshold based on all previous observations is provided at the end of the procedure. Thus, any changes in excitability occurring during the procedure would be ‘averaged out’ and remain undetected.

Meanwhile in threshold-tracking, the size of the response is important as the algorithm attempts to maintain it ‘on target’. Only the information from a single preceding response is required for the procedure to advance; thus, it adapts relatively quickly and can be continued indefinitely. Throughout this work, threshold estimates were obtained from linear regression fitted into stimulus – log-transformed response plots excluding responses outside the previously described linear range (Fisher et al., 2002). Thus effectively, threshold estimation by threshold-tracking was based on the slope of the linear part of the input-output curve obtained over a limited range of stimulus intensities. The main advantage of threshold-tracking over probabilistic methods is uninterrupted monitoring of CSE and flexibility in choosing the time intervals for the point estimate calculation.

In this work, RMT estimation by threshold-tracking using the conventional cut-off value of 0.05 mV was for the first time validated against the well-established relative frequency and best PEST methods (Experiment 1). In addition, optimal tracking duration needed to obtain a reliable threshold estimate was determined. It required only 12 stimuli on average and was significantly quicker than other methods without compromising the reliability of the estimates (Chapter 3). Furthermore, slow shifts in $RMT_{0.05mV}$ were demonstrated in some subjects with threshold-tracking which possibly represent fluctuations in CSE seen in Experiment 2.

7.3 Conventional vs threshold approach for SICI

Despite being first described nearly 20 years ago (Awiszus et al., 1999, Fisher et al., 2002), threshold approach in paired-pulse TMS paradigms could be considered as relatively novel. Apart from the initial reports, the majority of literature on threshold-tracking TMS available at the time of experiment planning in 2014 came from one research group based in Sydney (Vucic et al., 2006, Vucic and Kiernan, 2008, Vucic et al., 2008, Vucic et al., 2009, Menon et al., 2013). Over the years, this group has extensively used a standardised paired-pulse protocol for SICI and intracortical facilitation (ICF) mainly in the context of motoneuron disease, but no accounts on the reliability of their method were available until very recently (Matamala et al., 2018).

To the best of our knowledge, Experiment 3 is the first study to directly compare conventional and threshold-tracking SICI estimates and their test-retest reliability (Chapter 5). A strong linear relationship was found between the mean group SICI estimates across a range of conditioning stimulus intensities, which suggests that the two techniques likely reflect similar inhibitory mechanisms.

Similar observations have been recently reported by Amandusson and colleagues. In their study of 20 healthy volunteers, authors found a significant moderate inverse correlation between conventional and threshold SICI measurements at a single conditioning stimulus intensity of 80% RMT and an interstimulus interval of 3 ms (Amandusson et al., 2017). For conventional SICI, test stimulus intensity was set to 120% RMT, while threshold SICI was obtained using an adaptive best PEST algorithm with the cut-off value set at 0.5 mV.

Another group (Cirillo et al., 2018) has compared conventional and threshold SICI estimates across two interstimulus intervals (2 and 3 ms), a range of conditioning stimulus intensities (70-90% active MT) and two current directions (posterior-to-anterior and anterior-to-posterior). For threshold SICI estimates, maximum likelihood PEST paradigm with a threshold target of 0.2 mV was used, while the test stimulus intensity for conventional SICI was matched to the intensity of conditioned threshold determined with the adaptive threshold-hunting (PEST). A significant correlation between the two methods was found only at an interstimulus interval of 2 ms and posterior-to-anterior current direction. The authors concluded that the amplitude and threshold measurements of SICI may not be comparable across the interstimulus intervals and current directions, although lack of correlation could also be explained by a ‘floor’ effect or matched test intensities being suboptimal for the conventional method (Cirillo et al., 2018).

The reliability profile of threshold SICI estimates is comparable between the available reports (Matamala et al., 2018, Mooney et al., 2018, Samusyte et al., 2018). Despite good reproducibility, the agreement between repeated measurements was poor in all three healthy-volunteer studies (Appendix E). A combination of large coefficients of repeatability and high intraclass correlation coefficients suggests that a test cannot be reliably used for decision making in an individual and that the differences between individuals are likely large (as discussed in section 5.3).

7.4 Advantages and limitations of threshold-tracking

The interest in TMS measures based on thresholds seems to be increasing, particularly for paired-pulse paradigms. In the literature, terms ‘threshold-tracking’, ‘threshold-

hunting', 'adaptive threshold method' are often used as synonyms. However, there are important methodological differences between the threshold-tracking technique developed by H. Bostock and adaptive probabilistic threshold estimation method proposed by F. Awiszus (section 1.2) which will be discussed further.

7.4.1 Advantages

The main advantage of threshold-tracking over other threshold estimation methods, such as best PEST or relative frequency, is that it allows instantaneous and uninterrupted monitoring of corticospinal excitability which does not require processing of multiple responses. The tracking algorithm uses the size of a single preceding MEP to advance and could in theory be continued indefinitely. This possibility to monitor threshold proved to be a useful feature for quality control of the recordings, i.e. for recognising slow drifts of the coil position that would have otherwise remained un-noticed (seen as a constant and prolonged change in threshold in one direction). Some tracking 'noise' due to trial-to-trial variability of MEPs is unavoidable. However, the 'sensitivity' to this variability can be adjusted by changing tracking parameters such as acceptable tracking error, tracking mode and the maximum tracking step size. Thus overall, this paradigm is relatively robust against the trial-to-trial variability of MEPs.

