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IN VIVO GLX AND GLU MEASUREMENTS FROM GABA-EDITED MRS AT 3T

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KEYWORDS: Glx; Glutamate; PRESS; MEGA-PRESS; GABA-edited MRS.

ABBREVIATIONS: GABA, γ-aminobutyric acid; Glu, Glutmate; Gln, Glutamine; MRS, Magnetic Resonance Spectroscopy; PRESS, Point RESolved Spectroscopy; MEGA-PRESS, Mescher-Garwood PRESS; GABA+, GABA plus macromolecules; MM-Sup, Macromolecule Suppressed; TE, echo time.

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ABSTRACT:

In vivo quantification of glutamate (Glu) and GABA using magnetic resonance spectroscopy (MRS) is often achieved using two separate sequences; a short-echo PRESS acquisition for Glu and a MEGA-PRESS acquisition for GABA. The purpose of this study was to examine the agreement of Glu and Glx (the combined signal of Glutamate + Glutamine) quantified from two different GABA-edited MEGA-PRESS acquisitions (GABA+ TE=68 ms, and macromolecule suppressed, MMSup, TE=80 ms) with Glu and Glx quantified from a short-echo PRESS (PRESS-35, TE=35 ms) acquisition. Fifteen healthy male volunteers underwent a single scan session in which data were acquired using the three acquisitions (GABA+, MMSup and PRESS-35) in both the sensorimotor and anterior cingulate cortices using a voxel size of 3 x 3 x 3 cm^3. Glx and Glu were quantified from the MEGA-PRESS data using both the OFF sub-spectra and the difference (DIFF) spectra. Agreement was assessed using correlation analyses, Bland-Altman plots and intraclass correlation coefficients (ICC). Glx quantified from the OFF sub-spectra from both the GABA+ and MMSup acquisitions showed poor agreement with PRESS-35 in both brain regions. In the sensorimotor cortex, Glu quantified from the OFF sub-spectra of GABA+ showed moderate agreement with PRESS-35 data, but this finding was not replicated in the anterior cingulate cortex. Glx and Glu quantified using the DIFF spectra of either MEGA-PRESS sequence were in poor agreement with the PRESS-35 data in both brain regions. In conclusion, Glx and Glu measured from MEGA-PRESS data generally showed poor agreement with Glx and Glu measured using PRESS-35.

INTRODUCTION

There is increasing interest in measuring both Glutamate (Glu) and γ-aminobutyric acid (GABA) in the human brain *in vivo* due to their prominent roles in brain function. It is well accepted that these two neurotransmitters maintain the excitation/inhibition balance which is necessary for complex cognitive functions. Both Glu and GABA levels are related to cognitive functioning[1] and learning and plasticity [2,3], and altered GABA and Glu levels have been shown in numerous disorders such as schizophrenia,[4] autism,[5] psychosis[6] and Tourette's syndrome.[7]

Both Glu and GABA can be measured *in vivo* with proton magnetic resonance spectroscopy (MRS). Glu is most commonly measured using a standard short-echo point resolved spectroscopy sequence (PRESS, TE=30-35 ms). This sequence is chosen due to its availability and ease of implementation, relatively short scan time, high signal-to-noise ratio, and because it can provide data on multiple other metabolites.[8] In a typical PRESS spectrum, Glu appears as three multiplets at 3.75 ppm, 2.3 ppm and 2.1 ppm, and these resonances overlap with signals of other metabolites, in particular glutamine (Gln). Due to the challenges in separating Glu from Gln at 3 T, they are often quantified as Glx (the combined signal of Glu + Gln).[9]

GABA is at much lower concentration in the brain and the GABA resonances are overlapped by resonances from metabolites of higher concentration (such as creatine at 3.0 ppm), therefore the Mescher-Garwood PRESS (MEGA-PRESS) editing technique[10] is typically used. GABA also appears as three multiplets in the spectrum (3.0 ppm, 2.3 ppm and 1.9 ppm) which are coupled to one another. MEGA-PRESS consists of alternating two acquisition schemes which exploit this coupling to generate "ON" and "OFF" sub-spectra. The "ON" sub-spectrum is generated by applying an editing pulse at 1.9 ppm. As the GABA resonance at 1.9 ppm is coupled to the 3.0 ppm resonance, this pulse modulates the evolution

of the 3.0 ppm GABA resonance, without affecting the overlapping creatine. The "OFF" sub-spectra are generated by applying this pulse elsewhere (usually at 7.5. ppm) so that the evolution of the GABA resonance (and those of other metabolites, including creatine) are not affected by this pulse. Subtraction of the ON sub-spectrum from the OFF sub-spectrum to produce the difference spectrum (DIFF) removes resonances that are not affected by the 1.9 ppm editing pulse, therefore revealing the GABA at 3.0 ppm.[10,11]

