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Quantification of glutamate and glutamine using constant-time point-resolved spectroscopy at 3 T

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Abstract

Separate quantification of glutamate (Glu) and glutamine (Gln) using conventional MRS on clinical scanners is challenging. In previous work, constant-time point-resolved spectroscopy (CT-PRESS) was optimized at 3 T to detect Glu, but did not resolve Gln. To quantify Glu and Gln, a time-domain basis set was constructed taking into account metabolite T_2 relaxation times and dephasing from B_0 inhomogeneity. Metabolite concentrations were estimated by fitting the basis one-dimensional CT-PRESS diagonal magnitude spectra to the measured spectrum. This method was first validated using seven custom-built phantoms containing variable metabolite concentrations, and then applied to in vivo data acquired in rats exposed to vaporized ethanol and controls. Separate metabolite quantification revealed increased Gln after 16 weeks and increased Glu after 24 weeks of vaporized ethanol exposure in ethanol-treated compared with control rats. Without separate quantification, the signal from the combined resonances of Glu and Gln (Glx) showed an increase at both 16 and 24 weeks in ethanol-exposed rats, precluding the determination of the independent and differential contribution of each metabolite at each time.

Keywords

MRS; constant-time point-resolved spectroscopy (CT-PRESS); glutamate (Glu); glutamine (Gln); metabolite quantification

INTRODUCTION

Glutamate (Glu) and glutamine (Gln) are two major neurochemicals in the central nervous system. The quantification of Glu and Gln *in vivo* using MRS is of particular interest to a basic science understanding of neurotransmission and a clinical understanding of their independent contributions to the pathophysiology of various central nervous system disorders, including chronic pain, Alzheimer's disease, epilepsy and alcoholism (1–4). However, the quantification of Glu and Gln using conventional MRS at clinically available field strengths (e.g. 1.5–3 T) is difficult because of the overlapping multiplet structure of the coupled resonances.

Despite such obstacles, standard one-dimensional MRS data acquisition techniques, such as point-resolved spectroscopy (PRESS) (5) and stimulated echo acquisition mode (STEAM)

(6), have been used to study Glu and Gln at clinical field strengths. Such techniques typically rely on the linear combination model spectra (LCModel) for quantification (7). At the same time, modified PRESS and STEAM acquisition sequences have also been proposed for better resolution and quantification of Glu or Gln *in vivo*. Among them are a PRESS sequence with spectral-selective refocusing pulses for Glu quantification (8), multiple quantum coherence filtering (9) and a STEAM sequence with optimized timing parameters for Glu and Gln quantification (10). Although somewhat effective, these methods generally have the drawback of complicated implementation or a loss of signals from other metabolites.

Two-dimensional MRS techniques, such as *J*-resolved spectroscopy (11) and TE-averaged PRESS (12), provide a better solution than one-dimensional techniques for the detection of Glu because of reduced spectral overlap, as *J*-coupling information is encoded in the second dimension. Based on techniques similar to LCModel (7), quantification methods have been proposed using two-dimensional *J*-resolved MRS data (13,14). Another two-dimensional MRS technique, constant-time point-resolved spectroscopy (CT-PRESS), was developed for the better detection of coupled resonances with high signal-to-noise ratio (SNR) (15). In CT-PRESS, the temporal position of the last refocusing pulse is shifted in a series of excitations to encode the chemical shift (CS) information in the second time dimension (t_1). In the originally proposed sequence, the time interval between excitation and data acquisition is kept constant for all excitations. As the modulation caused by *J* coupling is the same for all encoding steps, the line splitting is suppressed in f_1 (effective homonuclear decoupling), reducing the spectral overlap. In addition, the evolution time t_c , i.e. the average TE of the CT-PRESS experiment, can be optimized in order to increase the SNR of a particular coupled resonance. Because the effectively decoupled CT-PRESS spectra are generated by integrating the two-dimensional spectra along the diagonal in magnitude mode to enhance SNR, linear least-squares fitting techniques with prior knowledge, e.g. LCModel, are not directly applicable to quantify overlapping resonances (7).

At 3 T, CT-PRESS has been optimized for the detection of the Glu C4 resonance at 2.35 ppm (16). At this field strength, however, resonances from Gln are not resolved. The Gln C2 resonance at 3.75 ppm overlaps with both the corresponding Glu resonance and the glutathione singlet, whereas the C4 resonance at 2.45 ppm only overlaps with the aspartate moiety of *N*-acetylaspartate (NAA).

However, by constructing the signal model in the time domain using the additional multiplet resonances of NAA at 2.67 ppm and the singlet at 2.01 ppm, in combination with prior knowledge of the spin systems, the contribution of NAA to the 2.45 ppm peak can be determined to give an estimate of the Gln concentration. The aims of this work were to implement this approach for the quantification of Glu and Gln from CT-PRESS data, to evaluate this quantification technique in phantom experiments and to apply it to *in vivo* data from a study on the effects of ethanol on rat brain chemistry (17).