The traditional target size for RMT determination by threshold-tracking is 0.2 mV, and ± 20% error is commonly allowed (Fisher et al., 2002, Vucic et al., 2006). In theory, threshold-tracking can be carried out at any target MEP size provided that it lies in the linear part of the stimulus – log-transformed response curve. This work has provided empirical evidence that threshold-tracking with the target set at 0.05 mV and 1 mV can be used to obtain valid TMS parameters such as conventional $RMT_{0.05mV}$ estimate or test stimulus intensity for control MEPs in paired-pulse TMS.

In paired-pulse TMS paradigms, threshold-tracking has the potential advantage of online adjustment of conditioning stimulus intensities. In the conventional paired-pulse TMS or threshold paradigms employing PEST algorithm, the active or resting motor thresholds are determined beforehand to individualise the conditioning stimulus intensities which are then maintained fixed throughout the paired-pulse recording with the assumption that CSE will remain constant during it. However, the findings of Experiment 2 suggest that slow fluctuations in RMT do occur during a 20-minute recording session (Chapter 4) which may result in the pre-defined conditioning stimulus intensities to become suboptimal for eliciting inhibition or facilitation. Meanwhile, in threshold-tracking paired-pulse paradigms, RMT is continuously monitored and conditioning stimulus intensities can be adjusted online to maintain them as a constant fraction of RMT. It was hypothesised that this could potentially improve the reliability of paired-pulse estimates.

On a group level, threshold SICI estimates obtained by threshold-tracking showed strong linear relationship with conventional 'amplitude' SICI estimates at most individual conditioning stimulus levels (Figure 5.3) as well as across the whole recruitment curve (Figure 5.4 A). The potential advantage of threshold-tracking for SICI estimation is that it is not limited by a 'floor' effect seen with conventional technique, thus allowing the inhibitory potential to be fully evaluated (Chapter 5). This would be an important advantage in studies exploring SICI-modulating interventions where enhancing effects are expected.

Lastly, threshold-tracking has a potential to shorten acquisition time for both single- and paired-pulse TMS measurements as shown in Experiments 1 and 3, thus it could be a preferred method where time constraints play an important role.

7.4.2 Limitations and further methodological considerations

Availability has probably been one of the main factors limiting a wider interest in the use of threshold-tracking for TMS studies. This method currently requires a specific software (QtracW). QtracW is mainly used for peripheral nerve excitability studies for which it has standardised recording protocols and inbuilt advanced data analysis, plotting, and modelling features. At the start of this work, none of this was available for TMS and all the stimulation and data analysis scripts were written anew for each experiment using software-specific code. However, in the process a number of new features tailored for TMS have been added to the software (both for recording and data analysis) and scripts developed during this work could provide a basis for standardised TMS protocols.

Threshold-tracking and other threshold estimation paradigms in QtracW are fully automated and do not require input from the operator. Generally, this is advantageous as it allows the operator to fully concentrate on coil positioning during the recording. Stimulation intensities can be over-written, and most parameters can be adjusted online, if needed; however, the response classification is done automatically by the software. For this reason, threshold-tracking may not be straightforward for measurements during voluntary contraction of the target muscle.

Experiment 1 showed that the duration of tracking is important for the validity of measurements, especially if tracking is started at intensities way above or below the measured threshold. How quickly the algorithm reaches the representative threshold depends not only on the tracking settings (i.e. tracking mode and maximum step size), but also on the individual excitability characteristics of the subject. We demonstrated that RMT tracking can be deemed valid after the response hits and/or crosses the target six times (Chapter 3). Shorter tracking may result in a significant overestimation (or

underestimation) of the thresholds. The stopping rule of six valid threshold estimates was used for threshold SICI estimation throughout this work, but whether this is the optimal duration of tracking for paired-pulse TMS remains to be explored experimentally.

Although T-SICI is not limited by a 'floor' effect (unlike conventional amplitude measurements), a 'ceiling' effect may be observed in subjects with high RMT and strong inhibition as the stimulator will run out of power to demonstrate its full extent (section 5.3.6). This occurred in Experiment 4, where one subject's T-SICI data had to be excluded from the analysis.

Threshold SICI estimates generally correlated well with conventional amplitude measurements, but this was not the case in all subjects. There were individuals in whom clear inhibition was seen with conventional method, but not threshold-tracking (e.g. c, h in Figure 5.4 C). In these instances, the discrepancy between the techniques could not be explained by differences in conditioning or test stimulus intensities. A-SICI showed a 'floor' effect in both cases, which suggests that CS intensities were optimal to elicit inhibition. A common finding in these two subjects was that the conditioned MEP amplitudes at peak A-SICI were approximately 0.4 mV, while in other subjects with a 'floor' effect in the conventional method and clearly present T-SICI (b, e, j in Figure 5.4 C) they ranged from 0.05 to 0.22 mV. This suggests that the target of 0.2 mV may be too low to demonstrate inhibition by threshold-tracking in some individuals. The reasons for this can only be speculated. One possible explanation is that the composition of descending corticospinal volleys resulting in MEPs of around 0.2 mV may differ between the subjects. SICI suppresses late I waves but does not affect I1 wave (Di Lazzaro et al., 1998a, Hanajima et al., 1998). Thus, if at the target MEP size the volley primarily consists of I1 or D wave[32], inhibitory effects will not be demonstrated by threshold-tracking.

The effect of the target size for paired-pulse threshold-tracking paradigms was investigated in one study (Van den Bos et al., 2018). Mean group SICI increased with the target size set at 1 mv instead of the usual 0.2 mV, but only at interstimulus intervals of 5-7 ms. Meanwhile, SICI at 2-3 ms remained the same, irrespective of the target level. Only mean group estimates are presented in the paper, so it remains unclear whether increasing the target level had any effect on the magnitude of SICI at 2-3 ms within individuals.

