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Investigating the long-term stability and neurochemical substrates of TMS and MRS

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Abstract

Transcranial magnetic stimulation (TMS) and magnetic resonance spectroscopy (MRS) are non-invasive techniques that allow the measurement of GABAergic and glutamatergic activity in the brain. TMS and MRS can be used to assess inhibitory and excitatory mechanisms, treatment response or disease presence and progression *in vivo*. However, despite their growing use in research and medical settings, ambiguity remains regarding their neurochemical substrates and long-term reproducibility. The goal of the present thesis is twofold. First, the long-term stability and reliability of various MRS and TMS measurements, obtained in the motor cortex, was investigated. Second, to better understand which aspects of the GABAergic network are targeted by the two techniques, TMS and MRS measures reflecting cortical inhibition and excitation were obtained following lorazepam administration using a placebo-controlled, double-blind, randomized, crossover design.

Two articles comprise this thesis. The first article is a longitudinal assessment of the stability and reliability of MRS-GABA and Glx (glutamate + glutamine) and TMS measures of cortical inhibition and facilitation in the sensorimotor (SMC) cortex of healthy adults. It was determined that MRS-GABA and MRS-Glx are stable over a three-month interval. TMS measures of resting motor threshold (rMT), cortical excitability (% maximum stimulator output; MSO) and cortical silent period (CSP) were also found to be stable and reliable. However, paired-pulse TMS measures such as short-interval cortical inhibition (SICI), long-interval cortical inhibition (LICI) and intracortical facilitation (ICF) had greater variability.

The second article aims to understand the differential sensitivity of TMS and MRS with respect to GABAergic activity in the primary motor cortex. It is based on the results and conclusions of a placebo-controlled, double-blind, randomized, crossover study, where benzodiazepine lorazepam was given to healthy adult volunteers. Magnetic resonance spectroscopy (GABA and Glx) was performed in the sensorimotor cortex and occipital cortex (OC). TMS measurements were acquired in the motor cortex only. MRS and TMS measures of cortical inhibition and excitability (rMT, input/output (I/O) curve, SICI, LICI, ICF, CSP) were

obtained following lorazepam or placebo administration. Lorazepam was found to decrease occipital GABA concentration, increase motor cortical inhibition and decrease cortical excitability. Lorazepam administration had no effect on other neurometabolites or TMS measurements. The effect of Lorazepam on short-interval cortical inhibition was found to depend on endogenous GABA levels in the SMC; higher GABA concentrations predicted a greater increase in SICI following drug intake.

Taken together, the studies presented in this thesis indicate that MRS neurometabolite levels are stable over time and may thus potentially serve as markers for the monitoring of disease progression and treatment response. However, while some TMS measures have good long-term stability (rMT, %MSO, CSP), others are not as reliable nor stable (SICI, LICI, ICF); care must be taken in clinical settings. Furthermore, the differential effects of lorazepam on MRS and TMS measures support the idea that the two techniques probe different aspects of the GABAergic system. Whereas TMS measures of cortical inhibition reflect phasic $GABA_A$ receptor activity, MRS-GABA primarily reflects intracellular, non-neurotransmitter metabolic GABA.

Keywords: Magnetic resonance spectroscopy, transcranial magnetic stimulation, GABA, glutamate, lorazepam, motor cortex.

Résumé

La stimulation magnétique transcrânienne (SMT) et la spectroscopie par résonance magnétique (SRM) sont des techniques non-invasives permettant de quantifier l'activité GABAergique et glutamatergique du cerveau. La SMT et la SRM ont plusieurs applications en clinique et en recherche. En effet, ces outils peuvent être utilisés afin de déterminer l'efficacité d'un traitement ou la progression d'un processus pathologique. Cependant, malgré leur utilisation croissante dans le domaine médical, une certaine incertitude demeure quant aux substrats neurochimiques de ces techniques et à la stabilité à long terme des données acquises par SMT et SRM. Donc, dans un premier temps, la stabilité à long terme de plusieurs mesures prises par SMT et par SRM a été étudiée. En second lieu, afin de mieux comprendre quelles composantes du système GABAergique sont ciblées par ces deux techniques, des mesures de SRM et de SMT ont été obtenues après l'administration d'une benzodiazépine, le lorazépam, selon un devis expérimental randomisé, croisé, à double-aveugle et contrôlé par placébo.

Deux articles composent cette thèse. Le premier article fait état d'une étude longitudinale, auprès d'adultes en santé, ayant pour but de déterminer la stabilité à long terme des concentrations de GABA et de Glx (glutamate + glutamine) obtenues par SRM ainsi que la stabilité des mesures d'inhibition et de facilitation corticale obtenues par SMT (rMT : seuil moteur au repos, %MSO : pourcentage d'intensité maximale du stimulateur, SICI : inhibition intra-corticale courte, LICI : inhibition intra-corticale longue, ICF : facilitation intra-corticale). Il a été démontré que les niveaux de GABA et de Glx sont stables au cours d'une période de trois mois. Alors que les mesures SMT de seuil moteur au repos, d'excitabilité corticale et de période corticale silencieuse sont stables à travers le temps, l'inhibition corticale à court intervalle et à long intervalle ainsi que la facilitation corticale sont beaucoup plus variables.

Le deuxième article vise à comprendre la dissociation dans la sensibilité des mesures de SMT et SRM à refléter différentes facettes de l'activité GABAergique du cortex moteur. L'article porte sur une étude dans laquelle du lorazépam a été administré à des participants adultes en santé selon un devis randomisé, croisé, à double-aveugle et contrôlé par placébo. Des

données SRM (GABA et Glx; cortex sensorimoteur et occipital) ainsi que des mesures SMT (cortex moteur) ont été obtenues suivant l'administration de lorazépam (ou de placébo). Il a été démontré que la prise de lorazépam réduisait les niveaux de GABA occipitaux, augmentait l'inhibition corticale et réduisait l'excitabilité du cortex moteur. La prise de médicament n'avait pas d'effet sur les autres mesures obtenues. De plus, il a été trouvé que l'effet du traitement sur l'inhibition corticale dépendait des concentrations endogènes de GABA dans le cortex sensorimoteur; une plus grande concentration de GABA étant prédictive d'une plus grande inhibition corticale suivant la prise de lorazépam.

Dans leur ensemble, les résultats provenant des deux articles présentés dans cette thèse permettent de conclure que les mesures SRM des divers neurométabolites sont stables à long terme dans le cortex moteur et pourraient potentiellement servir de marqueurs dans l'évaluation de l'efficacité d'un traitement ou de l'évolution de processus pathologiques. Par contre, bien que certaines mesures SMT soient stables à long terme (rMT, %MSO, CSP), d'autres sont beaucoup plus variables (SICI, LICI, ICF); ainsi, la prudence est conseillée dans l'interprétation de ces mesures lors d'études cliniques. De plus, les effets différents que produit la prise de lorazépam sur les mesures SRM et SMT supportent la théorie selon laquelle les deux techniques n'ont pas les mêmes substrats neurochimiques. En effet, alors que les mesures TMS d'inhibition corticale refléteraient l'activité phasique des récepteurs $GABA_A$, le signal SRM de GABA serait majoritairement intracellulaire et ne représenterait pas la neurotransmission GABAergique.

Mots-clés : Spectroscopie par résonnance magnétique, stimulation magnétique transcrânienne, GABA, glutamate, lorazépam, cortex moteur.

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Acronyms

%MSO: Percentage of the maximum stimulator output

5-HT: Serotonin

ACC: Anterior cingulate cortex

Ach: Acetylcholine

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate

BZD: Benzodiazepines

Cho: Choline

Cr: Creatine

CRLB: Cramér-Rao Lower Bound

CS: Conditioning stimulus

CSF: Cerebrospinal fluid

CSP: Cortical silent period

DA: Dopamine

GABA: γ-aminobutyric acid

$GABA_AR$: Type A GABA receptors

$GABA_BR$: Type B GABA receptors

Gln: Glutamine

Gln: Glutamate

Glx: Glutamate + Glutamine

GM : Gray matter

H_2O : Water

ICF: Intracortical facilitation

IPSP: Inhibitory post-synaptic potential

ISI: Interstimulus interval

LICI: Long-interval cortical inhibition

M1: Primary motor cortex

MEP: Motor evoked potential

MM: Macromolecules

MRI: Magnetic resonance imaging

MRS: Magnetic Resonance spectroscopy

NAA: N-acetyl aspartate

NE: Noradrenaline

NMDA: N-methyl-D-aspartate

ppTMS: paired-pulse transcranial magnetic stimulation

OC: Occipital cortex

RF: Radiofrequency

rMT: Resting motor threshold

ROI: Region of interest

SICI: Short-interval cortical inhibition

SMC: Sensorimotor cortex

tCr: Total creatine

TMS: Transcranial magnetic stimulation

TS: Test stimulus

VOI: Voxel of interest

WM: White matter

To my family

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Chapter 1 – Background Information

Overview

Transcranial magnetic stimulation and magnetic resonance spectroscopy can index GABAergic and glutamatergic activity in the brain. Whereas TMS indirectly probes inhibitory and excitatory activity through various protocols, MRS can directly measure neurotransmitter concentrations *in vivo* (Stagg, Bachtiar, & Johansen-Berg, 2011a; Tremblay et al., 2012). Due to their growing use and application in the medical field, several studies have assessed their short-term reliability. However, the long-term reliability of these techniques needs to be further studied to validate MRS and TMS as disease progressing and treatment response monitoring tools. Furthermore, although the underlying neurochemical mechanisms and substrates of the two techniques have been assessed, ambiguity remains as to how TMS and MRS measures probe the GABAergic and glutamatergic neurotransmitter systems. Shedding light on the neural underpinnings of these techniques is paramount in establishing their use in a clinical setting.

GABA and Glutamate Neurochemistry and BZD Modulation

Below is a section comprising an overview of the neurochemistry of GABAergic and glutamatergic systems as it pertains to the comprehension of the present thesis.

GABAergic Neurotransmission and Associated Receptors

Known as the primary inhibitory neurotransmitter in the brain, gamma-aminobutyric acid is involved in inhibitory signalling through the action of ionotropic and metabotropic receptors distributed pre, post and extra synaptically (Olsen & Sieghart, 2008). Due to the GABAergic system's far-reaching involvement in nervous system functioning, understanding how MRS and TMS can measure GABAergic activity is of considerable interest.

GABA Synthesis, Metabolism and Transport

GABA is synthesized within GABAergic neurons mainly from glutamate via the enzyme glutamate decarboxylase (GAD) and catabolized to succinic semi-aldehyde by GABA-transaminase. The latter process is termed the GABA shunt and is considered irreversible (Myers, Nutt, & Lingford-Hughes, 2016; Rae, 2014).

The majority of GABA lies within cell bodies, in a large GABA cytosolic metabolic pool (Patel, Rothman, Cline, & Behar, 2001; Paulsen, Odden, & Fonnum, 1988; Rae et al., 2003; Tapia & Gonzalez, 1978). As such, only a small portion is considered neurotransmitter GABA. Most neurotransmitter GABA is stored in vesicles. Cytosolic and vesicular GABA are in dynamic exchange (Bak, Schousboe, & Waagepetersen, 2006). Vesicular storing after synthesis is done through the action of vesicular GABA transporter (VGAT), and GABA can later be released into the synapse (Rae, 2014). When in the synaptic cleft, it can either bind to GABA receptors or be taken by GABA transporters. There are many classes of GABA transporters, with distinct pharmacological properties, which goes beyond the scope of the present work (Schousboe, 2000). Thus far, it is known that GATs can be found extrasynaptically on GABAergic neurons or on non-GABAergic neurons (Richerson & Wu, 2003; Yasumi, Sato, Shimada, Nishimura, & Tohyama, 1997). Thus, through GABA transporter (GAT) action, GABA can re-enter GABAergic neurons or be taken into glial cells where it will undergo catabolisis through the GABA shunt. Interestingly, GABA may even regulate transporter activity (Bernstein & Quick, 1999) and extrasynaptic transporters may sometimes operate in reverse, depending on the potential of the cell membrane. This would allow GABA to be released and in turn bind to extrasynaptic receptors, which contributes to tonic inhibition (Jackson, Esplin, & Čapek, 2000; Wu, Wang, Díez-Sampedro, & Richerson, 2007). The latter form of inhibitory signalling will be described in a later section.

GABA Receptors

Two main classes of GABA receptors, $GABA_A$ and $GABA_B$, have been identified, and can be differentiated through their molecular structure and pharmacological affinities. These receptors can be found throughout the brain, pre-, post- and extrasynaptically (Rae, 2014). While activation of both subtypes leads to cell hyperpolarization, $GABA_A$ receptors are fast-acting pentameric

ligand-gated ionotropic chloride-ion channel and $GABA_B$ receptors are slow-acting metabotropic heterodimeric G protein-coupled sites (Enna, 2007). A novel and lesser know ionotropic $GABA_C$ receptor has also been identified (Rae, Nasrallah, Griffin, & Balcar, 2009).

$GABA_A$ Receptor Structure and Function

Comprised of distinct molecular subunits, $GABA_A$ receptors are part of the cysteine-loop ligand-gated ion receptor family. These ionotropic receptors are pentameric assemblies of molecularly distinct subunits which cluster to form a central ion channel. While nineteen different subunits have thus far been cloned (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π and ρ_{1-3}) (Simon, Wakimoto, Fujita, Lalande, & Barnard, 2004), only a few of these combinations have been identified *in vivo* (Fritschy & Mohler, 1995; Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000; Wisden, Laurie, Monyer, & Seeburg, 1992). Nevertheless, several isoforms exist in humans or animals and, due to different subunit combinations and structural heterogeneity, there are a variety of $GABA_A$ receptors with distinct properties. Indeed, $GABA_A$ receptor sensitivity and activity is modulated by molecules acting at distinct sites on individual or multiple subunits (Möhler et al., 1997; Möhler, Fritschy, Crestani, Hensch, & Rudolph, 2004). Furthermore, $GABA_A$ receptor trafficking and localization are also determined by subunit composition (Nusser, Sieghart, & Somogyi, 1998; Wei, Zhang, Peng, Houser, & Mody, 2003). In addition, pharmacokinetic properties and responses to chemical modulation vary depending on subunit makeup. Biological and pharmacological receptor properties are thus determined by subunit composition (McKernan et al., 2000).

Most *in vivo* receptors are heteropentamers comprised of α- β- and γ- subunit isoforms (Barnard et al., 1998; Sieghart & Sperk, 2002) which assemble according to a 2 α, 2 β and 1 γ stochiometric ratio (Farrar, Whiting, Bonnert, & McKernan, 1999; Tretter, Ehya, Fuchs, & Sieghart, 1997). In the brain, most $GABA_A$ receptors are made using combinations of α_1 - α_2 - α_3 - α_5 - and β or γ subunits with the most prevalent receptor subtype being of the $\alpha_1\gamma_2\beta$ subunit combination, which is present throughout the brain with only the reticular nucleus of the thalamus, the spinal motoneurons and the granule cell layer of the olfactory bulb lacking this type of receptor (Martin & Olsen, 2000). $GABA_A$ receptor subunits can be found throughout the brain and peripheral organs. However, their role in the latter's functioning is largely unknown due to sparse

GABAergic innervation. In the brain, these receptors are associated with the major inhibitory neurotransmitter, GABA (Möhler, 2006).

These ion channel proteins open in response to binding of a chemical messenger, a ligand, to allow ion influx across the cell membrane. Activation of $GABA_A$ receptors leads to an increase in intra-neuronal chloride ion concentration causing cell hyperpolarization (Payne, Rivera, Voipio, & Kaila, 2003; Rivera, Voipio, & Kaila, 2005). This form of neurotransmission is termed phasic inhibition. It is the mode through which fundamental information is relayed within the CNS. It is mediated through fast-acting ionotropic $GABA_AR$ activity which leads to IPSP generation. When a local action potential arrives at the nerve terminal, a net calcium influx induces synaptic vesicular fusion with the presynaptic membrane at the site of release. GABA is then released into the synaptic cleft, reaching transient millimolar concentrations (Mody, De Koninck, Otis, & Soltesz, 1994), and allowing GABA binding to its receptors and ion channel opening. What further characterises this form of neurotransmission is the rapid time constant (< 500 μs) with which synaptic GABA levels decay (Farrant & Nusser, 2005).

Another form inhibitory signalling is also present. Tonic inhibition is a form of signalling, as opposed to phasic inhibition, that is not as temporally restricted and operates independently from phasic events (Mody, 2001). It would stem from GABA escaping from the synaptic cleft and activating extrasynaptic receptors in a constant or 'tonic' fashion (Farrant & Nusser, 2005). GABA levels in the extracellular space are low, in the micromolar range (Cavelier, Hamann, Rossi, Mobbs, & Attwell, 2005; Rae, 2014; Wu et al., 2007). Tonic inhibition may also occur due to GAT reversal, which operates against the cell concentration gradient, maintaining GABA in the extracellular space (Rae, 2014; Wu et al., 2007; Wu, Wang, & Richerson, 2003).

$GABA_B$ Receptor Structure and Function

$GABA_B$ receptors are the second major category of GABA receptors and are metabotropic in nature. They are found throughout the nervous system both pre- and post-synaptically (Enna, 2001a, 2001b). As opposed to $GABA_A$ receptors, they are coupled to G proteins (guanine

nucleotide-binding proteins), which can be found in an active (G_i) or inactive form (G_o), thereby acting as a signalling switch (Kaiser, Krieger, Lodish, & Berk, 2007). $GABA_B$ receptors are thus termed G-protein coupled receptors. $GABA_B$ receptor activation leads to a reduction of adenylyl cyclase activity, and an increase in cyclic AMP production, a secondary messenger which regulates ion (Ca^{2+}) channel function (Enna, 2001a; Kaiser et al., 2007). Stimulating these receptors results in potassium conductance increase, leading to cell hyperpolarization (Enna, 2001a). Activation of these receptors also leads to a reduction in excitatory postsynaptic potentials, through modulation of presynaptic calcium conductance, which leads to a reduction in excitatory neurotransmitter release (Dunlap, 1981; Newberry & Nicoll, 1985). This receptor system is thus inhibitory in nature. $GABA_B$ receptor-mediated changes in ion channel activity are thought to result from G-protein subunit liberation, which directly modulates K^+ channel function and secondary messenger production. The $GABA_B$ receptor is heterodimeric and is comprised of $GABA_{B1}$ and $GABA_{B2}$ subunits. The GABA recognition site is located on the $GABA_{B1}$ subunit, and the G-coupled effector system and allosteric modulation site is linked to the $GABA_{B2}$ subunit (Margeta-Mitrovic, Jan, & Jan, 2000; Robbins et al., 2001).

$GABA_c$ Receptor Structure and Function

Another class of ionotropic receptor has been identified; however, little is known about its functioning in the cerebral cortex. It is a pentameric assembly composed of ρ subunits (ρ_1 ρ_2 ρ_3) (Rae et al., 2009). Further information regarding this receptor type lies beyond the scope of the present thesis.

Glutamatergic System and Neurotransmission

Known as the major excitatory neurotransmitter in the brain, glutamate's role in the central nervous system is paramount. While the present thesis focuses primarily on GABA, below is a brief overview of the glutamatergic system.

Glutamate Synthesis, Metabolism and Transport

Glutamate synthesis may occur through several chemical processes which go beyond the scope of the present thesis. Glutamate exists in various metabolic pools in the brain with different turnover rates (Berl, Lajtha, & Waelsch, 1961; Shank, Leo, & Zielke, 1993). Furthermore, the distinction between metabolic and neurotransmitter glutamate (which was done for GABA) is not deemed useful (Rae, 2014). Intracellular glutamate is in the millimolar range while extracellular glutamate is in the micromolar range. This concentration gradient is kept to maintain optimal signal-to-noise ratios needed for efficient neurotransmission. Removal from the extracellular space is performed through the action of transporters located in neurons and glial cells (Rae, 2014) as well as by a cystine/glutamate antiporter (Albrecht et al., 2010).

Glutamate Receptor Overview

Glutamate has three ionotropic receptor subtypes: N-methyl-D-aspartate (NMDA), AMPA and Kainate as well as one metabotropic receptor subtype: mGluR (Rae, 2014). Further information on these receptors goes beyond the scope of the present work.

Benzodiazepine Mechanism of Action

Typically used as anxiolytics, benzodiazepines (BZD) are known to modulate inhibitory signalling through binding of an allosteric site on pentameric ionotropic gamma-aminobutyric acid A receptors ($GABA_AR$) (Di Lazzaro et al., 2006a). While GABA directly activates this receptor by binding on its recognition site, other pharmacological agents, such as benzodiazepines, act on other target receptor sites, indirectly facilitating $GABA_A$ receptor activity, and are thus termed allosteric modulators of $GABA_A$ receptor function. These drugs modulate $GABA_A$ receptor activity. Indeed, BZD binding induces conformational changes in the receptor, which enhances binding of its endogenous ligand (GABA), leading to increased GABA-sensitive chloride channel discharge frequency. This influx of chloride ions leads to cell hyperpolarization, triggering inhibitory action potentials. While the precise affinity profile of lorazepam, the drug chosen in the present work, for the different GABA receptor isoforms is unknown, this pharmacological agent is considered a classical benzodiazepine with a broad affinity for $\alpha_{1\text{-}2\text{-}3\text{-}5}$ subunits (Di Lazzaro et al., 2006a;

Fritschy & Mohler, 1995). Indeed, isoforms 1, 2, 3 and 5 of the alpha subunits all possess a histidine residue with a high BZD affinity profile. Inversely, isoforms 4 and 6 contain an arginine residue for which BZDs have no affinity (Griffin, Kaye, Bueno, & Kaye, 2013). It is believed that benzodiazepines do not modulate $GABA_B$ receptor activity directly as it is considered an exclusive $GABA_AR$ agonist. BZDs should also not directly modulate glutamatergic activity.

Transcranial Magnetic Stimulation

Overview

To induce magnetic stimulation, a changing magnetic field must be created. Through electromagnetic induction, this magnetic flux can generate an electrical current in the brain, which behaves as a conductor (Wassermann, Epstein, & Ziemann, 2008). To do so, TMS machines are equipped with a capacitor able to discharge rapidly across a copper coil placed on the scalp (Barker, Jalinous, & Freeston, 1985; Bohning, 2000; Ruohonen, 2003). This rapid discharge alters the magnetic field penetrating the scalp and produces a focalized electrical current in the area over which the coil was placed. This small electrical current targets cortical white matter where a transfer of charge along myelinyzed axons raises intracellular potential, thus triggering cellular membrane depolarization (Reilly, 1989; Roth & Basser, 1990). Since magnetic field intensity is inversely proportional to the coil-conductive material distance, it is possible to conclude that the depolarization is limited to the area under the coil. Furthermore, it can be assumed that TMS affects mainly white matter, which has lower impedance than gray matter (Wassermann et al., 2008).

In the motor cortex, TMS induces neuron depolarization and activates interneurons, leading to trans-synaptic activation of pyramidal cells. Descending volleys are induced in the corticospinal tract, where pyramidal axons project on spinal motoneurons (Klomjai, Katz, & Lackmy-Vallée, 2015). TMS-triggered motoneuron activation induces muscle responses called motor-evoked potentials (MEP) that can be recorded electromyographically using electrodes applied over the desired muscle. MEP peak-to-peak amplitude provides insight on corticospinal tract excitability and conduction in both healthy subjects and in patients suffering from central nervous system diseases (Wassermann et al., 2008). Furthermore, there are many different TMS techniques and

measures, with physiologically distinct mechanisms, which may be used to probe the brain and to evaluate treatment response (Cantello et al., 1991; Mills, 2003; Ziemann, Paulus, & Rothenberger, 1997a).

It is important to note that throughout TMS studies, pulse intensity refers to the percentage of maximum stimulator output (%MSO) and not to the peak magnetic field amplitude (in Tesla) induced by the coil. As neurophysiological responses are directly induced by peak magnetic field amplitudes and not TMS intensity, ensuring stable coil positioning throughout stimulation protocols is a critical methodological condition that must be met. Indeed, varying the distance and orientation of the coil throughout experiments may introduce significant error in the magnetic field strength that stimulates the brain, which would make measurement less reliable. Likewise, different TMS machines may emit pulses of varying power at the same %MSO, which partially limits comparisons between studies that use different equipment (Wassermann et al., 2008).

Stability of the Measurements

As previously stated, assessing the long-term stability of various TMS protocols is paramount in validating the technique's use in the medical field and to further its research applications. While there appears to be a consensus as to the reliability and stability of MT and cortical excitability (see below), ambiguity remains regarding test-retest stability of paired-pulse TMS protocols, and few studies have examined cortical silent period length stability. The following section will focus on studies performed on healthy gender-mixed adult (aged 19-83, average 29 ± 5 years) right-handed participants, with no history of neurological or psychiatric illnesses and no neuroactive medication intake, and with MEP measurements that were acquired from hand muscles. Only one study was performed on males only (Boroojerdi et al., 2000), one study included older adults (Kimiskidis et al., 2005), and a single known study examined gender effects on stability, and found none (Hermsen et al., 2016). While aging and sex were shown to affect TMS measures of motor excitability as well as cortical inhibition and facilitation, no known study aiming at assessing TMS reliability and stability examined aging effects (Ziemann et al., 2015).

Motor Threshold

The motor threshold (MT) refers to the minimum intensity that is necessary to elicit MEPs of >50μV in 5 out of 10 trials (Rossini et al., 2015). MT measurements can be obtained both at rest (no muscle contraction) or while active (tonic isometric muscle contraction). Several studies have investigated the short-term and long-term reliability of MT in healthy controls and have found good test-retest reliability over time (Hermsen et al., 2016; Malcolm et al., 2006; Ngomo, Leonard, Moffet, & Mercier, 2012; Plowman-Prine, Triggs, Malcolm, & Rosenbek, 2008). There thus appears to be a consensus that the motor threshold remains stable for time intervals as long as three months (Hermsen et al., 2016).

Cortical Excitability

Motor cortical excitability, often assessed by measuring the intensity required to induce MEPs of 1 mV amplitude (%MSO), was also found by several studies to possess good short- and long-term reliability (Hermsen et al., 2016; Maeda, Gangitano, Thall, & Pascual-Leone, 2002; Ngomo et al., 2012). Similarly to MT, cortical excitability, when assessed in healthy subjects shows little variation over extended periods of time (up to 3 months) (Hermsen et al., 2016).

Cortical Silent Period

The cortical silent period (CSP) refers to a TMS-induced interruption of voluntary activity in the EMG of a pre-contracted target muscle. When induced in a hand muscle, its duration is usually between 100 and 300 ms (Kimiskidis et al., 2005). Few studies have assessed CSP duration stability to date. However, a recent study by Hermsen and collaborators (2016), performed in a large (n = 93) sample of healthy subjects, demonstrated moderate test-retest reliability when assessed via automated software (r = 0.486) or visually (r = 0.466).

Short-Interval Intracortical Inhibition

Short-interval intracortical inhibition is a paired-pulse technique were a first conditioning sub-MT pulse (conditioning stimulus) is delivered and followed by a supra-MT pulse (test stimulus) at intervals between >1 ms and 6 ms (Rossini et al., 2015). The conditioning stimulus (CS) reduces

the MEP amplitude of the test stimulus (TS) (Kujirai et al., 1993). An inhibition ratio (CS-TS/TS MEP amplitude) can be computed, and its reliability can be assessed. Maeda and collaborators (2002) found SICI to have good test-retest reliability when assessed in the same day (r=0.88). Hermsen and collaborators (2016) have found SICI to have moderate test-retest reliability over a one-month interval (r = 0.383). Dyke and collaborators (2018) have also reported good test-retest same-day reliability. A study by Ngomo and collaborators (2012) reported good intra-class correlations in a relatively small sample (n = 12), for both short (r = 0.83) and long (0.91) time intervals, despite high between-session CVs for both short- and long-time intervals. Previous studies assessing reliability (Boroojerdi et al., 2000; Orth, Snijders, & Rothwell, 2003) have found similarly high between-session coefficient of variation for SICI ranging from 31 to 37%. Therefore, it appears that despite high coefficients of variation, reliability statistics, such as test-retest and intraclass correlations, point towards SICI being a fairly reliable parameter.

