Non-invasive markers of GABA and glutamate are related to radiological and clinical features of multiple sclerosis

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Table of Contents

List of Figures

List of Tables

Abstract

Clinical disability experienced by people with multiple sclerosis ranges from non-existent to severe motor and cognitive impairments. The heterogeneous symptoms of this chronic neurological disease are partially attributed to structural brain damage, which is variably disseminated throughout the brain and likely to worsen over the disease course. Converging lines of evidence suggest that multiple sclerosis-associated brain damage and disability may both be linked to the brain's most prevalent excitatory and inhibitory neurotransmitters, glutamate and γ-aminobutyric acid (GABA), respectively. Activity of these neurotransmitters can be estimated non-invasively in humans using transcranial magnetic stimulation (TMS)-derived biomarkers, involving analyses of evoked electromyographic data. Endogenous glutamate and GABA levels in various brain regions can also be safely estimated in living humans using proton magnetic resonance spectroscopy (^{1}H-MRS). Using a novel combination of non-invasive multimodal techniques, the hypothesis that GABA and glutamate are implicated in radiological and clinical features of multiple sclerosis was investigated, with a focus on motor function. The main findings were: (1) Considering a combination of multimodal biomarkers, inhibition may be increased overall during the stable remission phase of the relapsing-remitting form of multiple sclerosis, whereas inhibition may decrease in progressive disease sub-forms; (2) Several TMS- and ^{1}H-MRS-derived markers of intracortical activity or endogenous neurometabolite levels are moderately correlated with measures of structural brain damage assessed using advanced neuroimaging methods; (3) When adjusting for neuroimaging evidence of structural brain damage, markers of lower sensorimotor GABA level (estimated using ^{1}H-MRS) and higher intracortical inhibitory activity (estimated using TMS) are related to poorer upper limb motor

performance. This body of work provides novel insight into the involvement of glutamate and GABA in radiological and clinical features of multiple sclerosis, and points toward potential therapeutic targets and biomarkers relevant to motor function in this disease.

Résumé

Le fonctionnement moteur des personnes atteintes de sclérose en plaques varie entre une capacite normale et des déficiences graves. Les symptômes hétérogènes de ce maladie neurologique chronique sont dus en partie aux lésions cérébrales structurales, qui sont localisées de façon diffuse à travers le cerveau et risque de détériorer au cours de la maladie. La convergence des lignes de preuve suggèrent que les lésions cérébrales et l'invalidité associée à la sclérose en plaques peuvent être liées tout deux aux neurotransmetteurs excitateur et inhibiteur les plus prévalent du cerveau : le glutamate et l'acide γ-aminobutyrique (GABA). L'activité de ces neurotransmetteurs peuvent être estimé de façon non-invasive chez les humains en se servant de biomarqueurs dérivés de la stimulation magnétique transcrânienne, impliquant l'analyse des potentiels évoqués en électromyographie. Les niveaux de glutamate et de GABA endogènes dans différentes régions du cerveau peuvent également être évalués en toute sécurité chez les humains vivants utilisant la spectroscopie de résonance magnétique (^{1}H-MRS). En utilisant une nouvelle combinaison de techniques multimodales non invasives, ce projet a étudié l'hypothèse selon laquelle le GABA et le glutamate sont impliqués dans les caractéristiques radiologiques et cliniques de la sclérose en plaques, en mettant l'accent sur la fonction motrice. Les principaux résultats ont été les suivants: (1) Compte tenu d'une combinaison de biomarqueurs multimodaux, l'inhibition peut être augmentée globalement pendant la phase de remise à l'état stable de la forme rémittente de la sclérose en plaques, alors que l'inhibition peut diminuer dans les sous-formes de la maladie progressive; (2) Plusieurs marqueurs dérivés de SMT et de MRS de l'activité intracorticale ou des niveaux de neurometabolite endogènes sont modérément corrélés avec les mesures de lésions cérébrales structurelles évaluées à l'aide de méthodes avancées de

neuroimagerie; (3) Lors de l'ajustement pour l'ampleur des lésions cérébrales structurelles, les marqueurs du niveau de GABA dans le cortex sensorimoteur inférieur (estimé avec ^{1}H-MRS) et une activité inhibitrice intracorticale plus élevée (estimée avec la SMT) sont liés à une performance motrice manuelle plus faible. L'ensemble de cet oeuvre fournit un aperçu innovateur de l'implication du glutamate et du GABA dans les caractéristiques radiologiques et cliniques de la sclérose en plaques, et souligne des cibles thérapeutiques potentielles et les biomarqueurs pertinents pour la fonction motrice dans cette maladie.

Commonly Used Abbreviations

^{1}H-MRS = proton magnetic resonance spectroscopy

9HPT = Nine hole peg test

EMG = electromyography

GABA = γ-aminobutyric acid

CSP = cortical silent period

ICF = intracortical facilitation

LICI = long-interval intracortical inhibition

MEGA-PRESS = MEshcher–GArwood Point RESolved Spectroscopy

MEP = motor-evoked potential

MRI = magnetic resonance imaging

MS = multiple sclerosis

MTR = magnetization transfer ratio

PASAT = paced auditory serial additional test

SICI = short-interval intracortical inhibition

T25FW = timed 25-ft walk

TMS = transcranial magnetic stimulation

Acknowledgments

First and foremost, thank you to my supervisor, Dr. Lisa Koski, who provided me with the opportunity to take on this interesting thesis project, and has been a supportive mentor throughout all steps of my graduate studies. I also extend my gratitude to the colleagues in the Koski Lab who worked on this multimodal project most directly with me: Dr. Jidan Zhong, who was a post-doctoral fellow in our lab, and Scott Holmes, a doctoral student. As well, I've been fortunate to work with three excellent research assistants (Dr. Elena Lebedeva, Serge Gallant, and Dr. Rishanthi Sivakumaran) and many other lab mates over my years in graduate school who contributed to this research through lab management and data collection support. Furthermore, many thanks to the members of my thesis advisory committee, consisting of Dr. Sridar Narayanan, Dr. Douglas Arnold, and Dr. Alan Dagger, who have provided guidance on this project annually. I am also grateful to all other collaborators and co-authors, who are recognized for their specific contributions in the following section.

Several funding sources made the research presented in this thesis possible. Support for data collection and other aspects of the projects presented here were funded through operating grants from the Canadian Institutes of Health Research (CIHR) awarded to Dr. Koski (grant numbers: MOP-119428, MOP-97847). As a Master's student, my personal stipend support came from the CIHR grant that funded the project as a whole, as well as a graduate research studentship awarded by the CIHR Neuroinflammation Training Program. Since transferring into the doctoral program, I have been fortunate to receive financial support through the Vanier Canada Graduate Scholarship (CIHR). I am also thankful for having received travel awards

from several sources, including the MS Society of Canada, ECTRIMS, and McGill University, which alongside the CIHR grant supporting this project, have provided me to the opportunity present my research at several international conferences.

My development as a neuroscience researcher has been strengthened through various programs within McGill University and beyond. The Integrated Program in Neuroscience (IPN) has provided several courses, annual retreat, and other support. Thank you especially to the directors of the program during my time as a graduate student: Dr. Josephine Nalbantoglu and Dr. Joseph Rochford. Beyond my thesis project, the MS Society of Canada has supported additional interdisciplinary learning. This included funding my participation in three endMS Summer Schools occurring in different cities across Canada each year. The MS Society of Canada also provided me with the opportunity to participate in the endMS Scholar Program for Researchers IN Training (SPRINT), which brought me together with an interdisiplinary team of researchers to write a review article on an topic outside of my thesis focus, which has resulted in a publication in Autoimmue Reviews. Thank you to Dr. Luc Vallières and Ryder Whittaker Hawkins from Laval University, as well as Dr. Courtney Casserly from the University of Toronto, who collaborated with me on this additional project.

My graduate experience has been further enrichened through my time as a visiting student at the University of Oxford, where I worked on a project that extended on themes of this thesis research. Thank you to Dr. Charlotte Stagg for welcoming me into her research group at FMRIB, as well as Dr. Sarosh Irani, Dr. Adam Al-Diwani, and Emily Hinson, who I worked with most closely for my related project at Oxford. Furthermore, I am grateful for the to the funding sources that made this opportunity possible: The Osler Travel Award organized through

Brain@McGill and the Clinical Neurosciences Department at the University of Oxford, as well as the CIHR Michael Smith Foreign Study Supplement.

On a more personal note, I'd like to thank everyone who has made my time as a graduate student enjoyable beyond the laboratory. My time in Montreal has certainly been enhanced through my involvement in the Graduate Student Association for Neuroscience (GSAN), the Post-Graduate Student Association (PGSS), and BrainReach. I greatly appreciate the colleagues and friends who worked with me in these organizations. As well, I am very thankful to all the friends who I had the pleasure to join for regular post-research relaxation whether it was for trivia, festivals, or just good conversation. In particular, many thanks to Brent, who has been a wonderful support since meeting on the GSAN softball team my first summer in Montreal to finishing the writing of this thesis.

Preface & Contributions of Authors

Data collection

Collection of data for the projects presented in this thesis were equally shared by myself, post-doctoral fellow Dr. Jidan Zhong, and graduate student Scott Holmes. This included obtaining informed consent, TMS, magnetic resonance imaging and spectroscopy (with aid of radiographers), neuropsychological testing, and reviewing medical charts and clinical database information. Research assistants Elena Lebedeva and Serge Gallant also helped with data collection when needed. Undergraduate students and lab research assistants within our lab helped with participant recruitment, which was coordinated by Dr. Zhong. Stanley Hum provided assistance with extracting data from the clinical database at the MNI. Dr. Koski directly oversaw the early stages of data collection directly and assisted in data collection roles if ever needed throughout the projects. A subset of data presented in the manuscript in Chapter 2 was collected under CIHR grant MOP-97847 by earlier members of the Koski lab, including Ben Whately, Afiqah Yusef, and Rebecca Taylor-Sussex. This large, multimodal dataset was used for the manuscripts included in this thesis (for which I am first author), in addition to studies led by other lab members that explore distinct research questions from those presented here.

Data analyses

Analyses of TMS-evoked electromyographic data for all outcomes of interest was performed by myself using MATLAB, which was checked by Dr. Zhong.

Structural images contributing to semi-automatic lesion analyses were run through software by our collaborators in Dr. Douglas Arnold's laboratory. I performed the lesion mask editing and quality control steps of this process. Structural segmentation of T1-weighted images using the semi-automatic software, FreeSurfer, and non-linear registration to T1 images into MNI space, was performed primarily by Dr. Zhong. Pre-processing to create pseudo-T2 or MTR maps from collected images were performed by collaborators in Dr. Doug Arnold's group at the MNI. I personally performed all subsequent structural MRI data processing steps included to answer specific research questions of each manuscript of this thesis, as described in the manuscript-based chapters.

Analysis of the ^{1}H-MRS component of this project was conducted by myself, with training and from Sebastian Proulx. The basis sets for LCModel analyses were from Dr. Malgorzata Marjanska, Brice Tiret and the Unité de Neuroimagerie Fonctionelle of the Centre de Recherche de l'Institue Gériatrique de l'Université de Montréal. The original pre- and post-processing MATLAB code written by Sebastian Proulx was added to by myself to create a more complicated analysis that addressed for additional potential confounding factors in the presence of multiple sclerosis (e.g. lesions, relaxation time differences between patient groups, see Chapter 4). I also performed analyses to extract regional structural and microstructural imaging information within ^{1}H-MRS volumes of interest, as described in the manuscript.

Several lab members including myself, Dr. Zhong, Scott Holmes, as well as research assistants and undergraduate summer workers helped with marking neuropsychological tests and double checking this data.

Statistical analyses were designed and performed by myself using SPSS, with feedback from co-authors.

Writing of manuscripts and thesis chapters

I drafted all of the manuscripts included in this thesis. All co-authors on manuscripts provided feedback for intellectual content and approved the final version. Further changes were made to manuscripts in response to feedback provided by anonymous peer reviewers. Co-authors have approved inclusion of these published manuscript into my thesis. The abstract of this thesis was written by myself in English, and translated into French by Lisa Koski with help from Jessica the Neuromodualtion Unit of the MUHC. I independently wrote the Introduction, Background and Literature Review (Chapter 1), and Conclusion (Chapter 5) of this thesis, with some feedback from Dr. Koski. Members of my advisory committee (Dr. Sridar Narayanan, Dr. Douglas Arnold, and Dr. Alan Dagger) provided feedback on analysis and interpretation of data to be incorporated in my thesis, annually.

Demonstration of original scholarship

The work presented here displays original scholarship from myself and has made a distinct contribution to knowledge. I have created a project that extends greatly on the scope of the objectives and analyses proposed in the CIHR grants that funded this work. The multimodal combination of methods used in this work has not before been applied to study multiple sclerosis, and through this approach the research presented provides novel insights into this disease. This thesis project has generated a series of published peer-reviewed manuscripts, for which I am first-author (see subsequent section).

Publications & Presentations

Published peer-reviewed manuscripts – Thesis projects

Nantes JC, Proulx S, Zhong J, Holmes SA, Narayanan S, Brown RA, Hoge RD, & Koski L (2017). **GABA and glutamate levels correlate with MTR and clinical disability: Insights from multiple sclerosis.** *NeuroImage*, In Press.

Nantes JC, Zhong J, Holmes SA, Narayanan S, Lapierre Y, & Koski L (2016). **Cortical damage and disability in multiple sclerosis: Relation to intracortical inhibition and facilitation**. *Brain Stimulation,* 9(4): 556-73.

Nantes JC, Zhong J, Holmes SA, Whatley B, Narayanan S, Lapierre Y, Arnold DL, & Koski L (2016). **Intracortical inhibition abnormality during the remission phase of multiple sclerosis is related to upper limb dexterity and lesions.** *Clinical Neurophysiology,*127(2): 1503-11.

Published peer-reviewed manuscripts – Related work

Casserly CS[1], Nantes JC[1], Whittaker Hawkinsc RF, & Vallières L (2017). **Neutrophil perversion in demyelinating autoimmune diseases: mechanisms to medicine.** *Autoimmunity Reviews*, In Press.
[1]*Co-first authors contributed equally to this work*

Zhong J, Nantes JC, Holmes SA, Gallant S, Narayanan S, & Koski L (2016). **Abnormal Functional Connectivity and Cortical Integrity Influence Dominant Hand Motor Disability in Multiple Sclerosis: A Multi-modal Analysis.** *Human Brain Mapping,* 37(12):4262-4275.

Zhong J, Chen DQ, Nantes JC, Holmes SA, Hodaie M, & Koski L (2016). **Combined Structural and Functional Patterns Discriminating Motor Disability in Multiple Sclerosis Using Multivariate Approaches.** *Brain Imaging and Behahvior,* In Press.

Most Notable Thesis-related Conference Presentations

Linking neurotransmitters and functional connectivity: A multimodal study on relapsing-remitting and progressive MS. *Poster presentation at ECTRIMS Congress 2016* (London, September 2016).

Inhibition and functional connectivity in the healthy and multiple sclerosis brain: A combined TMS, MR spectroscopy and fMRI study. *Oral presentation at Brain Stimulation and Neuorimaging Meeting* (Geneva, June 2016).

GABA and glutamate levels are related to local demyelination and clinical symptoms of multiple sclerosis. *Poster presentation at CAN 2016* (Toronto, May 2016).

Intracortical inhibition in multiple sclerosis: Relation to clinical subtype, motor disability, and cortical damage. *Poster presentation at ECTRIMS Congress 2015* (Barcelona, October 2015).

Decoding cortical excitability measures in multiple sclerosis: A multimodal approach to linking cortical activity, brain damage, and disability. *Poster presentation at the 3rd North America Meeting on Brain Stimulation and Neuroimaging* (Montreal, April 2015).

Hyperactivity of corticospinal inhibitory neurotransmission: Relation to brain atrophy and motor function among RRMS patients in clinical remission. *Poster presentation at the endMS Conference* (Saint-Sauveur, Québec, December 2013).

Intracortical inhibition alteration during the remission phase of multiple sclerosis: Relation to white and grey matter damage. *Poster presentation at the Society for Neuroscience Conference* (San Diego, November 2013).

Introduction

The brain's main excitatory and inhibitory neurotransmitters, glutamate and γ-aminobutyric acid (GABA), respectively, are critical to brain function, and therefore, human life. These small molecules, involved in synaptic transmission among other functions, are key mediators of functional and structural plasticity of the brain [1-6]. The consequences of deregulated glutamate or GABA can be severe – leading to physical or psychological disability [1, 4, 7].

Uncovering roles of glutamate and GABA in neurological disease is of great interest as it could shed light on mechanisms of neural dysfunction and point toward optimal therapeutic targets. While insights into the contributions of these molecules to disease have largely come from *in vitro* and animal-based studies [4], technologies have been developed that can non-invasively estimate glutamate and GABA levels or activity in the human brain [8-10]. Considering limitations of disease models in appropriately representing human conditions, clinical research remains an extremely valuable step in the translation of scientific knowledge to medical advances [8, 11-14].

The **overarching objective** of this thesis is to apply a novel combination of non-invasive assessments to address how glutamate and GABA may be involved in clinical and neuropathological aspects of a chronic neurological condition, multiple sclerosis. This highly prevalent disease, of likely autoimmune origin, involves neuroinflammation and structural brain damage (i.e. myelin and axonal loss) throughout the central nervous system [15]. Clinical symptoms of multiple sclerosis vary greatly across those afflicted, and may include motor, cognitive, and psychological dysfunctions, among other debilitating disease consequences [16].

Over eighty percent of patients first experience a relapsing-remitting form of multiple sclerosis, involving periods of temporary clinical recovery between symptom relapses [17, 18]. For others, clinical deterioration is more progressive, as can occur from disease onset (primary progressive multiple sclerosis), or after having relapsing-remitting symptoms for several years (secondary progressive multiple sclerosis) [18, 19]. Noteworthy therapeutic advances have emerged that target the neuroinflammatory component of this disease, however, effective treatments for disability related to progressive neurodegeneration remain greatly limited [20-22].

Human research on neurotransmitter involvement in neurological disease, such as multiple sclerosis, is only in its infancy. Background provided in **Chapter 1** will outline technologies available to approach this problem, and will summarize evidence to date on this topic. The focus will specifically be on metrics derived using transcranial magnetic stimulation (TMS) and proton magnetic resonance spectroscopy (^{1}H-MRS), which can provide markers of glutamate and GABA intracortical activity and endogenous levels, respectively [8-10]. A literature review of previous studies applying these approaches to study multiple sclerosis and related conditions will be provided. This will be followed by an outline of the research questions and hypotheses to be addressed in the subsequent chapters of this manuscript-based thesis, each of which aim to piece together aspects of this puzzle not previously investigated. Specifically, the **main sub-objectives** will be to assess extent to which TMS- and ^{1}H-MRS-derived markers of glutamate and GABA activity or level are: (1) abnormal in certain clinically-defined multiple sclerosis subgroups, (2) related to multimodal markers of structural brain damage, and (3) related to clinical impairment when adjusting for the impact of structural brain damage, with a focus on motor function.

Chapters 2, **3**, and **4** each consist of a published manuscript investigating, in different ways, relationships between markers of glutamate and GABA activity or level, structural brain

damage, and clinical disability. A more focused background relevant to study-specific objectives, detailed descriptions of methodology and results, as well as in-depth discussions are provided within each of these manuscript-based chapters. All of these research studies **display original scholarship and make novel contributions** to this field of scientific knowledge.

To conclude, **Chapter 5** aims to integrate findings of the three studies presented and reflect on the overall outcomes of this body of work. The scientific and clinical impact of the novel findings of this thesis will be discussed.

Chapter 1

Background & Literature Review

Preface

This initial chapter summarizes background theory and literature relevant to the novel research investigations presented in subsequent chapters of this dissertation. The first two sections describe principles of the non-invasive methods used to estimate intracortical activity and endogenous levels of glutamate and GABA; specifically, applications of transcranial magnetic stimulation (TMS) and proton magnetic resonance spectroscopy (^{1}H-MRS). Literature reviews on previous research using these techniques to investigate abnormalities in glutamate and GABA intracortical activity or level in people multiple sclerosis are also included. In the following section, neuroimaging methods to assess structural integrity of the brain are discussed, and a summary of evidence to date linking the TMS- and ^{1}H-MRS-derived metrics of interest to markers of multiple sclerosis-related neurodegeneration and demyelination is provided. Measurement of clinical disability in multiple sclerosis, and how glutamate and GABA may have relevance to symptoms of this disease, is the focus on the subsequent section. Finally, the specific research questions and hypotheses to be addressed in this thesis, which fill important gaps in the current knowledge on this topic, are outlined.

1.1 Investigating intracortical activity with TMS

Clinical neurophysiology is a specialized discipline that has emerged following centuries of experimental advances in physics and medicine. Brain stimulation, in particular, has evolved into a clinically-feasible technique with both basic science and therapeutic applications [23-25]. As early as the 18th century, Luigi Galvani demonstrated that the transfer of electricity from a charged metal conductor to a nerve could cause a frog leg to twitch as if it were alive [26, 27]. In the early 20th century, Wilder Penfield among other neurosurgeons of the time, became pioneering cartographers of the human brain by assessing responses evoked from direct electrical stimulations of cortical tissue in awake surgical patients [28-30]. Brain stimulation techniques of the current era follow similar principles as Galvani's and Penfield's set-ups, though can now enable investigations to be performed non-invasively in humans [25]. TMS, first introduced by Baker *et al.* in 1985 [31], can safely and painlessly trigger electrical signaling in brain tissue underlying a stimulated scalp region [32]. Among its various uses, including neuromodulatory interventions [25], TMS enables biomarkers of intracortical activity to be derived by assessing properties of evoked electromyographic activity following delivery of an electromagnetic pulse to the scalp region overlying a motor cortical area [33]. Modern adaptations of this technique have helped to uncover pathophysiological aspects of human neurological disorders [24, 34, 35], including cortical excitability abnormalities in multiple sclerosis [36]. Its potential in this context has not, however, been fully explored as of yet.

TMS-derived biomarkers

To obtain biomarkers of intracortical activity using TMS, an electromagnetic stimulus can be delivered with a TMS coil to the scalp region overlying the cortical area corresponding to a targeted muscle (example in Figure 1.1-A). When the TMS pulse is strong enough, action potentials are triggered in the underlying brain tissue and then conducted through the corticospinal tract and downstream peripheral neural pathways, which can lead to a physical "twitch" in the corresponding muscle contralateral to the stimulation site [32, 33]. Surface electrodes placed on the skin overlying the targeted muscle can record a motor-evoked potential (MEP) resulting from the stimulation (Figure 1.1-B) [37]. This electromyographic data can be analyzed, ideally using computer-based software, to quantify physiologically-relevant factors such as MEP amplitude and latency [32, 33]. Variations on this basic protocol, such as measuring MEPs produced by paired-pulse, rather than single-pulse, TMS stimuli (Figure 1.1-C,D) [38], can also be performed to gain biomarkers that may more specifically reflect certain types of excitatory or inhibitory intracortical activity of interest [25].

Experiments involving pharmacological manipulation before TMS have, indirectly, linked the electromyographic characteristics elicited with specific stimulation protocols to the activity of neurotransmitters at neuronal receptors [10, 39]. A TMS protocol optimized to detect net excitatory cortical activity involves paired-pulse stimulation, whereby a sub-threshold (conditioning) magnetic pulse is delivered to the cortical area representing a muscle, followed by a subsequent supra-threshold (test) pulse in the same location [38]. The resting motor threshold (RMT), from which the sub- or supra-threshold stimulus intensity is defined, is determined prior to the paired-pulse protocol based on the minimum intensity of stimulation required that will

often (e.g. 5/10 times) evoke a MEP of a pre-defined size (e.g. ≥ 50 μ V) [10, 40]. When the interstimulus interval between the conditioning pulse and test pulse is between 7-20 ms, the amplitude of the paired-pulse MEP tends to be larger than the MEP amplitude generated from a single test pulse at the same intensity [38, 41]. This effect, coined as intracortical facilitation (ICF) (Figure 1.1-C) [42], can be represented numerically as a ratio of the average paired-pulse MEP amplitude compared to the average single-pulse MEP amplitude. Activity of glutamate at N-methyl-D-aspartate (NMDA) receptors is likely a major driver of the net facilitatory effect observed, as antagonists of this receptor decrease ICF [43, 44]. Noradrenergic and GABAergic activity likely contribute more modestly to the resulting relative MEP size [39, 45-50].

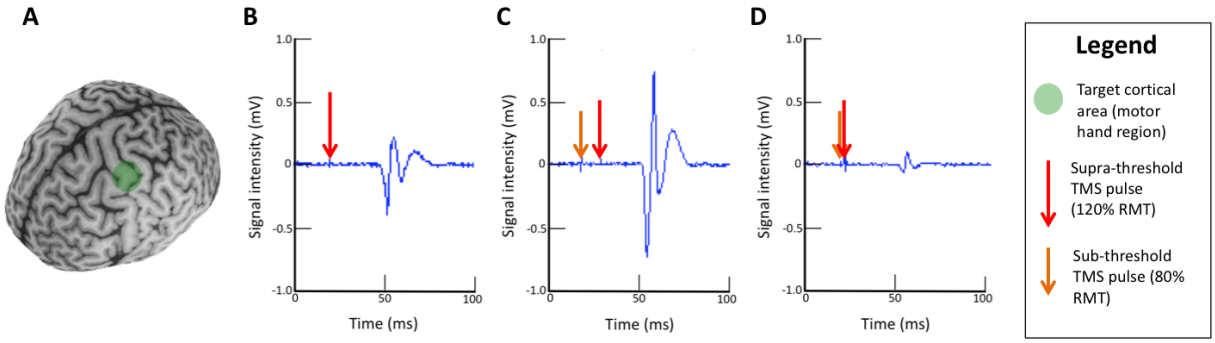

FIGURE 1.1 Overview of exemplary TMS paired-pulse protocols that can be used to obtain biomarkers of intracortical activity. The approximate location of an example target cortical area commonly stimulated for these applications of TMS is shown in **A** (i.e. within the motor hand region of the precentral gyrus, targeting the first dorsal interosseous muscle on the contralateral hand). In **B**, an example of a MEP produced from a single pulse suprathreshold TMS stimulus is presented. **C** and **D** show examples of MEPs recorded from the same muscle during intracortical facilitation (ICF) and short-interval intracortical inhibition (SICI) paired-pulse protocols, respectively. Location of arrows indicates timing of TMS pulses. Paired-pulses were applied at an inter-stimulus interval of 10 ms in **C** and of 2 ms in **D**.

Commonly used TMS-based metrics associated with inhibitory intracortical activity include short-interval intracortical inhibition (SICI), long-interval intracortical inhibition (LICI), and cortical silent period (CSP), each of which capture distinct aspects of inhibitory cortical activity [10, 39]. Variations on the paired-pulse technique described above are used to assess SICI and LICI. If the interstimulus interval between a subthreshold conditioning pulse and suprathreshold test pulse (delivered to the same primary motor location) is between 1-5 ms, the amplitude of the resulting paired-pulse MEP is usually inhibited (SICI: Figure 1.1-D) [38, 41]. For LICI, a suprathreshold stimulus following a prior MEP-producing suprathreshold stimulus 50-200 ms earlier at the same cortical location will generally produce a second MEP that is relatively inhibited in amplitude (Figure 1.2) [51-53]. Similar to ICF, these metrics can be described quantitatively through comparisons of average paired-pulse, relative to single-pulse, MEP amplitude. SICI is thought to reflect net inhibition, most largely mediated by activity at the fast-acting, ionotropic $GABA_A$ receptors [39]. This is supported by evidence that SICI is greatly influenced by drugs that selectively enhance the effect of these receptors (benzodiazepines) [10, 54, 55]. By contrast, pharmacological investigations suggest that LICI is more strongly linked to the slower-acting, metabotropic $GABA_B$ receptor-mediated activity [39, 56, 57].

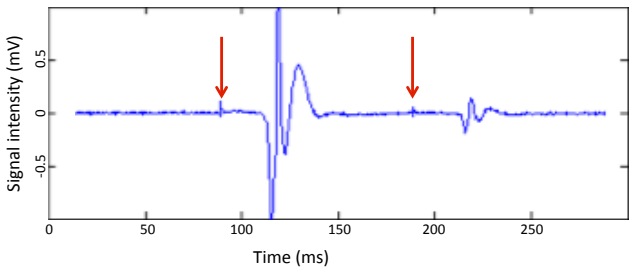

FIGURE 1.2 Example of electromyographic activity recorded from the first dorsal interosseous muscle during a LICI protocol. Red arrows indicate the timing of suprathreshold TMS stimuli, which are separated by 100 ms.

In contrast to the paired-pulse methods described above, CSP protocols involve delivery of a single suprathreshold test pulse while a muscle is voluntarily contracted. Following the MEP, electromyographic activity corresponding to tonic muscle contraction of the corresponding muscle becomes temporarily suppressed (Figure 1.3). The first 50-75 ms of this muscle activity suppression is mainly driven by spinal inhibition, whereas the later part is of supraspinal origin – likely intracortical [58-60]. While timing of this later CSP component suggests it could be mediated by $GABA_B$ receptors, it has been difficult to make this conclusion based on low-dose pharmacological studies in healthy people [46, 47, 57] because the blood brain barrier largely blocks entry of baclofen, a specific $GABA_B$ receptor agonist [39]. Notably, the hypothesized effect of a $GABA_B$ receptor agonist on CSP lengthening has been observed in patients given this drug as a treatment for severe spasticity resulting from spinal cord injury or progressive multiple sclerosis [61]. Overall, evidence to date indicates that CSP is largely influenced by motor cortical $GABA_B$ receptor activation, although $GABA_A$ activity and spinal mechanisms also partially contribute [39]. As the effects of CSP and LICI are dissociable [62, 63], these methods are complementary rather than representative of identical physiological phenomena.

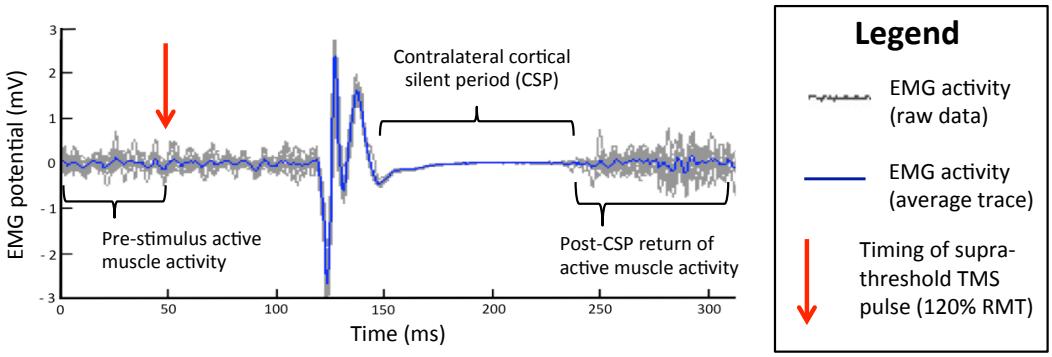

FIGURE 1.3 Overview of a TMS technique to obtain the CSP biomarker intracortical inhibition. Raw electromyography (EMG) data of 10 CSP trials, as well as the average of these trials are shown.

Exemplary data collection and analysis protocols of these TMS-based metrics are detailed in the *Materials and methods* sections of **Chapters 2** and **3**. While ICF, SICI, LICI, and CSP is far from a complete list of possible TMS-derived biomarkers to study intracortical excitatory or inhibitory activity, these metrics will be the focus of the present investigations. These were chosen because they are among most well-studied in terms of their physiological origin [33, 39] and there is some evidence of their clinical relevance in multiple sclerosis that warrants further investigation, as discussed in the following sections.

TMS intracortical activity studies on multiple sclerosis

Though relatively few TMS-based studies have been performed to investigate intracortical inhibition and facilitation in the presence of multiple sclerosis, evidence to date suggests that changes may occur over the course of this chronic condition. During acute symptom relapses, abnormally low SICI and short CSP, both indicators of low inhibition, have been observed in people with multiple sclerosis relative to that of healthy volunteers [64, 65]. During the stable clinical remission phases of relapsing-remitting multiple sclerosis, however, low intracortical inhibition has generally not been observed [66, 67]. A rebound may even occur during remission, wherein CSP becomes prolonged beyond durations of normal controls, as found by Caramia *et al.* (2004) who investigated treatment-naïve patients [65]. In **Chapter 2**, a study is presented that further seeks to resolve the extent to which intracortical inhibition may be elevated in clinical remission phases of relapsing-remitting multiple sclerosis, and explores factors that may contribute to why CSP prolongation occurs for some remitting patients, though not for others.

Changes in intracortical activity may also occur when progressive disability dominates. There is evidence that persons with secondary progressive multiple sclerosis may have lower SICI than both those with the earlier, relapsing-remitting form of the disease and healthy people [66-68]. Elevated ICF and lower LICI may also be implicated in the progression of multiple sclerosis, although this has not been observed consistently across investigations [66-68]. Of note, a study that excluded participants with upper limb impairment did not observe SICI, LICI, or ICF abnormalities among participants with multiple sclerosis [69], potentially suggesting that symptom type, rather than simply general disease course, may be of relevance. While fewer studies have assessed CSP in progressive forms of this disease, significant abnormalities have not been observed [67]. **Chapter 3** consists of a study that investigates physiological factors contributing to variability in these metrics of intracortical activity through a multimodal investigation on persons with various subtypes of multiple sclerosis, including progressive forms. Background on the potential radiological and clinical relevance of intracortical activity changes over the course of multiple sclerosis, driving hypotheses explored in the novel studies of this thesis, are discussed in *Sections 1.3* and *1.4*, respectively.

1.2 Investigating glutamate and GABA with ^{1}H-MRS

In parallel to advances in brain stimulation, non-invasive methods to study endogenous levels of metabolites within the brains of living organisms, including humans, have been developed. Regional concentrations of various neurochemicals, including glutamate and GABA, can be safely estimated in humans using proton magnetic resonance spectroscopy (^{1}H-MRS) [8, 70]. Evolved from a technique traditionally used in the field of chemistry to determine molecular

structures [71, 72], ^{1}H-MRS enables levels of known chemicals to be quantified using the same basic hardware as a standard MRI machine, with a few specialized adaptations [8].

An advantage of ^{1}H-MRS over brain stimulation-derived electromyographic biomarkers, is that it does not rely on a peripheral motor response. Therefore, ^{1}H-MRS can be used to investigate glutamate and GABA levels not only in regions directly associated with the motor system, but also areas of the brain responsible for other types neurological functions [35, 73]. It is important to remember, however, that while glutamate and GABA can act as neurotransmitters they also have many other functions outside of their synaptic duties [74-76]. Resonance signal intensities quantified using ^{1}H-MRS reflect levels of neurometabolites within both intracellular and extracellular pools of the brain tissue [72], a factor relevant to interpretation of ^{1}H-MRS data. For example, GABA is found not only within synaptic vesicles, but also within the cytoplasm of GABAergic interneurons, as well as within the extracellular fluid [77]. It has been proposed that GABA measured with ^{1}H-MRS may largely reflect extrasynaptic GABA tone [78], that is, extracellular GABA acting at certain receptor subtypes not directly associated with synapses [79]. For these reasons among others, ^{1}H-MRS should be considered as complementary to brain stimulation techniques, rather than as a method that could replace markers of intracortical activity derived with TMS [35]. Nonetheless, ^{1}H-MRS as a tool to investigate clinically-relevant neuropathological features of diseases, such as multiple sclerosis, has great potential that remains to be fully exploited.

^{1}H-MRS methodology

Spectroscopy provides the opportunity to investigate chemical levels based on *a priori* knowledge of the molecule-specific radiofrequency signals that create their spectral signature. ^{1}H-MRS takes advantage of the fact that the single proton of the hydrogen nucleus is both highly sensitive to the magnetic field and abundant in tissue [72]. Hydrogen nuclei of water molecules (Figure 1.4) will always resonate at the same frequency providing they are exposed to the same magnetic field [72]. In other types of molecules, such as glutamate and GABA (Figure 1.4), the electromagnetic environment of their hydrogen nuclei is dissimilar from that of water and other neurometabolites, therefore producing differing resonance frequencies. In these more complex molecules, the hydrogen nuclei in various positions in the same molecule impact each other causing a single molecule to have more than one frequency at which it resonances (a phenomenon known as scalar-, spin-spin- or J-coupling), resulting in various sub-peaks in its spectral pattern [72, 80, 81].

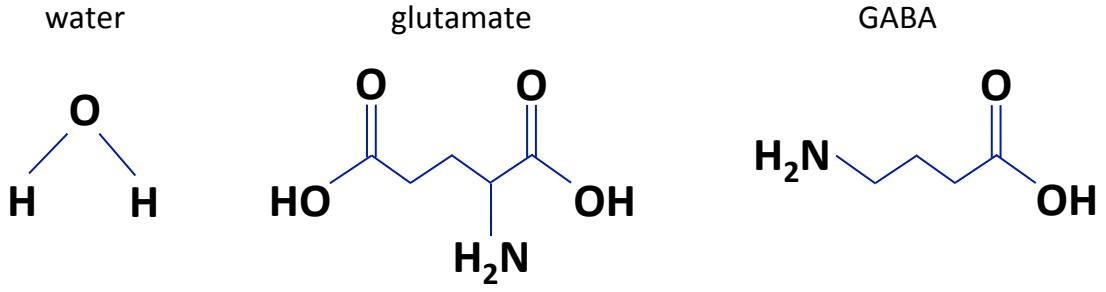

FIGURE 1.4 Chemical structures of water, glutamate, and GABA.