Though the majority of metabolites are unaffected by the 1.9 ppm editing pulse, this pulse also affects a macromolecule resonance at 1.7 ppm which is coupled to a macromolecule resonance at 3.0 ppm. Therefore, in a typical GABA-edited MEGA-PRESS acquisition, macromolecules "co-edit" with GABA (i.e. their behaviour is similarly affected); they are therefore also present in the DIFF spectrum at 3.0 ppm and are thought to account for roughly half of the GABA signal. For this reason, this GABA measure is often referred to as GABA+, to represent GABA+macromolecules.[12] An alternative approach is the macromolecule-suppressed (MMSup) MEGA-PRESS sequence. In this sequence, editing pulses are placed symmetrically around the 1.7 ppm macromolecule resonance peak (1.9 ppm for the ON sub-spectrum and 1.5 ppm for the OFF sub-spectrum). Therefore, the 1.7 ppm macromolecule signal is equally refocused in both the ON and OFF sub-spectra so the macromolecules resonances cancel out in the DIFF spectra.[12] The use of more selective editing pulses (20 ms for MMSup versus 14 ms for GABA+) requires a longer echo time (TE=80 ms) compared to TE = 68ms typically used in GABA+ measures. The MMSup acquisition provides a "purer" measure of GABA, but the MMSup GABA signal is roughly 50% smaller than the GABA+ signal and subsequently has a lower signal-to-noise ratio (SNR) and a higher model fit error. Additionally, the more selective editing pulses used in MMSup MEGA-PRESS are more sensitive to frequency drift.[13] Therefore, the selection of sequence to measure GABA (GABA+ versus MMSup) will depend on study-specific factors

such as the region of interest (which will affect overall data quality) and the maximum acceptable scan time (which will affect SNR).

Due to the need to use a specialised sequence to measure GABA, GABA and Glu (or Glx) are often measured with separate acquisitions. However, due to the complementary roles of GABA and Glu (GABA is an inhibitory neurotransmitter, Glu is an excitatory neurotransmitter), as well as the fact that Glu is a precursor to GABA,[14] the ability to measure GABA and Glu simultaneously is relevant to many studies. Though not designed to detect Glu (or Glx), this information is nevertheless present in MEGA-PRESS data. The MEGA-PRESS OFF sub-spectrum is similar to a PRESS spectrum (i.e., contains Glu signal), but has a longer echo time to accommodate the editing pulses. Therefore, the OFF sub-spectrum can be used to quantify Glu. Additionally, the editing pulses are not perfectly selective. Consequently, the editing pulse applied at 1.9 ppm for the ON sub-spectrum also edits the Glu and Gln resonances at 2.0-2.1 ppm, resulting in a co-edited Glu (and Glx) resonance at 3.75 ppm in the DIFF spectrum.[10,11] This co-edited Glu (and Glx) resonance has been shown to be stable across scan sessions.[15] Several studies have reported Glu or Glx measures using both the OFF sub-spectra and the DIFF spectra (e.g [16–18]), however there has been little validation for either method.

Using a phantom, Nezhad et al. (2017) demonstrated a linear relationship between the known concentration of Glu and its measurement from the DIFF spectrum of a MEGA-PRESS sequence.[19] This represents ideal conditions in which there is very little noise, no other metabolites or macromolecules present and no subject movement; *in vivo* Glu is more difficult to quantify. Indeed, van Veenendaal et al. (2018) showed strong correlations between levels of Glu and Glx measured using both the OFF sub-spectrum and the DIFF spectrum of a GABA+ sequence compared to known concentrations in a phantom. However, when comparing *in vivo* measures of Glu and Glx acquired using MEGA-PRESS with

measures acquired using PRESS, the correlations were substantially weaker.[20] Van Veenendaal et al. looked at Glu and Glx measured from a GABA+ sequence in the occipital-parietal lobe, however agreement may differ with voxel location and between MEGA-PRESS implementations (GABA+ versus MMsup).

