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RELIABILITY OF 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; reliability.

ABBREVIATIONS: GABA, γ-aminobutyric acid; Glu, Glutmate; Gln, Glutamine; MRS, Magnetic Resonance Spectroscopy; SE-PRESS, Short Echo 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, PRESS and MEGA-PRESS. The purpose of this study was to compare the measurement of Glu and Glx (Glu+ Glutamine; Gln) quantified from two different MEGA-PRESS protocols (GABA+, TE=68 ms, and macromolecule suppressed, MM-Sup, TE=80 ms) and short-echo (SE) PRESS (TE=35 ms). Fifteen healthy male volunteers underwent a single scan session in which data were acquired using the three sequences in both the sensorimotor cortex and the anterior cingulate cortex. Glx and Glu were quantified from the MEGA-PRESS data using both the difference (DIFF) spectra and the OFF sub-spectra. Glx and Glu measured using SE-PRESS was taken as de-facto standard due to its common use in the field. Reliability was assessed using repeated measures ANOVA, correlation analyses, intraclass correlation coefficients (ICC), and Bland-Altman plots. Glx and Glu quantified using the DIFF spectra of either MEGA-PRESS sequence were in poor agreement with the SE-PRESS data in both brain regions. Glx quantified from the OFF sub-spectra of both sequences and Glu quantified from the OFF sub-spectra of GABA+ MEGA-PRESS also showed poor agreement in both brain regions. In the sensorimotor cortex, Glu quantified from the OFF sub-spectra of MM-Sup MEGA-PRESS showed better agreement with SE-PRESS data, but this finding was not replicated in the anterior cingulate cortex data. In conclusion, Glx and Glu measured from MEGA-PRESS data generally shows poor agreement with Glx and Glu measured using SE-PRESS. Therefore, we recommend that a separate SE-PRESS 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.

INTRODUCTION

There is increasing interest in the ability to measure Glutamate (Glu) and γ-aminobutyric acid (GABA) in the human brain in vivo. Both Glu and GABA levels have been shown to be related to cognitive functioning[1] and altered GABA and Glu levels have been shown in numerous neuropsychiatric and neurological disorders such as schizophrenia, [2] autism [3], psychosis[4] and Tourette's syndrome.[5] Proton magnetic resonance spectroscopy (MRS) provides a way of measuring Glu and GABA levels *in vivo* in the human brain, however they are often studied in isolation despite the complementary information the two measurements offer.

Glu appears as three multiplets in the MR spectrum (3.75 ppm, 2.3 ppm and 2.1 ppm) and these peaks overlap with signals of other metabolites, in particular glutamine (Gln). Due to the challenges in separating Glu from Gln at 3T, they are often quantified as the combined signal Glx.[6] Glx can be measured using a short-echo point resolved spectroscopy sequence (SE-PRESS, TE=30-35 ms), which provides data on multiple metabolites owing to its high signal-to-noise ratio.[7]

GABA also appears as three multiplets in the spectrum (3.0 ppm, 2.3 ppm and 1.9 ppm). However, due to its low concentration in the brain and the presence of overlapping peaks from metabolites of much higher concentration (such as the 3.0 ppm creatine peak), GABA is typically measured using the Mescher-Garwood PRESS (MEGA-PRESS) editing technique.[8] GABA-edited MEGA-PRESS consists of alternating two pulse sequences. The "ON" sub-spectra are generated by applying an editing pulse at 1.9 ppm. Due to the effects of coupling, this modulates the evolution of the GABA peak at 3.0 ppm without affecting the overlapping creatine peak. The "OFF" sub-spectra are generated by applying this pulse elsewhere so GABA and the other metabolites (including creatine) are not affected by this editing pulse. The subtraction of the ON sub-spectrum from the OFF sub-spectrum to make the difference

(DIFF) spectrum removes the overlapping peaks, including creatine, so that a 3.0 ppm GABA peak is revealed.[8,9] Macromolecules also co-edit with GABA due to a resonance at 1.7 ppm which is coupled to a macromolecule peak at 3.0 ppm, and they are thought to account for roughly half of the GABA signal in a typical MEGA-PRESS sequence (TE=68 ms). For this reason, GABA measured using this sequence is referred to as GABA+.[10] One way to account for this is to use a macromolecule-suppressed (MM-Sup) MEGA-PRESS sequence. In this sequence, the editing pulses are placed symmetrically around the macromolecule resonance peak (1.9 ppm in the ON scans and 1.5 ppm in the OFF scans). This causes the 1.7 ppm macromolecule signal to be refocused in both the ON and OFF sub-spectra, subsequently the macromolecules are subtracted out in the DIFF spectra.[10] Though this provides a "purer" measure of GABA, the MM-Sup GABA signal is roughly 50% smaller than the GABA+ signal and subsequently has a higher model fit error and lower signal-to-noise ratio. Additionally, the more selective editing pulses used in MM-Sup MEGA-PRESS are more sensitive to frequency drift.[11]

As mentioned previously, it is often desirable to quantify both GABA and Glx to indicate inhibitory and excitatory tone. Most directly this is accomplished by using specific acquisitions for each metabolite (GABA-edited MEGA-PRESS and SE-PRESS, respectively). However, this may not be ideal as this results in a longer scan session. Additionally, potential differences in voxel localisation due to the likelihood of movement between sequences limits the ability to compare GABA and Glx levels from separate acquisitions.

The OFF sub-spectrum from MEGA-PRESS is similar to a SE-PRESS spectrum (i.e., contains Glx signal), but has a longer echo time as needed to accommodate the editing pulses. Therefore, the OFF sub-spectra could potentially be used to quantify Glx. Additionally, the GABA editing pulses are not perfectly selective and, consequently, in the ON sub-spectrum,

also affect the Glx peaks at 2.0-2.1 ppm. These peaks are coupled with the 3.75 ppm Glx peak and co-edit with GABA in the ON sub-spectrum and subsequently appear in the DIFF spectrum.[8,9] This co-edited Glx peak has been shown to be stable across scan sessions [12]. Several studies have reported Glx or Glu measures using both the OFF sub-spectra and the DIFF spectra (e.g [13–15]), however there has been little validation for either method.