To analyze these chemical-specific resonance signals, time-domain magnetic resonance data collected with ^{1}H-MRS is usually converted to frequency-domain data and expressed

relative to a reference resonance. The resulting unit, parts per million (ppm), is proportional to frequency (which is normally expressed in Hertz) while correcting for the fact that magnetic field strength influences chemical shift along the spectrum [72]. Locations on this ppm scale where resonances of certain metabolites have relatively strong resonance peaks [81], are labeled in Figure 1.5. Note that neruometabolites have, however, complex spectral patterns due to J-coupling, and therefore a single metabolite actually contributes to multiple peaks across the chemical shift range [72]. As well, their resonance peaks are not perfectly resolved from that of other metabolites in the brain, contributing to data analysis challenges discussed below.

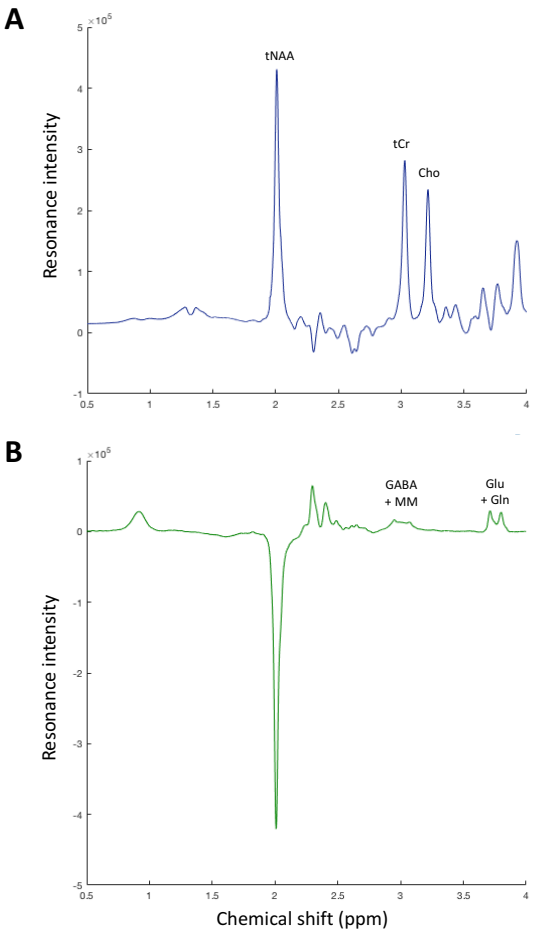

FIGURE 1.5 Example of single-voxel ^{1}H-MRS data acquired from a region of the human brain at 3T. **(A)** Exemplary ^{1}H-MRS spectra collected from a region of the human brain region. Some commonly-measured neruometabolites are labelled at their most prominent peaks on the ppm scale. **(B)** J-difference ^{1}H-MRS spectrum generated using the MEGA-PRESS method (described in the subsequent paragraphs of this section). Placement of labels indicates chemical shift locations where the resonance peaks of glutamate and GABA have prominent peaks observable after spectral editing. Note that even with this editing method, molecules in the brain that resonate at similar frequencies as that of glutamate and GABA (most notably glutamine and macromolecules, respectively) also contribute to the overall resonance intensity at these chemical shift locations, a factor that must be addressed in the analytic approach and data interpretation.

Legend: tNAA = total N-acetylaspartate, tCR – total creatine, Cho = choline, GABA = gamma-Aminobutyric acid,

The intensity of the resonance signal at a given spectral location is driven by the number of hydrogen nuclei resonating at that frequency. This enables relative levels of metabolites to be determined by comparing the intensity of resonance peaks corresponding to the metabolite of interest relative to that of a reference metabolite or water [72]. As water and lipids are several thousand fold more abundant in brain tissue than metabolites of interest to measure with ^{1}H-MRS, procedures to suppress their signals must be performed during data collection [72]. If water is to be used as a reference for metabolite quantification, separate water-unsuppressed ^{1}H-MRS scans must be collected [82].

Due to the presence of several detectable neurometabolites within brain tissue, many of which contribute resonance peaks that are not perfectly resolved from those of glutamate and GABA, the resulting spectrum will be quite complex to interpret [83]. Software packages, including those commercially available such as LCModel [84], can be used to analyze this data by modeling the contribution of each neurochemical to the resulting spectral peaks based on *a priori* knowledge (i.e. linear combination referencing "basis" spectra to assess probable levels of metabolites of interest). However, challenges still arise due to overlap of spectral peaks between neurometabolites along the spectrum, an issue which is intensified at lower magnetic field strengths as resonances will be less well resolved [81, 85, 86]. As it remains under debate whether glutamate and its precursor glutamine can be appropriately resolved at magnetic field strengths of 3 T and below, their combined contribution is often reported as Glx [72].

GABA is particularly difficult to measure because the concentration of this neurotransmitter in brain tissue (~1 mM) is very low in comparison to other metabolites that overlap with its multiplet J-coupled resonance peaks on the spectrum (e.g. creatine at 3ppm, see

Figure 1.5) [80, 81]. A popular approach to obtain reasonable estimates of brain GABA levels at moderate field strengths (e.g. 3 T) is through the use of a specialized spectral editing sequence, such as MEGA-PRESS (MEshcher–GArwood Point RESolved Spectroscopy) [87] (Figure 1.6).

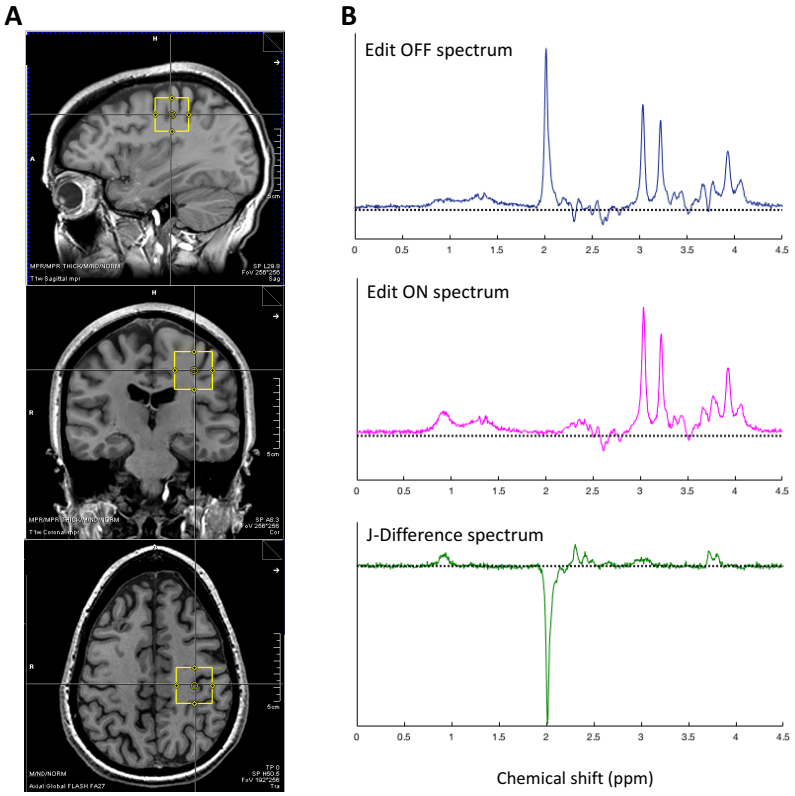

FIGURE 1.6 Summary of a data collection and analysis procedure for single-voxel ^{1}H-MRS data using a MEGA-PRESS sequence. **(A)** Example of voxel-placement immediately prior to a ^{1}H-MRS scan. The cubic voxel is placed in reference to a high-resolution T1-weighted image collected during the same scanning session. In this example, the voxel is centred over the primary cortical region known to be the locus of voluntary motor control of the right hand. **(B)** Example of averaged ^{1}H-MRS spectra acquired from a voxel of interest with a MEGA-PRESS sequence. Scans alternated between including editing pulses at 1.9 ppm (Edit ON, fuchsia) and those that did not have this resonance frequency edited (Edit OFF, blue), which were later processed to obtain the J-difference spectrum (green) to be analysed with software such as LCModel to obtain GABA level estimates.

The MEGA-PRESS sequence involves the inclusion of frequency-selective editing pulses that suppress resonance frequencies at strategic chemical shift locations. Specifically, this method can take advantage of the J-coupling pattern of GABA, as it differs from that of other metabolites that overlap with its most prominent resonance peak at 3 ppm [80]. Selectively suppressing resonances at 1.9 ppm during data collection will also modulate coupled resonances from GABA at 3 ppm, whereas it will not suppress that of other metabolites that resonate at 3 ppm which do not similarly have a J-coupled resonance peak at 1.9 ppm (e.g. creatine). By subtracting the spectrum acquired without a selective editing pulse at 1.9 ppm from the "Edit ON" spectrum, the resulting J-difference spectrum (Figure 1.5-B, Figure 1.6-B) enables GABA level to be better resolved from other metabolites than otherwise, and therefore more reliably quantified [81, 88].

In **Chapter 4**, further technical details relevant to data collection and analysis of this GABA-optimized ^{1}H-MRS procedure are provided. The analytic procedure described in the chapter's associated manuscript was developed with the aim of accounting for additional potentially confounding factors that could affect accuracy of neurometabolite quantification and data interpretation of that collected from individuals with neurological disease. For brevity, specifics of this will not be elaborated on further within this background section, as it is detailed in the later chapter.

^{1}H-MRS investigations on multiple sclerosis

Spectroscopy studies on multiple sclerosis have revealed abnormalities in endogenous neurometabolite levels, including glutamate and GABA, may occur in the brains of people with

this disease. Glutamate and Glx levels have generally been reported to be elevated within normal-appearing white matter and acute lesions [89-91], although not within chronic lesions [90], of people with multiple sclerosis. Within cortical and deep grey matter structures of people with multiple sclerosis, glutamate and Glx tend to be to be abnormally low [92-94]. Further, MacMillan *et al.* (2016) found glutamate measured from white matter regions has also been reported to decrease over time among those with progressive forms of the disease [95]. Fewer studies have sought to estimate GABA levels in the brains of people with multiple sclerosis, likely due to the additional technical challenges [81]. A small study on the relapsing-remitting form of this disease found GABA level measured from a sensorimotor brain region to not be significantly different in multiple sclerosis relative to that of healthy individuals [96]. Among those with secondary progressive multiple sclerosis, however, Cawley *et al.* (2015) found GABA level to be abnormally low in both the sensorimotor area and hippocampus [97]. Investigations into GABA levels in brain regions of people with multiple sclerosis have not previously included both relapsing-remitting and progressive forms the disease in the same study, which would better enable comparisons between these groups to be investigated more directly by using a consistent data collection and analysis protocol for both groups.

There remains a need to understand what may drive changes in regional endogenous glutamate and GABA levels, and what their ultimate consequences are in neurological disease. A summary of what is known so far is provided in the subsequent sections, and these important questions will be further addressed through the novel research investigations described in **Chapter 4** of this thesis.

1.3 Brain damage: Relations to glutamate and GABA

Research utilizing TMS- or ^{1}H-MRS-based approaches to study multiple sclerosis, summarized in the preceding sections, has indicated neurotransmitter-related abnormalities may occur over the disease course. While pathological factors related to such alterations are largely unexplored, structural damage is a strong candidate.

This hypothesis is drawn from converging lines of research. First, it is well-established that, in excess, glutamatergic activity triggers cell death through a cascade of reactions, known as excitotoxicity [4]. This pathophysiological process likely contributes to neural and glial damage occurring in various diseases of the brain [4, 98, 99]. Histological studies on multiple sclerosis brain tissue have provided evidence that glutamate dysregulation occurs in this disease [100-102]. This is further supported by research using animal models of multiple sclerosis, such as studies demonstrating that death of myelin-producing cells, oligodendrocytes, is prevented by administration of NMDA receptor antagonists [103, 104]. There is also some evidence that currently approved disease-modifying drugs may have neuroprotective effects, in part, through a mechanism that mitigates glutamate excitotoxicity [105, 106]. Through its interactions with glutamate mediating the excitatory/inhibitory balance, GABA may also be implicated in excitotoxicity [107].

Of further relevance, glutamate and GABA have key roles in structural plasticity of the brain [108, 109]. Despite glutamate's toxic effect when in excess, activity of this neurotransmitter is important for remyelination to occur as it instructs oligodendrocyte progenitor cells to differentiate into myelinating glial cells [109, 110]. Activity at GABA

receptors has also been shown to influence myelinating glial cells and their precursors [111, 112].

In this section, some non-invasive approaches to study multiple sclerosis-related brain damage are described. Further, human research to date on the possible link between such damage and markers of intracortical activity and regional neurometabolite levels is summarized.

Markers of multiple sclerosis-related brain damage

The extent of demyelination and neurodegeneration associated with multiple sclerosis cannot be measured directly in living humans; however, neuroimaging techniques provide markers of neural and glial integrity. Magnetic resonance imaging (MRI) methods can be used to detect macroscopic alterations indicative of lesions in the brain (Figure 1.7), even at the early stages of this disease [113-115]. These intensity abnormalities non-specifically indicate the presence of demyelination, cell-loss, inflammation, ischemia, edema, and/or gliosis [116].

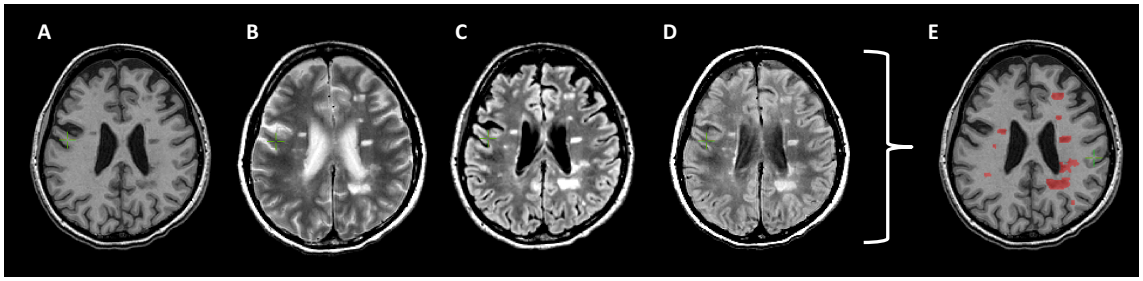

FIGURE 1.7 Examples of structural MRI imaging modalities that enable visualization of macroscopic lesions, including: **(A)** T1-weighted image, **(B)** T2-weighted image, **(C)** Fluid-attenuated inversion recovery (FLAIR) image, **(D)** Proton density-weighted image. These imaging modalities individually or, more ideally in combination, can be used to identify brain lesions characteristic of multiple sclerosis, as indicated by the red labels overlying the image in shown in **(E)**.

Neuroimaging evidence of brain lesions disseminated in time and space has become increasingly important for diagnosis of multiple sclerosis [115, 117, 118], a disease traditionally defined based on clinical presentation. Newly-appearing lesions identified with these methods provide markers of active neuroinflammatory events, which sometimes correspond to clinical relapses [119, 120]. Administration of a contrast agent (e.g. gadolinium) prior to neuroimaging enables active inflammatory lesions to be distinguished from chronic lesions in a single scan [115], though injection of a tracer makes this procedure more invasive. While conventional neuroimaging sequences used in clinical practice enable reasonable detection of lesioned white matter, they are much less sensitive to lesions within cortical and deep grey matter structures [115].

As focal white matter lesions identifiable with conventional neuroimaging techniques are not strongly associated with the severity of disability experienced by persons with multiple sclerosis [115, 121], this measure should ideally be complemented by other neuroimaging-derived metrics linked to clinical impairments. Brain atrophy, the irreversible loss of tissue, is also evident in people with multiple sclerosis from early disease stages and is moderately associated with disability severity [122-125]. This is usually measured through assessment of a T1-weighted image (e.g. Figure 1.7 A) because this provides relatively ideal contrast between cerebrospinal fluid, grey matter, and white matter [126]. A variety of semi-automated tools to segment tissue types (e.g. grey verses white matter) and functionally relevant regions (e.g. hippocampus, primary motor cortex), as well as measure volumes of the segmented images, have been developed and are freely available (e.g. FreeSurfer [127], Figure 1.8) [128].

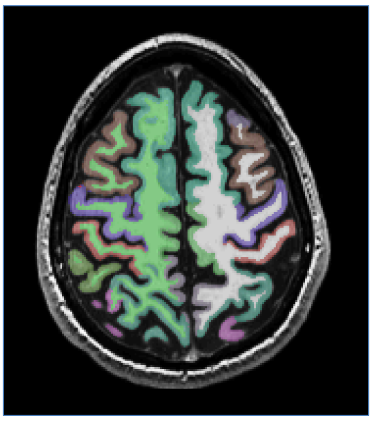

FIGURE 1.8 Segmentation of a T1-weighted brain image using FreeSurfer. Different colours correspond to specific brain regions identified with this semi-automated software. For example, the precentral gyri (loci of the primary motor cortices) are labelled in purple. Based on these segmentations, brain regions of interest can be measured for their volume and normalized (e.g. to skull size) to provide markers of regional atrophy.

Raw volumes measured using tools such as FreeSurfer should be adjusted to provide a more accurate marker of cell loss. This can include brain volume normalization based on cranial volume or, if longitudinal imaging data is available, comparing the image to a co-registered MRI acquired from the individual at a previous date [126, 129, 130]. While markers of whole brain atrophy using these methods have been linked to clinical disability of persons with multiple sclerosis, grey matter volume loss, specifically, is more clinically relevant [126, 131, 132]. Atrophy of grey matter structures estimated using neuroimaging techniques is predominantly driven by axonal loss [133, 134], though it is also influenced, in part, by demyelination of surviving axons [115].

Even within tissue that appears normal on conventional MRIs, microstructural damage, detectable using alternative modalities such as magnetization transfer (MT) neuroimaging, may be present [115, 135-137]. Interactions between free flowing protons and protons bound to macromolecule components of brain tissue drives the MT effect [138]. Comparing an image acquired with a MT pulse to a co-registered image acquired similarly though without an additional MT pulse, can generate the index MT ratio (MTR) (Figure 1.9), which reflects the

extent of magnetization exchange between free protons and those macromolecules bound to tissue. This semi-quantitative metric correlates with myelin content assessed with histological methods [139-146], and changes in MTR are similar to the biological time courses of demyelination and remyelination [139]. Other disease-related factors (e.g. axon loss, astrocytic proliferation) can also contribute to MTR abnormalities [115, 138]. Among people with multiple sclerosis, lower MTR is associated with more severe clinical impairments [147, 148].

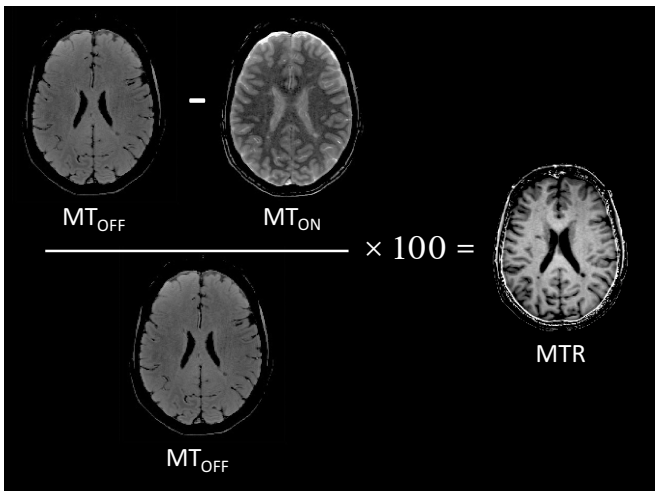

FIGURE 1.9 Formula to generate a MTR map of the brain. Examples of proton density-weighted FLASH sequence pair images acquired either with (MT_{ON}) or without (MT_{OFF}) an MT saturation pulse are shown on the left side of the equation. The resulting MTR image, for which intensities are indicative of microstructural integrity, is shown on the right side of the equation (expressed in percent units).

These various neuroimaging techniques are most valuable in combination. For example, masks reflecting segmentations of the brain defined through analyses of conventional neuroimaging sequences (e.g. lesions, grey matter) can be registered to MTR images (Figure 1.10), such that this metric can be extracted from specific regions of interest that had the benefit of being defined using independent, higher contrast images. Multimodal approaches including metrics of damage derived from various modalities may provide optimal descriptions of disease-related damage.

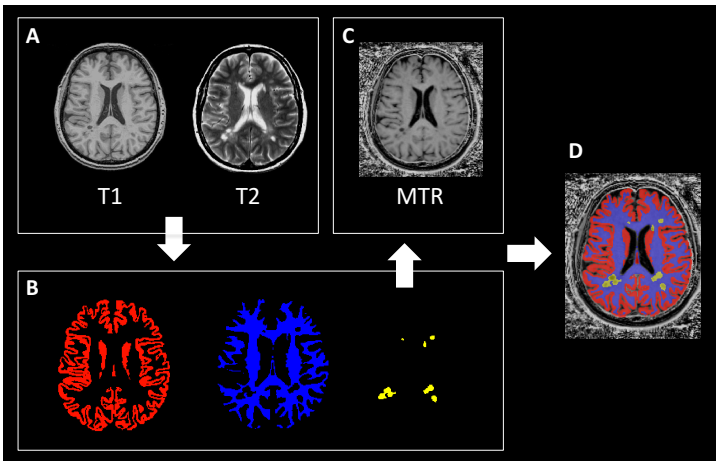

FIGURE 1.10 Example of a multimodal structural neuroimaging approach. Structural MRI images in **(A)** were used to create masks of segmented tissue types shown in **(B)**. These masks could be applied to a co-registered MTR images **(C)**, as shown in **(D)**, to gain information on regional mean MTR values, such as within normal-appearing white matter or grey matter regions of the brain.

Each novel research investigation described in the subsequent chapters of this thesis includes further details on these neuroimaging approaches. **Chapter 2** focuses on markers of macroscopic lesions and cortical atrophy in participants with relapsing-remitting multiple sclerosis. Studies presented in **Chapters 3** and **4** additionally include regional MTR assessments to evaluate how microstructural damage may be related to intracortical activity or neurometabolite concentrations across both relapsing-remitting and progressive subtypes of multiple sclerosis. It should be recognized that the methods described here have some limitations and do not represent the full scope of imaging modalities developed to assess structural or microstructural damage in the human brain, as will be discussed within the subsequent chapters. Nonetheless, combining these structural imaging methods with the TMS- and ^{1}H-MRS-derived metrics of interest described in the previous chapters, would provide novel insights into links between multiple sclerosis-related brain damage and GABA or glutamate, not previously investigated.

Relevance to TMS-based markers of inhibition and excitation

Few studies have investigated the potential link between multiple sclerosis-related brain damage and TMS measures of intracortical excitation and inhibition. As previous work examining relationships between white matter lesion load to ICF and SICI have yielded null results, it has been speculated that cortical damage may be the cause of abnormalities in TMS measures of excitability and inhibition among individuals with multiple sclerosis [66, 67]. Notably, abnormalities in ICF, SICI, and LICI have primarily been observed among those with a progressive disease course [66-68], a form of multiple sclerosis more strongly dominated by intracortical pathology than the relapsing-remitting subtype [131, 149, 150]. However, no study has yet used neuroimaging methods that are more specifically sensitive to grey matter damage, such as normalized cortical volume or cortical MTR, to answer this question more directly.

A further consideration is the influence of damage location, as lesions are variably disseminated across the brain and regions where cell loss dominates may change over the course of multiple sclerosis [115, 151]. Conte *et al.* (2009) reported lesion load assessed globally, nor specifically within the cortical spinal tract, to be related to SICI or ICF [66]. Nonetheless, further investigations on the relevance of damage location to TMS-based outcomes, integrating various other imaging approaches and brain regions of interest, are warranted.

In **Chapters 2** and **3**, studies are presented that seek to uncover the relationships between metrics of intracortical activity derived from TMS (e.g. ICF, CSP) and markers of structural damage from structural MRI analyses (e.g. cortical volume, MTR, lesions) in the brains of people with multiple sclerosis. These relationships will be examined for damage assessed

specifically within regions of interest with known relevance to motor function, as well as with brain damage assessed more globally.

Links to ^{1}H-MRS measures of glutamate and GABA levels

There has been a similar poverty of knowledge with respect to relationships between multiple sclerosis-related structural brain damage and ^{1}H-MRS measures of endogenous glutamate and GABA levels, although some recent studies have started to address this topic. Supporting the hypothesis that glutamate excitotoxicity contributes to brain atrophy in multiple sclerosis, Azevedo *et al.* (2014) found higher glutamate levels relative to *N*-acetylaspartate (NAA: another detectable neurometabolite, proving a marker of neuronal health [152]) level in normal appearing white matter are predictive of greater brain atrophy years later [153]. While Azevedo and colleagues had mainly assessed individuals with relapsing-remitting multiple sclerosis, MacMillan *et al.* (2016) found levels of glutamate and glutamine within white matter to decrease significantly over a period of two years among those with progressive forms of the disease [95]. While these authors did not find brain volume or total NAA level to similarly change significantly over this period [95], the possibility that glutamate level decreases could be linked to pathological processes that MTR is sensitive to was not ruled out. To date, one study has included both ^{1}H-MRS measures of glutamate levels and MTR measures in the same participants with multiple sclerosis [92]; however, the aim of this study had not been to directly assess relationships between these variables. Evidence from animal model-based research showing that glutamate level decreases with demyelination [154, 155] lends to the hypothesis

that lower glutamate concentrations may be linked to markers of microstructural integrity, such as MTR, in the brains of people with multiple sclerosis.

Even less is known regarding relationships between structural damage and brain GABA concentration. Cawley *et al.* (2015) [97] suggested that decreased GABA level observed in some brain regions of persons with secondary progressive multiple sclerosis may reflect decreased inhibitory interneuron density. However, no study on multiple sclerosis has previously investigated the extent to which GABA levels may be related to microstructural damage measured with techniques such as MTR.

To address these knowledge gaps, a novel research study is presented in **Chapter 4** that investigates relationships between regional neurometabolite levels, including glutamate and GABA, and microstructural integrity indicated by MTR in persons with multiple sclerosis.

1.4 Disability: Relations to glutamate and GABA

Beyond structural brain damage, uncovering neurochemical mechanisms underpinning, or even mitigating, various types of clinical disability occurring in neurological diseases such as multiple sclerosis remains an important challenge. This section includes descriptions of two commonly used clinical tools to assess disability in multiple sclerosis. Further, summaries of research that have begun to uncover links between multiple sclerosis-related clinical impairments and glutamate or GABA in the human brain, as well as important knowledge gaps remaining, are provided.

Measuring clinical disability in multiple sclerosis

The Expanded Disability Status Scale (EDSS) [156] is a traditionally popular ordinal scale to assess clinical impairment in multiple sclerosis. Disability is scored according to eight functional domains (i.e. pyramidal, cerebellar, brain stem, visual, sensory, bowel and bladder, cerebral/mental). A major critique of the EDSS is that the weighting of walking ability is over-emphasized [157, 158]. While it has limitations, EDSS remains widely used by neurologists and as a primary clinical outcome metric in clinical trials for this disease [159-162]. Most research manuscripts on multiple sclerosis include EDSS as, at the very least, a general descriptor of the clinical sample in a commonly understood dialect.

Since its introduction in the late 1990s, the Multiple Sclerosis Functional Composite (MSFC) has grown in popularity as a disability assessment tool for this disease, now challenging the EDSS as a primary outcome metric in clinical trials [161, 162]. This three-part test equally weights assessments of walking, upper limb motor function, and cognition evaluated with the timed 25-foot walking test (T25FW), 9-hole peg test (9HPT), and three-second version of the Paced Auditory Serial Addition Test (PASAT-3), respectively [163-165]. MSFC score correlates reasonably well with MRI-based measures of structural brain damage [166, 167] and patient-reported quality of life [168, 169]. Subscales of the MSFC can also be evaluated on their own to assess research questions on more specific functional domains rather than overall scores [162]. For example, the 9HPT, which on its own has been found to be valid and reliable measure of upper extremity function among people with multiple sclerosis, has been used widely to study the neural underpinnings of motor dexterity issues experienced by people with this disease [170].

In the novel studies of this thesis, clinical outcomes using the EDSS and MSFC (composite score and subscale outcomes) are reported. The 9HPT was chosen as a primary outcome measure, in part, because our TMS-based biomarkers of interest are inherently dependent on the activation of cortical and white matter regions of the motor system to produce the electromyographic activity that can be recorded (usually from a hand muscle) and analyzed. Further, motor impairment and its neurophysiological correlates are of interest due to the high percentage of persons with multiple sclerosis who experience motor symptoms, as well as the negative impact manual dexterity issues have on quality of life [171-175]. ^{1}H-MRS provides the opportunity to study glutamate and GABA levels in various brain regions, although these areas have low spatial resolution and must be selected *a priori*. It was chosen to investigate clinical correlates to neurometabolite concentrations within a volume of interest that included the TMS stimulation target, as well as a neighboring parietal area. Additional study-specific details and rationale are provided within the subsequent sub-section and upcoming chapters.

Clinical correlates of intracortical inhibition and facilitation

Few studies have investigated links between TMS-based markers of intracortical activity and clinical severity of multiple sclerosis. Lower SICI has been linked to greater disability in people with this disease, as measured with the EDSS [66, 67, 176]. However, a relationship between SICI and EDSS was not significant in a study that excluded those with upper limb impairment [69]. Significant correlations with overall EDSS score have not been found for LICI, CSP or ICF [66, 67, 69, 176]. However, one study suggested that longer CSP among people with multiple sclerosis may be been linked specifically to worse cerebellar dysfunction assessed as a

subcomponent of the EDSS [177]. Notably, abnormalities in cortical excitability and inhibition have also been observed in various neurological disorders involved motor dysfunction (e.g. Parkinson's disease, amyotrophic lateral sclerosis) [178, 179], and can become modulated in response to successful motor rehabilitation interventions including repetitive TMS, exercise, and pharmacological treatment [180-184].

While these studies indicate the potential clinical relevance of TMS-based biomarkers of intracortical inhibition and facilitation, there is much left to learn. In general, prior TMS research on multiple sclerosis has not separately investigated the role of specific functional domains (e.g. upper limb verses cognitive disability) despite the fact that this technique relies on the stimulation of the motor cortex, rather than areas more directly involved in other types of neurological functions. Additionally, previous relationships reported have not accounted for structural brain damage, such as neuroimaging markers of cortical atrophy or demyelination. This is of particular importance because without information on structural integrity of the brain, it is more difficult to infer whether links between TMS-based markers of intracortical inhibition (e.g. SICI) and clinical disability severity (e.g. EDSS) may reflect a functional change that could potentially mediate clinical impairments beyond that occurring due to brain damage. Further highlighting the importance of multimodal assessment, a 2015 review by Simpson and Macdonell concluded that while TMS is not sufficient on its own as a clinical tool for multiple sclerosis, it may provide useful diagnostic or prognostic information that could complement radiological and clinical disability measures, such as those already commonly used in clinical care [185].

In **Chapters 2** and **3**, novel research investigations are presented that assess the clinical relevance of TMS-derived markers of intracortical activity in multiple sclerosis, with a focus on

motor impairment. Beyond what has been demonstrated previously, the primary aim will be to assess the extent to which intracortical inhibitory and excitatory activity abnormalities are linked to clinical impairments when accounting for the impact of grey and white matter damage, assessing with multimodal non-invasive structural imaging techniques.

Clinical correlates of ^{1}H-MRS-measured neurometabolite levels

^{1}H-MRS studies on multiple sclerosis have revealed that higher Glx level within normal appearing white matter correlates with more severe disability, as assessed by the EDSS [94] and a closely related metric [89]. Further, higher glutamate/*N*-acetylaspartate level in normal appearing white matter has been linked to worsening of MSFC score over four years [153]. Within cortical structures, abnormally low glutamate level have been found to be related to poorer MSFC score [93]. Further, Muhlert *et al.* (2014) reported that within the hippocampus, cingulate cortex, and thalamus, glutamate deficits correlated with worse visuospatial memory of people with multiple sclerosis, even after accounting for the impact of lesions and MTR within the spectroscopic volume of interest [92]. Similar studies have not, however, been performed to investigate whether glutamate deficits have a similar impact in other disability domains, such as motor or executive function impairments experienced by people with multiple sclerosis. Further, these studies did not adjust for the potential impact of GABA concentrations in the same region.

To date, two studies have investigated the clinical relevance of endogenous GABA levels in multiple sclerosis. Bhattacharyya *et al.* (2013) reported that higher GABA level correlated with worse performance on the 9HPT among persons with relapsing-remitting multiple sclerosis, though not among the healthy volunteers [96]. Cawely *et al.* (2015), by contrast, found

abnormally low sensorimotor GABA level among people with secondary progressive multiple sclerosis to be strongly related poorer performance on tests strength, though more weakly related to poorer 9HPT performance (approached significance) [97]. These differing findings highlight the need for further investigations in both clinical sub-groups using consistent data collection and analysis approaches. As well, investigating these relationships while accounting for potentially confounding disease-related variables (e.g. microstructural damage) that can differ between these disease subgroups, could further help to understand the clinical relevance of GABA level.

Chapter 4 consists of a study that seeks to uncover clinical correlates of sensorimotor and parietal neurotransmitter levels. Importantly, these relationships were assessed accounting for neuroimaging markers of structural brain damage within the regions from which ^{1}H-MRS was measured, as well as global measures of atrophy.

1.5 Thesis Objective and Hypotheses

In an effort to fill important knowledge gaps outlined in the previous sections, this thesis includes three novel research studies, all of which make original contributions to knowledge. Through novel combinations of the methods described in this background chapter, each manuscript contributes to the overarching goal of this thesis:

OBJECTIVE

To investigate, using non-invasive tools, the extent to which glutamate and GABA are implicated in neuroradiological and clinical features of multiple sclerosis.

Each study will, in separate and novel ways, address aspects of the following research questions and hypotheses:

Question 1

To what extent are TMS-based markers of intracortical activity (ICF, SICI, CSP, LICI) or ^{1}H-MRS estimates of neurometabolite levels (glutamate, GABA) different between specific clinically-defined subgroups of people with multiple sclerosis and healthy individuals?

> ***Hypothesis:*** Clinically-defined subgroups of people with multiple sclerosis (e.g. those with motor impairment; those with progressively worsening disability) have differences in these TMS- or ^{1}H-MRS-derived outcomes as compared to other patient sub-groups (e.g. those with preserved motor function; those with a relapsing disease course) or healthy people.

Question 2

Are these TMS- and ^{1}H-MRS-derived variables of interest related to structural damage, as evidenced by neuroimaging metrics (e.g. lesion volume, cortical volume, MTR)?

> ***Hypothesis:*** These TMS- and ^{1}H-MRS-based variables found to be abnormal between groups will also correlate with more severe multiple sclerosis-related structural damage, as evidenced by MRI-based metrics.

Question 3

To what extent are these TMS- and ^{1}H-MRS-derived variables related to clinical disability (e.g. 9HPT performance, EDSS score), including when accounting for MRI evidence of structural damage?

Hypothesis: These TMS- and ^{1}H-MRS-based variables will explain variability between people with multiple sclerosis in clinical impairment severity, particularly motor disability, beyond that of MRI-based markers of structural damage.

Study-specific metrics and clinical sub-groups of focus for each of the three manuscripts of the thesis will be described within their respective chapters. In **Chapter 5**, these overarching research questions will be directly revisited to reflect on how the studies of this thesis have, together, provided answers and insights.

Chapter 2

Intracortical inhibition during remission

Preface

As a primary investigation to address the overarching research questions of this thesis, presented in the preceding chapter, two TMS-based markers of intracortical inhibition (SICI and CSP) were investigated among people with relapsing-remitting multiple sclerosis and matched healthy volunteers. The multiple sclerosis participants were divided into those who experienced upper limb motor impairment that persisted into the stable remission phase, and those with preserved motor function. Abnormalities in intracortical inhibition between these two clinically-defined multiple sclerosis sub-groups and controls were investigated to address *Question 1*. To answer *Question 2*, relationships between intracortical inhibition and regional neuroimaging markers of lesions and cortical integrity were examined. Finally, *Question 3* was addressed through the use of multivariate regression models, which aimed to investigate relationships between intracortical inhibition and clinical impairments when accounting for evidence of brain damage.

[†] The manuscript presented in this chapter is published in: Nantes JC, Zhong J, Holmes SA, Whatley B, Narayanan S, Lapierre Y, Arnold DL, & Koski L (2016). **Intracortical inhibition abnormality during the remission phase of multiple sclerosis is related to upper limb dexterity and lesions.** *Clinical Neurophysiology,*127(2): 1503-11.

2.1 Abstract

Objective. The impact of inhibitory cortical activity on motor impairment of people with relapsing-remitting multiple sclerosis (RRMS) has not been fully elucidated despite its relevance to neurorehabilitation. The present study assessed the extent to which transcranial magnetic stimulation (TMS)-based metrics of intracortical inhibition are related to motor disability and brain damage. **Methods.** Participants included forty-three persons with RRMS in the remitting phase and twenty-nine healthy controls. We stimulated the dominant hemisphere and recorded from the dominant hand to assess short-interval intracortical inhibition (SICI) and cortical silent period (CSP) duration. Disability was evaluated with the Multiple Sclerosis Functional Composite (MSFC). Regional cortical thickness and lesion volume were measured. **Results.** RRMS participants with dominant upper limb dexterity impairments had prolonged CSP, but equivalent SICI, compared to participants with preserved function. CSP was not related to walking or cognitive performance. Higher normalized lesion volume correlated with longer CSP duration. When adjusting for normalized lesion volume, longer CSP significantly predicted worse dominant upper extremity impairment. **Conclusions.** High intracortical inhibition possibly contributes to (or prevents remission from) motor impairment. Lesions may be associated with intracortical inhibition shifts. **Significance.** CSP duration and lesion burden should be considered when developing interventions aiming to mitigate motor impairment.

2.2 Introduction

Multiple sclerosis is a chronic neuroinflammatory disease that can cause motor impairments, among other debilitating symptoms [186]. For people with the relapsing-remitting course of multiple sclerosis (RRMS), symptoms associated with transient neuroinflammatory events greatly improve during spontaneous clinical remission phases despite the persistence of structural brain damage [17, 187]. Physiological mechanisms contributing to the severity of residual disability present during the remission phases of RRMS have not been fully elucidated.