The objective of the current study is to determine the agreement of Glu and Glx measured from the OFF and DIFF spectra from two GABA-edited MEGA-PRESS sequences (GABA+, TE=68 ms, and MMSup, TE=80 ms) with Glu and Glx measured from a typical short echo PRESS sequence (TE=35 ms, herein referred to as PRESS-35). The fundamental motivation for the study was the need for shorter scanning times when studying clinical populations, which can be achieved if only one of the two approaches is required. Though other sequences may be more accurate for measuring Glu (e.g. STEAM, Carr-Purcell PRESS, TE averaged PRESS[21], short echo phase-rotated STEAM[22]), PRESS-35 was chosen as it is the most popular sequence for clinical use due to its availability on MR scanners, relatively short scan time and its ability to measure multiple metabolites at once. Therefore, comparison of Glu and Glx values from MEGA-PRESS data (both the OFF-sub –spectra and the DIFF spectra) with PRESS-35 data will provide information on whether a separate PRESS sequence is needed to measure Glu, or whether MEGA-PRESS alone can be employed.

While this manuscript is not directly concerned with the measurement of GABA, we will follow standard abbreviations in the field; as a convention, in this manuscript we will refer to the Glu and Glx measurements using the OFF sub-spectra from the GABA+ MEGA-PRESS data as OFF-GABA+, and the measurements from the difference spectra from the GABA+ MEGA-PRESS data as DIFF-GABA+. Similarly, we will refer to the measurements using the OFF and the DIFF spectra from the MMSup MEGA-PRESS data as OFF-MMsup and DIFF-MMsup. Finally, as mentioned above, we will refer to the typical PRESS data acquired with an echo time of 35 ms as PRESS-35.

METHODS

Participants

Fifteen healthy, right-handed male participants between the ages of 18-30 were recruited. Data were collected with approval from the local research ethics board and with written informed consent from volunteers.

Data Acquisition

Data were collected on a 3T GE MR750w scanner (General Electric Healthcare, USA) with a 32-channel head coil. The scanning protocol included acquisition of a T_1-weighted BRAVO (BRAin VOlume imaging) scan for voxel placement (TR/TE=7.3/2.7 ms, 1 mm^3 isotropic voxels, flip angle=10°, TI=600 ms). Voxels were placed in the anterior cingulate cortex and the left sensorimotor cortex (Figure 1). The anterior cingulate cortex voxel was placed anterior to the genu of the corpus callosum, with the bottom edge parallel to the anterior cingulate-posterior cingulate line. The sensorimotor cortex voxel was centred at the hand-knob of the motor cortex and rotated such that the coronal and sagittal planes aligned with the cortical surface.[23] Voxel rotation for the sensorimotor voxel was achieved using a separate imaging prescription (that allows for rotation in all planes) to obtain a rotation and linking it to the voxel which thus enables an MRS voxel to be rotated in all planes.

The MEGA-PRESS implementation used was developed at King's College London and kindly made available by the co-authors from this institution. For each voxel location, two separate GABA-edited measurements were made, first, GABA+[24] (14 ms editing pulses placed at 1.9 ppm and 7.46 ppm in the ON and OFF conditions, respectively; TR/TE=1800/68 ms) and second, MMSup[25] (20 ms editing pulses placed at 1.9 ppm and 1.5 ppm in the ON and OFF conditions, respectively; TR/TE=1800/80 ms). Both sequences used

a voxel size of 3x3x3 cm^3, 320 averages and 4096 data points sampled at 5 kHz. This was followed by the PRESS-35 sequence (voxel size=3x3x3 cm^3; TR/TE=1800/35 ms; 64 averages; 4096 data points). Sixteen acquisitions without water suppression were also acquired for each sequence.

