Glutamate quantification by PRESS or MEGA-PRESS

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Original contribution

Glutamate quantification by PRESS or MEGA-PRESS: Validation, repeatability, and concordance

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ABSTRACT

Purpose: While PRESS is often employed to measure glutamate concentrations, MEGA-PRESS enables simultaneous Glx (glutamate and glutamine) and GABA measurements. This study aimed to compare validation, repeatability, and concordance of different approaches for glutamate quantification at 3 T to aid future studies in their selection of the appropriate sequence and quantification method.

Materials and methods: Nine phantoms with different glutamate and glutamine concentrations and five healthy participants were scanned twice to assess respectively the validation and repeatability of measurements with PRESS and MEGA-PRESS. To assess concordance between the different methods, results from 95 human participants were compared. PRESS, MEGA-PRESS (*i.e.* difference), and the MEGA-PRESS OFF spectra were analyzed with both LCModel and Gannet.

Results: In vitro, excellent agreement was shown between actual and measured glutamate concentrations for all measurements ($r > 0.98$). *In vivo* CVs were better for PRESS (2.9%) than MEGA-PRESS (4.9%) and MEGA-PRESS OFF (4.2%). However, the concordance between the sequences was low (PRESS and MEGA-PRESS OFF, $r = 0.3$) to modest (MEGA-PRESS *versus* MEGA-PRESS OFF, $r = 0.8$).

Conclusion: Both PRESS and MEGA-PRESS can be employed to measure *in vivo* glutamate concentrations, although PRESS shows a better repeatability. Comparisons between *in vivo* glutamate measures of different sequences however need to be interpreted cautiously.

1. Introduction

Clinical studies show increasing interest in *in vivo* measurements of glutamate levels, the most abundant excitatory neurotransmitter in the central nervous system [1,2]. Altered glutamate concentrations have been observed in several neurological and psychiatric brain diseases, such as epilepsy [3] or schizophrenia [4], and are related to cognitive or behavioral functioning [5]. Besides its function as neurotransmitter, glutamate is involved in several metabolic pathways and its concentration has been shown to be related to excitatory activity [1].

In vivo measurements of glutamate levels are enabled by proton MR spectroscopy (^{1}H MRS). However, due to J-coupling, the resonance frequency of glutamate resolves with a split pattern. Furthermore, its peaks are overlapped by other metabolites such as *N*-acetyl-aspartate and glutamine, making it challenging to measure glutamate levels, despite its high abundance in the brain [6]. As glutamate is especially difficult to disentangle from its precursor glutamine, the estimates of the two metabolites are often combined 'Glx'. However, the Glx approach makes the results of studies investigating glutamate ambiguous to interpret as possible shifts in balances between glutamate and glutamine may remain undetected [2,7].

Dedicated sequences have been developed to assess *in vivo* glutamate concentrations, including spectral editing of glutamate with multiple quantum coherence methods [8–10] or TE averaged point-resolved spectroscopy (TE averaged PRESS) [2,11]. Unfortunately, these sequences have several disadvantages: scan times are often too long for clinical applications, they might not be available or difficult to use on clinical MR scanners, or they only enable glutamate measurements, but no other possibly interesting neurometabolites. Glutamate concentrations are therefore frequently derived with commonly applied

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localized single voxel ^{1}H MRS such as PRESS, possibly with optimized TE for measuring glutamate [2]. To distinguish glutamate from other, overlapping metabolites, prior knowledge fitting can be applied, as for instance implemented in LCModel [12].

Recently, glutamate measurements have often been acquired with MEGA-PRESS [13,14]. MEGA-PRESS is a J-editing technique commonly used to measure the main inhibitory neurotransmitter γ-aminobutyric acid (GABA), which cannot be derived from standard PRESS scans due to overlapping metabolites. MEGA-PRESS includes editing pulses, selectively editing the GABA signal at 1.9 ppm, which refocuses the GABA signal at 3 ppm [15]. Also off-resonance spectra are acquired, including an editing pulse at 7.46 ppm, which is not expected to have an effect on the spectrum. The GABA concentration can be derived by subtracting these "OFF" spectra from the "ON" spectra (*i.e.* with editing pulse at 1.9 ppm). Although not originally designed for this purpose, MEGA-PRESS has also been used to estimate glutamate or Glx concentrations [16–19]. Glutamate and glutamine are chemically related to GABA and are known to co-edit with the MEGA-PRESS sequence, resulting in an additional Glx peak in the difference spectrum. Due to the editing, this Glx signal is no longer overlapped by other metabolites (in contrast with the PRESS sequence) and the area under this peak can simply be integrated to have an estimation of the Glx concentration. Some authors claim that this peak constitutes only glutamate [18,20,21], while others claim that glutamate and glutamine can be distinguished in the analysis of the spectra by using prior knowledge fitting [22].

Both PRESS and MEGA-PRESS sequences provide opportunities to investigate glutamate and glutamine concentrations. In addition, glutamate can also be acquired using only the spectra from the MEGA-PRESS acquisition without editing pulse. In clinical studies, time constraints often limit the number of sequences that can be used. When MEGA-PRESS is already applied for GABA quantification, one can wonder whether a separate PRESS scan is still needed for adequate glutamate quantification. However, despite the wide use of these techniques in literature, validation is limited to a small *in vitro* study at 4 T and a few reproducibility studies [22–26]. To our knowledge no comparisons between different *in vivo* approaches have been made. This study therefore aims to compare glutamate quantification for PRESS and MEGA-PRESS both in phantom as well as in *in vivo* with measurements at 3 T, and to investigate the claim that the Glx peak constitutes mainly glutamate, to aid future clinical studies in their selection of appropriate sequences and analysis methods.