Symptom recovery after neurological damage may be influenced by the brain's main inhibitory neurotransmitter, γ-aminobutyric acid (GABA), which has a key role in synaptic plasticity and motor learning [3, 188-191]. In humans, intracortical inhibitory activity can be studied non-invasively through a variety of protocols that involve analyzing peripheral electromyographic signals associated with transcranial magnetic stimulation (TMS) of the primary motor cortex (M1). Pharmacological evidence supports that the short-interval intracortical inhibition (SICI) metric is linked to inhibitory activity at ionotropic $GABA_A$ receptors, while the cortical silent period (CSP) primarily reflects metabotropic $GABA_B$ receptor activity [192].

The relationship between these TMS-based metrics and clinical manifestations of multiple sclerosis is not fully known. While CSP prolongation (indicating higher intracortical inhibition) has been reported to occur during the clinical remission phase of RRMS [65], it is not clear if this alteration is related to the preservation or impairment of function. As divergent areas of research support that plasticity and motor learning favor a low inhibitory state [3, 193, 194], it could be hypothesized that higher intracortical inhibition during remission is linked to more

severe persisting impairment. However, the reverse could also be true, as intracortical inhibition deficits are common during relapses [65] and among individuals with the later, secondary progressive form of multiple sclerosis [66, 67]. Moreover, it has not been directly assessed if intracortical inhibition is related specifically to impairment of the limb contralateral to the TMS stimulation site, as opposed to other neurological symptoms of this complex disease.

Of further consideration is the impact of brain damage on TMS-based outcomes. Conte et al. [66] reported that SICI of people with multiple sclerosis is not correlated with lesion load within the whole brain nor within the intracranial cortico-spinal tract (CST_i). While not previously assessed among people with multiple sclerosis, it is possible that neuroimaging analysis techniques that estimate atrophy and lesion impact near the stimulated cortex may provide metrics of damage relevant to intracortical inhibition abnormities. Understanding how inhibitory cortical activity may interact with brain damage to produce (or prevent) motor disability may support the development of optimal tools to assess disease burden and to treat symptoms of people with neurological conditions such as RRMS.

The primary objective of this study was to assess the extent to which TMS markers of intracortical inhibition (SICI, CSP) are abnormal among people with RRMS in remission that have upper limb dexterity impairments when compared to those with preserved upper limb function. Secondly, we investigated the specificity of the identified neurophysiological abnormalities in predicting poor performance of the limb contralateral to the TMS stimulation site compared to other types of disability. The relationship between intracortical inhibition and structural brain damage (as measured throughout the whole brain and near the stimulation site) was also investigated. We predicted that among RRMS participants, intracortical inhibition would be related to upper limb disability, as well as to damage around the cortical region

stimulated. Our additional multimodal analysis explored whether intracortical inhibition is related to disability independently of brain damage measured with MRI.

2.3 Methods

Participants

A random selection process was used to recruit people with RRMS from a clinical research database at the Montreal Neurological Institute and Hospital in Canada. Age- and sex-matched healthy control (HC) participants were recruited through advertising posters in the community. Age, sex, time since diagnosis, date of most recent relapse, medications, and Expanded Disability Status Scale (EDSS) score were extracted from the clinical database for RRMS participants, and applicable variables for HC participants were self-reported. People were not invited to participate if they: (1) had risk factors for undergoing TMS or MRI (e.g. medications lowering seizure threshold, history of seizure, pregnancy, ferromagnetic metal in body), (2) were taking medications known to affect intracortical inhibition (e.g. baclofen), (3) had a pre-existing health condition not attributed to MS (e.g. bipolar disorder, limb amputation), (4) had experienced a clinically-significant relapse within the three months prior to participation, or (5) were left-handed. Of people who took part in the study, two HC participants were excluded for abnormally poor motor performance (>2 standard deviations worse than published norms [195]). Two participants (1 RRMS, 1 HC) did not complete the study because their resting motor threshold was too high to assess SICI or CSP with our protocol. All participants provided informed consent. The Research Ethics Board at the Montreal Neurological Institute and

Hospital in Canada approved this study. The final sample included 29 HC and 43 RRMS participants.

Multiple Sclerosis Functional Composite

The Multiple Sclerosis Functional Composite (MSFC) [196], an assessment of performance across functional domains that has been validated among people with MS [165], was used to measure disability. The MSFC consists of three subscales: Timed 25-foot walk (T25FW), 9-hole peg test (9HPT), and the 3 second version of the Paced Auditory Serial Addition Test (PASAT), which measure leg function/ambulation, hand/arm dexterity, and cognitive function, respectively.

Neurophysiological assessments

TMS pulses were delivered with a Magstim 200^2 stimulator and figure-of-8 coil (outer wing diameter = 9.5cm) held against the head (left hemisphere) at a 45-degree angle to the sagittal plane (handle oriented posteriorly). Electromyographic data was collected with surface electrodes placed in a belly-tendon montage on the dominant hand (contralateral to the TMS stimulation site), with the recording electrode over the first dorsal interosseus (FDI) muscle. Data was amplified and filtered (bandwidth = 10-3000 Hz, Grass P511 AC Amplifiers) and collected at a sampling rate of 6 kHz. Using BrainSight 2 stereotaxic navigation software (Rogue Research Inc), the optimal target site to elicit an MEP from the target FDI was identified [197], marked, and referenced for all further stimulations. Resting motor threshold (RMT) was defined as the

lowest intensity of stimulation required to induce a motor evoked potential (MEP) of at least 50μV in 5 of 10 trials in the target FDI [40]. TMS data were analyzed semi-automatically with a MATLAB (Mathworks, MA, USA) analysis tool (dataWizard, version 0.7.7, A.D. Wu, UCLA) and manually edited by a researcher blinded to group membership.

The paired-pulse method (used to determine SICI) [38] required a second stimulator connected through a Bistim module. A conditioning stimulus (80% of RMT) was delivered, followed by the test stimulus (120% of RMT) at randomly ordered inter-stimulus intervals of 1, 2, and 3 ms. Single-pulse test stimuli at 120% RMT (with no conditioning stimulus) were interspersed throughout the paired-pulse procedure. For each interstimulus interval and for the single-pulse condition, eight MEPs were obtained and peak-to-peak amplitude was measured. For each paired-pulse interstimulus interval, SICI was expressed as (1 - (mean paired-pulse MEP amplitude / mean single-pulse MEP amplitude) x 100%) [184], such that higher SICI values would reflect a greater percentage of inhibition relative to the single-pulse MEP.

For the contralateral cortical silent period (CSP) technique [198] a single suprathreshold stimulus (120% of RMT) was delivered while the FDI muscle was voluntarily contracted at 40% of maximum voluntary pinch strength (determined by feedback from a Preston pinch gauge (Sammons Preston, Illinois, USA)), over 10 trials. Trials deemed invalid due to error in muscle contraction (monitored by a second researcher who was not conducting the stimulations) were repeated to ensure accuracy. For visual assessment of the CSP, the electromyographic traces of all valid trials were rectified, averaged, and highly magnified. The minimal absolute CSP duration was measured from the end of the MEP until the earliest onset of the contracted muscle EMG activity return [65, 199] (see Figure 2.1). To confirm that our main result did not rely solely on the conservative definition of the CSP chosen a priori, we performed additional

analyses using alternative definitions of CSP duration, including: (1) CSP end point defined as the EMG potential returning to 50μV on the average rectified trace, and (2) MEP onset used as the CSP start point.

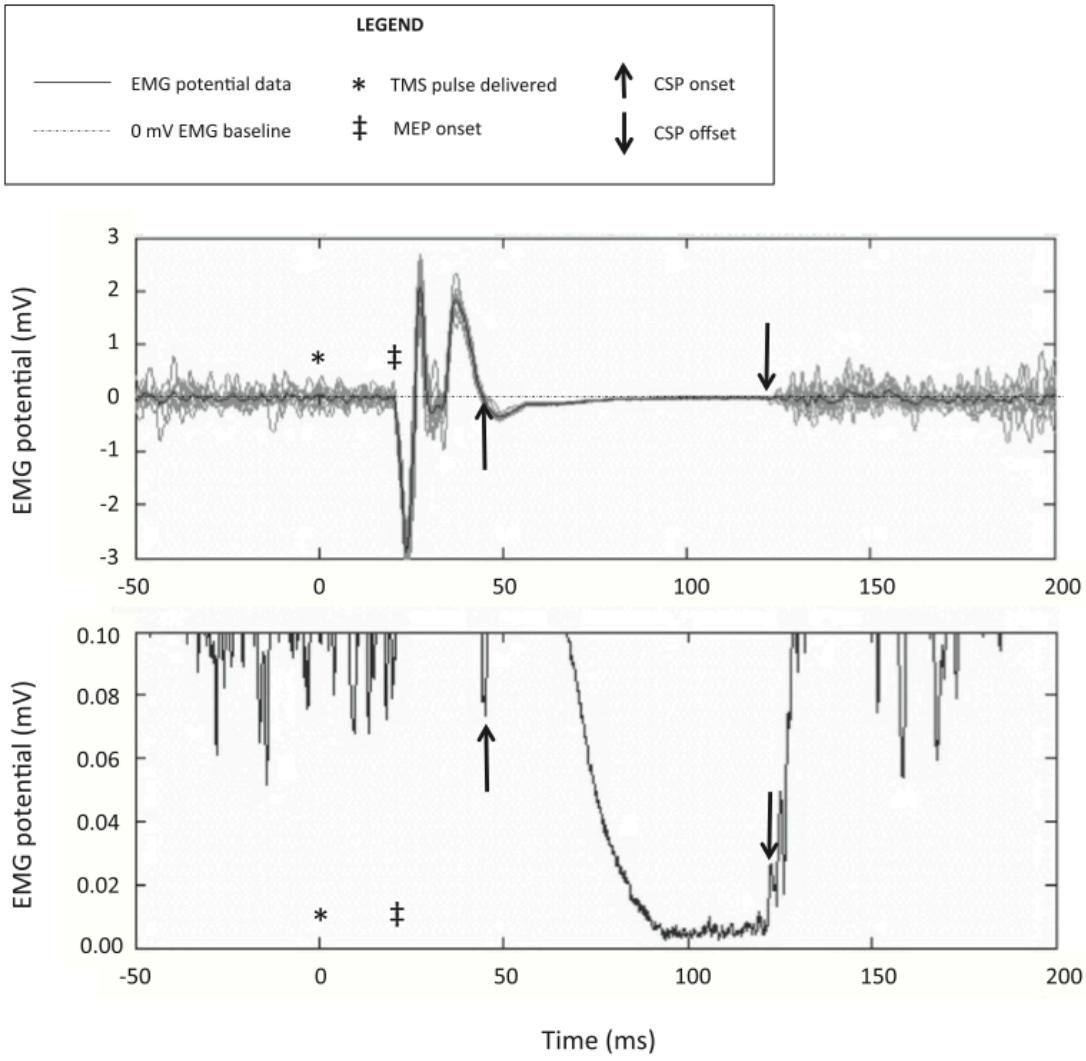

FIGURE 2.1 Example of CSP data from a participant with RRMS. The asterisk indicates the time point the TMS pulse was delivered, and the double dagger indicates the MEP onset location. The un-rectified average trace with raw data superimposed (upper panel), was used to assist identification of the MEP end point at the cross point of the EMG potential baseline. The rectified average trace, viewed at a high level of magnification (lower panel) was used to identify the earliest time point where contracted muscle EMG activity began to return to baseline. Upward and downward pointing arrows indicate the start and end points of the absolute CSP, respectively.

MEPs obtained during single pulse stimulations were measured for peak-to-peak amplitude and onset time to measure average MEP amplitude and latency, respectively. This was assessed separately for data collected from relaxed muscles and from active muscles (40% of maximum voluntary contraction).

Neuroimaging data collection and analysis

A subset of participants (15 HC, 38 RRMS) took part in the additional magnetic resonance imaging (MRI) protocol within three weeks of TMS and MSFC assessment. Neuroimaging was performed at the Montreal Neurological Institute and Hospital (Siemens TIM Trio scanner, 3 Tesla) using a 32-channel head coil[‡]. The protocol included acquisition of: (1) T1-weighted 3D fast low-angle shot sequence (repetition time (TR) = 20 ms, echo time (TE) = 5 ms, field of view (FOV) = 256 mm, number of slices = 192, slice thickness = 1 mm), (2) T2-weighted 3D fluid-attenuated inversion recovery (FLAIR) images turbo echo spin sequence (TR = 6000 ms, TE = 355 ms, FOV = 256 mm, slices number = 192, slice thickness = 1 mm), (3) proton density/T2-weighted dual spin echo sequence (TR = 2100 ms, TE = 17/76 ms, FOV = 256 mm, slices number = 60, slice thickness = 3 mm). Scans were co-registered during the preprocessing steps and coded to ensure blinding for imaging analysis steps.

T1-weighted images were processed in FreeSurfer (version 5.1.0, http://surfer.nmr.mgh.harvard.edu/), a validated semi-automated segmentation system allowing for the study of specific neuroanatomical regions [127, 200]. M1 of the left and right hemispheres were defined based on the precentral gyrus labels created with FreeSurfer. Based on

[‡] See published corrigendum in Appendix II

FreeSurfer segmentation, cortical thickness (within the whole brain and within each M1) was measured.

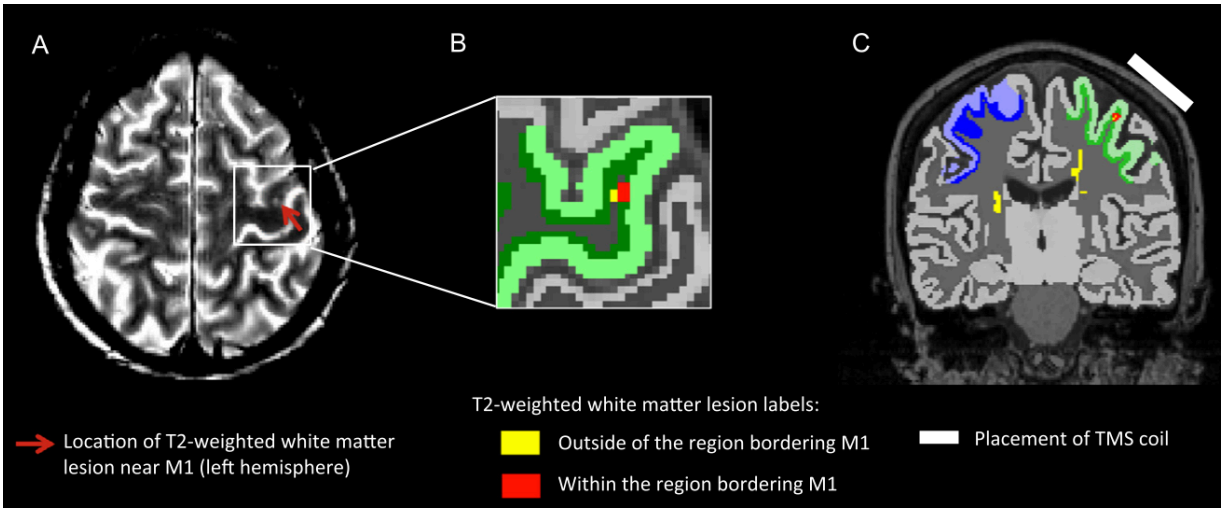

FIGURE 2.2 Example of a participant with RRMS who had a T2-weighted white matter lesion that bordered M1 of the left hemisphere. (A) A T2-weighted image of a RRMS participant with a white matter lesion identified near M1 of the left hemisphere is shown. (B, C) T1-weighted images with overlaying anatomical labels are shown. Grey matter labels (excluding M1) are shown in light grey. Light blue and light green indicate M1 of the right and left hemispheres, respectively. The dilated masks for M1 are shown in darker blue and darker green. A zoomed-in section from (A) is shown in (B). An example of the placement of the TMS coil relative to the participant's brain is also shown in (C).

T2-weighted lesions within the white matter were detected by a semi-automated lesion-detection software system [201] and manually corrected by a researcher who had been trained by a neuroradiologist. The subsequent analysis was designed to assess the amount of lesioned white matter in the region juxtacortical to M1 of the stimulated and non-stimulated hemispheres. Using the image dilation function in FSL [202], M1 masks were dilated by one voxel in each direction to define the region within the white matter that bordered the M1 cortical regions. The volume of

the white matter lesions that overlapped with the dilated M1 labels was measured (see Figure 2.2). Lesion volume within the CST_i of the left and right hemispheres (defined using the John Hopkins University diffusion tensor imaging-based white-matter atlas [203], which had been transformed into each participant's native space using ANTs [204]) was also measured. To produce inter-subject-comparable measures, the scaling factor generated from SienaX [129] was applied to all lesion volumes.

Statistical Analysis

9HPT completion times for the dominant upper limb were converted into z-scores based on a comparison to the performance of healthy participants. RRMS participants who performed within two standard deviations (SD) of the healthy participants' mean time were placed in the *preserved function* (RRMS-P) group, and those performing slower than two SD of the healthy participants' time were placed into the *impaired function* (RRMS-I) group.

For all statistical tests, data were analyzed with SPSS and considered statistically significant at $p < 0.05$.

Group analyses: Descriptive analyses were conducted to characterize the sample on all demographic, clinical, neurophysiological, and MRI-based variables. Chi-squared tests were used to compare groups on categorical variables including sex and use of immunomodulatory medication. To compare RRMS-P and RRMS-I groups on disease duration and EDSS score, Mann-Whitney U tests were used. The Shapiro-Wilk test of normality was conducted for each continuous variable. One-way Analysis of Variance (ANOVA) was used to compare HC, RRMS-P and RRMS-I participants for differences on normally distributed variables, followed by post-hoc Tukey t-tests tests. Non-normally distributed variables were compared with a Kruskall-

Wallis H test, followed by a post-hoc Mann-Whitney U tests as appropriate. A Bonferroni correction was used to account for the number of group comparisons for each post-hoc test.

Correlational analyses: As the goal of the study was to assess the extent to which abnormalities in TMS-based intracortical inhibition metrics are related to clinical disability and brain damage, only intracortical inhibition variables found to differ from HC participants in the group analyses were included in the subsequent correlational analyses. Spearman rank correlations were used to evaluate the relationships between: (1) TMS-based intracortical inhibition metrics and clinical outcomes, and (2) TMS-based intracortical inhibition metrics and MRI outcomes. We also assessed if EDSS score, disease duration, and MEP latency were similarly related to the neurophysiological and neuroimaging outcomes of interest.

Regression: We performed a series of multiple linear regression analyses to assess if TMS-based intracortical inhibition outcomes predicted disability independently of potentially confounding MRI-based or neurophysiological variables. Negative reciprocal transformations were applied to the 9HPT, EDSS and lesion volume data to ensure that assumptions of linear regression analysis were not violated due to non-normally distributed residuals.

2.4 Results

Clinical and demographic outcomes

Table 2.1 outlines the demographic and clinical characteristics of participants. Differences in age ($F_{2,71} = 1.01$, $p = 0.37$) and sex ($\chi^2_{(2)} = 3.51$, $p = 0.17$) were not statistically significant. RRMS-P and RRMS-I groups did not differ in disease duration ($U = 138$, $p = 0.87$) or in the

proportion of individuals taking immunomodulatory medications to treat MS ($\chi^2_{(1)} = 1.59$, $p = 0.21$).

TABLE 2.1 Demographic characteristics of participants.

	HC	RRMS-P	RRMS-I
Full Sample			
Total number of subjects	29	30	13
Women (n (%))	21 (72)	22 (73)	6 (46)
Taking immunomodulatory medication for MS (n (%))	0 (0)	17 (57)	10 (77)
Age, *years* (mean ± SD)	45.0 ± 13.0	42.9 ± 11.0	48.6 ± 12.6
Disease duration[a], *years* (median (Q1, Q3))	–	8.9 (3.2, 12.4)	8.3 (5.7, 11.6)
EDSS, *score* (median (Q1, Q3))	–	1.8 (1.0, 2.4)	3.0 (2.0, 3.5)
MSFC, *score* (mean ± SD)	0.65 ± 0.28	0.46 ± 0.41	-0.38 ± 0.59
MRI subgroup			
Total number of subjects	15	26	12
Women (n (%))	12 (80)	19 (73)	6 (50)
Taking immunomodulatory medication for MS (n (%))	0 (0)	16 (62)	10 (83)
Age, *years* (mean ± SD)	45.9 ± 15.3	41.5 ± 10.5	48.9 ± 13.2
Disease duration[a], *years* (median (Q1, Q3))	–	7.1 (2.8, 11.3)	8.0 (4.9, 10.4)
EDSS, *score* (median (Q1, Q3))	–	1.5 (1.0, 2.4)	2.5 (2.0, 3.5)
MSFC, *score* (mean ± SD)	0.72 ± 0.31	0.49 ± 0.40	-0.38 ± 0.62

EDSS = Expanded Disability Status Scale; MSFC = Multiple Sclerosis Functional Composite;
[a] Missing data from three RRMS-P and two RRMS-I participants

Outcomes of MSFC subscale scores, including significance of post-hoc assessments, are shown in Table 2.2. The T25FW test was not measured for one HC and two RRMS-P participants, because they did not return within 3 weeks for the subsequent session. Groups differed in 9HPT score (for both the dominant ($F_{2,71} = 73.6$, $p < 0.001$) and non-dominant hands

($\chi^2_{(2)} = 23.1$, $p < 0.001$), T25FW time ($\chi^2_{(2)} = 13.3$, $p = 0.001$), PASAT score ($\chi^2_{(2)} = 11.8$, $p = 0.003$), and MSFC score ($\chi^2_{(2)} = 25.1$, $p < 0.001$). The basis by which the groups were defined was confirmed, as dominant hand 9HPT time was longer for RRMS-I participants than RRMS-P participants, while HC and RRMS-P groups did not differ significantly. RRMS-I participants also performed worse on the non-dominant hand 9HPT and PASAT compared to RRMS-P and HC participants. For the T25FW, RRMS-P and RRMS-I groups did not differ, although both performed worse than controls. EDSS score was higher for RRMS-I participants compared to RRMS-P participants ($U = 188$, $p = 0.04$).

Neurophysiological outcomes

Outcomes of TMS-based measures assessed during muscle relaxation are shown in Table 2.2. The groups did not differ significantly in RMT ($\chi^2_{(2)} = 5.2$, $p = 0.08$), nor SICI at any of the inter-stimulus intervals (all $\chi^2_{(2)} < 2.3$, $ps > 0.05$). Group differences were however found for MEP amplitude ($\chi^2_{(2)} = 11.6$, $p = 0.003$) and MEP latency ($F_{2,71} = 13.2$, $p < 0.001$) of resting muscles, with RRMS-I participants having lower amplitudes and longer latencies than both HC and RRMS-P participants.

TABLE 2.2 Comparisons between HC and RRMS participants with preserved and impaired motor function.

	HC	RRMS-P	RRMS-I	HC vs. RRMS-P	HC vs. RRMS-I	RRMS-P vs. RRMS-I
Performance	**Median (Q1, Q3)**	**Median (Q1, Q3)**	**Median (Q1, Q3)**	**p**	**p**	**p**
9-HPT dominant hand time (s)	17.6 (17.0, 18.4)	19.0 (18.1, 19.9)	25.0 (22.9, 27.3)	–	‡‡‡	***
9-HPT non-dominant hand time (s)	18.6 (17.4, 19.7)	19.9 (19.1, 21.3)	23.1 (22.3, 28.9)	–	‡‡‡	**
T25FW time (s)	3.6 (3.3, 4.3)	4.5 (3.7, 4.8)	4.8 (4.1, 6.8)	†	‡	–
PASAT Score	52 (45, 55)	51 (39, 55)	35 (24, 44)	–	‡‡	**
Maximal pinch strength (kg)	5.4 (4.5, 6.1)	6.1 (4.1, 6.8)	5.9 (3.6, 6.8)	–	–	–
TMS – resting muscle conditions	**Median (Q1, Q3)**	**Median (Q1, Q3)**	**Median (Q1, Q3)**			
RMT (%)	42 (39, 47)	46 (39, 49)	51 (46, 53)	–	–	–
Single pulse MEP amplitude (mV)	0.71 (0.47, 1.54)	0.67 (0.38, 1.22)	0.33 (0.20, 0.42)	–	‡‡	*
MEP latency (ms)	22.0 (21.6, 23.2)	22.9 (22.3, 23.6)	24.9 (24.8, 26.0)	–	‡‡‡	***
SICI – 1 ms ISI (%)	76.8 (59.3, 88.1)	68.1 (56.2, 78.7)	68.1 (57.4, 74.3)	–	–	–
SICI – 2 ms ISI (%)	76.2 (45.8, 79.5)	66.2 (46.3, 77.7)	65.0 (44.2, 77.6)	–	–	–
SICI – 3 ms ISI (%)	71.0 (55.7, 79.6)	63.7 (42.0, 74.5)	65.5 (43.4, 73.6)	–	–	–
Normalized T2w white matter lesion volumes (mm^3)	**Median (Q1, Q3)**	**Median (Q1, Q3)**	**Median (Q1, Q3)**	**p**	**p**	**p**
Whole Brain	0 (0,0)	5341 (2157, 10058)	20357 (5656, 25115)	†††	‡‡‡	*
CST_i						
Left hemisphere	0 (0,0)	119 (0, 429)	500 (95, 1135)	†††	‡‡‡	–
Right hemisphere	0 (0,0)	145 (43, 248)	180 (55, 590)	†††	‡‡‡	–
Bordering M1						
Left hemisphere	0 (0,0)	0 (0, 24)	30 (16, 144)	††	‡‡‡	*
Right hemisphere	0 (0,0)	6 (0, 45)	34 (7, 198)	††	‡‡‡	–
Cortical thickness (mm)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**p**	**p**	**p**
Total cortex	2.58 (0.08)	2.51 (0.11)	2.41 (0.16)	–	‡‡	*
M1						
Left hemisphere	2.68 (0.18)	2.70 (0.16)	2.52 (0.33)	–	–	*
Right hemisphere	2.75 (0.15)	2.69 (0.16)	2.50 (0.43)	–	‡	–

Symbols indicate a significant differences identified from the post-hoc assessment comparing: HC and RRMS-P († $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$), HC and RRMS-I (‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$), and RRMS-P and RRMS-I (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Dashes (–) indicate non-significant outcomes ($p > 0.05$). SD, Q1, Q3 indicate standard deviation, lower quartile, and upper quartile, respectively.

While no between-group differences were found for maximal pinch strength ($F_{2,71} = 0.50$, $p > 0.05$) nor average MEP amplitude of a contracted muscle ($F_{2,71} = 0.84$, $p > 0.05$), between-group differences were found in MEP latency during muscle contraction ($F_{2,71} = 0.84$, $p > 0.05$) and CSP duration ($\chi^2_{(2)} = 9.02$, $p = 0.01$), with the RRMS-I group having significantly longer latencies and CSP duration than the other groups (Figure 2.3). These between-group differences in CSP duration remained significant even after removing the youngest females from the HC (n = 2) and RRMS-P (n = 3) groups, which minimized the trending differences in age and sex between groups.

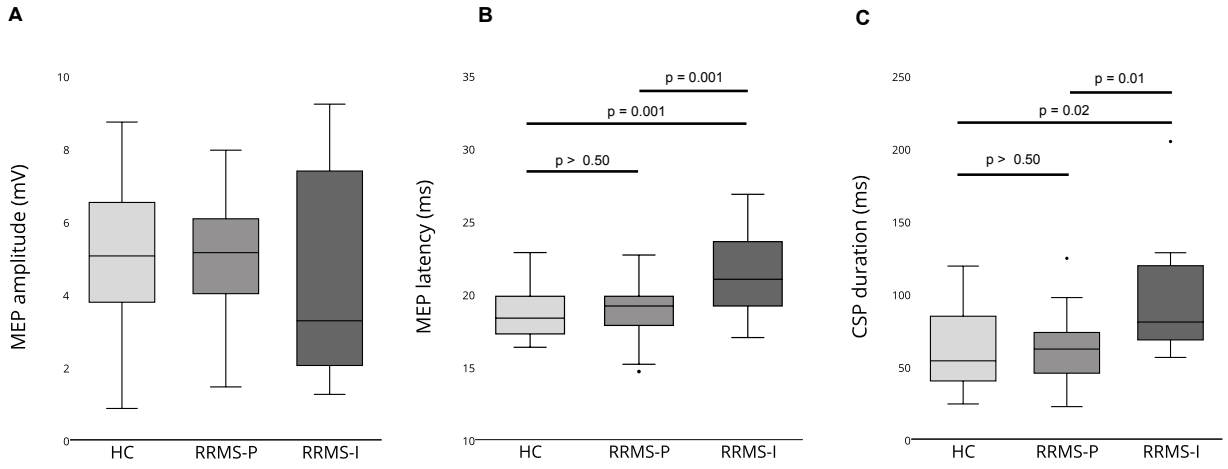

FIGURE 2.3 Outcomes of TMS-evoked electromyographic data collected during muscle contraction. When TMS was performed during muscle contraction, MEP amplitude **(A)** did not differ significantly between groups, although MEP latency **(B)** and CSP duration **(C)** were longer among RRMS-I participants compared to both other groups. Significance of non-parametric two-tailed post-hoc tests (corrected for multiple comparisons) is shown for the MEP latency and CSP duration data.

Confirming this result was not unique to the conservative definition of the CSP duration chosen a priori for the present study, we found that the observed between-group differences in

CSP duration remained significant when measuring the absolute CSP duration with the alternative, 50μV threshold-based definition of the CSP endpoint (HC: 68 ± 28ms, RRMS-P: 69 ±26ms, RRMS-I: 101 ±43ms), as well as when measuring the relative CSP beginning at the MEP onset (HC: 94 ± 29ms, RRMS-P: 96 ±28ms, RRMS-I: 130 ±43ms) (both $\chi^2_{(2)} < 8.6$, ps < 0.05).

The full sample of RRMS participants (RRMS-P and RRMS-I groups combined) did not differ from HC participants in age ($t_{(70)} = 0.12$, p = 0.91) or sex ($\chi^2 = 0.42$, p = 0.52). CSP duration of RRMS participants was positively correlated with 9HPT performance of the dominant upper limb ($r_{s(42)} = 0.40$, p = 0.04), but not the non-dominant upper limb ($r_{s(42)} = 0.18$, p > 0.05). Furthermore, CSP duration of MS participants was not correlated with walking speed, PASAT score, total MSFC score, or disease duration (ps > 0.05), but was modestly correlated with EDSS ($r_{s(42)} = 0.36$, p = 0.02). MEP latency during active muscle contraction correlated with longer 9HPT performance with either hand, as well as MSFC total score ($r_{s(42)} > 0.32$, ps < 0.05), but not other variables (ps > 0.05). None of the performance-based variables were significantly correlated with CSP duration or MEP latency among HC participants (ps > 0.05). A model with both CSP and MEP latency as predictors of dominant hand 9HPT performance was significant ($R^2 = 0.22$, $F_{2,42} = 5.65$, p = 0.007), with CSP significantly predicting performance (β = 0.36, p = 0.013) independently of MEP latency (β = 0.27, p = 0.063).

Neuroimaging outcomes

Demographic characteristics of participants who took part in the MRI component of the study are shown in Table 2.1, and group comparisons of neuroimaging outcomes are summarized in Table 2.2. Group differences in age ($F_{2,52} = 1.57$, p = 0.23), sex ($\chi^2_{(2)} = 3.11$, p = 0.21), disease

duration (U = 105, p = 0.70), and prevalence of participants taking immunomodulatory medications for MS ($\chi^2_{(1)}$ = 1.81, p = 0.18) remained non-significant when comparing participants of the subgroup. Group differences in cortical thickness occurred within the whole brain ($F_{2,52}$ = 7.19, p = 0.002), left hemisphere ($F_{2,52}$ = 3.23, p = 0.048), and right hemisphere M1 ($F_{2,52}$ = 3.68, p = 0.032). Differences were also found for normalized volume of T2-weighted white matter lesions within the whole brain, within the CST_i and in the regions bordering M1 of the left and right hemispheres (all $\chi^2_{(2)} > 19$, ps < 0.001).

Cortical thickness was lower among RRMS-I participants compared to RRMS-P participants within the whole brain and within M1 of the left hemisphere, but not within M1 of the right hemisphere. Compared to RRMS-P participants, RRMS-I participants had significantly higher normalized volumes for T2-weighted white matter lesions within the whole brain, CST_i of the left hemisphere, and CST_i of the right hemisphere. RRMS-I participants also had higher normalized volume of lesions in the region bordering M1 of the left and right hemispheres. EDSS score correlated with whole brain normalized lesion volume ($r_{s(37)}$ = 0.40 , ps = 0.013), but not with any of the other MRI-based outcomes (ps > 0.05).

In Table 2.3, the relationships between the neuroimaging outcomes and CSP duration are shown. Among the RRMS participants, higher normalized lesion volume (both within the whole brain and in the region bordering the left M1) was positively correlated with longer CSP after correcting for multiple comparisons, while no other significant relationships were found. By contrast, MEP latency was not significantly correlated with any of the MRI-based outcomes (ps > 0.05).

TABLE 2.3 Relationships between MRI outcomes and CSP duration.

Predictor	HC	RRMS
Cortical thickness (mm)		
Whole brain	-0.42	-0.22
M1		
Left hemisphere	-0.14	-0.32
Right hemisphere	-0.24	-0.24
T2w white matter lesion volume (mm^3)		
Whole brain	–	**0.51****
CST_i		
Left hemisphere	–	0.43
Right hemisphere	–	0.12
Bordering M1		
Left hemisphere	–	**0.44***
Right hemisphere	–	0.12

The correlation coefficient from Spearman's rank analysis is shown. Asterisks indicate 2-tailed significance after correcting for multiple comparisons (* $p < 0.05$, ** $p < 0.01$).

Multiple linear regression models including both CSP and normalized lesion volume as predictors of dominant hand 9HPT performance were significant when assessing lesion volume within the entire brain ($R^2 = 0.17$, $F_{2,37} = 3.55$, $p = 0.039$), as well as well as within the region bordering the left hemisphere M1 ($R^2 = 0.18$, $F_{2,37} = 3.94$, $p = 0.029$). In both models, CSP was found to be a significant predictor of 9HPT performance ($\beta s > 0.38$, $ps < 0.05$) independently of lesion volume ($\beta s < 0.14$, $ps > 0.05$). However, CSP did not predict EDSS independently of lesion volume in either region ($ps > 0.05$).

2.5 Discussion

The results of this study implicate CSP lengthening in specific manifestations of motor impairment present during remission phases of RRMS, and suggest that this neurophysiological irregularity is partially associated with lesion burden.

Intracortical inhibition and disability

The present study investigated the clinical implications of intracortical inhibition abnormalities in people with RRMS during the relatively stable remission phase. To obtain biomarkers of intracortical inhibition, we stimulated the dominant hemisphere and analyzed recordings from an electrode over a muscle on the contralateral (dominant) hand. On average, RRMS participants with impaired dominant upper extremity motor function had normal SICI, but abnormally long CSP duration. By contrast, RRMS participants with preserved motor abilities did not differ from healthy controls in either outcome. We further assessed the clinical specificity of this neurophysiological abnormality, finding that longer CSP was correlated with 9HPT performance of the dominant upper limb, while CSP did not correlate with non-dominant 9HPT completion time, walking speed, nor cognitive performance of RRMS participants.

Similar to the present study, others have reported that, outside of relapse, SICI is normal among people with RRMS [66, 67], while CSP prolongation has been reported among people with RRMS in remission [65] and among those with clinically isolated syndrome who later develop multiple sclerosis [205]. Our results further demonstrate that among remitting RRMS individuals, longer CSP is related to more severe upper extremity disability. This finding is consistent with studies of people in remission from a stroke, which support that higher GABA

impedes motor recovery [191, 206, 207], potentially by interfering with synaptic plasticity and motor learning [3, 193, 194, 208]. Nonetheless, the present study's results may be surprising considering that low intracortical inhibition has been reported during relapses [65] as well as during the later, secondary progressive stage of multiple sclerosis [66, 67]. Potentially, disability associated with CSP prolongation during remission could be related to a physiological mechanism that is largely distinct from intracortical inhibition deficits occurring in other disease phases.

Considering the previously described responses of SICI and CSP to specific neurotransmitter receptor agonists/antagonists and the temporal characteristics of these signals [192], a possible interpretation of the present result is a circumscribed increase in the long-lasting activity of $GABA_B$ receptors, as opposed to the faster-acting, and shorter-lived $GABA_A$ receptor activity, among individuals with dominant limb dexterity impairment. Alternatively, CSP prolongation in the absence of SICI change may be associated with neurophysiological alterations related to voluntary motor drive [209], motor attention [192, 210], or spinal mechanisms [59, 60], which could involve GABAergic activity directly or indirectly. While it is possible that factors other than inhibitory neurotransmission may contribute to CSP duration alterations, the present results are consistent with findings from a magnetic resonance spectroscopy study on people with RRMS by Bhattacharyya *et al.* (2013) [96], which found higher GABA concentration within a sensorimotor brain volume of the dominant hemisphere to predict worse performance on the 9HPT, but not to predict walking speed nor PASAT performance. Together with the present study, this work implicates a role for GABA associated with the dominant hemisphere motor region of people with RRMS in potentially mediating (or precluding recovery from) dominant upper limb dexterity impairment. Further investigation of

this mechanism is warranted, including assessing other forms of inhibition that can be measured with TMS (e.g. long-interval intracortical inhibition, ipsilateral cortical silent period).

Impact of lesion burden

Our subsequent neuroimaging analysis investigated possible physical origins of CSP prolongation observed among the motor-impaired individuals. To our knowledge, this is the first study to identify a significant relationship between MRI-based outcomes and TMS measures of intracortical inhibition among people with multiple sclerosis. Higher normalized volume of lesioned white matter within the whole brain and within the region bordering M1 of the stimulated hemisphere (but not the non-stimulated hemisphere) correlated with longer CSP duration.