Nezhad et al. (2017) used a phantom to demonstrate a linear relationship between the known concentration of Glu and its measurement from the DIFF spectra of a MEGA-PRESS sequence.[16] 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 Glx and Glu measured using both the OFF sub-spectra and the DIFF spectra of a GABA+ sequence in a phantom, however *in vivo* measures of Glx and Glu showed substantially weaker correlations.[17] Van Veenendaal et al. looked at Glx measured from a GABA+ sequence in the occipital-parietal lobe, however, reliability may differ with voxel location and between MEGA-PRESS implementations (GABA+ versus MM-sup).

The objective of the current study is to determine the agreement of Glx and Glu measured from the OFF and DIFF spectra from two GABA-edited MEGA-PRESS sequences (GABA+, TE=68 ms, and MM-Sup, TE=80ms) with Glx and Glu measured from a SE-PRESS sequence (TE = 35 ms). While this manuscript is not 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 Glx and Glu 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 MM-Sup MEGA-PRESS data as OFF-MM-sup and DIFF-MM-sup.

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 for voxel placement (TR/TE = 7.3/2.7 ms, 1 mm^3 isotropic voxels, flip angle = 10°, TI = 600ms). Voxels were placed in the anterior cingulate cortex and the left sensorimotor cortex (Figure 1). The anterior cingulate cortex voxel was placed anterior and superior to the corpus callosum 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.[18]

For each voxel, two separate GABA-edited measurements were made using parameters described previously.[19] First, GABA+ (14 ms editing pulses; ON/OFF = 1.9/7.46 ppm; TR/TE = 1800/68 ms) and second, macromolecule suppressed (MM-Sup; 20 ms editing pulses; ON/OFF = 1.9/1.5 ppm; TR/TE = 1800/80 ms).[20] Both sequences used a voxel size of 3x3x3 cm^3, 320 averages and 4096 data points sampled at 5 kHz. This was followed by a SE-PRESS sequence (voxel size = 3x3x3 cm^3; TR/TE = 1800/35 ms; 64 averages; 4096 data points). 16 water unsuppressed acquisitions were also acquired for each sequence.

Data Processing

The FID-A toolbox[21] 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. Eddy current correction was applied and Glx and Glu were quantified relative to water using LCModel[22] for all spectra, accounting for the T2 of water at each echo time. Basis sets for quantification were simulated using the FID-A toolbox based on exact sequence timings and real RF pulse shapes. Data quality was assessed by visual inspection and metabolite linewidth; spectra with a linewidth over 0.07 ppm (9 Hz) were excluded.[23]

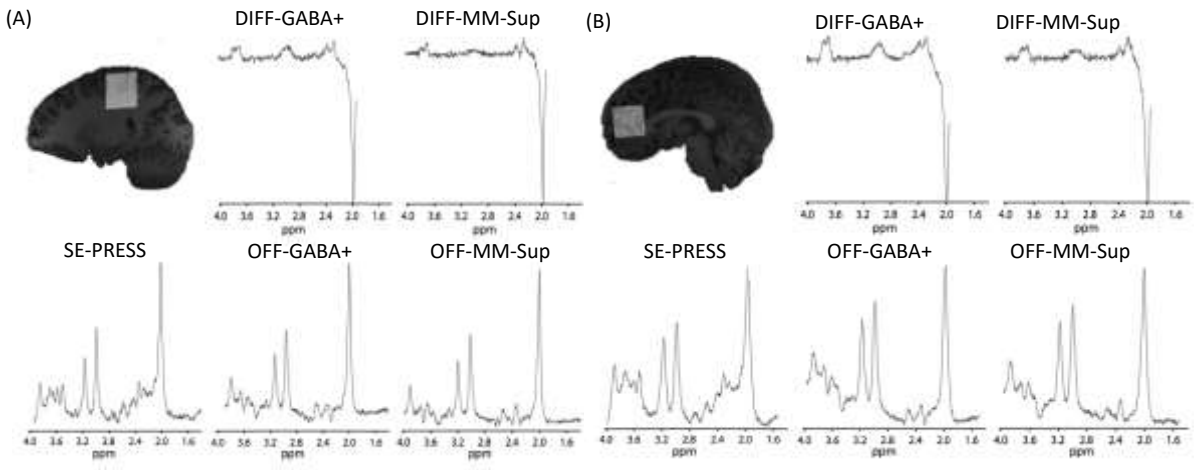

Figure 1: Voxel location and example spectra from a single volunteer in the sensorimotor cortex (A) and the anterior cingulate cortex (B).

Statistical Analysis

Statistical analyses were performed using SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Macintosh, Version 25.0. Armonk, NY: IBM Corp). To test for an effect of acquisition sequence on the mean metabolite value, repeated measures ANOVAs were used.

When assumptions of sphericity where violated, the Greenhouse-Geisser correction was applied. Post-hoc tests were conducted using the Bonferroni correction.

Correlation analysis was used as an initial step to assess the strength of the relationship between Glx and Glu measured using MEGA-PRESS compared to Glx and Glu measured from SE-PRESS. Reliability of Glx and Glu from the MEGA-PRESS data compared to the SE-PRESS data as de-facto standard (due to its common use in the field) was assessed using the intraclass correlation coefficient (ICC) and Bland-Altman plots.[24] The ICC is a modification of the Pearson correlation coefficient which takes into account both the degree of correlation and the overall agreement between measurements. A mixed-effects model was used and the level of reliability was assessed using the range of the 95% confidence intervals, values below 0.5 were considered poor reliability, values above 0.5 were considered moderate reliability, values above 0.7 were considered good reliability.[25] Bland-Altman plots were used to visualise differences between the two values[26] and were used in addition to regression analysis to test for a proportional bias.[27]

RESULTS

Data Quality

All spectra acquired from the sensorimotor cortex had sufficient quality for further analysis. Quality inspection resulted in exclusion of the following spectra from the anterior cingulate cortex data: three spectra from the SE-PRESS data set, two spectra from the GABA+ OFF data set, four spectra from the MM-Sup OFF data set, four spectra from the GABA+ DIFF data set and five spectra from the MM-Sup DIFF data set (Table 1).