2. Material and methods

2.1. Approach

This study consisted of three different experiments. The first experiment, a phantom study, aimed to determine and compare the validation and repeatability of PRESS and MEGA-PRESS for the quantification of glutamate (and glutamine) concentrations. The claim that the Glx peak signal from the MEGA-PRESS (*i.e.* difference) spectrum constitutes predominantly glutamate was also tested [20]. The second experiment aimed to assess the *in vivo* repeatability of the different methods. The third experiment aimed to assess concordance of glutamate (and glutamine) quantification of different methods in a large population.

2.2. MR measurements

All MR measurements were performed on a 3 T MR scanner (Philips Achieva TX, Philips Healthcare, the Netherlands) with a 32 channel head coil. MR spectroscopy included a MEGA-PRESS and a PRESS (Table 1) sequence for both the phantoms and human participants. A standard shim procedure was applied, optimizing the line width for the individual experiments. PRESS was performed with a TE of 35 ms, the shortest TE at our clinical scanner and commonly applied in clinical studies. The PRESS and MEGA-PRESS measurements were performed directly after each other (with and without water suppression) (Table 1). The excitation pulse for the MEGA-PRESS is a minimum-phase excitation pulse with a bandwidth of ~2 kHz, the refocusing pulses have a bandwidth of ~1.3 kHz. The sequence is shown in full in Fig. 1 of [27]. The CSDE between the GABA signals for the refocusing pulses is approximately 10%. The order of these scans was alternated between the phantoms in Experiment 1, while in Experiment 2 and 3, all measurements started with PRESS. A 3x3x3 cm^3 voxel-of-interest was placed, which was not repositioned between the scans. In the human participants, this voxel was positioned in the occipito-parietal lobe (Fig. 1), which was located on the T1-weighted image (1 mm cubic voxel, fast field echo sequence, TR/TE/TI 8.1/3.7/1008.6 ms, 170 sagittal slices).

2.3. Spectral analysis

The MRS measurements resulted in three different spectra: the PRESS spectrum and the two subspectra from the MEGA-PRESS scan, namely a spectrum with an editing pulse at 1.9 ppm ("ON") and a spectrum with an editing pulse at 7.46 ppm, which is not expected to have an effect on the spectrum ("OFF", *i.e.* a PRESS spectrum with TE 68 ms). Difference spectra were obtained by subtraction of "ON" and "OFF" spectra. The PRESS spectrum, MEGA-PRESS OFF, and MEGA-PRESS difference spectra were all analyzed with LCModel version 6.3-1 L (Fig. 2), which analyzes the spectrum as a linear combination of different simulated metabolite spectra [12]. Simulated basis sets were applied (kindly provided by Dr. Provencher), with sixteen simulated metabolites in the analyses of the PRESS and MEGA-PRESS OFF spectra, and six in the analyses of the MEGA-PRESS difference spectra. The basis sets were simulated using the gamma C++ library using published values for chemical shifts and J-couplings [6], updated with more accurate values [28]. Chemical shift displacement was not taken into consideration. In addition, thirteen simulated basis spectra were added to account for macromolecule and lipid contributions for PRESS and MEGA-PRESS OFF. For the phantom measurements, only simulated

Table 1
Procedures for MR spectroscopy.

Acquired spectrum	Sequence details	Analysis
PRESS	PRESS sequence: TE/TR: 35/2000 ms, 128 averages, VAPOR water suppression Separately acquired scan without water suppression: TE/TR: 35/2000 ms, 8 averages Total scan duration: 5 min	LCModel
MEGA-PRESS difference	MEGA-PRESS sequence: TE/TR 68/2000 ms, 320 averages, editing pulses (bandwidth 50 Hz) at 1.9 (ON) and 7.46 ppm (OFF), interleaved in 40 blocks, MOIST water suppression Separately acquired scan without water suppression: TE/TR 68/2000 ms, 8 averages Total scan duration: 11 min	LCModel Gannet
MEGA-PRESS OFF	MEGA-PRESS sequence: TE/TR 68/2000 ms, 160 averages, editing pulse at 7.46 ppm, MOIST water suppression Separately acquired scan without water suppression: TE/TR 68/2000 ms, 8 averages Total scan duration: 11 min	LCModel

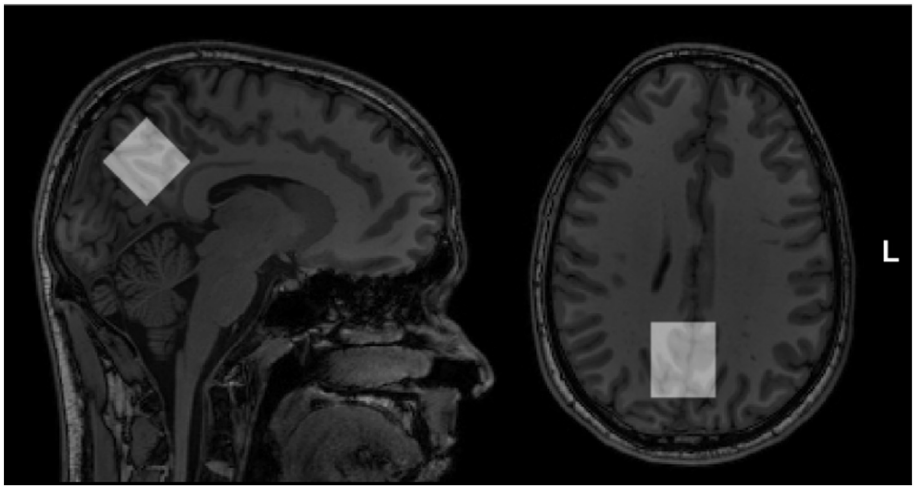

Fig. 1. Voxel placement in the *in vivo* experiments. L: left.