Volume of white matter lesions in the region near the stimulated cortex was found to be more strongly related to CSP duration compared to lesions in the parallel region of the opposite hemisphere. One caveat, however, is that juxtacortical lesions are often located close to lesioned grey matter tissue, to which conventional structural MRI techniques are mostly blind [211]. Thus, we cannot be certain if the relationship between lesions bordering M1 of the stimulated cortex with CSP was due to structural abnormalities within the white matter pathways leading to the cortex, or instead a direct consequence of cortical damage. Non-conventional imaging sequences (e.g. magnetization transfer, double-inversion recovery) are more sensitive to cortical damage than conventional MRI techniques [211, 212], and could thus help to elucidate this in future work. However, even the most sophisticated in vivo neuroimaging techniques cannot

detect a large proportion of grey matter lesions known to exist based on postmortem histology [213, 214].

Interestingly, it has been proposed in the stroke literature that CSP prolongation may be the result of the deafferentation of M1 due to lesions in various remote brain regions connected with the targeted cortical area, rather than to damage within the motor cortical region stimulated [215, 216]. As the present study also found higher total lesion volume to be correlated with longer CSP, it is possible that this holds true in multiple sclerosis. Since CSP was not significantly associated with lesions within the CST_i after correcting for multiple comparisons, it is possible that such deafferentation was more greatly influenced by damage within cortico-cortical, rather than cortico-spinal, pathways within the brain.

The interpretation that CSP prolongation is a direct consequence of structural brain damage does not, however, explain why CSP duration was not related to cortical thickness. While one possibility is that lesion volume is a more sensitive metric of structural damage relevant to CSP duration compared to cortical thickness, it should be noted that T2-weighted lesions are non-specific indicators of brain tissue that has been affected by demyelination, cell loss, inflammation, ischemia, edema or gliosis [116, 217]. Therefore, increases of any, or a combination, of these factors may have contributed to CSP prolongation. Interestingly, molecular immunology studies and research with animal models of neuroinflammatory disease have shown GABA to have immunosuppressant and anti-inflammatory properties [218-221]. Another possibility is that intracortical inhibition could be increased in response to glutamate [222], which has been found to be abnormally high in normal-appearing white matter and acute lesions of people with multiple sclerosis [89, 90]. Further research combining the current protocol with biomarkers of other disease-mediating mechanisms will be required to fully uncover whether

CSP prolongation, and the associated motor impairment, is direct consequence of structural damage, or rather linked to a neural mechanism responding to neuroinflammation or glutamate excitotoxicity.

Multimodal analysis

We further investigated the combined influence of lesion burden and neurophysiological alterations on disability. Longer CSP was found to be a significant predictor of 9HPT performance when adjusting for lesion volume, demonstrating that the link between CSP prolongation and motor impairment is not simply secondary to the impact of lesions. This result further highlights the need to investigate if other disease-related factors cause CSP prolongation in future work.

In contrast to dominant upper extremity motor performance, EDSS score was not related to CSP duration after adjusting for lesion volume. Alongside our finding that CSP duration is not correlated with total MSFC score, this outcome demonstrates the need for caution when interpreting TMS-based data in clinical populations, as certain metrics may be associated with specific symptoms rather than to general clinical decline. Nonetheless, biomarkers of neurophysiological changes related to specific symptoms may be useful in the development of more targeted and individualized therapies.

Conclusions/Implications

The novel findings of this study provide insight into the pathophysiology of upper extremity motor disability, and highlight factors to consider when interpreting TMS data in the

context of complex brain diseases such as multiple sclerosis. Our results warrant further investigation regarding clinical applications of using CSP as a biomarker of disease burden in neuromotor conditions and/or as a target for neuromodulatory therapies aiming to mitigate disability.

Chapter 3

Cortical damage and disability

Preface

In the preceding chapter, CSP, a form of intracortical inhibition abnormally high in some individuals in the remission phase of relapsing-remitting multiple sclerosis, was investigated with respect to its relationships with traditional radiological measures of brain damage and clinical correlates. Building on findings of this initial study, further knowledge gaps are addressed in the present chapter by: (1) including participants with progressive forms of multiple sclerosis, rather than only the relapsing-remitting subtype, (2) investigating markers of net excitatory activity, rather than only that of intracortical inhibition, and (3) analysing data from imaging sequences sensitive to microstructural grey and white matter damage, rather than only including more conventional MRI techniques. These analyses provide additional novel insights into the pathophysiology of motor impairment and highlight factors to consider when interpreting TMS-evoked electromyographic activity in a multi-stage neurological disease, such as multiple sclerosis.

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3.1 Abstract

Background. Multimodal research combining biomarkers of intracortical activity and cortical damage could shed light on pathophysiological and adaptive neural processes related to the clinical severity of neurological conditions such as multiple sclerosis (MS). **Objective.** Among people with relapsing-remitting and progressive forms of MS, we assessed the extent to which transcranial magnetic stimulation (TMS)-based biomarkers of excitatory and inhibitory cortical activity are related to cortical damage and clinical impairment. **Methods.** Participants included 18 healthy individuals and 36 people with MS who had a relapsing-remitting or progressive clinical course. Using TMS, intracortical facilitation (ICF), short-interval intracortical inhibition (SICI), long-interval intracortical inhibition (LICI), and cortical silent period (CSP) were obtained. Cortical volume and cortical magnetization transfer ratio (MTR) were quantified. Disability was assessed with Multiple Sclerosis Functional Composite (MSFC). **Results.** Lower mean MTR within the cerebral cortex correlated with shorter CSP among MS participants with a progressive, but not a relapsing-remitting, clinical course. Within the cortical hand knob region targeted with the TMS, lower MTR was correlated with lower SICI only among individuals with relapsing-remitting MS. Longer CSP, higher ICF, lower cortical MTR, and sex were all independent significant predictors of poor upper extremity motor performance, while only cortical MTR was a significant independent predictor of total MSFC score among people with MS. **Conclusions.** Cortical damage and cortical activity (both inhibitory and excitatory) may contribute to the severity of motor disability experienced by people with MS. When interpreting TMS-based outcomes, cortical integrity, clinical course, and symptom type should be considered.

3.2 Introduction

Multiple sclerosis (MS), a disease affecting the central nervous system, is a major cause of disability worldwide. Most people diagnosed with MS first experience a relapsing-remitting course, involving periods of clinical recovery between symptom relapses [17]. Others experience more continuous clinical deterioration, as can occur from disease onset (primary progressive MS) or after living with relapsing-remitting symptoms for several years (secondary progressive MS) [223]. While immunomodulatory and anti-inflammatory medications that mitigate relapsing-remitting symptoms are now widely available, effective therapeutic options for progressive disability and the associated neurodegeneration are less well established [21].

A potential contributor to neural and glial cell damage in many neurological conditions, including MS, is the over-activity of the brain's main excitatory neurotransmitter, glutamate [98, 224]. This pathophysiological process, known as excitotoxicity, is also influenced indirectly by inhibitory neurotransmission, which is primarily driven by γ-aminobutyric acid (GABA) [225, 226]. In humans, biomarkers of excitatory and inhibitory neurotransmission can be obtained non-invasively by analyzing characteristics of peripheral electromyographic activity evoked by transcranial magnetic stimulation (TMS) of the primary motor cortex [10]. Pharmacological studies involving the administration of neurotransmitter antagonists prior to TMS have linked glutamatergic activity at N-methyl-D-aspartate (NMDA) receptors to the TMS-derived measure of intracortical facilitation (ICF) [43, 44]. TMS-based metrics primarily associated with inhibitory cortical activity include short-interval intracortical inhibition (SICI), long-interval intracortical inhibition (LICI), and cortical silent period (CSP), each of which is believed to capture a relatively distinct aspect of inhibitory neurotransmission [10].

The relevance of excitatory and inhibitory cortical activity to structural brain damage, and ultimately to clinical impairment, is not fully understood. Notably, a disease-modifying therapy found to decrease ICF in people with MS, Fingolimod [227], has been demonstrated in animal models to modulate glutamatergic neurotransmission in addition to having remyelinating and neuroprotective effects [228-230]. Moreover, cortical damage has been suggested to cause the SICI deficits observed in people with secondary progressive MS [66, 67]. As previous studies have been limited to conventional neuroimaging sequences that are nearly blind to intracortical lesions and demyelination [211, 212], links between intracortical damage and TMS-based outcomes remain unclear.

Structural damage within regions that appear normal on T1- and T2-weighted images can, however, be estimated with the magnetization transfer ratio (MTR) imaging technique [231]. MTR reflects the fraction of water bound to macromolecules and has been shown to correlate with the severity of demyelination and axonal loss observed in postmortem histology studies of the MS brain [144, 214, 232]. Compared to relapsing-remitting MS, cortical MTR reduction in progressive forms of MS is more pronounced, and is less strongly co-localized with cortical atrophy [149]. Cortical MTR of people with MS has been linked to more severe disability [147], including motor impairment [148]. Therefore, in combination with TMS and atrophy assessments, MTR could be a powerful tool to elucidate the relationship between cortical damage and intracortical activity associated with the motor system.

Empirical information on cortical integrity could also clarify the meaning of previously reported relationships between intracortical activity and clinical outcomes (e.g. [66, 67, 69, 177, 233]). It could be hypothesized that by combating excitotoxicity, maintaining low excitatory or high inhibitory activity prevents cell damage and, as a result, prevents clinical disability. This is

challenged, however, by evidence that drugs that inhibit glutamatergic transmission have not been found to be clinically beneficial for people with MS [234-236], despite having neuroprotective effects [236]. Evidence that prolonged CSP (indicating high inhibition) is related to motor or cerebellar dysfunction in MS [177, 233], and demonstrations that lower GABA facilitates motor learning and motor symptom recovery [3, 191, 194, 206], further challenges this hypothesis. As excitatory and inhibitory activity and cortical damage may interact to produce, or prevent, clinical symptoms, assessing certain variables in isolation may conceal their contribution to clinical outcomes. A further important consideration regarding interpretation of TMS-based metrics, derived by stimulating the motor cortex, is that they are likely to be more closely related to motor system pathology than to more general disease progression.

Using a novel multimodal approach, we investigated the link between intracortical activity, cortical damage, and motor impairment. Our emphasis on motor dexterity was chosen due to its relevance to the primary motor cortex hand region stimulated in our TMS protocol, as well as its importance to daily activities and quality of life [171, 172]. We predicted that cortical damage would be related to inhibitory (SICI, CSP, LICI) and excitatory (ICF) intracortical activity of people with MS. We additionally hypothesized that combining assessments of cortical damage, intracortical inhibition, and intracortical facilitation would predict motor disability better than a single variable alone.

3.3 Material and methods

Participants

MS patients who had a relapsing-remitting, primary progressive, or secondary progressive course were randomly selected for recruitment from a clinical database at the Montreal Neurological Institute and Hospital in Canada. Poster advertisements were used to recruit age- and sex-matched healthy control (HC) participants. Screening (through telephone interviews and clinical chart review) further excluded people for: (1) left handedness, (2) health conditions other than MS (e.g. history of head trauma or cancer), (3) relative contraindications for undergoing MRI or TMS [237], (4) medications with documented effects on intracortical facilitation or inhibition (e.g. baclofen), and (5) relapse occurrence within 3 months prior to participation. Further exclusions occurred if valid TMS data could not be collected due to unacceptable noise in the electromyographic signal (i.e. movement artifact) and/or having a resting motor threshold (rMT) beyond the limit of our equipment when stimulating the central scalp region overlaying the left hemisphere (1 HC and 2 MS participants excluded). One HC participant was additionally excluded for being an extreme outlier (>3 standard deviations outside of mean) on several TMS-based outcome measures. Five MS participants were excluded from the analysis because they were taking *fingolimod*, which been shown to influence ICF [227]. The resulting sample included 18 HC and 36 MS participants. Data from some participants was also included in a study on a larger cohort of relapsing-remitting MS and healthy participants that focused on the relationship between conventional imaging metrics (i.e. white matter lesion volume), CSP prolongation, and clinical impairment persisting during remission [177].

All participants provided informed consent. The Research Ethics Board at the Montreal Neurological Institute and Hospital approved this protocol.

Demographic and clinical outcomes

Demographic and clinical variables (age, sex, diagnosis date, age of disease onset, clinical course, date of most recent relapse, EDSS, medications) of MS participants were extracted from the clinical database. Age, sex, and medications of healthy participants were self-reported. The Multiple Sclerosis Functional Composite (MSFC) was performed and scored according to standard procedure to assess clinical disability [164, 165]. This included the 9-hole peg test (9HPT), which is a valid and reliable measure of upper extremity function among people with MS [170]. Z-scores for the 9HPT and MSFC [164, 165] were used in analyses involving clinical outcomes.

TMS data collection

TMS was performed using a Magstim 200^2 stimulator and figure-of-8 coil (9.5cm outer wing diameter). With the handle oriented posteriorly, the coil was held against the left hemisphere at 45-degrees to the sagittal plane. Electromyographic data was recorded from surface electrodes in a belly-tendon montage, with the recording electrode over the first dorsal interosseus (FDI) muscle of the right (dominant) hand. The electromyographic signal was amplified, filtered (bandwidth = 10-3000 Hz) and collected at a sampling rate of 6000 Hz. A conventional hot-spotting technique was used to identify the target cortical region for the FDI muscle. Using BrainSight 2 stereotaxic navigation software (Rogue Research Inc), this location

was referenced as the stimulation site for all subsequent TMS protocols. For all protocols described below, invalid trials (e.g. movement artifact) were identified during the TMS procedure and replaced with additional trials at the end of each section, as necessary. Acceptable intra-individual reliability has been previously demonstrated for TMS-based metrics [238-241].

The standard rMT and 1mV threshold (rMT_{1mV}) were defined as the lowest stimulation intensity that in half of 10 trials elicited a motor-evoked potential (MEP) in a resting muscle ≥50μV [40] or ≥1mV, respectively.

SICI and ICF were assessed using the paired-pulse technique [38, 53], which involved an additional stimulator connected with a Bistim module. Conditioning pulses were delivered at 80% of rMT and test pulses at 120% of rMT. Paired-pulses (with inter-stimulus intervals between conditioning and test pulses set at 1ms, 2ms, and 3ms (for SICI) and 10ms (for ICF)) and non-paired single test pulses were delivered. The trials (8 per condition) were randomly sorted before the session to determine the order of delivery.

For LICI, we followed a protocol previously performed in people with other types of neurological conditions [242, 243]. Two paired-pulse stimulations (both at 100% rMT_{1mV}) were delivered 100ms apart. Eight single-pulse stimulations at 100% rMT_{1mV} were interspersed randomly between the eight paired-pulse trials. LICI was not collected from several participants (HC (11%), relapsing-remitting MS (27%), and progressive MS participants (36%)) because rMT_{1mV} was beyond the limits of our TMS unit, or a clean and consistent signal could not be acquired at this intensity during the TMS session.

Maximum voluntary contraction was determined using a Preston pinch gauge (Sammons Preston, Illinois, USA). For the cortical silent period (CSP) technique [198], a single suprathreshold stimulus (120% of rMT) was delivered during active contraction of the target

muscle (40% of maximum) over ten trials. As Lang et al. (2011) previously reported that time of day may influence CSP and LICI (but not other outcomes) [244], we extracted the data collection time of these protocols as recorded in the raw TMS data files.

TMS data analysis

A MATLAB-based analysis program (dataWizard, version 0.7.7, A.D. Wu, UCLA) was used for semi-automatic data processing. A researcher who was blinded to clinical status of participants performed quality control assessments. Among all valid trials within each condition, the average latency and peak-to-peak amplitude of the MEPs were measured. SICI and LICI were calculated according to the formula (1-(mean conditioned MEP amplitude)/(mean unconditioned MEP amplitude))×100% [184], and ICF according to (mean conditioned MEP amplitude)/(mean unconditioned MEP amplitude)×100%, such that higher numbers would reflect a higher percentage of inhibition and facilitation relative to the unconditioned MEP, respectively. SICI at the 2ms interstimulus interval was chosen as a main outcome, as it is close to the peak inhibitory activation believed to reflect synaptic $GABA_A$ receptor mediated activity [245, 246] and (unlike SICI at the 3ms inter-stimulus interval) had a distribution that satisfied the assumptions of analysis of covariance (ANCOVA). For CSP, the electromyographic trace was rectified, averaged over all valid trials, and highly magnified for visualization [247]. We measured the minimal absolute CSP duration, defined as the time from the end of the MEP until the earliest return of the electromyographic response [65, 247].

MRI data collection

Magnetic resonance imaging (MRI) data were collected at the Montreal Neurological Institute (Siemens TIM Trio scanner, 3 Tesla, 32-channel head coil) in Canada. A T1-weighted 3D fast low-angle shot (FLASH) sequence was collected with repetition time (TR) = 20ms, echo time (TE) = 5ms, and 192 slices (slice thickness = 1mm). T2-weighted 3D fluid-attenuated inversion recovery (FLAIR) images were acquired with TR = 6000ms, TE = 355ms, 2200ms inversion time, and 192 slices with 1 x 1 x 1 mm^3 voxel size. Proton density/T2-weighted dual spin echo sequence were collected with TR = 2100ms and TE = 17/76ms, for 60 slices (slice thickness = 3mm). For magnetization transfer (MT) imaging, we acquired a proton density-weighted FLASH sequence pair (TR = ~33ms, TE = 3.81ms, number of slices = 192, slice thickness = 1mm, flip angle = 10˚) both with (MT_{ON}) and without (MT_{OFF}) an MT saturation pulse. All images had a field of view of 256×256mm and in plane resolution of 1×1mm^2. One HC and three MS participants withdrew from the study without completing the neuroimaging protocol and were therefore not included in the analyses involving the MRI data.

MRI data analysis

Scans were coded to ensure blinding to group during image processing. All image modalities were linearly registered to the T1-weighted image of the participant. MTR within each voxel was calculated according to the formula: MTR (% units) = 100×([MT_{OFF} - MT_{ON}]/MT_{OFF}) [248].

For cortical segmentation [200], T1-weighted images were processed with FreeSurfer (version 5.1.0) [127]. Cortical segmentation was manually reviewed and corrected by the second

author. T2-weighted white matter lesions were marked using a semi-automated lesion-detection protocol [201]. By applying the scaling factor output from the skull-constrained registration to MNI standard space performed by SienaX [129], normalized lesion volumes were generated. The primary motor cortex hand knob region for the left hemisphere (M1-H_L) was labeled in the MNI-152 atlas space according to previously published anatomical landmarks [249]. The M1-H_L mask was subsequently transformed into each participant's native space using ANTs [204] and voxels of the transformed M1-H_L label that were outside of the primary motor cortex (based on FreeSurfer segmentation) were removed. We computed mean MTR within the entire cerebral cortex, M1-H_L (see Figure 3.1), and whole-brain white matter of each participant.

Statistical Analyses

HC, relapsing-remitting MS and progressive MS participants were compared on normally and non-normally distributed demographic variables using one-way Analysis of Variance (ANOVA) and Kruskal-Wallis H tests, respectively. Post-hoc Tukey HSD or Mann-Whitney U tests followed as needed. Chi-squared tests were used to compare groups on categorical variables. Unpaired T-tests and Mann-Whitney U tests, where appropriate, were used for two-group comparisons (i.e. HC vs. full MS sample). Analyses performed with the main purpose of replicating previous work (e.g. [66, 67, 69, 177, 233]) are described in *Appendix I*.

To address our primary objectives regarding the relationship between cortical damage and cortical activity, and ultimately their combined impact on disability, the following analyses were performed: The relationships between neuroimaging and TMS-based outcomes were assessed

with Spearman rank correlation analyses. Stepwise multiple linear regression analyses were performed to establish multivariable models that could best predict performance (9HPT, MSFC).

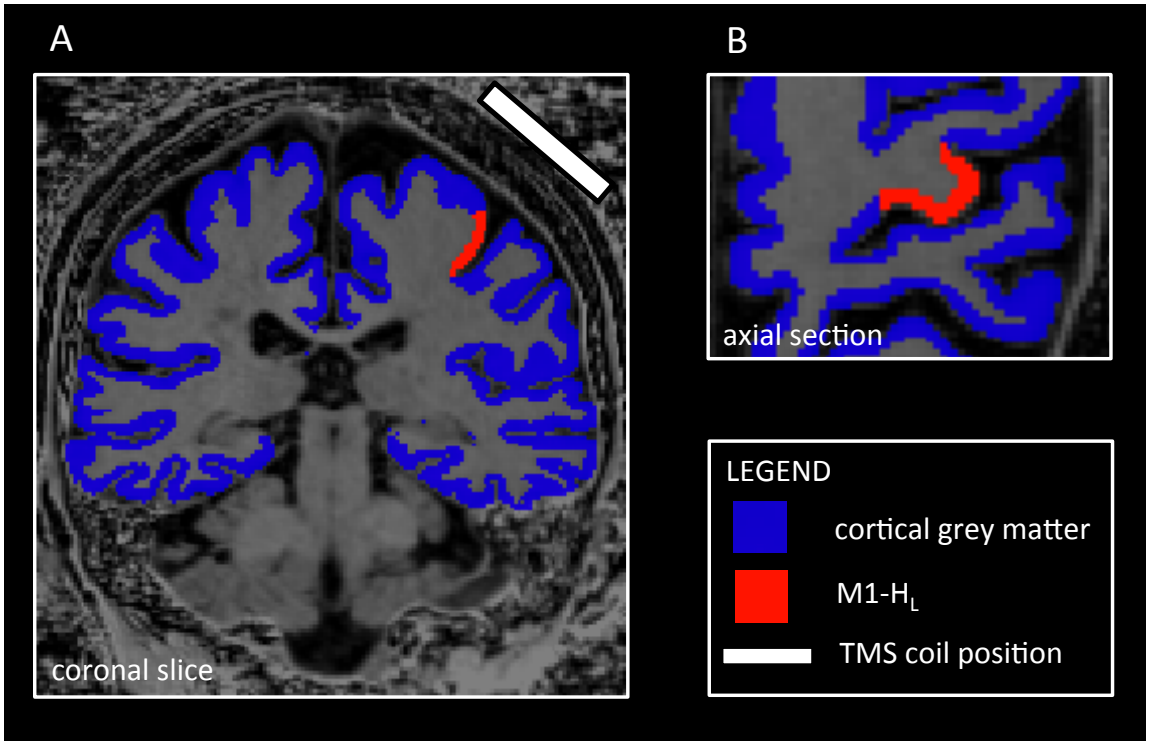

FIGURE 3.1 Magnetization transfer ratio (MTR) image of a participant with multiple sclerosis as viewed from a coronal slice (**A**) and a section of an axial slice centered over the left hemisphere motor hand knob (**B**). The regions of interest from which MTR was extracted are superimposed on the MTR images, including the cerebral cortex (blue and red regions), as well as specifically within the primary motor cortex hand knob region of the left (dominant) hemisphere (M1-H_L, red region). The approximate location of the transcranial magnetic stimulation (TMS) coil placement relative to the skull during neurophysiological data collection is shown in (**A**).

3.4 Results

Participant characteristics

Of participants with MS, 22 had the relapsing-remitting form of the disease and 14 had a progressive disease course (six primary progressive, eight secondary progressive). HC and the full MS sample (i.e. relapsing-remitting and progressive MS groups combined) did not differ in age ($t_{52} = -1.30$, $p > 0.05$) or in sex ($\chi^2_{(1)} = 0.04$, $p > 0.05$). Three-group comparisons (see Table 3.1) showed that progressive MS participants were significantly older than both healthy and relapsing-remitting participants (ps < 0.01), while relapsing-remitting MS and HC did not differ.

TABLE 3.1 Demographic characteristics of participants.

	HC	Relapsing-remitting MS	Progressive MS
Total number of subjects	18	22	14
Women, n *(%)*	13 (72)	15 (68)	10 (71)
Age, mean *years* ± *SD* **	45 ± 14	44 ± 12	60 ± 13
Taking immunomodulatory medication for MS, n *(%)* **	0 (0)	12 (55)	3 (21)
Age of MS onset[a], mean *years* ± *SD*	NA	31 ± 10	38 ± 13
Time since diagnosis[a], mean *years* ± *SD*	NA	10.2 ± 7.2	17.2 ± 16.3
Normalized lesion volume[b], *median* cm^3 *(Q1, Q3)* ***	0 (0,0)	5.3 (2.5, 16.6)	10.2 (1.50, 21.0)

Asterisks indicate significant differences between groups (* p < 0.05, ** p < 0.01, *** p < 0.001). HC = healthy control; MS = multiple sclerosis; SD = standard deviation; Q1 = lower quartile; Q3 = upper quartile; NA = not applicable. [a]Data unavailable for 2 participants in each group. [b]Data unavailable for 1 HC, 2 relapsing-remitting MS, and 1 progressive MS participant.

Compared to progressive MS participants, more relapsing-remitting MS participants were using immunomodulatory medications to treat MS (p < 0.01), trended toward having a shorter

time since diagnosis ($p = 0.05$), but did not differ significantly in normalized white matter lesion volume ($p > 0.05$) or in age of disease onset ($p > 0.05$). Average time of day of data collection for the CSP and LICI protocols did not differ between the groups (ps > 0.05). Age and sex-adjusted performance and neurophysiological outcomes are shown in Tables 3.2 and 3.3; see *Appendix I* for the full description of the related ANCOVA and regression analyses.

TABLE 3.2 Age- and sex-adjusted performance-based and TMS-based outcomes.

	HC	**Relapsing-remitting MS**	**Progressive MS**
Performance			
EDSS (score) **	N/A	2.1 ± 0.3	4.2 ± 0.4
MSFC total[a] (z-score) ***	0.65 ± 0.12	0.12 ± 0.11	-0.21 ± 0.15
9HPT (z-score) ***	0.95 ± 0.15	0.39 ± 0.14	-0.21 ± 0.19
TMS			
rMT (%)	48.5 ± 2.4	47.3 ± 2.2	49.9 ± 3.0
MEP latency (ms)			
relaxed muscle	23.0 ± 0.4	23.4 ± 0.3	24.5 ± 0.5
active muscle *	18.9 ± 0.4	18.6 ± 0.4	20.5 ± 0.5
MEP amplitude (mV)			
relaxed muscle	1.15 ± 0.21	0.85 ± 0.19	0.59 ± 0.26
active muscle	5.46 ± 0.43	5.14 ± 0.39	3.82 ± 0.53
ICF (%)	166 ± 15	150 ± 14	143 ± 19
SICI (%)	68 ± 6	64 ± 5	47 ± 7
CSP (ms)	77 ± 8	65 ± 7	75 ± 10

Mean ± standard error of the age and sex-adjusted outcomes are shown. Asterisks indicate significance differences found between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). HC = healthy control; MS = multiple sclerosis; EDSS = Expanded Disability Status Scale; MSFC = Multiple Sclerosis Functional Composite ; 9HPT = Nine Hole Peg Test; RMT= resting motor threshold; MEP = motor-evoked potential; ICF = intracortical facilitation; SICI = short-interval intracortical inhibition; CSP = cortical silent period; [a]Data unavailable for 3 participants.

TABLE 3.3 Age- and sex-adjusted TMS-based outcomes of MS participants with a progressive clinical course.

	Primary Progressive	Secondary Progressive
rMT (%)	49.2 ± 4.3	50.5 ± 3.9
MEP amplitude (mV)		
relaxed muscle	0.88 ± 0.37	0.34 ± 0.34 *
active muscle	4.51 ± 0.74	3.25 ± 0.68
ICF (%)	163 ± 27	127 ± 25
SICI (%)	61 ± 10	36 ± 9 *
CSP (ms)	83 ± 14	68 ± 13

Mean ± standard error of the age and sex-adjusted outcomes are shown. Asterisks indicate variables for which significant differences were found from a post-hoc test of the four-group ANCOVA analysis comparing healthy controls, relapsing-remitting MS, primary progressive MS, and secondary progressive MS.
MS = multiple sclerosis; RMT= resting motor threshold; MEP = motor-evoked potential; ICF = intracortical facilitation; SICI = short-interval intracortical inhibition, CSP = cortical silent period.

Neuroimaging outcomes

Compared to HC participants, MS participants had lower whole-brain cortical volume (mean ±SD: 486 ±41 vs. 450 ±43 cm^3, $t_{48} = 2.83$, $p < 0.01$) and MTR (mean ±SD: 36.2 ± 0.4 vs. 35.8 ± 0.7 % units, $t_{48} = 1.8$, $p < 0.05$). Within M1-H_L, however, MTR did not differ significantly between groups ($U = 264$, $p > 0.05$). MS participants also had lower mean white matter MTR (mean ±SD: 44.0 ± 0.6 vs. 43.3 ± 0.9 % units, $t_{48} = 2.9$, $p < 0.01$). Among all MS participants, but not healthy controls, lower MEP amplitude was nearly correlated with lower white matter MTR ($r_{s(32)} = 0.34$, $p = 0.06$) but not with cortical MTR or volume (ps > 0.05). None of the TMS-based markers of intracortical facilitation and inhibition (ICF, SICI, CSP, LICI) correlated

significantly with cortical volume or MTR in any region among people with MS or controls (all ps > 0.03).

We performed additional analyses separating relapsing-remitting and progressive MS participants (Table 3.4) to test an alternative hypothesis that groups may differ in the relationship between MRI- and TMS-based variables due to use of disease-modifying therapy, plasticity of the motor system[250], or other factors. MTR within M1-H_L correlated with lower SICI among relapsing-remitting MS participants. Lower mean whole brain cortical MTR correlated with shorter CSP duration among progressive MS participants. No other significant correlations were found between TMS-based variables and cortical MTR or cortical volume. To determine if these findings were specific to the cortex, we additionally assessed white matter MTR. Lower white matter MTR correlated with lower SICI among relapsing-remitting MS participants ($r_{s(19)} = 0.50$, p = 0.03), while no other relationships reached significance (ps > 0.05).

TABLE 3.4 Relationship between cortical MTR and TMS-based outcomes.

Region of interest	TMS	HC	Relapsing-remitting MS	Progressive MS
Whole brain	ICF	0.08	0.02	0.15
	SICI	0.05	0.30	0.26
	CSP	-0.21	0.14	**0.57 ***
	LICI[a]	-0.08	-0.01	0.18
M1-H_L	ICF	-0.01	-0.33	0.18
	SICI	0.37	**0.47 ***	0.01
	CSP	-0.13	0.29	0.05
	LICI[a]	-0.16	-0.34	-0.25

Spearman Rank correlation coefficients of the relationships between cortical MTR and the TMS-based outcomes are shown for each group. Significant correlations (two-tailed) between groups are indicated with asterisks (* p < 0.05). HC = healthy control; MS = multiple sclerosis; M1-H_L = primary motor cortex hand region of the left hemisphere; ICF = intracortical facilitation; SICI = short-interval intracortical inhibition, CSP = cortical silent period; LICI = long-interval intracortical inhibition. [a]Data missing from 2 HC, 4 relapsing-remitting MS, 6 progressive MS participants.

Multimodal predictors of motor and global disability

TMS-based measures of intracortical activity (CSP, ICF, SICI, MEP amplitude), neuroimaging markers of structural damage (cortical normalized volume, cortical MTR (entire cortex and $M1_{LH}$), white matter MTR, T2-weighted white matter lesion load), and demographic (age, sex) variables were entered into a stepwise multiple linear regression analysis to predict performance among all MS participants. LICI was not included due to the number of participants missing data on this variable. A model including CSP, ICF, cortical MTR and sex best predicted 9HPT (Table 3.5). Only cortical MTR was identified as a significant contributor to a model predicting MSFC total score ($R^2 = 0.21$, $F_{1,32} = 8.26$, $\beta = 0.46$ $p < 0.01$).

TABLE 3.5 Predictive model of 9HPT performance of MS participants based on stepwise linear regression analysis outcomes.

Step	Model summary	Predictor	β	t	*p*
1	$R^2 = 0.165$ F = 6.12 p = 0.019	CSP	-.406	-2.474	.019
2	$R^2 = 0.313$ F = 6.83 p = 0.004	CSP	-.422	-2.785	.009
		Sex	-.385	-2.541	.016
3	$R^2 = 0.419$ F = 6.98 p = 0.001	CSP	-.499	-3.427	.002
		Sex	-.371	-2.618	.014
		Cortical MTR	.336	2.306	.028
4	$R^2 = 0.502$ F = 7.07 p < 0.001	CSP	-.539	-3.896	.001
		Sex	-.413	-3.062	.005
		Cortical MTR	.310	2.252	.032
		ICF	-.296	-2.163	.039

ICF = intracortical facilitation; CSP = cortical silent period; MTR = magnetization transfer ratio;
β = standardized regression coefficient

3.5 Discussion

Using a novel multimodal approach, the present study investigated the relationship between intracortical activity, cortical integrity, and clinical disability. Cortical volume and MTR assessments showed that structural brain damage may contribute to variability in certain forms of intracortical activity. We also replicated previous reports of abnormally low SICI uniquely among people with secondary progressive MS, and found that other TMS-based metrics did not vary with general symptom progression. In combination with information on cortical integrity, however, ICF and CSP can contribute to our understanding of motor system pathology, which affects individuals of all MS subtypes to varying degrees.

Intracortical activity and damage

Compared to healthy participants, people with MS had significantly lower normalized cortical volume and MTR, indicating the presence of atrophy and demyelination [144, 232]. However, none of the MRI-based outcomes were related to ICF, SICI, CSP nor LICI when assessing all subtypes of MS together, indicating that cortical damage may not be the primary cause of variability in intracortical facilitation or inhibition in this population. As lower white matter MTR correlated close to significance with lower single pulse MEP amplitude, demyelination may contribute to motor evoked responses used as the baseline for SICI and ICF calculation.

We suspected that plasticity of the motor system [250], disease-modifying therapy, or other factors that tend to differ with clinical subtype could have masked the relationship between MTR and intracortical inhibition or facilitation in some participants. Indeed, further analyses

revealed that lower whole brain cortical MTR correlated with shorter CSP duration only among the progressive MS group. Importantly, this indicates that the relationship between poorer motor function and longer CSP is unlikely to have occurred because of cortical damage, but rather in spite of it. As we discuss in more detail in Nantes *et al.* (2016) [177], it is possible that high inhibition among motor-impaired individuals with relapsing-remitting MS may be secondary to disease-related factors such as inflammation or glutamate excitotoxicity [89, 90, 218-221, 251], rather than structural damage. Consistent with this, the present analyses implicate cortical damage in the shortening, rather than the prolongation, of the CSP. The net effect of competing mechanisms influencing CSP length in opposite directions could explain why CSP duration does not differ significantly between healthy and MS participants. Longitudinal multimodal research involving markers of inflammation, cortical damage, intracortical inhibition, and motor impairment will be needed to test this hypothesis more directly.

Predicting that intracortical inhibition and facilitation may be affected by damage in the vicinity of the primary motor cortical area targeted by TMS, we narrowed our MTR region of interest to the left hemisphere motor hand knob. Lower MTR within this area was related to lower SICI of relapsing-remitting MS participants, and this relationship trended toward significance among healthy participants. As SICI also correlated with white matter MTR, it is also possible that the integrity of white matter tracts could contribute to SICI variability during the relapsing-remitting phase. Thus, we speculate that SICI may be modestly sensitive to normal variability in the myelination of the stimulated cortical region and/or white matter tracts among minimally disabled people, while other disease-related factors may contribute to pathologically low SICI observed in secondary progressive MS. While beyond the scope of the present study to assess, low SICI among progressive MS participants could be a consequence of cortical motor

map re-organization [252], spinal cord damage [253], cortical lesions to which MTR is insensitive, or other disease-related factors for which the impact on intracortical inhibition is not known.

Multimodal predictors of motor disability

Our multivariate analysis revealed that motor impairment could be best predicted using a combination of TMS-based, neuroimaging, and demographic variables. Specifically, longer CSP, higher ICF, lower cortical MTR, and sex were independently related to upper extremity impairment. This demonstrates that biomarkers of excitatory and inhibitory cortical activity can provide information relevant to motor impairment that is complementary, rather than redundant, to measures of structural cortical integrity. By contrast, none of the TMS-based measures predicted overall MSFC score beyond the impact of cortical MTR. This suggests that, in the context of complex neurological conditions such as MS, electromyographic activity-derived biomarkers are more relevant to motor impairment than to general disability progression.

Of further interest is that higher ICF was significantly related to worse motor impairment only after adjusting for other variables including intracortical inhibition and cortical MTR. While ICF may reflect glutamatergic activity [43, 44], the clinical relevance of this may lie more in strength of excitatory activity relative to inhibition and damage, as opposed to a simple increase in ICF irrespective of other variables. This finding highlights the value of assessing both inhibitory and excitatory forms of cortical activity, and cortical damage, concurrently.

Limitations and future directions

Sample size, particularly among those with the primary progressive MS subtype, was a limitation to the present study. Additionally, outcomes involving LICI should be interpreted with caution considering that participant exclusion due to the high stimulation intensity required may have been non-random. We encourage others to assess LICI among people with progressive forms of MS using a protocol that requires a lower intensity of stimulation. Interpretation of CSP-related outcomes is difficult as longer CSP may have been selectively related to motor performance due to the relative sensitivity of this metric to physiological mechanisms related to spinal cord inhibition [59, 60] or motor attention [210]. Nonetheless, the interpretation that CSP reflects intracortical inhibition is consistent with studies linking lower GABA concentration around the motor cortex hand knob region to less severe motor impairment among individuals recovering from stroke [191, 206] and people with relapsing-remitting MS [96]. Additional studies are needed to determine if other factors that could influence impairment (e.g. spinal lesions, central motor conduction time, short-interval intracortical inhibition) are independent predictors of motor performance from CSP, ICF, and cortical MTR. Furthermore, the exploratory secondary outcomes included in this study should be replicated.