Table 1: Data quality of Glx and Glu from the sensorimotor cortex and anterior cingulate cortex

		PRESS	***OFF-GABA+***	***OFF-MM-Sup***	***DIFF-GABA+***	***DIFF-MM-Sup***
SM	*n*	15	15	15	15	15
	Linewidth (Hz)	4.22 ± 0.89	4.47 ± 0.89	3.96 ± 0.64	5.50 ± 1.66	03.83 ± 1.02
	Glx Mean	6.53 ± 0.83	7.5 ± 1.13	7.2- ± 1.15	3.27 ± 0.70	4.33 ± 0.98
	CRLB Range	5-8	6-9	6-10	2-4	3-7
	Glu Mean	5.80 ± 0.77	6.87 ± 0.91	7.13 ± 0.83	5.80 ± 0.86	6.00 ± 2.67
	CRLB Range	5-7	6-9	6-9	4-7	4-15
ACC	*n*	12	13	11	11	10
	Linewidth (Hz)	6.13 ± 0.89	5.49 ± 1.28	6.01 ± 01.41	6.40 ± 1.28	6.52 ± 1.41
	Glx Mean	5.67 ± 0.89	8.77 ± 1.79	7.91 ± 2.02	3.45 ± 0.52	4.80 ± 0.79
	CRLB Range	4-7	6-12	6-11	3-4	4-6
	Glu Mean	4.67 ±0.78	8.62 ± 1.85	7.82 ± 1.83	7.18 ± 1.77	6.20 ± 1.69
	CRLB Range	4-6	6-12	6-11	4-10	4-9

Mean ± standard deviation, SM = Sensorimotor Cortex, ACC = Anterior Cingulate Cortex

Sensorimotor Cortex Data

Sequence comparison

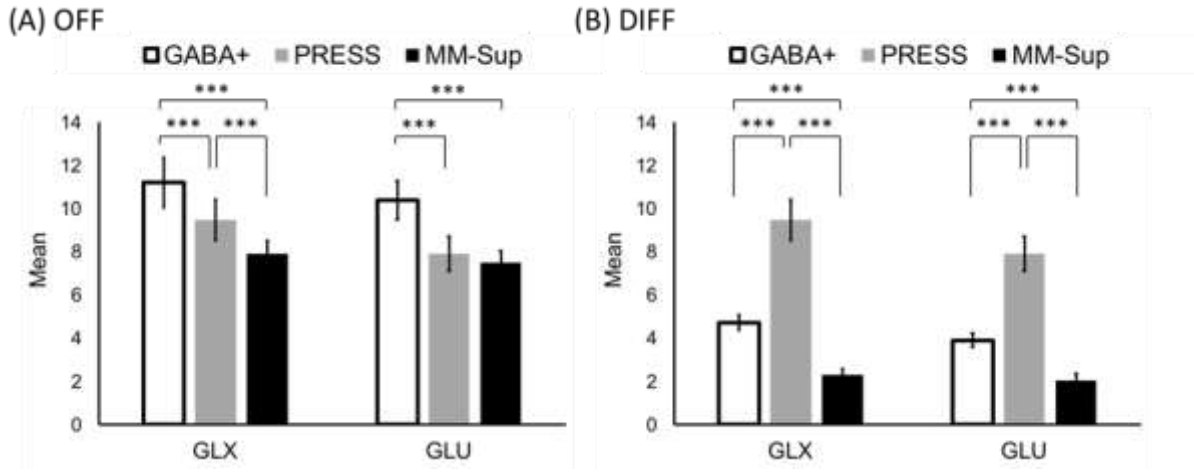

*Figure 2: Mean metabolite values using measured using the OFF sub-spectra (A) and the DIFF spectra (B) from each sequence, error bars represent standard deviation. ***p<0.001.*

There was a significant effect of sequence (SE-PRESS, OFF-GABA+, OFF-MM-Sup) on the mean of Glx measured using the OFF sub-spectra ($F(2,28)=59.150$, $p<0.001$; Figure 2A). Compared to the SE-PRESS, Glx was significantly higher when measured using OFF-GABA+ ($p<0.001$) and significantly lower using OFF-MM-Sup ($p<0.001$). There was also a significant effect of sequence on the mean of Glu measured using the OFF-sub-spectra ($F(2,28)=118.898$, $p<0.001$; Figure 2A). Glu measured using OFF-GABA+ was significantly higher ($p<0.001$) than the SE-PRESS, but there was no significant difference between Glu measured from OFF-MM-Sup and the SE-PRESS measurement ($p=0.110$).

There was a significant effect of sequence on the mean of Glx ($F(1.19,16.61)=770.014$, $p<0.001$, Greenhouse-Geisser corrected) and Glu ($F(1.320,18.477)=839.751$, $p<0.001$, Greenhouse-Geisser corrected) measured using the DIFF spectra (Figure 2B). Both the DIFF-GABA+ and the DIFF-MM-sup measures were significantly lower than the SE-PRESS ($p<0.001$). Figure 2B.

Correlation analyses

When comparing Glx measures, only Glx measured from DIFF-MM-Sup significantly correlated with Glx measured using SE-PRESS. In contrast, Glu measured using SE-PRESS significantly correlated with Glu measures from both MEGA-PRESS sequences (Figure 3).