[32] Normally D-wave should be recruited only at high stimulus intensities with posterior-to-anterior current flow in the motor cortex, but it is recruited early with lateromedial current direction (Figure 1.2). While coil position used in this experiment is considered optimal for inducing posterior-to-anterior current flow, it cannot be ruled out that this was not the case in some subjects due interindividual anatomical differences.

This suggests that target size could be important in quantifying inhibition by threshold-tracking (as test stimulus intensity (Garry and Thomson, 2009) and test MEP size (Roshan et al., 2003) are important in determining the magnitude of conventional SICI) and deserves further exploration.

7.5 Areas of application and practical considerations

This work was conducted with the consideration of two main potential areas of application for threshold-tracking TMS: routine clinical neurophysiology and development of novel therapies.

7.5.1 TMS as a diagnostic test

High variability and poor test-retest reliability of TMS parameters based on MEP amplitude measurements have probably been one of the major factors limiting the application of TMS in routine clinical practice. Time and resource constraints also play an important role. For instance, the IFCN guidelines recommended starting a routine central motor conduction time estimation procedure by determining patient's motor threshold to ensure that optimal stimulus intensities are used throughout (Groppa et al., 2012). However, motor estimation procedures proposed in these guidelines require delivery of more than 50 stimuli (Silbert et al., 2013) or a specific software (Awiszus and Borckardt, 2011) which essentially necessitates two operators to carry out the investigation. This would not be feasible in most busy clinical neurophysiology departments, especially given the limited diagnostic value of such measurements. Although abnormalities in paired-pulse TMS measures were observed in various CNS disorders (as discussed in section 1.3.4 of Chapter 1), large variability between patients and overlap with normal subjects limits their diagnostic utility (Berardelli et al., 2008, Chen et al., 2008).

Nevertheless, there has been an accumulating evidence that some paired-pulse paradigms may be helpful in certain conditions with challenging differential diagnosis. For example, combination of conventional short-interval intracortical inhibition-intracortical facilitation (SICI-ICF) and short-latency afferent inhibition (SAI) may aid in distinguishing types of atypical parkinsonism (impaired SAI was found in Alzheimer's disease and dementia with Lewy bodies, while impaired SICI-ICF – in dementia with Lewy bodies, corticobasal syndrome, and progressive supranuclear palsy; Benussi et al., 2018). The same group also showed that SICI-ICF and SAI abnormalities can differentiate between Alzheimer's disease and frontotemporal dementia with 91.8% sensitivity and 88.6% specificity (Benussi et al., 2017). However, the test-retest reliability of these measurements was not assessed in any of the studies.

A large body of evidence on potential utility of threshold-tracking TMS in the differential diagnosis of motoneuron disorders has come from the Sydney group (Vucic, 2009). Amyotrophic lateral sclerosis (ALS) is a disorder of the upper and lower motoneuron with a poor prognosis for which no effective treatment is currently available (Hardiman et al., 2017). While routine needle EMG provides evidence for the lower motoneuron dysfunction, demonstration of the upper motoneuron involvement crucial for the diagnosis of ALS (de Carvalho et al., 2008) may be challenging. With a standardised SICI-ICF protocol at CS 70% $RMT_{0.2mV}$ and interstimulus intervals of 1-7 ms and 10-30 ms, the Australian researchers observed that SICI is reduced in ALS, but not in mimic disorders (Vucic and Kiernan, 2008, Vucic et al., 2011, Menon et al., 2015a). A diagnostic cut-off value for averaged SICI of <5.5% $RMT_{0.2mV}$ has been proposed (Vucic et al., 2011) which in combination with inexcitable motor cortex showed a sensitivity of 73% and specificity of 81% in distinguishing ALS from mimic disorders (Menon et al., 2015a). Meanwhile, the diagnostic value of the central motor conduction time, a routine neurophysiological TMS measure of corticospinal tract integrity, was close to chance in the same study. Using the same threshold-tracking TMS protocol, the group reported that cortical hyperexcitability may precede the lower motoneuron involvement in ALS (Menon et al., 2015b), observed transient modulatory effects of riluzole on SICI (Geevasinga et al., 2016) and described prognostic value of SICI abnormalities (Shibuya et al., 2016b). Threshold-tracking TMS may also be useful in distinguishing syndromes with predominant upper motoneuron involvement (Geevasinga et al., 2015).

These reports make it very compelling to start using the above-mentioned TMS protocols in routine clinical practice. However, the test-retest reliability of the measurements becomes extremely important when dealing with an individual patient, particularly if the outcome of such test may determine further management and counselling. A healthy volunteer study showed that the standardised threshold-tracking SICI protocol had poor agreement of repeated measurements in an individual both on the same day and one week later (Matamala et al., 2018). A similar observation with a different protocol was also made in this thesis. However, no data on the reliability of these measurements in patient groups has been reported.

7.5.2 TMS measures as biomarkers for new drug development

The importance of biomarkers in the development of new therapies has been long recognised both by the pharmaceutical industry and regulatory authorities (Biomarkers Definitions Working Group, 2001, Colburn, 2003, Wagner, 2008). By definition, a biomarker is ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a

therapeutic intervention' (Biomarkers Definitions Working Group, 2001). It is not to be confused with a clinical endpoint which is 'a characteristic or variable that reflects how a patient feels, functions, or survives' (Biomarkers Definitions Working Group, 2001) and is the ultimate outcome measure of efficacy or hazard in clinical trials. However, certain biomarkers which have been proven to reliably predict the clinical outcome can be used as surrogate endpoints (Biomarkers Definitions Working Group, 2001) and can considerably improve the speed and efficiency of the development of new therapies (Wagner, 2008). Biomarker validation process entails demonstration of the reproducibility and accuracy of the assay, while biomarker qualification requires solid evidence of the link between the biomarker and the biological processes or clinical endpoints (Wagner, 2008).