Long-Interval Intracortical Inhibition

Long-interval cortical inhibition (LICI) consists of two suprathreshold pulses that are applied at an inter-pulse interval between 50 ms and 200 ms (Rossini et al., 2015). A study by Farzan (2010) has shown $LICI_{100ms}$ to be stable across a seven-day interval (Farzan et al., 2010). At the time of writing, it appears that no other study has examined the long-term stability of LICI.

Intracortical Facilitation

Intracortical facilitation (ICF) is similar in application to SICI except that the ISI is between 6 and 30 seconds, and the first sub-MT pulse facilitates the resulting TS MEP. While no consensus has yet been reached, it appears that intracortical facilitation shows poor stability across various time intervals. Indeed, Hermsen and collaborators (2016) have reported a null test-retest correlation (r = - 0.159) for ICF_{10ms} despite their large sample size (n = 93). Similarly, Dyke and collaborators (2018) have found poor to fair test-retest reliability, for same day measurement stability. Previous studies have found high between-session CVs for ICF ranging from 20 to 22.7 % (Boroojerdi et al., 2000; Orth et al., 2003). However, Maeda and collaborators (2002) have reported good test-retest reliability for ICF measures.

TMS Neurochemistry

Combining TMS protocols with central nervous system-active compounds with a known mechanism of action allows indirect evaluation of the underlying mechanisms through which TMS produces its effects (Teo, Terranova, Swayne, Greenwood, & Rothwell, 2009). Through studying these interactions, it is possible to infer the physiological mechanisms underlying the measurement. Below is a summary of the relevant measures and their proposed neurochemical substrates. It should be noted that no systematic assessment of pharmaco-TMS interactions of factors that may influence baseline TMS outcomes, such as age and sex, was performed (Ziemann et al., 2015).

Motor Threshold

Motor threshold (MT) reflects corticospinal excitability and depends on the excitability of axons activated by the TMS pulse, as well as excitability of synaptic connections to corticospinal neurons. MT thus appears to depend on glutamatergic synaptic activity (Paulus et al., 2008), where voltage-gated sodium channels are essential to axonal excitability regulation (Hodgkin & Huxley, 1952). Furthermore, ionotropic non-N-methyl-D-aspartate (non-NMDA) glutamate receptors are responsible for fast excitatory synaptic transmission (Douglas & Martin, 1998). Thus, pharmacological agents capable of blocking voltage-gated sodium channels, which reduces corticospinal excitability, influence MT. Indeed, MT was found to be elevated following the administration of anticonvulsants such as lacosamide, carbamazepine (Lang, Rothkegel, Peckolt, & Deuschl, 2013), phenytoin (Chen, Samii, Canos, Wassermann, & Hallett, 1997) and lamotrigine (Tergau et al., 2003). Motor threshold was found to be lower following the administration of NMDA receptor antagonist ketamine, which causes an indirect increase in glutamatergic transmission, mediated by the non-NMDA AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid) receptors (Lazzaro et al., 2003). Drugs targeting GABAergic neurotransmission as well as drugs targeting neuromodulators such as dopamine (DA), serotonin (5-HT), norepinephrine (NE) and acetylcholine (Ach) did not result in consistent effects on motor threshold (Paulus et al., 2008). Based on the following pharmacological profile, it was concluded

that MT relies on glutamatergic neurotransmission, and reflects axon excitability (Di Lazzaro, Ziemann, & Lemon, 2008).

Cortical Excitability

Cortical excitability can be measured using motor evoked potential (MEP) amplitude, which increases with stimulus intensity in a sigmoid fashion (Möller, Arai, Lücke, & Ziemann, 2009). While low intensity stimulation, at a slightly supra-threshold intensity, induces a corticospinal volley yielding a single indirect wave (I1-wave), high intensity stimulation yields multiple I-waves (I2-I4), which add up to produce higher amplitude MEPs (Di Lazzaro et al., 2008). The latter waves are thought to be produced through the activation of a cortical excitatory interneuron chain; whose circuitry is easily modifiable. Their activation leads to the activation of corticospinal neurons across synapses (Di Lazzaro & Ziemann, 2013). Thus, they are expected to be modulated by both neurotransmitter (GABA, glutamate) and neuromodulator (DA, NE, 5-HT, Ach) systems (Hasselmo & Barkai, 1995). Administrating compounds that alter these systems can influence MEP amplitude and thereby modulate cortical excitability.

Indeed, it was found that $GABA_A$ positive allosteric modulators such as benzodiazepines (midazolam (Schönle et al., 1989); lorazepam (Boroojerdi, Battaglia, Muellbacher, & Cohen, 2001; Di Lazzaro et al., 2000); diazepam (Heidegger, Krakow, & Ziemann, 2010)) and barbiturates (thiopental (Inghilleri, Berardelli, Marchetti, & Manfredi, 1996)) mainly or selectively reduce the high-amplitude MEP part of the input-output curve. Lorazepam-associated MEP amplitude reduction was found to be linked to late I-wave amplitude reduction (Di Lazzaro et al., 2000).

It was also found that several NE agonists (yohimbine (Plewnia, Bartels, Cohen, & Gerloff, 2001); reboxine (Plewnia et al., 2002); and a serotonin agonist (sertraline (Ilic, Korchounov, & Ziemann, 2002)) increases MEP amplitude. Furthermore, NMDA receptor antagonist ketamine was found to increase MEP amplitude (Lazzaro et al., 2003). Pharmacological agents affecting other neuromodulators as well as voltage-gated ion channel blockers were found to have inconsistent or nil effects on MEP amplitude (Paulus et al., 2008). It is thus possible to conclude that neuromodulator systems have a complex influence on inhibitory and excitatory synaptic

transmission. Furthermore, by studying the effects of these drugs, it was reported that changes in MEP could occur without changes in MT, and vice versa, suggesting that different neural mechanisms are at play (Ziemann et al., 2015).

Cortical Silent Period

The cortical silent period (CSP) refers to a TMS-induced interruption of voluntary activity in the EMG of the target muscle. When induced in a hand muscle, its duration plateaus around 200 to 300 ms (Kimiskidis et al., 2005). CSP duration was found to increase with stimulus intensity according to a sigmoid function. While it is believed that spinal inhibition contributes to the early part of CSP (the first 50 to 75 ms), it is believed that the late part of CSP reflects postsynaptic inhibitory processes ($GABA_AR$ and $GABA_BR$ activity) originating in the motor cortex (Fuhr, Agostino, & Hallett, 1991; Inghilleri, Berardelli, Cruccu, & Manfredi, 1993; Ziemann, Netz, Szelényi, & Hömberg, 1993).

Furthermore, CSP duration is consistent with that of animal inhibitory post-synaptic potentials (IPSP) following $GABA_B$ receptor activation in pyramidal cells (Connors, Malenka, & Silva, 1988). It was thus hypothesized that its late part can be attributed to a long-lasting cortical inhibition mediated is by $GABA_BRs$ (Nakamura, Kitagawa, Kawaguchi, & Tsuji, 1997). This hypothesis remains tentative as it was found that intravenous or oral administration of a $GABA_B$ agonist (baclofen) does not lengthen the CSP (Inghilleri et al., 1996; McDonnell, Orekhov, & Ziemann, 2006). However, the administered doses may have been too low for the desired effect to occur (Ziemann et al., 2015). Indeed, another study using high doses of intrathecal baclofen reported that it lengthens CSP duration (Stetkarova & Kofler, 2013). These discrepancies may be due to different drug administration routes (Rossini et al., 2015).

Greater CSP duration was observed after GABA reuptake inhibitor administration (tiagabine, vigabatrine). These drugs, which inhibit GABA transaminase, a GABA degrading enzyme, increase extracellular GABA concentration (Pierantozzi et al., 2004; Werhahn, Kunesch, Noachtar, Benecke, & Classen, 1999). However, these drugs are not $GABA_BR$ specific as they raise GABA neurotransmission in the synaptic cleft, which also causes $GABA_AR$ activation.

Furthermore, administration of benzodiazepines, which are positive $GABA_AR$ modulators, was found to lengthen short CSPs (<100 ms) obtained at low stimulation intensity (Kimiskidis et al., 2006; Ziemann, Lönnecker, Steinhoff, & Paulus, 1996b), and to shorten long CSPs (>100 ms) obtained at high stimulation intensity (Inghilleri et al., 1996; Kimiskidis et al., 2006). It was thus suggested that CSP tested with low-stimulus intensity reflects $GABA_AR$ activation, whereas $GABA_BRs$ become active at higher intensities (Paulus et al., 2008). Furthermore, administration of L-DOPA and DA agonists was found to lengthen CSP in some studies (Ziemann et al., 2015). Administration of other neuromodulators was found to have no consistent effect on CSP duration (Paulus et al., 2008).

Thus, these findings lead to the conclusion that CSP is comprised of three phases. While the early phase (50 ms) reflects spinal involvement, the short (<100 ms) and long (>100 ms) phases are mediated by GABAergic activity associated with inhibitory cortical mechanisms. More specifically, $GABA_AR$ mediate short CSPs and $GABA_BR$ mediate long CSPs.

Short-Interval Intracortical Inhibition

Short-interval intracortical inhibition (SICI) is a paired-pulse TMS protocol where a first conditioning sub-MT pulse (conditioning stimulus) is applied followed by a supra-MT pulse (test stimulus) at intervals between 1 and 6 ms (Rossini et al., 2015). The conditioning stimulus (CS) reduces the MEP amplitude of the test stimulus (TS) (Kujirai et al., 1993). Varying CS intensity yields a U-shaped SICI intensity curve. This finding suggests that low-intensity CS result in increased inhibition while high-intensity CS result in decreased inhibition (Ilić et al., 2002; Peurala, Müller-Dahlhaus, Arai, & Ziemann, 2008; Ziemann, Rothwell, & Ridding, 1996c). SICI is thought to activate a low-threshold cortical inhibitory circuit, which induces short IPSPs in corticospinal neurons (Ilić et al., 2002; Kujirai et al., 1993). Since SICI can be triggered using a pulse too low in intensity to activate corticospinal neurons, it is believed that SICI is due to intracortical inhibitory M1 interneuron activation, acting on excitatory neurons upstream of corticospinal neurons (Di Lazzaro et al., 1998). Furthermore, benzodiazepines, positive $GABA_AR$ modulators, were found to enhance SICI (Ziemann et al., 2015). This finding led to the hypothesis that SICI is dependent on $GABA_AR$ activity. Indeed, SICI duration is of approximately 20 ms

(Hanajima et al., 1998), which is consistent with $GABA_AR$-mediated fast IPSPs (Avoli et al., 1997).

Administration of a benzodiazepine-like compound (zolpidem) bearing an exclusive α_1 GABA subunit affinity has been shown not to alter SICI. This finding suggests that SICI represents α_2 or α_3 $GABA_AR$-mediated inhibition. It was also found that administration of a GABA reuptake inhibitor (tiagabine) (Werhahn et al., 1999) and a $GABA_BR$ agonist (baclofen) decreased SICI (McDonnell et al., 2006). These findings suggest that presynaptic $GABA_BR$-mediated inhibitory interneuron auto-inhibition modulates SICI (Müller-Dahlhaus, Liu, & Ziemann, 2008; Sanger, Garg, & Chen, 2001). Furthermore, administration of DA agonists resulted in increased SICI (Cabergoline (Korchounov, Ilić, & Ziemann, 2007); Pergolide (Ziemann, Bruns, & Paulus, 1996a)). The reverse was found for DA antagonists (Ziemann, Tergau, Bruns, Baudewig, & Paulus, 1997b) and NA agonists (Gilbert et al., 2006; Ilic, Korchounov, & Ziemann, 2003). SICI was unaffected by voltage-gated ion channel blockers (Paulus et al., 2008). Thus, SICI is not only affected by short-lasting $GABA_AR$-mediated postsynaptic inhibition, but also modulated by neuromodulator system activity.

Long-Interval Cortical Inhibition

Long-interval cortical inhibition (LICI) is another paired-pulse TMS protocol where two supra-MT TMS pulses are applied at an inter-pulse interval between 50 and 200 ms (Rossini et al., 2015). The first, conditioning pulse, inhibits the second, which leads to a diminished MEP. Inhibition intensity was found to increase with conditioning pulse intensity (Hammond, Bergman, & Brown, 2007). Due to the long intervals between pulses, LICI is thought to reflect $GABA_BR$-dependent slow IPSPs (Werhahn et al., 1999). This hypothesis is corroborated by the fact that the administration of baclofen, a $GABA_BR$ agonist, increases inhibition whereas benzodiazepines, which are positive allosteric $GABA_AR$ modulators, do not alter inhibition intensity (McDonnell et al., 2006; Teo et al., 2009). Furthermore, GABA reuptake inhibitor (vigabatrin and tiagabine) administration, which results in greater $GABA_B$ activation due to increased synaptic cleft GABA availability, was found to increase LICI (Werhahn et al., 1999). This form of inhibition is thus thought to be dependent on $GABA_BR$ activity.

Intracortical Facilitation

Intracortical facilitation (ICF) is another paired-pulse TMS protocol, where a first conditioning sub-MT pulse (conditioning stimulus) is applied followed by a supra-MT pulse (test stimulus) at intervals of 6 to 30 ms (Rossini et al., 2015). The conditioning stimulus (CS) increases MEP amplitude of the test stimulus (TS). While the mechanisms underlying ICF remain ambiguous, it is believed that it reflects motor cortex excitatory glutamatergic neuronal network activity (Ziemann et al., 1996c). It is hypothesized that ICF is the sum of glutamate-dependent excitatory processes as well as the end of the $GABA_A$-mediated IPSPs lasting for up to 20 seconds (Connors et al., 1988). As such, ICF is thus thought to be partially glutamate and $GABA_AR$-dependent (Rossini et al., 2015; Ziemann et al., 2015). Indeed, administration of benzodiazepines led to a reduction in ICF intensity (Inghilleri et al., 1996; Ziemann et al., 1996b). Furthermore, NA agonist and SSRI administration was found to increase ICF (Boroojerdi et al., 2001; Gerdelat-Mas et al., 2005; Plewnia et al., 2001; Plewnia et al., 2002). Therefore, ICF also depends on noradrenergic and serotoninergic activity.

Magnetic Resonance Spectroscopy

Overview

Proton magnetic resonance spectroscopy (H^1-MRS) allows direct non-invasive *in vivo* quantification of various neurometabolites by delivering radiofrequency (RF) pulses, which are composed of a magnetic field rotating at or near the spin Larmor frequency (the frequency of proton precession in a magnetic field). RF pulses are required in all magnetic resonance spectroscopy acquisitions, to perform the desired spin manipulations (excitation, editing, inversion, refocusing). Excitation pulses bring protons to a higher energy state so that they spin in phase. Following precession of the proton's spin, signals stemming from interactions with the magnetic field can be measured, after a specific period known as the spin echo time (the time between the excitation RF pulse and signal sampling). Refocusing and inversion pulses are used to improve signal characteristics; a more thorough explanation of these pulses lies outside the

scope of this thesis, and editing pulses are explained summarily later (De Graaf, 2019; Mullins et al., 2014).

Based on their molecular properties, chemical makeup, and their environment (the atoms or groups of atoms surrounding a proton), protons interact differently with the magnetic field, giving rise to signals comprising of a set of peaks at characteristic chemical shifts on the x-axis of the spectra, based on their spin Larmor frequency, which is specific to each nucleus. The location and area under the peaks provide information regarding the nature and concentration of the studied molecule. However, not all molecular signals can be resolved; some signals can only be differentiated in sufficiently high concentrations and/or field strengths (e.g. glutamate from glutamate/glutamine/glutathione). Editing techniques, which achieve separation of overlapping resonances, are often used to simplify the spectrum, and resolve specific signals (De Graaf, 2019).

When performed *in vivo*, brain metabolite levels can be obtained from a three-dimensional space termed voxel-of-interest. Due to the low concentration of the compounds, VOIs are often quite large, typically a few cubic centimetres (Mullins et al., 2014) to offset the lower signal-to-noise ratio. Depending on the magnetic field strength of the MR scanner, and the chosen acquisition sequence, a number of neurometabolites can be detected and quantified, where each give rise to characteristic signals (Currie et al., 2012). The following section will focus on the MEGA-PRESS sequence, as it is optimized for GABA detection.

GABA Detection Using MEGA-PRESS at 3T

Despite being present at concentrations within single voxel H^1-MRS detection limits (1 mmol in cortical grey matter), γ-aminobutyric acid (GABA) has proven difficult to reliably measure *in vivo* mainly due to the strong overlap with the signals occurring at similar chemical shifts stemming from other neurometabolites and macromolecules (MM) (Behar & Ogino, 1993; Behar, Rothman, Spencer, & Petroff, 1994; Waddell, Avison, Joers, & Gore, 2007). While its biochemical profile is not fully understood, the macromolecular signal is thought to stem from methyl and methylene resonances of cytosolic protein's amino acids, which have high molecular weight and tend to bias the overall spectrum (Behar & Ogino, 1993; Považan et al., 2015). J-difference spectral editing

sequences such as MEGA-PRESS (MEscher-Garwood Point RESolved Spectroscopy) take advantage of J-couplings – interactions through the electron network of non-equivalent protons with shared chemical bonds within a molecule – to resolve the γ-CH_2 GABA resonance occurring at 3 ppm from those of other metabolites (Mescher, Merkle, Kirsch, Garwood, & Gruetter, 1998; Mullins et al., 2014; Rae, 2014). Thus, J-editing techniques differentiate scalar-coupled from uncoupled spins, which respond differently to RF pulses, thereby allowing specific molecular signals to be resolved. Further explanation of NMR physics lies outside the scope of this thesis.

To allow the detection of GABA, MEGA-PRESS sequences involve applying two different interleaved RF pulses, an inversion pulse ("EDIT OFF"), and an editing pulse ("EDIT ON"), interact differently with GABA spin systems. The inversion pulse is applied elsewhere in the spectrum relative to the editing pulse to allow free J-coupling evolution throughout the echo time. The editing pulse of the MEGA-PRESS sequence is applied at the 1.9 ppm β-CH_2 GABA resonance to selectively refocus J-coupling evolution to the 3.0 ppm GABA spins. This pulse also affects the 1.7 ppm MM resonance, coediting MM with GABA and confounding the 3ppm peak. This MM contamination can be avoided by carefully choosing editing pulses, but at the price of reduced editing efficiency or increased echo times (Harris, Puts, Barker, & Edden, 2015; Henry, Dautry, Hantraye, & Bloch, 2001; Near, Simpson, Cowen, & Jezzard, 2011). Coedited MM are therefore either accepted as a confound in the so-called GABA+ measure, or dealt in post-processing by subtraction of an additionally acquired MM spectrum (Harris et al., 2015; Henry et al., 2001), or by fitting a model MM spectrum (Provencher, 1993, 2001), for pure GABA estimation. GABA measurements are then obtained by analyzing difference spectra ("EDIT DIFF") stemming from the subtraction of "EDIT ON" from "EDIT OFF" spectra. Despite difficulties caused by macromolecules, MEGA-PRESS has been found to adequately measure GABA+, and MM subtraction and fitting techniques can reliably estimate GABA *in vivo* (Bogner et al., 2010; Evans, McGonigle, & Edden, 2010; O'Gorman, Edden, Michels, Murdoch, & Martin, 2007; O'gorman, Michels, Edden, Murdoch, & Martin, 2011).

Glutamate Detection Using MEGA-PRESS at 3T

Using MEGA-PRESS, a composite peak stemming from glutamate, the primary excitatory neurotransmitter, and glutamine, a non-neuroactive amino acid neurotransmitter recycling intermediate and brain ammonia metabolic regulator (Waagepetersen, Sonnewald, & Schousboe, 2007), can be acquired. Despite their concentration being relatively high in the brain (< 12 mmol and 4-6 mmol respectively), glutamate and glutamine are notoriously difficult to assess, as they are hard to resolve from one another using H^1-MRS (Mullins et al., 2014; Rae, 2014). At moderate field strengths (≈ 3.0 T), using MEGA-PRESS, it is impossible to completely resolve and isolate the glutamate signal stemming from its γ-CH_2 resonance from that of glutamine occurring at similar chemical shifts (Rae, 2014). Hence, a composite glutamate + glutamine (Glx) signal at 3.75 ppm comprising the glutamate and glutamine resonances is obtained (Mullins et al., 2014). While few studies have assessed the stability of Glx concentrations obtained using MEGA-PRESS, it was reported that this editing technique leads to reproducible Glx measurements (O'Gorman et al., 2007; O'gorman et al., 2011).

Stability of the Measurements

Similarly to TMS, knowing the long-term stability of MRS measurements is of critical importance to determine if the technique may be used to evaluate pathologies and treatment response. The following sections will focus on GABA and Glutamate (Glx) measurements. The following section focuses on studies performed on healthy adult (aged 18-52, ≈ 26 ± 4 years), mostly gender balanced samples, with no history of psychiatric or neurological illness and no psychoactive substance intake. Two studies were restricted to male populations (Jang et al., 2005; Near, Ho, Sandberg, Kumaragamage, & Blicher, 2014) and one study demonstrated an influence of the menstrual cycle on GABA in some brain regions (Harada, Kubo, Nose, Nishitani, & Matsuda, 2011). It should be noted that the effects of the menstrual cycle on GABA was not examined in the sensorimotor cortex.

Measures of GABA

Previous studies assessing the short-term reproducibility and stability of GABA and GABA+, reported coefficients of variation ranging from ≈ 4 to 15% across several brain regions and editing techniques (Bogner et al., 2010; Dyke et al., 2017; Evans et al., 2010; Harada et al., 2011; Near et al., 2013; Near et al., 2014; O'gorman et al., 2011). A study by Evans (2010) reported a mean within-subject GABA/H_2O CV of 8.8% for a test-retest reliability study performed within a single day in the sensorimotor cortex. Another study by Dyke (2017) found a good intra-class correlation (0.62) and a low mean CV (11.91%) for same-day GABA/tCr measurements in the sensorimotor cortex. It thus appears GABA measurements are stable and reliable for same-day intervals across several VOIs, reference standards and spectroscopic sequences. However, at the time of writing, only one other study assessed the long-term stability of the technique. Indeed, a longitudinal (7-month) study of the occipital cortex using MEGA-PRESS at 3T, GABA/Cr levels were found to have a low CV (4.3%) and a fair level of absolute agreement (r = 0.52) (Near et al., 2014). Therefore, further studies are needed in different brain regions to firmly establish reliability estimates of GABA measurements.

Measures of Glutamate (Glx)

Reproducibility and short-term studies using various ROIs and MRS sequences have found small coefficients of variation, ranging from 3 to 10% (Dyke et al., 2017; Hurd et al., 2004; Jang et al., 2005; O'gorman et al., 2011). For example, O'Gorman and collaborators (2011) reported within-session CVs of 6% using LCModel values obtained from four scans of the DLPFC in a single session, while Hurd et al. (2004) found a CV <10% in the parietal cortex over multiple scans in a same-day reliability study. Dyke (2017) reported a good intra-class correlation (0.61) and a low mean CV (3.52%) over a same day interval in the sensorimotor cortex. Therefore, Glutamate (or Glx) measurements show little variability over time. Nevertheless, to our knowledge, the long-term stability of Glx has not been studied in healthy volunteers.

MRS Neurochemistry

MRS measurements, whether acquired using MEGA-PRESS or other sequences, are believed to reflect voxel-wide neurometabolite concentrations. As such, it is difficult to pinpoint the precise neurochemical substrates of the technique. Nevertheless, studies using active compounds with a known mechanism of action as well as knowledge from *in vitro* or *ex vivo* animal studies have helped determining what underlies MRS measurements. Studies on MRS neurochemistry are typically performed on adults aged 17 – 62 (35 ± 9) years, in either epileptic patients and/or controls. Only one study examined whether age modulated tiagabine's effects on GABA concentration and found no effect (Myers, Evans, Kalk, Edden, & Lingford-Hughes, 2014). None of the studies listed in the present section have examined the effects of gender on drug responses on neurometabolites.

MRS-GABA

As with other metabolites, MRS quantifies total GABA levels within the voxel of interest. It is thus impossible to precisely pinpoint what comprises MRS-GABA signals. Nevertheless, it is believed that MRS-GABA mainly reflects extrasynaptic rather than synaptic GABA (Dyke et al., 2017; Rae, 2014; Stagg, 2014). As such, MRS-GABA does not necessarily reflect GABAergic neurotransmission. It would rather represent either intracellular GABA or ambient, steady-state extracellular GABA. With respect to intracellular GABA, as it comes from various neurotransmitter pools due to different synthetic pathways, it is impossible to determine the extent with which the signal is proportional to a particular metabolic pool (Rae, 2014; Stagg et al., 2011a). However, pharmacological studies have helped further our understanding of what comprises the MRS-GABA signal.

Compounds that are capable of altering cellular concentrations such as vigabatrin (Mattson, Petroff, Rothman, & Behar, 1995; Verhoeff et al., 1999), topiramate, lamotrigine, and gabapentin (Kuzniecky et al., 2002; Petroff, Hyder, Mattson, & Rothman, 1999; Petroff, Hyder, Rothman, & Mattson, 2001) increase MRS-GABA signals. Furthermore, the administration of a novel GABA-aminotransferase inhibitor, which raises intra-cellular GABA concentrations by blocking enzymatic degradation of GABA, was found to reversibly increase MRS-GABA levels (Prescot et

al., 2018). However, when tiagabine, an agent which selectively increases extracellular concentrations (Fink-Jensen et al., 1992) by inhibiting GABA reuptake in the synaptic cleft through GAT transporter blockade, was administered, no alterations in GABA levels were found when assessed both *in-vivo* and *ex-vivo* (Myers et al., 2014; Patel, de Graaf, Rothman, & Behar, 2015). However, PET analysis revealed an increase in extracellular GABA by tiagabine (Stokes et al., 2013). Taken together these findings suggest that changes in extracellular GABA (without intracellular alterations) will not be detectable via MRS, unless these changes lead to a hundred-fold increase in extracellular GABA (Myers et al., 2016). As such, MRS signals probably mostly reflect intracellular levels.

Furthermore, BZD clonazepam administration was shown to decrease occipital GABA levels (Goddard et al., 2004) and non-BZD $GABA_AR$ agonist zolpidem led to a reduction in thalamic GABA (but not in the ACC) in healthy subjects (Licata et al., 2009). This is surprising, as benzodiazepines are not believed to directly alter GABA concentrations; rather they modulate receptor activity to increase its affinity for its endogenous ligand (Griffin et al., 2013). It was thus suggested that these alterations in MRS-GABA signals probably stem from a BZD-induced reduction in blood flow or in metabolism of the affected regions (Goddard et al., 2004; Licata et al., 2009). Nevertheless, these findings are surprising given the mechanism of action of BZD, and further studies are needed to better understand the region-dependent effects, if any, of BZD on MRS signals.

MRS-Glx

In a similar manner to GABA, ambiguity remains as to the precise substrates of MRS-Glx, which combines two signals stemming from neurometabolites involved in different processes. Glutamate and glutamine are related in the Glu/Gln cycle, where neurotransmitter glutamate is compartmentalized in synaptic vesicles at high concentrations, to be released into the synaptic space when triggered by nerve impulses. After binding to post-synaptic receptors, Glu is internalized into astrocytes, where it is converted to glutamine. After leaving the astrocytes and being taken on by neurons, glutaminase regenerates Glu and completes the Glu/Gln cycle (Kandel,

Schwartz, Jessell, Siegelbaum, & Hudspeth, 2000). Due to this dynamic exchange, coupled with the fact that the two neurotransmitters cannot be resolved using MEGA-PRESS at 3T, it is difficult to pinpoint what exactly comprises the MRS-Glx signals. Nevertheless, the majority of the Glx signal is from glutamate, and it has been shown that changes in glutamate concentrations are linked to metabolic activity. It is thus common to interpret differences in MRS-glutamate as being related to metabolic activity where an increase in MRS-Glu would indicate increased metabolism (Rae, 2014). Due to the still ambiguous role of Glutamine as it relates to central nervous system activity, further work is needed to understand how MRS-Gln changes relate to brain activity (Rae, 2014).