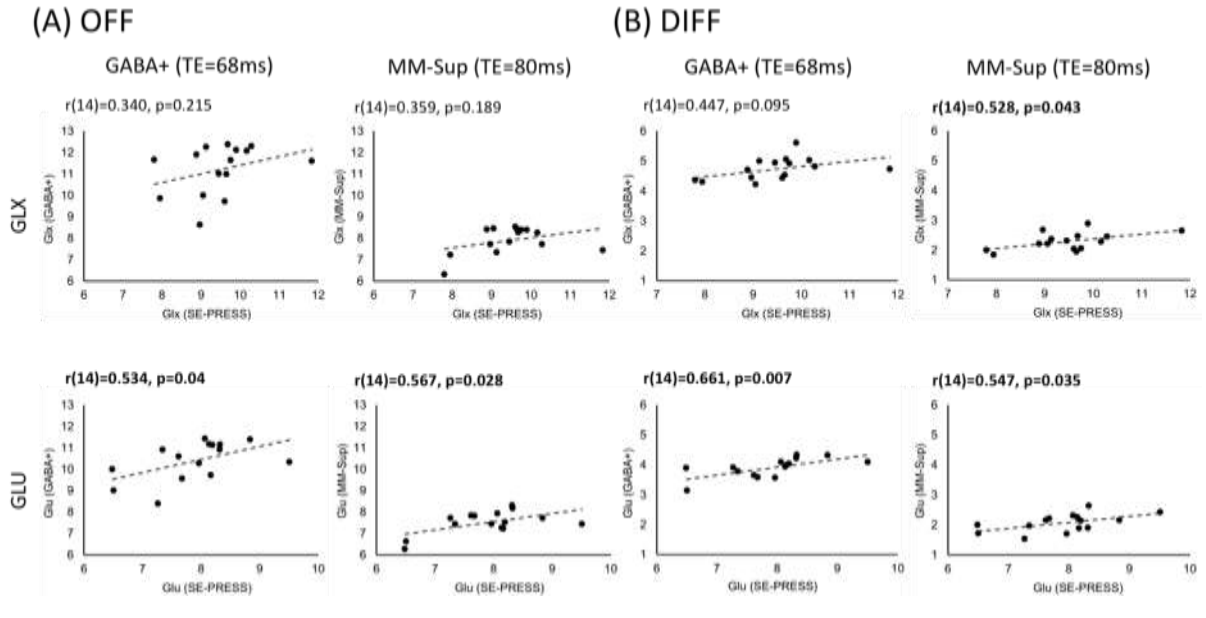

Figure 3: Scatter plots comparing metabolite values from the sensorimotor cortex measured using SE-PRESS and the MEGA-PRESS OFF sub-spectra (A) and the DIFF spectra (B).

Reliability analyses

The ICC indicates poor reliability for Glx measured using either OFF-GABA+ or OFF-MM-Sup. Glu measured using OFF-GABA+ also shows poor reliability. Glu measured using OFF-MM-Sup spectra shows poor-moderate reliability (Figure 4A). Both Glx and Glu measured from the DIFF spectra of either sequence show poor reliability (Figure 4B).

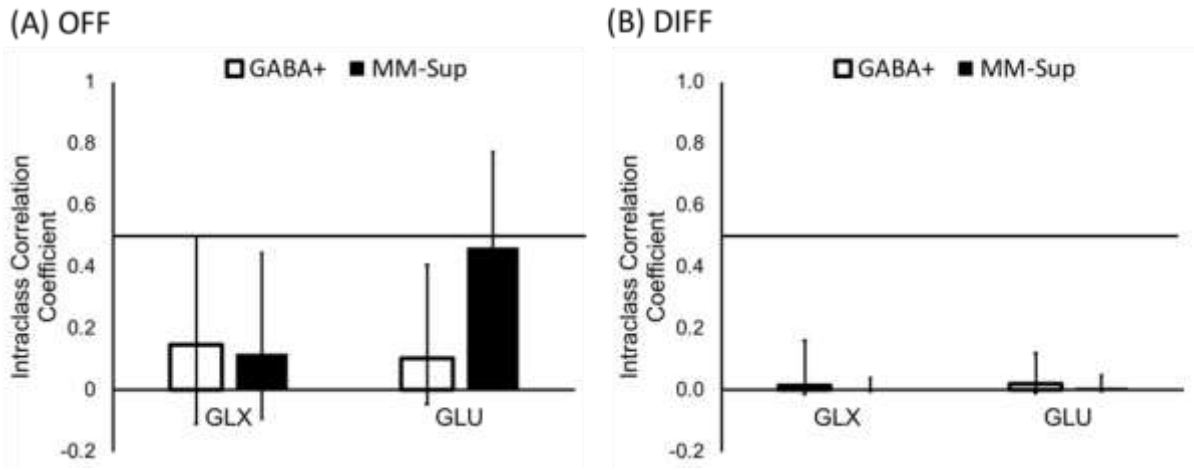

Figure 4: Intraclass correlation coefficient values and 95% confidence intervals showing the reliability between metabolite values measured from the MEGA-PRESS sequences compared to metabolite values measured from the SE-PRESS sequence. Values above 0.5 (solid line) indicate moderate reliability.

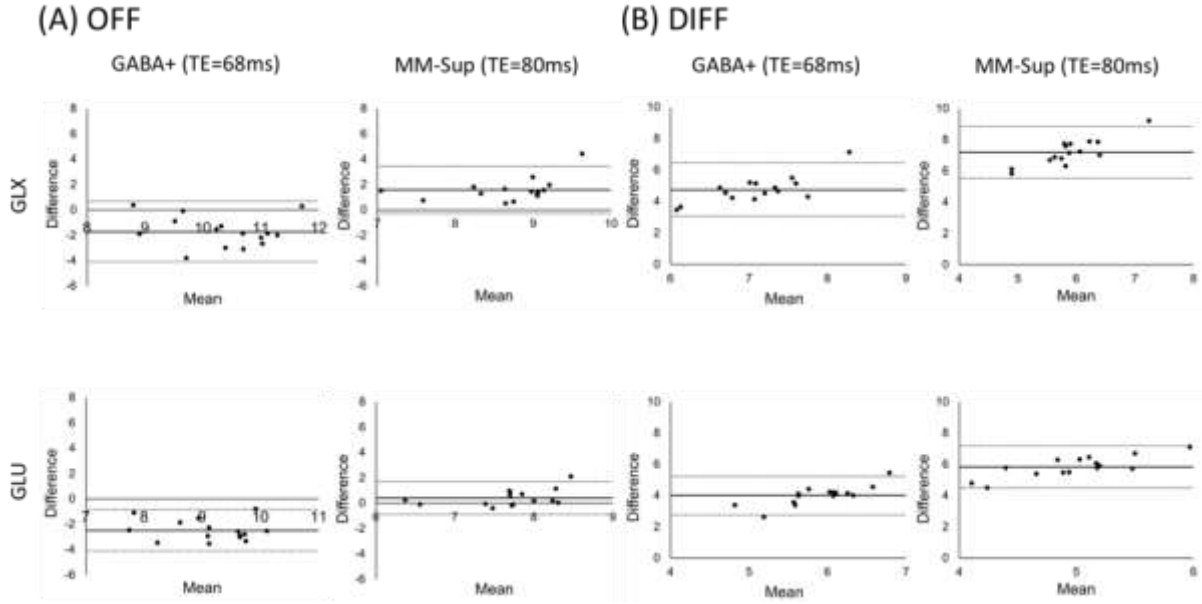

Figure 5: Bland-Altman plots comparing Glx and Glu values from the sensorimotor cortex measured using SE-PRESS and the MEGA-PRESS OFF sub-spectra (A) and DIFF spectra (B). The difference between the two paired measurements (SE-PRESS – MEGA-PRESS) is

