UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE FÍSICA

Reducing Motor Evoked Potential amplitude variability through normalization

Francisco Ruas Faro Viana

Mestrado Integrado em Engenharia Biomédica e Biofísica
Perfil em Sinais e Imagens Médicas

Dissertação orientada por:
Prof. Dr. Albino J. Oliveira-Maia
Prof. Dr. Pedro Cavaleiro Miranda

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Abstract

Transcranial Magnetic Stimulation (TMS) has several different applications, including *in vivo* assessment of motor cortical excitability in humans. When applied over the motor cortex, TMS single-pulses result in muscle responses that can be recorded with electromyography (EMG) as Motor Evoked Potentials (MEPs). These have been widely explored as potential biomarkers for neuropsychiatric disorders but methodological heterogeneity in their acquisition, and their inherent high variability have led to constraints in reproducibility. Normalization has been used to reduce variability of EMG measurements. Albeit being a standard practice to allow between-subject comparisons in EMG research, its effect on MEP variability has not been explored. In this study we aim to explore the impact of different normalization methods in MEP amplitude variability.

After validating our in-house built EMG acquisition system, Maximal Voluntary Isometric Contractions (MVICs) and MEPs were collected from 47 healthy volunteers. Four different strategies were used in MEP normalization: two based on external references and two based on internal references. Bootstrapping was used to define distributions of coefficients of variation (CV) for each normalization method. Specifically, iterative re-sampling of 30 MEPs per subject, repeated 5000 times, was performed and a distribution of CVs per method was obtained, allowing for statistical comparison of between-subject variability. A retest session was conducted to assess the impact of normalization on within-subject variability of MEP amplitude measurements, using intra-class correlation coefficients (ICC).

While normalization using external references did not reduce the CV, internal reference normalization resulted in a reduction of approximately 67% of between-subject variability. Normalization did not reduce within subject variability as measured by the ICCs.

Our findings suggest that internal reference normalization reduces between-subject variability and has a minimal impact on within-subject variability. Additional research is necessary to further improve internal reference normalization methods towards potential use of MEPs as biomarkers of neuropsychiatric disorders.

Keywords: Transcranial Magnetic Stimulation; Motor Evoked Potentials; Normalization; Variability; Electromyography

Resumo

A Estimulação Magnética Transcraniana (EMT) é uma técnica que permite estimulação cerebral indolor e não-invasiva, sendo utilizada para diferentes fins tais como a avaliação da excitabilidade cortical em humanos *in vivo*. Quando aplicada sobre o córtex, a EMT resulta na despolarização dos neurónios próximos do local de estimulação sendo possível estudar o efeito imediato da estimulação recorrendo a diferentes técnicas como a ressonância magnética funcional e a eletroencefalografia. Em particular, a eletromiografia (EMG) pode ser utilizada para registar a atividade muscular resultante da estimulação do córtex motor. Ao ser aplicada sobre esta área, a EMT provoca a propagação de potenciais de ação nos neurónios piramidais dando origem a respostas musculares involuntárias a cujo sinal registado com EMG se dá o nome de Potenciais Evocados Motores (PEMs). A amplitude dos PEMs tem sido amplamente explorada como um potencial biomarcador de perturbações neurológicas e psiquiátricas tais como a esclerose múltipla, a doença de Parkinson e a perturbação depressiva major. No entanto, a heterogeneidade metodológica na recolha de medidas baseadas em EMT entre grupos de investigação, bem como a variabilidade inerentemente elevada dos PEMs, tem levantado problemas de reprodutibilidade nesta área de investigação. Fatores como a intensidade da estimulação ou o intervalo de tempo entra a aplicação dos pulsos de EMT tem impacto na amplitude dos PEMs. Adicionalmente, as medidas de EMG são vulneráveis a fontes de variabilidade associadas às condições da aquisição, tais como a temperatura e humidade ou distância entre os elétrodos, e ainda a fontes de variabilidade relacionadas com os sujeitos tais como o tamanho dos músculos e das fibras musculares em estudo. A normalização tem sido utilizada como um instrumento para reduzir a variabilidade das medições com EMG e, embora seja necessária para permitir comparações entre sujeitos, o estudo do seu efeito sobre a variabilidade dos PEM ainda não foi explorado.

Nesta dissertação propomos o estudo do impacto de diferentes técnicas de normalização na variabilidade da amplitude dos PEMs entre sujeitos e na estabilidade destas medidas. Contudo, uma vez que o sistema de aquisição de EMG utilizado na Fundação Champalimaud para medir as respostas a EMT foi desenvolvido neste instituto, foi necessário proceder à sua validação, comparando-o com uma solução comercial.

Com estes objetivos em mente, duas experiências distintas foram desenvolvidas no contexto desta dissertação de mestrado. Em primeiro lugar, dez participantes foram recrutados na Fundação Champalimaud para a validação do sistema de aquisição de EMG. O grau de semelhança entre os sinais recolhidos pelo sistema de aquisição de EMG da Fundação Champalimaud e a solução comercial foi verificada através da recolha simultânea de contrações máximas isométricas voluntárias e de 40 PEMs. A solução comercial utilizada para proceder a esta validação foi o sistema de aquisição BIOPAC MP36 (BIOPAC ® Systems, Inc.), um equipamento que permite a recolha de diferentes sinais biomédicos tais como o eletrocardiograma, o eletroencefalograma e EMG. A análise da semelhança entre os sinais recolhidos em ambos os sistemas baseou-se em duas técnicas que nos permitem comparar os sinais ao longo de toda

a sua extensão: o *Linear Fit Method* e a análise dos *Integrated Pointwise Indices*. Estas análises demonstraram uma excelente concordância entre os sistemas de aquisição de EMG desenvolvido na Fundação Champalimaud e a solução comercial, sendo que os coeficientes da *Linear Fit Method* se encontram próximos dos valores de referência ($a \rightarrow 1$, $b \rightarrow 0$ and $R^2 \rightarrow 1$) e os valores dos *Integrated Pointwise Indices* são superiores a 0,9. Tendo em conta os resultados observados, conclui-se que o sistema de aquisição de EMG desenvolvido na Fundação Champalimaud pode ser utilizado para a medição de contrações máximas isométricas voluntárias e de amplitude de PEM resultantes da aplicação de pulsos de EMT.

Em segundo lugar, 47 participantes foram recrutados na Fundação Champalimaud com o objetivo de estudar o impacto de diferentes métodos de normalização na variabilidade da amplitude dos PEM. Os participantes realizaram até quatro sessões experimentais em que foram recolhidas três contrações máximas isométricas voluntárias antes da aplicação de 40 pulsos de EMT sobre o córtex motor. Nas duas primeiras sessões, a estimulação foi aplicada sobre o hemisfério esquerdo ou direito, resultando em PEM recolhidos na mão direita ou na mão esquerda, respetivamente. A ordem destas sessões foi aleatorizada de modo a garantir a recolha dum número equivalente de participantes a iniciar a participação nesta experiência com sessões de estimulação do hemisfério esquerdo ou direito. Estas sessões foram realizadas num intervalo de 5 dias separados por pelo menos 48h para evitar a contaminação dos resultados observados pelas sessões anteriores. A amplitude dos PEM foi normalizada segundo duas estratégias diferentes. Estas basearam-se em sinais recolhidos durante a realização das contrações máximas isométricas voluntárias (referência externa) ou nos próprios PEM (referência interna). Para cada uma destas estratégias, definiram-se dois tipos de fatores de normalização baseados na amplitude máxima dos sinais ou na amplitude máxima após a retificação do sinal de EMG. De forma a estudar o efeito da normalização na variabilidade entre sujeitos definimos a amplitude dos PEM por sujeito como a média aritmética de 30 PEMs selecionados aleatoriamente. Em seguida, o coeficiente de variação dado pela divisão do desvio padrão da amplitude média dos sujeitos pela amplitude média desta população foi calculado para cada método de normalização. Este processo foi repetido 5000 vezes através da reamostragem iterativa de 30 PEMs por sujeito obtendo-se uma distribuição de coeficientes de variação por método. Desta forma foi possível realizar uma comparação estatística da variabilidade entre sujeitos resultante das diferentes estratégias de normalização. Quatro a oito semanas depois, as sessões experimentais foram repetidas permitindo-nos analisar a estabilidade das medidas de amplitude dos PEM utilizando os coeficientes de correlação intra-classe.

Na ausência de normalização o intervalo de confiança de 95% para os coeficientes de variação dos potenciais evocados motores recolhidos da mão direita foi [1.0567, 1.0577]. Enquanto a normalização utilizando referências externas não resultou na diminuição do coeficiente de variação, a utilização de referências internas resultou numa redução da variabilidade entre sujeitos de aproximadamente 65% sendo [0.3653, 0.3660] o intervalo de confiança de 95% para o método que resultou na maior redução da variabilidade entre sujeitos. O efeito da normalização utilizando referências internas na variabilidade entre sujeitos foi confirmada recorrendo aos PEMs recolhidos na mão direita. Quanto ao efeito da normalização na estabilidade da amplitude dos PEM não encontramos um aumento significativo dos coeficientes de correlação intra-classe. No entanto, os métodos de normalização utilizando referências internas apresentam valores mais próximos dos obtidos na ausência de normalização sendo que a normalização utilizando referências externas reduziu marginalmente os coeficientes de correlação intra-classe. Para todos os métodos de normalização propostos e na ausência de normalização, a estabilidade da medida encontra-se próxima dos valores encontrados na literatura podendo ser classificada como moderada a boa.

Após termos validado com sucesso o sistema de aquisição de EMG desenvolvido na Fundação Champalimaud, demonstramos que, apesar da normalização usando referências externas não reduzir a variabilidade entre sujeitos e, em alguns casos, diminuir a estabilidade das medidas de amplitude dos PEMs, a normalização usando referências internas reduz a variabilidade entre sujeitos num fator de até 65% sem ter um impacto considerável na variabilidade entre sujeitos da medida. No entanto, é necessário aprofundar estes resultados realizando mais experiências para validar a sua utilização como uma medida de interesse clínico e de investigação.