Data Processing

The FID-A toolbox[26] was used to apply the following pre-processing steps to all data: coil combination, removal of motion-corrupted averages, frequency drift correction and zero order phase correction. FID-A was also used to isolate and average the OFF sub-spectra and calculate the DIFF spectra from the MEGA-PRESS data. LCModel Version 6.3-1J[27] was used to apply eddy current correction and quantification relative to water for all spectra. To account for the T_2 decay of water at each echo time the ATT_{H2O} parameter settings for each acquisition were as follows: MMSup=0.37, GABA+=0.43, PRESS-35=0.65, as per the LCModel manual. The concentration of water (WCONC) was set to 55510. Basis sets for quantification were simulated using the FID-A toolbox based on exact sequence timings and RF pulse shapes for each sequence. Basis sets for the OFF-GABA+, OFF-MMSup and PRESS-35 data included the following metabolites: alanine, aspartate, glycerophosphocholine, phosphocholine, creatine, phosphocreatine, GABA, Glu, Gln, lactate, inositol, n-acetyl aspartate, n-acetylaspartylglutamate, scyllo-inositol, glutathione, glucose and taurine. Basis sets for the DIFF-GABA+ and DIFF-MMSup data included: GABA, Glu, Gln, n-acetyl aspartate, n-acetylaspartylglutamate and glutathione. Additionally, the setting ‘mega-press-3’ was used for the DIFF-GABA+ and DIFF-MMSup data. Macromolecules were fitted using the default parameterized macromolecule resonances in LCModel. All other LCModel parameters (in particular ATT_{MET}=1.0) were left at their default values.

A voxel mask was generated and segmented using the tissue segmentation functions in ‘Gannet’.[28] The grey matter, white matter and cerebrospinal fluid fractions were

subsequently used to perform tissue correction as per the Gasparovic method[29] according to the equation:

$$C_{metab} = \frac{met_{LCM} \text{ x } (f_{GM} \text{ x } R_{GM} + f_{WM} \text{ x } R_{WM} + f_{CSF} \text{ x } R_{CSF})}{(1 - R_{CSF}) \text{ x } R_{met}}$$

where

$$R_x = e^{\frac{-TR}{T2x}} \text{ x } (1 - e^{\frac{-TE}{T1x}})$$

and

$$f_x = \frac{f_{x_vol} \text{ x } d_x}{f_{GM_vol} \text{ x } d_{GM} + f_{WM_vol} \text{ x } d_{WM} + f_{CSF_vol} \text{ x } d_{CSF}}$$

C_{metab} is the molal concentration of a given metabolite, met_{LCM} is the metabolite level obtained from LCModel, T_{1x} and T_{2x} are the assumed T_1 and T_2 relation times for each tissue type and metabolite, TR and TE are the repetition and echo times, respectively. f_{x_vol} is the tissue fraction obtained from the segmentation analysis, d_x refers to the density of MR-visible water (GM = 0.78, WM = 0.65, CSF = 0.97).

Data quality was assessed by visual inspection and metabolite linewidth; spectra with a linewidth over 0.07 ppm (9 Hz) based on the data quality recommendations as per Kreis .[30]

Statistical Analysis

Statistical analyses were performed using SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Macintosh, Version 25.0. Armonk, NY: IBM Corp). Correlation analysis was used as an initial step to assess the strength of the relationship between Glx and Glu measured using the two MEGA-PRESS acquisitions compared to Glx and Glu measured from PRESS-35. Agreement between Glx and Glu from the MEGA-PRESS data compared to Glx and Glu from the PRESS-35 data was assessed using Bland-Altman plots and the

intraclass correlation coefficient (ICC).[31] Bland-Altman plots were used to visualise differences between the two values[32] and were used in addition to regression analysis to test for a systematic and proportional bias.[33] The ICC is a modification of the Pearson correlation coefficient that takes into account both the degree of correlation and the overall agreement between measurements. A mixed-effects ICC model was used and the level of agreement was assessed using the range of the 95% confidence intervals, values below 0.5 are considered poor agreement, values between 0.5-0.7 are considered moderate agreement, values above 0.7 are considered good agreement.[34]

RESULTS

Data Quality

All spectra acquired from the sensorimotor cortex had sufficient quality for further analysis. Quality inspection resulted in exclusion of PRESS-35 spectra from the anterior cingulate cortex data in three participants. Subsequently all data for these participants were excluded. Additionally, quality inspection resulted in exclusion of the following spectra from the anterior cingulate cortex: participant 8, DIFF-GABA+, OFF-MMSup, DIFF MM-Sup; participant 9, OFF-MMSup, DIFF MM-Sup; participant 11, DIFF-MMsup.