spectra of glutamate, glutamine, GABA, creatine, inositol, *N*-acetylaspartate, and phosphorylcholine were included in the basis set. Settings of LCModel were as described in the LCModel manual [29]: spectra were analyzed from 0.2 to 4.0 ppm for PRESS and MEGA-PRESS OFF and from 1.95 to 4.2 ppm for the MEGA-PRESS scans. Eddy current correction was included in all analyses; the MEGA-PRESS option was applied in the analyses of the MEGA-PRESS data. Frequency and phase correction is implemented in LCModel and applied to the averaged spectrum. Gannet (version 2.0) was applied to estimate the surface area of the 'Glx peak' in the MEGA-PRESS difference spectrum using a double Gaussian fit (Fig. 2) [30]. Preprocessing in Gannet included frequency and phase correction, including spectral registration between each block of subspectra [31,32]. The concentrations were considered relative to the separately acquired water signal. No correction for tissue composition was included, as only within-subject variations within the same voxel were compared. All statistical analyses described in the following paragraphs were performed with R version 3.2.2 [33].

2.4. Experiment 1

Nine 350 mL phantom solutions were made with a stock solution as described in the LCModel manual [29], including sodium azide, DSS, sodium formate, and a phosphate buffer. The stock solution furthermore contained physiological concentrations of creatine (7.8 mM), myo-Inositol (5.9 mM), *N*-acetylaspartate (10.3 mM) and choline (1.7 mM) [6]. Glutamate, glutamine and GABA were added to the stock solution in five different concentrations, which were based on physiological concentrations, *i.e.* approximately 6.0–12.5 mM, 3.0–5.8 mM, and 1.3–1.9 mM for glutamate, glutamine, and GABA, respectively [6]. The concentrations varied from 0 (1 phantom), within the physiological range (3 phantoms), to twice the mean value of the physiological range (1 phantom, Table 2). The pH of the solutions was set to 7.2 using NaOH.

All phantoms were prepared on the day of the MR experiments. Each phantom was measured twice and temperature-controlled at 36.5–38.0 °C using a water bath which was continuously refilled with water heated outside the MR room.

To assess the validation, the Pearson correlation coefficient was calculated between actual and measured concentrations of glutamate, glutamine, Glx, and GABA. To assess relative contributions of glutamate, glutamine, and GABA concentrations to the Glx peak in the MEGA-PRESS spectrum, linear regression analysis was applied with the measured Glx peak as dependent variable, and present metabolite concentrations as independent variables. In both analyses, mean concentrations of the two repeated measurements were considered.

2.5. Experiment 2

To assess *in vivo* repeatability, five healthy participants (age 29 ± 4 year, four male) were recruited. All participants provided written informed consent before participation in the experiment. The MR spectroscopy protocol was directly repeated to limit physiological variations within the participants. In this experiment, the coefficient of variation (CV) was calculated, which was defined as the $\frac{1}{n}\sum_{i=1}^{n}(SD_i/\overline{x}_i)$, with i the participant, n the number of participants (*i.e.* $n = 5$), SD_i the sample standard deviation of the two repeated measurements, and $\overline{x}_i$ the average of the two measurements. Additionally, Bland-Altman analyses were performed. Therefore, the standard deviation of the difference between the two repeated measurements (s_c) was computed [34]. The 95% limits of agreement (LoAs) were equal to $1.96 \cdot s_c$.

Three different CVs and LOAs for glutamate, glutamine, Glx, and GABA were obtained based on the PRESS, MEGA-PRESS OFF, and MEGA-PRESS difference spectra, all analyzed with LCModel. In addition, a fourth CV and LoA (only Glx and GABA) was calculated based on the MEGA-PRESS difference spectrum analyzed with Gannet.

2.6. Experiment 3

Concordance between the different methods was tested using MRS data of 106 participants, who were included in a study on type 2 diabetes mellitus [35]. The study population comprised 41 healthy participants, 47 patients with type 2 diabetes, and 18 patients with metabolic syndrome (which is considered a prestage of type 2 diabetes). Fifty-nine percent of the participants were male (41% female) and the age of all participants was 63 ± 8 years (mean ± std). The study was approved by the local medical ethical committee and all participants gave their written informed consent. This study is registered at clinicaltrials.gov (with identifier NCT01705210).

To test concordance between the three spectra, the Pearson's correlation coefficient was calculated. Similar to Experiment 2, the correlation between four different measurements was calculated. In addition, correlations between glutamate and Glx estimates were calculated, as Glx estimates are often used as a proxy for glutamate estimates.