Palavras-chave: Estimulação Magnética Transcraniana; Potenciais Evocados Motores; Normalização; Variabilidade; Electromiografia

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List of Abbreviations

ACh	Acetylcholine
CCU	Champalimaud Centre for the Unknown
CV	Coefficient of Variation
EMG	Electromyography
FDI	*first digiti interosseous*
ICC	Intra-class correlation coefficient
IPI	Integrated Pointwise Indices
LFM	Linear Fit Method
MSO	Maximum Stimulator Output
MRI	Magnetic Resonance Imaging
MVIC	Maximum Voluntary Isometric Contraction
MEP	Motor Evoked Potential
MUAP	Muscle Unit Action Potential
nMEP	Normalized Motor Evoked Potential
RMT	Resting Motor Threshold
TMS	Transcranial Magnetic Stimulation

Chapter 1

Introduction

1.1 Transcranial Magnetic Stimulation

Transcranial magnetic stimulation (TMS) is a non-invasive brain stimulation technique with several applications in the study of brain electrophysiology. A typical TMS device discharges capacitors that send electrical current through a coil of conductive wire, generating a time-varying magnetic field described by field lines which lie in a plane perpendicular to the coil forming closed loops. This magnetic field leads to the creation of a time-varying electrical field perpendicularly which in turn will interact with nearby conductive material (eg. brain tissue) generating an electrical current parallel to the coil surface (Figure 1.1). The use of magnetic fields to induce electrical current at a distance was was first described in the 1830s by Michael Faraday (Faraday, 1831) and since then is referred to as Faraday's law of electromagnetic induction.

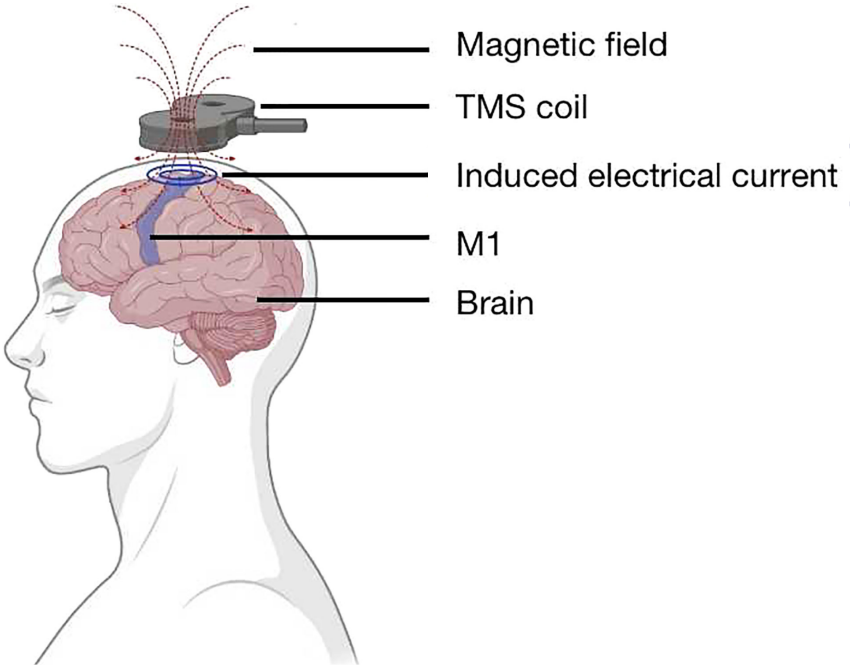

Figure 1.1: **Principles of trancranial magnetic stimulation**. A coil of conductive wire placed tangentialy on the scalp generates a time-varying magnetic field described by field lines which lie in a plane perpendicular to the coil forming closed loops. This magnetic field leads to the creation of a time-varying electrical field perpendicularly which in turn will interact with nearby conductive material (brain tissue) generating an electrical current parallel to the coil surface. Figure adapted from Zhou et al. (2022)

First reports of non-invasive magnetic stimulation arise in 1896 when a French physician, Jacques-Arsène d'Arsonval, reported volunteers seeing flickers of light when passing alternate current through a coil placed around their heads (D'Arsonval, 1896), percepts that were later termed phosphenes. These findings were replicated and expanded upon by several researchers in the following years (Thompson, 1910; Dunlap, 1911; Magnusson & Stevens, 1911, 1914). Indeed, in 1947 Barlow argued that the phosphenes resulted from the stimulation of the retina, rather than the nervous tissue (Barlow et al., 1947).

Interest in magnetic stimulation did not waver and experiments with animal models later showed that it was possible to stimulate nerve and skeletal muscle by means of alternating magnetic fields (Kolin et al., 1959; Bickford & Fremming, 1965). Finally, in 1985, Anthony Barker and his colleagues developed the first transcranial magnetic stimulator capable of stimulating the human cortex non-invasively (Barker et al., 1985). In their experiment, after placing surface electrodes on the hand abductor digiti minimi muscle to measure muscle responses using electromyography (EMG), researchers positioned the edge of the magnetic coil on the scalp of volunteers over the contralateral motor cortex (MC) and on the elbow over the ulnar nerve to apply pulsed magnetic fields. Examples of the resulting EMG trace are presented in Figure 1.2 and represent the first recording of Motor Evoked Potentials (MEP) elicited by TMS. Subsequently, inaugural TMS clinical studies were performed to distinguish conduction time in central motor pathways between patients with Multiple Sclerosis and healthy controls (Barker et al., 1986).

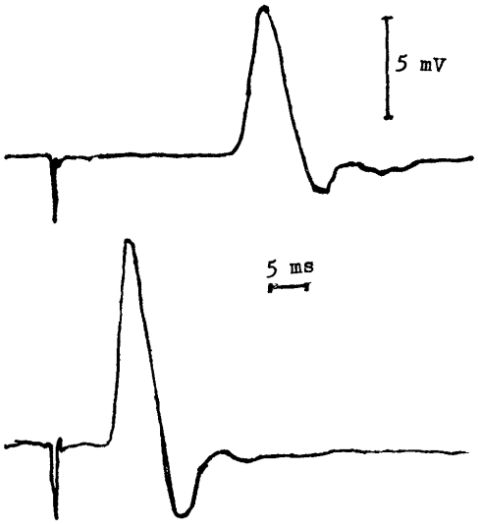

Figure 1.2: **First recording of a Motor Evoked Potential**. Motor Evoked Potentials recorded from the hand abductor digiti minimi resulting from a magnetic pulses delivered over the contralateral motor cortex (**top**) and ipsilateral ulnar nerve at the elbow (**bottom**). Figure adapted from Barker et al. (1985)

1.2 Electromyography and the measurement of Motor Evoked Potentials

Electromyography is a technique that allows the recording of myoelectric activity resulting from the depolarization of muscle fibers. Muscle fibers are organized into functional groups innervated by lower motor neurons, an arrangement designated as the motor unit. Motor units constitute the basic element that allows muscle control by the nervous system. When an action potential travels down the axon of a lower motor neuron and reaches the neuromuscular junction, acetylcholine (ACh) is released in the

synaptic cleft and binds to ACh receptors on the muscle fiber membrane, resulting in the propagation of an action potential and, consequently, in muscle fiber contraction. Since motor neuron axons branch out to several muscle fibers, action potentials add up to form a motor unit action potential (MUAP) (Cram & Kasman, 2011). The measurement of the change in voltage potential across multiple muscle fibers is the basis of EMG recordings.

In surface EMG, electrodes are placed on the skin over a target muscle and record the electrical activity that results from the summation of several MUAPs happening in the area below the electrodes. While an action potential measured on a single cell membrane can reach a peak to peak amplitude of $110\,\mathrm{mV}$ Hall & Hall (2021), unfiltered surface EMG peak to peak amplitude ranges from $0\,\mathrm{mV}$ to $10\,\mathrm{mV}$ (Basmajian & De Luca, 1985). This loss of energy is due to the impedance of the body tissue between the muscles and the sensing electrodes (Basmajian & De Luca, 1985). To measure these signals without further attenuation and distortion, it is important to maintain electrode-skin interface impedance at least 10 to 100 times lower than the acquisition system's input impedance (Cram & Rommen, 1989). This is normally achieved by introducing a saline medium between the skin and electrode that facilitates current flow and minimizes the susceptibility of the electrode-skin interface to other factors such as changes in temperature, sweat or presence of hair.

Pairing surface EMG with external stimulation of nerves and muscle provides important insights into the integrity of the corticomotor pathway and serves as a diagnostic tool for several syndromes and disorders. TMS pulses applied over the motor cortex depolarize piramidal neurons, triggering action potentials that propagate down the motor pathway. The recording of these action potentials, or descending volleys, along the spinal cord has shown that different sub-populations of motor neurons are recruited when the direction and intensity of the applied magnetic pulses varies, leading to changes in MU recruitment that may be reflected in MEP features such as their peak-to-peak amplitude or latency (Groppa et al., 2012; Bestmann & Krakauer, 2015). As is expected, cortical motor areas do not function in an isolated manner and receive constant input from other systems that relay information ranging from sensory inputs required for smooth and coordinated movement to information regarding emotional status that may influence the way a particular action is carried through (hastily in response to fear, for example) (Cram & Kasman, 2011). Motor evoked potentials thus provide a tool to tap into a wide range of neural and cognitive processes that influence muscle control and consequently MU recruitment.

1.3 Motor Evoked Potentials amplitude as a biomarker: Examples and Limitations

Several different features can be extracted from MEPs and one of the most studied features is their peak-to-peak amplitude. Changes in MEP peak-to-peak amplitude can be associated with dysfunction at several levels of the corticomotor pathway allowing us to gain insights into the physiological characteristics underlying a variety of neurological and neuropsychiatric disorders.

In multiple sclerosis, for example, several authors have shown decreased MEP amplitude when compared to healthy controls (Gagliardo et al., 2007; Bridoux et al., 2015; Nantes et al., 2016; Mordillo-Mateos et al., 2019; Mohy et al., 2020) possibly reflecting loss of axonal integrity that impairs corticospinal pathways. In Parkinson's disease, higher values of MEP amplitude have been demonstrated, which may be associated with aberrant muscle activity during stimulation (Valls-Solé et al., 1994; Bologna et al., 2018). MEP amplitude has also shown potential to characterize neuropsychiatric disorders. Decreased MEP amplitude has been shown to be associated with major depressive disorder and to also be

present in children with autism spectrum disorder (Shajahan et al., 1999; Fitzgerald et al., 2004; Jannati et al., 2020). However, the establishment of MEP amplitude as a biomarker has also been contested due to conflicting evidence and lack of reproducibility of the findings (Grunhaus et al., 2003; Kühn et al., 2004; Liepert et al., 2005; Thickbroom et al., 2008; Enticott et al., 2013; Gündüz & Kiziltan, 2015; Pedapati et al., 2016).

One possible reason for the lack of reproducibility in this field is the variability of MEP amplitude measurements. Between-subject variability can be measured using the Coefficient of Variation (CV), a measure of dispersion that allows us to compare the spread of data independently of its mean or unit of measurement. It is defined as the ratio of the standard deviation to the mean of a given sample. Within- subject variability, one the other hand, can be measured by studying the test-retest stability of the measurements when repeated in the same subject across time using the intra-class correlation coefficient (ICC). MEPs collected in healthy participant cohorts present diverse values of CVs, in many cases larger than 1 (Chang et al., 2016; Chroni et al., 2002; Nantes et al., 2016; Mohy et al., 2020), and also quite diverse reliability (Schilberg et al., 2017; Pellegrini et al., 2018; Herbert & Burke, 2021).