shown on the y-axis and the mean of the two measures is shown on the x-axis. The solid lines represent the overall mean difference and the dashed lines represent the limits of agreement.

The Bland-Altman plots in Figure 5 echo the findings of the ANOVA. Glx measured using OFF-GABA+ was systematically higher than Glx measured using SE-PRESS, in contrast Glx measured using OFF-MM-Sup was systematically lower. Glu measured using OFF-GABA+ was also systematically higher than the SE-PRESS measurement, but there was no systematic bias in Glu measured from OFF MM-Sup. All measures from the DIFF spectra were systematically lower than the SE-PRESS measurements.

Regression analysis showed no proportional bias in either Glx or Glu values measured from the OFF sub-spectra of either MEGA-PRESS sequence. There was no significant relationship between the difference of the measures (OFF-SE-PRESS – OFF-MEGA-PRESS) and the mean for either MEGA-PRESS acquisition for both Glx (OFF-GABA+: ß=-0.18, p = 0.52; OFF-MM-Sup: ß=0.427, p=0.113) and Glu (OFF-GABA+: ß=-0.159, p = 0.572; OFF-MM-Sup: ß=0.441, p=0.099).

A proportional bias was seen in Glx and Glu measured using the DIFF sub-spectra from both MEGA-PRESS sequences. There was a significant relationship between the difference of the measures and the mean for both MEGA-PRESS sequences for both Glx (DIFF-GABA+: ß=0.779, p=0.001; DIFF-MM-Sup: ß=0.865, p<0.001) and Glu (DIFF-GABA+: ß=0.811, p<0.001; DIFF-MM-Sup: ß=0.813 p<0.001). This can be seen in Figure 5B, as Glx (and Glu) levels increase, the difference between the two values also increases.

Anterior Cingulate Cortex Data

Sequence Comparison

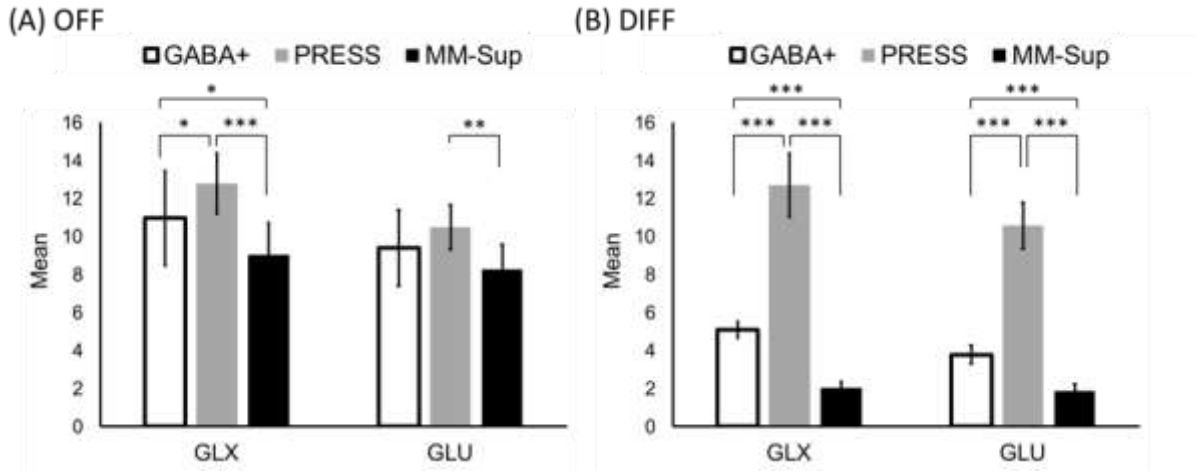

*Figure 6: Mean metabolite values using measured using the OFF sub-spectra (A) and the DIFF spectra (B) from each sequence, error bars represent standard deviation. ***p<0.001, **p<0.01, **p<0.05.*

There was a significant effect of sequence on the mean of Glx measured using the OFF sub-spectra ($F(2,18)=20.871$, $p<0.001$). Glx measured using both OFF-GABA+ ($p=0.025$) and OFF-MM-Sup ($p<0.001$) were significantly lower than the SE-PRESS ($p<0.001$). Glx measured using OFF-GABA+ was significantly higher than Glx measured using OFF-MM-Sup ($p=0.039$). There was also a significant effect of sequence on the mean of Glu ($F(2,18)=9.132$, $p=0.002$).There was no significant difference between Glu measured from OFF-GABA+ and Glu measured using SE-PRESS ($p=0.271$); however, Glu measured using OFF-MM-Sup was significantly lower that the SE-PRESS ($p=0.004$). Glu measured using OFF-GABA+ was not significantly different from Glu measured using OFF-MM-Sup ($p=0.151$; Figure 6A).

There was a significant effect of sequence on the mean of Glx ($F(1.08,8.638)=2.4.072$, $p<0.001$, Greenhouse-Geisser corrected) and Glu ($F(1.08,8.641)=445.378$, $p<0.001$,

Greenhouse-Geisser corrected) measured using the DIFF spectra. Both Glx and Glu measured using DIFF-GABA+ and DIFF-MM-Sup were significantly lower than SE-PRESS ($p<0.001$). Glx and Glu were also significantly lower when measured using DIFF-MM-Sup compared to DIFF-GABA+ ($p<0.001$; Figure 6B).