In early clinical stages of drug development, the availability of target-engagement and well-defined disease-related biomarkers is important in understanding the mechanism of action and facilitating the 'proof of concept' (Wagner, 2008). Positron Emission Tomography (PET) or Single Photon Emission Computed Tomography (SPECT) with target-specific radioligands can be utilised to non-invasively demonstrate that the drug reaches the intended target and binds to it in the brain *in vivo* (as reviewed by Grimwood and Hartig, 2009). Although the extent of the target occupancy can be used to guide the dosing regimen and, in some cases, may help predict the clinical outcome, physiological biomarkers are necessary to ascertain the drug's efficacy at the target and a given receptor occupancy.

'Effects of AZD7325 on SICI' clinical trial (Chapter 6) was a part of a research programme exploring $GABA_A$ α2,3 signalling as a novel therapy for focal dystonia. The aim of this phase I clinical trial was to explore whether SICI can be used as a target-engagement biomarker for the $GABA_A$ α2,3 receptor signalling. Had the enhancement of SICI after a single oral dose of AZD7325 been observed in healthy volunteers, its use in the subsequent patient study as a disease-related biomarker may have helped to elucidate the role of impaired $GABA_A$ α2,3 receptor signalling in the motor cortex in the pathophysiology of this condition[33].

However, the phase I clinical trial failed to demonstrate a significant enhancement of SICI measured with both conventional and threshold-tracking methods. It could be concluded that SICI is not mediated by α2,3 receptor signalling and other $GABA_A$ receptor subtypes are required to modulate SICI, but the lack of an active comparator and the general concern of the efficacy of the study medication complicates the interpretation of this outcome (as discussed in section 6.3.2 of Chapter 6). The story of

[33] As phase I trial was negative, it was decided not to proceed with the patient study.

the development of AZD7325 serves as an example that high receptor occupancy by a compound does not necessarily translate into a significant biological effect[34] and underscores the need for objective physiological biomarkers of target-engagement.

7.6 Future considerations

Despite relative robustness against the variability of MEPs and possibility to continuously monitor and adapt to the changes in corticospinal excitability, SICI estimates obtained by threshold-tracking did not prove to be reliable enough to be used for individual decision making. However, they may provide improved reproducibility on a group level which would potentially reduce the sample sizes required for interventional studies (Samusyte et al., 2018). Unfortunately, the interventional study with selective $GABA_A$ α2,3 receptor modulator did not provide further insight into the comparability of the two techniques, likely due to the lack of efficacy of the study medication. Therefore, the sensitivity of threshold SICI to an intervention remains to be determined, preferably in studies employing classical benzodiazepines which have shown a robust effect on conventional measurements in the past.

This work has not explored the significance of SICI overlap with short-interval intracortical facilitation (SICF). Dissection of inhibitory and faciliatory circuits may be particularly important in disease conditions (e.g. ALS) where it can provide insight into the underlying pathophysiology, or pharmacological studies of drugs with poorly understood mechanisms of action. Threshold-tracking has been shown to have the potential to discern these two overlapping circuits (Awiszus et al., 1999, Fisher et al., 2002), and an optimised protocol looked promising in pilot recordings carried out in our lab.

Although it is generally accepted that corticospinal excitability is not static and is influenced by multiple technical and biological factors, the extent and the pattern of its fluctuations observed during a standard recording session was somewhat unexpected (Chapter 4). While the source of these extremely slow changes is yet to be determined, they could in part explain the large within-subject variability of TMS measurements despite attempts to optimise the estimation methods and control for technical factors.

In light of these observations, the main potential of the threshold-tracking technique is that it provides the means to seamlessly record these fluctuations and could be used to

[34] It was shown that generally $GABA_A$ positive allosteric modulators (e.g. classical benzodiazepines, zolpidem) exert their therapeutic effects at a rather low receptor occupancy (5-30%, as reviewed in Grimwood and Hartig, 2009). Given that much higher receptor occupancy was reached with the doses of AZD7325 used in Experiment 4 (Jucaite et al., 2017), it is likely that the intrinsic ligand activity of AZD7325 is probably much lower than that of classical drugs of this class.

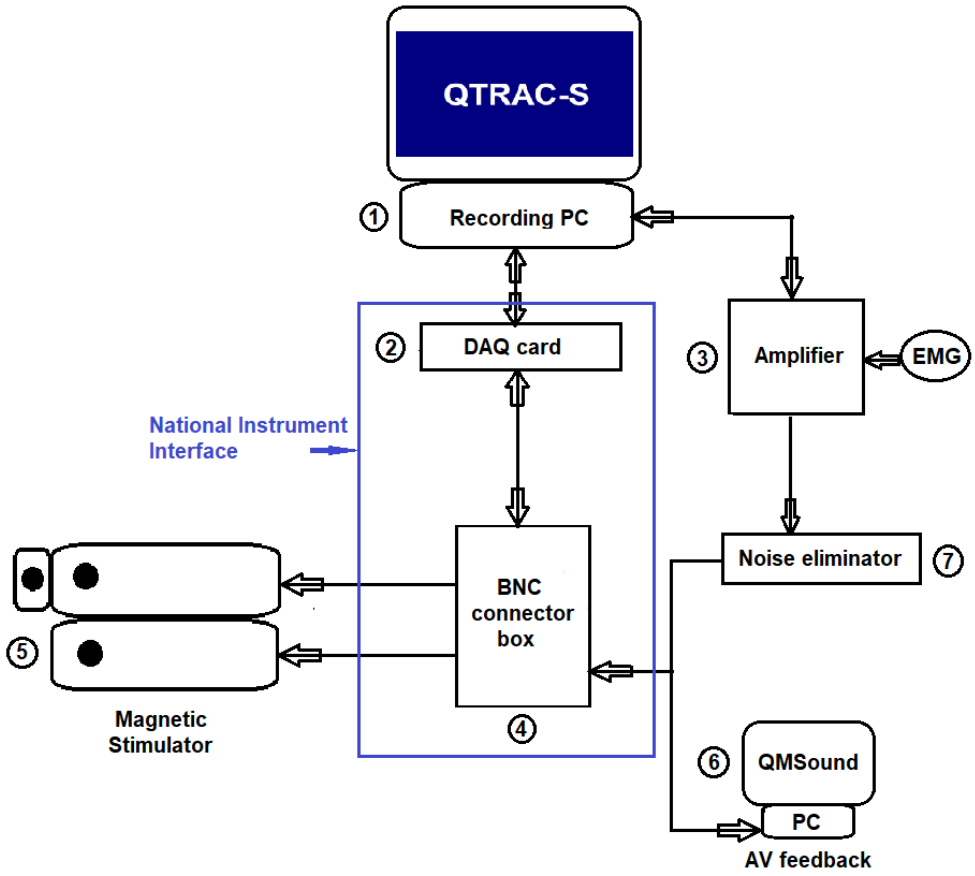
observe the dynamics between corticospinal excitability and various inhibitory and facilitatory circuits in real-time (e.g. in behavioural experiments or studies of CNS pathology). It would also allow to detect the immediate effects of modulatory interventions (such as fast-acting CNS drugs, transcranial direct current stimulation, motor tasks, etc.) with a minimal confounding impact of MEP variability, thus opening new avenues in TMS research.