Variability of MEP measurements has been attributed to various factors. First of all, MEPs are known to have large trial-to-trial variability due to naturally occurring rapid and spontaneous fluctuations of corticospinal excitability (Kiers et al., 1993; Groppa et al., 2012). Consequently, MEP amplitude estimation has relied on averaging multiple MEPs in order to obtain a more robust measure (Cuypers et al., 2014; Rossini et al., 2015), with a variable number of MEPs being used to calculate this average (Jannati et al., 2019; Sui et al., 2020). In 2016, Chang et al. have shown that at least 21 MEPs should be collected to achieve an accurate estimate of MEP amplitude.

Other factors have been reported to impact MEP amplitude measurements such as inter-pulse interval and muscle activation. When collecting multiple MEPs, the inter-pulse interval should be larger than six seconds to avoid carry-over effects from previous stimuli and pulses should be delivered in random intervals to avoid stimulus anticipation impacting the measurement (Julkunen et al., 2012; Pellicciari et al., 2016). The presence of muscle activity in the period leading to the application of TMS pulses on the motor cortex has been shown to increase the amplitude of MEPs (Darling & Butler, 2006). Thus, it is common practice to monitor the EMG activity leading to the application of the TMS pulse and exclude from subsequent analyses MEPs where muscle activity could be detected prior to the application of the TMS pulse (Hinder et al., 2014; McCambridge et al., 2020; Castricum et al., 2022).

Aside from TMS-specific sources of variability, MEP measurements are also sensitive to general sources of variability common to any technique dependent on surface EMG. These include session-specific variables like electrode configuration and placement (Mohr et al., 2018), skin preparation and impedance (Cram & Rommen, 1989), temperature and humidity (De Luca, 2002, 2006), as well as subject specific parameters such as the number of active motor neurons, muscle fiber diameter and the amount of tissue between the surface of the muscle and the sensing electrodes (De Luca, 1997). These factors vary between and within individuals across different acquisition sessions bringing uncertainty to the interpretation of the EMG that may be addresses when a normalization procedure is performed.

1.4 Signal normalization in electromyography

Normalization in EMG refers to the conversion of the signal to a scale relative to a known and repeatable value or factor extracted from the same muscle group (Halaki & Ginn, 2012). Normalization can be performed following a three-step process. First, a reference signal must be chosen. Reference signals can be divided into two types: internal references where the signal is extracted from the same

action as the one under investigation, or external references which are signals obtained from the same muscle while another action is being performed. Second, mean or maximum values are taken from the reference signals and used to calculate a normalization factor. Some authors calculate the normalization factor by averaging several reference values of the same type as it can improve the repeatability of the normalization factor. Finally, normalization is achieved by dividing the raw EMG signal by the normalization factor.

A large number of EMG normalization methods have been proposed (Besomi et al., 2020). The most common external reference used in EMG normalization is the Maximum Voluntary Isometric Contractions (Balshaw & Hunter, 2012). MVICs are collected to reflect the maximal neural activation of the target muscle but there is no consensus on which actions produce maximal activation in any given muscle across all individuals. Furthermore, when studying clinical populations the collection of signals under maximal effort may be impossible due to the presence of pain or other limiting factors such as medication. Normalization to the maximum M-wave (M-max) has also been applied to EMG signals (Lanza et al., 2018). The M-wave is the resulting EMG signal detected after transcutaneous electrical stimulation of peripheral motor nerve at a point proximal to the target muscle. M-max is defined as the signal collected when EMG amplitude ceases to increase while increasing the stimulation intensity. However, the repeatability of M-max values has been questioned, making this technique less useful in the reduction of variability of EMG signals (Halaki & Ginn, 2012). Finally, the use of peak activation levels obtained during the task under investigation has been shown to decrease between-subject variability. However, this reduction in variability is in part achieved by removing real biological variation from the studied signals impairing our capability to compare muscle activity across individuals (Besomi et al., 2020).

1.5 Aims and Outline

MEP peak-to-peak amplitude has been frequently used to assess changes in corticospinal excitability, but its inherently large variability has limited its establishment as a biomarker. Although some authors have reported the ratio MEP peak-to-peak to M-max amplitude (Gagliardo et al., 2007; Mordillo-Mateos et al., 2019; Latella et al., 2020), the impact of this normalization on the variability of MEPs has not been studied. Hence, the primary aim of the current work is to study the impact of different external and internal reference normalization techniques on MEP amplitude variability. We hypothesize that normalization will decrease between-subject variability of MEP amplitude and increase the test-retest stability of this measure. Since at the Champalimaud Centre for the Unknown an in-house built EMG acquisition system is used to collect TMS-EMG measures, it was crucial to validate our acquisition system against a well-established commercial solution before progressing into the study of the impact of normalization in MEP variability. Thus, the following chapters in this dissertation will be divided into two major parts, each dedicated to one of the two experiments developed within its scope:

- The **Validation of the in-house EMG acquisition system** where we have tested the agreement between the in house EMG acquisition system against EMG signals simultaneously collected using an existing commercial solution, and;
- The **Reduction of motor evoked potential variability through normalization** where several MEP amplitude normalization methods are proposed and assessed according to their impact on between- and within-subject variability of MEP amplitude.

Chapter 2

Methods

2.1 General Methods

2.1.1 Participants

Two separate cohorts were recruited in the context of this dissertation, one for the EMG acquisition system validation and another for the study of the impact of normalization on MEP amplitude variability (see Tables 2.2 and 2.3 for details). Participants were recruited at the Champalimaud Centre for the Unknown in Lisbon, Portugal, after collection of informed consent, as approved by the local ethics committee. Eligibility for TMS was assessed through a safety questionnaire adapted from previously published guidelines (see Rossi et al., 2009). This document included items regarding history of psychiatric and neurological disorder, loss of consciousness, hearing impairment as well as presence of metallic or magnetic implants, ongoing medication and pregnancy status. Answers to the questionnaire were reviewed by a physician and, if no safety concerns were raised, participants would be enrolled in the study.

2.1.2 Experimental Procedures

Participants were seated in a comfortable chair with both forearms at rest. In order to improve the quality of the electrode-skin interface, dead skin cells and oils present over the target muscle were removed using water sandpaper followed by a quick rinse with an alcohol embedded gauze swab (De Luca, 2006). Electrodes were placed over the *first digiti interosseous* (FDI) of the right hand and a lycra swimming cap was fitted on the participants' head, to draw references to determine the location of the TMS stimulation site (see details below).

2.1.3 Electromyography Recordings

The in-house EMG acquisition system was developed by the Scientific Hardware Development Platform at the Champalimaud Centre for the Unkown which provides support in the electronic and mechanical fields to aid internal research projects as well as external projects (https://www.cf-hw.org/). This system incorporates five independent EMG channels in addition to one channel which receives direct input from the TMS machine signalling pulse delivery. The EMG acquisition parameters were based on international guidelines for the collection of TMS-EMG data (Groppa et al., 2012). Data streaming was done through Bluetooth, integrated using a custom-made BONSAI script (Lopes et al., 2015) and saved onto binary (.bin) files.

Electromyography recordings were performed using medical grade, Ag/AgCl based disposable cutaneous electrodes with a snap-on connector (Kendall™ H124SG). These electrodes do not require the application of any conductive paste or gels since the sensor area and surroundings are coated with conductive and adhesive hydrogel. MEP measurements follow the same principles as compound muscle action potential recordings during peripheral motor nerve conduction studies (Groppa et al., 2012), therefore a bipolar electrode montage was chosen. One electrode was placed over the belly of the right FDI while another electrode was placed distally with its center at approximately $2.5\,\mathrm{cm}$ in the direction of the muscle tendon (Figure 2.1). Additionally, a ground electrode was placed over the left elbow to provide a zero voltage reference point. Since EMG recordings are highly sensitive to changes in inter-electrode distance as well as electrode positioning, standardization of electrode placement across participants is strongly recommended and was performed here.

Recordings began with the collection of Maximum Voluntary Isometric Contractions (MVICs), according to the procedures for each experiment (see details below), after which TMS procedures were conducted.

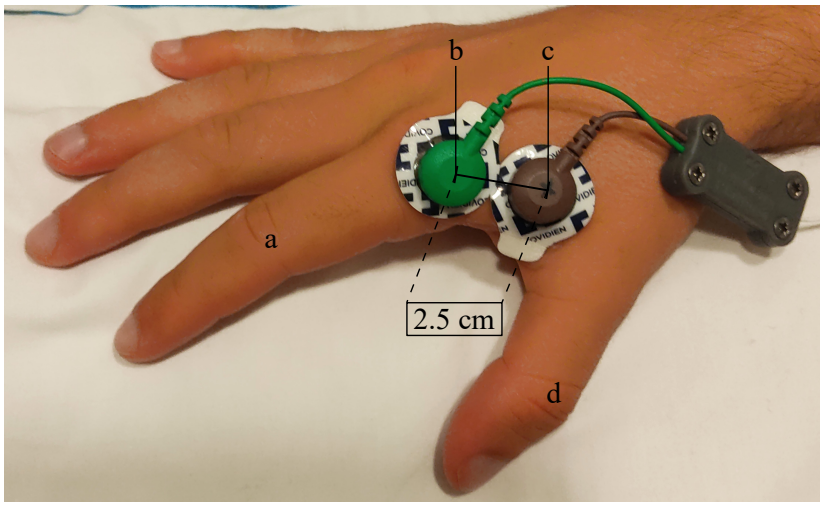

Figure 2.1: **An example of EMG electrode placement on one of the researchers hand. a)** index finger; **b)** muscle tendon; **c)** belly of the FDI; **d)** thumb. One electrode placed over the belly of the FDI while another electrode is placed distally with its center at approximately 2.5 cm in the direction of the muscle tendon.
Abbreviations: EMG - Electromyography; FDI - *first digiti interosseous*

2.1.4 Transcranial Magnetic Stimulation

Transcranial Magnetic Stimulation procedures were performed under the guidelines recommended by the International Federation of Clinical Neurophysiology (Rossini et al., 2015) using the MagPro X100 magnetic stimulator distributed by MagVenture with a figure eight coil (Cool-B65).