3. Results

3.1. Experiment 1

All estimated concentrations can be found in Table S1. The obtained line widths were 4.67 ± 0.97 Hz and 7.23 ± 1.64 Hz, for PRESS and MEGA-PRESS in the phantom experiments, respectively. With a Pearson's correlation coefficient larger than or equal to 0.98 in all

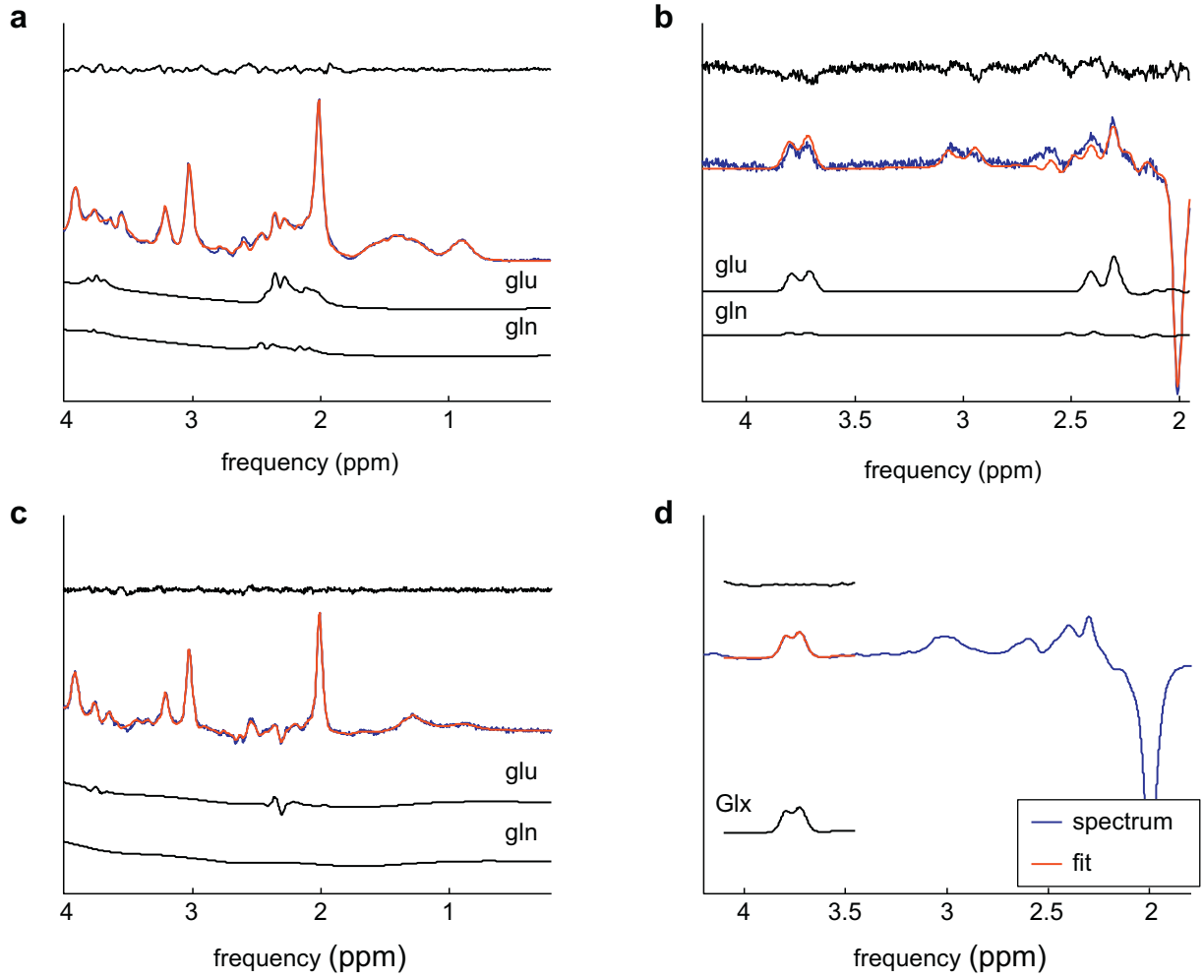

Fig. 2. Examples of the different spectra measured *in vivo*. LCModel analyses of the PRESS (a), MEGA-PRESS (b), and MEGA-PRESS OFF scans (c), which show the measured spectrum (blue), total fit (red), individual glutamate (glu) and glutamine (gln) estimates (black, on bottom), and residuals (black, on top). An example of the Gannet analysis of a MEGA-PRESS scan is displayed in d, in which the spectrum (after frequency and phase corrections), Glx fit, and residuals are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Glutamate, glutamine and γ-aminobutyric acid (GABA) concentrations in the different phantoms.

Phantom	Glutamate (mM)	Glutamine (mM)	GABA (mM)
1	18.2	0.0	0.0
2	0.0	9.2	0.0
3	0.0	0.0	3.5
4	6.2	4.5	1.9
5	6.0	6.1	1.6
6	9.0	2.9	1.9
7	9.0	6.0	1.3
8	12.1	3.0	1.5
9	12.0	4.6	1.3

measurements ($p < 0.001$), the individual LCModel estimates showed a high correlation with the prepared glutamate, glutamine, Glx, and GABA concentrations, except for the GABA estimates in PRESS ($r = 0.55$, $p = 0.12$) and MEGA-PRESS OFF ($r = 0.88$, $p = 0.002$).

The regression analysis, performed to assess the relative contribution of glutamate, glutamine, and GABA to the Glx peak measured with MEGA-PRESS, showed significant associations of both glutamate and glutamine with this Glx peak (Table 3). The regression coefficients were comparable, indicating that the estimated glutamate/glutamine ratio is similar in the Glx peak as the actual proportion. GABA was not significantly associated to the Glx peak.