7.7 Acknowledgements of contributions

This research was funded by Medical Research Council (Grant Reference MR/K015222/1). Prof Martin Koltzenburg (Chief Investigator), Prof John Rothwell, Dr Lorraine Webber (AstraZeneca) developed the concept and the protocol for 'Effects of AZD7325 on SICI trial' and were in the trial team (Experiment 4). Prof Martin Koltzenburg developed the initial QtracW script which served as a template in the subsequent work. Prof Hugh Bostock kindly implemented new features in QtracW required for the experimental work, provided continuous guidance for script development, data analysis, and troubleshooting. Gareth Bahlke, biomedical engineer at the National Hospital for Neurology and Neurosurgery, assisted with the technical part of the experimental setup. Charlotte Havill was one of the operators in the Experiment 2 and presented some data in her MSc thesis; the dataset in the current thesis was analysed independently.

Appendix A - A detailed description of the hardware setup

A.1 Schematic diagram of the hardware setup for TMS using QtracS software



① Viking Select EMG Unit (Nicolet Biomedical Inc., Madison, WI, USA) running QtracS software;

② Data Acquisition Card – NI PCI-6221 (National Instruments, Austin, TX, USA);

③ Nicolet EA-2 (Nicolet Biomedical Inc., Madison, WI, USA);

④ NI BNC-2110 (National Instruments, Austin, TX, USA);

⑤ BiStim^2 (Magstim, Whitland, UK);

⑥ Personal computer for audiovisual (AV) feedback running QMSound software;

⑦ HumBug 50/60 Hz Noise Eliminator (Quest Scientific Instruments Inc., North Vancouver, BC, Canada).

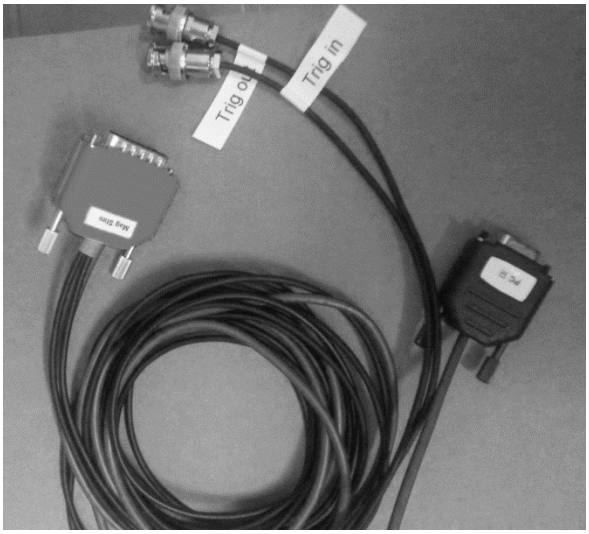
The National Instrument Interface consists of a DAQ card fitted to the Viking Select EMG Unit and a BNC connector box. Alternatively, all-in-one USB boxes (with DAQ cards and BNC sockets), such as NI USB-6221-BNC or USB-6251-BNC (National Instruments, Austin, TX, USA), can be used.

A.2 Types of connections between the devices

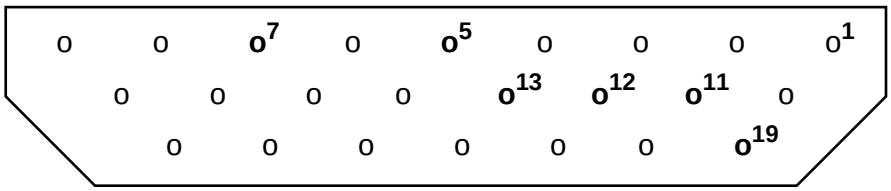
Port/connector on Device 1	Cable		Port/connector on Device 2
	Connector type (to Device 1)	**Connector type (to Device 2)**	
COM serial port RS-232 of ① →	9-Pin D-type Female	26 Way HD D-type Male	→ Isolated interface of ⑤ (Master Unit)
Digital signal from ② via (P0.0) User 1 of ④ →	BNC	26 Way HD D-type Male	→ Isolated interface of ⑤ (Master Unit)
Digital signal from ② via (P0.1) User 2 of ④ →	BNC	BNC	→ Trigger In on Stimulator Interface Module connected to ⑤ (Slave Unit)
Analog signal from Analog out of ③ →	3.5 mm Mono Jack plug	BNC	→ Input of ⑦
Analog signal from Output of ⑦ →	BNC	BNC	→ AI0 of ④
Analog signal from Output of ⑦ →	BNC	3.5 mm Stereo Jack plug	→ Line In of ⑥
② ↔	SHC68-68-EPM (68 HD D-type to VHDCI)		↔ ④
① ↔	Fire Wire*		↔ ③

HD – high-density; BNC - Bayonet Neill–Concelman; VHDCI – very high-density cable interconnect; circled numbers refer to the device number in A.1.