While a comprehensive review of glutamatergic activity modulation through pharmacological compounds lies beyond the scope of the present thesis, it has been shown that BZD administration does not alter Glu signals. Indeed, benzodiazepines midazolam (Yildiz et al., 2010), lorazepam (Brambilla et al., 2002), alprazolam (Henry et al., 2010) and clonazepam (Goddard et al., 2004; Henry et al., 2010) do not modulate glutamate levels in healthy individuals.

Linking MRS and TMS Measures

Despite their parallel development and complimentary application, previous studies consistently point to a clear dissociation between TMS and MRS measures.

Measures Relying on GABA

Stagg and collaborators (2011a) reported no correlation between MRS-derived measures of GABA neurotransmitter levels, obtained using a SPECIAL sequence, and TMS measures of cortical inhibition reflecting synaptic $GABA_A$ (SICI) and $GABA_B$ (LICI) receptor activity in M1. However, they reported that SICI at a 1 ms ISI, which has a different, not fully understood, mechanism of action than SICI at longer ISIs, did correlate with MRS-GABA. This finding was not replicated by later studies (Dyke et al., 2017; Hermans et al., 2018) with a larger sample and a different spectroscopic sequence. Furthermore, Tremblay and collaborators (2012) found that TMS measures of $GABA_AR$ or $GABA_BR$-dependent intracortical inhibition are not linked to

global MRS-GABA concentrations in M1 measured using MEGA-PRESS at 3T, and that the cortical silent period is not linked to MRS-GABA levels. Dyke and collaborators (2017) also found that MRS-GABA signals obtained with a STEAM sequence at 7T were not related to any TMS parameter including measures of the poorly understood cortical facilitation (ICF), which replicates both earlier findings (Stagg et al., 2011b). A study examining MRS-GABA levels, obtained using MEGA-PRESS at 3T, and TMS measures of inhibition and facilitation in young or older adults also reported no correlation between measures obtained with both techniques (Hermans et al., 2018).

Measures Relying on Glutamate (Glx)

MRS-glutamate, assessed with the SPECIAL sequence, which can resolve Glu from Gln, was found to not correlate with ICF (12 ms ISI). Dyke (2017) did, however, find a relationship with MRS-Glu and the 10 ms ICF but not the 12 ms ISI ICF. This is a surprising finding, as the same mechanism appears to underlie ICF across ISIs. Thus, further work is needed to fully determine the extent to which MRS-measured glutamate plays a role in ICF. Furthermore, previous work reported a correlation between MRS-Glx and CSP duration (Tremblay et al., 2012), which is surprising given the mechanism underlying CSP duration, which is thought to be glutamate independent (Ziemann et al., 2015). However, the relationship between global cortical excitability and MRS-Glu remains ambiguous. Indeed, previous studies have reported conflicting results with respect to the relationship between MRS-glutamate and the slope of the input/output curve (Dyke et al., 2017; Stagg et al., 2011b). While Stagg (2011) found that greater glutamate concentration in the SMC was correlated with the slope of the I/O curve, Dyke (2017) found that glutamate concentrations was negatively correlated with the I/O plateau.

Summary of the Current Knowledge and Future Research Avenues

It is well established that MRS offers short-term reliable GABA and Glu (Glx) level assessment across several brain regions, spectroscopic sequences, and internal standards (Bogner et al., 2010; Evans et al., 2010; Harada et al., 2011; Hurd et al., 2004; Near et al., 2013; Near et al., 2014;

O'gorman et al., 2011). However, only one other study has assessed the long-term stability of GABA+ concentrations using MRS in healthy subjects (Near et al., 2014) and the long-term stability of glutamate has never been assessed, to our knowledge. Furthermore, studies on the short-term stability of TMS measures reveal that some parameters possess good intrinsic stability (motor thresholds, cortical silent period). However, there is a lack of consensus regarding the stability of other parameters (paired-pulse measures of cortical inhibition and excitation) (Boroojerdi et al., 2000; Hermsen et al., 2016; Ngomo et al., 2012; Orth & Rothwell, 2004). In addition, further data is needed to understand how stable TMS measurements are across longer time intervals. Having such longitudinal studies will help validate the techniques' use in the medical field. To our knowledge, investigating the long-term stability of motor-cortical MRS and TMS measurements, obtained in the same cohort of healthy individuals, has never been attempted before.

Furthermore, it is known that both TMS and MRS may index GABAergic and glutamatergic activity. However, the precise neurochemical substrates of these techniques are not yet fully understood. So far, with respect to GABA, it is believed that TMS measurements reflect receptor activity whereas MRS-GABA levels originate from extrasynaptic GABA pools. Furthermore, it is well established in the literature that baseline MRS and TMS values are not linked, hinting at a clear disassociation between the neurochemistry underlying both techniques' functioning. However, we do not yet know how TMS and MRS measures may be modulated following pharmacological intervention in a same participant cohort. Investigating such a link would prove useful in understanding the differential nature of TMS and MRS measurements. To our knowledge, it has never been attempted to use a pharmacological agent, such as a benzodiazepine, to study its effects on MRS and TMS measurements, in the same group of participants.

Objectives and hypothesis

The present thesis has two main objectives:

1) Evaluate the long-term reliability of TMS and MRS measures of GABA and glutamate activity.

2) Investigate the neurochemical substrates of TMS and MRS measures of GABA and glutamate activity.

For *Objective 1*, healthy adults underwent two sessions of TMS and MRS at a three-month interval. TMS and MRS measures of GABA and Glutamate (Glx) were obtained at the two time points to determine their long-term reliability. For *Objective 2*, healthy adults underwent TMS and MRS testing after the administration of classical benzodiazepine lorazepam (or placebo). TMS and MRS measures of GABA and Glutamate (Glx) were obtained in the same individual after active or placebo challenge and their effects compared.

Objective 1: Longitudinal assessment of ^{1}H-MRS (GABA & Glx) and TMS measures of cortical inhibition and facilitation in the sensorimotor cortex

The primary hypotheses are the following:

1) MRS GABA and Glx measurements will be stable across a three-month interval.
2) TMS measures of MT, %MSO and CSP duration will be stable across a three-month interval.
3) Paired pulse TMS measures will show greater variability and lesser stability across time.

The secondary hypothesis is the following:

1) MRS measures of GABA and Glx will not correlate with TMS measures of cortical inhibition or facilitation.

Objective 2: TMS and H^{1}-MRS measures of excitation and inhibition following lorazepam administration

The main hypotheses are the following:

1) MRS-GABA and MRS-Glutamate (Glx) concentrations will be unaffected by lorazepam in either VOI.

2) Lorazepam will lower cortical excitability (MEP amplitude), increase short-interval cortical inhibition (SICI) and reduce cortical facilitation (ICF).

3) Lorazepam will have no effect on MT, CSP duration and LICI.

The secondary hypothesis is the following:

1) MRS measures of GABA and Glx will not correlate with TMS measures of cortical inhibition or facilitation.

Chapter 2

Article 1: Longitudinal assessment of ^{1}H-MRS (GABA & Glx) and TMS measures of cortical inhibition and facilitation in the sensorimotor cortex

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Abstract

The purpose of the present study was to investigate the long-term stability of water-referenced GABA and Glx neurometabolite concentrations in the sensorimotor cortex using MRS and to assess the long-term stability of GABA- and glutamate-related intracortical excitability using transcranial magnetic stimulation (TMS). Healthy individuals underwent two sessions of MRS and TMS at a three-month interval. A MEGA-PRESS sequence was used at 3T to acquire MRS signals in the sensorimotor cortex. Metabolites were quantified by basis spectra fitting and metabolite concentrations were derived using unsuppressed water reference scans accounting for relaxation and partial volume effects. TMS was performed using published standards. After performing stability and reliability analyses for MRS and TMS, reliable change indexes were computed for all measures with a statistically significant test-retest correlation. No significant effect of time was found for GABA, Glx and TMS measures. There was an excellent ICC and a strong correlation across time for GABA and Glx. Analysis of TMS measure stability revealed an excellent ICC for rMT CSP and %MSO and a fair ICC for 2ms SICI. There was no significant correlation between MRS and TMS measures at any time point. This study shows that MRS-GABA and MRS-Glx of the sensorimotor cortex have good stability over a three-month period, with variability across time comparable to that reported in other brain areas. While resting motor threshold, %MSO and CSP were found to be stable and reliable, other TMS measures have greater variability and lesser reliability.

Keywords: Magnetic resonance, MEGA-PRESS, motor cortex, transcranial magnetic stimulation, GABA, glutamate

Introduction

Proton magnetic resonance spectroscopy and transcranial magnetic stimulation are non-invasive techniques used to assess GABAergic and glutamatergic activity in the brain. Despite their parallel and complementary applications, studies have found that measurements obtained through both techniques do not correlate, and it was thus hypothesized that MRS and TMS have different neurochemical substrates and mechanisms of action (Stagg et al. 2011a; Tremblay et al. 2013b; Dyke et al. 2017a). Indeed, it is believed that MRS can directly measure extra-synaptic GABA and glutamate concentrations, and that TMS indirectly reflects GABA and glutamate receptor function (Stagg et al., 2011).

MRS allows for direct *in vivo* quantification of various neurometabolites by taking advantage of their molecular properties. Depending on their chemical environment, protons interact differently with the magnetic field, giving rise to signals at characteristic chemical shifts at which corresponding peaks or set of peaks can be seen on the spectra. The location and area under the peaks provide information regarding the nature and concentration of the molecules of interest. By using J-difference spectral editing sequences such as MEGA-PRESS, which take advantage of J-couplings – interactions through the electron network of non-equivalent protons with shared chemical bonds within a molecule – it is possible to obtain a 3 ppm GABA signal (Mescher et al. 1998; Mullins et al. 2014; Rae 2014). Also using MEGA-PRESS, a composite glutamate/glutamine (Glx) signal at 3.75 ppm, comprising glutamate and glutamine resonances that can't be resolved at moderate field strengths (≈ 3.0 T), can also be obtained (Mullins et al. 2014).

TMS has been used to modulate and probe neurophysiological mechanisms underlying cortical function and plasticity (Hallett 2007). When applied to the motor cortex, the TMS-triggered depolarization activates interneurons, leading to trans-synaptic activation of pyramidal cells, which in turn induces descending volleys in the corticospinal tract, where pyramidal axons project on spinal motoneurons (Klomjai et al. 2015). The subsequent motoneuron activation induces muscle responses called motor-evoked potentials (MEP). Using different techniques, various TMS measures can be obtained and are believed to rely on different receptor-dependent physiological mechanisms reflecting inhibitory and excitatory processes in the brain.

Despite their distinct methodological properties, MRS and TMS can be used in a clinical setting to assess disease progression and treatment response (Cantello et al. 1991; Ziemann et al. 1997; Mills 2003). Indeed, abnormal GABA and glutamate signalling has been found to be implicated in various neurological and psychiatric conditions, such as amyotrophic lateral sclerosis, Parkinson's disease, and schizophrenia (Han and Ma 2010; Emir et al. 2012; Foerster et al. 2012; Rowland et al. 2012). Monitoring metabolite concentration using MRS can thus provide insight as to the presence and evolution of disease and permit objective evaluation of treatment response. Furthermore, various pathological processes such as epilepsy, amyotrophic lateral sclerosis, Parkinson's disease, multiple sclerosis and cerebral lesions were found to alter TMS responses across a wide variety of measures (Mills and Nithi 1997; Rossini et al. 2015).

However, to increase the validity and use of MRS and TMS as diagnostic and disease-monitoring tools, the long-term stability and reliability of both techniques must be further examined. With respect to MRS, few studies have investigated the long term stability of GABA or glutamate concentrations in healthy individuals (Near et al. 2014). As for TMS, while motor thresholds (MT) were found to be stable across a number of studies (Mills and Nithi 1997; Carroll et al. 2001; Ngomo et al. 2012; Hermsen et al. 2016), inconsistent results have been reported with regards to paired-pulse paradigms. Indeed, while some studies have found paired-pulse techniques (ppTMS) to be stable and reliable (Ngomo et al. 2012; Hermsen et al. 2016), others have reported high variability across sessions (Boroojerdi et al. 2000; Maeda et al. 2002; Wassermann 2002; Orth et al. 2003). Importantly, the long-term stability of TMS and MRS measures of inhibitory and excitatory activity has not yet been reported within the same individual. This is of significant interest to determine whether intraindividual variance of GABA/glutamate extrasynaptic concentration and receptor function share a common factor.

The goal of the present study was threefold. First, it aimed at assessing if single voxel MRS GABA and Glx concentrations within the sensorimotor cortex are stable across time. Water was chosen as a reference instead of total creatine or *n*-acetylaspartate as it is readily quantifiable (Gasparovic et al. 2006) and may possess greater usefulness for expressing neurometabolite levels due to varying NAA and Cr concentrations in clinical conditions (Martin 2007). Second, it aimed at determining the long-term stability of various TMS measures (rMT, %MSO, SICI, ICF, LICI,

CSP) that have been used in clinical settings. Third, it aimed at further exploring the ambiguous link between MRS and TMS measures of GABA and glutamate and determining whether intra-individual variation in one technique can predict variation in another.

Methods

The study consisted of two MRI sessions lasting approximately 60 minutes immediately followed by TMS sessions lasting approximately 30 minutes at a three-month interval (t1 and t2).

Participants

Fourteen healthy right-handed participants (8 male, 6 female) aged 18 – 40 years were recruited using advertisements posted on campus and social media. Exclusion criteria were the following: neurological or psychiatric conditions, psychoactive medication (past or present intake), history of traumatic brain injury, history of fainting or seizures, substance abuse, and any contraindications to MR scanning or transcranial magnetic stimulation. All participants provided written informed consent prior to testing, and the experiments were performed with the approval of the local ethics committee (*Comité mixte d'éthique de la recherche du RNQ*). Participants were instructed to refrain from alcohol consumption 48 hours before each session and from consumption of psychoactive drugs for the duration of the study. Two participants abandoned the study after one session, and one was excluded after the second session due to an anatomical anomaly. Their data were excluded from the final sample.

Magnetic resonance imaging

Magnetic resonance imaging sessions were performed at the Unité de Neurimagerie Fonctionnelle, Centre de Recherche de l'Institut Universitaire de Gériatrie de Montréal. MR acquisitions were performed using a 3T whole-body system scanner (MAGNETOM Trio, Siemens, Erlangen, Germany) using a 32-channel receive-only head coil. Anatomical images were acquired using a T_1-weighted MPRAGE sequence according to the following parameters: T_R (repetition time) = 2,300 ms; T_E (echo time) = 2.98 ms; FA (flip angle) = 90°; FOV (field of view) = 256 mm, matrix = 256 × 256 x 176; T_I (inversion time) = 900 ms; number of slices= 176; slice thickness = 1 mm; orientation: sagittal; voxel size 1.0 x 1.0 x 1.0 mm^3; acquisition time: 9:50 min.

Magnetic resonance spectroscopy

Magnetic resonance spectroscopy was performed according to previously published procedures. (Lefebvre et al. 2018). Data were acquired by first manually placing a voxel-of-interest (30 x 30 x 30 mm^3, Fig. 1) over the left sensorimotor area according to published anatomical landmarks (Figure 1; Yousry et al. 1997). Voxel placement at t2 was performed using images showing voxel placement at t1 with axial, coronal and sagittal views to ensure adequate stability throughout sessions. Shimming was done using FAST(EST)MAP (Gruetter & Tkác, 2000) to ensure that the linewidth of water was under 10 Hz. A MEGA-PRESS sequence (Mescher et al. 1996; Mescher et al. 1998) was used to acquire neurometabolite signals according to the following parameters: T_R = 3,000 ms; T_E = 68 ms; Excite FA = 90°; Refocus FA = 180°. Double-banded pulses were used to simultaneously suppress water signal and edit the 3 ppm GABA γ–CH_2 resonance. The water-suppressing band was applied at 4.7 ppm while the editing band was applied at 1.9 ppm (EDIT ON) or at 7.5 ppm (EDIT OFF). Additional water suppression using variable power with optimized relaxation delays (VAPOR) and outer volume suppression (OVS) techniques (Tkáč et al. 1999), optimized for the human 3T system, were incorporated prior to running the MEGA-PRESS sequence. The acquisition frequency was centered on GABA at 3ppm (delta frequency = -1.7ppm). To minimize frequency drift and maintain editing efficiency, MEGA-PRESS data were acquired in blocks of 32 'EDIT OFF' and 32 'EDIT ON' interleaved scans with frequency adjustments performed before each block. Four blocks were acquired for a total acquisition time of 12 min. Individual FIDs were stored for offline processing. The water reference required for absolute metabolite quantification was obtained from a separate acquisition using the same MEGA-PRESS sequence and voxel prescription, but without MEGA and VAPOR water suppression (both set to "only RF off"), and centered on water at 4.7ppm (delta frequency = 0). A single block of 4 averages was acquired (acquisition time: 42 sec).

Frequency and phase of individual averages were corrected offline and then averaged, independently for 'EDIT OFF' and 'EDIT ON', to produce the 'EDIT OFF' and 'EDIT ON' subspectra. Small frequency errors between the 'EDIT OFF' and 'EDIT ON' subspectra were manually corrected by minimizing subtraction error in the difference spectra around the 3.9-ppm creatine and the 3.2-ppm choline resonance. The final difference spectra ('EDIT DIFF') were obtained by subtracting the 'EDIT OFF' from the 'EDIT ON' subspectra.

MRS data analysis

Both 'EDIT OFF' and 'EDIT DIFF' subspectra were analyzed using LCModel 6.2-1A, which calculated the best fit for these spectra as a linear combination of model spectra (Provencher 1993, 2001). The basis set for the 'EDIT OFF' spectra was comprised of an experimentally measured metabolite-nulled macromolecular spectrum acquired from the occipital region of an independent cohort of 11 healthy adults (no medical, neurological, or psychiatric conditions and not receiving medication) as well as simulated metabolite spectra. A MATLAB-operated home-written software based on density matrix formalism (Henry et al. 2001) was used to simulate the basis set for 'EDIT OFF' metabolite spectra according to known chemical shifts and J couplings (Govindaraju et al. 2000). The basis set was comprised of simulated spectra of the following metabolites: alanine, ascorbate, aspartate, creatine (CH2 moiety), creatine (CH3 moiety), GABA, glucose, glutamate (Glu), glutamine (Gln), glycerophosphorylcholine, glycine, glutathione, lactate, myo-ionositol, N-actetylaspartate, N-acetylaspartylglutamate, phosphocreatine (CH2 moiety), phosphocreatine (CH3 moiety), phosphorylcholine, phosphorylethanolamine, scyllo-inositol and taurine. LCModel fitting was performed across the 0.2 to 4.0 ppm range for the 'EDIT OFF' spectra. The basis set for 'EDIT DIFF' difference spectra included an experimentally measured metabolite-nulled macromolecular (MM) spectrum from the occipital region (averaged across 11 subjects) as well as experimentally measured spectra from 100 mM NAA, GABA, Glu and Gln phantoms at a 7.2 pH and at 37 °C. Fitting was performed over the 0.5 – 4.0 ppm spectral range for the 'EDIT DIFF' spectra. The occipital region was chosen for both the 'EDIT OFF' and 'EDIT DIFF' basis sets, because of its high signal-to-noise ratio. An example fitted difference spectra can be seen on Figure 2. LCModel spline baseline modeling was deactivated for both 'EDIT OFF' and 'EDIT DIFF' spectra analysis with the NOBASE = T input parameter. Default LCModel simulations of lipid and MM resonances were also deactivated. No baseline correction, zero-filling, or apodization functions were applied to the *in vivo* data prior to LCModel analysis. The independently acquired water signal was used as an internal standard reference for metabolite quantification. Spectra with GABA Cramér-Rao lower bounds (CRLB) > 30% were excluded from further analysis.

For the correction of relaxation and partial volume effects on water-referenced metabolite concentrations, the proportion of gray matter (GM), white matter (WM) and cerebrospinal fluid (CSF) within the MRS voxel was obtained following tissue segmentation performed on anatomical

MPRAGE images from each participant using the automated *FreeSurfer* pipeline (V 5.3.0). The T_1 and T_2 water relaxation times used in the calculation of attenuation factors were taken from published reports [T_1(GM) = 1.29 s, T_1(WM) = 0.87 s, T_1(CSF) = 4 s, T_2(GM) = 110 ms, T_2(WM) = 80 ms, and T_2(CSF) = 400 ms] (Wansapura et al. 1999; Rooney et al. 2007). Water attenuation was computed using the fractional volume of each compartment (Gasparovic et al. 2006).

Water-referenced GABA, Glu, Gln, NAA, and MM values were obtained based on the segmentation-corrected 'EDIT DIFF' output (referred to as GABA/H_2O, Glu/H_2O, Gln/H_2O, NAA/H_2O and MM/H_2O). As glutamate cannot be resolved from glutamine at 3T, Glx/H_2O values were computed (Glu/H_2O +Gln/H_2O) and interpreted as an indicator of glutamate concentrations. GABA/Glx ratios, which reflect the balance of inhibitory and excitatory neurotransmitter concentrations, were also calculated as follows: (GABA/H_2O) / (Glx/H_2O) = GABA/Glx.

For better comparison with other studies, we also expressed metabolite concentrations as ratios to NAA and total creatine (tCr). For ratios to NAA (GABA/NAA and Glx/NAA), 'EDIT DIFF' water-referenced concentrations were divided by the 'EDIT DIFF' water-referenced NAA concentrations, following: GABA/NAA = (GABA/H_2O) / (NAA/H_2O) and Glx/NAA = (Glx/H_2O) / (NAA/H_2O). For ratios to tCr, water-referenced concentrations were divided by 'EDIT OFF' water-referenced tCr concentrations. Total creatine followed: tCr/H_2O = Cr/H_2O + PCr/H_2O, where Cr/H_2O = ($CrCH_3$/H_2O + $CrCH_2$/H_2O)/2 and PCr/H_2O = ($PCrCH_3$/H_2O + $PCrCH_2$/H_2O)/2 since the CH_2 and CH_3 moieties of creatine (Cr) and phospho-creatine (PCr), producing the 3.9-ppm ($CrCH_2$ and $PCrCH_2$) and 3.0-ppm ($CrCH_3$ and $PCrCH_3$) peaks, were fitted and quantified separately with four basis spectra ($CrCH_3$, $CrCH_2$, $PCrCH_3$ and $PCrCH_2$). GABA and Glx ratios to tCr therefore followed: GABA/tCr = (GABA/H_2O) / (tCr/H_2O) and Glx/tCr = (Glx/H_2O) / (tCr/H_2O), where Glx/H_2O and GABA/H_2O were obtained from 'EDIT DIFF' subspectra, for internal consistency and better comparison to other studies.

Transcranial magnetic stimulation

During TMS experiments, participants were seated comfortably on a chair, and instructed to remain relaxed, alert, still, and to keep their hands and feet uncrossed and palms facing slightly upwards. Electromyographic (EMG) activity was recorded using two self-adhesive electrodes

placed on the right first dorsal interosseous (FDI) muscle and the side of the index finger to measure muscle contraction. A ground electrode was positioned over the right forearm muscle. The EMG signal was filtered with a bandwidth of 20-1,000 Hz and digitized at a sampling rate of 4 kHz with a Powerlab 4/30 system (ADInstruments, Colorado Springs, CO). Motor evoked potentials (MEPs) were recorded with Scope v4.0 software (ADInstruments, Colorado Springs, CO) and stored offline for analysis.

TMS was delivered over the left primary motor cortex through an 8-cm figure-of-eight coil connected to a MagPro stimulator (MagVenture, Farum, Denmark). The coil was positioned flat on the head of participants with a 45° angle from the midline, with the handle pointing backwards to deliver biphasic currents in an anterior-posterior direction in the coil. Throughout the experiment, TMS pulses were delivered at a frequency of 0.1-0.2 Hz to avoid long-lasting modulation of M1 excitability (Chen et al. 1997). Resting motor threshold, paired-pulse and cortical silent period protocols were performed during both experimental sessions using published protocols (Rossini et al. 2015). The optimal site of stimulation was defined as the coil position from which TMS produced MEPs of maximum amplitude in the target muscle of the contralateral hand and marked on the participant's scalp using a water-soluble wax crayon to ensure stable coil positioning throughout the experiment.

Resting motor threshold. The resting motor threshold (rMT) was defined as the minimum stimulus intensity required to elicit MEPs of at least 50 μV in 5 of 10 trials in a resting muscle.

Paired-pulse measures. Paired-pulse stimulation was performed with a test-stimulus (TS) intensity that elicited MEPs ranging from ≈ 0.6 – 1.2 mV amplitude and a conditioning stimulus (CS) intensity set at 70% rMT. For short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF), 10 CS-TS pairs and 10 TS-only pulses were delivered at different interstimulus intervals (ISI) in a randomized order for each participant. SICI was assessed at ISI_{2ms} and ISI_{3ms} (referred to as $SICI_{2ms}$ and $SICI_{3ms}$ throughout the text), and ICF was assessed at ISI_{9ms} and ISI_{12ms} (referred to as ICF_{9ms} and ICF_{12ms} throughout the text). The percentage of maximum stimulator output (%MSO) required to elicit MEPs of 1mV average amplitude for the test stimuli was used as a marker of corticospinal excitability. For long interval intracortical inhibition (LICI), two

successive pulses at TS intensity were delivered at an ISI_{100ms} until ten EMG recordings where the first MEP had a peak-to-peak amplitude between 0.5 and 1.5 mV were obtained and recorded.
Cortical silent period (CSP): To induce a CSP, a single TMS pulse with an intensity equivalent to 120% rMT was delivered while participants maintained a voluntary isometric muscle contraction of the right FDI at ≈20% maximum strength. To determine contraction strength, EMG signals were monitored as participants were first asked to briefly maintain maximum isometric muscle contraction while grasping a pencil with the thumb and index finger, and then relax until the peak-to-peak EMG signal amplitude was approximately 20% of the maximum. Participants were instructed to maintain this level of muscle contraction and were continuously instructed to increase or decrease muscle contraction, as needed, to ensure adequate stability of the tonic EMG signal.

Data analysis was performed in the same way at both time points (t1 and t2). MEPs (test stimulus, $ISI_{2ms,}$ ISI_{3ms}, ISI_{9ms}, ISI_{12ms}, ISI_{100ms}) were visually inspected and trials with EMG activity reflecting muscle contraction in the 500 ms prior to stimulation were excluded from analysis. Outlier values (± 3 SD) were also excluded. After averaging peak-to-peak MEP amplitudes for the TS-alone (TS-MEP) and paired pulse measures, inhibition and facilitation indexes were computed as the ratio of the average MEP amplitude for each ISI ($SICI_{2ms}$, $SICI_{3ms}$, ICF_{9ms}, ICF_{12ms}) over the average TS-MEP amplitude. For LICI, the ratio of the average peak-to-peak amplitude of the second MEPs elicited over the average peak-to-peak MEP amplitude at test stimulus intensity was computed. CSP duration was measured manually from TMS pulse to resumption of sustained EMG activity, as shown on Figure 3 (Groppa et al. 2012). Outliers (± 3 SD) and CSPs without a clear delimitation were excluded, and remaining CSP durations were averaged.