The motor hotspot for maximal stimulation of the FDI was determined on the scalp using a standardized non-neuronavigated strategy, as described previously (Cotovio et al., 2021). After placing the lycra swimming cap on the participants' head, the cranial vertex was determined by drawing the intersection between the midsagittal and intertragus lines. From there, $5\,\mathrm{cm}$ were measured laterally on the intertragus line marking an initial estimate of the motor hotspot. Then, the TMS coil was placed tangentially to the participant's scalp with the handle pointing posteriorly at a 45º angle relative to the midsagital line

and its centre over the marked location. After instructing participants to remain silent and relaxed (an instruction that was repeated several times throughout TMS related measurements), several TMS single pulses were applied at 30% of the maximum stimulator output (MSO) over the initial motor hotspot estimate. Stimulation intensity was subsequently incremented in steps of 5-10% of MSO until a consistent motor response was observed. Thereafter, while systematically applying single pulses at several locations around the initial estimate, the motor hotspot for the FDI was determined as the location that most consistently resulted in higher amplitude MEPs and, when possible, where the visible contraction was limited to the FDI.

Resting Motor Threshold (RMT) was then determined based on EMG recordings of the MEPs. RMT was defined as the minimum intensity needed to elicit MEPs of at least $50\,\mu\mathrm{V}$ amplitude in 5 out of 10 single-pulses, separated by a random inter-pulse interval of more than $6\,\mathrm{s}$. Indeed, after marking the motor hotspot on participants' swimming caps, stimulation intensity was reduced in steps of 2% of MSO until failure to comply with the above-mentioned criteria. Finally, a 1% increment on the MSO was made until the criteria was once again met with the resulting MSO representing the subject's RMT.

Following RMT determination, MEPs were collected by applying single pulses over the motor hotspot at 120% of the RMT. A total of 40 TMS single-pulses were applied, ensuring that a sufficient number of MEPs were elicited to allow for a reliable estimate of MEP amplitude per subject (see Chang et al., 2016). A random inter-pulse interval of at least $6\,\mathrm{s}$ was used to avoid carry-over effects from previous stimulus (Pellicciari et al., 2016) as well as making the pulse sequence unpredictable, minimizing target muscle pre-activation caused by the anticipation of TMS pulse application.

2.2 Validation of the in-house EMG acquisition system

2.2.1 Study design & experimental procedures

This study was designed to assess the agreement between an in-house built EMG acquisition system and a commonly used commercial EMG system. Several commercial devices are available for the collection of EMG with some TMS stimulators allowing for EMG system integration, but typically at a high cost (Fuentes et al., 2019). Consequently, several research groups have aimed to design and validate low-cost prototypes (Supuk et al., 2014; Heywood et al., 2018; Fortune et al., 2019). However, validation protocols have been pointed out to be limited, with the agreement between systems being dependent on the action performed while simultaneously recording EMG from two acquisition systems (Tecchio et al., 2021).

To address these limitations and adequately validate our own local equipment, we developed a strictly controlled protocol where participants were first asked to perform five MVICs by pushing their index finger laterally against the researcher's hand while their forearm was being supported to minimize signal contamination from other muscle sources. Each MVIC had a duration of approximately $3\,\mathrm{s}$ with $1\,\mathrm{min}$ of rest between them. Then, the experimental session proceeded with the determination of the motor hotspot and RMT for the right FDI followed by the application of 40 TMS pulses over the left motor cortex at an intensity of 120% RMT.

The signals collected using the in-house EMG acquisition system were tested against data collected with the BIOPAC MP36 acquisition system (BIOPAC ® Systems, Inc.), a commercial solution which features built-in universal amplifiers capable of recording a wide range of physiological signals such as the electrocardiogram, electroencephalogram and EMG. An acquisition profile most suitable for the acquisition of TMS-EMG data was selected and the resulting data was sent to BIOPAC Student Lab

software and saved into MATLAB files (.mat). A summary of the EMG acquisition parameters for both systems can be found in Table 2.1. Albeit having a smaller maximum input voltage range, no saturation was observed in the signals collected with the in-house EMG acquisition system. To carry out simultaneous EMG acquisition both systems were connected to each electrode by means of a splitter cable (Figure 2.2). Otherwise, between-system differences in wave-shape would be expected due to the differences in geometry from target muscle to electrodes.

The assessment of the agreement between both systems was performed using two forms of wave-similarity analysis: the **linear fit method** and the **integrated pointwise indices** (Iosa et al., 2018; Pini et al., 2019). Instead of relying on the extraction of discrete measures such as amplitude and activation time, these methods allow us to evaluate the agreement between both signals throughout their whole extent, providing a more complete comparison of the systems.

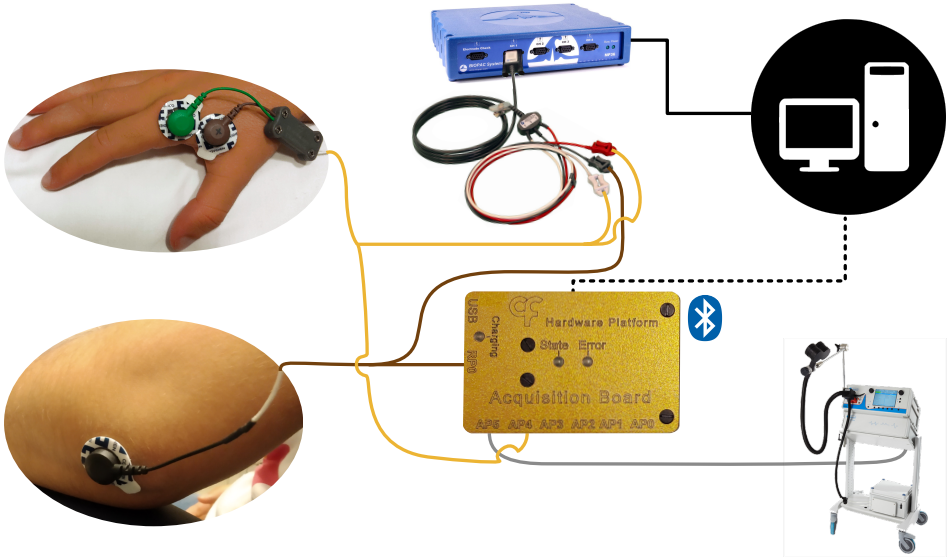

Figure 2.2: **Board Validation Setup and Signal Processing Diagram**. Simultaneous EMG acquisition was achieve by connecting both systems to electrode sensors with a splitter cable.

Table 2.1: **Summary of EMG acquisition system parameters**. Abbreviations: ADC - Analog to Digital Converter

	In-House EMG acquisition system	**Commercial System**
Gain	206	200
Sampling Frequency	5000 Hz	5000 Hz
Hardware Passband	5 Hz - 1000 Hz	5 Hz - 20 kHz
ADC Sampling resolution	16 bits	24 bits
Max Input voltage range	$\pm$ 7.3 mV	$\pm$ 2 V

2.2.2 Data Processing & Analysis

Data processing and analysis was performed using custom-written scripts in Python3 employing several conventional libraries such as numpy (https://numpy.org/), pandas (https://pandas.pydata.org/), scipy (https://www.scipy.org/) and statsmodels (https://www.statsmodels.org/).

Table 2.2: **In-house EMG acquisition system validation population characterisation**. Abbreviations: SD - standard deviation.

N	Age (mean ± SD)	Female (%)
10	28.5 ± 8.9	50.0

Four data-files were generated for each subject, two .bin files and two .mat files corresponding to the collection of raw MVICs and MEPs with the in-house and commercial systems, respectively. EMG data was imported into Python3 and the time-series were aligned using cross-correlation to estimate the relative time-shift between both systems. Then, data was segmented into epochs containing single MVICs and MEPs, excluding epochs where MEP amplitude was smaller than $50\,\mu V$. Finally, each epoch was linearly detrended. This procedure was performed to deal with possible differences in DC amplifier offset and with the differential profile of the TMS pulse artifact in both systems. Since the goal of this experiment was to compare the agreement between the in-house acquisition system and the commercial EMG systems, no further data processing was performed.

Agreement between both systems was first assessed through wave-similarity analysis. Two distinct strategies were used: the **linear fit method** (Iosa et al., 2018) and the **integrated pointwise indices** (Pini et al., 2019).

The **linear fit method** is calculated by plotting a test signal S_{test} against a reference signal S_{ref}, characterizing a set of points in a Cartesian coordinate system ($(S_{ref}(i), S_{test}(i))$ for $i = 1, ..., N$ where N represents the number of samples). Then, a linear fit is applied to this set of points defining:

$$\widehat{Y} = a \cdot S_{ref} + b \tag{2.1}$$

where $\widehat{Y}$ represents the best approximation of S_{test} values by means of a linear transformation of S_{ref} values; a represents a scaling factor, *i.e.* a factor by which S_{ref} should be multiplied to match S_{test} except for a scalar offset; and b which represents the scalar offset.

The linear fit method (Figure 2.3) relies on the interpretation of the coefficient of determination (R^2) and the linear regression coefficients (a and b), calculated by the following formulas:

$$\begin{aligned} a &= \frac{\sum_{i=1}^{N} \left(S_{ref}(i) - \overline{S_{ref}}\right) \cdot \left(S_{test}(i) - \overline{S_{test}}\right)}{\sum_{i=1}^{N} \left(S_{ref}(i) - \overline{S_{ref}}\right)^2} \\ b &= \overline{S_{test}} - a \cdot \overline{S_{ref}} \\ R^2 &= \frac{\sum_{i=1}^{N} \left(a_i \cdot S_{ref}(i) + b - \overline{S_{test}}\right)}{\sum_{i=1}^{N} \left(S_{test}(i) - \overline{S_{test}}\right)^2} \end{aligned} \tag{2.2}$$

From equations 2.2* we can estimate the optimal values of a, b and R^2. If $S_{test} \simeq S_{ref}$, $a \to 1$,

*Equations 2.1 and 2.2 adapted from Iosa et al., 2018

$b \to 0$ and $R^2 \to 1$. In this study, MVIC and MEP epochs collected with the in-house EMG acquisition system will be considered as test signals while there BIOPAC MP36 counterparts will be considered reference signals. Then, the 95% confidence intervals (CI) of the LFM coefficients were calculated for all MEP epochs, for all MVIC epochs and for all epochs together to quantify the agreement between both systems.