Sensorimotor Cortex Data

Glx measures in the sensorimotor cortex from the OFF sub-spectra from either MEGA-PRESS sequence did not significantly correlate with PRESS-35 Glx measures at a significance level of $p \leq 0.05$, though a trend towards a positive correlation was seen between OFF-MMSup and PRESS-35 ($p=0.058$; Figure 2A). Glu measures from both OFF-GABA+ and OFF-MMSup significantly correlated with PRESS-35 Glu measures (Figure 2B). Glx (Figure 2C) and Glu (Figure 2D) measured from both DIFF-GABA+ and DIFF-MMSup also significantly correlated with Glx and Glu measured using PRESS-35.

The Bland-Altman analysis indicates no systemic bias when comparing both Glx and Glu measured in the sensorimotor cortex using OFF-GABA+ and Glu measured using OFF-MMSup with PRESS-35 measures, as zero is within the limits of agreement (Figure 3A). Glx measured using OFF-MMSup is on average 3.5 units lower than PRESS-35 values (Figure 3B). All measures from the MEGA-PRESS DIFF spectra were systematically lower than the PRESS-35 measurements (Figure 3C and Figure 3D).

To further investigate the results of the Bland-Altman plots, regression analysis was used to examine the relationship between the difference of the two measures (i.e., the y-axis of the

Bland-Altman plot, which shows the values from the OFF-MEGA-PRESS acquisitions subtracted from the values from the PRESS-35 acquisition) and the mean of the two measures (i.e., the x-axis of the Bland-Altman plot). A significant relationship between the measures indicates a proportional bias; as the mean of the two measures increases, the difference between the two measures also increases. There was no proportional bias in either Glx (Figure 3A) or Glu (Figure 3B) values measured from OFF-GABA+ (Glx: ß=0.302, p = 0.274; Glu: ß=0.354, p=0.195; Figure 3A). There was, however, a significant proportional bias in both Glx and Glu values measured from OFF-MMSup (Glx: ß=0.543, p=0.036, Figure 3A Glu: ß=0.566, p=0.028, Figure 3B). A significant proportional bias was also seen in both Glx (Figure 3C) and Glu (Figure 3D) measured using the DIFF sub-spectra (DIFF-GABA+ Glx: ß=0.871, p<0.001; Glu: ß=0.864, p<0.001; DIFF-MMSup Glx: ß=0.916, p<0.001; Glu: ß=0.917 p<0.001).

Due to the large systematic and proportional bias observed in Glx and Glu values from the DIFF spectra, ICC values were only calculated for measures from the OFF-GABA+ and OFF-MMSup sub-spectra compared to the PRESS-35 data. Glx and Glu measured from OFF-GABA+ and Glu measured from OFF-MMSup all show poor-to-moderate agreement with PRESS-35 measures, as the mean was below an ICC of 0.5 but the 95% confidence interval crosses 0.5.The mean and the confidence intervals of the ICC comparing Glx measured from OFF-MMSup and Glx from PRESS-35 were below 0.5 indicating poor agreement (Figure *4*).

Anterior Cingulate Cortex Data

Glx and Glu measured in the anterior cingulate cortex using OFF-GABA+ or OFF-MMsup did not significantly correlate with PRESS-35 values (Figure 5A and Figure 5B, respectively). Only Glu measured using DIFF-GABA+ significantly correlated with PRESS-

35 values, all other Glx and Glu values obtained from the DIFF spectra did not correlated with PRESS-35 values (Figure 5C and Figure 5D, respectively).

The Bland-Altman analysis indicates no systemic bias when comparing both Glx and Glu measured in the anterior cingulate cortex using either OFF-GABA+ with PRESS-35 measures, as zero is within the limits of agreement, however, there is a large range in the limits of agreement. Glx measured using OFF-MMSup was on average 6 units lower than the PRESS-35 values. There was no systematic bias in Glu measured using OFF-MMSup (Figure 6A and Figure 6B). All measures from the DIFF spectra (both DIFF-GABA+ and DIFF-MM-Sup) were systematically lower than the PRESS-35 measurements (Figure 6C and Figure 6D). Regression analysis showed no proportional bias in either Glx or Glu values measured using OFF-GABA+ (Glx: ß=-0.300, p=0.344, Figure 6A; Glu; ß=-0.241, p=0.451, Figure 6B). In contrast, there was a significant proportional bias in both Glx measured using OFF-MMSup (Glx: ß=0.060, p=0.003, Figure 6A). There was no proportional bias in Gly measured using OFF-MMSup (Glu: ß=0.060, p=0.870, Figure 6B). Additionally, proportional biases were seen for both Glx (Figure 6C) and Glu (Figure 6D) measures from the DIFF-GABA+ and the DIFF-MMSup (DIFF-GABA+ Glx: ß=0.843, p=0.001; Glu: ß=0.831, p=0.002; DIFF-MMSup Glx: ß=0.894, p=0.001; Glu ß=0.853, p=0.003; Figure 6C and D).