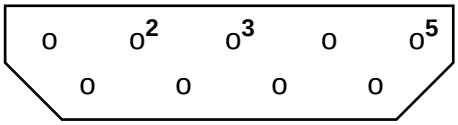
A.3 Custom-made cable for connection to Magstim200^2



26-Way High-Density D-type Male Connector



9-Pin Female RS-232 Connector



26-Way High-Density D-type Male Connector	9-Pin Female RS-232 Connector	BNC
Pin 5		Trigger In (to Magstim200^2) on (+) pulse
Pin 7		Trigger Out (from Magstim200^2) on (+) pulse
Pin 11 (Ground)	Pin 5 (Ground)	
Pin 12 (Rx to Magstim200^2)	Pin 3 (Tx from PC)	
Pin 13 (Tx from Magstim200^2)	Pin 2 (Rx to PC)	
Pin 19		Auxillary Ground

BNC - Bayonet Neill–Concelman; Rx – Receive; Tx – Transmit; PC – recording personal computer. Courtesy of Gareth Bahlke, a biomedical engineer at the National Hospital for Neurology and Neurosurgery.

Appendix B - Clinical trial assessments (Experiment 4)

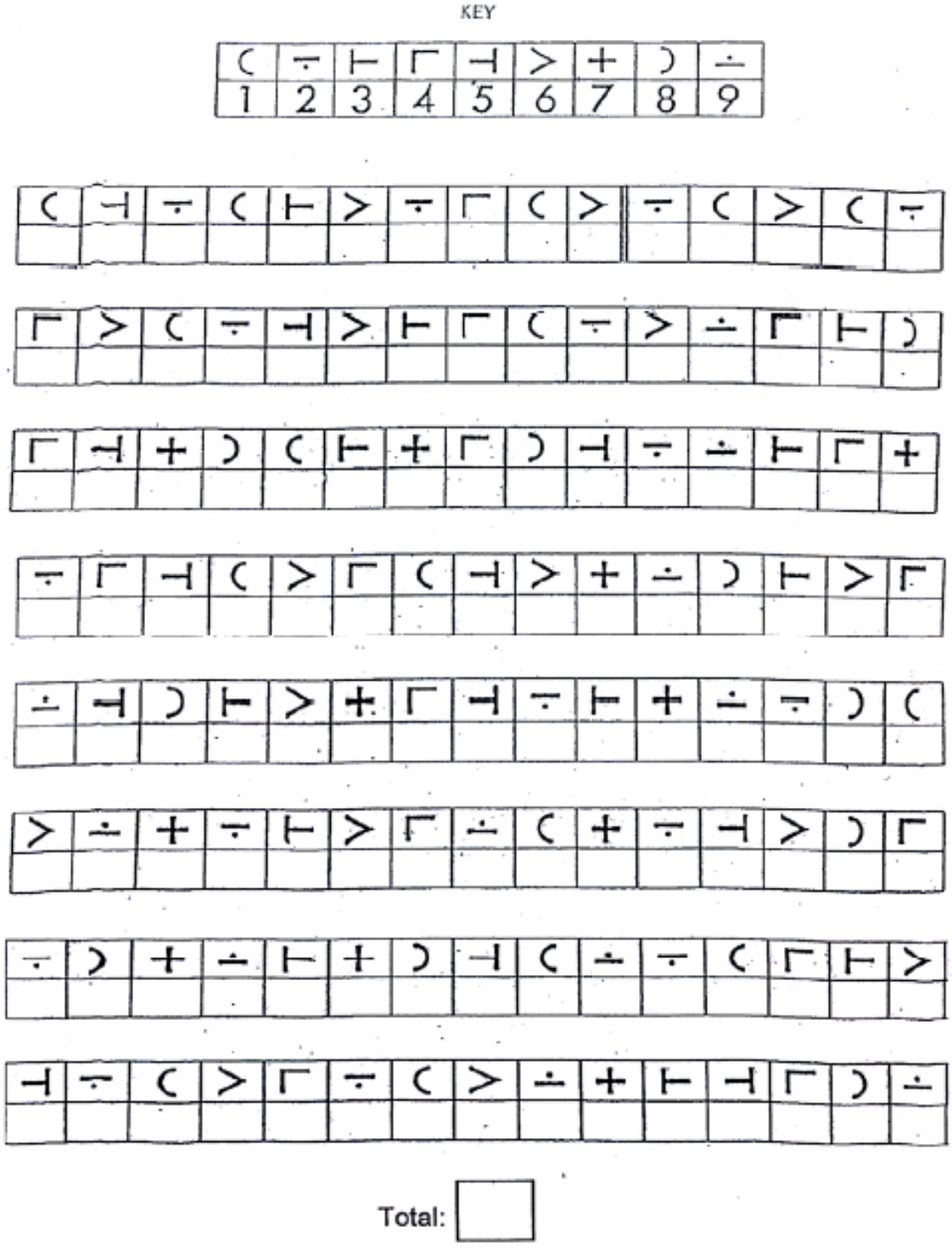
B.1 TMS safety questionnaire

	Yes	No
Do you have epilepsy or have you ever had a convulsion or a seizure?		
Have you ever had a fainting spell or syncope?		
Have you ever had a head trauma that was diagnosed as a concussion or was associated with loss of consciousness?		
Do you have any hearing problems or ringing in your ears?		
Do you have any implanted medical device?		
Do you have ferromagnetic metal in your body (e.g. splinters, fragments, clips, etc.) other than dental fillings?		
Did you ever undergo TMS in the past?		
If yes, were there any problems?		
Did you ever undergo MRI in the past?		
If yes, were there any problems other than claustrophobia?		