Table 3
Results of the linear regression analysis with the measured Glx peak (Glx as measured with MEGA-PRESS and analyzed with Gannet) as dependent variable, and the known phantom concentrations as dependent variables. The table shows the (not standardized) regression coefficient (β) and its 95%-confidence interval.

	β	95%-CI
[Glutamate]	0.106*	0.093-0.119
[Glutamine]	0.087*	0.061-0.112
[GABA]	− 0.001	− 0.040-0.037

* $p \leq 0.001$.

3.2. Experiment 2

As five participants were scanned twice, 5 × 2 PRESS and MEGA-PRESS difference spectra were obtained. All acquired spectra had sufficient quality for further analyses (visually inspected) and had an SNR ≥ 20, although not all metabolites could be detected in all spectra. The obtained line widths were 10.30 ± 2.98 Hz and 12.66 ± 1.86 Hz for the *in vivo* PRESS and MEGA-PRESS experiments, respectively. In the PRESS scan, the CRLBs were ≤ 5 for glutamate and Glx, and ≤ 20 for glutamine. Glutamate and Glx had CRLBs ≤ 7 and ≤ 5 in MEGA-

Table 4
Repeatability of *in vivo* measurements ($n = 5$) with PRESS, MEGA-PRESS OFF, and MEGA-PRESS. The Limits of Agreement are displayed relative to the mean concentrations (as can be found in Table S1). All concentrations are estimated with LCModel, unless indicated differently.

	Glutamate	Glutamine	Glx	GABA +[a]
Coefficient of variation				
PRESS	2.9%	8.8%	3.1%	NR
MEGA-PRESS OFF	4.2%	9.7%[b]	4.5%	NR
MEGA-PRESS	4.9%	NR	4.3%	6.1%
MEGA-PRESS (Gannet)	–	–	5.2%	8.1%
Limits of agreement				
PRESS	1 ± 9%	−3 ± 34%	0 ± 12%	NR
MEGA-PRESS OFF	−1 ± 13%	−12 ± 24%[b]	−3 ± 13%	NR
MEGA-PRESS	−1 ± 19%	NR	−6 ± 9%	−2 ± 21%
MEGA-PRESS (Gannet)	–	–	−5 ± 21%	−9 ± 31%

NR: no reliable estimates could be given for these metabolites.
[a] GABA including possible macromolecules, which can add up to 50% of the total GABA + signal [34].
[b] Based on four out of five participants.

PRESS scans (OFF and difference spectra, respectively). Quantification of glutamine failed in seven out of ten difference spectra, and in one out of ten OFF spectra (zero glutamine detected), which were subsequently excluded from further analyses. In none of the participants two glutamine estimations from MEGA-PRESS difference were available, therefore these results were further disregarded. In the OFF spectra of four remaining participants, the CRLB of glutamine was between 20 and 30.

GABA could only be estimated from the MEGA-PRESS difference spectra and had a CRLB ≤ 6 in the LCModel analyses. In the MEGA-PRESS scan, the average absolute drift of the water peak between two subsequent blocks was 0.22 ± 0.08 Hz.

CVs and LoAs of the *in vivo* experiments are displayed in Table 4. The lowest CV was shown in the PRESS spectrum, while the CVs were comparable between the MEGA-PRESS difference and OFF spectra. The Bland-Altman analyses showed, in case of glutamate, the most narrow LoA for the PRESS spectrum and larger LoAs for both MEGA-PRESS difference spectra (Fig. 3). For Glx, the LoA was most narrow in the MEGA-PRESS difference spectrum (Table 4). Except for the MEGA-PRESS difference spectra, all LoAs were more narrow for glutamate than for Glx.

3.3. Experiment 3

Data of 95 participants was included in this experiment. In five of the 106 participants, no complete PRESS/MEGA-PRESS data sets were acquired. In another six participants, data was excluded for insufficient spectral quality ($n = 3$) and because of high drifts in the MEGA-PRESS scans ($n = 3$, > 2 ppm difference in resonance frequency of the water peak between two subsequent blocks).

In the included 95 participants, CRLBs of glutamate and Glx were ≤ 6 in the PRESS scan and ≤ 20 in both MEGA-PRESS scans. Again, quantification of glutamine was difficult, with only 72 participants with a CRLB ≤ 20 in the PRESS scan, nineteen in the MEGA-PRESS scan and five in the MEGA-PRESS OFF scan. Therefore, glutamine was disregarded in further analyses. The average absolute drift of the water peak was $4.2 \cdot 10^{-3} \pm 4.9 \cdot 10^{-3}$ ppm between two subsequent blocks in the MEGA-PRESS scan.

The Pearson's correlation coefficients, calculated between glutamate and Glx as measured with the different methods, are displayed in Table 5. The concordance between PRESS and MEGA-PRESS was low, albeit more beneficial for glutamate than for Glx (Fig. 4). Furthermore, the concordance between the two spectra (difference and OFF) acquired with the MEGA-PRESS sequence was high, and better than between the PRESS and MEGA-PRESS sequence. Strikingly is the high correlation of glutamate and Glx in the MEGA-PRESS OFF scan, compared with this correlation in the PRESS or MEGA-PRESS scans. Finally, the correlation of the Glx estimate from GANNET and LCModel is modest (r = 0.65, p < 0.001), but was expected to be higher considering the fact that exactly the same data were analyzed with different programs.