Statistical Analysis

To verify uniformity of voxel placement across sessions, a repeated measures *t*-test as well as a test-retest Pearson's correlation and intra-class correlation (two-way mixed model) of the absolute agreement of single measures across time was performed for %GM, %WM and %CSF. Coefficients of variation $\left(COV = \frac{SD}{M} \times 100\%\right)$ were computed separately across time (intra-subject) and across subjects (inter-subject) to describe within- and between-subject variability for

GABA, Glx, GABA/Glx, and MM water-referenced metabolite levels, rMT, %MSO, TS-MEP, and CSP measures, as well as all paired-pulse ($SICI_{2ms}$, $SICI_{3ms}$, ICF_{9ms}, ICF_{12ms}, $LICI_{100ms}$) ratios. Despite not being the primary focus of this study, the same descriptive and reliability statistics were computed for water-referenced NAA and tCr, as well as NAA and tCr referenced GABA and Glx concentrations. Furthermore, a repeated measures *t*-test was performed for these same variables to test for systematic effects. Repeated measures *t*-tests were also performed to compare the test stimulus average MEP amplitudes and each of the average MEP amplitudes for $SICI_{2ms}$, $SICI_{3ms}$, ICF_{9ms}, ICF_{12ms}, $LICI_{100ms}$ to assess paired-pulse effects on MEPs.

Test-retest reliability was assessed using Pearson's correlations between t1 and t2. Intra-class correlation coefficients (ICC) of the absolute agreement for metabolites of interest and TMS single measurements across time were computed using a two-way mixed model. ICC values were classified as poor (< 0.40), fair (0.40 – 0.59), good (0.60 – 0.74) or excellent (0.75 – 1.00) based on accepted guidelines (Cicchetti 1994; McGraw and Wong 1996). Reliable change indexes (RCI), which indicate the change in the quantitative variable that can be expected by random variation, were computed for MRS and TMS measures, with the rest-retest correlation used as the coefficient of reliability. The following formula was used to determine RCI:

$$RCI = \sqrt{2 \times (standard\ error\ of\ measurement)^2}$$

$$Standard\ error\ of\ measurement = SD \times \sqrt{1 - coefficient\ of\ reliability}$$

Correlations were computed between water-referenced metabolite concentrations (GABA, Glx, GABA/Glx) and TMS measures (rMT, %MSO, SICI-ISI_{2ms}, SICI-ISI_{3ms}, ICF-ISI_{9ms}, ICF-ISI_{12ms}, $LICI_{100ms}$, and CSP) at t1 and t2 to assess the relationship between MRS and TMS measures. An analysis of the correlations between the COVs of MRS and TMS measures as well as between GABA and Glx levels and the ratio of the rMT needed to produce MEPs averaging 1mV (%MSO/%rMT) was also performed.

Statistical analyses were performed using a standard statistical software package (SPSS 24, IBM, NY, USA). A p value ≤ 0.05 was considered statistically significant, and a Bonferroni correction was applied to correct for multiple comparisons.

Results

Following analysis, one subject with CRLB = 37% was excluded from the sample. The final sample comprised 10 right-handed adults (5 males, 5 females), aged 19 – 35 (26 ± 4) years. Session 1 and 2 were separated by an average of 96 ± 7 days. Outlier data for one subject at ISI_{12ms} and another subject at ISI_{100ms} were excluded.

Magnetic resonance spectroscopy

The averages and standard deviations of CSF, GM, and WM percentages at t1 and t2 are shown in Table 1. Paired samples *t*-tests revealed no significant effect of time for CSF ($t_{(9)}$ = 0.923, p = 0.380), GM ($t_{(9)}$ = 0.021, p = 0.983), and WM ($t_{(9)}$ = 0.112, p = 0.913) ratios. Strong test-retest correlations and good to excellent ICCs were obtained for CSF ($r_{(10)}$=0.852, p = 0.002; r = 0.854, 95% CI [0.539, 0.961], $F_{(9, 9)}$ = 12.530, p < 0.001), GM ($r_{(10)}$ = 0.673, p = 0.033; r = 0.675, 95% CI [0.090, 0.909], $F_{(9, 9)}$ = 4.738, p = 0.015), and WM ($r_{(10)}$ = 0.741, p = 0.014; r = 0.749, 95% CI [0.246, 0.932], $F_{(9, 9)}$ = 6.386, p =0.005) percentages. These results suggest that voxel positioning did not significantly differ between sessions.

Table 2 shows the mean (M) and the standard deviation (SD) across subjects of water, NAA, and creatine (tCr) referenced metabolite values for each session. Average CRLBs were 18.6 ± 5.6 (range: 12 – 29) for GABA, 2.90 ± 0.32 (range: 2 – 3) for Glu and 12.7 ± 2.8 (range: 9 – 18) for Gln at t1 and 17.4 ± 54 for GABA (range: 11 – 26), 3.00 ± 0 for Glu (range: 3) and 12.5 ± 1.3 (range: 9 – 18) for Gln at t2.

Paired samples *t*-tests revealed no significant effect of time for GABA/H_2O ($t_{(9)}$ = 1.136, p = 0.285), Glx/H_2O ($t_{(9)}$ = 1.163, p = 0.275) and MM/H_2O ($t_{(9)}$ = 0.088, p = 0.931) levels and GABA/Glx ($t_{(9)}$ = 1.838, p = 0.099) ratios. Descriptive and reliability statistics for the main metabolites of interest (GABA/H_2O, Glx/H_2O), MM/H_2O, GABA/Glx ratios, and supplementary metabolites (NAA/H_2O, tCr/H_2O, GABA/NAA, Glx/NAA, GABA/tCr, Glx/tCr), are shown in Table 2. Furthermore, GABA/H_2O presented a strong test-retest correlation ($r_{(10)}$ = 0.815, p = 0.004) and an excellent ICC (r = 0.809, 95% CI [0.432, 0.948], $F_{(9, 9)}$ = 9.715, p ≤ 0.001), Glx/H_2O presented a strong test-rest correlation ($r_{(10)}$=0.741, p = 0.014) and a good ICC ($r_{(10)}$=0.641, 95%

CI [0.105, 0.895], $F_{(9, 9)} = 4.693$, $p = 0.015$), and MM/H_2O presented a non-significant test-retest correlation ($r_{(10)}$=0.353, $p = 0.317$) and a poor ICC ($r_{(10)}$=0.374, 95% CI [-0.368, 0.803], $F_{(9, 9)} = 2.075$, $p = 0.146$). GABA/Glx ratios presented a strong test-retest correlation ($r_{(10)} = 0.832$, $p = 0.003$) and an excellent ICC ($r = 0.780$, 95% CI [0.339, 0.940], $F_{(9, 9)} = 9.797$, $p \leq 0.001$). Intra- and inter-subject average COVs and RCIs are also shown in Table 2. In general, inter-subject COVs were larger than intra-subject COVs. It can also be seen that stability and reliability statistics for NAA- and tCr-referenced metabolites are generally equivalent or poorer than H_2O-referenced metabolite values.

Transcranial magnetic stimulation

After scanning individual MEP trials for outliers (± 3 SD), 1.65% of all trials were removed across all participants and TMS variables. After excluding outlier trials, the lowest number of trials used for analysis was 9. The means (M), standard deviations (SD), intra- and inter-subject coefficients of variation, test-retest correlations, ICC and RCI values for rMT, %MSO, CSP, SICI, ICF and LICI are presented in Table 2. Overall, inter-subject COVs were larger than intra-subject COVs. Furthermore, TS-MEP were sufficiently stable across time (intra-subject COVs of 16%) for %MSO to be considered an adequate measure of corticospinal excitability. It can also be seen that, at both time points and compared to the MEP amplitude at test stimulus intensity, $SICI_{2ms}$, $SICI_{3ms}$, and $LICI_{100ms}$ reduced MEP amplitudes and ICF_{9ms} and ICF_{12ms} increased MEP amplitudes. However, inhibitory (SICI and LICI) effects were statistically significant at the Bonferroni-corrected significance level (all $p \leq 0.05/5 = 0.01$) while facilitatory (ICF) effects were not statistically significant (all $p \geq 0.226$) due to higher variability and smaller effect sizes, at both time points.

Paired samples *t*-tests revealed no significant effect of time for rMT ($t_{(9)} = 0.114$, $p = 0.912$), %MSO ($t_{(9)} = 0.258$, $p = 0.803$), $SICI_{2ms}$ ($t_{(9)} = 0.759$, $p = 0.467$), $SICI_{3ms}$ ($t_{(9)} = 0.167$, $p = 0.871$), ICF_{9ms} ($t_{(9)} = 0.543$, $p = 0.600$), ICF_{12ms} ($t_{(8)} = -0.940$, $p = 0.375$), and $LICI_{100ms}$ ($t_{(8)} = 0.909$, $p = 0.390$), and CSP ($t_{(9)} = -0.528$, $p = 0.610$). Further reliability analyses revealed near-perfect test-retest correlations and excellent ICCs for rMT ($r_{(10)} = 0.965$, $p < 0.001$; $r_{(10)}$=0.968, 95% CI [0.875, 0.992], $F_{(9, 9)} = 54.864$, $p < 0.001$) and %MSO ($r_{(10)} = 0.978$, $p < 0.001$; $r_{(10)}$=0.977, 95% CI [0.9135, 0.994], $F_{(9, 9)} = 79.776$, $p < 0.001$) measurements. No significant test-retest correlations (all $p >$

0.05) and poor ICCs were found for all paired-pulse measures, except for $SICI_{2ms}$, which showed a fair, but non-significant ICC ($r_{(10)} = 0.417$, 95% CI [-0.255, 0.815, $F_{(9, 9)} = 2.372$, $p = 0.107$). CSPs showed a strong test-retest correlation ($r_{(10)} = 0.799$, $p = 0.006$) and an excellent ICC ($r_{(10)} = 0.810$, 95% CI [0.409, 0.949, $F_{(9, 9)} = 8.936$, $p = 0.002$).

Relationship between MRS and TMS measures

The systematic examination of correlations between three water-referenced MRS measures (GABA, Glx, GABA/Glx) and eight TMS measures (rMT, %MSO, CSP, and $SICI_{2ms}$, $SICI_{3ms}$, ICF_{9ms}, ICF_{12ms} and $LICI_{100ms}$ indexes) using a Bonferroni-corrected significance level ($\alpha = 0.05/8 = 0.00625$) for multiple comparisons revealed no significant effect (all $p \geq 0.041$). The statistical values for the systematic examination of correlations between MRS and TMS measures are shown in Table 3.

Correlation analysis between the intra-subject COV of three MRS measures (COV_GABA, COV_Glx, COV_GABA/Glx) and eight TMS measures (COV_rMT, COV_%MSO, COV_CSP, and COV_$SICI_{2ms}$, COV_$SICI_{3ms}$, COV_ICF_{9ms}, COV_ICF_{12ms} and COV_$LICI_{100ms}$ indexes) using a Bonferroni-corrected significance level ($\alpha = 0.05/8 = 0.00625$) for multiple comparisons revealed no significant result. Using an uncorrected significance level ($\alpha = 0.05$), a strong positive correlation was found between the coefficient of variation of Glx and %MSO ($r_{(10)} = 0.682$, $p = 0.030$), suggesting that participants that showed greater Glx variability also showed greater variability in cortical excitability. A positive and weak, but not statistically significant ($p \geq 0.653$) correlation was found between %MSO/rMT ratios and metabolite (GABA, GABA/Glx) levels at both time points, suggesting that higher GABA levels and GABA/Glx ratios may be associated with higher intensities, relative to the rMT, needed to produce MEPs of 1 mV.

Discussion

The goal of the present study was to assess the long-term stability of TMS measures of GABA and glutamate synaptic activity and MRS measures of GABA/H_2O and Glx/H_2O concentration in sensorimotor cortex of healthy individuals. While MRS measures were stable over time, TMS measures were found to be reliable for rMT, %MSO, and CSP only. Among paired-pulse TMS

measures, $SICI_{2ms}$ yielded fair, but not statistically significant reliability statistics. Additionally, correlation analysis revealed no significant relationship between TMS and MRS measures at any timepoint. Furthermore, stability and reliability statistics of tCr and NAA-referenced metabolites were also obtained and found to be equivalent or poorer than water-referenced values, which is expected as the protocol used in this study is optimized for water-referenced GABA detection. Therefore, we limit the following discussion to water-referenced spectroscopy values.

Magnetic resonance spectroscopy

Despite differences in regions of interest, between-sessions intervals, data referencing, editing and processing techniques, within-subject COVs (10%) in the present study do not diverge significantly from those reported in studies investigating the short-term reproducibility and stability of GABA and GABA+ (GABA + MM), where COVs of ≈ 4 to 15% have been reported (Bogner et al. 2010; Evans et al. 2010; Harada et al. 2011; O'gorman et al. 2011; Near et al. 2013; Near et al. 2014; Greenhouse et al. 2016). This suggests that variations in GABA levels measured here are likely attributable to measurement error rather than long-term changes in metabolite concentration. Indeed, Evans and collaborators (2010) reported similar within- (8.8%) and between-subject (0.5%-19.7%) single-day COVs for sensorimotor GABA/H_2O. In addition, Greenhouse and collaborators (2016) have found a within-subject creatine-referenced COV of 3.9 ± 1.0% and a strong test-retest correlation (r = 0.64) over two scans across an approximate two-week period in the sensorimotor region, which is similar to the present findings. Furthermore, a 7-month longitudinal study reported GABA+/Cr levels with a low COV (4.3%) and a fair level of absolute agreement (r=0.52) in the occipital cortex (Near et al. 2014). The excellent absolute agreement between single measurements across time (r = 0.75) suggests that GABA measurements in the present study are highly reliable despite COVs that are higher than those reported by Near et al. (2014) in the occipital cortex. Furthermore, ICC values for GABA are similar to that of another study, which reported a good ICC for GABA/tCr when assessing its short-term (3 hours) reliability (Dyke et al. 2017a).

An average within-subject COV of 4% and a fair level of absolute agreement was found for Glx, suggesting that concentrations vary little over time. Indeed, Glx COVs were consistent with those reported in reproducibility and short-term studies using different ROIs and MRS

sequences (Hurd et al. 2004; Jang et al. 2005; O'gorman et al. 2011). For example, in the dorsolateral prefrontal cortex, O'gorman and collaborators (2011) reported within-session COVs of 6% between four measurements acquired within the same scanning session while Hurd et al. (2004) reported a COV of <10% in the parietal cortex over multiple scans. This suggests that variability estimates for Glx, as was the case for GABA, were likely due to measurement error.

The present data thus show that GABA/H_2O and Glx/H_2O concentrations in the sensorimotor cortex of healthy individuals are stable over a three-month period. Furthermore, specialized acquisition (MEGA-PRESS) and analysis techniques (LCModel) allow stable and precise measurements of GABA and Glx at moderate field strengths (3T) and minimize the impact of macromolecular contamination of GABA signals. In addition, no significant difference was found in GABA concentration in the sensorimotor area between older and younger adults, indicating that a significant change in individual GABA levels would not stem from aging (Mooney et al. 2017; Hermans et al. 2018a). However, glutamate concentration does decrease during adulthood (Grachev and Apkarian 2001). By taking into account the latter findings as well as the present results, it appears that GABA concentrations could be used as markers for monitoring disease progression and treatment effects in neurological and psychiatric populations, and Glx may also be used over shorter intervals or after taking into account the impact of aging on neurochemical concentrations. Furthermore, RCI analysis suggests that a change in water-referenced GABA levels of ≈0.15 or a change in water-referenced Glx levels of ≈0.72 across time, using the present protocol, would most likely reflect a significant alteration in metabolite concentration.

Transcranial magnetic stimulation

The excellent reliability of rMT measures found in the present study support previous reports where test-retest stability were found to be excellent within a one-month (Hermsen et al. 2016) or three-month period (Ngomo et al. 2012). Similarly, the intensity required to induced MEPs of 1mV amplitude (%MSO) was also very stable across time, which is consistent with previous reports (Maeda et al. 2002; Ngomo et al. 2012; Hermsen et al. 2016). Taken together with previous studies, the present data clearly show that rMT and %MSO values reflecting cortical excitability are very stable over periods of at least three months. Indeed, reliable change index values indicate that a

variation greater than 3% for both rMT and %MSO can be a reliable indicator of a change in excitability within primary motor cortex.

In the present study, paired pulse techniques were found to be highly variable between sessions and between subjects. With respect to coefficients of variation, the present findings are in general agreement with those previously reported. Indeed, between-sessions COVs were found to be lower than between-subjects COVs. Furthermore, between-sessions COVs for all ISIs were similar to those reported in previous studies, where time intervals between sessions ranged from minutes to months (Boroojerdi et al. 2000; Orth et al. 2003; Ngomo et al. 2012). However, discrepancies between the present findings and those of previous studies are found in other reliability parameters. For short-interval cortical inhibition, a fair level of reliability was observed for $SICI_{2ms}$ (ICC=0.417) only; $SICI_{3ms}$ showed a poor reliability. The latter finding is in disagreement with previous studies where significant and excellent test-retest correlations for $SICI_{2ms}$ and $SICI_{3ms}$ (Maeda et al. ; Ngomo et al. 2012; Hermsen et al. 2016; Dyke et al. 2018) were reported. Furthermore, it is unclear why only $SICI_{2ms}$ was found to be reliable, as both 2 and 3ms SICI are thought to share the same mechanism of action (Ziemann et al. 2015a). Small sample size in the present study may partly explain this finding. In addition, the above discrepancy may also stem from contamination of facilitatory processes which may reduce net inhibitory responses following $SICI_{3ms}$ to a greater extent than $SICI_{2ms}$ (Peurala et al. 2008). With respect to intracortical facilitation, statistically non-significant and poor test-retest correlations were obtained for both ICF_{9ms} (r=0.267) and ICF_{12ms} (r=0.379), which is not surprising given that facilitatory protocols did not produce robust effects on MEP amplitudes in our study. This result is in partial agreement with previous findings, where test-retest correlations ranged from strong to non-existent (Maeda et al. ; Ngomo et al. 2012; Hermsen et al. 2016; Dyke et al. 2018). As for $LICI_{100ms}$, the obtained low reproducibility (r = -0.052) may be partially explained by a statistical floor effect.

The lack of consensus across studies reporting paired pulse TMS reliability measures emphasizes the fact that care should be taken when interpreting paired-pulse measures across time or following interventions. However, discrepancies in outcome may be due to methodological differences between studies. For example, it has been shown that increasing the number of TMS-induced MEPs to at least 20 diminishes trial-to-trial variability and leads to more stable measures

(Chang et al. 2016; Goldsworthy et al. 2016). It is therefore possible that collecting additional MEPs would have increased TMS sensitivity and reproducibility in the present study. Other studies have demonstrated that methodologies assessing cortical inhibition and facilitation based on threshold tracking techniques (TTT) instead of paired-pulse MEP ratios yield more stable findings (Murase et al. 2015; Mooney et al. 2017; Samusyte et al. 2018). In addition, a systematic examination of the effect of varying CS intensity in paired-pulse paradigms showed that response variability between individuals at a specific ISI was substantial across CS intensities (Orth et al. 2003). Indeed, individuals that showed strong inhibition at ISI_{2ms} at a CS intensity of 60% rMT did not necessarily show strong inhibition for the same ISI at 70% rMT. Using different %rMT as a CS would have been interesting in determining which %rMT yields more stable and reliable ppTMS measures. Furthermore, performing a similar reliability assessment using the active motor threshold (%AMT) as a basis for TMS measurement would have been of interest since intracortical inhibition and facilitation were found to be strongly correlated to %AMT (Orth et al. 2003). Therefore, due to the great heterogeneity between paired-pulse protocols, care should be taken when comparing paired-pulse studies with different methodologies. Lastly, it must be noted that reliable change indexes ranging from 0.17 to 1.05 for paired-pulse indexes were obtained in the present study, which may be appropriate for clinical purposes. However, the absence of statistical significance for reliability statistics used in calculating ppTMS RCIs suggests that those should be used carefully.

Finally, for CSP measurements, a previous study (Hermsen et al. 2016) reported that the reliability of CSP length, which was measured visually or automatically, yielded similar test-retest correlation coefficients (visual r=0.466; automated r=0.486). The results of the present work support these findings, as an excellent absolute agreement was found across time (r=0.81) when CSP length was examined visually. Furthermore, both intra- (COV = 5%) and inter-subject (COV = 13%) variability were found to be low. Thus, CSP length seems to be stable across time over a minimal time interval of three months. As a result, the reliable change index can be duly interpreted and suggests that an inter-session variation of at least 15 ms is significant.

MRS and TMS

In the present study, no correlations were found between MRS and TMS measures at either time point, giving further credence to the idea that MRS and TMS have different neurochemical substrates. Furthermore, exploratory analyses revealed no significant relationship between intra-subject variability of TMS and MRS measures.

Transcranial magnetic stimulation is believed to mainly reflect receptor-dependent activity. Indeed, it has been shown that that MT is a measure of corticospinal excitability and is thought to depend on glutamatergic synaptic activity (Hodgkin and Huxley 1952; Paulus et al. 2008). Furthermore, SICI appears to rely on fast-acting $GABA_A$ receptor-mediated inhibition (Di Lazzaro et al. 1998) while LICI involves slow-acting $GABA_B$ receptor-mediated inhibition (Ziemann et al. 2015b) and ICF is believed to implicate both glutamatergic and GABA-ergic receptor networks (Ziemann et al. 2015b). While it is believed spinal mechanisms contribute to the early part of CSP (the first 50 to 75 ms), its late part is thought to reflect motor cortical postsynaptic inhibition ($GABA_AR$ and $GABA_BR$ activity). (Fuhr et al. 1991; Inghilleri et al. 1993; Ziemann et al. 1993; Ziemann et al. 2015b).

Magnetic resonance spectroscopy, on the other hand, quantifies total neurometabolite concentrations within an area of interest and does not represent receptor activity. It is believed that MRS-GABA mainly reflects extrasynaptic concentrations (Rae 2014; Stagg 2014; Dyke et al. 2017a). Extrasynaptic GABA is thought to mediate tonic inhibition, and is involved in regulating tonic and phasic activity in GABAergic circuits (Wu et al. 2007; Glykys et al. 2008). In a similar manner to GABA, MRS-Glx also measures total Glx concentration in a given area. However, ambiguity remains as to the precise substrates of MRS-Glx, which combines two signals stemming from Glu and Gln that can't be resolved using MEGA-PRESS at 3T. Furthermore, since these two neurometabolites are involved in different neurobiological processes and constantly undergo dynamic exchange through the Glu/Gln cycle (Bak et al. 2006; McKenna 2007), it is difficult to precisely pinpoint what comprises MRS-Glx, and how it relates to neurophysiological functioning.

Due to their very different modes of action, it is not surprising that receptor-activity dependent TMS measures do not to correlate with MRS-GABA and MRS-Glx. Indeed, no

correlations were found between MRS-GABA and MRS-Glx (or MRS-Glu) concentrations and TMS measures of cortical inhibition or facilitation in previous studies (Stagg et al. 2011c; Tremblay et al. 2013a; Dyke et al. 2017b; Hermans et al. 2018b). However, Tremblay and collaborators (2013) reported a significant correlation between MRS-Glx and cortical silent period length, which was not replicated in the present study. The present results are, however, in agreement with the suggested mechanism of action underlying CSP duration, which is thought to be glutamate-independent (Ziemann et al. 2015b).

The absence of significant correlations between MRS-GABA and TMS measures thus appear to be replicated across MR sequences, field strength and sample size, but also across TMS techniques, giving further credence to the idea that MRS measures of GABA do not reflect TMS-derived measures of cortical inhibition or facilitation. Furthermore, given that GABA levels were found be similar in both young and older adults, while some TMS measures of cortical excitability and inhibition were found to be modulated by age (Mooney et al. 2017; Hermans et al. 2018a), it is likely that both techniques possess different neurochemical substrates, with respect to GABA and its associated receptors. The lack of correlation between MRS-Glu (or MRS-Glx) and TMS measures of cortical inhibition, facilitation and silent period has also been replicated across studies (Stagg et al. 2011b; Tremblay et al. 2013b; Dyke et al. 2017b). However, the relationship between global cortical excitability and MRS-Glu remains ambiguous. Indeed, previous studies have reported conflicting results with respect to the relationship between MRS-glutamate and the slope of the input/output curve, which indexes global corticospinal activity (Stagg et al. 2011a; Dyke et al. 2017a). Another study has found a positive correlation between MEP amplitudes and motor cortical GABA/Cr concentrations, which was not replicated in the present study (Greenhouse et al. 2017). Finally, the present study showed that the intraindividual variability of TMS measures does not appear to be predictive of intraindividual variability of MRS measures. Indeed, no statistically significant correlation between MRS-COVs and TMS-COVs was found for any measurement. This suggests that TMS and MRS variations in measurement stability are independent from each other, further strengthening the argument that distinct inhibitory/excitatory mechanisms can be assessed by the two techniques.

Conclusion

This study revealed that water-referenced MRS-GABA and MRS-Glx have good stability over a three-month period, with variability across time comparable to that of other studies where measurements were taken at different time-intervals and in different brain areas. (Near et al. 2014; Dyke et al. 2017a). While rMT, %MSO and CSP were found to be stable over time, paired pulse TMS measures showed greater variability and lesser reliability. Therefore, MRS (GABA, Glx) and some TMS (rMT, %MSO, CSP) measures possess robust methodological properties that make them reliable markers of disease progression and treatment effects. The present study also added to the existing literature suggesting that MRS and TMS measures do not reflect the same neurochemical events, while showing for the first time that the long-term stability of the two techniques are independent of each other.

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Disclosures

The authors report no conflicts of interest.

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Tables

Table 1. Cerebrospinal Fluid (CSF), Grey Matter (GM) and White Matter (WM) Ratios

	Time 1 (M ± SD)[a]	Time 2 (M ± SD)[a]	*P*[b]	*r*[c]	ICC[d]	RCI
CSF	0.033 ± 0.010	0.035 ± 0.010	0.38	0.85**	0.85**	0.006
GM	0.224 ± 0.040	0.224 ± 0.052	0.98	0.67*	0.68*	0.036
WM	0.743 ± 0.048	0.742 ± 0.058	0.91	0.74*	0.75**	0.037

[a] The sum of average (M) ratios may not be exactly equal to 1.00 due to rounding.

[b] P-value of the repeated measures *t*-test.

[c] Pearson's correlation coefficient between time 1 and time 2.

[d] Intra-class correlation coefficient of the absolute agreement between single measures of time 1 and time 2 using a two-way mixed model.

* $p \leq 0.05$; ** $p \leq 0.01$.

Table 2. Descriptive, Stability and Reliability Statistics for MRS and TMS Variables

	Time 1 (M ± SD)	Time 2 (M ± SD)	Within-Subject COV (%)	Between-Subject COV (%)	r[a]	ICC[b]	RCI
Segmented MRS Measures							
[GABA/H_2O]	0.834 ± 0.239	0.888 ± 0.254	10	29	0.815**	0.809**	0.147
[Glx/H_2O]	14.495 ± 1.232	14.183 ± 0.728	4	7	0.741*	0.641*	0.718
[NAA/H_2O]	22.268 ± 0.470	22.170 ± 0.758	1	3	0.722*	0.661*	0.459
[MM/H_2O]	4.462 ± 0.212	4.455 ± 0.185	3	4	0.353	0.374	0.220
[GABA/Glx]	0.057 ± 0.014	0.062 ± 0.017	10	25	0.832**	0.780**	0.009
[GABA/NAA]	0.037 ± 0.011	0.040 ± 0.012	11	30	0.798**	0.788**	0.007
[Glx/NAA]	0.652 ± 0.062	0.641 ± 0.044	3	8	0.703*	0.676*	0.041
[tCr/H_2O]	17.259 ± 1.647	17.695 ± 0.857	4	7	0.398	0.330	1.424
[GABA/tCr]	0.049 ± 0.016	0.050 ± 0.015	14	31	0.651*	0.669*	0.013
[Glx/tCr]	0.850 ± 0.137	0.803 ± 0.050	6	11	0.614	0.379	0.091
TMS measures							
rMT (%)	39 ± 10	39 ± 11	4	26	0.965**	0.968**	3
%MSO (%)	47 ± 17	47 ± 15	4	34	0.978**	0.976**	3
TS (mV)	0.93 ± 0.23	0.96 ± 0.26	16	26	0.388	0.407	0.27
CSP (ms)	184 ± 24	187 ± 25	5	13	0.799**	0.810**	15
$SICI_{2ms}$	0.31 ± 0.21	0.37 ± 0.27	42	69	0.421	0.417	0.25
$SICI_{3ms}$	0.25 ± 0.18	0.26 ± 0.14	51	65	-0.187	-0.207	0.25
ICF_{9ms}	1.16 ± 0.53	1.04 ± 0.63	34	54	0.267	0.278	0.70
ICF_{12ms}	1.10 ± 0.60	1.52 ± 0.89	44	57	0.095	0.089	1.05
$LICI_{100ms}$	0.18 ± 0.16	0.12 ± 0.11	55	93	0.217	0.209	0.17

[a] Pearson's correlation coefficient between time 1 and time 2.
[b] Intra-class correlation coefficient of the absolute agreement between single measures of time 1 and time 2 using a two-way mixed model.
* $p \leq 0.05$; ** $p \leq 0.01$.