B.2 Visual analogue scale for sedation

Mark a point on the line below that represents how sedated you feel at the moment:

B.3 Symbol Digit Modalities Test



B.4 Urine Drug Screen Test

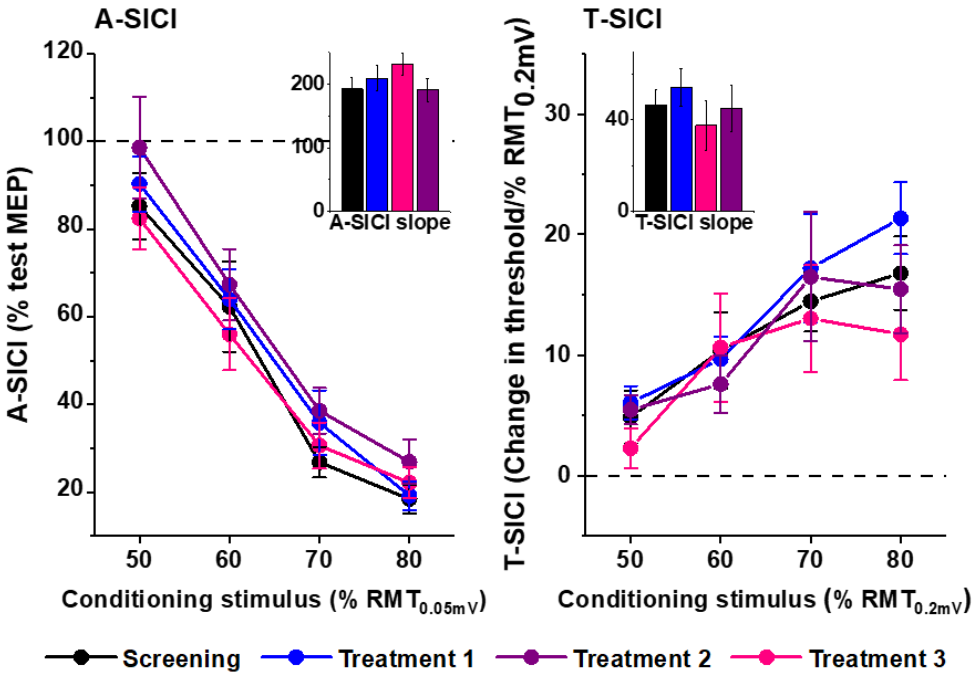
Drug-Screen-Cup II (Nal Von Minden GmbH, Moers, Germany)

Drug and detection cut-off	**Drug and detection cut-off**
Benzodiazepines 300 ng/ml	Tetrahydrocannabinol 50 ng/ml
Amphetamine 1000 ng/ml	Methadone 300 ng/ml
Morphine/opioids 300 ng/ml	Ecstasy 500 ng/ml
Cocaine 300 ng/ml	Buprenorphine 10 ng/ml
Metamphetamine 1000 mg/ml	Tricyclic antidepressants 1000 ng/ml

Negative test was indicated by a T-line, positive test – by the absence of a T-line (Nal Von Minden, 2014).

Appendix C - Reliability of TMS measurements (Experiment 4)

C.1 Mean group SICI (pre-dose)



Baseline SICI recruitment curves (Experiment 6). *No change in mean group SICI was observed throughout the trial. Measurements from four sessions are presented (Screening visit and baselines of three Treatment visits). There was no difference between the sessions neither in individual SICI conditions nor combined slope measurement (inset) with either technique (repeated measures ANOVA, p>0.05; Friedman's test, p≥0.139). Error bars represent standard error of the mean. Dashed lines represent the control condition (100% test MEP for A-SICI, 0% RMT$_{0.2mV}$ for T-SICI).*

C.2 Reliability of TMS parameters

Parameter	Mean ± SD*	ICC(2,1)	SD_{WS}	Coefficient of repeatability (SDC)	SDC_{group}
$RMT_{0.05mV}$	47.7 ± 8.8	0.923 (0.829-0.974)	2.5	7.0	2.0
$RMT_{0.2mV}$	49.2 ± 8.3	0.853 (0.697-0.949)	3.4	9.3	2.7
TS_{1mV}	61.8 ± 15.9	0.806 (0.615-0.930)	7.6	21.0	6.1
A-SICI50	89.1 ± 18.4	0.209 (0-0.573)	25.6	70.9	20.5
A-SICI60	62.4 ± 22.3	0.448 (0.159-0.753)	21.6	59.8	17.3
A-SICI70	32.9 ± 16.7 26.8 (19.6) †	0.654 (0.394-0.863)	11.5	32.0	9.2
A-SICI80	21.6 ± 9.9	0.390 (0.116-0.711)	10.6	29.3	8.5
A-SICI slope	206.0 ± 52.4	0.548 (0.273-0.808)	43.7	121.2	35.0
peak A-SICI	19.1 ± 9.5	0.616 (0.350-0.845)	7.0	19.5	5.6
T-SICI50	4.7 ± 4.6	0.511 (0.234-0.787)	4.1	11.4	3.3
T-SICI60	9.6 ± 9.6 6.5 (9.2) †	0.715 (0.475-0.892)	5.8	16.1	4.6
T-SICI70	15.3 ± 13.0 11.2 (13.5) †	0.678 (0.423-0.875)	8.5	23.4	6.8
T-SICI80	16.3 ± 9.4	0.490 (0.214-0.775)	8.7	24.1	7.0
T-SICI slope	45.8 ± 29.0	0.760 (0.543-0.912)	15.7	43.4	12.5
peak T-SICI	22.5 ± 13.9	0.803 (0.611-0.929)	6.7	18.5	5.3