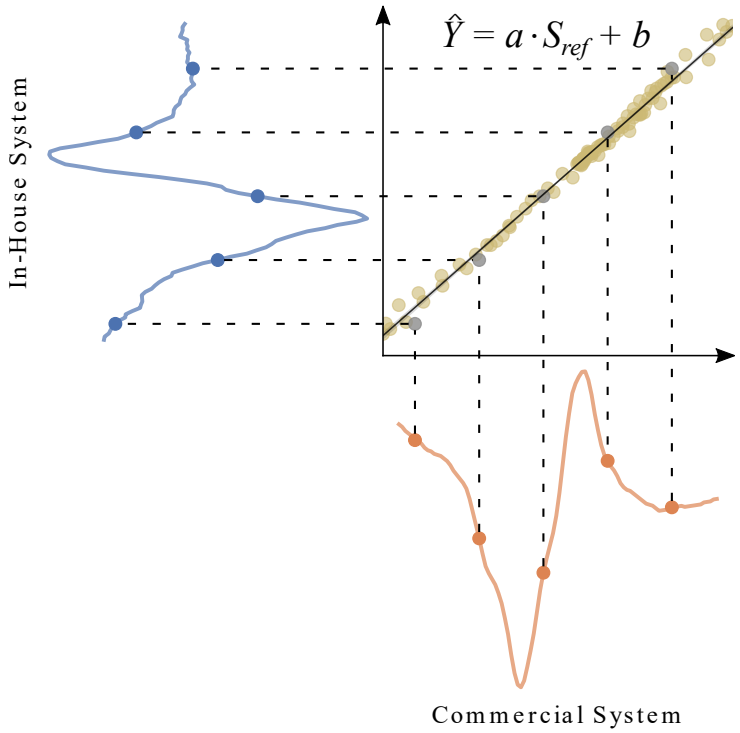

Figure 2.3: **Illustration of the Linear Fit Method applied to an example motor evoked potential**.

Furthermore, each epoch was used to calculate an **Integrated Pointwise Index** (IPI_{icc}). This technique follows an extension of common reliability measures for univariate analysis integrated over each data point of the EMG trace:

$$IPI_{icc} = \frac{\sum_i^N ICC(i)}{N-1} \tag{2.3}$$

The chosen reliability measure was the intraclass correlation coefficient for absolute agreement (ICC). While similar to a correlation, ICCs for absolute agreement have the advantage that they take into account the similarity of the measured values across the two samples and not only their tendency. They can be calculated using variance components of a two-way mixed effect model without interactions (Trevethan, 2017). According to this model, the total sum of squares of each data point i can be divided into three factors: individual ($SS_{ind}(i)$), repetition ($SS_{rep}(i)$) and residual ($SS_{res}(i)$) sum of squares. Dividing each sum of squares by its corresponding degree of freedom we obtain the mean sum of squares for each factor: $MS_{ind}(i)$, $MS_{rep}(i)$ and $MS_{res}(i)$, respectively. $ICC(i)$ calculation for each data point is made by applying equation 2.4[†]:

$$ICC_{aa}(i) = \frac{MS_{ind}(i) - MS_{res}(i)}{MS_{ind}(i) + MS_{res}(i) + \frac{2}{N} \cdot [MS_{rep}(i) + MS_{res}(i)]} \tag{2.4}$$

[†]Equations 2.3 and 2.4 adapted from Pini et al., 2019

Mean IPI_{icc} across subjects were calculated for MEP and MVIC epochs separately.

As a confirmatory analysis, ICCs for absolute agreement were calculated using a two-way mixed effect model for subject mean MEP and MVIC amplitudes (Koo & Li, 2016). Mean MEP amplitude was estimated from the mean difference between minimum and maximum values for all valid MEP epochs. Mean MVIC amplitude estimation was made by averaging the three largest MVIC epochs. MVIC amplitude for each contraction was obtained by applying a $200\,\mathrm{ms}$ moving window (Farfán et al., 2010) to calculate the root mean squared value of each timepoint and finding its maximum.

2.3 Reduction of Motor Evoked Potential variability through normalization

2.3.1 Study design & experimental procedures

The following data was collected as part of an ongoing project which aims to study the test-retest stability of TMS-EMG cortical excitability and plasticity-like measurements as well as their applicability as potential biomarkers for Major Depressive Disorder.

In this study, participants were asked to perform up to four TMS experimental sessions. The first two sessions were performed within a 5-day interval, separated by at least 48h to ensure that TMS stimulation would not lead to any carry-over effects impacting the following session. In these sessions, MEPs were either collected by applying TMS pulses over the left motor cortex or over the right motor cortex with the acquisition order being randomly defined for each participant. Four to eight weeks later, these experimental sessions were repeated following the same acquisition order as in the initial sessions. In another group of subjects, only two experimental sessions were performed with TMS pulses applied exclusively over the left motor cortex. The second session took place four to eight weeks after the first one.

EMG and TMS-related procedures were similar to the ones described in section 2.1.2 aside from a few changes. First, the number of performed MVICs was reduced from five to three, minimizing the possible impact of this action on our main measure of interest (MEPs) but still accounting for the learning process required to properly elicit reliable MVICs. Additionally, the action chosen to measure MVICs was changed in order to produce higher levels of muscle activation. In this protocol, researchers asked participants to make the shape of a circle by pushing their index fingernail against the belly of their thumb with maximum strength. MVICs were only collected from the right hand, independently of the assessed hemisphere. Second, EMG was acquired using the in-house system only and the sampling frequency and hardware band-pass filter parameters were adjusted to 4000 Hz and 1 Hz to 2 kHz, respectively. In the first experiment, sampling frequency and low cutoff frequency were chosen to match the commercial systems acquisition parameters. The sampling frequency chosen in this experiment is considered to be sufficient for the collection of TMS-EMG measurements (Groppa et al., 2012) and, by lowering the low cutoff frequency to 1 Hz, a larger proportion of the signal is preserved. The high cutoff frequency was chosen to perform simple anti-aliasing filtering. Third, after motor hotspot determination, coil positioning was aided by the Visor2™ Neuronavigation System (ANT Neuro, Enschede, Netherlands). This system uses an infrared camera to track reflective spheres placed on the stimulator coil and on participants' heads. Neuronavigation improves the ability to precisely revisit the same stimulation site within a session (Gugino et al., 2001; Sondergaard et al., 2021), thus improving our ability to reliably elicit MEPs.

Table 2.3: **Reduction of MEP variability population characterisation**. Abbreviations: MEP - Motor Evoked Potential; SD - standard deviation.

N	Age (mean $\pm$ SD)	Female (%)
47	36.7 $\pm$ 13.4	51.1

2.3.2 Data Processing & Analysis

Each TMS session generated one .bin file which was imported into Python3 for preprocessing. Since conventional filtering strategies produced distortions to the signal of interest, preprocessing was performed by applying a 3-level adaptive wavelet filter based on a Daubechies family wavelet (*db1*). This process consists in the application of the discrete wavelet transform resulting in a multilevel decomposition of the raw data. At a first level, raw data was represented by "approximation" and "detail" coefficients, that hold low and high frequency information, respectively. In following levels, the discrete wavelet transform was applied to the "approximation" coefficients, resulting in an increasingly lower resolution decomposition of the signal. The BayesShrink algorithm (Chang et al., 2000), which estimates an unique threshold for each wavelet subband, was then used to apply soft thresholding to the resulting wavelet coefficients. In soft thresholding, coefficients below the threshold are set to 0 while the remaining coefficients are scaled down to a factor relative to the threshold, thus avoiding signal discontinuities that would result in artifacts during time series reconstruction. Wavelet filtering has been shown to be especially useful when dealing with non-stationary signals as well as in the separation of noise sources with overlapping frequency contents (Samann & Schanze, 2019; Wahab & O'Haver, 2020; Machetanz et al., 2021). Subsequently, further smoothing was performed through the application of a third order Savitsky-Golay filter with a window size of 5 (Luo et al., 2005).This filter follows a similar approach to moving average smoothing but instead of replacing each data point by the average of the time points within a desired time-window, it uses polynomial least-squares approximation to calculate the new data points.

After signal denoising, a supervised algorithm was applied to isolate MVICs and MEPs and check for muscle pre-activation prior to the application of the TMS pulse. Pre-activation was identified following two criteria. First, the $50\,\mathrm{ms}$ leading to the application of the TMS pulse were compared with a reference portion of the EMG signal where no contraction was observed: if the rectified mean amplitude of the signal exceeded the rectified reference mean value by 2 times its standard deviation (McCambridge et al., 2020), the MEP was marked for pre-activation. If the first criterion was met and the root mean squared amplitude was larger than $15\,\mu\mathrm{V}$ (Hinder et al., 2014), the MEP in question was rejected from the analysis. MEPs smaller than $50\,\mu\mathrm{V}$ were also excluded from subsequent analyses.

Motor Evoked Potential normalization

Several different normalization methods were employed in the interest of testing their differential effects on MEP amplitude variability. For each subject, reference signals were determined using four distinct approaches, two of them based on external references, and two of them based on internal references (see Figure 2.4).

External references were derived from MVICs collected in the beginning of the acquisition protocol. To obtain the first external reference signal we applied a 200 ms moving average to the positive and negative portions of MVICs and drew an envelope around each contraction. We then calculated the envelope amplitude, defined as the peak to peak distance between maximum and minimum values - MVIC amplitude maximum after wave envelope (ENVnorm; Figure 2.4A - top). The other external reference signal was inspired in common EMG strategies to determine signal amplitude. First, signal was rectified by taking the absolute value of the EMG trace; then, we applied a $200\,\mathrm{ms}$ moving average on the resulting signal to determine peak amplitude - MVIC amplitude maximum after wave rectification (RECTnorm; Figure 2.4A - bottom). Internal references were based on MEP recordings. One was based on the peak to peak amplitude of MEPs (P2Pnorm; Figure 2.4B - left), while the other were based on

MEPs' absolute maximum - (ABSnorm; Figure 2.4B - right). To calculate the normalization factor for each one of the 4 methods, we took the largest or the average of the two or three largest reference signals. Hence, we defined a total of twelve normalization methods (4 types of reference signal extraction × 3 types of factor computation = 12 normalization methods).