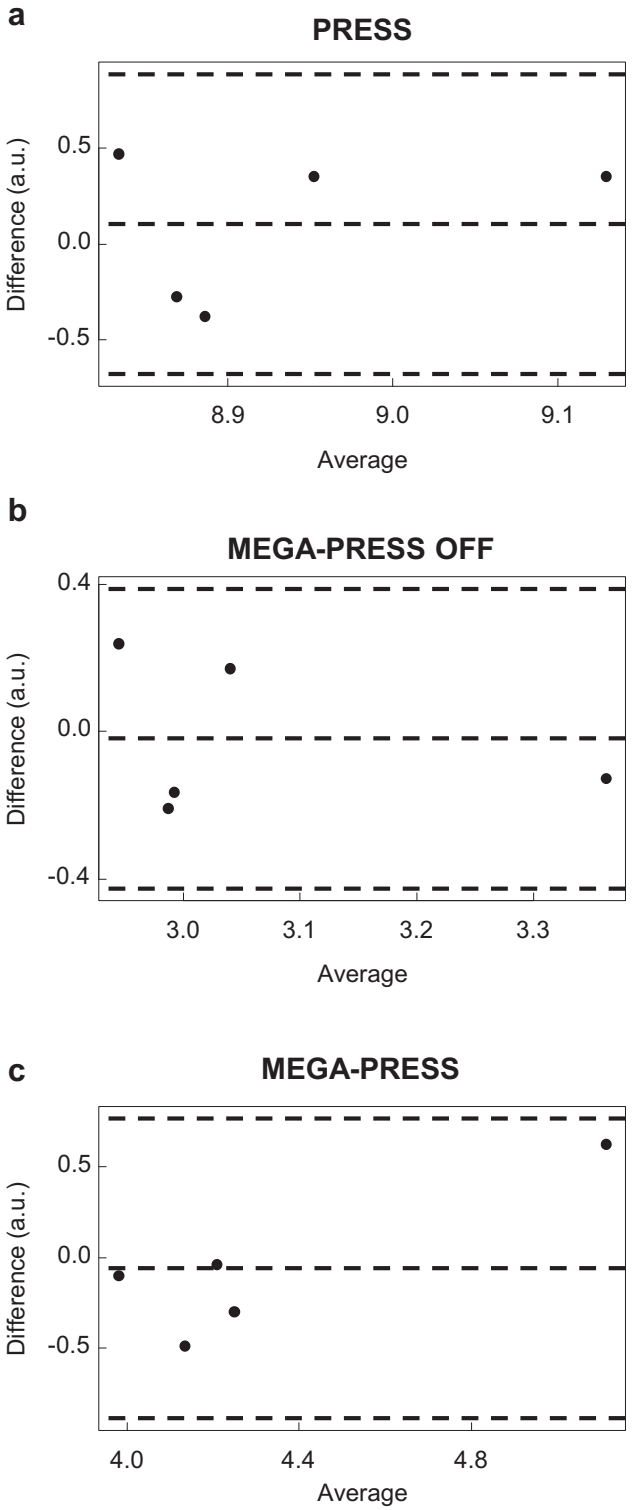

Fig. 3. Bland-Altman plots showing the *in vivo* repeatability of glutamate estimates with PRESS (a), MEGA-PRESS OFF (b) and MEGA-PRESS (c).

Table 5
Pearson's correlation coefficients between the different *in vivo* applied MRS methods. The concentrations are analyzed with LCModel (except for Glx_{MP} (Gannet), which was analyzed with Gannet) and expressed relative to the water signal. Results are ordered according to the sequence used, indicated by the shaded boxes.

	Glu_{PRESS}	Glx_{PRESS}	Glu_{MP}	Glx_{MP}	Glx_{MP} (Gannet)	Glu_{MPO}	Glx_{MPO}
Glu_{PRESS}	–	0.83**	0.40**	0.39**	0.21	0.35*	0.37*
Glx_{PRESS}	0.83**	–	0.25	n.s.	n.s.	0.27*	0.24
Glu_{MP}	0.40**	0.25	–	0.86**	0.63**	0.78**	0.79**
Glx_{MP}	0.39**	n.s.	0.86**	–	0.65**	0.75**	0.86**
Glx_{MP} (Gannet)	0.21	n.s.	0.63**	0.65**	–	0.87**	0.83**
Glu_{MPO}	0.35*	0.27*	0.78**	0.75**	0.87**	–	0.94**
Glx_{MPO}	0.37*	0.24	0.79**	0.86**	0.83**	0.94**	–

MP: MEGA-PRESS, MPO: MEGA-PRESS OFF.
n.s.: non-significant correlation.
*$p < 0.01$.
**$p < 0.0001$.

4. Discussion

In this study, three different experiments were performed to assess the quantification of glutamate. In all these experiments, four different combinations of acquisition and analysis methods were compared to distinguish glutamate from the other metabolites: (i) a PRESS sequence, of which the spectrum was analyzed with LCModel, (ii) a MEGA-PRESS difference sequence with analysis in LCModel, (iii) the off-spectrum from MEGA-PRESS, analyzed in LCModel, and (iv) MEGA-PRESS difference with analysis of the 'Glx' peak using Gannet. The first experiment, a phantom study, showed a high correlation between measured and actual glutamate concentrations. The second experiment showed better CVs and LoAs for *in vivo* glutamate measurements with PRESS than with MEGA-PRESS OFF or MEGA-PRESS. When comparing the quantitative estimates themselves of the different methods *in vivo*, as performed in the third experiment, the correlation between the methods was only modest (varying from r = 0.3 for PRESS and MEGA-PRESS OFF to r = 0.8 between MEGA-PRESS and MEGA-PRESS OFF).