Correlation Analysis

There were no significant correlations between Glx measures. Only Glu measured from DIFF-GABA+ correlated with SE-PRESS, there were no other significant correlations between Glu measures (Figure 7).

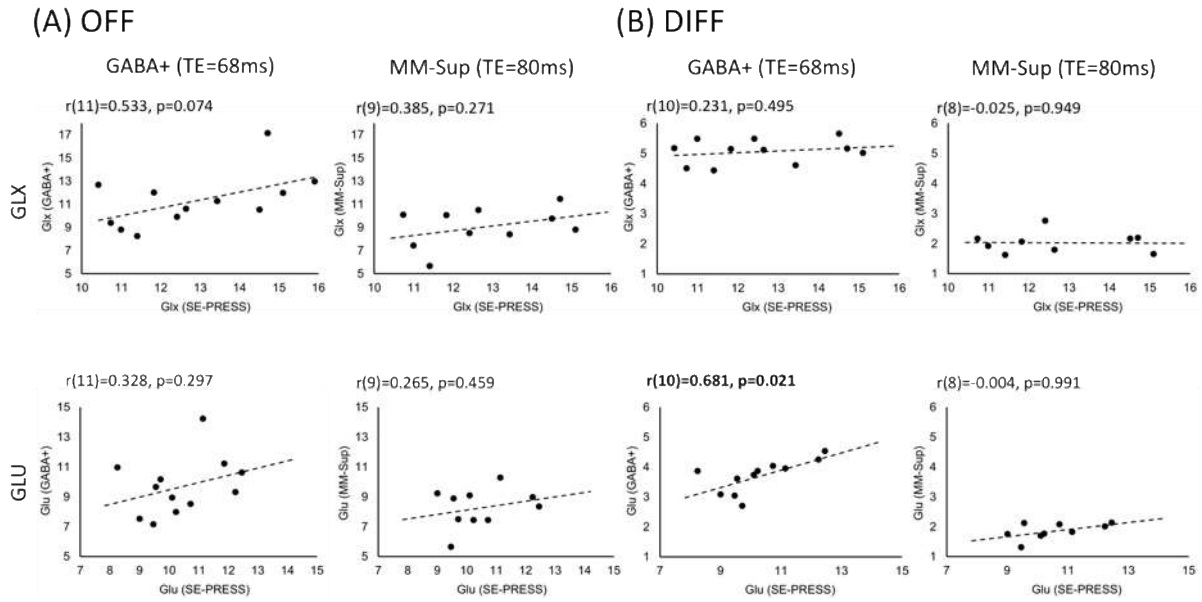

Figure 7: Scatter plots comparing metabolite values from the anterior cingulate cortex measured using SE-PRESS and the MEGA-PRESS OFF sub-spectra (A) and DIFF spectra (B).

Reliability Analysis

The ICC indicates poor-moderate reliability for Glx and Glu measured from OFF-GABA+. Glx and Glu measured from OFF-MM-Sup shows poor reliability. (Figure 8A). Both Glx and Glu measured from the DIFF spectra of either sequence show poor reliability (Figure 8B).

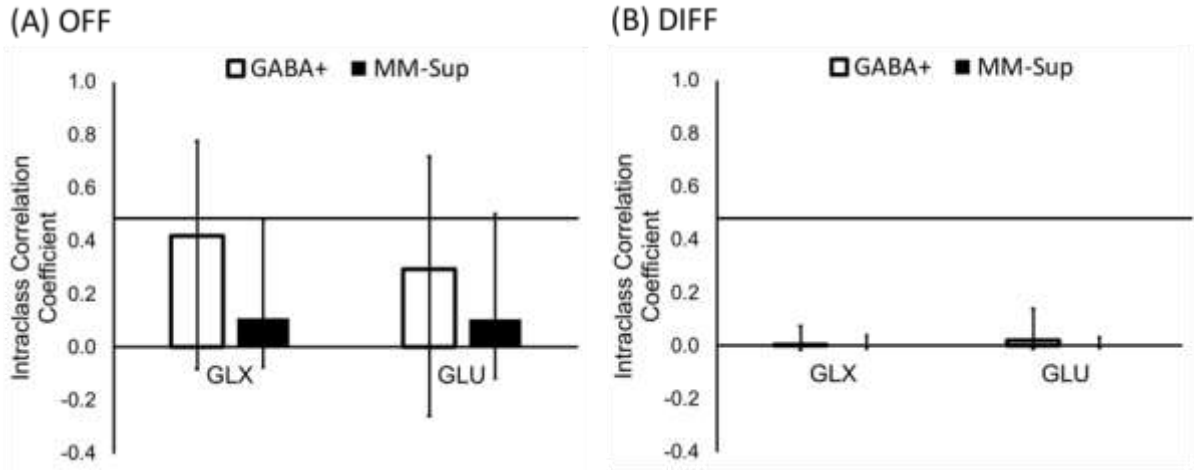

Figure 8: Intraclass correlation coefficient values and 95% confidence intervals showing the reliability between metabolite values measured from the MEGA-PRESS sequences compared to metabolite values measured from the SE-PRESS sequence. Values above 0.5 (solid line) indicate moderate reliability.