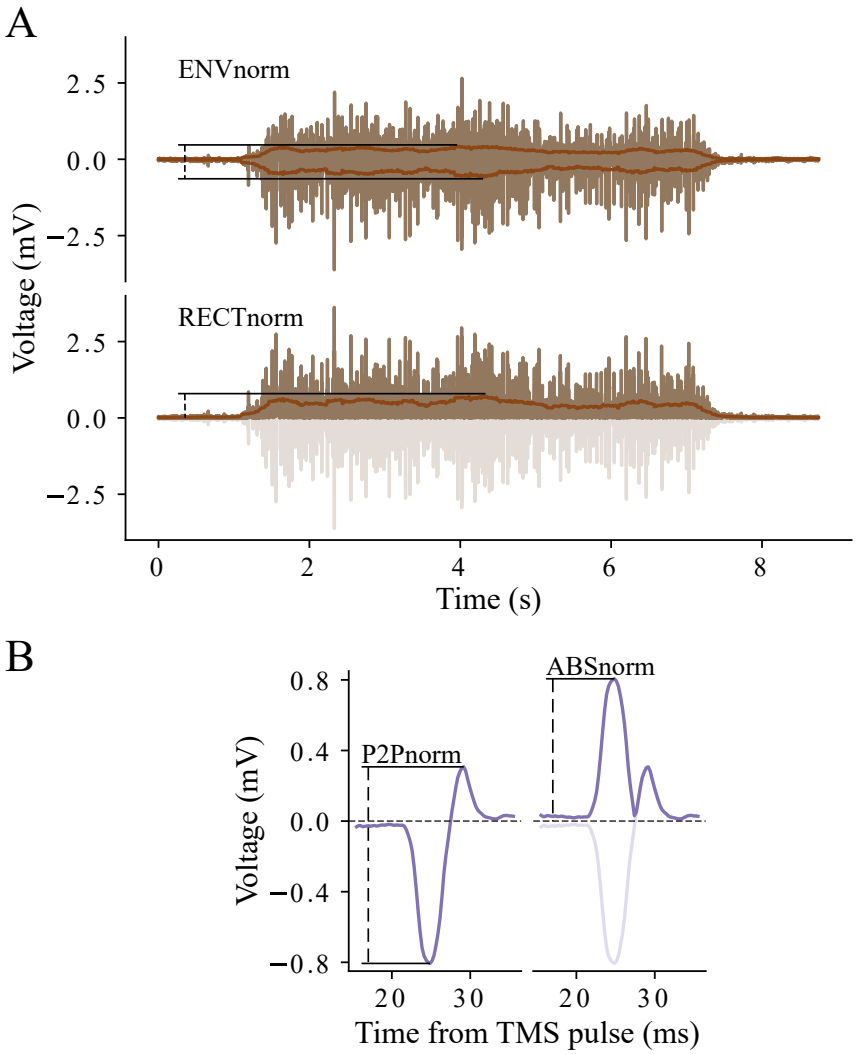

Figure 2.4: **Normalization methods. A**) External references: MVIC amplitude maximum after wave envelope (ENVnorm - top); MVIC amplitude maximum after wave rectification (RECTnorm - bottom); **B**) Internal references: MEP peak-to-peak amplitude (P2Pnorm - left); MEP absolute maximum (ABSnorm - right).

Abbreviations: MVIC – Maximum Voluntary Isometric Contraction; MEP – Motor Evoked Potential.

Statistical analysis

The effect of the different normalization methods on MEP amplitude variability was first assessed on MEPs collected on the right hand resulting from the stimulation of the left motor cortex. After, and to confirm the effect of normalization on MEP amplitude variability, between- and within-subject analysis was applied to the MEPs collected from the left hand resulting from the application of TMS pulses over the right motor cortex.

The impact of normalization methods in between-subject variability was assessed using the coef-

ficient of variation (CV). To allow for statistical comparison of CVs, a bootstrapping paradigm was applied as follows. First we performed iterative re-sampling of 30 MEPs per subject (5000 iterations), with MEPs used for normalization factors excluded from the average when using internal references normalization methods. One participant was excluded from this analysis because less than 30 MEPs were available. Then, for each iteration, after normalizing each MEP (nMEP), per subject MEP amplitude was defined as the average of the randomly selected nMEPs (Chang et al., 2016). Finally, for each iteration, CV was calculated using the cohort mean and standard deviation of MEP amplitude across subjects. By performing these steps for each normalization method, a CV was calculated for each method 5000 times, creating a distribution of CVs that was used to compare their performance. Since subjects repeated the MEP assessments four to eight weeks after the first session, we explored the test-retest stability of each method by calculating the intra-class correlation coefficients for absolute agreement (ICCs) using a two-way mixed-effects model (Koo & Li, 2016).

Nevertheless, these analyses may be limited by two factors. First, bootstrap analysis may be constrained by the fact that only a small number of MEPs per subject are available after the removal of invalid MEPs. In these subjects, repeated sampling of the same group of MEPs would render bootstrapping fruitless as approximately the same value would be reached in each iteration. Hence, a sensitivity analysis was performed excluding subjects for whom less than 37 MEPs were not available. This threshold was chosen as it ensures that for each subject the number of combinations available when sampling MEPs is at least one order of magnitude larger than 5000 iterations even after removal of three MEPs used in the calculation of the normalization factors. Second, as in internal reference normalization the largest MEPs are not included in the calculation of mean nMEP peak to peak amplitude per subject, the comparison between normalization strategies could favor methods in which a larger number of MEPs are excluded due to its use in the calculation of normalization factors. Thus, the analysis was repeated while removing all MEPs used to calculate internal normalization factors in all normalization approaches, including external reference normalization. Furthermore, since the number of TMS single-pulse applied to estimate MEP peak to peak amplitude per subject is variable across different research centers, CVs and ICCs were also calculated when considering only the first 10, 15, 21 and 30 TMS pulses. Since bootstrapping could not be applied in this analysis, a single value of CV was calculated per method.

Author's contributions

In the development of this master thesis I had the opportunity to provide major contributions at several levels of the research process. Alongside a group of researchers from the Neuropsychiatry Unit of the Champalimaud Centre for the Unknown I participated in the study design for both experiments and was responsible for a large part of participant recruitment and data acquisition as a TMS technician. Data acquisition and data processing pipelines were developed by me and are currently being used in TMS-EMG research at the CCU. Finally, I developed the analysis pipeline which benefited from regular discussions with other researchers and supervisors.

Chapter 3

Results & Discussion

In the following chapter, the results for the two experiments developed in the context of this dissertation are presented and discussed.

3.1 Validation of the in-house EMG acquisition system

Ten right-handed participants were enrolled in the validation of the in-house EMG acquisition system. Age and sex are described in Table 3.1.

Table 3.1: **In-house EMG acquisition system validation population characterisation**. Abbreviations: SD - standard deviation.

N	**Age (mean $\pm$ SD)**	**Female (%)**
10	28.5 $\pm$ 8.9	50.0

Table 3.2 reports the reliability scores for the wave-similarity analyses. LFM and IPI analysis both reveal high agreement between the in-house and commercial acquisition systems. LFM coefficients were close to reference values ($a \rightarrow 1$, $b \rightarrow 0$ and $R^2 \rightarrow 1$) and IPI_{icc} results were higher than 0.90, indicating that excellent agreement can be found between the in-house and commercial EMG systems (Koo & Li, 2016). The confirmatory analysis using ICCs of subject mean MEP and MVIC amplitude also revealed excellent agreement between both systems with ICCs of 0.999 and 0.965 for mean MEPs and MVICs, respectively.

Table 3.2: **Wave-similarity analyses results**. Linear fit method coefficients are represented by the 95% confidence interval. Integrated pointwise indices using the intra-class correlation coefficient for absolute agreement as reliability measure showed excellent agreement (larger than 0.90). Abbreviations: MEP – Motor Evoked Potential; MVIC – Maximum Voluntary Isometric Contraction.

		Both	**MEP epochs**	**MVIC epochs**
Linear Fit Method	a	[0.924, 0.934]	[0.929, 0.940]	[0.878, 0.901]
	b	[-0.471, -0.214]	[-0.539, -0.246]	[-0.045, 0.036]
	R^2	[0.953, 0.964]	[0.962, 0.972]	[0.882, 0.920]
Integrated Pointwise Index	IPI_{icc}		0.951	0.933

Albeit being very close to one, the scaling factor a indicated that signals collected using the in-house EMG acquisition system present a smaller amplitude when compared to those collected with the commercial system. This may be explained by their different filtering characteristics, namely the low-pass frequency (Table 2.1). As the commercial system has a higher high cutoff frequency, the signal suffers from less amplitude distortion. This effect is more noticeable in the MVIC epochs as their EMG representation is composed by a wider and more complex frequency band. In line with these results, the coefficient of determination R^2 was closer to the reference value in MEP epochs.

On the other hand, the scalar offset b is closer to 0 in the MVIC epochs. This can be explained by the fact that the size of MVIC epochs time-window being about one order of magnitude larger than that of MEP epochs thus resulting in a more effective linear detrending of the signals. However, in both cases we observed that linear detrending was effective in eliminating differences in DC amplifier offset.

This experiment was not without limitations. First, albeit the connection between both systems via a splitter cable allowed for simultaneous EMG acquisition using the same electrodes and therefore solving the issue of differences in waveform due to distinct electrode placement, it introduces a source of non-physiological noise that could impair our capability to extract information from the timeseries. Second, and as previously discussed, acquisition parameters are not equal across both systems and differences in filtering can explain the slight mismatch between the signals. Nevertheless, the analyses performed in this work have shown that the differences between systems are negligible. Finally, the validation of the in-house acquisition system for other muscle groups used in TMS-EMG studies is lacking (Tecchio et al., 2021). However, the FDI has been one of the most commonly used muscles to collect MEPs and is the main target of current research being developed at the CCU using TMS-EMG set-ups.

Overall, this work validates the use of the in-house EMG acquisition system for current and future TMS-EMG research performed at the CCU.

3.2 Reduction of Motor Evoked Potential variability through normalization

The following section presents the results obtained in the study of the effect of the proposed normalization methods on MEP amplitude variability. Forty seven participants, two of them left-handed, were enrolled in this study. Age and sex are described in Table 3.3.

Table 3.3: **Reduction of MEP variability population characterisation**. Abbreviations: MEP - Motor Evoked Potential; SD - standard deviation.

N	Age (mean $\pm$ SD)	Female (%)
47	36.7 $\pm$ 13.4	51.1

The effect of normalization on between-subject variability of MEPs collected in the right hand (left motor cortex) is represented in 3.1A.In the absence of normalization, 95% confidence intervals (CI) of the CVs were [1.0567, 1.0577]. When considering normalization methods, we found differences in between-subject variability between external and internal reference strategies. Specifically, except for RECTnorm, when using one and two references to calculate normalization factor, external reference methods increased between-subject variability. On the other hand, both internal reference normalization strategies resulted in a significant reduction of between-subject variability, particularly when using three references to calculate the normalization factor. The CVs 95% CI when normalizing to ABSnorm and P2Pnorm using three references were [0.3886, 0.3892] and [0.3653, 0.3660], respectively. This represents a reduction of approximately 65% in between-subject variability.

Test-retest stability of MEP amplitude collected following stimulation of left motor cortex is represented in Figure 3.1B. In the absence of normalization, stability of MEP amplitude is good ($ICC_{Average} = 0.77$, 95% CI [0.59, 0.87]). Normalization to MVIC amplitude maximum after wave rectification reduces stability, while other normalization strategies have only a slight impact. In fact, normalization to MEP absolute maximum using three references marginally improved MEP amplitude stability ($ICC_{Average} = 0.79$, 95% CI [0.61, 0.88]).