Previous studies already showed sufficient accuracy in glutamate measurements with 30 ms PRESS and the MEGA-PRESS OFF spectra at 4 T [23]. Also appropriate repeatability [24] and reproducibility values of PRESS [25,26] or MEGA-PRESS [22] for measuring glutamate in humans was shown previously. Corresponding to our results, the reproducibility of glutamate and Glx was comparable [22,24], while a much better reproducibility of glutamate than of glutamine was shown (only with PRESS [25]). This latter might be explained by the higher *in*

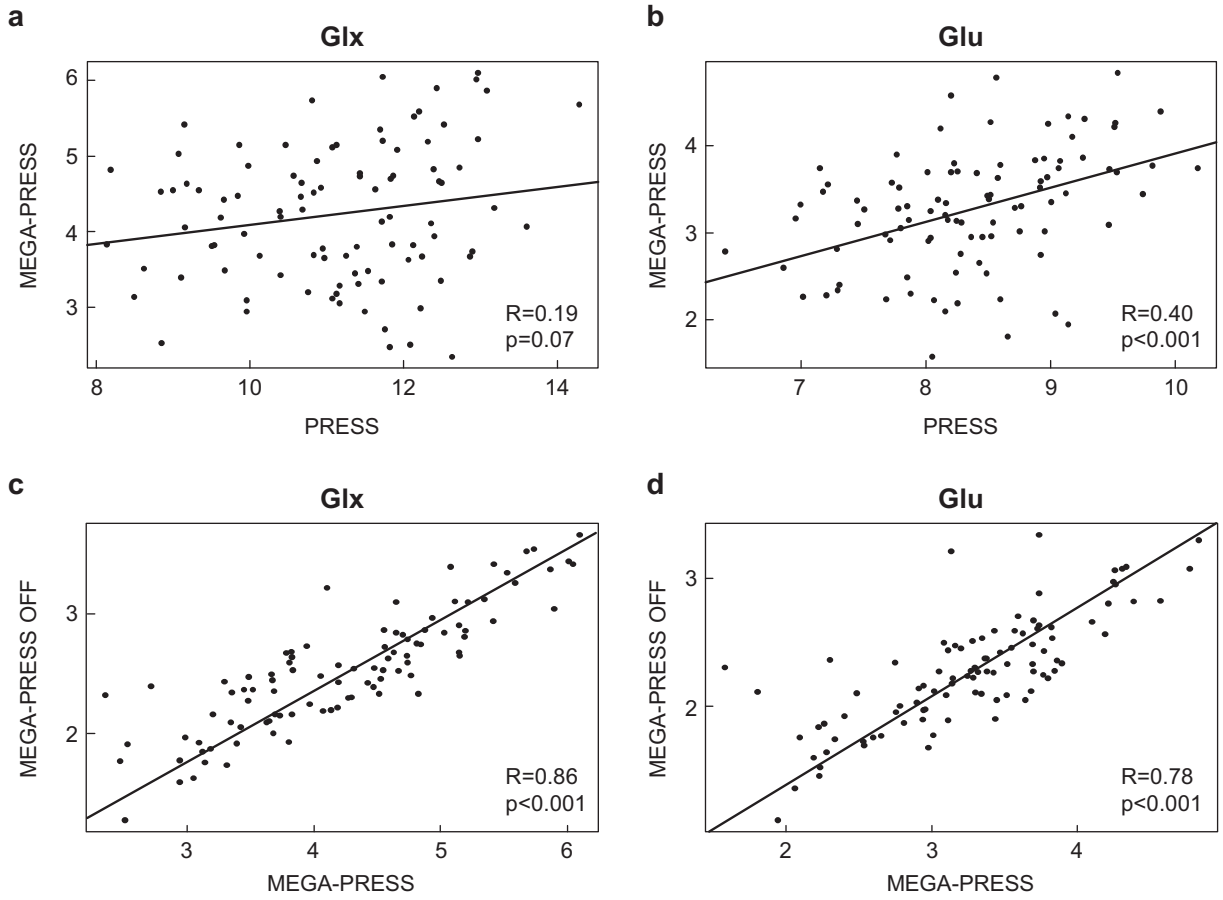

Fig. 4. Both the Glx (a) as the glutamate (b) concentrations (relative to water) measured *in vivo* with PRESS and MEGA-PRESS show a low correlation, despite being measured in the same participants and directly after each other. Higher correlations were measured between the MEGA-PRESS and MEGA-PRESS OFF estimates of Glx (c) and glutamate (d).

vivo glutamate than glutamine concentrations, leading to better signal-to-noise ratios in glutamate measurements.