MTR may also be useful to investigate the link between structural damage and other TMS-based variables linked to disability in people with MS, including inter-hemispheric excitability [254] and short-interval intracortical facilitation [69]. Alternative MRI techniques sensitive to cortical damage, such as diffusion imaging and ultrahigh-field MRI [255] could also help to elucidate the relationship between cortical damage and activity. Such studies may consider

defining the cortical region relevant to the TMS hot-spot functionally, rather than anatomically, as shifts in the functional hot-spot could occur in response to brain injury [252]. Future higher-powered studies should investigate how damage within various structures of the motor network, as well as the integrity of structural and functional connections between these regions, impacts the various forms of intracortical inhibition and facilitation that can be measured with TMS.

Conclusions

The present study sheds light on the clinical value, and limitations, of using TMS-based metrics in the context of neurological disease, and demonstrates the importance of multimodal assessments. While we confirm SICI to be abnormally low in secondary progressive MS, our data support that this is may not be simply a consequence of cortical damage. We also find evidence that motor system dysfunction may be driven by physiological factors related to CSP and ICF that are independent of cortical damage. Longitudinal and interventional studies are needed.

Chapter 4

Insights from ^{1}H-MRS

Preface

Moving beyond TMS-based outcomes examined in the preceding chapters, the manuscript presented here uses ^{1}H-MRS to investigate the extent to which glutamate and GABA levels within two regions of the human brain are related to microstructural damage and clinical impairment. This was investigated in a mixed group of patients with relapsing-remitting, primary progressive, and secondary progressive forms of multiple sclerosis. An analytic approach optimized for accurate measurement of these neurometabolites, even in the presence of this multistage neurological disease, is presented. The novel results yielded from taking this approach largely resolve apparent discrepancies in the very limited literature relevant to this topic published previously. Moreover, this is the first study to our knowledge to report a relationship between MTR and glutamate or GABA levels assessed with ^{1}H-MRS. Thus, this manuscript contains significant methodological development beyond the current literature and furthers our understanding of the radiological and clinical implications of glutamate and GABA in multiple sclerosis.

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4.1 Abstract

Converging areas of research have implicated glutamate and γ-aminobutyric acid (GABA) as key players in neuronal signalling and other central functions. Further research is needed, however, to identify microstructural and behavioral links to regional variability in levels of these neurometabolites, particularly in the presence of demyelinating disease. Thus, we sought to investigate the extent to which regional glutamate and GABA levels are related to a neuroimaging marker of microstructural damage and to motor and cognitive performance. Twenty-one healthy volunteers and 47 people with multiple sclerosis (all right-handed) participated in this study. Motor and cognitive abilities were assessed with standard tests used in the study of multiple sclerosis. Proton magnetic resonance spectroscopy data were acquired from sensorimotor and parietal regions of the brains' left cerebral hemisphere using a MEGA-PRESS sequence. Our analysis protocol for the spectroscopy data was designed to account for confounding factors that could contaminate the measurement of neurometabolite levels due to disease, such as the macromolecule signal, partial volume effects, and relaxation effects. Glutamate levels in both regions of interest were lower in people with multiple sclerosis. In the sensorimotor (though not the parietal) region, GABA concentration was higher in the multiple sclerosis group compared to controls. Lower magnetization transfer ratio within grey and white matter regions from which spectroscopy data were acquired was linked to neurometabolite levels. When adjusting for age, normalized brain volume, MTR, total N-acetylaspartate level, and glutamate level, significant relationships were found between lower sensorimotor GABA level and worse performance on several tests, including one of upper limb motor function. This work highlights important methodological considerations relevant to analysis of spectroscopy

data, particularly in the afflicted human brain. These findings support that regional neurotransmitter levels are linked to local microstructural integrity and specific behavioral abilities that can be affected in diseases such as multiple sclerosis.

4.2 Introduction

The brain's main molecules that can act as excitatory and inhibitory neurotransmitters, glutamate and γ-aminobutyric acid (GABA), respectively, are involved in several critical functions of the central nervous system. The link between regional levels of these neurometabolites, integrity of brain structures, and behavior, however, is largely unknown for humans. Studying the brain in the presence of neurological disease can help to uncover these relationships. In particular, the high degree of radiological and symptom heterogeneity of multiple sclerosis [223, 256] makes this condition useful for investigating these questions within various functional systems of the brain. While the relevance of GABA and glutamate levels have begun to be explored in multiple sclerosis and related conditions, methodological limitations have greatly restricted our understanding thus far.

Concentrations of metabolites within various brain regions can be measured non-invasively in humans using proton magnetic resonance spectroscopy (^{1}H-MRS) [8]. ^{1}H-MRS studies in people with multiple sclerosis have reported that glutamate and the glutamate + glutamine complex (Glx) in grey matter structures tend to be abnormally low, and these reductions have been linked to more severe clinical impairments [93, 94, 257]. Within acute lesions and normal-appearing white matter of the brain of people with multiple sclerosis, glutamate and Glx are typically unaltered or even elevated [89, 90, 93, 94, 153]. However, glutamate in white matter has been found to decrease over time among people with the secondary progressive form of multiple sclerosis, even in the absence of clinical progression [95]. Further research is needed to determine if glutamate level in specific cortical regions may contribute to motor or cognitive

function, or conversely, if such behavioral abilities may be more directly attributed to GABA or tissue integrity.

Few studies have investigated GABA levels in the brains of people with multiple sclerosis despite its close structural and functional relationship with glutamate. This can likely be attributed to technical challenges associated with measuring GABA levels at standard field strengths (e.g. 3T), as specialized data acquisition and analysis protocols are required [70]. Data analysis and interpretation is additionally complicated in the context of neurological disease due to factors such as the relaxation time of water, known to be abnormal in lesions and normal appearing brain tissue of people with multiple sclerosis (e.g. [258]). Among people with the relapsing-remitting form of multiple sclerosis, Bhattacharyya *et al.* [96] found that higher GABA within a sensorimotor brain region correlated with worse fine motor performance. Differences in GABA concentration between the multiple sclerosis and healthy subjects in this initial small study did not, however, reach significance. Conversely, Cawley *et al.* [97] reported that sensorimotor GABA concentration may be abnormally low among people with the secondary progressive form of multiple sclerosis, and that lower GABA may be implicated in worse motor function. Macromolecule alteration due to the disease could not, however, be ruled out as the cause of the apparent GABA deficits in the latter study. The apparently conflicting outcomes of these initial studies may also be attributed to differences in patient characteristics (e.g. relapsing-remitting vs. secondary progressive subtypes). Further investigation into regional GABA level is needed using methods optimized to adjust for potentially confounding factors and to better understand this complex, multi-phase disease.

Appropriate interpretation of ^{1}H-MRS data may be improved by determining the extent to which microstructural integrity is related to metabolite levels. Notably, demyelination has been

shown to co-occur with glutamate deficits in mouse models of neurological disease [154, 155, 259]. While such damage can not be measured directly in living human patients, neuroimaging measures such as magnetization transfer ratio (MTR) can estimate the amount of microstructural damage even within regions that appear normal on images generated with conventional neuroimaging sequences [231, 260]. Postmortem analyses of the multiple sclerosis brain have found lower MTR to be linked to myelin loss in both white matter and grey matter regions [144, 214, 261]. The magnetization transfer signal can also be influenced by other aspects of the disease such as axonal damage and inflammation [260]. Of note, MTR is lower in histologically defined cortical lesions compared to normal appearing grey matter in patient brains [261-263]. Considering the challenges of cortical lesion segmentation at standard field strengths such as 3T [213, 264, 265], MTR provides the advantage of being a relatively clinically feasible method sensitive to intracortical pathology [214]. Therefore, in combination with atrophy assessment, analysis of tissue integrity using MTR could provide valuable information relevant to the interpretation of metabolite concentrations, including when assessed in the brains of individuals with neurological damage.

Using a method optimized for accurate measurement of neurometabolite levels in both the healthy and diseased human brain, our study investigated microstructural and behavioral correlates to regional glutamate and GABA levels. We assessed the relationships between neurometabolite levels and MTR, as we suspected the amount of microstructural damage might co-occur with variability in local neurochemical levels, as would be particularly evident by studying the brain in a diseased state. MTR measured within the region that ^{1}H-MRS was collected was predicted to be more strongly linked to neurometabolite levels than MTR measured globally. In addition, we sought to assess the extent to which glutamate and GABA

concentrations in two distinct regions of interest are related to performance on tests of fine motor and cognitive function. We chose to investigate the area including the hand region of the primary motor cortex due to its relevance to motor dexterity and the opportunity to extend on previous work investigating GABA in this area (i.e. Bhattacharyya *et al.,* 2013; Cawley *et al.,* 2015). We predicted that performance on a test of manual motor dexterity would correlate with glutamate and GABA concentrations in this region, including when statistically adjusting for markers of local neuronal health and myelination. Whether such a relationship may be regionally specific to the sensorimotor area was explored by assessing relationships between neurometabolite levels in a parietal region and motor performance. As structural and functional MRI studies have found the parietal area to be one of several brain regions implicated in cognitive function of people with MS [266-268], we also investigated relationships between neurotransmitter levels in this area and performance on a test of cognitive ability. Our analyses help to elucidate the links between neurochemical levels, structural integrity of the brain, and motor and cognitive ability.

4.3 Material and methods

Study Population

People with multiple sclerosis were recruited through the *Montreal Neurological Institute and Hospital Multiple Sclerosis Clinical Research Database*, consisting of registered patients who consented to having their charts available and to be contacted for research recruitment purposes. Healthy individuals were recruited to participate through word of mouth and poster advertisements. Persons with contraindications for undergoing MRI (e.g. pregnancy, pacemaker,

ferromagnetic metal in body), or who had health conditions not associated with their multiple sclerosis diagnosis (e.g. cancer) were not invited to participate in the study. Furthermore, individuals taking medications with known direct effects on GABAergic activity (e.g. baclofen for treatment of spasticity) were excluded from participating. Two multiple sclerosis participants were recruited who had been taking levothyroxine, but were included in the analyses as we confirmed excluding them had a negligible effect on outcomes. All participants were right-handed and fluent in either English or French. Informed consent was obtained according to the Declaration of Helsinki and individuals were compensated for participating. The Research Ethics Board at the Montreal Neurological Institute and Hospital approved this protocol.

Clinical and performance-based assessments

Based on information from the clinical database, age, sex, date of most recent relapse, disease-modifying therapies, and most recent Expanded Disability Status Scale (EDSS) score assessed by a neurologist were recorded. Age, sex, and medical history of healthy participants were self-reported. Participants filled out the Hospital Anxiety and Depression Scale (HADS) [269] due to the known relationship between GABA deficits and mental illness [270, 271]. The Multiple Sclerosis Functional Composite was performed according to standard procedure [165, 272]. This test consists of three sub-scales: Timed 25-foot walk, 9-hole peg test (9HPT), and the three-second-interval version of the paced auditory serial addition test (PASAT), which assess leg function/ambulation, hand/arm dexterity, and attention/working memory, respectively. Z-scores were calculated in reference to the National Multiple Sclerosis Society Task Force database [165, 272]. The oral version of the Symbol Digit Modality Test (SDMT) was also

performed [273]. Performance-based assessments were carried out as close as possible to the MRI scan (average of 5 days apart for completion of all study components), though not in a single session to avoid over-burdening a clinical population known to suffer from fatigue [274].

MRI and ^{1}H-MRS Data Collection

MR scanning occurred at the Brain Imaging Centre of the Montreal Neurological Institute in Canada using a Siemens Tim Trio scanner at 3 Tesla with a 32-channel receive-only head coil. Structural images acquired included: (1) T1-weighted 3D fast low-angle shot (FLASH) sequence (repetition time (TR) = 20 ms, echo time (TE) = 5 ms, number of slices = 192, slice thickness = 1 mm); (2) T2-weighted fluid-attenuated inversion recovery (FLAIR) images using a turbo spin echo sequence (TR/TE = 6000/355 ms, FOV = 256 mm, number of slices = 192), and (3) proton density/T2-weighted dual-echo turbo spin echo (TR = 2100 ms, TE_1/TE_2 = 17/76 ms, FOV = 256 mm, number of slices = 60, slice thickness = 3 mm). Additionally, magnetization transfer (MT) was acquired using a pair of gradient echo sequences (TR/TE = ~33/3.81 ms, FOV = 256 mm, slices number = 192, slice thickness = 1 mm) with (MT_{ON}), and without (MT_{OFF}), an MT saturation pulse. All images had a field of view of 256 × 256 mm^2 and in-plane resolution of 1 × 1 mm^2.

Single voxel ^{1}H-MRS was acquired from two cubic VOIs (27 × 27 × 27 mm^3). Both VOIs were placed in the left (dominant) hemisphere, with the *sensorimotor* VOI positioned over the primary motor hand knob cortical region (localized by referencing anatomical landmarks [249]) and the *parietal* VOI placed in the parietal lobe without overlapping with the sensorimotor VOI (shown in Figure 4.1). Positioning of these VOIs was performed while viewing the participant's

high-resolution T1-weighted image. An additional T1-weighted sequence was collected immediately before ^{1}H-MRS, and VOI re-positioning was performed before commencing ^{1}H-MRS data collection if the participant's head had moved between scans.

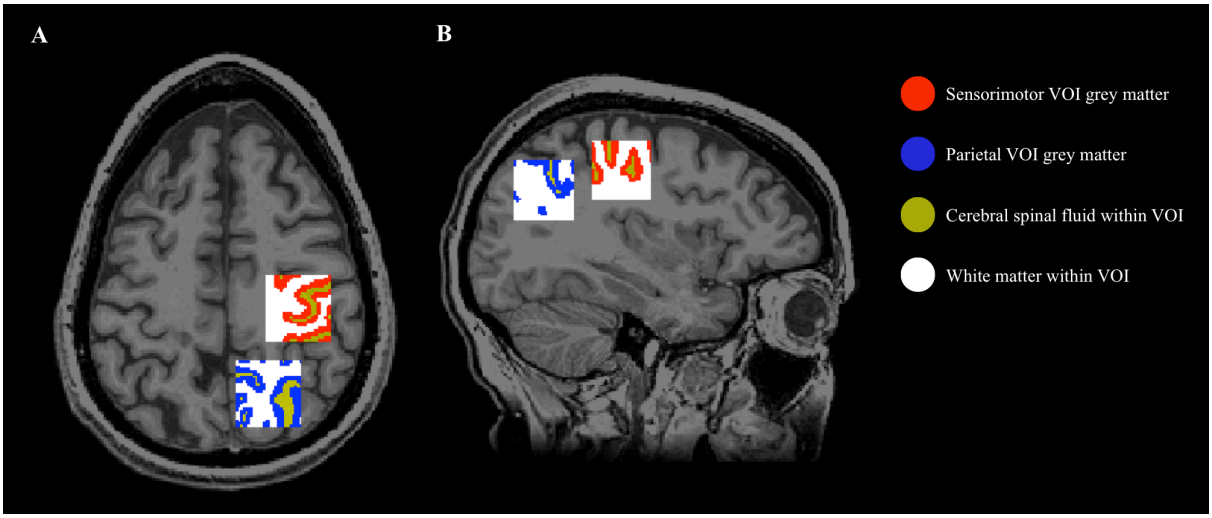

FIGURE 4.1 The placement of the cubic sensorimotor and parietal volumes of interest (VOIs) in the left cerebral hemisphere, from which ^{1}H-MRS data were collected, are shown from an axial (A) and a sagittal (B) slice of a study participant. Segmentation of grey matter, white matter, and CSF within each VOI, used for partial volume correction and regional MTR calculation, are shown.

Before ^{1}H-MRS scanning commenced, FASTESTMAP shimming [275] was performed. Acceptable field homogeneity of the water signal was confirmed (full width at half-maximum < 10 Hz). A MEGA-PRESS (MEshcher–GArwood Point RESolved Spectroscopy) sequence [87] (TR/TE = 3000/68 ms) was used for water suppression and editing of J-coupled resonances. GABA levels measured using the MEGA-PRESS method have previously been found to be reliable in healthy populations [276]. The sequence included double-banded suppression pulses centered on water at 4.7 ppm and alternating between 7.5 ppm and 1.9 ppm for the $Edit_{OFF}$ and $Edit_{ON}$ spectra, respectively. Data for each VOI were collected in four blocks of 64 scans, with

each scan alternating between the $Edit_{ON}$ and $Edit_{OFF}$ parameters (scanning time = 17 min/VOI). VAPOR (variable power optimized relaxation delays) for additional water suppression and an outer volume suppression sequence to saturate spins outside of the VOIs [277] were also incorporated into the MEGA-PRESS sequence. Additionally, a water-unsuppressed spectrum was obtained at each VOI using the same parameters as for the metabolite spectrum, except that both the MEGA water suppression band (4.7 ppm) and VAPOR were turned off. Incomplete collection of ^{1}H-MRS data during scanning session led to the exclusion of one participant for the sensorimotor VOI and four participants for the parietal VOI.

Structural MRI processing and analysis

Neuroimaging data were coded such that the researchers performing the post-processing steps would be blinded to age, sex, group membership, and clinical test scores. T2-weighted lesions were detected in the white matter using a semi-automated lesion-detection program [201], with manual correction by a single trained researcher as needed. T1-weighted images were segmented semi-automatically using FreeSurfer version 5.1.0 (http://surfer.nmr.mgh.harvard.edu/). Segmentation errors due to lesion presence or image quality were manually corrected to ensure reliable segmentation of white and grey matter.

The anatomic regions corresponding to the VOIs of each participant were masked according to the anatomic coordinates recorded during ^{1}H-MRS data collection. Grey matter, white matter, and cerebral spinal fluid (CSF) fractions within each VOI (see Figure 4.1 for example), as well as within-VOI lesion volume, were quantified.

MTR, in percent units (pu), within each voxel was calculated according to Eq. (1):

$$MTR = 100 \times \frac{MT_{OFF} - MT_{ON}}{MT_{OFF}} \quad \textbf{(1)}$$

Mean regional MTR was computed for grey and white matter regions within each VOI and throughout the whole brain.

^{1}H-MRS processing and analysis

A custom MATLAB-based program, similar to that reported elsewhere [222, 278], enabled re-alignment of the frequency and phase of single-shot ^{1}H-MRS scans based on the spectral range covering creatine and choline, as well as the removal of motion-corrupted averages. Acceptable averages were included in the spectra quantified by LCModel (Version 6.3) [84]. For each VOI of each participant, the $Edit_{ON}$ spectrum was subtracted from the $Edit_{OFF}$ spectrum to generate the difference spectrum. The basis set for the difference spectrum (same as previously reported by Tremblay *et al*. (2013, 2014), had been created using experimentally measured phantoms of *N*-acetylaspartate (NAA), GABA, glutamate, and glutamine, and an experimentally measured metabolite-nulled macromolecule spectrum. This enabled explicit modeling of the macromolecule contributions to minimize contamination of the GABA signal by macromolecules at 3 ppm [279-281]. Spectral data on GABA and glutamate (the primary metabolites of interest), as well as macromolecules and NAA (a marker of neuronal health [152]) were extracted from the difference spectra. NAA levels reported here likely include smaller contributions from other acetylated compounds [282], which can be referred to as "total NAA". Our post-processing protocol was designed to adjust for factors associated with neurological disease that could confound metabolite level measurements, including relaxation time abnormalities known to occur in both lesions and normal appearing brain tissue of people with

multiple sclerosis (e.g. Jurcoane *et al.* (2013) [258]). Corrections for relaxation and partial volume effects were performed using methods adapted from Gasparovic and colleagues (2006, 2009) [82, 283] and Harris *et al.* (2015) [284], as described below.

The relaxation attenuation factor for the ^{1}H-MRS-visible water signal was calculated in each of four compartments of the VOI: cerebral spinal fluid (CSF), grey matter (GM), normal-appearing white matter (NAWM), and white matter lesion (WML), according to the formula:

$$R_{H_20_y} = (1 - exp(-TR/T1_{H_20_y}) \times (exp(-TE/T2_{H_20_y}) \quad \textbf{(2)}$$

where R_{H20y} is the attenuation factor for water in compartment *y*, *TR* is the repetition time, *TE* is the echo time, and $T1_{H20y}$ and $T2_{H20y}$ are the T1 and T2 relaxation times for water in compartment *y*, respectively. $T1_{H20y}$ in tissue was estimated using values from of a study by Jurcoane *et al* [258], which found relaxation time differences between normal controls and people with the relapsing-remitting and progressive forms of multiple sclerosis in GM, NAWM, and WML (values assigned based on group membership). $T2_{H20y}$ was estimated at the individual level within each compartment using a two-point estimate ("pseudo-T2") computed from the dual-echo sequence [285, 286], according to the formula:

$$T2_{H_20_y} \approx pT2_y = \frac{TE_2 - TE_1}{ln\frac{S_{1y}}{S_{2y}}} \quad \textbf{(3)}$$

where $pT2_y$ is the pseudo-T2 relaxation time in compartment *y*, and S_{1y} and S_{2y} are image intensities in compartment *y* at the respective echo times TE_1 and TE_2. For all participants, T1 and T2 relaxation times of water in CSF were assumed to be 3.817 s [287] and 0.503 s [288], respectively (as used in AD Harris, NA Puts and RA Edden [284]).

Molar water fractions in each compartment (f_y) were calculated according to the formula:

$$f_y = \frac{f_{yVOL} \times D_y}{f_{CSF_{VOL}} \times D_{CSF} + f_{GM_{VOL}} \times D_{GM} + f_{NAWM_{VOL}} \times D_{NAWM} + f_{WML_{VOL}} \times D_{WML}} \quad \textbf{(4)}$$

where f_{CSFVOL}, f_{GMVOL}, $f_{NAWMVOL}$, and f_{WMLVOL} are the within-VOI fractions (determined by the present study's segmentation) of CSF, GM, NAWM, and WML, respectively, and D_{CSF}, D_{GM}, D_{NAWM}, and D_{WML} are the relative densities of MR-visible water in each compartment (literature-based values used in Gasparovic *et al.* (2009) [283]). f_{yVOL} and D_y are the within-VOI fractions and relative densities of MR-visible water in the compartment for which f_y is being calculated.

The attenuation factor for water (R_{H20}) in the VOI was calculated:

$$R_{H_20} = f_{CSF} \times R_{H_20_{CSF}} + f_{GM} \times R_{H_20_{GM}} + f_{NAWM} \times R_{H_20_{NAWM}} + f_{WML} \times R_{H_20_{WML}} \quad \textbf{(5)}$$

The relaxation attenuation factor for each metabolite (R_M) was also calculated:

$$R_M = (1 - exp(-TR/T1_M) \times (exp(-TE/T2_M) \quad \textbf{(6)}$$

where $T1_M$ and $T2_M$ are the relaxation times of metabolite M. $T2_M$ was estimated using literature-based values: $T2_{NAA}$ = 0.309 s [289], $T2_{glutamate}$ = 0.180 s [289], $T2_{GABA}$ = 0.088 s [290], and $T2_{macromolecule}$ = 0.040 s [290]. Since $T1_M$ is negligible when TR is long [84], a value of 1 was assigned to this parameter for all metabolites.

To account for differences in metabolite concentrations between GM and WM, we used a method adapted from that proposed by Harris *et al.* (2015) [284]. The tissue correction factor for each metabolite (T_M) was calculated using the formula:

$$T_M = \frac{1}{f_{GM_{VOL}} + \alpha f_{WM_{VOL}}} \times \frac{\mu_{GM} + \alpha\mu_{WM}}{\mu_{GM} + \mu_{WM}} \quad \textbf{(7)}$$

where μ_{GM} and μ_{WM} are the averaged GM and WM (i.e. NAWM + WML) fractions of healthy participants for the VOI, respectively. α is the WM:GM ratio of metabolite M. Since it has been

estimated that GABA is ~7x greater in GM than in white matter and glutamate is ~2x greater in GM than WM [291], an α of 0.14 (i.e. 1/7) and 0.5 (i.e. 1/2) were assigned to calculate T_{GABA} and $T_{glutamate}$, respectively. An α of 1 was assigned to correct concentrations of NAA and macromolecules, since these metabolites are similarly concentrated in grey and white matter [291-293] (i.e. regular CSF-correction applied).

The final corrected concentration of each metabolite ($[M]_{Cor}$) in each VOI was calculated:

$$[M]_{Cor} = \frac{S_M}{S_{H_20}} \times \frac{\#H_{H_20}}{\#H_{met}} \times [H_20] \times \frac{R_{H_20}}{R_M} \times T_M \quad \textbf{(8)}$$

where S_M/S_{H20} is the ratio between the resonance signal of metabolite *M* and unsuppressed water signal. $\#H_{H2O}$ is the number of water protons (i.e. 2), $\#H_{met}$ is the number of equivalent protons that contribute to the resonance of the singlet used for water scaling (i.e. 3 for NAA singlet at ~2.01 ppm), and $[H_20]$ is the ^{1}H-MRS-visible water concentration in the VOI, which was estimated to be 55510 mM [82]. We report metabolite levels in institutional units (iu).

Data were excluded from further analysis if GABA levels were undetectable or if data were identified during visual inspection as being of unacceptable quality, for example due to subject motion or subscapular lipid signal contamination (sensorimotor VOI: four multiple sclerosis and four control participants excluded; parietal VOI: four multiple sclerosis and three control participants excluded). Cramér-Rao lower bounds (CRLB) of glutamate, NAA, and macromolecule concentrations were ≤ 5 in each VOI for all participants. An arbitrary CRLB threshold for GABA was not used as a sole determinant to exclude data.

Statistical analyses

Descriptive analyses were conducted to characterize the sample on demographic, clinical, performance-based, and neuroimaging-based variables. Chi-squared tests were used to compare groups on categorical variables. Between-group comparisons on normally distributed and non-normally distributed continuous variables were assessed with unpaired t-tests and Mann-Whitney U tests, respectively.

Age was included as a covariate in general linear models due to the large age-range of participants in the sample and evidence that age may influence ^{1}H-MRS measures of metabolite levels [294, 295]. General linear models were used to compare age-adjusted metabolite concentrations between healthy control and multiple sclerosis groups. For models involving GABA, we performed additional analyses adjusting for HADS score, as depression and anxiety are associated with GABA deficits [270, 271]. Due to previous reports that sensorimotor GABA may be altered in opposite directions in individuals with relapsing-remitting and progressive forms of multiple sclerosis [96, 97], we performed additional three-group ANCOVA models (adjusting for age and HADS score) to assess if GABA concentration differed between these subgroups.

Among multiple sclerosis participants, multiple linear regression analyses were performed to assess relationships between structural MTR in the VOI (assessed separately for grey and white matter regions to avoid issues of multicolinearity) and metabolite concentrations, adjusting for age. Significance was adjusted to account for multiple comparisons (i.e. four separate models due to two VOIs (sensorimotor, parietal) and two regions MTR was measured in (grey matter, white matter) using a Bonferroni correction. We additionally performed the same multiple linear

regression analyses as above substituting whole-brain MTR for within-VOI measures as a predictor of metabolite level. One-tailed paired t-tests were performed to determine if the strength of partial correlations between MTR and levels of metabolites were stronger when MTR was measured within the VOI compared to globally. The relationship between MTR within the VOIs and metabolite levels across all participants (healthy and multiple sclerosis) were also plotted and assessed with Pearson correlation analyses.

As a primary clinical outcome, the 9HPT was chosen such that we could investigate previously reported relationships between fine motor impairment and sensorimotor neurotransmitter levels that appear to be conflicting (*see Introduction*). The PASAT was also chosen as a primary outcome because it is sensitive to working memory, attention, and processing speed deficits [296], and neuroimaging studies on multiple sclerosis have suggested that the parietal region is one of the brain regions implicated in PASAT performance [266-268]. The extent to which neurometabolite concentrations (GABA, glutamate) predicted performance (9HPT, PASAT) was assessed with linear regression models (adjusting for age). We performed additional models also adjusting for normalized brain volume, regional total NAA and regional MTR to help account for the impact of local neuronal health and myelination on performance. Further exploratory analyses were also performed to investigate relationships between regional neurotransmitter levels and other assessments typical in the study of multiple sclerosis: SDMT, T25FW, EDSS. These analyses were intended to complement and inform the results obtained for our *a priori* specified outcomes.

4.4 Results

Participant characteristics

Demographic, clinical, and whole brain structural MRI characteristics of participants are displayed in Table 5.1. Healthy control and multiple sclerosis groups did not differ significantly in age or sex. Compared to healthy controls, multiple sclerosis participants performed significantly worse on all of the clinical tests, and also had lower normalized brain volume, and lower MTR within grey matter and white matter regions.

Metabolite concentrations and MTR in VOIs

Table 5.2 displays the corrected metabolite concentrations as well as structural information on the sensorimotor and parietal VOIs. For both VOIs, levels of total NAA and glutamate of multiple sclerosis participants were significantly lower than those of healthy volunteers. Macromolecule levels were significantly lower among multiple sclerosis participants in the sensorimotor VOI, but differences did not reach significance in the parietal VOI. Glutamine was not significantly different between groups in either VOI ($p > 0.1$). In contrast to the other metabolites, GABA concentration was higher among multiple sclerosis participants within the sensorimotor VOI ($p = 0.06$), a difference that reached significance when adjusting for HADS score in addition to age ($p = 0.02$).

TABLE 4.1 Participant characteristics.

Group	*Healthy control*	*Multiple sclerosis*
Demographic and clinical outcomes		
Number of subjects	21	47
Women, n (*%)* [a]	12 (57)	29 (62)
Age, mean *years* ± *SD* [b]	44 ± 14	49 ± 13
Clinical subtype (RRMS:SPMS:PPMS), n	--	27 : 14 : 6
Taking immunomodulatory medication, n (*%)*	--	21 (45)
EDSS, median *score* (Q1, Q3)	--	3.0 (2.0, 4.0)
HADS, median *score* (Q1, Q3) [b]	6 (3, 7)	11 (6, 14) ***
Performance-based outcomes, median (Q1, Q3)		
MSFC total, *z-score* [b c]	0.7 (0.3, 0.8)	-0.1 (-0.6, 0.5) ***
9HPT, *z-score* [b c]	1.1 (0.6, 1.4)	0.2 (-0.6, 0.7) ***
T25FW, *z-score* [d]	0.5 (0.5, 0.5)	0.4 (0.2, 0.4) ***
PASAT, *z-score* [d]	0.6 (0.0, 0.8)	-0.3 (-1.5, 0.6) *
SDMT, *score* [b c]	58 (51, 64)	50 (44, 61) *
Structural MRI outcomes, median (Q1, Q3)		
Normalized brain volume, cm^3 [b]	1562 (1515, 1597)	1452 (1369, 1512) ***
Normalized T2w white matter lesion volume, cm^3 [d]	0 (0, 0)	7.1 (2.2, 19.8) ***
Grey matter MTR, *pu* [b]	36.5 (36.1, 36.8)	36.0 (35.4, 36.2) **
NAWM MTR, *pu* [b]	44.1 (43.8, 44.3)	43.3 (42.6, 43.9) ***
Lesion MTR, *pu*	--	39.2 (37.7, 41.1)

Asterisks indicate significant differences between healthy control and multiple sclerosis groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Superscript letters indicate the statistical test performed to compare healthy and multiple sclerosis participant groups and other relevant information: [a] chi-squared test; [b] unpaired t-test (one-tailed); [c] data missing from one participant; [d] Mann-Whitney U-test (one-tailed).
SD = standard deviation; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; PPMS = primary progressive multiple sclerosis; Q1 = lower quartile; Q3 = upper quartile; -- = not applicable; EDSS = Expanded Disability Status Scale; HADS = Hospital Anxiety and Depression Scale; MSFC = Multiple Sclerosis Functional Composite; 9HPT = 9-Hole Peg Test; T25FW = Timed 25 ft Walk; PASAT = Paced Auditory Serial Addition Test; SDMT = symbol digit modalities test; MTR = magnetization transfer ratio; NAWM = normal appearing white matter; pu = percent units

TABLE 4.2 Neurochemical levels and structural characteristics of VOIs.

	Sensorimotor VOI		**Parietal VOI**	
	Healthy control	*Multiple sclerosis*	*Healthy control*	*Multiple sclerosis*
N (% female)	18 (50)	44 (59)	19 (63)	42 (60)
Corrected concentrations (*iu*), *mean ± SE* [a]				
GABA	0.44 ± 0.07	0.60 ± 0.04 [†]	0.59 ± 0.05	0.54 ± 0.03
Glutamate	10.8 ± 0.21	9.9 ± 0.14 **	12.2 ± 0.26	11.4 ± 0.18 *
Total *N*-acetylaspartate	21.2 ± 0.47	19.4 ± 0.30 **	21.2 ± 0.42	19.9 ± 0.28 **
Macromolecules	14.2 ± 0.27	13.2 ± 0.17 **	14.0 ± 0.27	13.6 ± 0.18
Structural composition				
Grey matter MTR (pu), *mean ± SD* [b]	36.3 ± 0.7	35.9 ± 1.3	36.6 ± 0.7	35.9 ± 1.3 *
White matter MTR (pu), *median (Q1, Q3)* [c]	43.9 (43.6, 44.3)	43.1 (42.0, 43.8) ***	43.8 (43.6, 44.5)	42.9 (41.6, 43.7) ***
Lesion (% of VOI), *median (Q1, Q3)*	--	1.3 (0.5, 6.7)	--	2.4 (0.2, 5.5)

Asterisks indicate significant differences between healthy control and multiple sclerosis groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
[†] Indicates between group differences reached significance only after adjusting for HADS score. N denotes the number of subjects with data for the VOI after data quality exclusions. Superscript letters indicate the statistical test performed to compare healthy and multiple sclerosis participant groups: [a] ANOCVA; [b] unpaired t-test (1-tailed); [c] Mann-Whitney U-test (1-tailed)
VOI = volume of interest; HC = healthy control; MS = multiple sclerosis; SE = standard error; SD = standard deviation; Q1 = lower quartile; Q3 = upper quartile; -- = not applicable; ^{1}H-MRS = proton magnetic resonance spectroscopy; iu = institutional units; GABA = γ-aminobutyric acid; MTR = magnetization transfer ratio

Three-group ANCOVA analysis separating multiple sclerosis participants into relapsing-remitting and progressive (i.e. primary and secondary progressive subtypes combined) groups based on clinical course revealed that the sensorimotor GABA increase in multiple sclerosis participants was primarily driven by those with the relapsing-remitting subtype (GABA control: 0.41 ± 0.08 iu, relapsing-remitting multiple sclerosis: 0.64 ± 0.06 iu, progressive multiple sclerosis: 0.57 ± 0.08 iu), as post-hoc assessments found sensorimotor GABA levels of relapsing-remitting multiple sclerosis participants differed significantly from healthy controls ($p = 0.02$), while other between-group contrasts did not reach significance ($p > 0.1$). GABA did not differ between groups in the parietal VOI even when HADS score was included as a covariate (p

> 0.05). In white and grey matter of both VOIs, mean MTR was lower for multiple sclerosis participants than controls (Table 5.2).

Relationships between MTR and metabolite level

Assessing all healthy and multiple sclerosis participants as a single group, Pearson correlations found regional MTR (average of mean MTR in white matter and mean MTR in grey matter of the VOI) was significantly related to lower glutamate and lower total NAA level in both VOIs, as well as higher GABA level in the sensorimotor VOI (Figure 4.2). Among only healthy participants, however, age-adjusted MTR in white matter and grey matter regions were not significantly related to regional total NAA, GABA, nor glutamate level in either VOI ($p > 0.05$).

Age-adjusted relationships between MTR within the VOI and the corrected concentrations of the metabolites of interest among multiple sclerosis participants alone are shown in Table 5.3. Lower within-VOI grey matter MTR and white matter MTR were strongly correlated with lower total NAA level among multiple sclerosis participants in both VOIs. Lower glutamate levels were significantly related to lower white matter MTR for the parietal VOI. Higher sensorimotor GABA level was significantly correlated with lower within-VOI grey matter MTR. No other relationships reached significance after multiple comparison correction. Across metabolites and VOIs, partial correlations of the age-adjusted relationships between MTR and metabolite levels were stronger when MTR was assessed within the VOI compared to globally for both white matter (within-VOI: mean $|r| \pm SD = 0.37 \pm 21$ vs. whole brain: mean $|r| \pm SD = 0.30 \pm 21$; $p =$

0.02) and grey matter (within-VOI: mean |r| ± SD = 0.36 ± 23 vs. whole brain: mean |r| ± SD = 0.30 ± 25; p = 0.01) regions.