As mentioned in the previous section, bootstrapping analysis could be constrained by the fact that only a small number of MEPs per subject remain after the removal of invalid MEPs. However, when limiting the analysis to subjects for whom at least 37 MEPs (n=44) were available, ensuring a large enough number of combinations is available to generate a distribution of CVs with 5000 iterations, the results were not qualitatively different (Figure 3.2A and B). Additionally, the reduction of variability observed with internal reference normalization could be due to the removal of the largest MEPs in the calculation of mean nMEP peak to peak amplitude per subject. Nonetheless, when the MEPs used to calculate internal normalization factors were removed in all normalization approaches, including external reference normalization, the results were very similar (Figure 3.2C and D). In this analysis, CV mean values for ABSnorm and P2Pnorm normalization using three references are distinct from does observed in Figure 3.1A. This difference arises from the fact that different MEPs are used in the calculation of ABSnorm and P2Pnorm normalization factors since larger MEP absolute amplitude does not necessarily imply larger MEP peak to peak amplitude.

To validate the results obtained when stimulating the left M1, a confirmatory analysis was applied to MEPs resulting from the left hand, i.e., right M1 stimulation. From the total sample, 30 individuals also collected MEPs from the right M1 (50.0% female, 38.1 ± 14.5 years old, 6.7% left-handed). Since

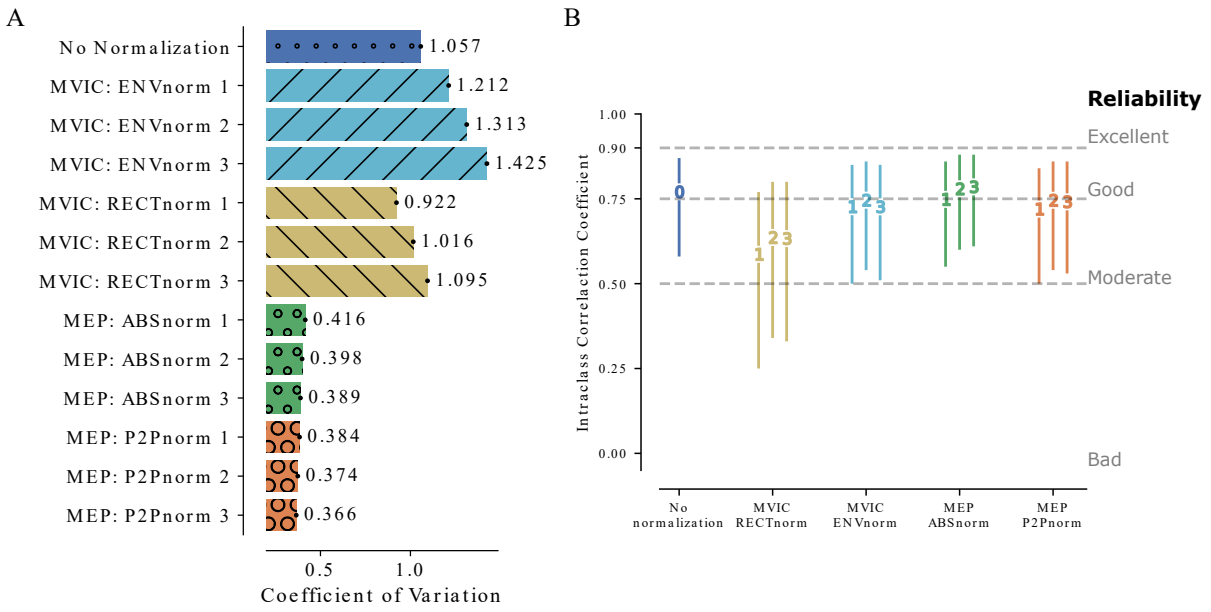

Figure 3.1: **Variability for MEPs collected in the right hand. A**) Between-subject variability: Bars represent 95% Confidence Interval. Numbers next to the bars represent mean CV. **B**) Test-retest stability: Bars represent 95% Confidence Interval. Numbers represent number of references used to calculate the normalization factor.

Abbreviations: CV - Coefficient of Variation; MVIC - Maximum Voluntary Isometric Contraction; MEP – Motor Evoked Potential.

MVICs were exclusively collected from the right hand, the effect of external reference normalization in right M1 MEPs variability was not tested. The impact of normalization in between-subject variability of MEPs collected from the left hand (right M1) can be seen in Figure 3.3A. In the absence of normalization, CVs 95% CI was [0.6955, 0.6964]. As described above for the left M1, internal reference normalization significantly reduced between subject variability, with 95% CI CVs using three references being [0.4055, 0.4064] for ABSnorm and [0.3921, 0.3930] for P2Pnorm. In the absence of normalization, test-retest stability for MEPs collected from the right M1 showed good to excellent values ($ICC_{Average} = 0.86$, 95% CI [0.70, 0.93]) while normalization to internal references had only a slight impact on test-retest stability of MEPs (Figure 3.3B). Despite not showing an increase in ICCs, normalization to ABSnorm using three references showed the least impact on test-retest stability ($ICC_{Average} = 0.78$, 95% CI [0.54, 0.89]).

The impact of normalization on MEP variability when reducing the number of TMS pulses applied for estimation of MEP peak to peak amplitude per subject can be seen in Figure 3.4. The effect on between-subject variability follows the same pattern and values as the ones observed when using all of the available pulses (Figure 3.4A). Regarding test-retest stability it is noteworthy that similar values were observed when the number of TMS pulses was reduced (Figure 3.4B). Furthermore, normalization resulted in a small impact on ICCs that was accentuated when the number of applied pulses fell below 21, the number of pulses required for accurate estimation of mean MEP peak to peak amplitude as recommended by Chang et al. (2016).

The findings presented here reveal that normalization, depending on reference type, impacted between-subject variability of MEP amplitude, while having a small impact on test-retest stability.

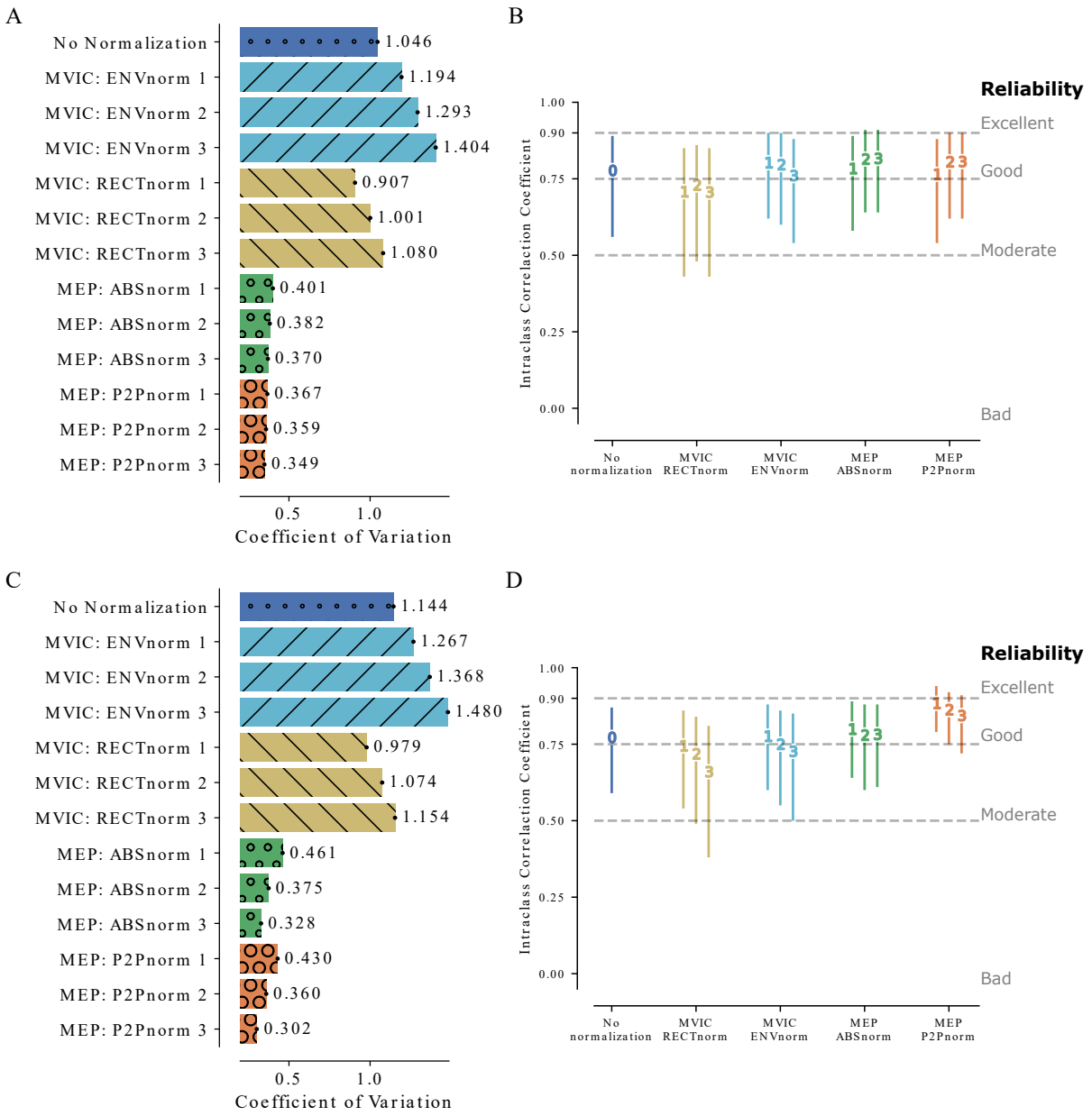

Figure 3.2: **Variability for MEPs collected in the left hand in subjects for whom at least 37 MEPs were available (A and B) and after removal of internal references across all normalization methods (C and D)**. Between-subject variability represented in panels A and C: Bars represent 95% Confidence Interval. Numbers next to the bars represent mean CV. Test-retest stability represented in panels B and D: Bars represent 95% Confidence Interval. Numbers represent number of references used to calculate the normalization factor.

Abbreviations: CV - Coefficient of Variation; MVIC - Maximum Voluntary Isometric Contraction; MEP – Motor Evoked Potential.