Due to the large systematic and proportional bias observed in the values measured using the DIFF spectra, ICC values were only calculated for measures from the OFF sub-spectra. The ICC indicates poor-moderate agreement between Glx and Glu measured using OFF-GABA+ with the PRESS-35 values, and poor agreement between Glx and Glu measured using OFF-MMSup and the PRESS-35 values (Figure 7).

DISCUSSION

In the present study, we examined the agreement of Glx and Glu measured from the OFF and DIFF spectra from two GABA-edited MEGA-PRESS sequences (GABA+: TE=68 ms, MMSup: TE=80 ms) across two voxels (sensorimotor cortex and anterior cingulate cortex). Overall, there is limited agreement in measuring Glx and Glu from GABA-edited MEGA-PRESS data compared to the Glx and Glu measures obtained from a PRESS-35 acquisition, which leads us to conclude that quantifying Glx and/or Glu from either the OFF-sub-spectrum or the co-edited resonance from the DIFF spectrum of GABA-edited MEGA-PRESS data cannot replace the measure obtained from a short echo PRESS acquisition.

When considering the results from the correlation analyses, Bland-Altman plots and the ICC analyses, the strongest agreement with the PRESS-35 measures was for Glu measured using OFF-GABA+ in the sensorimotor cortex; however, this was not replicated in the anterior cingulate cortex data. This regional difference is likely in part due to the overall lower data quality in the anterior cingulate cortex, resulting from poorer B_0 and B_1 homogeneity and challenges shimming in this region, particularly for large voxels. For example, the linewidth of the DIFF-MMSup from the anterior cingulate cortex is almost double the linewidth of DIFF-MMSup from the sensorimotor cortex. Therefore, if data quality is sufficient, Glu measured using OFF-GABA+ could potentially be used as a substitute for a PRESS-35 acquisition, however this method should not be applied when data quality is expected to be lower, such as in the anterior cingulate cortex.

All Glx measures from the MEGA-PRESS data (OFF-GABA+ and OFF-MMSup, DIFF-GABA+ and DIFF-MMSup) showed poor agreement with PRESS-35 data in both brain regions. This discrepancy between Glu and Glx may be due to the phase evolution of Gln at the different echo times. Using simulations, Mullins et al. (2008) showed suppression of Gln resonance intensity at TE=40-80 ms, with the greatest Gln suppression at 80 ms.[9]

Therefore, the overall Glx signal (Glu+Gln) will vary across echo times due to the different Gln signal contributions. Consistent with these simulations, Schubert et al. (2004) showed that Glx resonances obtained using a PRESS sequence with TE = 80 ms *in vivo* resembled a Glu spectrum obtained from a phantom.[35] Interestingly, our data show Glu measured using OFF-MMSup (TE=80 ms) was slightly lower than Glu measured using PRESS-35 (though this was not classed as a significant bias as 0 was still within the limits of agreement). This may be due to certain factors related to working with *in vivo* data. The OFF-MMSup data has an editing pulse applied at 1.5 ppm, although this shouldn't affect the evolution of the Glu signal, it likely will affect the evolution of the underlying macromolecules. Additionally, the contamination of the macromolecule baseline is likely to be higher at shorter echo-time acquisition (i.e. PRESS-35) due to macromolecules' short T_2 relaxation times[36], which may explain the difference seen here. Indeed, there is evidence that PRESS with TE=35 ms overestimates Glu.[21] This reiterates the need in the MRS field as a whole for a better understanding of the contribution of macromolecule baseline to MRS data.