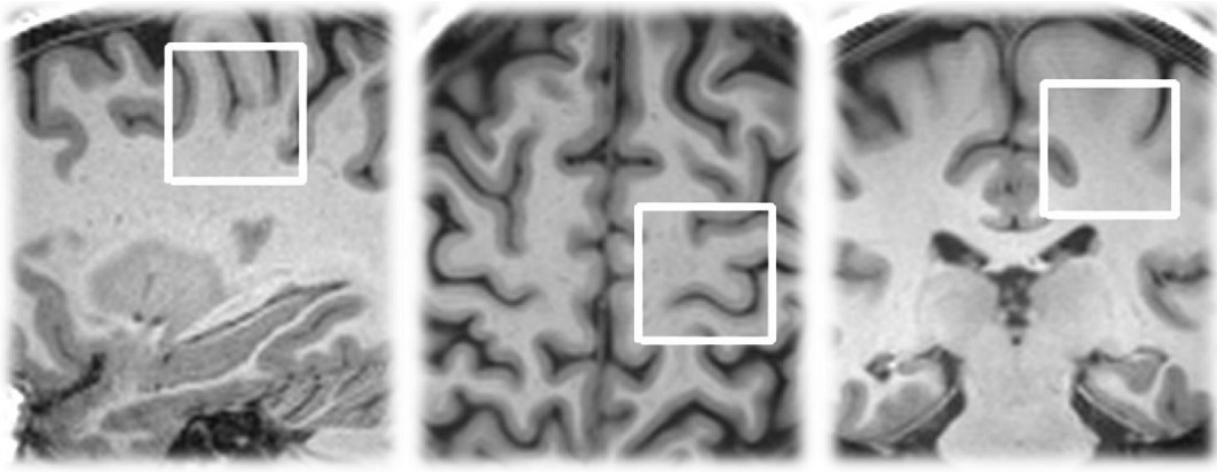
Table 3. Correlation Coefficients between water-referenced metabolite and TMS Measures at T1 and T2

	Time 1			Time 2		
	GABA	Glx	GABA/Glx	GABA	Glx	GABA/Glx
RMT	-0.04	0.18	-0.14	0.11	0.54	0.01
%MSO	0.05	0.16	-0.03	0.13	0.52	0.03
%MSO/RMT	0.16	0.05	0.14	0.16	0.18	0.13
$SICI_{2ms}$	-0.26	-0.17	-0.25	-0.42	-0.65*	-0.32
$SICI_{3ms}$	-0.29	-0.37	-0.21	-0.01	0.04	-0.03
ICF_{9ms}	0.12	-0.02	0.18	-0.08	-0.65*	0.05
ICF_{12ms}	-0.29	-0.05	-0.31	0.25	-0.16	0.29
$LICI_{100ms}$	-0.09	0.08	-0.18	0.63	0.10	0.64*
CSP	0.20	0.37	0.09	-0.33	0.41	-0.42

* $p \leq 0.05$; ** $p \leq 0.00625$ (Bonferroni-corrected significance level)

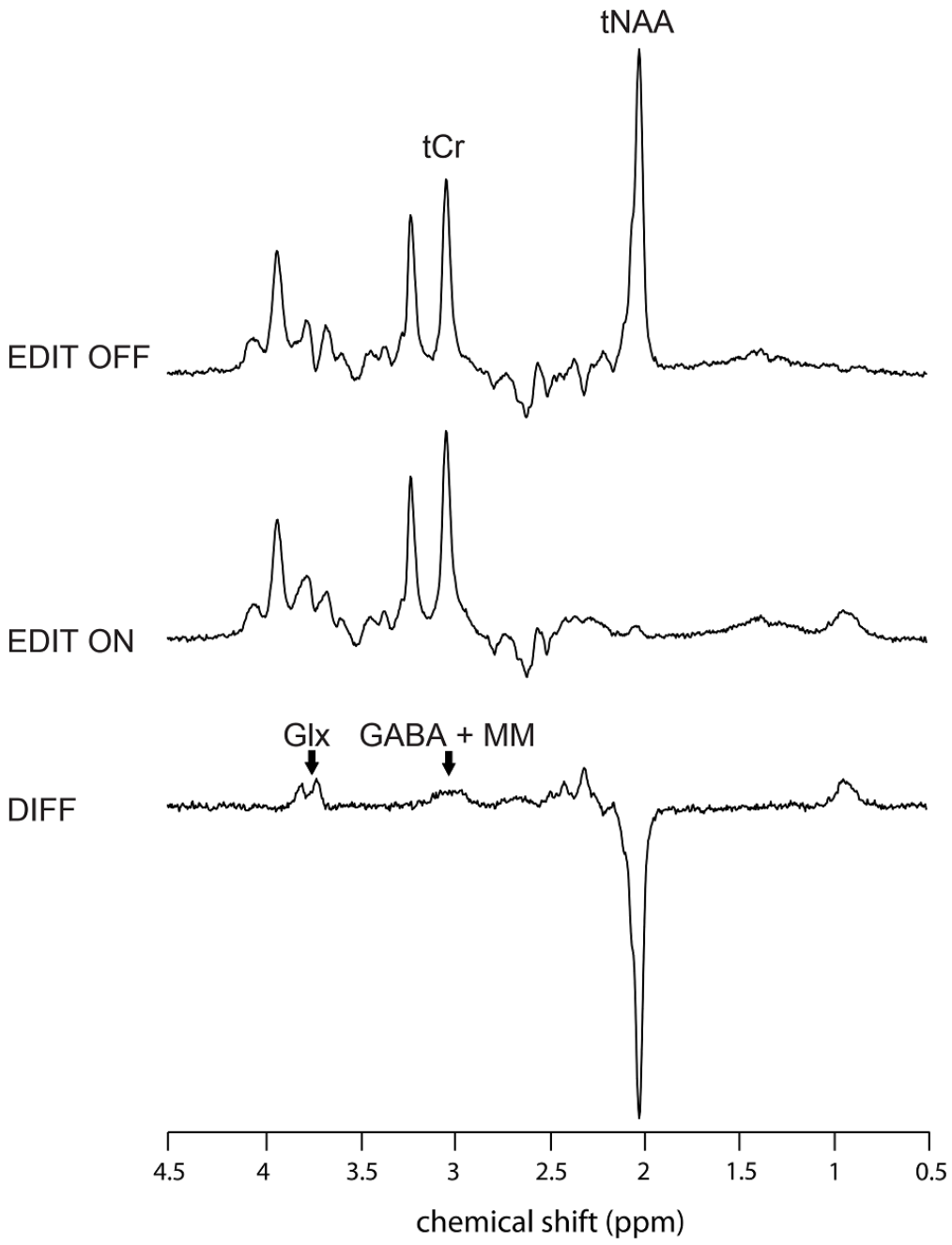
Figures

Figure 1. Position of the voxel of interest over left sensorimotor cortex.



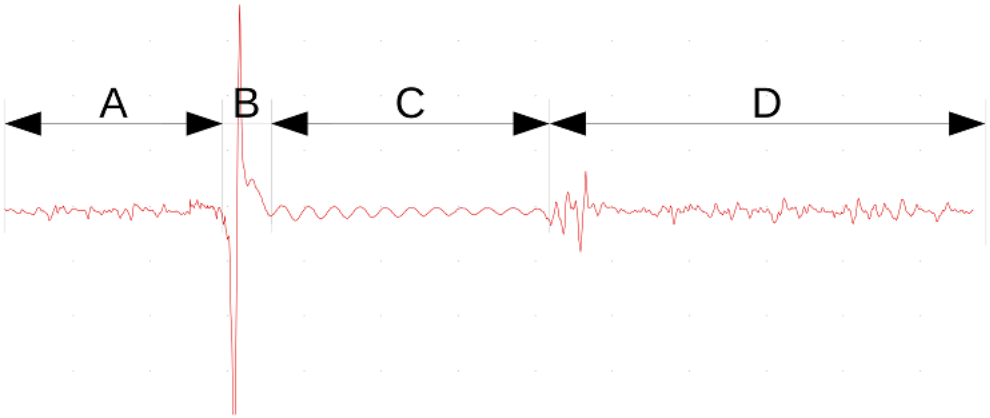
Legend: The voxel of interest (30 x 30 x 30 mm^3) is shown in sagittal, coronal, and axial views, respectively.

Figure 2. Fitted spectra for EDIT OFF, EDIT ON and DIFF spectra



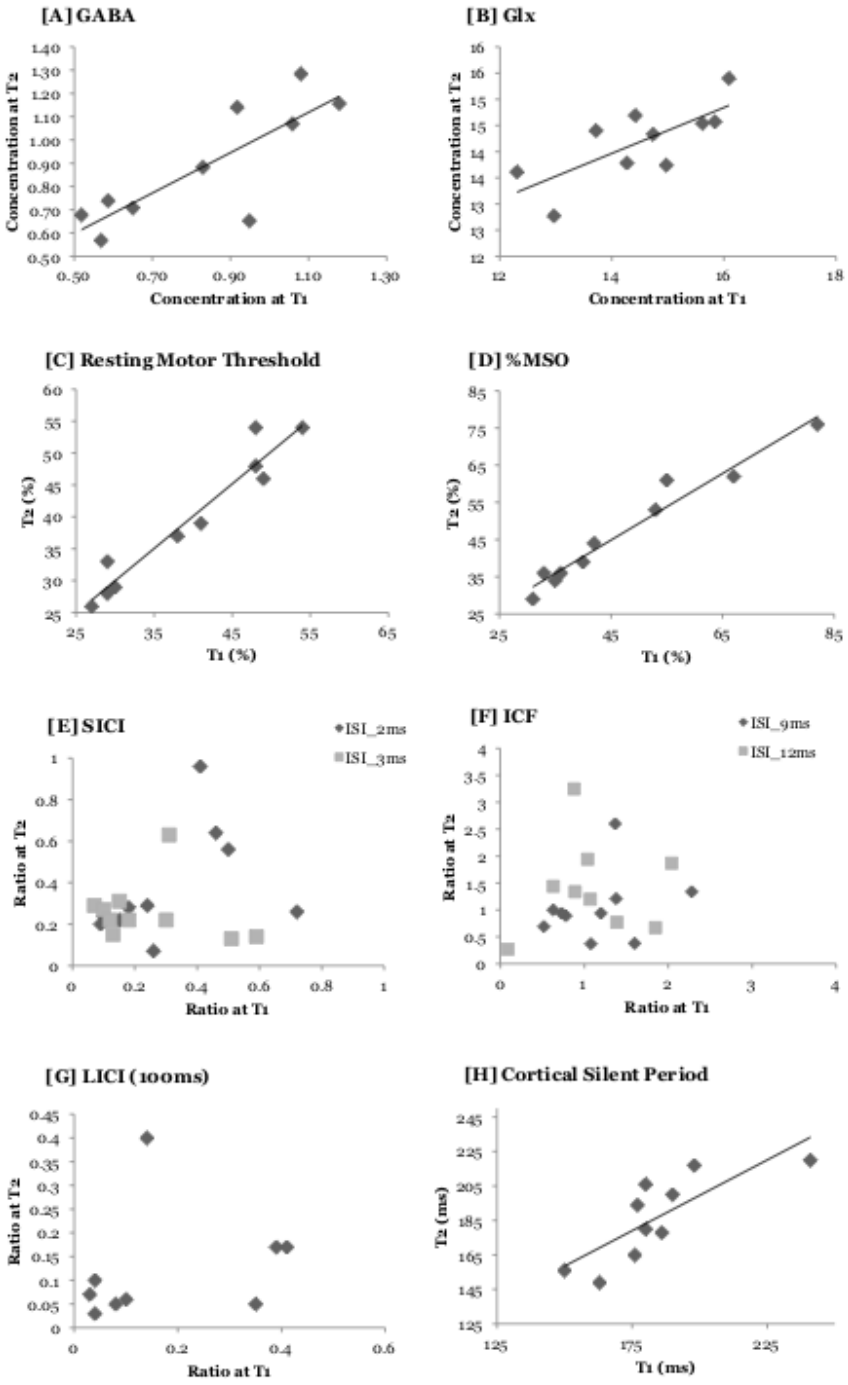
Legend: The characteristic peaks of Glx, and GABA+MM are shown on the difference (DIFF) spectra.

Figure 3. EMG signal for the cortical silent period.



Legend: (A) Period of tonic muscle contraction maintained at approximately 20% of maximum contraction. (B) MEP elicited from a TMS pulse at 120%rMT. (C) Period of EMG inactivity. (D) Resumption of tonic EMG activity.

Figure 4. Test-retest correlations for MRS and TMS measurements



Legend: Scatter plots illustrating the association between measures for MRS for (A) GABA, (B) Glx and TMS for (C) RMT, (D) %MSO, (E) SICI, (F) ICF, (G) LICI, and (H) CSP. Statistically significant correlations are identified by a trendline.

Chapter 3

Article 2: TMS and H^1-MRS measures of excitation and inhibition following lorazepam administration

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Highlights

- Lorazepam decreases motor cortical excitability and increases cortical inhibition.
- Lorazepam has no effect on motor cortex GABA and Glx levels.
- Lorazepam may decrease occipital GABA by an activity-dependent metabolic mechanism
- TMS reflects synaptic activity while MRS reflects extrasynaptic metabolite levels.
- Higher motor cortical GABA increases the effect of lorazepam on cortical inhibition.

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Abstract

This study aimed at better understanding the neurochemistry underlying TMS and MRS measurements as it pertains to GABAergic activity following administration of allosteric GABAA receptor agonist lorazepam. Seventeen healthy adults (8 females, 26.0 ± 5.4 years old) participated in a double-blind, crossover, placebo-controlled study, where participants underwent TMS and MRS two hours after drug intake (placebo or lorazepam; 2.5 mg). Neuronavigated TMS measures reflecting cortical inhibition and excitation were obtained in the left primary motor cortex. Sensorimotor cortex and occipital cortex MRS data were acquired using a 3T scanner with a MEGA-PRESS sequence, allowing water-referenced [GABA] and [Glx] (glutamate+glutamine) quantification. Lorazepam administration decreased occipital [GABA], decreased motor cortex excitability and increased GABAA-receptor mediated motor cortex inhibition (SICI). Lorazepam intake did not modulate sensorimotor [GABA] and TMS measures of intra-cortical facilitation, long-interval cortical inhibition, cortical silent period, and resting motor threshold. Furthermore, higher sensorimotor [GABA] was associated with higher cortical inhibition (SICI) following lorazepam administration, suggesting that baseline sensorimotor [GABA] may be valuable in predicting pharmacological or neuromodulatory treatment response. Finally, the differential effects of lorazepam on MRS and TMS measures, with respect to GABA, support the idea that TMS measures of cortical inhibition reflect synaptic GABAergic phasic inhibitory activity while MRS reflects extrasynaptic GABA.

Key words: Magnetic resonance spectroscopy (MRS); Gamma aminobutyric acid (GABA); Sensorimotor cortex; Transcranial magnetic stimulation (TMS)**;** Lorazepam

Introduction

Transcranial magnetic stimulation (TMS) and magnetic resonance spectroscopy (MRS) can probe the GABAergic system in the human brain (Puts & Edden, 2012; Ziemann et al., 2015). While MRS allows direct *in vivo* quantification of GABA and other metabolite levels in a chosen area of the brain (Mullins et al., 2014), TMS measures of intracortical inhibition, obtained in the sensorimotor cortex (SMC), indirectly reflect GABAergic inhibition (Di Lazzaro et al., 2006; Kujirai et al., 1993). Furthermore, recent studies have shown that measures of MRS-GABA and TMS-GABA obtained in the SMC of the same individual do not correlate, hinting at a dissociation between the neurochemical substrates of both techniques (Cuypers et al., 2020; Dyke et al., 2017; Ferland et al., 2019; Hermans et al., 2018; Stagg et al., 2011; Tremblay et al., 2012).

Several studies have shown that benzodiazepines (BZD) increase TMS-derived short intracortical inhibition (SICI), indicating that SICI depends on ionotropic $GABA_A$ receptors ($GABA_AR$) (Di Lazzaro et al., 2006; Kujirai et al., 1993; Ziemann et al., 1996; Ziemann et al., 2015). This theory is consistent with the mechanism of action of BZD, which are believed to modulate inhibitory signalling through binding on an allosteric site on pentameric $GABA_AR$. BZD induce conformational changes in the receptor, promoting GABA binding, chloride channel opening, and ion entry into the cell, leading to hyperpolarisation and inhibitory post-synaptic potential (IPSP) generation (Griffin et al., 2013; Möhler et al., 2002). This mechanism is linked to temporally restricted phasic inhibition on which most GABAergic transmission relies (Farrant & Nusser, 2005). TMS measures of cortical inhibition may thus be considered synaptic GABAergic activity markers.

MRS signals are believed to reflect extrasynaptic [GABA] (Mason et al., 2001; Rae, 2014a; Stagg et al., 2011; Waagepetersen et al., 1999) which is composed of intracellular GABA, mostly involved in cell metabolism, and extracellular GABA, involved in tonic inhibition, a non-temporally restricted form of GABA signalling stemming from GABA spillover and transporter reversal (Farrant & Nusser, 2005; Myers et al., 2016; Rae, 2014a; Semyanov et al., 2003; Stagg et al., 2011; Wu et al., 2007). Since intracellular [GABA] is in the millimolar range while extracellular levels lie in the micromolar range (Cavelier et al., 2005; Rae, 2014a; Wu et al., 2007), MRS-GABA signals would mainly reflect intracellular [GABA] (Myers et al., 2016;

Waagepetersen et al., 1999). Indeed, pharmacological agents that selectively increase extracellular concentrations (tiagabine) do not appear to alter [GABA] (Myers et al., 2014), while compounds that positively modulate intracellular concentrations (vigabatrin, gabapentin) increase [GABA] (Cai et al., 2012; Mueller et al., 2001). Furthermore, $GABA_AR$ agonists, which do not directly modulate cellular GABA concentrations, were found to have varied effects on [GABA]. BZD (clonazepam) administration reduced occipital [GABA] (Goddard et al., 2004) and non-BZD $GABA_AR$ agonist zolpidem lowered thalamic [GABA] while having no effect on anterior cingulate cortex [GABA] (Licata et al., 2009). These findings are difficult to explain considering the proposed neurochemistry underlying MRS measurements, and few studies have examined the effect of benzodiazepines on [GABA].

To examine the differential sensitivity of MRS and TMS with respect to GABAergic activity in the primary motor cortex, lorazepam, a classical benzodiazepine, was administered to healthy individuals before assessing GABAergic activity in the left motor cortex with TMS, and GABA levels in the left SMC and occipital cortex (OC) with MRS. In this randomized, placebo-controlled, double-blind crossover study, each participant underwent MRS and TMS testing following drug intake. It was hypothesized that 1) As previously reported, lorazepam will increase intracortical inhibition and decrease cortical excitability; 2) Lorazepam will not modulate GABA levels in SMC, but will lower it in the OC, as previously reported; 3) Due to the heterogeneous spatial distribution of $GABA_AR$ subunits, there will be no correlation between GABA levels in SMC and OC; and 4) As previously reported, TMS-GABA and MRS-GABA measures will not correlate. However, coupling between the two measures of GABAergic activity could be present at the individual level, where baseline SMC [GABA] would be associated with lorazepam modulation of GABA synaptic activity.

Materials and Methods

Participants

Twenty-two healthy, right-handed, adult participants were recruited using word of mouth and advertisements posted on campus and social media. Exclusion criteria were the following: neurological or psychiatric conditions, psychoactive medication, history of traumatic brain injury,

fainting or seizures, substance abuse, and any contraindications to MR scanning or transcranial magnetic stimulation. Participants were evaluated by a neurologist to exclude lorazepam contraindications. Handedness was assessed based on participant writing hand preference. Participants provided written informed consent prior to testing, and experiments were performed with the approval of the local ethics committee (*Comité d'éthique de la recherche vieillissement-neuroimagerie,* Centre intégré Universitaire de santé et de services sociaux du Centre-Sud-de-l'Île-de-Montréal*)*.

Experimental design

In this double-blind, randomized, crossover, placebo-controlled study, each participant underwent two TMS sessions (45 minutes each) and two MRI sessions (75 minutes each) on different days, separated by at least 72 hours in a randomized order. Randomization, blinding, and drug distribution were managed by the hospital pharmacy department (*Institut Universitaire de Gériatrie de Montréal)*. Lorazepam was administered orally at a dose (2.5 mg) known to alter cortical excitability (Di Lazzaro et al., 2006; Ziemann et al., 1996). All measurements were performed two hours after drug or placebo intake according to lorazepam pharmacokinetics (Di Lazzaro et al., 2000; Di Lazzaro et al., 2006; Kyriakopoulos et al., 1978). To control for circadian variability in response (Lang et al., 2011; Soreni et al., 2006), TMS and MRS data collection began between 1:00 and 2:00 PM.

After each session, participants were asked if they believed they received the placebo or the active treatment. A visual analog wakefulness scale was also completed by each participant after each session to evaluate sedation, which consisted of a 100mm, non-graded horizontal line with 0mm : « very sleepy » and 100mm : «very alert» (Di Lazzaro et al., 2006; Kyriakopoulos et al., 1978). Participants were asked to place a mark on the scale representing their wakefulness level.

Magnetic Resonance Imaging and Spectroscopy

Magnetic resonance imaging sessions were performed at the Unité de Neuriomagerie Fonctionnelle, Centre de Recherche de l'Institut Universitaire de Gériatrie de Montréal. A 3T

whole-body system scanner (MAGNETOM Trio, Siemens, Erlangen, Germany) with a 32-channel receive-only head coil were used for MR acquisition. While in the scanner, participants were shown a variety of background images on a slideshow.

Anatomical Imaging

Whole-brain T_1-weighted MPRAGE anatomical images were acquired to position the spectroscopic voxels-of-interest (VOI; 30 x 30 x 30 mm^3) using the following parameters: T_R (repetition time) = 2,300 ms; T_E (echo time) = 2.98 ms; FA (flip angle) = 90°; FOV (field of view) = 256 mm, matrix = 256 × 256 x 176; T_I (inversion time) = 900 ms; number of slices= 176; slice thickness = 1 mm; orientation: sagittal; voxel size 1.0 x 1.0 x 1.0 mm^3; acquisition time: 9:50 min.

Magnetic Resonance Spectroscopy

Spectroscopic measurements were acquired first of the sensorimotor cortex, and then of the occipital cortex. The first spectroscopic VOI (30 x 30 x 30 mm^3) was situated in the left SMC according to published anatomical landmarks (Yousry et al., 1997) and the second (30 x 30 x 30 mm^3) in the OC at the midline of the occipital lobe (Figure 1). Voxel placement during the second session (T2) was based on axial, coronal and sagittal views obtained during the first session (T1). Shimming was done using FAST(EST)MAP (Gruetter & Tkáč, 2000) to ensure a water linewidth under 10 Hz. Metabolite signals were acquired using MEGA-PRESS (Mescher et al., 1996, 199) with the following parameters: T_R = 3,000ms; T_E = 68ms; Excite FA = 90°; Refocus FA = 180°. Water signal suppression and 3ppm GABA γ–CH_2 resonance editing was done simultaneously using double-banded pulses. The water-suppressing band was applied at 4.7 ppm while the editing band was applied at 1.9 ppm (EDIT ON) or at 7.5 ppm (EDIT OFF). Before running MEGA-PRESS, additional water suppression using variable power with optimized relaxation delays (VAPOR) and outer volume suppression (OVS) techniques were incorporated (Tkáč et al., 1999). The acquisition frequency was centered on GABA at 3 ppm (δ frequency = – 1.7 ppm). MEGA-PRESS data were acquired in blocks of 32 ‘EDIT OFF’ and 32 ‘EDIT ON’ interleaved scans (4 blocks; 12-minute acquisition time) with frequency adjustments performed before each block. Individual free induction decays (FIDs) were stored for offline processing. The same MEGA-

PRESS sequence (without MEGA and VAPOR water suppression) and voxel coordinates were used to acquire the water signal, which serves as a reference for metabolite quantification. Acquisition was centered on water at 4.7ppm (δ frequency = 0) and single block of 4 averages was acquired (acquisition time: 42 sec).

MRS Analysis

A researcher blind to drug condition analyzed MRS data. Prior to analysing spectra, tissue segmentation to correct for fractional volume composition of gray matter (GM), white matter (WM) and cerebrospinal fluid (CSF) within voxels was performed using *FreeSurfer* 5.3.0 to allow for the correction of relaxation and partial volume effects on water-referenced metabolite concentrations. Water attenuation was computed using the fractional volume of each compartment (Gasparovic et al., 2006). The T_1 and T_2 water relaxation times used in the attenuation factor calculations were taken from published reports [T_1(GM) = 1.29 s, T_1(WM) = 0.87 s, T_1(CSF) = 4 s, T_2(GM) = 110 ms, T_2(WM) = 80 ms, and T_2(CSF) = 400 ms] (Rooney et al., 2007; Wansapura, Holland, Dunn, & Ball Jr, 1999).

Individual averages were frequency and phase corrected offline and then averaged independently for 'EDIT OFF' and 'EDIT ON' acquisitions, to generate the 'EDIT OFF' and 'EDIT ON' subspectra. Small frequency errors between 'EDIT OFF' and 'EDIT ON' subspectra were manually corrected in LCModel 6.2-1A (Provencher, 1993, 2001) by minimizing subtraction error in the difference spectra around the 3.9-ppm creatine and the 3.2-ppm choline resonance. The final difference spectra ('EDIT DIFF') were obtained by subtracting the 'EDIT OFF' from the 'EDIT ON' subspectra.

LCModel 6.2-1A (Provencher, 1993, 2001) was used to analyse the 'EDIT DIFF' spectra. LCModel spline baseline modeling was deactivated with the NOBASE = T input parameter. Lipid and MM resonances simulations were also deactivated. No baseline correction, zero-filling, or apodization functions were applied to the *in vivo* data prior to LCModel analysis. Spectra with GABA Cramér-Rao lower bounds (CRLB) > 35% were excluded from further analysis.

The basis set for 'EDIT DIFF' was comprised of both an experimentally measured metabolite-nulled macromolecular (MM) spectrum acquired from the occipital region of the same independent healthy adult cohort, as well as experimentally measured spectra from 100 mM NAA, GABA, Glu and Gln phantoms (7.2 pH and at 37 °C). Fitting was performed over the 0.5 – 4.0 ppm spectral range.

The water signal was used as an internal standard reference for metabolite quantification. Water-referenced GABA, Glu and Gln values were obtained based on the segmentation-corrected 'EDIT DIFF' output for both SMC and OC spectra and analyzed using LCModel 6.2-1A (Provencher, 1993, 2001). Since at 3T glutamate cannot be resolved from glutamine, [Glx] was computed ([Glu] +[Gln]) and interpreted as reflective of [glutamate].