The lack of strong concordance between the PRESS and MEGA-PRESS estimates found in our study is surprising. A possible explanation is the attribution of other molecules to the glutamate and glutamine signal, most likely macromolecules. In general, contribution of macromolecules is lower at longer TEs due to the relatively short T_2 relaxation times of macromolecules [36]. This could also explain the low concordance between PRESS (TE = 35) and both MEGA-PRESS (TE = 68) and MEGA-PRESS OFF (TE = 68), while the concordance between the latter two was much higher. Less contamination by macromolecules can therefore be expected in the MEGA-PRESS difference than in PRESS spectra, although it is unknown how the editing pulse affects the relative ratio between Glx and macromolecules. In MEGA-PRESS, up to 50% of the GABA + peak constitutes macromolecules [37], but it has been suggested that no macromolecules are present in the Glx peak [22]. More research is needed to assess the contributions of these macromolecules to the Glx signals in both PRESS as MEGA-PRESS.

Interestingly, there is not a large correlation between the Glx estimate obtained from Gannet and LCModel. This might be due to the inherently difference underpinnings of the Gannet and LCModel software: Firstly, different methods are applied to estimate metabolites (peak fitting and prior knowledge, respectively). Secondly, different preprocessing options are included in the analysis. Gannet includes frequency and phase correction between individual spectra, while these corrections are applied on the difference spectrum in LCModel. Both of these differences might contribute to the moderate correlation between the Glx estimates. Possibly, peak fitting is less accurate than using prior knowledge, which explains the higher coefficient of variation and limits of agreement in the Gannet analyses. In LCModel, the results might be less accurate due to subtraction artifacts. The combination of these factors might results in a lower than expected correlation between these measurements.

A secondary question in this study was, whether the Glx peak measured with a MEGA-PRESS sequence constitutes mainly glutamate, as claimed by others [18,21,38]. Our phantom results show that both glutamate and glutamine contribute strongly to this Glx peak. Previous *in vivo* work, concluded that the Glx peak in MEGA-PRESS constitutes primarily glutamate, based on a comparison between the Glx measure from MEGA-PRESS and glutamate and combined glutamate and glutamine measures from CT-PRESS [20], and argued that Glx measurements can be interpreted as glutamate. However, as the glutamate and Glx concentration are inherently correlated, the independent contribution of glutamate to Glx cannot be estimated *in vivo*. Thus, solely measuring the 'Glx' peak does not yield an unambiguous estimate of glutamate, and Glx alterations in patient studies should not automatically be attributed to changes in glutamate only. The concordance between PRESS and MEGA-PRESS was more beneficial for glutamate than for Glx, possibly as the glutamine estimation is not very reliable, and Glx is glutamate and glutamine combined.

An important issue is that some authors report reservations whether short-echo PRESS scans can reliably separate the glutamate and glutamine signals. For instance, Henry, Lauriat, Shanahan, Renshaw, Jensen [23] showed in simulations (at 4 T) that with increasing linewidths, rises in glutamate estimations are accompanied with large drops in glutamine estimations, suggesting unreliable separation. Contrasting, glutamate concentrations estimated at 4 T were similar to those at 7 T, with higher reliability at 7 T [39]. Our phantom experiment also showed good separation of the two signals, but these results cannot be directly translated to *in vivo* experiments as line widths are smaller in phantoms than *in vivo*. The high correlation of glutamate and Glx, as seen in MEGA-PRESS OFF (r = 0.94), likely indicates that glutamine was (potentially incorrectly) attributed to glutamate in these measurements.

Although in all three experiments, all scans were made in a single session without repositioning of the participant (or phantom), it is possible that small differences in voxel position due to head movement might have occurred. This could explain some random error, but is not expected to cause the lack of concordance seen in experiment 3, as the MEGA-PRESS scan was performed directly after PRESS scan.

The main limitation of this study is the gap between phantom experiments, in which actual concentrations are known, and the *in vivo* situation which includes unknown confounding factors such as possible macromolecules and movement artifacts. This is illustrated by the low concordance shown *in vivo* between the different methods despite their good validation metrics and repeatability. Furthermore, we chose to only compare PRESS with TE = 35 ms, with MEGA-PRESS with TE = 68 ms, as these settings are commonly applied to measure glutamate/Glx. However, also other TEs can be used, which might be more beneficial to measure glutamate [2]. Additionally, we did not convert concentrations to mM, as absolute concentrations requires accurate knowledge of T1 and T2 relaxation times (*in vivo* or *in vitro*) [40]. Therefore, we opted to report concentrations in institutional units, which unfortunately precludes a true 'accuracy' assessment. However, the applied correlation analyses still provide useful information regarding validation and concordance.

Another limitation is that glutamate and glutamine concentrations were only assessed within normal range. It is possible that one method is more favorable when glutamate concentrations are below this range, as might be the case in some conditions. Due to time restrictions, we did not acquire metabolite-nulled macromolecules spectra, to properly account for macromolecules in the LCModel analysis. Future studies should incorporate the acquisition of these macromolecule spectra and perform simulations in which the macromolecule contribution can be varied. Finally, only LCModel and Gannet were considered in this study, while other analysis software (Tarquin, JMRUI [41,42]) are available as well.

5. Conclusion

In conclusion, both PRESS as MEGA-PRESS, in combination with prior knowledge fitting such as implemented in LCModel, enable reliable glutamate measurements. This means that researchers interested in both GABA and glutamate do not require additional PRESS measurements for glutamate, albeit at the cost of a lower repeatability. In case MEGA-PRESS is applied, it is recommended to use the difference spectrum for glutamate detection, as the OFF spectrum does not seem to separate glutamate and glutamine correctly. Further studies are prompted into the effects of macromolecules on glutamate quantification.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mri.2017.12.029.

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