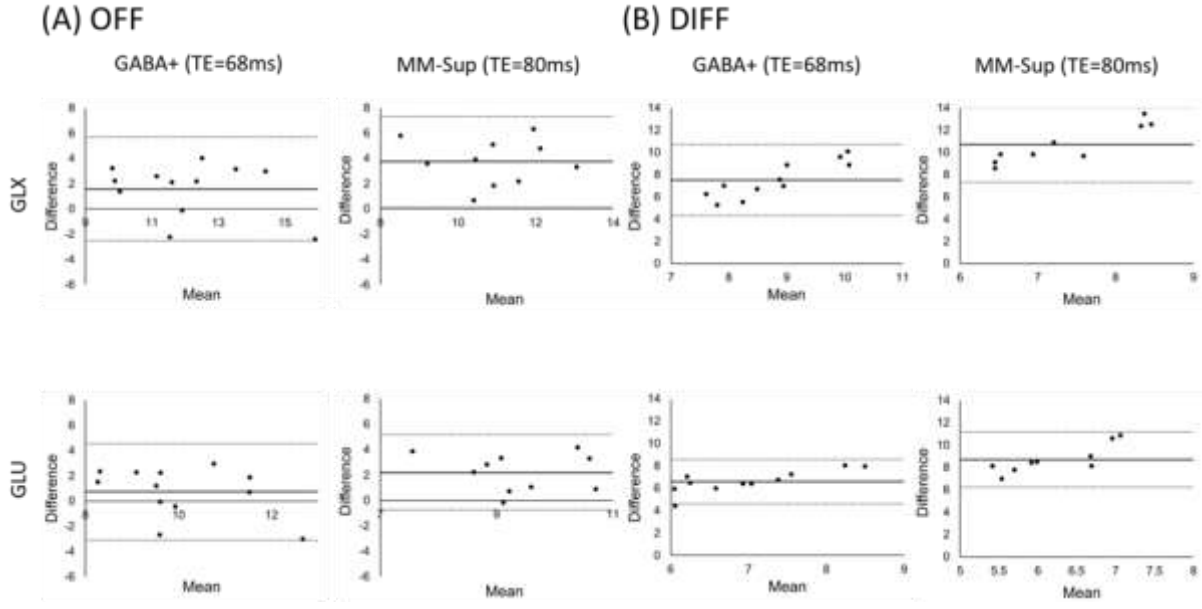

Figure 9: Bland-Altman plots comparing Glx and Glu values from the anterior cingulate cortex measured using SE-PRESS and the MEGA-PRESS OFF sub-spectra (A) and the DIFF spectra (B) from MEGA-PRESS. The difference between the two paired measurements is shown on the y-axis and the mean of the two measures is shown on the x-axis. The solid lines represent the overall mean difference and the dashed lines represent the limits of agreement.

The Bland-Altman plots in Figure 9 echo the findings of the ANOVA. Glx measured using OFF-GABA+ and OFF-MM-Sup were systematically lower than Glx measured using SE-PRESS. There was no systematic bias in Glu measured using OFF GABA+, though it should be noted that there is a large spread in the difference values. In contrast, Glu measured using OFF-MM-Sup was systematically lower than the SE-PRESS measurement. All measures from the DIFF spectra were systematically lower than the SE-PRESS measurements.

Regression analysis showed no proportional bias in either Glx or Glu values measured from the OFF sub-spectra of either the GABA+ or MM-sup sequence. There was no significant relationship between the difference of the measures (SE-PRESS – OFF-MEGA-PRESS) and the mean for either MEGA-PRESS acquisition for both Glx (OFF-GABA+: ß=-0.279, p=0.379; OFF-MM-Sup: ß=-0.055, p=0.881). and Glu (OFF-GABA+: ß=-0.388, p=0.213; OFF-MM-Sup: ß=-0.115, p=0.752).

In contrast, a proportional bias was seen in Glx and Glu measured using the DIFF sub-spectra from both MEGA-PRESS sequences. There was a significant relationship between the difference of the measures and the mean for both MEGA-PRESS sequences for both Glx (DIFF-GABA+: ß=0.891, $p<0.001$; DIFF-MM-Sup: ß=0.916, p=0.001) and Glu (DIFF-GABA+: ß=0.796, $p = 0.003$; DIFF-MM-Sup: ß=0.826, $p = 0.006$). This can be seen in Figure 9B, as Glx (and Glu) levels increase, the difference between the two values also increases.

DISCUSSION

In the present study, using an SE-PRESS (TE = 35 ms) as a de-facto standard, we used correlation and reliability analyses to examine the agreement of Glx and Glu measured from the OFF and DIFF spectra from two GABA-edited MEGA-PRESS sequences (GABA+: TE=68 ms, 14 ms editing pulses at 1.9 ppm and 7.5 ppm; MM-Sup: TE=80 ms, 20 ms editing pulses at 1.9 ppm and 1.5 ppm). Overall, Glx and Glu measured using either sequence (GABA+ or MM-Sup) or either metric (OFF sub-spectra or DIFF spectra) showed poor agreement with Glx and Glu measured using SE-PRESS.

Glx and Glu values measured using the DIFF spectra from both sequences (GABA+ and MM-Sup) in both brain areas (sensorimotor cortex and anterior cingulate cortex) were significantly lower than values from SE-PRESS. Though Glu measured from both sequences did correlate in the sensorimotor cortex, only Glu measured from DIFF-GABA+ correlated with SE-PRESS measures in the anterior cingulate cortex. Additionally, the presence of a proportional bias and the poor reliability indicated by the ICC all contribute to the conclusion that Glx and Glu measured using the DIFF spectra of GABA+ or MM-Sup acquisitions is not in agreement with SE-PRESS measures.

Our data is consistent with van Veenendaal et al. (2018) who also showed a correlation between glutamate measured from the DIFF spectrum and a SE-PRESS acquisition. However, here we build on their results and show that, while a correlation exists and the correlation between the DIFF spectrum and the SE-PRESS is stronger than the correlation of the OFF-subspectrum and the SE-PRESS, the systematic bias as well as the proportional bias present in the DIFF data make it unsuitable for substitution of a SE-PRESS acquisition. The presence of bias in the DIFF data is unsurprising because this is an edited measure. Glu and Gln are present in the SE-PRESS spectra as several multiplets (Glx: 3.75 ppm, 2.4 ppm and 2.1 ppm; Glu: 3.75 ppm, 2.3 ppm and 2.1 ppm).[28] The 1.9 ppm editing pulse used to target

GABA is broad and also partially affects the 2.1 ppm Glu multiplet which is coupled to the other multiplets.[9] As the editing pulse does not directly target the 2.1 ppm peak, the Glu peaks are only partially edited, resulting in a fraction of the possible Glu (and therefore Glx) signal present in the DIFF spectra.[8,9,12] As the levels of Glu increase, only a proportion of this increase will be reflected in the DIFF spectra, therefore, Glu (and Glx) values obtained using SE-PRESS will increase at a faster rate than Glu (and Glx) values in the DIFF spectra, resulting in a proportional bias in addition to the systematic bias. Because Gln is edited less effectively than Glu as it is further away from the editing pulse,[16] less Gln is present in the Glx signal. This may explain the stronger correlations for the Glu data compared to the Glx data. Additionally, the more selective editing pulse used in the MM-Sup acquisition results in less Glx and Glu in the DIFF spectra of MM-Sup data compared to GABA+.