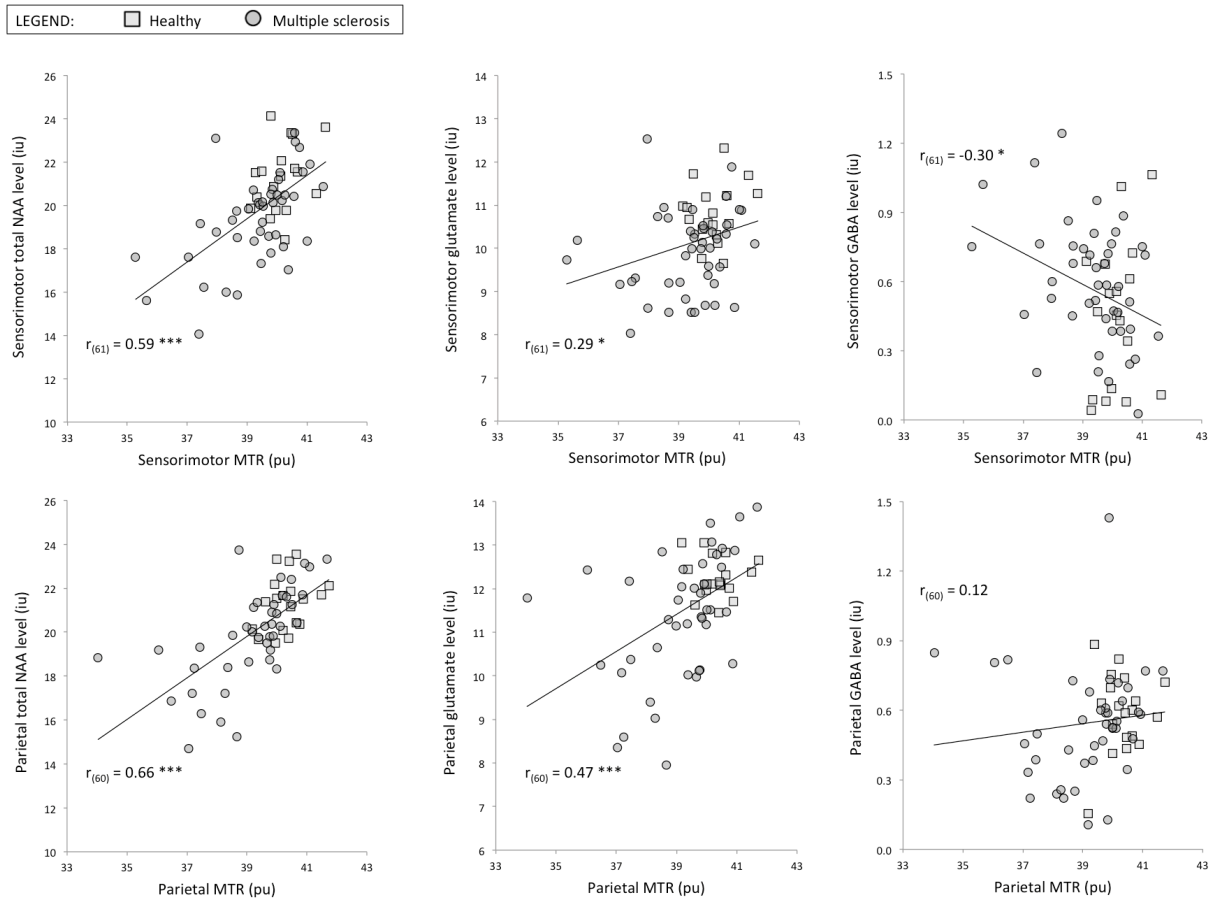

FIGURE 4.2 Relationships between MTR within the regions that the chemical spectra was obtained and metabolite concentrations of people with multiple sclerosis and healthy individuals. Pearson correlation coefficients (r) are indicated on each plot. Significant relationships (two-tailed) are indicated with asterisks (* $p < 0.05$, *** $p < 0.001$).

TABLE 4.3 Relationships between MTR within the regions that the chemical spectra was obtained and metabolite concentrations of people with multiple sclerosis.

VOI	Metabolite	*Grey matter MTR*	*White matter MTR*
Sensorimotor	GABA	-0.45 *	-0.31
	Glutamate	0.07	0.24
	Total NAA	0.55 ***	0.54 ***
Parietal	GABA	0.09	0.04
	Glutamate	0.37	0.44 *
	Total NAA	0.63 ***	0.63 ***

Partial correlations of the relationship between mean regional MTR and metabolite level, adjusting for age, are shown. Significant relationships (two-tailed, corrected for multiple comparisons) are indicated with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

VOI = volume of interest; GABA = γ-aminobutyric acid; MTR = magnetization transfer ratio; NAA = *N*-acetylaspartate

Relation to clinical outcomes

Multiple regression analyses investigating the relationships between GABA and glutamate levels and performance (adjusting age, total NAA, MTR, and normalized brain volume) are shown in Tables 5.4 and 5.5. Higher GABA level, total NAA level, and MTR measured from the sensorimotor VOI were found to be significant predictors of better 9HPT score (Table 5.4). Outcomes of additional exploratory analyses with alternative dependent variables (T25FW, EDSS, SDMT, PASAT) found higher sensorimotor GABA to also be linked to better EDSS and SDMT scores (Table 5.4), while no other models showed a significant link between GABA or glutamate levels and performance. Although our preliminary investigations found parietal glutamate to be a significant predictor of PASAT and SDMT performance (but not 9HPT score)

when adjusting for only regional GABA level and age (Table S1), this relationship did not remain significant when MTR, total NAA, and normalized brain volume were included as covariates (Table 5.5). Similar models performed on the data obtained from healthy volunteers found neither glutamate nor GABA in either VOI were predictive of normal variability on any of the performance-based outcomes (all $p > 0.05$).

Table 4.4 Relationships between neurometabolite levels in the sensorimotor VOI and clinical function of participants with multiple sclerosis adjusting for markers of structural integrity and age.

Dependent variable	Model summary	Predictor	r	*p*
9HPT	$R^2 = 0.40$ $F_{6,42} = 3.95$ $p = 0.004$	GABA	0.423	0.008
		Glutamate	-0.156	0.351
		Total NAA	0.381	0.018
		MTR	0.387	0.016
		NBV	-0.196	0.238
		Age	-0.243	0.141
PASAT	$R^2 = 0.26$ $F_{6,43} = 2.18$ $p = 0.07$	GABA	0.274	0.091
		Glutamate	-0.198	0.227
		Total NAA	0.363	0.023
		MTR	0.172	0.294
		NBV	-0.023	0.889
		Age	-0.039	0.816
T25FW	$R^2 = 0.30$ $F_{6,41} = 2.56$ $p = 0.04$	GABA	0.276	0.098
		Glutamate	-0.274	0.100
		Total NAA	0.230	0.171
		MTR	0.161	0.341
		NBV	0.210	0.211
		Age	-0.167	0.322
SDMT	$R^2 = 0.52$ $F_{6,43} = 6.59$ $p < 0.001$	GABA	0.454	0.004
		Glutamate	-0.324	0.044
		Total NAA	0.433	0.006
		MTR	0.200	0.221
		NBV	0.279	0.085
		Age	-0.226	0.167
EDSS	$R^2 = 0.27$ $F_{5,43} = 2.31$ $p = 0.05$	GABA	-0.342	0.033
		Glutamate	0.148	0.367
		Total NAA	-0.168	0.306
		MTR	-0.136	0.411
		NBV	-0.074	0.655
		Age	0.291	0.072

r = partial correlation; VOI = volume of interest; GABA = γ-aminobutyric acid; MTR = magnetization transfer ratio; NAA = *N*-acetylaspartate; NBV = normalized brain volume; 9HPT = nine hole peg test; PASAT = Paced Auditory Serial Addition Test; T25FW = timed 25-ft walk; EDSS = Expanded Disability Status Scale; SDMT = symbol digit modalities test

Note: Higher scores for 9HPT, PASAT, T25FW, and SDMT indicaate better performance, whereas higher scores on the EDSS indicate worse disability. For the model with T25FW as the dependent variable, two outliers were removed such that normally distributed residuals could be obtained.

Table 4.5 Relationships between neurometabolite levels in the parietal VOI and clinical function of participants with multiple sclerosis adjusting for markers of structural integrity and age.

Dependent variable	Model summary	Predictor	r	*p*
9HPT	$R^2 = 0.31$ $F_{6,41} = 2.62$ $p = 0.033$	GABA	-0.147	0.386
		Glutamate	-0.102	0.548
		Total NAA	0.292	0.079
		MTR	0.248	0.139
		NBV	-0.183	0.278
		Age	-0.184	0.276
PASAT	$R^2 = 0.39$ $F_{6,41} = 3.74$ $p = 0.006$	GABA	0.122	0.472
		Glutamate	0.040	0.816
		Total NAA	0.336	0.042
		MTR	0.266	0.111
		NBV	-0.265	0.113
		Age	0.095	0.574
T25FW	$R^2 = 0.33$ $F_{6,41} = 2.82$ $p = 0.024$	GABA	-0.062	0.716
		Glutamate	-0.123	0.469
		Total NAA	0.064	0.708
		MTR	0.216	0.200
		NBV	0.105	0.537
		Age	-0.352	0.033
SDMT	$R^2 = 0.53$ $F_{6,41} = 6.52$ $p < 0.001$	GABA	0.029	0.867
		Glutamate	-0.124	0.466
		Total NAA	0.326	0.049
		MTR	0.235	0.162
		NBV	0.088	0.605
		Age	-0.227	0.176
EDSS	$R^2 = 0.23$ $F_{6,41} = 3.05$ $p = 0.017$	GABA	0.071	0.675
		Glutamate	-0.001	0.996
		Total NAA	0.136	0.422
		MTR	-0.354	0.032
		NBV	0.055	0.746
		Age	0.502	0.002

r = partial correlation; VOI = volume of interest; GABA = γ-aminobutyric acid; MTR = magnetization transfer ratio; NAA = *N*-acetylaspartate; NBV = normalized brain volume; 9HPT = nine hole peg test; PASAT = Paced Auditory Serial Addition Test; T25FW = timed 25-ft walk; EDSS = Expanded Disability Status Scale; SDMT = symbol digit modalities test

Note: Higher scores for 9HPT, PASAT, T25FW, and SDMT indicate better performance, whereas higher scores on the EDSS indicate worse disability.

4.5 Discussion

By assessing GABA and glutamate levels in a clinical population that is highly heterogeneous in structural damage and symptoms, this study supports that levels of these neurometabolites in the human brain may have regionally specific relationships to microstructural integrity and behavioral abilities. Moreover, this manuscript highlights factors important to consider when analyzing and interpreting ^{1}H-MRS-measured metabolite levels, including in the presence of neurological disease.

Neurometabolite concentrations and MTR

To accurately estimate metabolite levels in the presence of neurological injury, our protocol was optimized to account for potential confounding factors such as macromolecule contamination and relaxation effects. We further assessed how metabolite concentrations are related to MTR, an indicator of microstructural damage even within brain tissue that appears normal when visualized with T1- and T2-weighted MRI [144, 214, 231]. Lower total NAA, a marker of neuronal health [152], was related to lower regional MTR, confirming that these measures likely reflect related pathological phenomena. Demonstrating the regional specificity of microstructural damage on local metabolite levels detected with ^{1}H-MRS, MTR within the regions from which the chemical spectra were obtained proved to be a stronger predictor of metabolite levels than MTR assessed globally.

Glutamate concentration was abnormally low in both VOIs for multiple sclerosis participants. Moreover, relationships between lower glutamate and lower MTR were observed, suggesting that microstructural damage may be linked to pathologically low glutamate levels.

Notably, this finding is consistent with research on the cuprizone mouse model of human multiple sclerosis, which found that decreases in both glutamate and NAA co-occur with demyelination [154, 155]. It is possible that microstructural damage, such as demyelination, could have also contributed to previously observed glutamate level decreases over time in white matter of people with secondary progressive multiple sclerosis [95]. Of further interest, Gautier *et al.,* (2015) demonstrated in an *in vivo* remyelination model that glutamate is involved in signaling to instruct the differentiation of oligodendrocyte progenitor cells into myelinating glial cells [110], thus suggesting that low glutamate could impede the remyelination healing process. Together, these studies raise the non-mutually exclusive possibilities that glutamate levels may be low because myelin is damaged, or that remyelination may be prevented because of low glutamatergic neural transmission. Considering that the MTR signal can also be influenced by other aspects of multiple sclerosis such as axonal damage [260], it is also possible that disease-related factors other than myelin content may have contributed to the observed relationship between MTR and glutamate level.

In contrast to glutamate, GABA concentration was relatively high among multiple sclerosis participants within the sensorimotor (though not the parietal) VOI, suggesting a regional increase in this neurometabolite due to the disease. This result differs from that of a study by Bhattacharyya *et al.,* (2013), which did not find significant between-group differences in sensorimotor GABA concentration in a comparatively small sample of relapsing-remitting multiple sclerosis participants (n = 13) and healthy controls (n = 10) [96]. Moreover, our results are in contrast to the conclusions of Cawley *et al.* (2015), suggesting that sensorimotor GABA concentration is abnormally low among individuals with secondary progressive multiple sclerosis [97]. As further examination of our data revealed that participants with the relapsing-

remitting, rather than the progressive, form of multiple sclerosis primarily drove the observed GABA level increase, difference in the clinical cohorts assessed is the most likely reason for the between-study differences in GABA level outcomes. Of further note, the present study took additional steps to adjust for other factors that could confound measurements of metabolite concentrations in the context of neurological disease including relaxation time abnormalities in normal appearing brain tissue and lesions [258, 297], age [295], anxiety/depression [270, 271], and the fact that GABA concentration is many times greater in grey matter compared to white matter [284, 291].

Various non-mutually exclusive mechanisms may have contributed to the elevation in GABA level observed in the sensorimotor region of multiple sclerosis participants. Notably, GABA and its receptors exist not only in neurons and glia [298], but also in immune cells [220]. The immunosuppressant and anti-inflammatory properties of GABA have been demonstrated in studies on immune cells of multiple sclerosis patients and animal models of neuroinflammatory disease [218, 220, 299-301]. Thus, higher GABA could reflect a corrective response aimed at mitigating inflammation. Another possibility is that the regionally elevated GABA levels may reflect increased 'tonic' inhibition occurring to mitigate hyperexcitability. Such a mechanism has previously been proposed as an explanation for a similar regional GABA level increase in people with Tourette's syndrome, as somatosensory GABA level correlated negatively with both cortical excitability and the functional MRI blood-oxygen-level dependent response among people with this condition [302].

Of further importance, explanations for the abnormal GABA elevation observed here must take into account the regional specificity of the finding, i.e., the between-group difference was observed in the sensorimotor, but not the parietal, VOI. Therefore, the cause of the GABA

increase would likely have regional specificity. Considering that total NAA level and MTR were decreased in both regions, it seems unlikely that GABA was increased regionally as a direct response to neuronal health or structural damage. One possibility is that the presence of disease-related factors not assessed in the present study (e.g. markers of active inflammatory lesions requiring more invasive methods) could have disproportionately affected one of the VOIs in the present cohort, inducing a relatively localized GABAergic response. Another possibility is that the regional increase occurred because of a specific compensatory strategy of the brain to increase GABA locally rather than on a global level. Regional GABA level increases likely have specific downstream effects on connected neural pathways, considering evidence that local neurotransmitter levels are linked to functional connectivity within its associated network, though not globally [303]. Interestingly, our subsequent analysis highlighted the potential clinical benefit of higher sensorimotor (though not parietal) GABA.

Neurometabolite levels and clinical outcomes

We further investigated the relationships between regional GABA and glutamate concentrations and performance on tests of fine motor and cognitive function of people with multiple sclerosis. While our initial analyses suggested glutamate level in the parietal region to be related to cognitive ability, our later multimodal analyses found that its impact was not independent of markers of structural damage (see *Appendix I* for discussion). Interestingly, higher sensorimotor GABA concentration was found to be significantly related to better 9HPT performance among multiple sclerosis participants when adjusting for glutamate level, total NAA level, MTR, normalized brain volume, and age. This indicates that elevated GABA could

be associated with a neurophysiological process that mitigates the impact of pathological factors that would otherwise cause disability. While our findings are consistent with a report by Cawely *et al.* (2015) from secondary progressive multiple sclerosis participants [97], they contrast with evidence from studies on other populations suggesting that motor learning and motor symptom recovery are supported by a reduction of GABA [3, 191, 194, 206]. Thus, the benefit of higher GABA level in the presence of multiple sclerosis likely occurs by a mechanism that is independent, and even overrides the benefit, of the GABAergic suppression that has been shown to support motor learning. Of note, *in vitro* and animal-based research has revealed that GABAergic activity can reduce the negative impact of disease-mediating processes implicated in multiple sclerosis, such as neuroinflammation [218, 299, 300] or excitotoxicity [225, 226]. In the current study, exploratory regression analyses also showed a similar relationship between higher sensorimotor GABA level and performance on other tests of disability (i.e. EDSS and SDMT), suggesting elevated GABA or mechanisms associated with it may have a more generalized beneficial impact beyond the motor system. We could not rule out the possibility that GABA may have been similarly altered in other clinically relevant brain regions which we had not selected to assess *a priori* for the present study.

As we unravel the mechanism by which regional glutamate and GABA may influence behavior outcomes, it is important to remember that individual regions are part of larger, interconnected neural networks that can become altered in the presence of disease. Considering evidence of neural network dysfunction occurring in the multiple sclerosis (e.g. [304-307]), it is notable that there is building evidence that GABA and glutamate are linked to functional neural activity in the human brain (for review see Duncan *et al.,* (2014) [73]). Thus, regional levels of these neurometabolites may influence activity within associated neural networks, which

ultimately contributes to specific behavioral outcomes or clinical disability of people with multiple sclerosis.

Study limitations and future directions

Our methodology was designed to optimize accurate measurement of GABA levels at 3T, including in the presence of neurological disease. Limitations beyond those mentioned in the previous sections remain. Metabolites were measured from relatively large VOIs, preventing us from separately assessing metabolite concentrations in grey matter, normal appearing white matter, and lesions. In future work, others may also consider directly measuring, rather than modeling, the macromolecule contribution [70], as well as measuring relaxation times and water density in individual participants as opposed to estimating these parameters using literature-based values and pseudo-T2. However, as doing so would require lengthening the scanning time of an already long protocol, this may be undesirable particularly in studies involving clinical populations that suffer from fatigue, such as multiple sclerosis [274]. It should also be noted that the present study's heterogeneous cohort was of a modest sample size, and therefore outcomes of highly multivariate analyses that did not reach significance should be considered carefully and may warrant further investigation in larger-scale studies.

Appropriate interpretation of metrics derived from magnetic resonance spectroscopy and imaging studies is a general challenge in the field. The extent to which GABA and glutamate measured with ^{1}H-MRS reflect their roles in neurotransmission as opposed to other functions remains unclear [72, 73]. In regards to structural MRI, several histological studies suggest that MTR is reflective largely of myelination [144, 214, 261]. However, this metric is also sensitive

to other aspects of the disease such as axonal damage and inflammation [260]. Future studies may also consider comparing the findings with MTR to that using alternative imaging markers of myelin (e.g. diffusion tensor imaging, myelin water fraction) [260] or cortical integrity (e.g. cortical lesion load). Such studies should consider using higher magnetic field strengths, as this would enable better resolution of metabolite signals and cortical damage and/or shorten data acquisition times [265, 308, 309]. Longitudinal research assessing the extent to which GABA and glutamate levels change with not only structural integrity, but also with markers of inflammation (e.g. gadolinium-enhancing lesions [310]), would further inform our understanding of their involvement across the disease course.

4.6 Conclusions

The novel multimodal analyses presented here informs our understanding of the complex interplay between GABA and glutamate, microstructural brain damage, and behavior. Using non-invasive methods, we provide evidence from humans that markers of local myelination and specific behavioral abilities are linked to regional neurometabolite levels. Moreover, this work highlights that myelin and macromolecule levels, among other factors, are important to consider when analyzing GABA and glutamate levels assessed with ^{1}H-MRS. As such, the methodological and theoretical contributions of this work greatly contribute and enrich emerging theory on the pathophysiology of multiple sclerosis with respect to GABA and glutamate involvement. This could support the development and assessment of new therapeutic strategies involving glutamate or GABA modulation to treat symptoms of multiple sclerosis and related diseases.

Chapter 5

Conclusions

Preface

Together, the three novel studies presented in this dissertation make up an important body of work that provides insight into how glutamate and GABA are implicated in radiological and clinical features of multiple sclerosis. The preceding chapters of this manuscript-based thesis provided in-depth discussions regarding interpretations of study-specific outcomes, the importance and limitations of each investigation, as well as recommendations for future work. In this concluding chapter, the overarching research questions (outlined in **Chapter 1**, *Section 1.5*) are directly addressed with respect how these research studies have, as a whole, provided answers.

5.1 Answers to overarching research questions

Question 1

The primary research question asked the extent to which markers of glutamate and GABA (activities and endogenous levels) reveal abnormalities among those with clinically-defined sub-forms of multiple sclerosis and healthy people. In **Chapters 2** and **3**, TMS was used as a tool to probe information on intracortical excitability, specifically focusing on biomarkers that have been linked to activity at receptors for glutamate and GABA. **Chapter 2** specifically assessed patients with relapsing-remitting multiple sclerosis, dividing patient groups by whether or not they experienced motor impairment during the clinical remission phase. Differences between people with relapsing-remitting multiple sclerosis to those with primary progressive and secondary progressive multiple sclerosis were next addressed in **Chapter 3**. ^{1}H-MRS was used in **Chapter 4** to study endogenous levels of glutamate and GABA within sensorimotor and parietal regions of the left hemisphere between multiple sclerosis patients of various clinical subtypes.

The hypothesis that differences would be found between these groups in our TMS- and ^{1}H-MRS markers of interest was generally supported. With respect to markers of GABA activity and endogenous levels, these abnormalities depended on the type of intracortical inhibition and the region of the brain assessed, respectively. The most interesting novel finding was the regional elevation in sensorimotor GABA level, which was driven by those with a relapsing-remitting, rather than progressive, form of multiple sclerosis. There was also some evidence of CSP lengthening (indicative of increased inhibition) among those with the relapsing-remitting subtype, although this was limited to participants with fine motor impairment that persisted into

the clinical remission phase. Among those with progressive forms of multiple sclerosis, abnormalities in GABA level and CSP were not observed. However, SICI was found to be abnormally low among persons with secondary progressive multiple sclerosis, consistent with previous reports [66-68]. For technical reasons discussed in **Chapter 3**, findings with LICI were inconclusive, although recent evidence from others suggest that LICI is abnormally low in patients with progressive forms of multiple sclerosis, a factor that could be normalized with a medication given for spasticity [68]. Taken together, there appears to be an overall decrease in inhibition among those with progressive multiple sclerosis relative to clinical remission phases of the relapsing-remitting form of this disease. As discussed in the preceding chapters, this could indicate that certain forms of inhibition become augmented in response to pathological aspects generally dominating in the earlier part of the disease, such as inflammation, whereas factors more closely linked to progressive disease processes may drive inhibition deficits.

Markers of excitatory brain activity and glutamate level were also investigated. Abnormalities in ICF were not observed among participants with multiple sclerosis in any of the clinically-defined sub-groups assessed. Nonetheless, glutamate level was abnormally low in both brain regions from which ^{1}H-MRS was collected among people with multiple sclerosis with both relapsing-remitting and progressive disease subtypes. These findings were generally consistent with previous research on multiple sclerosis [66, 67, 69, 92-94], and confirmed the discordance between information gained about glutamate with ^{1}H-MRS and TMS for the first time in largely overlapping multiple sclerosis patient groups. Overall, more evidence was found for markers of inhibition, or GABA, in discriminating between clinically-defined subgroups of people with multiple sclerosis than that of glutamate, indicating that GABA-related biomarkers may have greater potential clinical utility as disease biomarkers for multiple sclerosis.

Question 2

All novel studies of this thesis aimed, in different ways, to evaluate the extent to which radiologically-derived markers of structural brain damage could explain variability in the TMS- or ^{1}H-MRS metrics of interest. Overall, evidence supported the hypothesis that abnormalities observed with these metrics in some people with multiple sclerosis (identified in *Question 1*) may be attributed, at least in part, to types of brain damage detectable with non-invasive neuroimaging methods. For example, both glutamate and GABA levels correlated with MTR, a marker of microstructural integrity such as myelination [139-146]. Further, CSP prolongation, observed in some relapsing-remitting patients, was linked to higher volumes of white matter brain lesions [113-115].

A few notable expectations to this rule were observed. While it has been proposed by others that SICI abnormality in progressive forms of the disease is driven by intracortical damage [66, 67], direct evidence of this was not found. Specifically, correlations between SICI and cortical volume, as well as between SICI and cortical MTR, did not reach significance among participants with progressive multiple sclerosis. Further, CSP was not abnormal relative to controls in progressive multiple sclerosis participants, yet, this variable correlated directly with cortical MTR in this patient group. This suggested that in those with progressive forms of multiple sclerosis, cortical microstructural damage may counter factors that would otherwise lead to the observation of CSP prolongation. Both of these unexpected findings further emphasized the need for advanced multimodal imaging to appropriately interpret TMS-based markers of intracortical inhibition, particularly in the presence of neurological disease.

Question 3

The final question investigated was the extent to which markers of glutamate and GABA activity and level could explain variability in clinical function, particularly motor ability, when accounting for the impact of structural damage. All studies found evidence that certain TMS- or ^{1}H-MRS-derived variables are significant predictors of performance on a test of upper limb motor dexterity when including MRI-derived markers of structural brain damage in the model as potentially confounding covariates.

Analyses that included TMS data demonstrated that when accounting for various neuroimaging metrics (including markers of lesions, atrophy, and microstructural damage) longer CSP (indicative of higher intracortical inhibition [39]) predicted worse motor impairment. By contrast, ^{1}H-MRS analyses revealed that higher GABA level in the sensorimotor region was linked to better performance on the same test of motor ability when adjusting for markers of structural damage. While both findings demonstrate the functional impact of these metrics beyond structural imaging outcomes, the striking differences in directionality of clinical correlates indicate that the TMS-derived CSP and ^{1}H-MRS measures of GABA level reflect very different physiological entities. As discussed within their respective chapters of focus, it is possible that high activity of GABA indicated by CSP prolongation may prevent motor learning and other forms of plasticity [3, 193, 194, 208]. Higher GABA level may, by contrast, reflect extrasynaptic GABAergic tone rather than synaptic neurotransmitter activity [78, 311]. Potentially, higher GABA here could have beneficial effects through interactions with immune-mediated activity [218, 299, 300] or mitigating the impact of harmful excitotoxicity [225, 226].

Of note, further multimodal analyses combining these methods have confirmed that CSP and GABA level are not correlated among people with multiple sclerosis (see *Appendix I*), and that both remain independent predictors of 9HPT performance when included in the same multiple regression model.

Evidence indicating links between clinical disability and markers of glutamate level or activity was less strong. While lower parietal glutamate level was correlated with performance on a test of motor function, its impact was not independent from that of structural damage. Higher ICF was modestly linked to motor performance independently of structural damage, although this was only found when also including CSP in the model. Overall, markers of GABA intracortical activity and endogenous levels had the greatest clinical relevance, a factor of importance with respect to potential future applications of these non-invasive tools in treatment trials or clinical settings.

5.2 Conclusion and Impact

Using non-invasive multimodal investigations, the original research presented in this dissertation provides new evidence on how glutamate and GABA are implicated in neuroradiological and clinical features of multiple sclerosis. These studies deliver novel information on neurophysiological aspects of multiple sclerosis, particularly those relevant to upper limb motor function. Furthermore, these findings highlight important methodological considerations relevant to the analysis and appropriate interpretation of TMS and ^{1}H-MRS data, which likely has relevance across various neurological diseases. The research outcomes of this body of work may be hypothesis-driving for future studies investigating therapeutic strategies to

mitigate the severity of motor impairment in multiple sclerosis by targeting inhibition (e.g. through repetitive TMS or pharmaceutical interventions), and could contribute to the development of more optimal clinical tools that take advantage of multimodal evaluations.

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Appendix I

AI-1 Supplementary materials related to Chapter 3

Supplementary Methods

To replicate previous findings (e.g. [1-5]) while taking into account additional factors not fully considered in some other studies (i.e. age and sex; relevance to motor impairment vs. global disability; primary vs. secondary progressive MS), the following analyses were performed: ANCOVA compared groups on neurophysiological and performance-based outcomes (adjusting for age and sex), followed by Fisher's LSD post-hoc tests as needed. A series of hierarchical linear regression analyses assessed the extent to which TMS-based variables predicted performance (9HPT, MSFC, EDSS) independently of age and sex. Assumptions of ANCOVA and linear regression were assessed for each analysis, and the data were transformed or outliers were removed if necessary.

Supplementary Results

Age and sex-adjusted performance and neurophysiological outcomes are shown in Table 2. Performance differed between groups, with post-hoc tests finding performance of HC participants was better than relapsing-remitting MS participants, which was better than progressive MS participants on both the 9HPT and overall MSFC (ps < 0.05). EDSS was higher among progressive compared to relapsing-remitting MS participants. MEP latency (active muscles) was significantly longer among progressive MS participants compared to the other

groups (ps < 0.05), but no other between-group differences in the neurophysiological variables reached significance. Groups did not differ in maximum voluntary contraction (p > 0.05). Of the participants with LICI data collected, the mean ± standard error of HC, relapsing-remitting MS and progressive MS participants was 81 ±7, 75 ±9, and 63 ±10 %, respectively. However, we were not able to attain normally distrusted residuals required for a valid ANCOVA, even after data transformation of LICI. A Kruskal-Wallis H test (which did not take into account age and sex), however, did not find differences between groups to reach significance for LICI ($\chi 2(2) = 2.4$, $p > 0.05$).

As it has previously been reported that SICI is abnormally low among people with secondary progressive MS[3, 4], we considered that our null results might have been due to treating primary and secondary progressive participants as a single group (see Table 3 for outcomes of the separate subgroups). We therefore performed additional four-group ANCOVA analyses (i.e. HC vs. relapsing-remitting MS vs. primary progressive vs. secondary progressive), which found SICI ($F3,48 = 3.24$, $p < 0.05$) and log-transformed MEP amplitude ($F3,48 = 4.13$, $p < 0.05$), but not other TMS-based outcomes (ps > 0.05), to differ significantly between the four groups. Post-hoc assessment found secondary progressive MS participants had significantly lower SICI compared to HC ($p < 0.01$), relapsing-remitting MS ($p < 0.01$), and primary progressive MS participants ($p < 0.05$). MEP amplitude was significantly lower among secondary progressive MS participants compared to the healthy and relapsing-remitting MS groups (ps < 0.05). No other post-hoc contrasts reached significance.

Among all participants with MS, a model including CSP predicted 9HPT better than a model including age and sex alone ($R2$ change = 0.17, $F1,32$ change = 8.36, $p < 0.01$), with higher CSP predicting worse 9HPT score independently of age and sex ($\beta = -0.42$, $p = 0.007$).

This finding remained significant when additionally adjusting for time of TMS data collection. However, 9HPT score was not related to SICI, LICI, or ICF when adjusting for age and sex (ps > 0.05). None of the TMS-based biomarkers of intracortical inhibition or facilitation significantly predicted EDSS or total MSFC score better than age and sex alone among MS participants (all ps > 0.05). Among HC participants, inclusion of any of the TMS-based outcomes did not significantly improve the model to predict total MSFC score or 9HPT score beyond the impact of age and sex (all ps > 0.05).

AI-2 Supplementary materials related to Chapter 4

Supplementary Results

Multiple regression analyses investigating age-adjusted relationships between GABA and glutamate levels and performance-based outcomes of multiple sclerosis participants are shown in Table S.1. For both VOIs, GABA and glutamate levels were not significantly associated with 9HPT performance. Lower glutamate level in the parietal (though not the sensorimotor) VOI was significantly associated with worse PASAT performance. To confirm this outcome was similar when using an alternate measure of cognitive function, we repeated these analyses replacing PASAT with SDMT as the dependent variable. Consistently, lower parietal (though not sensorimotor) glutamate level was related to worse SDMT performance when adjusting for GABA level and age. However, the relationship between parietal glutamate

level and performance on the PASAT and SDMT did not remain significant when additionally including total NAA, MTR, and normalized brain volume into the model (Table 5.5).

TABLE S.1 Relationships between regional GABA and glutamate levels and performance of participants with multiple sclerosis.

VOI	Dependent variable	Model summary	Predictor	r	p
Parietal	9HPT	$R^2 = 0.11$ $F_{3,41} = 1.60$ $p = 0.21$	GABA	-0.24	0.15
			Glutamate	0.27	0.12
			Age	-0.12	0.49
	PASAT	$R^2 = 0.12$ $F_{3,41} = 2.77$ $p = 0.06$	GABA	-0.004	0.98
			Glutamate	0.46	0.008
			Age	0.20	0.23
	SDMT	$R^2 = 0.27$ $F_{3,41} = 4.70$ $p = 0.007$	GABA	-0.07	0.62
			Glutamate	0.40	0.02
			Age	-0.24	0.12
Sensorimotor	9HPT	$R^2 = 0.07$ $F_{3,42} = 0.97$ $p = 0.42$	GABA	0.02	0.89
			Glutamate	0.18	0.26
			Age	-0.16	0.33
	PASAT	$R^2 = 0.03$ $F_{3,42} = 0.46$ $p = 0.71$	GABA	-0.14	0.39
			Glutamate	0.13	0.43
			Age	-0.03	0.88
	SDMT	$R^2 = 0.11$ $F_{3,43} = 1.72$ $p = 0.18$	GABA	-0.06	0.71
			Glutamate	0.12	0.45
			Age	-0.30	0.06

r = partial correlation; VOI = volume of interest; GABA = γ-aminobutyric acid; MTR = magnetization transfer ratio; NAA = *N*-acetylaspartate; 9HPT = nine hole peg test; PASAT = Paced Auditory Serial Addition Test; SDMT = symbol digit modality test

Supplementary Discussion

While it has been reported previously that glutamate deficits in the parietal region are not related to visual or verbal memory of people with multiple sclerosis [6], we assessed participants on the PASAT: a test of cognitive function that is widely used in the field of multiple sclerosis and that is mainly sensitive to attention, working memory, and processing speed deficits [7]. The PASAT's sensitivity to practice effects [8] could theoretically confer an advantage on patients who have encountered the task previously; yet, the multiple sclerosis group as a whole still performed below the level of the healthy control group on the PASAT. Similarly, visual attention/processing speed measured with the SDMT was worse in the multiple sclerosis group compared to the normal controls. Our initial age-adjusted models found lower parietal (though not sensorimotor) glutamate level of multiple sclerosis participants was related to worse cognitive performance when adjusting for GABA level and age. Notably, previous functional MRI evidence has shown regions of the left parietal lobe to be among the brain areas strongly activated when people with multiple sclerosis perform these cognitive tasks [9-11].

Glutamate concentration did not, however, remain a significant independent predictor of PASAT performance after adjusting for markers of structural damage (i.e. partial correlations of glutamate level, MTR, and normalized brain volume did not reach significance). Thus, it is difficult to determine whether impaired cognitive performance is directly related to glutamate deficits, or is simply a consequence of structural brain damage. The modest sample size of multiple sclerosis participants may have limited our ability to detect independent effects of these variables. Considering this study's findings presented in the main text, it remains possible that

demyelination and neurodegeneration may ultimately lead to both net decreases in glutamate detectable by ^{1}H-MRS (intracellular + extracellular [12]) as well as cognitive decline. It may be interest for future studies to investigate the possible independent contributions of glutamate and structural damage to clinical outcomes using larger sample sizes and more participants with progressive forms of the disease.

AI-3 Supplementary materials related to Chapter 5

Supplementary Results

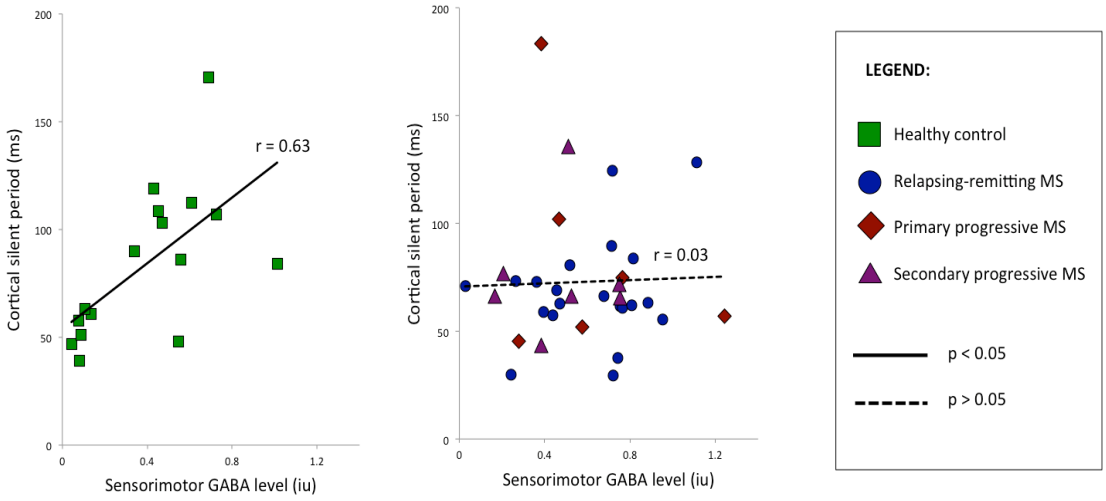

FIGURE S.1. Relationships between sensorimotor GABA level and cortical silent period duration among healthy volunteers and people with multiple sclerosis (MS).

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Appendix II

Reprints of Published Manuscripts Included in Thesis

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Intracortical inhibition abnormality during the remission phase of multiple sclerosis is related to upper limb dexterity and lesions

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HIGHLIGHTS

- Cortical silent period (CSP) prolongation is related to upper extremity impairment of people with relapsing-remitting multiple sclerosis (RRMS) in remission.
- Normalized lesion volume is correlated with longer CSP duration, while cortical thickness is not.
- CSP duration predicts motor impairment independently of lesion volume.

ABSTRACT

Objective: The impact of inhibitory cortical activity on motor impairment of people with relapsing-remitting multiple sclerosis (RRMS) has not been fully elucidated despite its relevance to neurorehabilitation. The present study assessed the extent to which transcranial magnetic stimulation (TMS)-based metrics of intracortical inhibition are related to motor disability and brain damage.
Methods: Participants included forty-three persons with RRMS in the remitting phase and twenty-nine healthy controls. We stimulated the dominant hemisphere and recorded from the dominant hand to assess short-interval intracortical inhibition (SICI) and cortical silent period (CSP) duration. Disability was evaluated with the Multiple Sclerosis Functional Composite (MSFC). Regional cortical thickness and lesion volume were measured.
Results: RRMS participants with dominant upper limb dexterity impairments had prolonged CSP, but equivalent SICI, compared to participants with preserved function. CSP was not related to walking or cognitive performance. Higher normalized lesion volume correlated with longer CSP duration. When adjusting for normalized lesion volume, longer CSP significantly predicted worse dominant upper extremity impairment.
Conclusions: High intracortical inhibition possibly contributes to (or prevents remission from) motor impairment. Lesions may be associated with intracortical inhibition shifts.
Significance: CSP duration and lesion burden should be considered when developing interventions aiming to mitigate motor impairment.