Reliability of baseline TMS parameters (Experiment 6). *The reliability parameters were calculated from four recordings – Screening visit and baselines of three Treatment visits. * Averaged across four sessions; † median (interquartile range). Mean ± SD, coefficient of repeatability and SDC_{group} are expressed as % MSO for RMTs and TS_{1mV}, % test MEP for A-SICI and % $RMT_{0.2mV}$ for T-SICI parameters; intraclass correlation coefficient (ICC) is a dimensionless measure (95% confidence intervals are indicated in brackets). A-SICI – short interval intracortical inhibition obtained with conventional technique, numbers indicate the intensity of conditioning stimulus; T-SICI – short interval intracortical inhibition obtained with threshold-tracking, numbers indicate the intensity of conditioning stimulus; SD – standard deviation; SD_{WS} – within-subject standard deviation; SDC - smallest detectable change for an individual; SDC_{group} (calculated as SDC/√n) indicates the difference in measurements that would be considered a true change in a sample of twelve subjects.*

Appendix D - Adverse events (Experiment 4)

AE term	Not related	Placebo	2 mg AZD7325	10 mg AZD7325
Nervous system disorders				
Somnolence	7	9	8	9
Sedation	0	0	0	1
Dizziness	0	2	1	5
Headache	2	1	0	0
Hypoaesthesia	1	0	0	2
Paraesthesia	0	0	0	2
Coordination abnormal	0	0	0	1
Disturbance in attention	0	0	1	0
Dreamy state	0	0	0	1
Dysarthria	0	0	0	1
Head discomfort	0	0	1	0
Hyperaesthesia	0	0	1	0
Hypotonia	0	0	0	1
Parosmia	0	0	0	1
General disorders and administration site conditions				
Application site rash	1	0	0	0
Fatigue	0	1	1	2
Feeling abnormal	0	0	0	1
Feeling drunk	0	0	0	3
Feeling of relaxation	0	0	1	1
Hunger	0	1	0	2
Influenza like illness	1	0	0	0
Sluggishness	0	0	1	1
Vaccination site bruising	1	0	0	0
Vessel puncture site bruise	4	0	0	0
Psychiatric disorders				
Abnormal dreams	0	0	1	0
Agitation	0	0	0	1
Anxiety	0	1	0	0
Euphoric mood	0	0	0	3
Time perception altered	0	0	0	1
Gastrointestinal disorders				
Abdominal discomfort	0	0	0	1
Abdominal distention	0	0	0	1
Abdominal pain	0	0	1	0

Continued on the next page

Continued from the previous page

AE term	Not related	Placebo	2 mg AZD7325	10 mg AZD7325
Eructation	0	0	0	1
Mouth ulceration	1	0	0	0
Nausea	1	1	0	0
Investigations				
Aspartate aminotransferase increased	0	0	0	1
Blood urine present	1	0	0	0
Lymphocyte count decreased	0	0	1	0
Neutrophil count decreased	0	0	1	0
Protein urine present	1	0	0	0
Skin and subcutaneous tissue disorders				
Eczema	1	0	0	0
Hyperhidrosis	0	1	0	1
Pain of skin	0	1	0	0
Eye disorders				
Visual impairment	0	0	0	2
Respiratory, thoracic and mediastinal disorders				
Dysphonia	0	0	0	1
Cough	1	0	0	0
Infections and infestations				
Nasopharyngitis	1	0	0	0
Injury, poisoning and procedural complications				
Limb injury	1	0	0	0
Vascular disorders				
Hypotension	0	0	0	1
Surgical and medical procedures				
Analgesic therapy*	0	0	0	1

A complete adverse event list (Experiment 4). *All adverse events reported by 12 subjects who completed the trial are listed using Preferred Terms from MedDRA version 17.0; number of subjects exposed is indicated in the columns. AEs reported at Screening visit and Treatment visits prior dosing are also included. * Post-exercise muscle ache relief.*

Appendix E - Reliability of threshold SICI

Parameter		Experiment 3 (Samusyte et al., 2018)	(Matamala et al., 2018)	(Mooney et al., 2018)
SICI	Protocol	TT, ISI 2.5 ms, CS 70% $RMT_{0.2mV}$	TT, ISI 3 ms, CS 70% $RMT_{0.2mV}$	PEST, ISI 3 ms, CS 90% $AMT_{0.1mV}$
	Mean ± SD	20.4 ± 15.9	14.6 ± 10.6 – 21.1 ± 12.7	8 ± 2 – 9 ± 2
Reproducibility (ICC)	Intraday	0.960	0.72	0.911
	Interday	0.761	0.91	0.930
Repeatability (CR/SDC)	Intraday	14	20.96	9
	Interday	27	14.07	11

Reliability profile of threshold SICI estimates. *In all studies, SICI was measured with the control threshold target set at 0.2 mV, group means are presented as percentage change in threshold. TT- threshold-tracking, PEST – parameter estimation by sequential testing, ISI – interstimulus interval, CS – conditioning stimulus,* $RMT_{0.2mV}$ *– resting motor threshold, target set at 0.2 mV, AMT – active motor threshold, cut-off value 0.1 mV, ICC – intraclass correlation coefficient, values of average measures models are presented, CR/SDC – coefficient of repeatability/smallest detectable change.*

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