Our findings are consistent with van Veenendaal et al. (2018) who also showed a correlation between glutamate measured from the DIFF spectrum and a PRESS-35 acquisition.[20] Here we additinally show systematic and proportional biases are present in Glx and Glu measures from the DIFF spectra, as seen in the Bland-Altman plots. The presence of bias in the data from the DIFF spectra is unsurprising because this is an edited measure. The 1.9 ppm editing pulse is broad and therefore also modulates the 2.1 ppm Glu multiplet that is coupled at 3.75 ppm.[11,37] As the editing pulse does not directly target 2.1 ppm, the Glu and Gln resonances are only partially refocussed, resulting in a fraction of the possible signal present in the DIFF spectra.[10,11,15] As the concentration of Glu increases, only a proportion of this increase will be reflected in the DIFF spectra, but all of this increase will be directly reflected in the PRESS-35 measures. Therefore, at higher concentrations, Glu (and Glx)

measurements will be lower in the DIFF spectra, resulting in a proportional bias in addition to the systematic bias. Furthermore, Gln is edited less effectively than Glu as it is further away from the editing pulse,[19] which may explain the stronger correlations for the Glu data compared to the Glx data. The effects of the editing pulse not completely targeting Glu and Gln are augmented in the MMSup results as this editing pulse is more selective.

One limitation of this study is that the ground truth of the Glx/Glu concentration is not known, making it impossible to say for certain which sequence represents the correct value. Alternative sequences (e.g. STEAM, Carr-Purcell PRESS, TE averaged PRESS,[21] short echo phase-rotated STEAM[22]), and different PRESS echo times (e.g. 40 ms, 80 ms[9,21]) have been suggested to be more optimal for measuring Glu. In an alternative comparison, Maddock et al. (2018) demonstrated strong correlations between Glx and Glu measured using PRESS with TE=80 ms and OFF-GABA+ in the dorsolateral prefrontal cortex.[39] The suppression of Gln is more similar at echo times of 68 ms and 80 ms compared to the difference in suppression of Gln at 35 ms and 68 ms, which explains the strong Glx correlation reported by Maddock et al compared to the weak correlations observed in this current study. We chose PRESS-35 (a standard PRESS acquisition with TE=35 ms) as our reference as it is the most commonly used approach in the literature to measure Glx and Glu. Hancu (2009) showed Glx and Glu measured using PRESS-35 data have lower coefficients of variation and CRLBs than when measured using a PRESS acquisition with a TE=80 ms,[21] suggesting that PRESS-35 Glx/Glu measurements are more reproducible than PRESS data with TE=80 ms .

A second limitation of this study is that the order of the sequences was not counterbalanced. There was always a time delay between the GABA+ acquisition and the PRESS-35, and the sensorimotor cortex was always scanned after the anterior cingulate cortex. Lastly, our sample size was small and included only a healthy, male population; quantification of Glx and Glu may be more difficult in clinical populations.

In conclusion, the results of this study generally show poor agreement between Glx and Glu measured using the OFF sub-spectra or the DIFF spectra from GABA+ or MMSup data and Glx and Glu measured using PRESS-35. Therefore, we recommend that a separate PRESS-35 be used when studying Glx, Glu and GABA, rather than using Glx or Glu metrics from the MEGA-PRESS data, particularly in areas of the brain with lower signal quality.

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Table 1: Summary of data acquired in the sensorimotor and anterior cingulate cortices, including number of datasets included, average linewidth, mean and standard deviation of the quantified Glx and Glu concentrations, and fit metrics (CRLB) for each metabolite.

		PRESS-35	***OFF-GABA+***	***OFF-MM-Sup***	***DIFF-GABA+***	***DIFF-MM-Sup***
SM	*n*	15	15	15	15	15
	FWHM (Hz)	4.22 ± 0.89	4.47 ± 0.89	3.96 ± 0.64	5.50 ± 1.66	03.83 ± 1.02
	Glx					
	Mean ± SD (mol/kg)	13.05 ± 1.53	13.80 ± 1.24	9.57 ± 0.96	6.08 ± 0.51	2.79 ± 0.44
	%CRLB Range	5-8	6-9	6-10	2-4	3-7
	CoV	0.12	0.10	0.10	0.08	0.15
	Glu					
	Mean ± SD (mol/kg)	10.92 ± 1.31	12.77 ± 1.05	9.10 ± 0.85	5.02 ± 0.54	2.50 ± 0.35
	%CRLB Range	5-7	6-9	6-9	4-7	4-15
	CoV	0.12	0.08	0.09	0.11	0.14
ACC	*n*	12	12	10	11	9
	FWHM (Hz)	6.13 ± 0.89	5.49 ± 1.28	6.01 ± 01.41	6.40 ± 1.28	6.52 ± 1.41
	Glx					
	Mean ± SD (mol/kg)	18.84 ± 2.26	15.61 ± 3.04	12.78 ± 2.24	7.40 ± 0.66	2.87 ± 0.52
	%CRLB Range	4-7	6-12	6-11	3-4	4-6
	CV	0.12	0.19	0.18	0.09	0.19
	Glu					
	Mean ± SD (mol/kg)	15.51 ±1.97	13.78 ± 2.92	11.7 9 ± 1.68	5.31 ± 0.82	2.63 ± 0.52
	%CRLB Range	4-6	6-12	6-11	4-10	4-9
	CoV	0.13	0.21	0.14	0.16	0.20