Abbreviations: RRMS, relapsing-remitting multiple sclerosis; RRMS-P, RRMS participant with preserved motor function; RRMS-I, RRMS participant with impaired motor function; HC, healthy control; GABA, γ-aminobutyric acid; TMS, transcranial magnetic stimulation; M1, primary motor cortex; SICI, short-interval intracortical inhibition; CSP, cortical silent period; EDSS, Expanded Disability Status Scale; CST[1], intracortical cortico-spinal tract; MSFC, Multiple Sclerosis Functional Composite; T25FW, Timed 25-foot walk; 9HPT, 9-hole peg test; PASAT, the Paced Auditory Serial Addition Test; RMT, resting motor threshold; MEP, motor-evoked potential.

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1. Introduction

Multiple sclerosis is a chronic neuroinflammatory disease that can cause motor impairments, among other debilitating symptoms (Compston and Coles, 2008). For people with the relapsing-remitting course of multiple sclerosis (RRMS), symptoms associated with transient neuroinflammatory events greatly improve during spontaneous clinical remission phases despite the persistence of structural brain damage (Hauser and Oksenberg, 2006; Steinman, 2014). Physiological mechanisms contributing to the severity of residual disability present during the remission phases of RRMS have not been fully elucidated.

Symptom recovery after neurological damage may be influenced by the brain's main inhibitory neurotransmitter, γ-aminobutyric acid (GABA), which has a key role in synaptic plasticity and motor learning (Sanes and Donoghue, 2000; Stagg et al., 2011; Kim et al., 2014; Sampaio-Baptista et al., 2014; Blicher et al., 2015). In humans, intracortical inhibitory activity can be studied non-invasively through a variety of protocols that involve analyzing peripheral electromyographic signals associated with transcranial magnetic stimulation (TMS) of the primary motor cortex (M1). Pharmacological evidence supports that the short-interval intracortical inhibition (SICI) metric is linked to inhibitory activity at ionotropic $GABA_A$ receptors, while the cortical silent period (CSP) primarily reflects metabotropic $GABA_B$ receptor activity (Ziemann, 2013).

The relationship between these TMS-based metrics and clinical manifestations of multiple sclerosis is not fully known. While CSP prolongation (indicating higher intracortical inhibition) has been reported to occur during the clinical remission phase of RRMS (Caramia et al., 2004), it is not clear if this alteration is related to the preservation or impairment of function. As divergent areas of research support that plasticity and motor learning favor a low inhibitory state (Levy et al., 2002; Floyer-Lea et al., 2006; Stagg et al., 2011), it could be hypothesized that higher intracortical inhibition during remission is linked to more severe persisting impairment. However, the reverse could also be true, as intracortical inhibition deficits are common during relapses (Caramia et al., 2004) and among individuals with the later, secondary progressive form of multiple sclerosis (Conte et al., 2009; Vucic et al., 2012). Moreover, it has not been directly assessed if intracortical inhibition is related specifically to impairment of the limb contralateral to the TMS stimulation site, as opposed to other neurological symptoms of this complex disease.

Of further consideration is the impact of brain damage on TMS-based outcomes. Conte et al. (2009) reported that SICI of people with multiple sclerosis is not correlated with lesion load within the whole brain nor within the intracranial cortico-spinal tract (CST_i). While not previously assessed among people with multiple sclerosis, it is possible that neuroimaging analysis techniques that estimate atrophy and lesion impact near the stimulated cortex may provide metrics of damage relevant to intracortical inhibition abnormities. Understanding how inhibitory cortical activity may interact with brain damage to produce (or prevent) motor disability may support the development of optimal tools to assess disease burden and to treat symptoms of people with neurological conditions such as RRMS.

The primary objective of this study was to assess the extent to which TMS markers of intracortical inhibition (SICI, CSP) are abnormal among people with RRMS in remission that have upper limb dexterity impairments when compared to those with preserved upper limb function. Secondly, we investigated the specificity of the identified neurophysiological abnormalities in predicting poor performance of the limb contralateral to the TMS stimulation site compared to other types of disability. The relationship between intracortical inhibition and structural brain damage (as measured throughout the whole brain and near the stimulation site) was also investigated. We predicted that among RRMS participants, intracortical inhibition would be related to upper limb disability, as well as to damage around the cortical region stimulated. Our additional multimodal analysis explored whether intracortical inhibition is related to disability independently of brain damage measured with MRI.

2. Methods

2.1. Participants

A random selection process was used to recruit people with RRMS from a clinical research database at the Montreal Neurological Institute and Hospital in Canada. Age- and sex-matched healthy control (HC) participants were recruited through advertising posters in the community. Age, sex, time since diagnosis, date of most recent relapse, medications, and Expanded Disability Status Scale (EDSS) score were extracted from the clinical database for RRMS participants, and applicable variables for HC participants were self-reported. People were not invited to participate if they: (1) had risk factors for undergoing TMS or MRI (e.g. medications lowering seizure threshold, history of seizure, pregnancy, ferromagnetic metal in body), (2) were taking medications known to affect intracortical inhibition (e.g. baclofen), (3) had a pre-existing health condition not attributed to MS (e.g. bipolar disorder, limb amputation), (4) had experienced a clinically-significant relapse within the three months prior to participation, or (5) were left-handed. Of people who took part in the study, two HC participants were excluded for abnormally poor motor performance (>2 standard deviations worse than published norms (Oxford Grice et al., 2003)). Two participants (1 RRMS, 1 HC) did not complete the study because their resting motor threshold was too high to assess SICI or CSP with our protocol. All participants provided informed consent. The Research Ethics Board at the Montreal Neurological Institute and Hospital in Canada approved this study. The final sample included 29 HC and 43 RRMS participants.

2.2. Multiple Sclerosis Functional Composite

The Multiple Sclerosis Functional Composite (MSFC) (Fischer et al., 1999), an assessment of performance across functional domains that has been validated among people with MS (Cutter et al., 1999), was used to measure disability. The MSFC consists of three subscales: Timed 25-foot walk (T25FW), 9-hole peg test (9HPT), and the 3 second version of the Paced Auditory Serial Addition Test (PASAT), which measure leg function/ambulation, hand/arm dexterity, and cognitive function, respectively.

2.3. Neurophysiological assessments

TMS pulses were delivered with a Magstim 200^2 stimulator and figure-of-8 coil (outer wing diameter = 9.5 cm) held against the head (left hemisphere) at a 45-degree angle to the sagittal plane (handle oriented posteriorly). Electromyographic data was collected with surface electrodes placed in a belly-tendon montage on the dominant hand (contralateral to the TMS stimulation site), with the recording electrode over the first dorsal interosseus (FDI) muscle. Data was amplified and filtered (bandwidth = 10–3000 Hz, Grass P511 AC Amplifiers) and collected at a sampling rate of 6 kHz. Using BrainSight 2 stereotaxic navigation software (Rogue Research Inc), the optimal target site to elicit an MEP from the target FDI was identified (Thielscher and Kammer, 2002), marked, and referenced for all further stimulations. Resting motor threshold (RMT) was defined as the lowest intensity of stimulation

required to induce a motor evoked potential (MEP) of at least 50 μV in 5 of 10 trials in the target FDI (Rossini et al., 1994). TMS data were analyzed semi-automatically with a MATLAB (Mathworks, MA, USA) analysis tool (dataWizard, version 0.7.7, A.D. Wu, UCLA) and manually edited by a researcher blinded to group membership.

The paired-pulse method (used to determine SICI) (Kujirai et al., 1993) required a second stimulator connected through a Bistim module. A conditioning stimulus (80% of RMT) was delivered, followed by the test stimulus (120% of RMT) at randomly ordered inter-stimulus intervals of 1, 2, and 3 ms. Single-pulse test stimuli at 120% RMT (with no conditioning stimulus) were interspersed throughout the paired-pulse procedure. For each interstimulus interval and for the single-pulse condition, eight MEPs were obtained and peak-to-peak amplitude was measured. For each paired-pulse interstimulus interval, SICI was expressed as (1 − (mean paired-pulse MEP amplitude/mean single-pulse MEP amplitude) × 100%) (Ayache et al., 2014), such that higher SICI values would reflect a greater percentage of inhibition relative to the single-pulse MEP.

For the contralateral cortical silent period (CSP) technique (Cantello et al., 1992) a single suprathreshold stimulus (120% of RMT) was delivered while the FDI muscle was voluntarily contracted at 40% of maximum voluntary pinch strength (determined by feedback from a Preston pinch gauge (Sammons Preston, Illinois, USA)), over 10 trials. Trials deemed invalid due to error in muscle contraction (monitored by a second researcher who was not conducting the stimulations) were repeated to ensure accuracy. For visual assessment of the CSP, the electromyographic traces of all valid trials were rectified, averaged, and highly magnified. The minimal absolute CSP duration was measured from the end of the MEP until the earliest onset of the contracted muscle EMG activity return (Caramia et al., 2004; Kallioniemi et al., 2014) (see Fig. 1). To confirm that our main result did not rely solely on the conservative definition of the CSP chosen a priori, we performed additional analyses using alternative definitions of CSP duration, including: (1) CSP end point defined as the EMG potential returning to 50 μV on the average rectified trace, and (2) MEP onset used as the CSP start point.

MEPs obtained during single pulse stimulations were measured for peak-to-peak amplitude and onset time to measure average MEP amplitude and latency, respectively. This was assessed separately for data collected from relaxed muscles and from active muscles (40% of maximum voluntary contraction).

2.4. Neuroimaging data collection and analysis

A subset of participants (15 HC, 38 RRMS) took part in the additional magnetic resonance imaging (MRI) protocol within 45 days of TMS and MSFC assessment. Neuroimaging was performed at the Montreal Neurological Institute and Hospital (Siemens TIM Trio scanner, 3 Tesla) using a 32-channel head coil. The protocol included acquisition of: (1) T1-weighted 3D fast low-angle shot sequence (repetition time (TR) = 20 ms, echo time (TE) = 5 ms, field of view (FOV) = 256 mm, number of slices = 192, slice thickness = 1 mm), (2) T2-weighted 3D fluid-attenuated inversion recovery (FLAIR) images turbo echo spin sequence (TR = 6000 ms, TE = 355 ms, FOV = 256 mm, slices number = 192, slice thickness = 1 mm), (3) proton density/T2-weighted dual spin echo sequence (TR = 2100 ms, TE = 17/76 ms, FOV = 256 mm, slices number = 60, slice thickness = 3 mm). Scans were co-registered during the preprocessing steps and coded to ensure blinding for imaging analysis steps.

T1-weighted images were processed in FreeSurfer (version 5.1.0, http://surfer.nmr.mgh.harvard.edu/), a validated semi-automated segmentation system allowing for the study of specific neuroanatomical regions (Fischl et al., 2002; Derakhshan et al., 2010). M1 of the left and right hemispheres were defined based on the precentral gyrus labels created with FreeSurfer. Based on FreeSurfer segmentation, cortical thickness (within the whole brain and within each M1) was measured.

T2-weighted lesions within the white matter were detected by a semi-automated lesion-detection software system (Francis, 2004) and manually corrected by a researcher who had been trained by a neuroradiologist. The subsequent analysis was designed to assess the amount of lesioned white matter in the region juxtacortical to M1 of the stimulated and non-stimulated hemispheres. Using the image dilation function in FSL (Jenkinson et al., 2012), M1 masks were dilated by one voxel in each direction to define the region within the white matter that bordered the M1 cortical regions. The volume of the white matter lesions that overlapped with the dilated M1 labels was measured (see Fig. 2). Lesion volume within the CST_i of the left and right hemispheres (defined using the John Hopkins University diffusion tensor imaging-based white-matter atlas (Hua et al., 2008), which had been transformed into each participant's native space using ANTs (Avants et al., 2011)) was also measured. To produce inter-subject-comparable measures, the scaling factor generated from SienaX (Smith, 2002) was applied to all lesion volumes.

2.5. Statistical analysis

9HPT completion times for the dominant upper limb were converted into *z*-scores based on a comparison to the performance of healthy participants. RRMS participants who performed within two standard deviations (SD) of the healthy participants' mean time were placed in the *preserved function* (RRMS-P) group, and those performing slower than two SD of the healthy participants' time were placed into the *impaired function* (RRMS-I) group.

For all statistical tests, data were analyzed with SPSS and considered statistically significant at $p < 0.05$.

2.5.1. Group analyses

Descriptive analyses were conducted to characterize the sample on all demographic, clinical, neurophysiological, and MRI-based variables. Chi-squared tests were used to compare groups on categorical variables including sex and use of immunomodulatory medication. To compare RRMS-P and RRMS-I groups on disease duration and EDSS score, Mann–Whitney *U* tests were used. The Shapiro–Wilk test of normality was conducted for each continuous variable. One-way Analysis of Variance (ANOVA) was used to compare HC, RRMS-P and RRMS-I participants for differences on normally distributed variables, followed by post hoc Tukey *t*-tests tests. Non-normally distributed variables were compared with a Kruskall-–Wallis *H* test, followed by a post hoc Mann–Whitney *U* tests as appropriate. A Bonferroni correction was used to account for the number of group comparisons for each post hoc test.

2.5.2. Correlational analyses

As the goal of the study was to assess the extent to which abnormalities in TMS-based intracortical inhibition metrics are related to clinical disability and brain damage, only intracortical inhibition variables found to differ from HC participants in the group analyses were included in the subsequent correlational analyses. Spearman rank correlations were used to evaluate the relationships between: (1) TMS-based intracortical inhibition metrics and clinical outcomes, and (2) TMS-based intracortical inhibition metrics and MRI outcomes. We also assessed if EDSS score, disease duration, and MEP latency were similarly related to the neurophysiological and neuroimaging outcomes of interest.

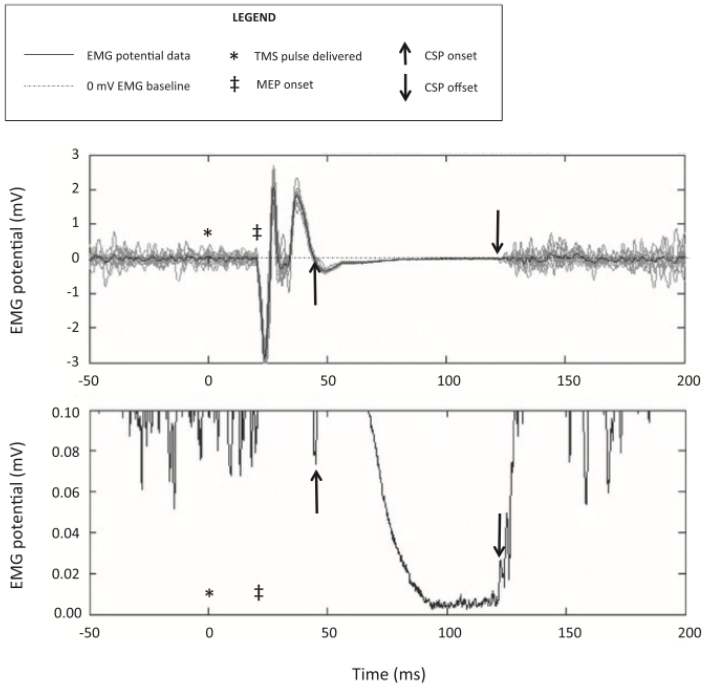

Fig. 1. Example of CSP data from a participant with RRMS. The asterisk indicates the time point the TMS pulse was delivered, and the double dagger indicates the MEP onset location. The un-rectified average trace with raw data superimposed (upper panel), was used to assist identification of the MEP end point at the cross point of the EMG potential baseline. The rectified average trace, viewed at a high level of magnification (lower panel) was used to identify the earliest time point where contracted muscle EMG activity began to return to baseline. Upward and downward pointing arrows indicate the start and end points of the absolute CSP, respectively.

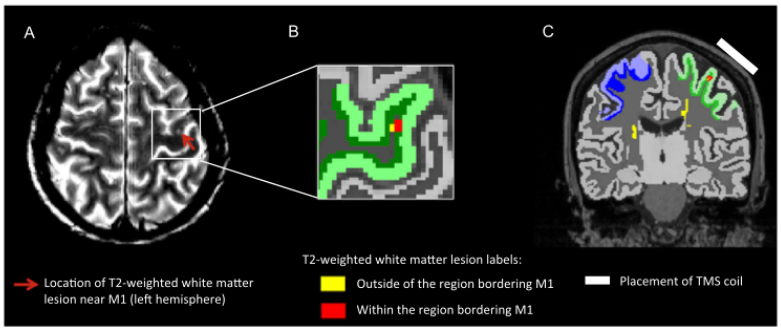

Fig. 2. Example of a participant with RRMS who had a T2-weighted white matter lesion that bordered M1 of the left hemisphere. (A) A T2-weighted image of a RRMS participant with a white matter lesion identified near M1 of the left hemisphere is shown. (B,C) T1-weighted images with overlaying anatomical labels are shown. Grey matter labels (excluding M1) are shown in light grey. Light blue and light green indicate M1 of the right and left hemispheres, respectively. The dilated masks for M1 are shown in darker blue and darker green. A zoomed-in section from (A) is shown in (B). An example of the placement of the TMS coil relative to the participant's brain is also shown in (C).

2.5.3. Regression

We performed a series of multiple linear regression analyses to assess if TMS-based intracortical inhibition outcomes predicted disability independently of potentially confounding MRI-based or neurophysiological variables. Negative reciprocal transformations were applied to the 9HPT, EDSS and lesion volume data to ensure that assumptions of linear regression analysis were not violated due to non-normally distributed residuals.

3. Results

3.1. Clinical and demographic outcomes

Table 1 outlines the demographic and clinical characteristics of participants. Differences in age ($F_{2,71} = 1.01$, $p = 0.37$) and sex ($\chi^2_{(2)} = 3.51$, $p = 0.17$) were not statistically significant. RRMS-P and RRMS-I groups did not differ in disease duration ($U = 138$, $p = 0.87$)

Table 1
Demographic characteristics of participants.

	HC	RRMS-P	RRMS-I
Full sample			
Total number of subjects	29	30	13
Women (*n* (%))	21 (72)	22 (73)	6 (46)
Taking immunomodulatory medication for MS (*n* (%))	0 (0)	17 (57)	10 (77)
Age, *years* (mean ± SD)	45.0 ± 13.0	42.9 ± 11.0	48.6 ± 12.6
Disease duration[a], *years* (median (Q1,Q3))	–	8.9 (3.2,12.4)	8.3 (5.7,11.6)
EDSS, *score* (median (Q1,Q3))	–	1.8 (1.0,2.4)	3.0 (2.0,3.5)
MSFC, *score* (mean ± SD)	0.65 ± 0.28	0.46 ± 0.41	−0.38 ± 0.59
MRI subgroup			
Total number of subjects	15	26	12
Women (*n* (%))	12 (80)	19 (73)	6 (50)
Taking immunomodulatory medication for MS (*n* (%))	0 (0)	16 (62)	10 (83)
Age, *years* (mean ± SD)	45.9 ± 15.3	41.5 ± 10.5	48.9 ± 13.2
Disease duration[a], *years* (median (Q1,Q3))	–	7.1 (2.8,11.3)	8.0 (4.9,10.4)
EDSS, *score* (median (Q1,Q3))	–	1.5 (1.0,2.4)	2.5 (2.0,3.5)
MSFC, *score* (mean ± SD)	0.72 ± 0.31	0.49 ± 0.40	−0.38 ± 0.62

EDSS = Expanded Disability Status Scale; MSFC = Multiple Sclerosis Functional Composite.
[a] Missing data from three RRMS-P and two RRMS-I participants.

or in the proportion of individuals taking immunomodulatory medications to treat MS ($\chi^2_{(1)} = 1.59$, $p = 0.21$).

Outcomes of MSFC subscale scores, including significance of post hoc assessments, are shown in Table 2. The T25FW test was not measured for one HC and two RRMS-P participants, because they did not return within 3 weeks for the subsequent session. Groups differed in 9HPT score (for both the dominant ($F_{2,71} = 73.6$, $p < 0.001$) and non-dominant hands ($\chi^2_{(2)} = 23.1$, $p < 0.001$), T25FW time ($\chi^2_{(2)} = 13.3$, $p = 0.001$), PASAT score ($\chi^2_{(2)} = 11.8$, $p = 0.003$), and MSFC score ($\chi^2_{(2)} = 25.1$, $p < 0.001$). The basis by which the groups were defined was confirmed, as dominant hand 9HPT time was longer for RRMS-I participants than RRMS-P participants, while HC and RRMS-P groups did not differ significantly. RRMS-I participants also performed worse on the non-dominant hand 9HPT and PASAT compared to RRMS-P and HC participants. For the T25FW, RRMS-P and RRMS-I groups did not differ, although both performed worse than controls. EDSS score was higher for RRMS-I participants compared to RRMS-P participants ($U = 119$, $p = 0.04$).

3.2. Neurophysiological outcomes

Outcomes of TMS-based measures assessed during muscle relaxation are shown in Table 2. The groups did not differ significantly in RMT ($\chi^2_{(2)} = 5.2$, $p = 0.08$), nor SICI at any of the inter-stimulus intervals (all $\chi^2_{(2)} < 2.3$, $ps > 0.05$). Group differences were however found for MEP amplitude ($\chi^2_{(2)} = 11.6$, $p = 0.003$) and MEP latency ($F_{2,71} = 13.2$, $p < 0.001$) of resting muscles, with RRMS-I participants having lower amplitudes and longer latencies than both HC and RRMS-P participants. While no between-group differences were found for maximal pinch strength ($F_{2,71} = 0.50$, $p > 0.05$) nor average MEP amplitude of a contracted muscle ($F_{2,71} = 0.84$, $p > 0.05$), between-group differences were found in MEP latency during muscle contraction ($F_{2,71} = 8.9$, $p < 0.001$) and CSP duration ($\chi^2_{(2)} = 9.02$, $p = 0.01$), with the RRMS-I group having significantly longer latencies and CSP duration than the other groups (Fig. 3). These between-group differences in CSP duration remained significant even after removing the youngest females from the HC ($n = 2$) and RRMS-P ($n = 3$) groups, which minimized the trending differences in age and sex between groups. Confirming this result was not unique to the conservative definition of the CSP duration chosen a priori for the present study, we found that the observed between-group differences in CSP duration remained significant when measuring the absolute CSP duration with the alternative, 50 μV threshold-based definition of the CSP endpoint (HC: 68 ± 28 ms, RRMS-P: 69 ± 26 ms, RRMS-I: 101 ± 43 ms), as well as when measuring the relative CSP beginning at the MEP onset (HC: 94 ± 29 ms, RRMS-P: 96 ± 28 ms, RRMS-I: 130 ± 43 ms) (both $\chi^2_{(2)} > 8.5$, $ps < 0.05$).

The full sample of RRMS participants (RRMS-P and RRMS-I groups combined) did not differ from HC participants in age ($t_{(70)} = 0.12$, $p = 0.91$) or sex ($\chi^2 = 0.42$, $p = 0.52$). CSP duration of RRMS participants was positively correlated with 9HPT performance of the dominant upper limb ($r_{s(42)} = 0.37$, $p = 0.01$), but not the non-dominant upper limb ($r_{s(42)} = 0.18$, $p > 0.05$). Furthermore, CSP duration of MS participants was not correlated with walking speed, PASAT score, total MSFC score, or disease duration ($ps > 0.05$), but was modestly correlated with EDSS ($r_{s(42)} = 0.36$, $p = 0.02$). MEP latency during active muscle contraction correlated with longer 9HPT performance with either hand, as well as MSFC total score ($r_{s(42)} > 0.32$, $ps < 0.05$), but not other variables ($ps > 0.05$). None of the performance-based variables were significantly correlated with CSP duration or MEP latency among HC participants ($ps > 0.05$). A model with both CSP and MEP latency as predictors of dominant hand 9HPT performance was significant ($R^2 = 0.22$, $F_{2,42} = 5.65$, $p = 0.007$), with CSP significantly predicting performance ($\beta = 0.36$, $p = 0.013$) independently of MEP latency ($\beta = 0.27$, $p = 0.063$).

3.3. Neuroimaging outcomes

Demographic characteristics of participants who took part in the MRI component of the study are shown in Table 1, and group comparisons of neuroimaging outcomes are summarized in Table 2. Group differences in age ($F_{2,52} = 1.54$, $p = 0.23$), sex ($\chi^2_{(2)} = 3.11$, $p = 0.21$), disease duration ($U = 105$, $p = 0.70$), and prevalence of participants taking immunomodulatory medications for MS ($\chi^2_{(1)} = 1.81$, $p = 0.18$) remained non-significant when comparing participants of the subgroup. Group differences in cortical thickness occurred within the whole brain ($F_{2,52} = 7.19$, $p = 0.002$), left hemisphere ($F_{2,52} = 3.23$, $p = 0.048$), and right hemisphere M1 ($F_{2,52} = 3.68$, $p = 0.032$). Differences were also found for normalized volume of T2-weighted white matter lesions within the whole brain, within the CST_i and in the regions bordering M1 of the left and right hemispheres (all $\chi^2_{(2)} > 19$, $ps < 0.001$).

Cortical thickness was lower among RRMS-I participants compared to RRMS-P participants within the whole brain and within M1 of the left hemisphere, but not within M1 of the right hemisphere. Compared to RRMS-P participants, RRMS-I participants

Table 2
Comparisons between HC participants and RRMS participants with preserved and impaired motor function.

	HC	RRMS-P	RRMS-I	HC vs. RRMS-P	HC vs. RRMS-I	RRMS-P vs. RRMS-I
Performance	Median (Q1,Q3)	Median (Q1,Q3)	Median (Q1,Q3)	*p*	*p*	*p*
9-HPT dominant hand time (s)	17.6 (17.0,18.4)	19.0 (18.1,19.9)	25.0 (22.9,27.3)	–	‡‡‡	***
9-HPT non-dominant hand time (s)	18.6 (17.4,19.7)	19.9 (19.1,21.3)	23.1 (22.3,28.9)	–	‡‡‡	**
T25FW time (s)	3.6 (3.3,4.3)	4.5 (3.7,4.8)	4.8 (4.1,6.8)	†	‡	–
PASAT score	52 (45,55)	51 (39,55)	35 (24,44)	–	‡‡	**
Maximal pinch strength (kg)	5.4 (4.5, 6.1)	6.1 (4.1, 6.8)	5.9 (3.6, 6.8)	–	–	–
TMS – resting muscle conditions	Median (Q1,Q3)	Median (Q1,Q3)	Median (Q1,Q3)			
RMT (%)	42 (39,47)	46 (39,49)	51 (46,53)	–	–	–
Single pulse MEP amplitude (mV)	0.71 (0.47,1.54)	0.67 (0.38,1.22)	0.33 (0.20,0.42)	–	‡‡	*
MEP latency (ms)	22.0 (21.6,23.2)	22.9 (22.3,23.6)	24.9 (24.8,26.0)	–	‡‡‡	***
SICI – 1 ms ISI (%)	76.8 (59.3,88.1)	68.1 (56.2,78.7)	68.1 (57.4,74.3)	–	–	–
SICI – 2 ms ISI (%)	67.2 (45.8,79.5)	66.2 (46.3,77.7)	65.0 (44.2,77.6)	–	–	–
SICI – 3 ms ISI (%)	71.0 (55.7,79.6)	63.7 (42.0,74.5)	65.5 (43.4,73.6)	–	–	–
Normalized T2w white matter lesion volumes (mm^3)	Median (Q1,Q3)	Median (Q1,Q3)	Median (Q1,Q3)	*p*	*p*	*p*
Whole brain	0 (0,0)	5341 (2157,10058)	20357 (5656,25115)	†††	‡‡‡	*
CST_i						
Left hemisphere	0 (0,0)	119 (0,429)	500 (95,1135)	†††	‡‡‡	–
Right hemisphere	0 (0,0)	145 (43,248)	180 (55,590)	†††	‡‡‡	–
Bordering M1						
Left hemisphere	0 (0,0)	0 (0,24)	30 (16,144)	††	‡‡‡	*
Right hemisphere	0 (0,0)	6 (0,45)	34 (7,198)	††	‡‡‡	–
Cortical thickness (mm)	Mean (SD)	Mean (SD)	Mean (SD)	*p*	*p*	*p*
Total cortex	2.58 (0.08)	2.51 (0.11)	2.41 (0.16)	–	‡‡	*
M1						
Left hemisphere	2.68 (0.18)	2.70 (0.16)	2.52 (0.33)	–	–	*
Right hemisphere	2.75 (0.15)	2.69 (0.16)	2.50 (0.43)	–	‡	–

Symbols indicate a significant differences identified from the post hoc assessment comparing: HC and RRMS-P ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$), HC and RRMS-I ($^{\ddagger}p < 0.05$, $^{\ddagger\ddagger}p < 0.01$, $^{\ddagger\ddagger\ddagger}p < 0.001$), and RRMS-P and RRMS-I ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Dashes (–) indicate non-significant outcomes ($p > 0.05$). SD, Q1, Q3 indicate standard deviation, lower quartile, and upper quartile, respectively.

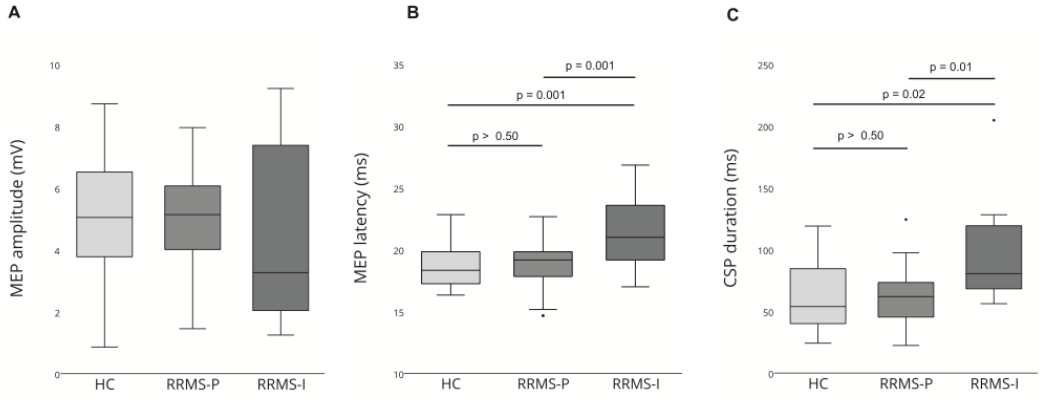

Fig. 3. Outcomes of TMS-evoked electromyographic data collected during muscle contraction. When TMS was performed during muscle contraction, MEP amplitude (A) did not differ significantly between groups, although MEP latency (B) and CSP duration (C) were longer among RRMS-I participants compared to both other groups. Significance of non-parametric two-tailed post hoc tests (corrected for multiple comparisons) is shown for the MEP latency and CSP duration data.

had significantly higher normalized volumes for T2-weighted white matter lesions within the whole brain and region bordering M1 of the left hemisphere, but not other regions. EDSS score correlated with whole brain normalized lesion volume ($r_{s(37)} = 0.40$, $ps = 0.013$), but not with any of the other MRI-based outcomes ($ps > 0.05$).

In Table 3, the relationships between the neuroimaging outcomes and CSP duration are shown. Among the RRMS participants, higher normalized lesion volume (both within the whole brain and in the region bordering the left M1) was positively correlated with longer CSP after correcting for multiple comparisons, while no other significant relationships were found. By contrast, MEP latency was not significantly correlated with any of the MRI-based outcomes ($ps > 0.05$).

Multiple linear regression models including both CSP and normalized lesion volume as predictors of dominant hand 9HPT performance were significant when assessing lesion volume within the entire brain ($R^2 = 0.17$, $F_{2,37} = 3.55$, $p = 0.039$), as well as well as

within the region bordering the left hemisphere M1 ($R^2 = 0.18$, $F_{2,37} = 3.94$, $p = 0.029$). In both models, CSP was found to be a significant predictor of 9HPT performance ($\beta s > 0.38$, $ps < 0.05$) independently of lesion volume ($\beta s < 0.14$, $ps > 0.05$). However, CSP did not predict EDSS independently of lesion volume in either region ($ps > 0.05$).

4. Discussion

The results of this study implicate CSP lengthening in specific manifestations of motor impairment present during remission phases of RRMS, and suggest that this neurophysiological irregularity is partially associated with lesion burden.

4.1. Intracortical inhibition and disability

The present study investigated the clinical implications of intracortical inhibition abnormalities in people with RRMS during the relatively stable remission phase. To obtain biomarkers of intracortical inhibition, we stimulated the dominant hemisphere and analyzed recordings from an electrode over a muscle on the contralateral (dominant) hand. On average, RRMS participants with impaired dominant upper extremity motor function had normal SICI, but abnormally long CSP duration. By contrast, RRMS participants with preserved motor abilities did not differ from healthy controls in either outcome. We further assessed the clinical specificity of this neurophysiological abnormality, finding that longer CSP was correlated with 9HPT performance of the dominant upper limb, while CSP did not correlate with non-dominant 9HPT completion time, walking speed, nor cognitive performance of RRMS participants.

Similar to the present study, others have reported that, outside of relapse, SICI is normal among people with RRMS (Conte et al., 2009; Vucic et al., 2012), while CSP prolongation has been reported among people with RRMS in remission (Caramia et al., 2004) and among those with clinically isolated syndrome who later develop multiple sclerosis (Pallix-Guyot et al., 2011). Our results further demonstrate that among remitting RRMS individuals, longer CSP is related to more severe upper extremity disability. This finding is consistent with studies of people in remission from a stroke, which support that higher GABA impedes motor recovery (Lazar et al., 2010; Bachtiar and Stagg, 2014; Blicher et al., 2015), potentially by interfering with synaptic plasticity and motor learning (Levy et al., 2002; Floyer-Lea et al., 2006; Krakauer, 2006; Stagg et al., 2011). Nonetheless, the present study's results may be surprising considering that low intracortical inhibition has been reported during relapses (Caramia et al., 2004) as well as during the later, secondary progressive stage of multiple sclerosis (Conte et al., 2009; Vucic et al., 2012). Potentially, disability associated with CSP prolongation during remission could be related to a physiological mechanism that is largely distinct from intracortical inhibition deficits occurring in other disease phases.

Considering the previously described responses of SICI and CSP to specific neurotransmitter receptor agonists/antagonists and the temporal characteristics of these signals (Ziemann, 2013), a possible interpretation of the present result is a circumscribed increase in the long-lasting activity of $GABA_B$ receptors, as opposed to the faster-acting, and shorter-lived $GABA_A$ receptor activity, among individuals with dominant limb dexterity impairment. Alternatively, CSP prolongation in the absence of SICI change may be associated with neurophysiological alterations related to voluntary motor drive (Tergau et al., 1999), motor attention (Hoshiyama and Kakigi, 1999; Ziemann, 2013), or spinal mechanisms (Inghilleri et al., 1993; Ziemann et al., 1993), which could involve GABAergic activity directly or indirectly. While it is possible that factors other than inhibitory neurotransmission may contribute to CSP duration alterations, the present results are consistent with findings from a magnetic resonance spectroscopy study on people with RRMS by Bhattacharyya et al. (2013), which found higher GABA concentration within a sensorimotor brain volume of the dominant hemisphere to predict worse performance on the 9HPT, but not to predict walking speed nor PASAT performance. Together with the present study, this work implicates a role for GABA associated with the dominant hemisphere motor region of people with RRMS in potentially mediating (or precluding recovery from) dominant upper limb dexterity impairment. Further investigation of this mechanism is warranted, including assessing other forms of inhibition that can be measured with TMS (e.g. long-interval intracortical inhibition, ipsilateral cortical silent period).

Table 3
Relationships between MRI outcomes and CSP duration.

Predictor	HC	RRMS
Cortical thickness (mm)		
Whole brain	−0.42	−0.22
M1		
Left hemisphere	−0.14	−0.32
Right hemisphere	−0.24	−0.24
T2w white matter lesion volume (mm^3)		
Whole brain	–	0.51**
CST_i		
Left hemisphere	–	0.43
Right hemisphere	–	0.12
Bordering M1		
Left hemisphere	–	0.44*
Right hemisphere	–	0.12

The correlation coefficient from Spearman's rank analysis is shown.
Asterisks indicate 2-tailed significance after correcting for multiple comparisons (*$p < 0.05$, **$p < 0.01$).

4.2. Impact of lesion burden

Our subsequent neuroimaging analysis investigated possible physical origins of CSP prolongation observed among the motor-impaired individuals. To our knowledge, this is the first study to identify a significant relationship between MRI-based outcomes and TMS measures of intracortical inhibition among people with multiple sclerosis. Higher normalized volume of lesioned white matter within the whole brain and within the region bordering M1 of the stimulated hemisphere (but not the non-stimulated hemisphere) correlated with longer CSP duration.

Volume of white matter lesions in the region near the stimulated cortex was found to be more strongly related to CSP duration compared to lesions in the parallel region of the opposite hemisphere. One caveat, however, is that juxtacortical lesions are often located close to lesioned grey matter tissue, to which conventional structural MRI techniques are mostly blind (Vural et al., 2013). Thus, we cannot be certain if the relationship between lesions bordering M1 of the stimulated cortex with CSP was due to structural abnormalities within the white matter pathways leading to the cortex, or instead a direct consequence of cortical damage. Non-conventional imaging sequences (e.g. magnetization transfer, double-inversion recovery) are more sensitive to cortical damage than conventional MRI techniques (Vural et al., 2013; Filippi et al., 2014), and could thus help to elucidate this in future work. However, even the most sophisticated in vivo neuroimaging techniques cannot detect a large proportion of grey matter lesions known to exist based on postmortem histology (Geurts et al., 2005; Chen et al., 2013).