TMS Experiments and EMG Recording

During TMS experiments, participants were seated and instructed to remain relaxed, alert, still, and to keep their hands and feet uncrossed and palms facing slightly upwards. Electromyographic (EMG) activity was recorded using two self-adhesive electrodes placed over the right first dorsal interosseous (FDI) muscle and the side of the right index finger. A ground electrode was positioned over the right forearm. The EMG signal was filtered with a bandwidth of 20-1000 Hz and digitized at a 4 kHz sampling rate using a Powerlab 4/30 system (ADInstruments, Colorado Springs, CO). Motor evoked potentials (MEPs) were recorded with Scope v4.0 software (ADInstruments, Colorado Springs, CO) and stored offline for analysis.

TMS was delivered using an 8-cm figure-of-eight coil connected to a Magstim 200^2 stimulator (Magstim Company Ltd, Spring Gardens, UK). The coil was positioned flat on the head at a 45º angle from the midline to deliver anterior-posterior currents. Resting motor threshold, cortical excitability, paired pulse and cortical silent period (CSP) acquisitions were performed on the optimal site of stimulation in the left hemisphere during both experimental sessions using previously published methods (Ferland et al., 2019; Rossini et al., 2015). The optimal stimulation site was identified as the area located approximately at a 45° angle laterally along the central sulcus of the left hemisphere where the highest amplitude MEPs and a visible hand movement were elicited with the minimal stimulation. The site was marked on the participant's scalp using a water-

soluble wax crayon and registered through a stereotactic neuronavigation system (Brainsight; NeuroConn GmbH, Ilmenau, Germany) and both points of reference were monitored continuously to ensure stable positioning. Throughout TMS experiments, MEPs were carefully monitored online, and EMG signals showing pre-stimulation activity were immediately discarded and reacquired after instructing subjects to relax their arm.

Resting motor threshold. Determined by progressively adjusting TMS intensity to the lowest that elicits MEPs ≥50μV in at least 5 out of 10 trials (Ferland et al., 2019; Rossini et al., 2015).

Cortical Excitability (I/O curve). Acquired by delivering 10 pulses for each of the following intensities, in a randomized order: 100%rMT, 110%rMT, 120%rMT, 130%rMT, and 140%rMT.

Paired pulse. Test stimulus (TS) intensity was adjusted to elicit MEPs with 1mV peak-to-peak amplitude (range: ≈ 0.6 – 1.2 mV). Ten pulses were administered at that intensity (TS intensity) and MEPs were recorded (TS-MEP). For short intra-cortical inhibition (SICI) and intracortical facilitation (ICF), the conditioning-stimulus (CS) intensity was at 60%rMT to minimize floor effects (Kujirai et al., 1993). Ten CS-TS pairs were delivered and recorded at different interstimulus intervals (ISI) in a randomized order for each participant. SICI was assessed at ISI_{2ms} and $ISI_{3ms,}$ and ICF was assessed at ISI_{9ms} and ISI_{15ms}. Long-interval cortical inhibition (LICI) was assessed with ISI_{100ms}, with both pulses at TS intensity. Ten LICI pairs of stimuli were recorded, and the second MEP was analyzed.

Cortical silent period (CSP): Fifteen silent period measures were obtained by administering TMS pulses at 120%rMT while participants maintained a voluntary isometric muscle contraction at approximately 20% of their maximum. The cortical silent period was measured manually as the total period (starting at the TMS pulse) until the resumption of tonic EMG signal.

TMS data analysis

TMS data were analyzed by a researcher blind to drug condition. Average peak-to-peak MEP amplitudes were calculated for rMT100%, rMT110%, rMT120%, rMT130%, rMT140%, TS-MEP, $ISI_{2ms,}$ ISI_{3ms}, ISI_{9ms}, ISI_{15ms}, and ISI_{100ms}. The input-output (I/O) curve slope was computed

using a standard function with the average peak-to-peak MEP amplitudes for rMT100% to rMT140% as known *y*'s and 100% to 140% as known *x*'s.

Paired-pulse inhibition or facilitation indexes were computed as ratios of the average peak-to-peak MEP amplitude at each ISI over the average TS peak-to-peak MEP amplitude. CSP duration was measured manually (Ferland et al., 2019).

Statistical analysis

All statistical analyses were performed using a standard statistical package (SPSS 25, IBM, NY, USA). A significance level of $\alpha=0.05$ was used throughout, with a Bonferroni correction for multiple comparisons when appropriate. Normality assumptions were verified with Shapiro-Wilk's test, and the corresponding non-parametric statistics were used when assumptions were violated.

Blinding was assessed with a one-variable χ^2 test where subjects were expected to guess the correct treatment with an accuracy of 50%. Responses of "I don't know" were treated as incorrect. Sedation effects were assessed with a 2x2 repeated measures ANOVA with *treatment* (Placebo, Lorazepam) and *condition* (TMS, MRS) as within-subject factors.

Treatment effects were analyzed across treatments (Placebo, Lorazepam), intensities (100%rMT, 110%rMT, 120%rMT, 130%rMT, 140%rMT), and ISIs ($SICI_{2ms}$, $SICI_{3ms}$, ICF_{9ms}, ICF_{15ms}, $LICI_{100ms}$), and for rMT, the I/O slope, and CSP durations, with a repeated-measures model.

Baseline (placebo) neurometabolite values were analyzed as independent variables to predict response ratios for TMS variables found to be modulated by lorazepam administration, to investigate a potential relationship between baseline GABA levels and post-BZD SICI response ratios and between baseline Glx levels and post-BZD cortical excitability.

Correlational analyses between baseline SMC neurometabolite (GABA & Glx) levels and main TMS variables (rMT, I/O curve slope, $SICI_{2ms}$, $SICI_{3ms}$, ICF_{9ms}, ICF_{15ms}, LICI, CSP) were

performed separately within both treatment conditions (placebo, lorazepam) with a Bonferroni-adjusted significance level of 0.00625 (α=0.05/8) to assess their independence. Lastly, correlations between SMC and OC metabolite values were computed to assess regional differences in metabolite concentrations.

Effect size statistics and correlation coefficients were interpreted according to published standards (Bakeman, 2005; Cohen, 1988, 1992). For Wilcoxon's signed-rank test, effect sizes (r) were calculated by dividing the Z value by the square root of the number of pairings (Pallant, 2007).

Results

Participant data and exclusions

Two subjects abandoned the study before data acquisition. Three subjects were excluded from all analyses: one with $[GABA]_{SMC}$ Cramér-Rao lower bound (CRLB) = 54% and experimental error in TMS acquisitions, one with lipid contamination of the sensorimotor cortex signal and failure to comply with experimental conditions, and one due to experimental complications that lead to an excessively long delay between lorazepam intake and the completion of experiments. Two subjects were excluded from occipital MRS analyses only: one due to technical difficulties and another due to lipid contamination of the signal. One subject was excluded from I/O analyses due to experimental error. After general exclusions, the sample consisted of 17 right-handed adults (8 females) aged 19-43 (26.0 ± 5.4) years. After specific exclusions, there were 15 subjects for OC analyses, 16 for IO analyses, and 17 for all other analyses.

There was an average of 10 days between both TMS sessions and both MRI sessions, and an average of 37 days between TMS and MRI sessions. Participants guessed the experimental condition with an overall accuracy of 81%, exceeding chance [$\chi^2(3, N = 68) = 16.588, p < 0.001$].

Lorazepam effects on MRS neurometabolite concentrations

As shown in Table 1 and Figure 2, no treatment effects were found for $[GABA]_{SMC}$ ($t_{(16)} = 1.160$, $p = 0.263$, d = 0.28) and $[Glx]_{SMC}$ ($t_{(16)} = 0.159$, $p = 0.876$, d = 0.04). Lorazepam administration

was found to moderately decrease $[GABA]_{OC}$ ($t_{(14)} = 2.381$, $p = 0.032$, d = 0.61), while having no effect on $[Glx]_{OC}$ ($t_{(14)} = 1.380$, $p = 0.189$, d = 0.36).

Lorazepam effects on TMS-derived measures of cortical excitability

As shown in Table 1, no treatment effect was found for rMT ($t_{(16)} = 0.226$, $p = 0.824$, d = 0.05), TS intensity ($t_{(16)} = 1.854$, $p = 0.082$, d = 0.45), or TS-MEP amplitude ($t_{(16)} = 0.259$, $p = 0.799$, d = 0.06). Since I/O curve data (slope and MEPs) did not meet the normality assumption, their corresponding treatment effects were examined with the Wilcoxon signed rank test. No effect was observed on the global I/O curve slope (W = 35, $p = 0.087$, r = 0.30), as well as on average MEPs at 100%rMT (W = 49, $p = 0.535$, r = 0.11), 110%rMT (W = 59, $p = 0.638$, r = 0.08), 120%rMT (W = 35, $p = 0.087$, r = 0.30), and 130%rMT (W = 35, $p = 0.087$, r = 0.30). Lorazepam moderately reduced MEPs at 140%rMT (W = 29, $p = 0.043$, r = 0.36; Figure 3).

Lorazepam effects on TMS-derived paired-pulse inhibitory and facilitatory measures

Paired-pulse indexes did not respect the normality assumption. Therefore, treatment effects were assessed with non-parametric tests. Lorazepam administration moderately increased SICI, as demonstrated by decreased ratios at 2ms (W = 26, $p = 0.030$., r = 0.37), but not at 3ms (W = 50.5, $p = 0.219$, r = 0.21). For ICF, no significant effect was observed for ICF_{9ms} (W = 56.5, $p = 0.342$, r = 0.16) or ICF_{15ms} (W = 56, $p = 0.332$, r = 0.17). Finally, lorazepam administration had no effect on $GABA_B$ receptor dependent $LICI_{100ms}$ ratios (W = 51, $p = .610$, r = 0.09). These results are illustrated in Figure 4.

Lorazepam effects on the cortical silent period

Lorazepam administration had no effect on $GABA_A$ and $GABA_B$ receptor-dependent CSP duration ($t_{(16)} = 0.329$, p = 0.747, d = 0.08).

Predicting the effects of lorazepam from MRS measurements

A potential link between BZD-modulated cortical excitability (140%rMT) and cortical inhibition ($SICI_{2ms}$) response ratios and baseline $[Glx]_{SMC}$ and $[GABA]_{SMC}$, respectively, was investigated. The Shapiro-Wilk test revealed that cortical excitability ($p = 0.035$), but not the $SICI_{2ms}$ ($p = 0.131$) response ratio violated the normality assumption. However, after removing one extreme value ($Z = 2.73$) from the 140%rMT data set, normality was respected ($p = 0.157$). It was found that baseline $[GABA]_{SMC}$ negatively correlated with $SICI_{2ms}$ response ratios ($r_{(15)} = -0.49$, $p = 0.047$), indicating that the effect of lorazepam on the $SICI_{2ms}$ measure was stronger in subjects with higher baseline $[GABA]_{SMC}$. A trend between baseline $[Glx]_{SMC}$ and cortical excitability at 140%rMT ($r_{(13)} = 0.50$, $p = 0.059$) was also found. These results are illustrated in Figure 5.

Correlations between TMS and MRS measurements

No correlation was found between baseline SMC [GABA] and OC [GABA] ($r_{(15)} = 0.322$, $p = 0.242$), suggesting regional differences in GABA expression. A strong positive correlation was found between baseline SMC [Glx] and OC [Glx] ($r_{(15)} = 0.804$, $p < 0.001$), suggesting spatial homogeneity in Glx expression. Systematic correlation analysis of baseline MRS_{SMC} (GABA, Glx) and TMS measures (rMT, I/O curve, $SICI_{2ms}$, $SICI_{3ms}$, ICF_{9ms}, ICF_{15ms}, $LICI_{100ms}$, and CSP) revealed no significant correlation. Following a report by Hermans and collaborators (2018), exploratory, unplanned correlation analysis between the main inhibitory and excitatory TMS measures ($SICI_{2ms}$, $SICI_{3ms}$, ICF_{9ms}, ICF_{15ms}) in the lorazepam and placebo conditions was performed. In the placebo condition, ICF_{9ms} and ICF_{15ms} were significantly correlated ($r_{(16)} = 0.702$, $p = .002$). In the lorazepam condition, $SICI_{2ms}$ and $SICI_{3ms}$ were significantly correlated ($r_{(16)} = 0.600$, $p = .011$) but it did not survive multiple comparisons correction. Finally, a second exploratory, unplanned correlation analysis was performed with the two TMS measures that were significantly affected by lorazepam ($SICI_{2ms}$ and TMS 140% rMT). No significant correlation was found for the placebo ($r_{(15)} = 0.175$, $p = 0.518$) or lorazepam ($r_{(15)} = 0.293$, $p = 0.271$) conditions.

Sedation effects of lorazepam

Sedation response analysis revealed no significant *measurement* x *treatment* interaction ($F_{(1,15)} = 3.333$, $p = 0.088$), no measurement (imaging or TMS) effect ($F_{(1, 15)} = 0.786$, $p = 0.389$), and a

strong treatment effect ($F_{(1, 15)} = 36.544$, $p < 0.001$, $\eta_p^2 = 0.709$). A large sedation effect (d = 1.51) was observed between placebo ($M = 61.406$) and lorazepam administration ($M = 24.281$).

Discussion

In the present study, lorazepam did not alter $[Glx]_{SMC\ \&\ OC}$ or $[GABA]_{SMC}$ levels when compared to placebo. However, $[GABA]_{OC}$ decreased by 9% following lorazepam administration. Furthermore, lorazepam moderately decreased cortical excitability at the higher end of the I/O curve and moderately increased $GABA_AR$-mediated $SICI_{2ms}$. Lorazepam had no effect $SICI_{3ms}$, rMT, ICF, LICI, or CSP measures. In addition, higher $[GABA]_{SMC}$ was associated with a greater increase in cortical inhibition following lorazepam administration, suggesting that $[GABA]_{SMC}$ may be a marker for benzodiazepine sensitivity, specifically for its effects that are mediated by the GABAergic system.

The effects of lorazepam on MRS-Glx measures in SMC and OC

In the present study, lorazepam administration was found to have no effect on $[Glx]_{SMC}$ and $[Glx]_{OC}$. This is in line with previous studies that have shown that benzodiazepines midazolam (Yildiz et al., 2010), lorazepam (Brambilla et al., 2002), alprazolam (Henry et al., 2010) and clonazepam (Goddard et al., 2004; Henry et al., 2010) do not modulate glutamate levels in healthy individuals. The present data therefore provide important confirmatory evidence for the absence of MRS-detectable effects of benzodiazepines on acute glutamate (Glx) levels, as other studies were either not controlled by placebo, or reported data collected from ten participants or less (Brambilla et al., 2002; Goddard et al., 2004; Henry et al., 2010). However, while it is known that MRS quantifies total tissue concentrations, Glx signals acquired at 3T comprise those of both Glu and Gln, two neurometabolites engaged in a dynamic exchange as part of the Glu/Gln cycle and involved in different processes (Bak et al., 2006; McKenna, 2007). Therefore, ambiguity remains as to what precisely comprises MRS-Glx signals, the extent with which glutamate modulation can be perceived and its involvement in neurophysiological functioning. Nevertheless, the majority of the Glx signal stems from glutamate, and it has been shown that changes in [Glu] are linked to metabolic activity. It is thus common to interpret differences in [Glu] as being related to metabolic activity where an increase in [Glu] would indicate increased metabolism (Rae, 2014b).

The effects of lorazepam on MRS-GABA measures in SMC and OC

Similarly, lorazepam did not significantly modulate $[GABA]_{SMC}$. However, lorazepam decreased $[GABA]_{OC}$ by 9%, in relative agreement with a study where a 24% decrease in occipital [GABA] following clonazepam intake was observed (Goddard et al., 2004). In addition, it was found that zolpidem, a non-benzodiazepine allosteric $GABA_AR$ agonist, lowered [GABA] by 25% in the thalamus, but not in the ACC (Licata et al., 2009). Therefore, $GABA_AR$ agonists appear to modulate [GABA] in a region-dependent manner, and the chosen pharmacological agent may also play a role. Indeed, zolpidem binds almost exclusively to $GABA_ARs$ bearing an a1 subunit, while classical benzodiazepines bind to $GABA_ARs$ bearing a1, a2, a3, or a5 subunits. Such receptor subtypes are distributed heterogeneously throughout the brain (Crestani et al., 2001; Rudolph et al., 2000; Sieghart, 1994), and each pharmacological agent has a different affinity profile for $GABA_AR$ isoforms (Griffin et al., 2013). In addition, the lack of correlation in [GABA] found between brain regions (Greenhouse et al., 2016), also observed in the present study, hints at distinct GABAergic activity across brain regions, supporting the theory that $GABA_AR$ agonists produce region-dependent effects.

The decrease in $[GABA]_{OC}$ found in the present study is surprising given the mechanism of action of BZD, which directly modulate $GABA_AR$ activity (Griffin et al., 2013). Such modulation is believed not to be reflected in MRS-GABA signals, which reflect extrasynaptic concentrations (Myers et al., 2016; Rae, 2014a; Charlotte J Stagg et al., 2011; Waagepetersen et al., 1999). Furthermore, in light of the sensitivity of *in vivo* MRS, which detects changes in the millimolar range and across long acquisition times (Mullins et al., 2014; Shungu et al., 2016), and given that GABA released in the synaptic cleft as part of phasic inhibition has a decay time constant under 500μs (Farrant & Nusser, 2005), it is likely that any measured changes in [GABA] following drug administration reflect relatively longer lasting intracellular metabolic changes and not transient synaptic activity. Indeed, the reduction in $[GABA]_{OC}$ may be caused by BZD-induced changes in blood flow and reduced metabolism, possibly leading to reduced GABA synthesis. Indeed, positron-emission tomography (PET) studies have demonstrated a decrease in blood flow and metabolism following benzodiazepine administration (Gene-Jack et al., 1996; Goddard et al., 2004; Licata et al., 2009; Matthew et al., 1995; Veselis et al., 1997; Volkow et al., 1995). PET

studies have also shown that BZD administration produces region- and activity-dependent effects, where the greatest decrease in metabolism and cerebral blood flow were found in more activated brain regions (Gene-Jack et al., 1996; Veselis et al., 1997). In the present study, visual stimuli were presented during scanning, possibly activating the occipital cortex, and subjects were instructed to remain perfectly still, presumably minimizing SMC activation. Therefore, the lorazepam-induced reduction in $[GABA]_{OC}$ could be partly explained by specific regional changes in blood flow and metabolism brought upon by visual activation. In addition, BZD may downregulate glutamic acid decarboxylase (GAD), an enzymatic precursor to GABA synthesis (Raol et al., 2005), which may compound [GABA] reduction by a metabolic mechanism.

Lorazepam effects on TMS measures of cortical excitation and inhibition

The present findings indicate that lorazepam administration decreases cortical excitability at the higher end of the I/O curve, in broad agreement with the literature, where BZDs were found to mainly or selectively reduce the high-amplitude part of the input-output curve, by suppressing late I-waves (Di Lazzaro et al., 2000; Di Lazzaro et al., 2004; Ziemann et al., 2015).

In agreement with previous work (Di Lazzaro et al., 2000; Di Lazzaro et al., 2005; Di Lazzaro et al., 2006; Fritschy & Mohler, 1995; Ziemann et al., 1996; Ziemann et al., 2015), lorazepam was shown here to increase $GABA_AR_{\alpha 2\text{-}\alpha 3}$-dependant SICI. Knowing that $\alpha 2$ or $\alpha 3$ subunits have a high affinity for BZD and are involved in phasic inhibition (Farrant & Nusser, 2005), inhibitory TMS measures would reflect thus phasic inhibition. However, it is unclear why the present effect is specific to $SICI_{2ms}$, as both $SICI_{2ms}$ and $SICI_{3ms}$ are believed to be mediated by $GABA_A$ receptors. The high variability of paired pulse measures (Boroojerdi et al., 2000; Ferland et al., 2019; Orth et al., 2003) and differences in methodology (Di Lazzaro et al., 2000; Di Lazzaro et al., 2005) could partially explain this difference. Furthermore, contamination by facilitatory mechanisms could have interfered with net inhibitory response, which has been shown to be greater for $SICI_{3ms}$ than $SICI_{2ms}$ (Peurala, et al., 2008). As a result, intrinsic differences between $SICI_{2ms}$ and $SICI_{3ms}$ with regards to facilitatory interactions may modulate their response to lorazepam.

Regarding ICF, which likely reflects motor cortex excitatory glutamatergic neuronal network activity (Ziemann et al., 1996), the present findings are inconsistent with previous studies

showing that benzodiazepines suppressed facilitation effects (Mohammadi et al., 2006; Ziemann et al., 1996). This discrepancy may be explained by methodological differences between paired-pulse protocols as well as the high variability of ICF measures which could mask statistical significance (Dyke et al., 2018; Ferland et al., 2019; Hermsen et al., 2016; Maeda et al., 2002; Ngomo et al., 2012). Lastly, no treatment effects were seen for CSP duration. CSPs were induced with TMS pulses of moderate intensity (120%rMT), leading to CSP durations (average duration = 160 ms) outside the ranges found to be modulated with benzodiazepines. Indeed BZD were found to shorten long (>200 ms) CSP and to prolong short CSP (<100 ms) duration (Inghilleri et al., 1996; Kimiskidis et al., 2006; Ziemann et al., 1996).

Relationship between TMS and MRS measures

The absence of correlation between TMS and MRS-GABA measures, also previously observed (Cuypers et al., 2020; Dyke et al., 2017; Ferland et al., 2019; Hermans et al., 2018; Stagg et al., 2011; Tremblay et al., 2012), supports the idea that MRS and TMS target different aspects of the GABAergic system. However, the effects of lorazepam on synaptic inhibition was found to depend on baseline [GABA]. Previous studies have shown that higher GABA levels reflect increased intracellular GABA (Myers et al., 2016) potentially available for release into the synaptic cleft. Knowing that BZD increase $GABA_AR$ sensitivity to their endogenous ligand (Griffin et al., 2013), individuals with higher GABA levels could be more sensitive to the effects of BZD agonists such as lorazepam. Interestingly, in a condition where lower GABA levels are reported (panic disorder), a lower receptor sensitivity to BZD was found (Bremner et al., 2000; Goddard et al., 2004; Kaschka et al., 1995), establishing a link between low [GABA] and BZD sensitivity. Since SICI relies on $GABA_AR$ action, a greater BZD-derived receptor sensitivity to GABA may lead to increased inhibition.

Similarly to previous studies (Dyke et al., 2017; Ferland et al., 2019; Stagg et al., 2011; Tremblay et al., 2012), no association was found between MRS [Glx] and TMS measures of cortical excitability, inhibition or facilitation within baseline or lorazepam conditions. However, a possible association was found between baseline [Glx] and BZD-induced cortical excitability reduction, where a higher [Glx] was associated with a lesser decrease in cortical excitability following lorazepam intake. While the present result should be interpreted cautiously, previous

work report an ambiguous relationship between motor cortical glutamate and corticospinal excitability (Dyke et al., 2017; Stagg et al., 2011). Further studies are needed to shed light on the relationship between glutamate, cortical excitability, and lorazepam.

With regards to the relationship between TMS measures, exploratory analysis showed that ICF_{9ms} and ICF_{15ms} were strongly correlated at baseline, but this coupling disappeared following administration of lorazepam. Intracortical facilitation has been shown to be linked to both glutamatergic excitatory mechanisms and the suppression of GABAergic activity (Ziemann et al., 1996). Indeed, it is believed that ICF partly reflects lasting GABAergic inhibition as I_3 waves, which are associated with SICI, are suppressed up to 20ms after stimulation (Hanajima et al., 1998). Although preliminary, these data suggest that modulation of $GABA_AR$ activity with lorazepam may have indirect effects on ICF.

Methodological considerations and limitations

To our knowledge, this is the first placebo controlled pharmacological study that examined the effects of lorazepam administration on MRS and TMS measures in the same participants. While this study was double-blind in design, the drug dosage, shown to produce consistent effects on TMS measures (Ziemann et al., 1996), also reduces wakefulness although participants were awake throughout experimental procedures. As a result, it was not possible to achieve adequate subject blinding. The present study also assessed MRS and TMS values following lorazepam or placebo administration without pre-treatment comparisons. MRS-GABA and MRS-Glx measurements have been shown to be highly stable across time, with similar coefficients of variation for within-day and between-days measurements (Bogner et al., 2010; Evans, McGonigle, & Edden, 2010; Ferland et al., 2019; Greenhouse et al., 2016; Harada et al., 2011; Near et al., 2013; Near et al., 2014; O'gorman et al., 2011). Similar findings have been reported for TMS, where inter-session variability is similar across testing intervals (Boroojerdi et al., 2000; Dyke et al., 2018; Ferland et al., 2019; Hermsen et al., 2016; Maeda et al., 2002; Ngomo et al., 2012; Orth & Rothwell, 2004; Orth et al., 2003). This suggests that comparing pre- and post-treatment values would not have significantly reduced measurement error. Finally, it has been shown that trial-to-trial MEP amplitude variations can be relatively high (Pitcher et al., 2003; Jung et al., 2010). As a result, ten MEPs per condition may not be optimal for obtaining reliable measurements of corticospinal

excitability and intracortical inhibition/facilitation (Bashir et al., 2017; Chang et al., 2016). Thus, despite the fact that ten MEPs appear to be sufficient to obtain Cronbach's alpha values above 0.9 (Change et al., 2016), at least 20 TMS pulses would have been needed to achieve maximum reliability (Bashir et al., 2017; Chang et al., 2016).

Conclusion

This study revealed a differential effect of classical benzodiazepine lorazepam on TMS and H^1-MRS measures of GABA activity in the sensorimotor cortex and occipital cortex. Whereas lorazepam induced no detectable changes in sensorimotor [Glx] and [GABA], it reduced cortical excitability, at the higher stimulator output intensities, and increased intracortical inhibition, as assessed with TMS. No relationship was found between baseline MRS measures of GABA and glutamate and TMS measures in either experimental condition. Altogether, these findings support the idea that the two techniques measure different aspects of the GABAergic system. TMS may thus reflect synaptic activity while MRS measures overall cellular concentrations which do not necessarily translate to inhibitory neurotransmission. Furthermore, higher motor cortical [GABA] were associated with greater post-BZD increases in cortical inhibition. Therefore, endogenous neurometabolite concentrations may predict treatment response.

CRediT authorship contribution statement

Marie Chantal Ferland: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Review & Editing, Visualization, and Project administration. **Jean-Marc Therrien-Blanchet:** Formal analysis, Investigation, Data Curation, Writing – Original Draft, Review & Editing, Visualization. **Sébastien Proulx:** Software, Writing – Review & Editing. **Gabrielle Klees-Themens:** Investigation, Data Curation. **Benoit-Antoine Bacon:** Writing – Review & Editing. **Thien Thanh Dang Vu:** Validation, Investigation, Writing – Review & Editing. **Hugo Théoret:** Conceptualization, Methodology, Investigation, Resources, Writing – Review & Editing, Supervision, Funding acquisition.

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Conflicts of Interest

Dr. Thien Thanh Dang Vu received consultant fees from Eisai. The other authors report no conflicts of interest.