Mean ± standard deviation, FWHM = full width half maximum estimated by LCModel, CRLB = Cramér-Rao lower bounds as calculated by LCModel, CoV = coefficient of variation, SM = Sensorimotor Cortex, ACC = Anterior Cingulate Cortex, Glx = Glutamate+Glutamine, Glu = Glutamate, GABA+=GABA plus macromolecules (TE=68 ms), MMSup=macromolecule suppressed (TE=80 ms), OFF=data quantified from the OFF sub-spectra, DIFF=data quantified from the difference spectra.

Figure 1: Voxel location and example spectra from a single volunteer in the sensorimotor cortex (A) and the anterior cingulate cortex (B). GABA and Glx resonances in the DIFF spectra have been scaled for visualisation.

Figure 2: Scatter plots comparing Glx and Glu quantification methods in the sensorimotor cortex, with significant p-values in bold. (A) Glx and (B) Glu measurements from OFF-GABA+ and OFF-MMSup compared to the PRESS-35 measurements. (C) Glx and (D) Glu measured from the DIFF-GABA+ and DIFF-MMSup acquisitions compared to the PRESS-35 measurements. GABA+=GABA plus macromolecules (TE=68 ms), MMSup=macromolecule suppressed (TE=80 ms).

Figure 3: Bland-Altman plots comparing Glx and Glu quantification methods in the sensorimotor cortex. (A) Glx quantified from OFF MEGA-PRESS compared with PRESS-35, (B) Glu quantified from OFF MEGA-PRESS compared with PRESS-35, (C) Glx quantified from DIFF MEGA-PRESS compared with PRESS-35, (D) Glu quantified from DIFF MEGA-PRESS compared with PRESS-35. The difference between the two paired measurements (PRESS-35 – MEGA-PRESS) is shown on the y-axis and the mean of the two measures is shown on the x-axis. Grey triangles show data from the GABA+ acquisition and black circles show data from the MMSup acquisition. Solid lines represent the overall mean difference and the dashed lines represent the limits of agreement based on the 95% confidence intervals.

Figure 4: Intraclass correlation coefficient values and 95% confidence intervals comparing Glx and Glu quantified from OFF MEGA-PRESS data and PRESS-35 data for the sensorimotor cortex. Values of 0.5 (dashed line) indicate moderate agreement.

Figure 5: Scatter plots comparing Glu and Glx quantification methods in the anterior cingulate cortex. (A) Glx and (B) Glu measurements from OFF-GABA+ and OFF-MMSup compared to the PRESS-35 measurements. (C) Glx and (D) Glu measured from the DIFF-GABA+ and DIFF-MMSup acquisitions compared to the PRESS-35 measurements.

Figure 6: Bland-Altman plots comparing Glx and Glu quantification methods in the anterior cingulate cortex. (A) Glx quantified from OFF MEGA-PRESS compared with PRESS-35, (B) Glu quantified from OFF MEGA-PRESS compared with PRESS-35, (C) Glx quantified from DIFF MEGA-PRESS compared with PRESS-35, (D) Glu quantified from DIFF MEGA-PRESS compared with PRESS-35. The difference between the two paired measurements (PRESS-35 – MEGA-PRESS) is shown on the y-axis and the mean of the two measures is shown on the x-axis. Grey triangles show data from the GABA+ acquisition and black circles show data from the MM-Sup acquisition. Solid lines represent the overall mean difference and the dashed lines represent the limits of agreement based on the 95% confidence intervals.

Figure 7: Intraclass correlation coefficient values and 95% confidence intervals comparing Glx and Glu quantified from OFF MEGA-PRESS data and PRESS-35 data for the sensorimotor cortex. Values of 0.5 (dashed line) indicate moderate agreement.