Overall, external reference normalization methods did not reduce between-subject variability. In fact, in most instances they showed higher CVs, particularly when the normalization factor was calculated as the mean of different MVICs. These results suggests that participants do not perform equally across MVIC trials, emphasizing the need to repeat MVIC trials several times in order to achieve a reliable measure of maximal voluntary neuronal activation (Alenabi et al., 2018). Additionally, in the best scenario, only negligible impact on between-subject variability can be obtained when using any of

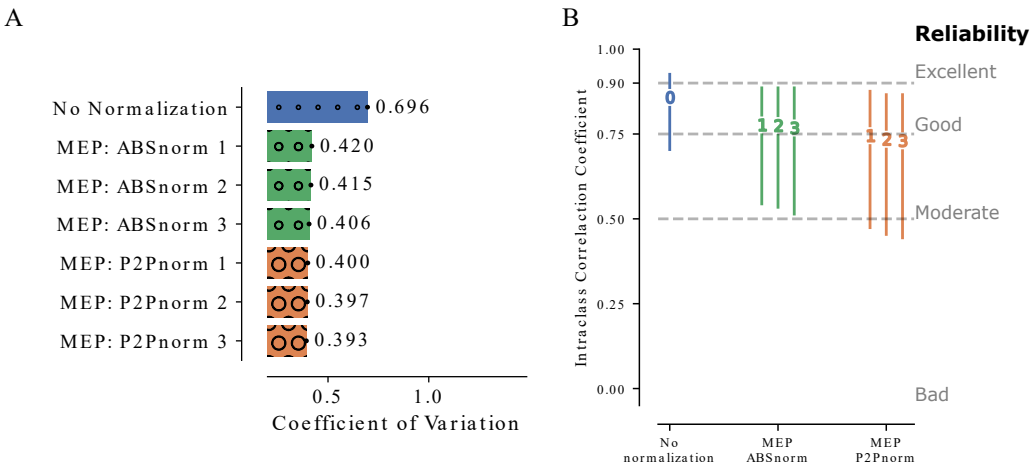

Figure 3.3: **Variability for MEPs collected in the left hand.** **A**) Between-subject variability: Bars represent 95% Confidence Interval. Numbers next to the bars represent mean CV. **B**) Test-retest stability: Bars represent 95% Confidence Interval. Numbers represent number of references used to calculate the normalization factor.

Abbreviations: CV - Coefficient of Variation; MVIC - Maximum Voluntary Isometric Contraction; MEP – Motor Evoked Potential.

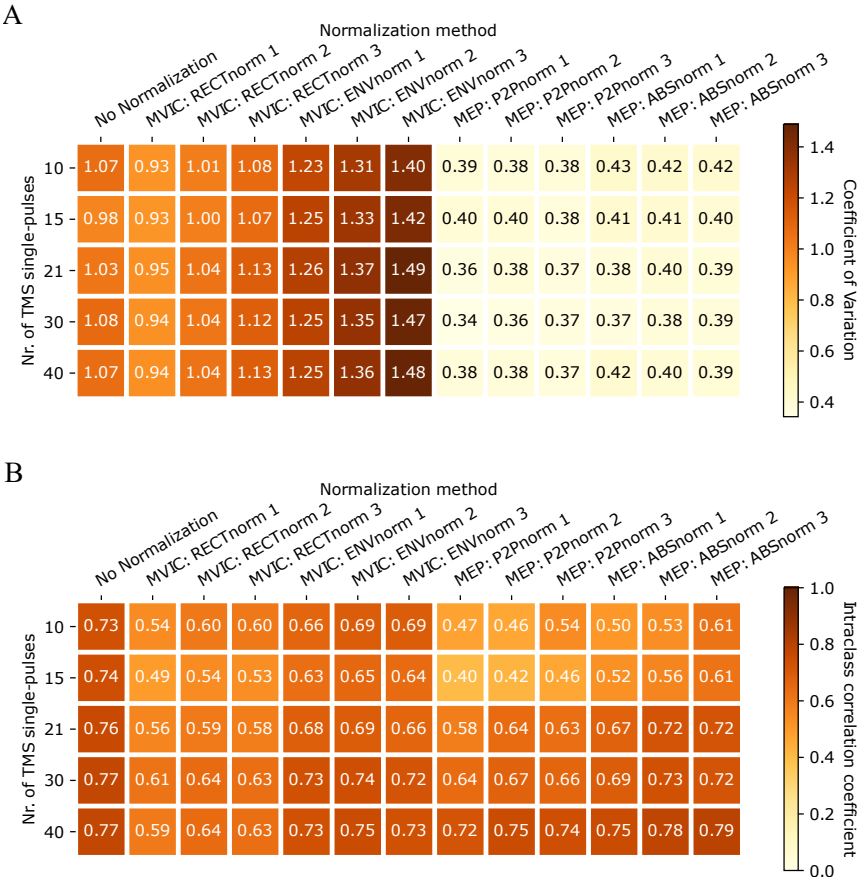

Figure 3.4: **MEP variability measures per number of applied TMS single-pulses.** **A**) Between-subject variability. **B**) Test-retest stability.

Abbreviations: MVIC - Maximum Voluntary Isometric Contraction; MEP – Motor Evoked Potential.

the methods of external reference normalization used here. Although Everaert et al. have shown that there is a positive correlation between MVICs and MEPs collected from the tibialis anterior muscle, the increase in between-subject variability and decrease in test-retest stability caused by external reference normalization suggests that there is no stable relationship between the amplitude of the MVICs and MEPs collected in this study, further suggesting that the action chosen to elicit MVICs does not produce maximum activation levels of the target muscles stimulated by TMS. On the other hand, internal reference normalization has shown a clear reduction of MEP amplitude between-subject variability even if a reduced number of TMS single-pulses is applied for estimation of MEP peak to peak amplitude. Based on the reduction of between-subject variability and on its impact in test-retest stability, normalization to ABSnorm using three references should be considered as the best candidate for reducing MEP amplitude variability.

In the absence of normalization, between-subject variability and test-retest stability for MEPs collected in both hands fall within reported values for MEPs collected in the FDI (Schilberg et al., 2017; Fried et al., 2017; Jannati et al., 2019; Mordillo-Mateos et al., 2019). Interestingly, when normalization was not performed, MEPs collected in the left hand (right M1) revealed lower between-subject variability and test-retest stability than that for MEPs collected from right hand (left M1). This finding was still observed when recomputing the analysis for right hand MEPs but only considering the group of subjects who performed sessions on both right and left hand (N=30; data not shown). Such difference may result from our methods, with collection of MVICs in the right, but not left hand, prior to eliciting MEPs. In fact, exercise has been shown to modulate MEP amplitude (Darling & Butler, 2006; El-Sayes et al., 2019; Nicolini et al., 2020; Moscatelli et al., 2020). This effect is dependent on exercise type (MacDonald et al., 2019), level of fitness (Lulic et al., 2017) or presence of medical disorders (Samii et al., 1997; Shajahan et al., 1999; Cerri et al., 2010; Palmer et al., 2016). Thus, the impact of MVIC collection on the measurment of MEP peak to peak amplitude should be taken into consideration in future studies. Nevertheless, it is noteworthy that, upon internal reference normalization, MEP CVs were comparable. In fact, the effect of both external and internal normalization methods on between-subject variability was consistent even when the analysis focused only on the first 10, 15, 21 and 30 collected MEPs. In the absence of normalization neither CVs or ICCs were impacted by the number of MEPs used for amplitude estimation. However, when less than the recommended number of MEPs was used for MEP amplitude estimation (Chang et al., 2016), test-retest stability of internal normalization methods was slightly reduced. Hence, if internal reference normalization is applied at least 21 single-pulses should be collected.

Our results should be interpreted considering potential limitations. First, creating a distribution of CVs through bootstrapping could be constrained by the fact that only a small number of MEPs per subject may be available after the removal of invalid MEPs. However, limiting this analysis to subject for whom at least 37 MEPs were available did not impact the results presented above. Second, as in internal reference normalization MEPs are not included in the calculation of mean nMEP peak to peak amplitude per subject, the comparison between normalization strategies could have favored methods in which a larger number of MEPs are excluded. However, when the MEPs used to calculate internal normalization factors were removed in all normalization approaches, results were identical. Finally, the reduction of MEP amplitude between-subject variability resulting from using internal normalization methods may be due to removal of true physiological variability impairing our capability to discriminate between healthy and diseased populations using nMEPs (Besomi et al., 2020). Further research should be conducted in other to clarify the impact of internal reference normalization methods in distinguishing different populations.

Chapter 4

Conclusion

The work presented in this dissertation was developed to respond to two main objectives. First we aimed to validate our in-house built EMG acquisition system against a well established commercial solution. Through wave-similarity and reliability analyses, we have shown that there is excellent agreement between the in-house system and the commercial solution. These findings bring assurance in the use of our acquisition for extracting meaningful conclusions from TMS-EMG experiments at the Champalimaud Centre for the Unknown.

Second, we aimed to test the effect of different normalization procedures in MEP amplitude variability. Two types of references were used for this purpose: internal and external references. We demonstrated that the latter, external reference normalization, does not reduce between-subject stability and that normalization using MVIC rectified amplitude worsens its test-retest stability. In opposition, internal reference normalization only shows a slight impact on test-retest stability while demonstrating a considerable impact in between-subject variability of MEP amplitude.

By analysing the impact on MEP amplitude variability, we suggest that normalization to ABSnorm using three references is a viable approach to normalize MEPs. Further research should be performed to validate its use in clinical populations and for clinical questions.

Other contributions

The work detailed in this dissertation has been presented as a poster at the 34th European College of Neuropsychopharmacology Congress and is currently under review for publication. Additionally, while at the Neuropsychiatry Unit I had the opportunity to participate in several other projects related to TMS. I was responsible for setting up the neuronavigation system and providing brief training in its use to other TMS technicians from the Neuropsychiatry Unit. Shortly after setting up this system I was responsible for participant recruitment and data acquisition in an experiment that was included in the 2021 paper published in Brain Stimulation by Cotovio & Oliveira-Maia et al., of which I am a coauthor. This experiment aimed at studying the between-session reliability of a TMS technician to find the TMS motor hotspot and treatment target using the localization method applied at the CCU. More recently, following FDA clearance of MagVenture TMS Therapy® for adjunct treatment of Obsessive-Compulsive Disorder I was involved in the formulation of a training in symptom provocation protocol for TMS technicians (Maia et al., 2022, of which I am also a coauthor).

In parallel with the previously mentioned work, I have been actively involved in the implementation of lesional network mapping approaches that explore how brain lesions causing neuropsychiatric syndromes, in particular mania and obsessive-compulsive disorder, may be affecting functionally connected regions of the brain. This technique is based on average resting-state functional connectivity from large neuroimaging databases of healthy individuals, i.e., connectomes. We have recently shown that results of lesion network mapping of mania is consistent and reproducible across several different connectomes (Cotovio et al., 2022, of which I am also a coauthor).

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