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Tables

Table 1. Results of Lorazepam and Placebo Treatments on MRS and TMS Measures

Table 1: Results of Lorazepam and Placebo Treatments on MRS and TMS Measures

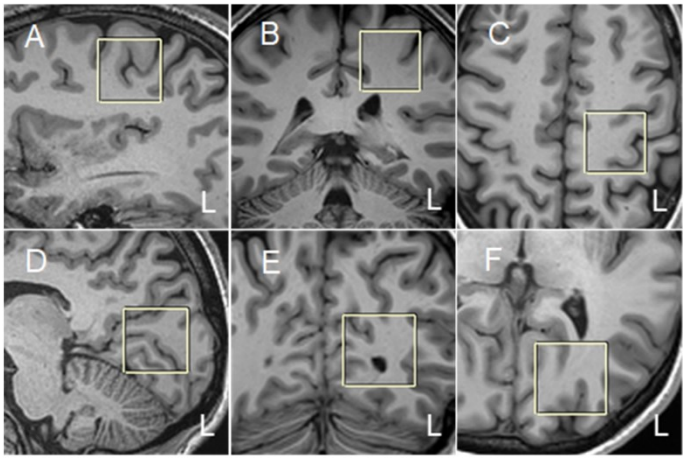
	Lorazepam			Placebo			Treatment Ratio[†]	*N*	*P*[‡]	Effect size
	Md	*M*	*SD*	*Md*	*M*	*SD*				
MRS Measures										
SMC_GABA/H_2O	0.65	0.63	0.10	0.56	0.59	0.13	1.07	17	.263	0.28
SMC_Glx/H_2O	12.90	12.97	0.70	12.98	12.99	0.75	1.00	17	.876	0.04
OC_GABA/H_2O	0.50	0.52	0.10	0.57	0.57	0.13	0.91	15	.032*	0.61
OC_Glx/H_2O	13.08	13.04	0.98	13.06	13.25	0.92	0.98	15	.189	0.36
TMS Measures										
rMT%	39.00	41.59	7.46	41.00	41.71	7.81	1.00	17	.824	0.05
100%rMT (mV)	0.08	0.10	0.08	0.08	0.11	0.12	0.91	16	.535	0.11
110%rMT (mV)	0.32	0.49	0.54	0.24	0.45	0.50	1.09	16	.638	0.08
120%rMT (mV)	0.60	0.80	0.71	0.75	1.07	0.92	0.74	16	.087	0.30
130%rMT (mV)	0.78	1.21	1.32	1.21	1.79	1.52	0.68	16	.087	0.30
140%rMT (mV)	0.99	1.53	1.39	1.37	2.43	2.32	0.63	16	.043*	0.36
I/O slope (mV/100%rMT)	2.24	3.58	3.54	3.65	5.96	5.84	0.60	16	.087	0.35
TS%	57.00	54.94	11.84	54.00	52.06	12.39	1.06	17	.082	0.45
TS-MEP (mV)	0.89	0.86	0.26	0.89	0.87	0.15	0.98	17	.799	0.06
$SICI_{2ms}$ ratio	0.38	0.52	0.34	0.66	0.84	0.50	0.62	17	.030*	0.37
$SICI_{3ms}$ ratio	0.49	0.57	0.37	0.55	0.74	0.50	0.77	17	.219	0.21
ICF_{9ms} ratio	1.13	1.10	0.42	1.27	1.26	0.51	0.88	17	.342	0.16
ICF_{15ms} ratio	1.03	1.06	0.67	1.01	1.11	0.60	0.96	17	.332	0.17
$LICI_{100ms}$ ratio	0.06	0.13	0.15	0.08	0.18	0.27	0.74	17	.610	0.09
CSP (ms)	168	162	27	164	159	27	1.02	17	.747	0.08

[†] Lorazepam/Placebo treatment ratio.

[‡] *P*-value for the appropriate paired-samples hypothesis test. Statistically significant ($p < .05$) results are indicated with an asterisk (*).

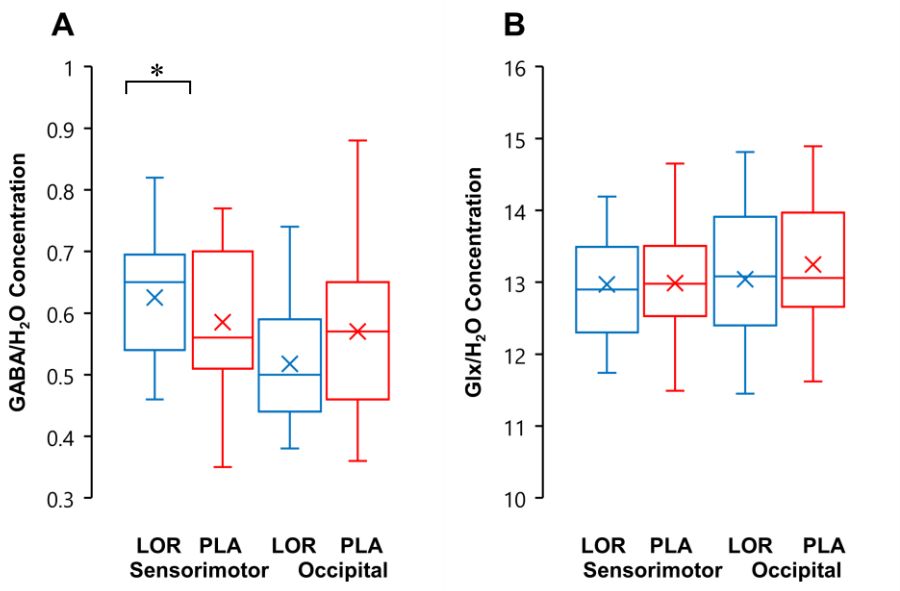
Figures

Figure 1. Voxels of interest



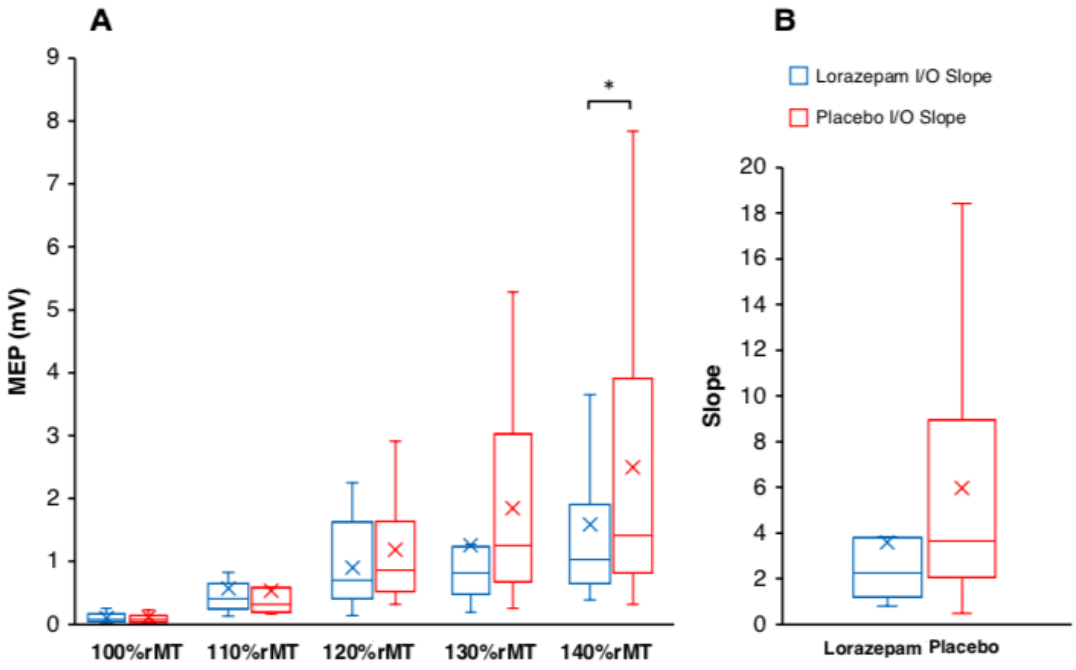
Legend: Spectrosopic voxel of interest (30 x 30 x 30 mm^3) of a typical subject, as illustrated in sensorimotor (A) sagittal, (B) coronal, and (C) axial views and occipital (D) sagittal, (E) coronal, and (F) axial views. L: left.

Figure 2. Effects of lorazepam on GABA and Glx by VOI



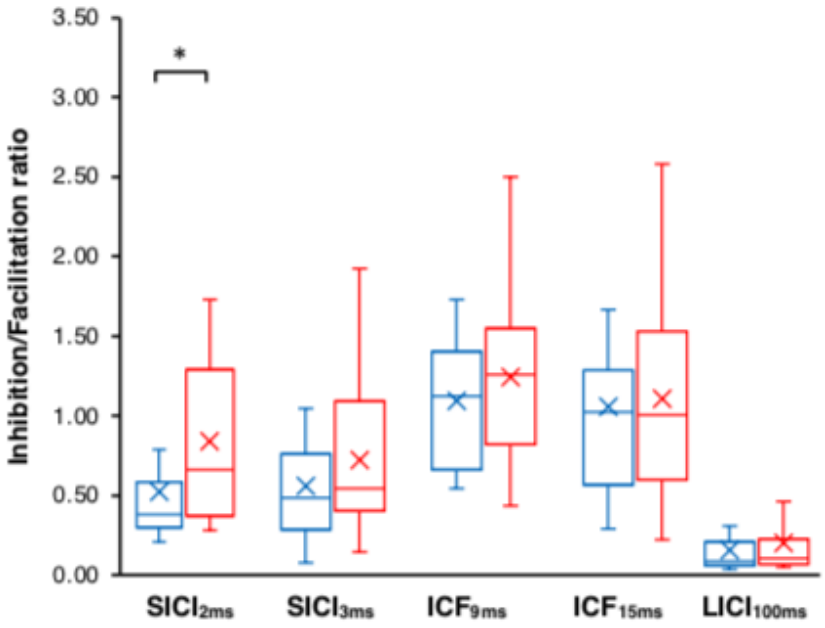
Legend: Box plots illustrating the effects of lorazepam on **(A)** GABA and **(B)** Glx concentrations by region. Statistically significant differences ($p < 0.05$) are shown with an asterisk (*).

Figure 3. Effects of lorazepam on the I/O curve



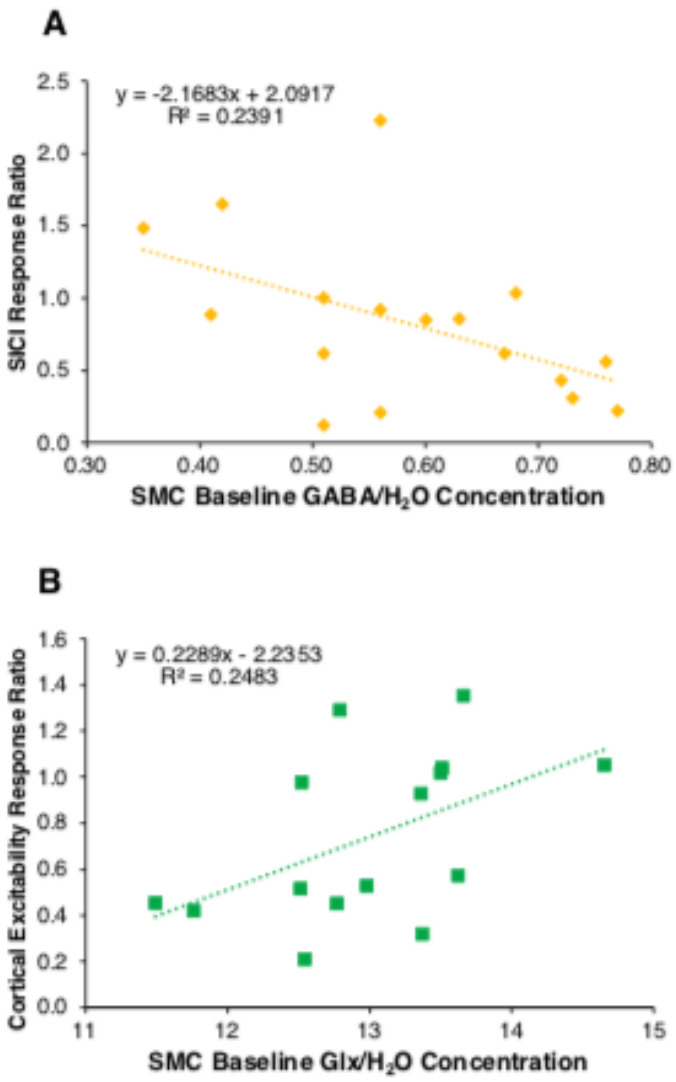
Legend: Box plots illustrating effects of lorazepam on **(A)** the Input output curve at 5 TMS intensities, **(B)** the input output curve slope. The × symbol represents the mean. Statistically significant ($p < .05$) differences in medians between placebo and lorazepam conditions are shown with an asterisk (*). The placebo group is identified in red and the lorazepam group is identified in blue.

Figure 4. Effects of lorazepam of paired-pulse measures



Legend: Box plots illustrating effects of lorazepam on paired-pulse ratios for average SICI, ICF, and LICI ratios. The × symbol represents the mean.Statistically significant ($p < .05$) differences in medians between placebo and lorazepam conditions are shown with an asterisk (*). The placebo group is identified in red and the lorazepam group is identified in blue.

Figure 5. Relationship between baseline metabolite levels and TMS measures



Legend: Relationship between individual baseline **(A)** GABA and SICI (at ISI_{2ms}), as well as **(B)** Glx and cortical excitability (140%rMT) lorazepam/placebo response ratios.

Chapter 4 – General Discussion

The present thesis aimed at determining if TMS and MRS measures reflecting GABAergic and glutamatergic activity are stable across time to better understand their utility in assessing treatment response and disease progression, especially in pathologies of the motor cortex. To our knowledge, this was the first time that a longitudinal multimodal TMS and MRS study was undertaken in the same participants. Another objective of the present thesis was to further study, through pharmacological challenge, the neurochemical substrates underlying TMS and MRS measures of excitation and inhibition and how the two techniques relate to GABAergic and glutamatergic activity. To date, no previous study had reported MRS measures of neurometabolite concentration and TMS measures of cortical excitation and inhibition following pharmacological intervention in the same participants.

TMS and MRS Long-Term Stability

The study presented in chapter 2 of the current thesis has shown that water-referenced GABA and Glx measurements, obtained using MEGA-PRESS at 3T and analyzed using LCModel, are stable across time when measured in the SMC of healthy volunteers. Regarding TMS measurements, cortical excitability (rMT and %MSO) and cortical silent period values obtained in the motor cortex are stable across time. Furthermore, despite showing high between- and intra-subject variability, $SICI_{2ms}$ measures were found to be fairly reliable. Other paired-pulse measures ($SICI_{3ms}$, ICF_{9ms}, ICF_{12ms} and LICI) demonstrated high overall variability as well as poor to nil reproducibility over time.

To our knowledge, this was the first time that a combined MRS and TMS reliability analysis was performed in the same cohort of participants and enabled us to determine that MRS and TMS measures of cortical excitation, inhibition, and facilitation do not covary across time. Indeed, while all MRS parameters are stable across a three-month interval, paired pulse TMS indexes were highly variable, when measured in the same individual.

Magnetic Resonance Spectroscopy

The study presented in chapter 2 of the current thesis revealed that water-referenced GABA and Glx measurements, obtained using MEGA-PRESS at 3T and analyzed using LCModel, are stable across time when measured in the sensorimotor cortex of healthy volunteers.

Regarding GABA, the reported variability and reliability statistics (COVs and ICCs) are in line with previous work that assessed the short-term stability of the technique when measuring GABA or GABA+ levels (Bogner et al., 2010; Evans et al., 2010; Greenhouse, Noah, Maddock, & Ivry, 2016; Harada et al., 2011; Near et al., 2014; Neuling, Rach, & Herrmann, 2013; O'gorman et al., 2011). As variations in GABA concentrations across a long-term period do not exceed those of reliability studies, variations in GABA concentrations are thus likely to be explained by measurement error as opposed to GABA changes across time. Importantly, stability statistics reported in the present work are also in accordance with a previous longitudinal study performed in the occipital cortex of healthy volunteers (Near et al., 2014). Since GABA levels were shown to be stable both in the occipital and sensorimotor cortices, it is possible that this long-term stability can be generalized to other brain regions as well. Regarding Glx, the variability of measurements reported in chapter 2 is similar to those reported in previous studies where either Glu or Glx was measured (Hurd et al., 2004; Jang et al., 2005; O'gorman et al., 2011). In addition, the reported variability did not exceed that of short-term reliability studies indicating that variation in Glx concentrations can be attributable to measurement error. Therefore, both GABA and Glx concentrations were found to be stable over a long-term period (3 months) in the sensorimotor cortex of healthy adults. Likewise, fair to good stability statistics were obtained for Glx levels. To date, to our knowledge, there are no studies that have attempted to assess the long-term stability of MRS-Glx in other brain regions.

Reliable change indexes for GABA and Glx were also reported in chapter 2 of the present thesis to determine the threshold at which a change in metabolite concentration may become reflective of a pathological process. This analysis revealed that a change in water-referenced GABA levels of ≈0.15 or a change in water-referenced Glx levels of ≈0.72 across a three-month period would most likely reflect a significant alteration in metabolite concentration. However,

further studies are needed to determine if changes in concentration outside these bounds are reflective of an ongoing motor cortical pathology.

An important issue related to the use of MRS-derived concentrations as disease progression markers is age. Previous studies have reported age-related reductions in prefrontal, parietal, and sensorimotor GABA levels (Gao et al., 2013; Grachev & Apkarian, 2001; Porges et al., 2017). However, Hermans and collaborators (2018) have shown no difference in sensorimotor GABA levels between younger (19 to 34 years old) and older (63 to 74 years old) individuals. A more recent study reported that GABA concentrations decline with age in the sensorimotor cortex (Cuypers et al., 2020). In addition, it was found that glutamate declines with age throughout childhood and between young and older adults (Grachev & Apkarian, 2001). The difference between age thus appears to be considerable, and to our knowledge, no longitudinal study has been performed to assess the rate at which GABA or Glu concentrations decline. Care must be taken when using water-referenced GABA or Glx to study treatment responses or monitor motor-cortical pathological processes over prolonged time intervals.

The various neurological and psychiatric conditions, and treatment response assessments, where MRS may be used, likely produce changes on a more rapid scale than what would follow from the normal aging process. For example, patients with amyotrophic lateral sclerosis (ALS), a neurodegenerative motor neuron disorder, display elevated Glx level in the sensorimotor cortex when compared to age-matched controls (Han & Ma, 2010). Furthermore, Parkinson's disease, which is characterized by nigrostriatal dopaminergic neuron degeneration, results in elevated GABA in the pons and putamen, when compared to age-matched controls (Emir, Tuite, & Öz, 2012). Regarding treatment response, the administration of topiramate, an epilepsy medication, resulted in higher GABA levels in the occipital cortex of patients suffering from epilepsy (Petroff et al., 2001). Gabapentin, another medication used to treat epilepsy, which acts by raising central GABA, also induces MRS-detectable increases in GABA concentrations; a 48% increase in occipital GABA following treatment was reported in previous work (Kuzniecky et al., 1998; Kuzniecky et al., 2002). Thus, given the good reliability of MRS assessment and since several pathologies show MRS-detectable effects, this technique may be of considerable clinical usefulness.

Transcranial Magnetic Stimulation

In the present work, cortical excitability (rMT and % MSO) and cortical silent period measures were found to be stable, which is in line with previous work (Hermsen et al., 2016; Maeda et al., 2002; Ngomo et al., 2012). For paired-pulse measurements of cortical inhibition and facilitation, the high variability across time is broadly consistent with the literature, which investigated the stability of such measurements across intervals spanning from minutes to months (Boroojerdi et al., 2000; Ngomo et al., 2012; Orth et al., 2003). Furthermore, marginally stable measures were obtained for $SICI_{2ms}$ but not for $SICI_{3ms,}$ partially contradicting previous work where adequate to excellent stability was reported for both $SICI_{2ms}$ and $SICI_{3ms}$ (Dyke, Kim, Jackson, & Jackson, 2018; Hermsen et al., 2016; Maeda et al., 2002; Ngomo et al., 2012). Since $SICI_{2ms}$ and $SICI_{3ms}$ are thought to share similar mechanisms, this discrepancy is difficult to explain. It is however possible that facilitatory processes may reduce net inhibitory responses following $SICI_{3ms}$ to a greater extent than for $SICI_{2ms}$ (Peurala et al., 2008). Regarding ICF, analysis revealed that facilitatory protocols did not produce robust effects in MEP amplitudes; as such, it is unsurprising that non-statistically significant and poor test-retest correlations were reported for both ICF_{9ms} and ICF_{12ms}. This finding is in line with the majority of previous work, where poor reliability statistics are reported (Dyke et al., 2018; Hermsen et al., 2016). Indeed, only one previous study, which set its CS intensity at 80%rMT, found a strong test-retest correlation for ICF (Maeda et al., 2002). Furthermore, the present work was the first to assess $LICI_{100ms}$ long-term reliability; the measurement was found not to be reproducible, probably in part due to a statistical floor effect. However, a previous study by Farzan (2010) had demonstrated that LICI has good short-term test-retest reliability. These meausrements were obtained by delivering 100 TMS stimuli per condition (TS, CS+TS). The high number of delivered pulses likely explain this increased stability. Therefore, while the reliability of single-pulse TMS parameters is well established in the literature, including the present work, results differ across studies aiming to investigate ppTMS reliability. Heterogeneity in paired-pulse protocol parameters may also explain differing findings. For example, response variability between individuals at a specific ISI was substantial across different CS intensities (Orth et al., 2003); individuals that showed strong inhibition at ISI_{2ms} at a CS intensity of 60% rMT did not necessarily show strong inhibition for the

same ISI at 70% rMT. Furthermore, a recent study has demonstrated that 20 MEPs are needed to maximize reliability (Chang et al., 2016; Goldsworthy, Hordacre, & Ridding, 2016).

Since motor threshold, % MSO and CSP measures were found to be stable across a three-month interval, these parameters may thus be of value when assessing treatment response or disease progression in the motor cortex. Indeed, it was found that a variation across time greater than 3% of rMT or %MSO is considered a significant variation, which suggests an abnormal variation in motor cortical neurophysiology. Likewise, a variation in CSP length across time of at least 15 ms suggests a potentially abnormal process. The computed RCIs for paired-pulse indexes reported in chapter 2 may still be appropriate for clinical purposes but should be interpreted carefully due to their high variability, and further work is needed to validate these indexes as well as their clinical significance. Further work is also needed to investigate the stability of these indexes as well as their clinical significance. Nevertheless, according to the present work as well as previous studies, SICI seems to be the most promising in terms of clinical monitoring. However, the intrinsic variability of SICI measurements remains high; as such, utmost care should be taken when using SICI, or other paired-pulse measurements, as markers of disease progression or treatment response.

Similarly to MRS, it is important to note the effects of normal aging on TMS measurements. A significant age-related increase in motor threshold has been reported (Rossini, Desiato, & Caramia, 1992) as well as MEP amplitude reduction (Oliviero et al., 2006; Pitcher, Ogston, & Miles, 2003). No consensus has yet been reached regarding age-related effects as it pertains to GABAergic-dependent TMS measurements obtained in M1. While some studies have found decreased inhibition in older adults (Heise et al., 2013; Peinemann, Lehner, Conrad, & Siebner, 2001), others have shown no changes (Stevens-Lapsley, Thomas, Hedgecock, & Kluger, 2013; Wassermann, 2002) or even increased inhibition (Kossev, Schrader, Däuper, Dengler, & Rollnik, 2002). A recent study by Hermans and collaborators (2018) reported a reduction in SICI and LICI in older adults when compared to younger individuals. Therefore, one should be mindful of age-related changes when using TMS as a clinical tool.

Despite its high variability, transcranial magnetic stimulation has been extensively used in the medical field, especially to monitor or assess pathological changes which may stem from motor cortical pathophysiology. Indeed, alterations across several TMS protocols were observed in epilepsy, ALS, Parkinson's disease, multiple sclerosis and cerebral lesions, to name a few (Chen et al., 2008; Rossini et al., 2015). For example, alterations in motor threshold and MEP amplitude have been reported in multiple sclerosis, stroke, cervical myelopathy and ALS (Chen et al., 2008; Rossini et al., 2015). In addition, a twofold increase in SICI was found in ALS patients compared to age-matched controls, despite high variability (Ziemann et al., 1997c). Furthermore, for treatment monitoring, several TMS parameters were found to be consistently modulated by pharmacological agents (Ziemann et al., 2015). For example, benzodiazepines consistently increased $GABA_AR$-dependent SICI as reported in previous work and chapter 3 of the present thesis (Di Lazzaro et al., 2000; Ziemann et al., 1996b). Thus, despite high variability in certain TMS measurements, transcranial magnetic stimulation protocols can detect pathological processes or treatment effects. However, to maximize reliability, it might be best to obtain several MEP measurements (>20) and collect several measurements in a given day (Chang et al., 2016).

Neurochemical Correlates of TMS and MRS

The work presented in chapter 3 of the present thesis found that lorazepam administration modulates short-interval cortical inhibition as well as MEP amplitudes at the highest stimulator intensities. No other TMS parameters were altered by BZD administration. Furthermore, Glx levels were unchanged in either the sensorimotor or occipital cortex. However, GABA levels decreased in the occipital cortex while remaining stable in the SMC. Interestingly, while no link has been found between TMS or MRS baseline values, higher baseline GABA levels were found to correlate with greater BZD-induced changes in SICI. A trend was also found between endogenous Glx concentrations and MEP amplitude reduction, where higher Glx levels lessened the BZD-induced MEP reduction. These findings represent novel associations between endogenous metabolite levels and TMS responses following pharmacological intervention.

Transcranial Magnetic Stimulation Neurochemistry

The article presented in chapter 3 of this thesis showed that lorazepam administration is associated with reduced cortical excitability, as well as increased short interval cortical inhibition. Lorazepam administration had no effect on rMT, LICI, ICF and CSP duration.

In line with previous work, the study presented in chapter 3 of this thesis suggests that drugs targeting GABAergic neurotransmission, including benzodiazepines, has no effect on corticospinal excitability-dependent motor threshold (Paulus et al., 2008; Ziemann et al., 2015). Indeed, MT can be influenced by pharmacological compounds which act on the excitability of the basic neural elements in the motor cortical system. For example, drugs acting on voltage-gated sodium channels, which regulate axonal excitability, such as some anti-epileptic drugs, modulate MT (Ziemann et al., 2015). Since lorazepam acts on GABA receptors themselves, while leaving basic neural elements' excitability unchanged (Griffin et al., 2013), it is to be expected that BZDs do not modulate motor threshold.

In the study presented in chapter 3, lorazepam was found to decrease cortical excitability selectively at the highest intensities, which is largely in agreement with previous pharmacological studies (midazolam (Schönle et al., 1989); lorazepam (Di Lazzaro et al., 2000); diazepam (Heidegger et al., 2010)). These findings highlight the impact of $GABA_AR$ modulation on cortical excitability. MEP at higher amplitudes are the product of a sum of multiple excitatory descending volleys, namely later I-waves as well as D-waves (Di Lazzaro et al., 2004), where the former are selectively suppressed by BZD modulation of inhibitory interneurons (Di Lazzaro et al., 2000). Indeed, in contrast to early I-waves and D-waves, these later I-waves are thought to originate from transsynaptic activation of cortical spinal neurons through excitatory interneuron circuits which are also regulated by GABAergic and neuromodulator connections. Since lorazepam acts upon GABA receptors, resulting in increased inhibition, it is not surprising that the MEPs comprised of later I-waves, i.e., the higher parts of the I/O curve, would be selectively reduced.

Furthermore, the study presented in chapter 3 replicates previous findings where BZDs capable of modulating α2- or α3-$GABA_A$ receptors were found to increase short interval cortical inhibition (Di Lazzaro et al., 1998; Ilić et al., 2002; Kujirai et al., 1993; Ziemann et al., 2015). The

present work thus provides important confirmatory evidence that SICI likely reflects receptor activity, and relies on activation of a low threshold intracortical inhibitory circuit mediated by fast-acting ionotropic $GABA_A$ receptors bearing a α2- or α3 subunit, which induces short IPSPs in corticospinal neurons (Di Lazzaro et al., 1998; Ilić et al., 2002; Kujirai et al., 1993; Ziemann et al., 2015). As such, since SICI depends on $GABA_AR$ with α2 or α3 subunits, which have a high affinity for BZD and are involved in phasic inhibition (Di Lazzaro et al., 2006b; Farrant & Nusser, 2005), inhibitory TMS measures likely reflect phasic inhibition.