Glx measured using the OFF sub-spectra from both sequences (GABA+ and MM-Sup) in both regions was significantly different to values from the SE-PRESS sequence and did not correlate with SE-PRESS measures. In both locations with both sequences, Glx showed poor reliability as assessed by ICC analysis with the exception of Glx measured using OFF GABA+ from the anterior cingulate cortex, which was only slightly better showing poor-moderate reliability. Taken together Glx measured using the OFF sub-spectra of GABA+ or MM-Sup acquisitions is also not in agreement with SE-PRESS measures.

In the sensorimotor cortex, Glu measured using OFF-GABA+ was significantly higher than SE-PRESS measures while there was no significant difference between Glu measured using OFF-MM-Sup and SE-PRESS. In contrast, in the anterior cingulate cortex data, there was no significant difference between Glu measured using OFF-GABA+ and SE-PRESS, howeverGlu measured using OFF-MM-Sup was significantly lower. In the sensorimotor cortex, The OFF-spectrum Glucorrelated with the SE-PRESS data using both sequences, but neither correlation was seen in the anterior cingulate data. In the sensorimotor cortex, ICC

analysis showed poor reliability between Glu measured using OFF-GABA+ and SE-PRESS, likely due to the systematic bias preset. Glu measured using OFF-MM-Sup showed poor-moderate reliability. In contrast to the sensorimotor cortex, Glu measured in the anterior cingulate cortex showed poor-moderate reliability using OFF GABA+ and poor reliability using OFF-MM-Sup.

The results suggest that, in areas of high signal-to-noise ratio such as the sensorimotor cortex, Glu measured using OFF-MM-Sup could potentially be used as a substitute for a SE-PRESS acquisition, although caution is required if wishing to to compare with the literature values. However, this method should not be applied in areas of low signal-to-noise ratio such as the anterior cingulate cortex.

This difference between glutamate measures from the GABA+ and MM-Sup sequences is likely due to the phase evolution from j-coupling at the various echo times. Using simulated data, Mullins et al. (2008) showed a decrease in both the Glu and Gln peak intensity at TE=60-70ms.[6] The suppression of both Glu and Gln in the GABA+ OFF-spectra (TE=68 ms) results in Glx and Glu values that are not in agreement with Glx and Glu obtained at SE-PRESS (TE=35 ms). At TE=80 ms, Gln is suppressed, whilst Glu is at a a similar intensity to the signal at TE=30-40 ms.[6] Indeed, Schubert et al. (2004) showed that, at TE=80 ms, the Glx peaks from in vivo data resembled that of a Glu spectra obtained from a phantom.[29] Our significant correlation of Glu (but not Glx) from the OFF-MM-sup (TE = 80 ms) with the SE-PRESS data is consistent with the characteristic signal evolution of Glu across TEs. 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.[30] At TE=80 ms there is increased accuracy for separating Glu and Gln, based on the reasons discussed above, and therefore can be used in a PRESS sequence to more accurately quantify Glu. The signal intensity for Gln at TE=68 ms is close to that at TE=80ms,[6] therefore strong correlations

between Glx measures would be expected. SE-PRESS was chosen for the present study as this is the most commonly used PRESS sequence owing to minimal signal loss from T_2 relaxation, which increases the SNR, and therefore can be used to collect data on a variety of metabolites.[7] However, based on the evidence discussed above, this is not the optimal TE for detecting glutamate. Therefore, it is feasible that the Glx and Glu values from the MEGA-PRESS data are more accurate than those from the SE-PRESS data. Hancu (2009) showed both Glu and Glx had lower coefficients of variation and lower Cramer-Rao Lower Bounds when measured using SE-PRESS (TE=35 ms) compared to TE=80 ms,[31] suggesting that data acquired at TE=35 ms is more reproducible than data acquired at TE=80 ms.

The ICCs for Glx and Glu from the OFF-GABA+ in the anterior cingulate cortex were higher than those in the sensorimotor cortex. Initially, this was surprising due to the lack of correlation of the MEGA-PRESS data with the SE-PRESS data in the anterior cingulate cortex. It may be due to the calculation of the ICC; the ICC represents a comparison of the within-subject variance and the between subject variance, a small within subject variance will lead to a larger ICC. However, if the between subject variance increases but the within subject variance remains the same, the ICC will also increase.[32] The range of data is greater in the anterior cingulate cortex than the sensorimotor cortex, and higher for Glx and Glu measured using both the OFF- and DIFF-GABA+ compared to MM-Sup. When combined with the information from the correlation analysis and the Bland-Altman plots, Glx and Glu measured using the OFF sub-spectra are in higher agreement with SE-PRESS data in the sensorimotor cortex compared to the anterior cingulate cortex. Thus, we suggest the larger ICCs for Glx and Glu from the OFF-GABA+ anterior cingulate cortex data must not be interpreted in isolation and are likely to be a result of the dynamic range of these data.

A limitation of this study is that the order of the sequences was not counterbalanced. Therefore, there was always a time delay between the GABA+ acquisition and the SE-

PRESS, as the GABA+ acquisition was followed by MM-Sup. This means there was a higher chance of movement between GABA+ and SE-PRESS than between MM-Sup and SE-PRESS. Additionally, the sensorimotor cortex was always scanned after the anterior cingulate cortex. To compensate for this, the SE-PRESS could be acquired between the two MEGA-PRESS scans, with the order of the MEGA-PRESS scans and the order of the brain regions scanned counter balanced across participants. Lastly, our sample size was small and included a healthy, male population. Quantification of Glx and Glu may be less accurate in clinical populations, such as those with stroke, which may affect signal quality.

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 MM-Sup data and Glx and Glu measured using SE-PRESS. Therefore, we recommend that a separate SE-PRESS 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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