Interestingly, it has been proposed in the stroke literature that CSP prolongation may be the result of the deafferentation of M1

due to lesions in various remote brain regions connected with the targeted cortical area, rather than to damage within the motor cortical region stimulated (von Giesen et al., 1994; Classen et al., 1997). As the present study also found higher total lesion volume to be correlated with longer CSP, it is possible that this holds true in multiple sclerosis. Since CSP was not significantly associated with lesions within the CST_i after correcting for multiple comparisons, it is possible that such deafferentation was more greatly influenced by damage within cortico-cortical, rather than cortico-spinal, pathways within the brain.

The interpretation that CSP prolongation is a direct consequence of structural brain damage does not, however, explain why CSP duration was not related to cortical thickness. While one possibility is that lesion volume is a more sensitive metric of structural damage relevant to CSP duration compared to cortical thickness, it should be noted that T2-weighted lesions are non-specific indicators of brain tissue that has been affected by demyelination, cell loss, inflammation, ischemia, edema or gliosis (Ferguson et al., 1997; Fox et al., 2011). Therefore, increases of any, or a combination, of these factors may have contributed to CSP prolongation. Interestingly, molecular immunology studies and research with animal models of neuroinflammatory disease have shown GABA to have immunosuppressant and anti-inflammatory properties (Tian et al., 2004; Bhat et al., 2010; Jin et al., 2013; Paul et al., 2014). Another possibility is that intracortical inhibition could be increased in response to glutamate (Tremblay et al., 2013), which has been found to be abnormally high in normal-appearing white matter and acute lesions of people with multiple sclerosis (Srinivasan et al., 2005; Tisell et al., 2013). Further research combining the current protocol with biomarkers of other disease-mediating mechanisms will be required to fully uncover whether CSP prolongation, and the associated motor impairment, is direct consequence of structural damage, or rather linked to a neural mechanism responding to neuroinflammation or glutamate excitotoxicity.

4.3. Multimodal analysis

We further investigated the combined influence of lesion burden and neurophysiological alterations on disability. Longer CSP was found to be a significant predictor of 9HPT performance when adjusting for lesion volume, demonstrating that the link between CSP prolongation and motor impairment is not simply secondary to the impact of lesions. This result further highlights the need to investigate if other disease-related factors cause CSP prolongation in future work.

In contrast to dominant upper extremity motor performance, EDSS score was not related to CSP duration after adjusting for lesion volume. Alongside our finding that CSP duration is not correlated with total MSFC score, this outcome demonstrates the need for caution when interpreting TMS-based data in clinical populations, as certain metrics may be associated with specific symptoms rather than to general clinical decline. Nonetheless, biomarkers of neurophysiological changes related to specific symptoms may be useful in the development of more targeted and individualized therapies.

4.4. Conclusions/implications

The novel findings of this study provide insight into the pathophysiology of upper extremity motor disability, and highlight factors to consider when interpreting TMS data in the context of complex brain diseases such as multiple sclerosis. Our results warrant further investigation regarding clinical applications of using CSP as a biomarker of disease burden in neuromotor conditions and/or as a target for neuromodulatory therapies aiming to mitigate disability.

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Corrigendum

Corrigendum to "Intracortical inhibition abnormality during the remission phase of multiple sclerosis is related to upper limb dexterity and lesions" [Clin. Neurophysiol. 127 (2016) 1503–1511]

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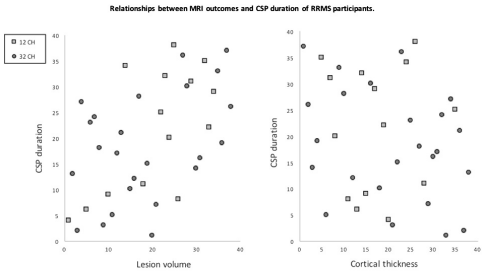
We recently detected an error in the MRI protocol we reported in "Intracortical inhibition abnormality during the remission phase of multiple sclerosis is related to upper limb dexterity and lesions", published this year in Clinical Neurophysiology. A subset of the relapsing-remitting multiple sclerosis (RRMS) participants had data collected with a 12-channel head-coil, rather than the 32-channel head coil reported for all participants. This error is relevant only to data used in the third objective of this multimodal study.

In testing for a possible impact of head-coil type on the results and conclusions of the study we found:

1. Participants with data collected with the 12-channel head coil ($n = 14$) were representative of the full sample in terms of demographic and clinical characteristics. Eight had been classified as motor function persevered (RRMS-P) and six had been classified as motor-function impaired (RRMS-I).
2. The figure shows the location of participants scanned with the 12-channel vs. 32-channel head coil, within the scatterplots representing the relationships between MRI outcomes and CSP duration. No systematic bias is seen.
3. Including coil-type as a covariate into our multiple regression analysis did not change our finding that CSP duration is a significant predictor of 9-HPT score independently of lesion volume.

Based on this examination of our data, we conclude that it is unlikely that the difference in head coils biased outcomes or impacted the conclusions of this manuscript. We apologise for this error.



Spearman's rank correlation coefficients related to the ranked data of "whole brain" outcomes presented here are reported in Table 3 of the manuscript. Results do not appear to be influenced by whether the participant had data collected with the 12 channel head coil (12 CH) or the 32 channel head coil (32 CH).

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Cortical Damage and Disability in Multiple Sclerosis: Relation to Intracortical Inhibition and Facilitation

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ABSTRACT

Background: Multimodal research combining biomarkers of intracortical activity and cortical damage could shed light on pathophysiological and adaptive neural processes related to the clinical severity of neurological conditions such as multiple sclerosis (MS).
Objective: Among people with relapsing-remitting and progressive forms of MS, we assessed the extent to which transcranial magnetic stimulation (TMS)-based biomarkers of excitatory and inhibitory cortical activity are related to cortical damage and clinical impairment.
Methods: Participants included 18 healthy individuals and 36 people with MS who had a relapsing-remitting or progressive clinical course. Using TMS, intracortical facilitation (ICF), short-interval intracortical inhibition (SICI), long-interval intracortical inhibition (LICI), and cortical silent period (CSP) were obtained. Cortical volume and cortical magnetization transfer ratio (MTR) were quantified. Disability was assessed with Multiple Sclerosis Functional Composite (MSFC).
Results: Lower mean MTR within the cerebral cortex correlated with shorter CSP among MS participants with a progressive, but not a relapsing-remitting, clinical course. Within the cortical hand knob region targeted with TMS, lower MTR was correlated with lower SICI only among individuals with relapsing-remitting MS. Longer CSP, higher ICF, lower cortical MTR, and sex were all independent significant predictors of poor upper extremity motor performance, while only cortical MTR was a significant independent predictor of total MSFC score among people with MS.
Conclusions: Cortical damage and cortical activity (both inhibitory and excitatory) may contribute to the severity of motor disability experienced by people with MS. When interpreting TMS-based outcomes, cortical integrity, clinical course, and symptom type should be considered.

Introduction

Multiple sclerosis (MS), a disease affecting the central nervous system, is a major cause of disability worldwide. Most people diagnosed with MS first experience a relapsing-remitting course, involving periods of clinical recovery between symptom relapses [1]. Others experience more continuous clinical deterioration, as can occur from disease onset (primary progressive MS) or after living with relapsing-remitting symptoms for several years (secondary progressive MS) [2]. While immunomodulatory and anti-inflammatory medications that mitigate relapsing-remitting symptoms are now widely available, effective therapeutic options for progressive disability and the associated neurodegeneration are less well established [3].

A potential contributor to neural and glial cell damage in many neurological conditions, including MS, is the over-activity of the brain's main excitatory neurotransmitter, glutamate [4,5]. This pathophysiological process, known as excitotoxicity, is also influenced indirectly by inhibitory neurotransmission, which is primarily driven by γ-aminobutyric acid (GABA) [6,7]. In humans, biomarkers of excitatory and inhibitory neurotransmission can be obtained non-invasively by analyzing characteristics of peripheral electromyographic activity evoked by transcranial magnetic stimulation (TMS) of the primary motor cortex [8]. Pharmacological studies involving the

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administration of neurotransmitter antagonists prior to TMS have linked glutamatergic activity at N-methyl-D-aspartate (NMDA) receptors to the TMS-derived measure of intracortical facilitation (ICF) [9,10]. TMS-based metrics primarily associated with inhibitory cortical activity include short-interval intracortical inhibition (SICI), long-interval intracortical inhibition (LICI), and cortical silent period (CSP), each of which is believed to capture a relatively distinct aspect of inhibitory neurotransmission [8].

The relevance of excitatory and inhibitory cortical activity to structural brain damage, and ultimately to clinical impairment, is not fully understood. Notably, a disease-modifying therapy found to decrease ICF in people with MS, fingolimod [11], has been demonstrated in animal models to modulate glutamatergic neurotransmission in addition to having remyelinating and neuroprotective effects [12–14]. Moreover, cortical damage has been suggested to cause the SICI deficits observed in people with secondary progressive MS [15,16]. As previous studies have been limited to conventional neuroimaging sequences that are nearly blind to intracortical lesions and demyelination [17,18], links between intracortical damage and TMS-based outcomes remain unclear.

Structural damage within regions that appear normal on T1- and T2-weighted images can, however, be estimated with the magnetization transfer ratio (MTR) imaging technique [19]. MTR reflects the fraction of water bound to macromolecules and has been shown to correlate with the severity of demyelination and axonal loss observed in postmortem histology studies of the MS brain [20–22]. Compared to relapsing-remitting MS, cortical MTR reduction in progressive forms of MS is more pronounced, and is less strongly co-localized with cortical atrophy [23]. Cortical MTR of people with MS has been linked to more severe disability [24], including motor impairment [25]. Therefore, in combination with TMS and atrophy assessments, MTR could be a powerful tool to elucidate the relationship between cortical damage and intracortical activity associated with the motor system.

Empirical information on cortical integrity could also clarify the meaning of previously reported relationships between intracortical activity and clinical outcomes (e.g. [15,16,26–28]). It could be hypothesized that by combating excitotoxicity, maintaining low excitatory or high inhibitory activity prevents cell damage, and as a result, prevents clinical disability. This is challenged, however, by evidence that drugs that inhibit glutamatergic transmission have not been found to be clinically beneficial for people with MS [29–31], despite having neuroprotective effects [31]. Evidence that prolonged CSP (indicating high inhibition) is related to motor or cerebellar dysfunction in MS [26,27], and demonstrations that lower GABA facilitates motor learning and motor symptom recovery [32–35], further challenges this hypothesis. As excitatory and inhibitory activity and cortical damage may interact to produce, or prevent, clinical symptoms, assessing certain variables in isolation may conceal their contribution to clinical outcomes. A further important consideration regarding interpretation of TMS-based metrics, derived by stimulating the motor cortex, is that they are likely to be more closely related to motor system pathology than to more general disease progression.

Using a novel multimodal approach, we investigated the link between intracortical activity, cortical damage, and motor impairment. Our emphasis on motor dexterity was chosen due to its relevance to the primary motor cortex hand region stimulated in our TMS protocol, as well as its importance to daily activities and quality of life [36,37]. We predicted that cortical damage would be related to inhibitory (SICI, CSP, LICI) and excitatory (ICF) intracortical activity of people with MS. We additionally hypothesized that combining assessments of cortical damage, intracortical inhibition, and intracortical facilitation would predict motor disability better than a single variable alone.

Material and methods

Participants

MS patients who had a relapsing-remitting, primary progressive, or secondary progressive course were randomly selected for recruitment from a clinical database at the Montreal Neurological Institute and Hospital in Canada. Poster advertisements were used to recruit age- and sex-matched healthy control (HC) participants. Screening (through telephone interviews and clinical chart review) further excluded people for: (1) left handedness, (2) health conditions other than MS (e.g. history of head trauma or cancer), (3) relative contraindications for undergoing MRI or TMS [38], (4) medications with documented effects on intracortical facilitation or inhibition (e.g. baclofen), and (5) relapse occurrence within 3 months prior to participation. Further exclusions occurred if valid TMS data could not be collected due to unacceptable noise in the electromyographic signal (i.e. movement artifact) and/or having a resting motor threshold (rMT) beyond the limit of our equipment when stimulating the central scalp region overlaying the left hemisphere (1 HC and 2 MS participants excluded). One HC participant was additionally excluded for being an extreme outlier (>3 standard deviations outside of mean) on several TMS-based outcome measures. Five MS participants were excluded from the analysis because they were taking fingolimod, which has been shown to influence ICF [11]. The resulting sample included 18 HC and 36 MS participants. Data from some participants was also included in a study on a larger cohort of relapsing-remitting MS and healthy participants that focused on the relationship between conventional imaging metrics (i.e. white matter lesion volume), CSP prolongation, and clinical impairment persisting during remission [26].

All participants provided informed consent. The Research Ethics Board at the Montreal Neurological Institute and Hospital approved this protocol.

Demographic and clinical outcomes

Demographic and clinical variables (age, sex, diagnosis date, age of disease onset, clinical course, date of most recent relapse, EDSS, medications) of MS participants were extracted from the clinical database. Age, sex, and medications of healthy participants were self-reported. The Multiple Sclerosis Functional Composite (MSFC) was performed and scored according to standard procedure to assess clinical disability [39,40]. This included the 9-hole peg test (9HPT), which is a valid and reliable measure of upper extremity function among people with MS [41]. Z-scores for the 9HPT and MSFC [39,40] were used in analyses involving clinical outcomes.

TMS data collection

TMS was performed using a Magstim 200[2] stimulator and figure-of-8 coil (9.5 cm outer wing diameter). With the handle oriented posteriorly, the coil was held against the left hemisphere at 45-degrees to the sagittal plane. Electromyographic data was recorded from surface electrodes in a belly-tendon montage, with the recording electrode over the first dorsal interosseus (FDI) muscle of the right (dominant) hand. The electromyographic signal was amplified, filtered (bandwidth = 10–3000 Hz) and collected at a sampling rate of 6000 Hz. A conventional hot-spotting technique was used to identify the target cortical region for the FDI muscle. Using Brainsight 2 stereotaxic navigation software (Rogue Research, Inc.), this location was referenced as the stimulation site for all subsequent TMS protocols. For all protocols described below, invalid trials (e.g. movement artifact) were identified during the TMS procedure and replaced with additional trials at the end of each section,

as necessary. Acceptable intra-individual reliability has been previously demonstrated for TMS-based metrics [42–45].

The standard rMT and 1 mV threshold (rMT_{1mV}) were defined as the lowest stimulation intensity that in half of 10 trials elicited a motor-evoked potential (MEP) in a resting muscle ≥50 μV [46] or ≥1 mV, respectively.

SICI and ICF were assessed using the paired-pulse technique [47,48], which involved an additional stimulator connected with a BiStim² module. Conditioning pulses were delivered at 80% of rMT and test pulses at 120% of rMT. Paired-pulses (with inter-stimulus intervals between conditioning and test pulses set at 1 ms, 2 ms, and 3 ms (for SICI) and 10 ms (for ICF)) and non-paired single test pulses were delivered. The trials (8 per condition) were randomly sorted before the session to determine the order of delivery.

For LICI, we followed a protocol previously performed in people with other types of neurological conditions [49,50]. Two paired-pulse stimulations (both at 100% rMT_{1mV}) were delivered 100 ms apart. Eight single-pulse stimulations at 100% rMT_{1mV} were interspersed randomly between the eight paired-pulse trials. LICI was not collected from several participants (HC (11%), relapsing-remitting MS (27%), and progressive MS participants (36%)) because rMT_{1mV} was beyond the limits of our TMS unit, or a clean and consistent signal could not be acquired at this intensity during the TMS session.

Maximum voluntary contraction was determined using a Preston pinch gauge (Sammons Preston, Illinois, USA). For the cortical silent period (CSP) technique [51], a single suprathreshold stimulus (120% of rMT) was delivered during active contraction of the target muscle (40% of maximum) over ten trials. As Lang et al. previously reported that time of day may influence CSP and LICI (but not other outcomes) [52], we extracted the data collection time of these protocols as recorded in the raw TMS data files.

TMS data analysis

A MATLAB-based analysis program (dataWizard, version 0.7.7, A.D. Wu, UCLA) was used for semi-automatic data processing. A researcher who was blinded to clinical status of participants performed quality control assessments. Among all valid trials within each condition, the average latency and peak-to-peak amplitude of the MEPs were measured. SICI and LICI were calculated according to the formula (1-(mean conditioned MEP amplitude)/(mean unconditioned MEP amplitude)) × 100% [53], and ICF according to (mean conditioned MEP amplitude)/(mean unconditioned MEP amplitude) × 100%, such that higher numbers would reflect a higher percentage of inhibition and facilitation relative to the unconditioned MEP, respectively. SICI at the 2ms interstimulus interval was chosen as a main outcome, as it is close to the peak inhibitory activation believed to reflect synaptic $GABA_A$ receptor mediated activity [54,55] and (unlike SICI at the 3ms inter-stimulus interval) had a distribution that satisfied the assumptions of analysis of covariance (ANCOVA). For CSP, the electromyographic trace was rectified, averaged over all valid trials, and highly magnified for visualization [26]. We measured the minimal absolute CSP duration, defined as the time from the end of the MEP until the earliest return of the electromyographic response [26,56].

MRI data collection

Magnetic resonance imaging (MRI) data were collected at the Montreal Neurological Institute (Siemens TIM Trio scanner, 3 Tesla, 32-channel head coil) in Canada. A T1-weighted 3D fast low-angle shot (FLASH) sequence was collected with repetition time (TR) = 20 ms, echo time (TE) = 5 ms, and 192 slices (slice thickness = 1 mm). T2-weighted 3D fluid-attenuated inversion recovery (FLAIR) images were acquired with TR = 6000ms, TE = 355 ms, 2200 ms inversion time, and 192 slices with $1 \times 1 \times 1$ mm^3 voxel size. Proton density/T2-weighted dual spin echo sequence were collected with TR = 2100 ms and TE = 17/76 ms, for 60 slices (slice thickness = 3 mm). For magnetization transfer (MT) imaging, we acquired a proton density-weighted FLASH sequence pair (TR = ~33 ms, TE = 3.81 ms, number of slices = 192, slice thickness = 1 mm, flip angle = 10°) both with (MT_{ON}) and without (MT_{OFF}) an MT saturation pulse. All images had a field of view of 256×256 mm and in plane resolution of 1×1 mm^2. One HC and three MS participants withdrew from the study without completing the neuroimaging protocol and were therefore not included in the analyses involving the MRI data.

MRI data analysis

Scans were coded to ensure blinding to group during image processing. All image modalities were linearly registered to the T1-weighted image of the participant. MTR within each voxel was calculated according to the formula: MTR (percent units) = $100 \times ([MT_{OFF} - MT_{ON}]/MT_{OFF})$ [57].

For cortical segmentation [58], T1-weighted images were processed with FreeSurfer (version 5.1.0) [59]. Cortical segmentation was manually reviewed and corrected by the second author. T2-weighted white matter lesions were marked using a semi-automated lesion-detection protocol [60]. By applying the scaling factor output from the skull-constrained registration to MNI standard space performed by SienaX [61], normalized lesion volumes were generated. The primary motor cortex hand knob region for the left hemisphere ($M1\text{-}H_L$) was labeled in the MNI-152 atlas space according to previously published anatomical landmarks [62]. The $M1\text{-}H_L$ mask was subsequently transformed into each participant's native space using ANTs [63] and voxels of the transformed $M1\text{-}H_L$ label that were outside of the primary motor cortex (based on FreeSurfer segmentation) were removed. We computed mean MTR within the entire cerebral cortex, $M1\text{-}H_L$ (see Fig. 1), and whole-brain white matter of each participant.

Statistical analyses

HC, relapsing-remitting MS and progressive MS participants were compared on normally and non-normally distributed demographic variables using one-way analysis of variance (ANOVA) and Kruskal–Wallis H-tests, respectively. Post-hoc Tukey HSD or Mann–Whitney U-tests followed as needed. Chi-squared tests were used to compare groups on categorical variables. Unpaired T-tests and Mann–Whitney U-tests, where appropriate, were used for two-group comparisons (i.e. HC vs. full MS sample). Analyses performed with the main purpose of replicating previous work (e.g. [15,16,26–28]) are described in Supplementary Methods.

To address our primary objectives regarding the relationship between cortical damage and cortical activity, and ultimately their combined impact on disability, the following analyses were performed: The relationships between neuroimaging and TMS-based outcomes were assessed with Spearman rank correlation analyses. Stepwise multiple linear regression analyses were performed to establish multivariable models that could best predict performance (9HPT, MSFC).

Results

Participant characteristics

Of participants with MS, 22 had the relapsing-remitting form of the disease and 14 had a progressive disease course (six primary

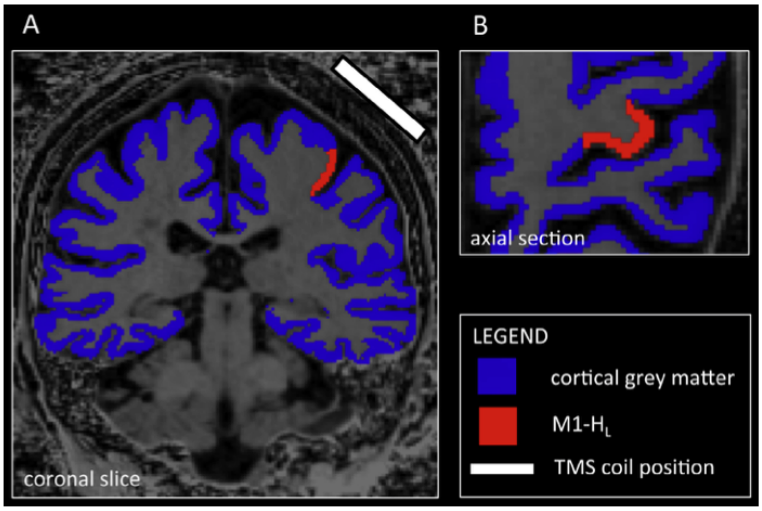

Figure 1. Magnetization transfer ratio (MTR) image of a participant with multiple sclerosis as viewed from a coronal slice (A) and a section of an axial slice centered over the left hemisphere motor hand knob (B). The regions of interest from which MTR was extracted are superimposed on the MTR images, including the cerebral cortex (blue and red regions), as well as specifically within the primary motor cortex hand knob region of the left (dominant) hemisphere (M1-H_L, red region). The approximate location of the transcranial magnetic stimulation (TMS) coil placement relative to the skull during neurophysiological data collection is shown in (A).

progressive, eight secondary progressive). HC and the full MS sample (i.e. relapsing-remitting and progressive MS groups combined) did not differ in age ($t_{52} = -1.30$, $p > 0.05$) or in sex ($\chi^2_{(1)} = 0.04$, $p > 0.05$). Three-group comparisons (see Table 1) showed that progressive MS participants were significantly older than both healthy and relapsing-remitting participants (ps < 0.01), while relapsing-remitting MS and HC groups did not differ. Compared to progressive MS participants, relapsing-remitting MS participants were more likely to be using immunomodulatory medications to treat MS ($p < 0.01$), trended toward having a shorter time since diagnosis ($p = 0.05$), but did not differ significantly in normalized white matter lesion volume ($p > 0.05$) or in age of disease onset ($p > 0.05$). Average time of day of data collection for the CSP and LICI protocols did not differ between the groups (ps > 0.05). Age and sex-adjusted performance and neurophysiological outcomes are shown in Tables 2 and 3; see Supplementary Results for the full description of the related ANCOVA and regression analyses.

Table 1
Demographic characteristics of participants.

	HC	Relapsing-remitting MS	Progressive MS
Total number of subjects	18	22	14
Women, n (%)	13 (72)	15 (68)	10 (71)
Age, mean *years* ± SD**	45 ± 14	44 ± 12	60 ± 13
Taking immunomodulatory medication for MS, n (%)**	0 (0)	12 (55)	3 (21)
Age of MS onset[a], mean *years* ± SD	NA	31 ± 10	38 ± 13
Time since diagnosis[a], mean *years* ± SD	NA	10 ± 7	17 ± 16
Normalized lesion volume[b], *median cm*^{3} (Q1, Q3)***	0 (0, 0)	5.3 (2.5, 16.6)	10.2 (1.5, 21.0)

Asterisks indicate significant differences between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). HC = healthy control; MS = multiple sclerosis; SD = standard deviation; Q1 = lower quartile; Q3 = upper quartile; NA = not applicable. [a] Data unavailable for 2 participants in each group. [b] Data unavailable for 1 HC, 2 relapsing-remitting MS, and 1 progressive MS participant.

Neuroimaging outcomes

Compared to HC participants, MS participants had lower whole-brain cortical volume (mean ± SD: 486 ± 41 vs. 450 ± 43 cm^3, $t_{48} = 2.83$, $p < 0.01$) and MTR (mean ± SD: 36.2 ± 0.4 vs. 35.8 ± 0.7 percent units, $t_{48} = 1.8$, $p < 0.05$). Within M1-H_L, however, MTR did not differ significantly between groups ($U = 264$, $p > 0.05$). MS participants also had lower mean white matter MTR (mean ± SD: 44.0 ± 0.6 vs. 43.3 ± 0.9 percent units, $t_{48} = 2.9$, $p < 0.01$). Among all MS participants, but not healthy controls, lower MEP amplitude was nearly significantly correlated with lower white matter MTR ($r_{(34)} = 0.34$, $p = 0.06$), but not with cortical MTR or volume (ps > 0.3). None of the TMS-based

Table 2
Age- and sex-adjusted performance-based and TMS-based outcomes.

	HC	Relapsing-remitting MS	Progressive MS
Performance			
EDSS (score)**	NA	2.1 ± 0.3	4.2 ± 0.4
MSFC total[a] (z-score)***	0.65 ± 0.12	0.12 ± 0.11	–0.21 ± 0.15
9HPT (z-score)***	0.95 ± 0.15	0.39 ± 0.14	–0.12 ± 0.19
TMS			
rMT (%)	48.5 ± 2.4	47.3 ± 2.2	49.9 ± 3.0
MEP latency (ms)			
Relaxed muscle	23.0 ± 0.4	23.4 ± 0.3	24.5 ± 0.5
Active muscle*	18.9 ± 0.4	18.6 ± 0.4	20.5 ± 0.5
MEP amplitude (mV)			
Relaxed muscle	1.15 ± 0.21	0.85 ± 0.19	0.59 ± 0.26
Active muscle	5.46 ± 0.43	5.14 ± 0.39	3.82 ± 0.53
ICF (%)	166 ± 15	150 ± 14	143 ± 19
SICI (%)	68 ± 6	64 ± 5	47 ± 7
CSP (ms)	77 ± 8	65 ± 7	75 ± 10

Mean ± standard error of the age and sex-adjusted outcomes are shown. Asterisks indicate significant differences found between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). HC = healthy control; MS = multiple sclerosis; EDSS = Expanded Disability Status Scale; MSFC = Multiple Sclerosis Functional Composite; 9HPT = Nine Hole Peg Test; rMT = resting motor threshold; MEP = motor-evoked potential; ICF = intracortical facilitation; SICI = short-interval intracortical inhibition; CSP = cortical silent period; NA = not applicable; [a] Data unavailable for 3 participants.

Table 3
Age- and sex-adjusted TMS-based outcomes of MS participants with a progressive clinical course.

	Primary progressive	Secondary progressive
rMT (%)	49.2 ± 4.3	50.5 ± 3.9
MEP amplitude (mV)		
Relaxed muscle	0.88 ± 0.37	0.34 ± 0.34*
Active muscle	4.51 ± 0.74	3.25 ± 0.68
ICF (%)	163 ± 27	127 ± 25
SICI (%)	61 ± 10	36 ± 9*
CSP (ms)	83 ± 14	68 ± 13

Standard error of the age and sex-adjusted outcomes are shown. Asterisks indicate variables for which significant differences were found from a post-hoc test of the four-group ANCOVA analysis comparing healthy controls, relapsing-remitting MS, primary progressive MS, and secondary progressive MS. MS = multiple sclerosis; rMT= resting motor threshold; MEP = motor-evoked potential; ICF = intracortical facilitation; SICI = short-interval intracortical inhibition, CSP = cortical silent period.

markers of intracortical facilitation and inhibition (ICF, SICI, CSP, LICI) correlated significantly with cortical volume or MTR in any region among people with MS or controls (all ps > 0.05).

We performed additional analyses separating relapsing-remitting and progressive MS participants (Table 4) to test an alternative hypothesis that groups may differ in the relationship between MRI- and TMS-based variables due to use of disease-modifying therapy, plasticity of the motor system [64], or other factors. MTR within M1-H_L correlated with lower SICI among relapsing-remitting MS participants. Lower mean whole brain cortical MTR correlated with shorter CSP duration among progressive MS participants. No other significant correlations were found between TMS-based variables and cortical MTR or cortical volume. To determine if these findings were specific to the cortex, we additionally assessed white matter MTR. Lower white matter MTR correlated with lower SICI among relapsing-remitting MS participants ($r_{(19)} = 0.50$, $p = 0.03$), while no other relationships reached significance (ps > 0.05).

Multimodal predictors of motor and global disability

TMS-based measures of intracortical activity (CSP, ICF, SICI, MEP amplitude), neuroimaging markers of structural damage (cortical volume, cortical MTR (entire cortex and M1-H_L), white matter MTR, T2-weighted white matter lesion load), and demographic (age, sex) variables were entered into a stepwise multiple linear regression analysis to predict performance among all MS participants. LICI was not included due to the number of participants missing data on this variable. A model including CSP, ICF, cortical MTR and sex best predicted 9HPT performance (Table 5). Only cortical MTR was identified as a significant contributor to a model predicting MSFC total score ($R^2 = 0.21$, $F_{1,32} = 8.26$, $\beta = 0.46$ $p < 0.01$).

Table 4
Relationship between cortical MTR and TMS-based outcomes.

Region of interest	TMS	HC	Relapsing-remitting MS	Progressive MS
Whole brain	ICF	0.08	0.02	0.15
	SICI	0.05	0.3	0.26
	CSP	–0.21	0.14	0.57*
	LICI[a]	–0.08	–0.01	0.18
M1-H_L	ICF	–0.01	–0.33	0.18
	SICI	0.37	0.47*	0.01
	CSP	–0.13	0.29	0.05
	LICI[a]	–0.16	–0.34	–0.25

Spearman Rank correlation coefficients of the relationships between cortical MTR and the TMS-based outcomes are shown for each group. Significant correlations (two-tailed) between groups are indicated with asterisks (* p < 0.05). HC = healthy control; MS = multiple sclerosis; M1-H_L = primary motor cortex hand region of the left hemisphere; ICF = intracortical facilitation; SICI = short-interval intracortical inhibition, CSP = cortical silent period; LICI = long-interval intracortical inhibition. [a] Data missing from 2 HC, 4 relapsing-remitting MS, and 6 progressive MS participants.

Table 5
Predictive model of 9HPT performance of MS participants based on stepwise linear regression analysis outcomes.

Step	Model summary	Predictor	β	t	*p*
1	$R^2 = 0.165$	CSP	–0.406	–2.474	0.019
	$F = 6.12$				
	$p = 0.019$				
2	$R^2 = 0.313$	CSP	–0.422	–2.785	0.009
	$F = 6.83$				
	$p = 0.004$	Sex	–0.385	–2.541	0.016
3	$R^2 = 0.419$	CSP	–0.499	–3.427	0.002
	$F = 6.98$	Sex	–0.371	–2.618	0.014
	$p = 0.001$	Cortical MTR	0.336	2.306	0.028
4	$R^2 = 0.502$	CSP	–0.539	–3.896	0.001
	$F = 7.07$	Sex	–0.413	–3.062	0.005
	$p < 0.001$	Cortical MTR	0.310	2.252	0.032
		ICF	–0.296	–2.163	0.039

ICF = intracortical facilitation; CSP = cortical silent period; MTR = magnetization transfer ratio; β = standardized regression coefficient.

Discussion

Using a novel multimodal approach, the present study investigated the relationship between intracortical activity, cortical integrity, and clinical disability. Cortical volume and MTR assessments showed that structural brain damage may contribute to variability in certain forms of intracortical activity. We also replicated previous reports of abnormally low SICI uniquely among people with secondary progressive MS, and found that other TMS-based metrics did not vary with general symptom progression. In combination with information on cortical integrity, however, ICF and CSP can contribute to our understanding of motor system pathology, which affects individuals of all MS subtypes to varying degrees.

Intracortical activity and damage

Compared to healthy participants, people with MS had significantly lower normalized cortical volume and MTR, indicating the presence of atrophy and demyelination [20,21]. However, none of the MRI-based outcomes were related to ICF, SICI, CSP nor LICI when assessing all subtypes of MS together, indicating that cortical damage may not be the primary cause of variability in intracortical facilitation or inhibition in this population. As lower white matter MTR correlated close to significance with lower single pulse MEP amplitude, demyelination may contribute to motor evoked responses used as the baseline for SICI and ICF calculation.

We suspected that plasticity of the motor system [64], disease-modifying therapy, or other factors that tend to differ with clinical subtype could have masked the relationship between MTR and intracortical inhibition or facilitation in some participants. Indeed, further analyses revealed that lower whole brain cortical MTR correlated with shorter CSP duration only among the progressive MS group. Importantly, this indicates that the relationship between poorer motor function and longer CSP is unlikely to have occurred because of cortical damage, but rather in spite of it. As we discuss in more detail in Nantes et al. [26], it is possible that high inhibition among motor-impaired individuals with relapsing-remitting MS may be secondary to disease-related factors such as inflammation or glutamate excitotoxicity [65–71], rather than structural damage. Consistent with this, the present analyses implicate cortical damage in the shortening, rather than the prolongation, of the CSP. The net effect of competing mechanisms influencing CSP length in opposite directions could explain why CSP duration does not differ significantly between healthy and MS participants. Longitudinal multimodal research involving markers of inflammation, cortical

damage, intracortical inhibition, and motor impairment will be needed to test this hypothesis more directly.

Predicting that intracortical inhibition and facilitation may be affected by damage in the vicinity of the primary motor cortical area targeted by TMS, we narrowed our MTR region of interest to the left hemisphere motor hand knob. Lower MTR within this area was related to lower SICI of relapsing-remitting MS participants, and this relationship trended toward significance among healthy participants. As SICI also correlated with white matter MTR, it is also possible that the integrity of white matter tracts could contribute to SICI variability during the relapsing-remitting phase. Thus, we speculate that SICI may be modestly sensitive to normal variability in the myelination of the stimulated cortical region and/or white matter tracts among minimally disabled people, while other disease-related factors may contribute to pathologically low SICI observed in secondary progressive MS. While beyond the scope of the present study to assess, low SICI among progressive MS participants could be a consequence of cortical motor map re-organization [72], spinal cord damage [73], cortical lesions to which MTR is insensitive, or other disease-related factors for which the impact on intracortical inhibition is not known.

Multimodal predictors of motor disability

Our multivariate analysis revealed that motor impairment could be best predicted using a combination of TMS-based, neuroimaging, and demographic variables. Specifically, longer CSP, higher ICF, lower cortical MTR, and sex were independently related to upper extremity impairment. This demonstrates that biomarkers of excitatory and inhibitory cortical activity can provide information relevant to motor impairment that is complementary, rather than redundant, to measures of structural cortical integrity. By contrast, none of the TMS-based measures predicted overall MSFC score beyond the impact of cortical MTR. This suggests that, in the context of complex neurological conditions such as MS, electromyographic activity-derived biomarkers are more relevant to motor impairment than to general disability progression.

Of further interest is that higher ICF was significantly related to worse motor impairment only after adjusting for other variables including intracortical inhibition and cortical MTR. While ICF may reflect glutamatergic activity [9,10], the clinical relevance of this may lie more in strength of excitatory activity relative to inhibition and damage, as opposed to a simple increase in ICF irrespective of other variables. This finding highlights the value of assessing both inhibitory and excitatory forms of cortical activity, and cortical damage, concurrently.

Limitations and future directions

Sample size, particularly among those with the primary progressive MS subtype, was a limitation to the present study. Additionally, outcomes involving LICI should be interpreted with caution considering that participant exclusion due to the high stimulation intensity required may have been non-random. We encourage others to assess LICI among people with progressive forms of MS using a protocol that requires a lower intensity of stimulation. Interpretation of CSP-related outcomes is difficult as longer CSP may have been selectively related to motor performance due to the relative sensitivity of this metric to physiological mechanisms related to spinal cord inhibition [74,75] or motor attention [76]. Nonetheless, the interpretation that CSP reflects intracortical inhibition is consistent with studies linking lower GABA concentration around the motor cortex hand knob region to less severe motor impairment among individuals recovering from stroke [34,35] and people with relapsing-remitting MS [77]. Additional studies are needed to determine if other factors that could influence impairment (e.g. spinal lesions, central motor conduction time, short-interval intracortical inhibition) are independent predictors of motor performance from CSP, ICF, and cortical MTR. Furthermore, the exploratory secondary outcomes included in this study should be replicated.

MTR may also be useful to investigate the link between structural damage and other TMS-based variables linked to disability in people with MS, including inter-hemispheric excitability [78] and short-interval intracortical facilitation [28]. Alternative MRI techniques sensitive to cortical damage, such as diffusion recovery imaging and ultrahigh-field MRI [79] could also help to elucidate the relationship between cortical damage and activity. Such studies may consider defining the cortical region relevant to the TMS hot-spot functionally, rather than anatomically, as shifts in the functional hot-spot could occur in response to brain injury [72]. Future higher-powered studies should investigate how damage within various structures of the motor network, as well as the integrity of structural and functional connections between these regions, impacts the various forms of intracortical inhibition and facilitation that can be measured with TMS.

Conclusions

The present study sheds light on the clinical value, and limitations, of using TMS-based metrics in the context of neurological disease, and demonstrates the importance of multimodal assessments. While we confirm SICI to be abnormally low in secondary progressive MS, our data support that this may not be simply a consequence of cortical damage. We also find evidence that motor system dysfunction may be driven by physiological factors related to CSP and ICF that are independent of cortical damage. Longitudinal and interventional studies are needed to assess the potential clinical utility of the present study's multimodal approach.

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Appendix: Supplementary material

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