Long-interval cortical inhibition was also found to be independent of $GABA_AR$ action as lorazepam did not modulate its effects. This is in line with previous work suggesting that LICI is dependent upon $GABA_B$ receptor activity (McDonnell et al., 2006; Mohammadi et al., 2006; Teo et al., 2009).

While poorly understood, it is believed that ICF is the net sum of excitatory glutamatergic-dependent processes as well as the tail of the $GABA_AR$-mediated SICI (Ziemann et al., 2015). This hypothesis suggests that BZD modulation may be observed in ICF protocols. However, the work presented in the current thesis showed no BZD effect on ICF while previous work has shown that benzodiazepines can reduce facilitation effects (Mohammadi et al., 2006; Ziemann et al., 1996b). While partially contradictory with previous studies, the results presented in chapter 3 are nevertheless in agreement with the theory that ICF is mainly reliant on glutamatergic and noradrenergic circuitry (Di Lazzaro & Rothwell, 2014). Differences in protocol parameters may also explain the discrepancies (Rossini et al., 2015). It is possible that depending on the parameters used it is possible that either the GABAergic or glutamatergic circuitry, which may underly ICF, is solicited, explaining differing results.

Lastly, benzodiazepines were found to lengthen short CSPs (<100 ms) obtained at low stimulation intensity and to shorten long CSPs (>200 ms) obtained at high stimulation intensity (Inghilleri et al., 1996; Kimiskidis et al., 2006; Ziemann et al., 1996b). However, in the present work, CSPs were induced with TMS pulses of moderate intensity (120%rMT), leading to CSPs durations, of 160 ms on average, which is outside the ranges that can be modulated by BZD action.

Magnetic Resonance Spectroscopy Neurochemistry

Lorazepam administration was found to have no effect on Glx levels in either VOIs. However, GABA levels were reduced in the occipital cortex but were unchanged in the sensorimotor cortex following BZD intake.

Pharmacological Modulation of MRS-Glx

The work presented in chapter 3 of this thesis confirms previous reports where no effect of BZD administration was found on acute glutamate levels assessed with MRS in healthy individuals. Indeed, lorazepam did not modulate occipital or sensorimotor cortical Glx concentrations. This result provides important confirmatory evidence since previous studies either had less than 10 participants or were not placebo-controlled (Brambilla et al., 2002; Goddard et al., 2004; Henry et al., 2010). Our finding is unsurprising given that BZDs such as lorazepam act on GABA receptors, therefore not directly modulating glutamate (Glx) levels. Nevertheless, despite the findings presented in chapter 3, ambiguity remains as to the neurochemical substrates of MRS-Glx since this measurement is a combination of signals stemming from both Glu and Gln. However, since glutamate comprises most of the signal, and it has been suggested that MRS-Glu relates to metabolic glutamate (Rae, 2014), the findings reported in the present thesis are in agreement with the proposed theory.

Pharmacological Modulation of MRS-GABA

The data presented in chapter 3 of this thesis shows no effect of lorazepam administration on sensorimotor cortex MRS-GABA levels, but a reduction in occipital GABA was observed. This latter finding replicates previous results from Goddard (2004), where a reduction in occipital GABA was reported following clonazepam intake, as well as findings from Licata (2009), where a reduction in MRS-GABA levels was seen in the thalamus, but not in the ACC of healthy individuals following administration of zolpidem, a non-BZD $GABA_AR$ agonist. Thus, results presented in this thesis suggest that $GABA_AR$ allosteric modulators have region-dependent effects on MRS-GABA concentrations, which is not surprising given that no correlation was found between GABA levels in different brain regions (Greenhouse et al., 2016), suggesting independent

GABAergic activity between areas. However, the potential differential effects may also be partly due to the chosen pharmacological agent. Indeed, CNS-active compounds may have different affinities for $GABA_A$ receptor isoforms, which are distributed heterogeneously throughout the brain (Farrant & Nusser, 2005; Griffin et al., 2013; Möhler, 2006). Indeed, α_1 isoforms are highly concentrated in the cortex, thalamus and cerebellum and are responsible for BZDs' sedative effects. In contrast α_2 receptors are found in high concentration in the limbic system, motor neurons and in the dorsal horn of the spinal cord and are responsible for the myorelaxant effects of BZDs (Griffin et al., 2013). Furthermore, while the precise affinity profile for each GABA receptor isoform is not always known, not all BZD share the same affinity. For example, Clonazepam has a weaker binding affinity of $GABA_A$ receptors when compared to other highly potent benzodiazepines such as lorazepam (Chouinard, Young, & Annable, 1983). Therefore, it is important to consider the pharmacological affinity profile of the administered compound when assessing MRS responses.

MRS-GABA Levels: Phasic, Tonic or Metabolic Activity Markers

It is believed that MRS-GABA signals are not reflective of phasic or synaptic activity and as such do not reflect GABAergic neurotransmission per se (Dyke et al., 2017; Myers et al., 2016; Stagg et al., 2011a; Tremblay et al., 2012). While quantifying total VOI concentrations, MRS measures likely reflect extrasynaptic GABA. Furthermore, since spectroscopic measures are acquired over a prolonged period, neurometabolite levels would thus reflect steady-state concentrations, not punctual spikes in GABA due to phasic activity. Indeed, as discussed previously, phasic inhibition leads to increased GABA in the synaptic cleft for roughly 500 us which is extremely brief, and lies outside of the temporal resolution of MRS (Farrant & Nusser, 2005; Myers et al., 2016).

Previous studies have often referenced MRS-GABA signals as proportional to tonic GABAergic inhibition, as this form of GABA signalling is not as temporally restricted as phasic activity (Rae, 2014; Stagg et al., 2011a). However, GABA concentrations in the extracellular space are in the micromolar range while intracellular concentrations are in the millimolar range (Cavelier et al., 2005; Rae, 2014; Wu et al., 2007); as such, MRS-GABA signals would mostly stem from intracellular GABA concentrations (Myers et al., 2016; Rae, 2014). This is consistent with

pharmacological studies which demonstrated that administering agents that modulate intracellular GABA (vigabatrin, gabapentin, CPP-115) (Kuzniecky et al., 2002; Mattson et al., 1995; Petroff et al., 1999; Prescot et al., 2018) increase MRS-GABA while agents that specifically modulate extracellular concentrations (tiagabine) do not modulate MRS-GABA levels (Myers et al., 2014), despite PET evidence showing higher extracellular GABA concentrations following tiagabine intake (Stokes et al., 2013). This is also consistent with the sensitivity of MEGA-PRESS at 3T, where concentrations in the millimolar range can be measured (Mullins et al., 2014; Rae, 2014). Pharmacological modulation of extracellular GABA would need to cause over a hundred-fold increase in concentration for it to yield an MRS-detectable effect (Myers et al., 2016). As such, one can hypothesize that MRS-GABA mainly reflects intracellular metabolic GABA.

Explaining the Reduction of Occipital GABA Levels: a Metabolic Hypothesis

The reduction in occipital GABA reported in the present work and in previous studies (Goddard et al., 2004; Licata et al., 2009) remains surprising given the mechanism of action of $GABA_AR$ receptor agonists, which take action on the receptor itself (Griffin et al., 2013). Such receptor activity is believed not to be reflected in MRS levels (Goddard et al., 2004; Myers et al., 2016). This suggests that the observed reduction may stem from a BZD-induced metabolic activity downregulation. Indeed, previous PET studies have demonstrated reduced blood flow and glucose metabolism following BZD administration, which would lead to less GABA being synthesized (Matthew et al. 1995; Volkow et al. 1995; Gene-Jack et al. 1996; Veselis et al. 1997; Goddard et al. 2004; Licata et al. 2009). This reduced metabolic activity, observed by PET imaging, would decrease GABA synthesis and may explain the observed reduction in GABA levels measured using MRS. Furthermore, the greater the activation of a region, the greater the decrease in metabolism and blood flow observed via PET (Matthew et al., 1995; Veselis et al., 1997; Volkow et al., 1995). In the study presented in chapter 3, we can presume that the occipital cortex showed greater activation, when compared to the SMC, as visual stimuli were presented throughout the scanning session to increase wakefulness, while participants were instructed to remain still. This disparity in brain region activation could thus explain the differential effects of BZD on occipital versus motor cortical GABA levels. Thus, the work presented in this thesis suggests that BZD lowers GABA levels in a region-dependent fashion, through a metabolic explanation, were activated brain regions show the greatest decrease.

Furthermore, a lorazepam-induced downregulation of glutamic acid decarboxylase, an enzymatic precursor in GABA synthesis, may also potentially explain the findings reported in chapter 3. Indeed, Raol (2005) highlighted that BZD administration can lead to GAD downregulation after prolonged diazepam use. Such a reduction in a synthetic precursor would lead to reduced GABA being produced. However, such downregulation is the result of long-time BZD use and the short-term effects of BZD on GAD expression has not been examined. It is thus not clear how a short-term effect, such as in chapter 3, would impact GAD expression.

Clinical applications

When designing studies aiming at measuring MRS-GABA, researchers who aim to induce MR-detectable changes should use drugs that raise intracellular concentrations, such as anti-epileptics vigabatrin (Mattson et al., 1995; Verhoeff et al., 1999), topiramate, lamotrigine and gabapentin (Kuzniecky et al., 2002; Petroff et al., 1999). Conversely, drugs that only alter extracellular or synaptic GABA such as tiagabine, an agent which selectively increases extracellular concentrations (Fink-Jensen et al., 1992), are expected to yield null results if studied using spectroscopy (Myers et al., 2016). Likewise, researchers should interpret a positive finding on an MRS study where a BZD or other $GABA_AR$ agonists or antagonists as reflecting primarily metabolic changes in GABA levels and not altered extracellular or synaptic GABA. Changes in MRS-Glx are also expected to reflect altered metabolic activity and not neurotransmission per se (Rae, 2014).

Linking TMS and MRS Measures

In the work comprising the present thesis, no link between baseline TMS and baseline MRS measures was found, which is in line with previous work. However, a novel relationship emerged between endogenous MRS-GABA levels and BZD-induced SICI increase. Furthermore, a trend was found between baseline MRS-Glx levels and MEP amplitude reduction, where greater Glx baseline levels were associated with a weaker MEP amplitude reduction following BZD intake. These results will be discussed below.

MRS and TMS: Probing Different Aspects of the GABAergic and Glutamatergic Systems

Taken together, findings presented in this thesis and previous work show no association between MRS-GABA and TMS measures. Such findings are robust not only across MR sequences, field strength and sample size, but also across TMS techniques (Dyke et al., 2017; Stagg et al., 2011b; Tremblay et al., 2012). This further supports the idea that MRS measures of GABA do not reflect TMS-derived measures of cortical inhibition or facilitation. Indeed, while MRS likely reflects intracellular concentrations involved in metabolic activity, TMS measures, such as SICI, are believed to reflect receptor activity involved in phasic events.

Similarly to previous findings (Dyke et al., 2017; Stagg et al., 2011b; Tremblay et al., 2012), in the works comprising this thesis, no link was found between Glx levels and MT, SICI, ICF, LICI and CSP. However, studies suggest an ambiguous relationship exists between some cortical excitability (I/O curve) measures and motor cortical glutamate levels (Dyke et al., 2017; Stagg et al., 2011b). More precisely, Stagg (2011) found a positive association between glutamate concentrations and the I/O slope, which would entail that subjects with increased glutamate have greater corticospinal excitability. However, Dyke (2017) found a negative link between glutamate and the I/O plateau, suggesting that higher glutamate is linked to lower maximum cortical excitability. The study presented in chapter 3 found no such association between endogenous Glx levels and global cortical excitability. Both previous studies had considerably differing methodologies and were able to resolve the glutamate signal, which may explain the discrepancies in findings. The differing findings may also be attributed to the dynamic synthetic cycling between glutamate/glutamine and GABA, which is synthesised from glutamine (Rae, 2014). Indeed, a linear combination of Gln/Glu and GABA/Glu ratios was reported to predict MEP amplitudes (Dyke et al., 2017). These findings hint at a complex relationship between these neurometabolites with respect to their impact on cortical excitability.

MRS Neurometabolite Levels as Potential BZD Response Predictive Factors of TMS measurements

In light of the dissociation between endogenous MRS levels and TMS measurements, chapter 3 highlights a peculiar finding: a greater MRS-GABA level predicted a greater increase in cortical inhibition following BZD intake. In addition, a trend was found between higher endogenous Glx levels and resistance to BZD-induced cortical excitability depression. To our knowledge, this is the first time that such associations have been reported.

Knowing that BZD increase $GABA_A$ receptor affinity for its endogenous ligand (Griffin et al., 2013), individuals with higher GABA concentration, potentially available for release in the synaptic cleft, may further benefit from the increased receptor affinity for GABA induced by BZD action when compared to individuals with lower GABA levels. Knowing that SICI is potentiated by $GABA_AR$ receptor action, greater BZD-triggered GABA binding would likely result in increased cortical inhibition in subjects with greater GABA concentrations. Another possible explanation for this finding is that there may be an association between GABA concentrations and $GABA_A$ receptor affinities to benzodiazepines. Interestingly, in panic disorder, a condition associated with lower GABA concentrations in the occipital cortex possibly due to a GAD enzyme dysfunction, a lower receptor sensitivity to benzodiazepines is also reported (Bremner et al., 2000; Goddard et al., 2004; Kaschka, Feistel, & Ebert, 1995). Indeed, neuroreceptor imaging studies have highlighted lower levels of cortical and hippocampal BZD receptive binding or affinity in panic disorder (Bremner et al., 2000; Kaschka et al., 1995; Kuikka et al., 1995; Schlegel et al., 1994). This establishes a link between lower GABA concentration and benzodiazepine sensitivity which may potentially explain our finding. Based on the assumption that GABA levels are tied to BZD-receptor binding affinity, a greater increase in $GABA_AR$ dependent SICI may thus be observed in individuals with higher endogenous GABA levels.

Furthermore, a possible association was reported in chapter 3 between baseline Glx levels and BZD-induced cortical excitability reduction, where a higher Glx concentration potentially predicted a lesser decrease in cortical excitability following lorazepam intake. While the present result is a statistical trend, it remains of note since, as mentioned before, previous works report an

ambiguous relationship between motor cortical glutamate and corticospinal excitability (Dyke et al., 2017; Stagg et al., 2011b). A possible explanation for this finding is that individuals with higher glutamate levels may have higher global cortical excitability as reported in Stagg (2011). This would in turn suggest that higher glutamate levels may lessen the ability of benzodiazepines to depress cortical excitability. Nevertheless, further studies are needed to shed light on the relationship between glutamate, cortical excitability, and lorazepam.

Limits and methodological considerations of the present work

TMS data acquisition and processing

A common limitation of TMS studies is the statistical abnormality of many TMS measurements, which impedes parametric analysis. Indeed, as demonstrated in the study presented in chapter 3, significant deviations from normality were found in paired-pulse and cortical excitability measurements, which compromises parametric analyses with smaller ($n<30$) sample sizes. Therefore, using non-parametric statistics would yield more robust findings in studies constrained to a smaller sample. Another common pitfall of TMS protocols is the high intra- and inter-subject variability as well as the poor reproducibility of some TMS measures. Indeed, while motor threshold and cortical silent period measurements have demonstrated excellent reliability and stability, cortical excitability measures, obtained with input-output curve protocols, and intracortical inhibition and facilitation measures, obtained with paired-pulse protocols, have varying reliability and stability (Boroojerdi et al., 2000; Hermsen et al., 2016; Ngomo et al., 2012; Orth et al., 2003).

One approach taken by TMS studies to remedy these methodological issues is to explore variations on the input output curve and paired-pulse paradigms. For example, the use of threshold tracking techniques (TTT), which involve tracking the motor threshold and test stimulus and continually adjusting their intensity so that their elicited MEPs remain at a set number, yielded more stable paired-pulse findings (Mooney, Cirillo, & Byblow, 2017; Murase, Cengiz, & Rothwell, 2015; Samusyte, Bostock, Rothwell, & Koltzenburg, 2018). Other studies vary the conditioning stimulus intensity within paired-pulse protocols or determine the values of the input-

output curve differently, and thereby obtain satisfactory stability. Future studies varying the intensity of the conditioning stimulus to determine which intensity, expressed as a percentage of the rMT, yields the most stable paired-pulse measures would be a significant contribution to this field. Furthermore, performing a similar reliability assessment using the active motor threshold (%aMT) as a basis for TMS measurement would be of interest since intracortical inhibition and facilitation were found to be strongly correlated to %aMT (Orth et al., 2003). However, varying data acquisition protocols has limitations. Notably, excessive divergence from specific protocols used in previous studies can potentially compromise the generalizability of important results in the field. For instance, the effects of lorazepam on cortical excitability and measures of cortical inhibition were initially discovered with protocols not too dissimilar to those used in the study presented in chapter 3 (Di Lazzaro et al., 2000; Di Lazzaro et al., 2005; Kimiskidis et al., 2006). Small differences in protocol may have led to a divergence in results regarding the effects of lorazepam on SICI. Indeed, a specific increase in $SICI_{2ms}$ was observed in the study presented in chapter 3, which used a CS set at 60%rMT, while a specific increase in $SICI_{3ms}$ was found in a previous study which used CS set at 70%rMT (Di Lazzaro et al., 2005). Therefore, altering protocols to increase the reliability of measurements may compromise the generalizability of previous results in the literature.

Another approach that may increase the reliability of paired-pulse and cortical excitability TMS protocols would be to increase the number of MEPs obtained for each measurement. Indeed, it has been shown that increasing the number of TMS-induced MEPs to at least 20 produces more stable results (Chang et al., 2016; Goldsworthy et al., 2016). TMS measurements obtained using 20 MEPs were shown to have excellent reliability coefficients (Cronbach's alpha ≥ 0.95). Therefore, it is possible that the protocol used here might not have been optimal as only 10 pulses were used in each condition. However, low frequency repeated transcranial magnetic stimulation has been shown to reduce cortical excitability. Considering that same study demonstrated that measurements obtained with 10 MEPs had adequate reliability (Cronbach's alpha ≈ 0.90), obtaining TMS measurements with 10 MEPs appears to be a satisfactory compromise between optimal reliability and mitigating the risk of reducing cortical excitability.

MRS data acquisition and processing

As explained in a previous section, when acquiring GABA, the signal is contaminated by the presence of a macromolecular signal, which may be corrected using several techniques (Mullins et al., 2014), which is a limitation of MRS. Coedited MM are either accepted as a confound, or dealt with by subtracting an additionally acquired MM spectrum (Harris et al., 2015; Henry et al., 2001), or by fitting a model MM spectrum (Provencher, 1993, 2001), for pure GABA estimation as was done in both studies. Nevertheless, MEGA-PRESS has been found to adequately measure GABA+, and MM subtraction and fitting techniques can reliably estimate GABA *in vivo* (Bogner et al., 2010; Evans et al., 2010; O'Gorman et al., 2007; O'gorman et al., 2011). While the corrections provided are accurate and yield reliable results when estimating *in vivo* GABA, the possibility of MM contaminating the signal is important to consider when interpreting MRS-GABA signals, especially so in clinical settings.

While the present thesis mainly focused on GABA, the topic of glutamate stability and drug effects was also broached. However, MEGA-PRESS at 3T is unable to resolve Glu from Gln (O'gorman et al., 2011) which thus limits the interpretation of Glx levels. It would have been ideal to obtain an isolated Glu signal for the performed assessments. Future studies aiming at assessing Glu long-term stability or drug intake effects should use a spectroscopic sequence yielding isolated Glu signals, without glutamine contamination, thereby drastically improving the validity of such measurements.

MRS data in the articles presented in Chapters 2 and 3 were filtered using a quality threshold based on relative Cramér-Rao Lower Bounds (CRLB%), where scans with CRLB >30% were rejected. However, recent advances in MRS analysis methods have demonstrated that using %CRLB as a filtering method may bias group data. Therefore, it is suggested that MRS studies use absolute values such as signal to noise ratios to filter data (Kreis, 2016; Near et al., 2020). Regardless, in the studies presented in this thesis, two subjects (one per study) were excluded based on %CRLB, which should limit bias to a minimum.

Drug responses

While the design of the study presented in chapter 3 was double-blind in nature, participants were able to infer the treatment with near-perfect accuracy based on the drug's sedating effects. Therefore, it was impossible to achieve adequate subject blinding with the present dosage. While a lower dosage may have rendered blinding possible, the chosen dose has been shown to produce consistent effects on TMS measures (Ziemann et al., 1996b). Indeed, it is known that lorazepam's effects on cortical inhibition are mediated by $\alpha_{2\text{-}3}$ and its sedating effects are mediated by α_2 (Griffin et al., 2013). Therefore, this drug's effects on cortical inhibition and on wakefulness are inextricably tied.

In addition, no spectroscopic measurements were made before drug administration, which would be a potential limitation of the study presented in chapter 3. However, as stated before, MRS-GABA and MRS-Glx measurements are stable across time, with similar within-day and between-day measurements COVs (Bogner et al., 2010; Evans et al., 2010; Greenhouse et al., 2016; Harada et al., 2011; Near et al., 2013; Near et al., 2014; O'gorman et al., 2011). For TMS, inter-session variability is similar across testing intervals (Boroojerdi et al., 2000; Dyke et al., 2018; Hermsen et al., 2016; Maeda et al., 2002; Ngomo et al., 2012; Orth & Rothwell, 2004; Orth et al., 2003). Thus, comparing pre- and post-treatment values would not have significantly reduced measurement error. Furthermore, cortical excitability varies significantly across the circadian cycle (Ly et al., 2016); the between-day design allowed all experiments to be conducted at approximately the same time, controlled for intra-day variations. Nevertheless, future studies may choose to incorporate pre-drug treatment data in addition to placebo control. These pre-drug intake measures should be done across time intervals where circadian cycle variations will not impact TMS measurements.

Statistical Power

The statistical power of the results presented in both studies included in this thesis was analysed to evaluate the overall validity of the conclusions. Regarding the study presented in Chapter 2, despite a seemingly small sample size ($n = 10$), adequate power ($1\text{-}\beta = .80$) at the chosen statistical significance level ($\alpha = .05$) is achieved for large effect sizes ($|\rho| = 0.71$) or larger.

Therefore, all statistically significant findings pertaining to the stability of MRS measures ([GABA/H_2O], [Glx/H_2O]) and TMS measures (rMT, %MSO, CSP) are adequately powered and can be considered valid. Considering that this study's primary objective was the examination of the stability and reliability of selected MRS and TMS measures, there is, arguably, only scientific interest in demonstrating statistical significance for measurements whose reproducibility parameters are sufficiently high. Indeed, while this study's sample size is on the lower end of the sample sizes of previous studies examining the reliability of MRS measures, where sample sizes ranged from 8 to 28, statistical power is sufficient for fulfilling this objective (Bogner et al., 2010; Evans et al., 2010; Greenhouse et al., 2016; Harada et al., 2011; Mooney et al., 2017; Near et al., 2014; O'Gorman et al., 2007; O'gorman et al., 2011). With regards to the reproducibility of TMS measures, the study presented in Chapter 2's sample size falls within the range of typical studies examining this research question, namely, 4 to 15, with the exception of a single study by Hermsen et al. (2016) with a very large (n = 93) sample size (Boroojerdi et al., 2000; Dyke et al., 2018; Hermsen et al., 2016; Maeda et al., 2002; Mooney et al., 2017; Ngomo et al., 2012; Orth & Rothwell, 2004; Orth et al., 2003).

Fulfilling the objectives of the study presented in Chapter 3 required reproducing the known effects of lorazepam administration on corticospinal excitability and $GABA_A$-R-mediated SICI (Di Lazzaro et al., 2005; Kimiskidis et al., 2006). Therefore, it was necessary to ensure sufficient statistical power for this study to be able to discern neurophysiological effects. *Post-hoc* power analyses using effect sizes found in the previously cited studies examining the effects of lorazepam on SICI and cortical excitability suggest that the study presented in Chapter 3 is sufficiently powered with regards to lorazepam's effects on SICI ($d = 0.79$, $\alpha = 0.05$, $n = 17$, $1-\beta = 0.86$) and the cortical excitability curve slope ($d = 1.07$, $\alpha = 0.05$, $n = 16$, $1-\beta = 0.98$).

Examining the relationship between MRS and TMS measures was a secondary objective of both studies presented in this thesis. Most findings from both studies in the present thesis agree with the literature, as previously discussed, and the sample size of the second study falls within the range of previous comparable studies, which reported sample sizes between 12 – 29 (Dyke et al., 2017; Mooney et al., 2017; Stagg et al., 2011b; Tremblay et al., 2012). Therefore, conclusions

made in this thesis regarding the relationship between MRS and TMS measures are sound and in accordance with previous works.

The study presented in Chapter 3 also found an interesting correlation between baseline MRS measures and TMS response ratios following lorazepam administration, suggesting that endogenous MRS measures could serve as markers of response sensitivity. This novel result has not been replicated and there are no studies with analogous results that could serve as a basis of comparison. *Post-hoc* power analyses revealed that the correlation between baseline $[GABA]_{SMC}$ and $SICI_{2ms}$ response ratios ($r_{(15)} = -0.49$, $p = 0.047$) achieved relatively low power ($1-\beta=0.58$). Therefore, care should be taken in its interpretation; it should be replicated in a higher-powered study before fully accepting it as a true positive result. Likewise, the study presented in Chapter 3 of this thesis demonstrates a moderate ($d = 0.61$) reduction in occipital GABA levels following lorazepam administration, which is slightly underpowered given its sample and effect size ($n = 15$, $1-\beta = 0.73$). While it was not possible to use effect sizes found in previously published studies that found a similar effect due to lack of descriptive statistics, the previous result is in line with these two studies that found a reduction of GABA in the occipital cortex and thalamus following $GABA_A$-R receptor agonist administration (Goddard et al., 2004; Licata et al., 2009). Therefore, the reduction in occipital GABA following lorazepam administration reported in the study included in Chapter 3 is likely to be a true positive result.

Future Research Avenues

To further validate the use of TMS and MRS in the medical field, it would be interesting in future studies to assess the stability of TMS protocols and MRS acquisitions over longer time intervals and with a greater number of measurement time points. For instance, a two-year assessment with measurements taken every two months with participants segregated into different age groups would yield valuable data regarding the impact of aging on the stability of TMS responses and MRS-derived neurometabolite levels. In addition, as previously suggested, further validating the RCI values reported in the present study may guide clinicians in determining if changes outside these bounds reflect a pathological process.

While the work presented in the current thesis sheds light on the neurochemical substrates of MRS-GABA, it would be interesting to use other pharmacological agents, such as tiagabine, gabapentin or vigabatrin, in a randomized placebo-controlled study. This would enable researchers to further pinpoint what comprises MRS-GABA signal. Due to the peculiar drop in MRS-GABA levels in the occipital cortex that was reported in chapter 3 of the present thesis, it would be of clinical interest to attempt to reproduce this finding using other benzodiazepines and in other brain regions, both while such regions are engaged in tasks and at rest to confirm the metabolic hypothesis posited in the present and in previous work.

Finally, while the link or lack thereof between MRS and TMS baseline measures has been extensively studied, the potential association between baseline GABA levels and BZD-induced SICI enhancement is novel and has, to our knowledge, never been reported or studied previously. Therefore, further studies are needed to replicate the present results and to determine if greater GABA concentrations are predictive of overall increased BZD sensitivity. Similarly, the trend found between baseline Glx levels and BZD-induced cortical excitability reduction, where a higher Glx concentration potentially predicted a lesser decrease in cortical excitability following lorazepam intake, needs to be further explored.

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