MATERIALS AND METHODS

Sequence and reconstruction

All data were acquired using a CT-PRESS sequence optimized for the detection of the Glu C4 resonance at 2.35 ppm on a 3-T Signa MR scanner (GE Healthcare, Waukesha, WI, USA) with software release version 14M5 (16). The sequence consisted of a standard CS-selective water suppression module followed by a PRESS module for volume selection. The excitation pulse was a linear phase Shinnar-Le Roux radiofrequency (RF) pulse with a pulse width of 3.6 ms, and both refocusing RF pulses (167° flip angle) had a pulse width of 5.2 ms. The timing of the CT-PRESS sequence is shown in Fig. 1. The last refocusing RF pulse

in the PRESS module was shifted by 129 steps with a step size of $\Delta t_1/2 = 0.8$ ms, corresponding to a spectral bandwidth in f_1 of 625 Hz. For each CS step, 2048 complex data points in t_2 were acquired at a spectral bandwidth of 5000 Hz. The constant time t_c was set at 139 ms to achieve maximum SNR for the Glu C4 resonance. In a variant of the originally published CT-PRESS sequence, the data acquisition started immediately after the crusher gradient of the last refocusing RF pulse to increase the SNR (18,19). After apodization with sine-bell functions in both t_1 and t_2 dimensions, a two-dimensional Fourier transform was performed. To compensate for the different start times of the data acquisitions, a t_1 -dependent linear phase was applied in the t_2 dimension. The one-dimensional effectively decoupled spectrum, the so-called diagonal spectrum, was generated by integrating the two-dimensional magnitude spectrum along f_2 within a 313 Hz interval around the spectral diagonal.

For each voxel, a second acquisition was performed with the same sequence, but without water suppression and only 17 CS encoding steps ($\Delta t_1/2 = 6.4$ ms) for the estimation of the B_0 inhomogeneity effects. For the *in vivo* experiment, the amount of tissue water in the voxel was estimated from this dataset and used for normalization of the metabolite data.

Quantification

Neglecting T_1 relaxation, the two-dimensional time-domain data $S(t_1, t_2)$ of the CT-PRESS experiment can be described by the following signal model:

$$S(t_1, t_2) = \sum_i C_i M_i(t_1, t_2) \exp\left(-\frac{t_{12} + (t_c - t_1)/2 + dt + t_2}{T_{2i}} - \frac{|t_2'|}{T_2'}\right)$$

As shown in Fig. 1, t_{12} is the time from the center of the excitation pulse to the center of the first refocusing pulse, dt is the time from the center of the last refocusing pulse to the start of the data acquisition and t_c is the constant time for the CT-PRESS sequence. C_i is the concentration weight of the i th metabolite, t_1 is the temporal position of the last refocusing pulse shifted in a series of excitations ranging from -51.2 to 51.2 ms and t_2 is the time from the start of the acquisition window. T_{2i} is the spin-spin relaxation time constant of a particular resonance, T_2' is the time constant corresponding to the additional decay induced by B_0 inhomogeneity in the voxel and t_2' is the acquisition time relative to the respective echo time. $M_i(t_1, t_2)$ is the signal modulation of an individual metabolite caused by the evolution of CS and J coupling.

For quantification, $M_i(t_1, t_2)$ of the metabolites NAA, choline (Cho), creatine (Cre), myo-inositol (mI), Glu and Gln were simulated with full density matrices, i.e. the Hamiltonian included the full J -coupling terms, using the GAMMA NMR software library with the same timing parameters as used for the measured data (20). In the simulation, ideal, i.e. infinitely short, RF pulses were employed, as the bandwidths of the RF pulses used in the sequence were one order of magnitude higher than the CS range of Glu and Gln, and pulse widths were one order of magnitude shorter than $1/J$ for Glu and Gln (21,22). The singlet and coupled resonances of NAA were simulated separately to allow for different T_2 values in the signal model. CSs and J -coupling constants used in the simulations were taken from Govindaraju *et al.* (23). The signal decay caused by B_0 inhomogeneity and the amount of tissue water in the voxel were estimated from the water-unsuppressed dataset assuming a two-compartment model: brain tissue and cerebrospinal fluid. The percentage of tissue water in the voxel, T_2 of tissue water and T_2' for the B_0 inhomogeneity dephasing were estimated by fitting the time-domain data from the 17 excitations. For each acquisition, only data from the time of the echo onward were used for the estimation and T_2 of cerebrospinal fluid was

fixed at 500 ms to increase the numerical stability of the fit (24). The procedure for estimating the T_2 relaxation time of each resonance is described later for both phantom and *in vivo* studies.

With estimated T_2' and T_2 for each resonance and initially assuming equal concentration weights for all metabolites, the time-domain data $S(t_1, t_2)$ were calculated and the diagonal spectrum was reconstructed in the same way as for the experimental data. Quantification was achieved by fitting the reconstructed one-dimensional diagonal magnitude spectrum from the signal model with the measured one-dimensional diagonal magnitude spectrum. Concentrations of NAA (2.01 ppm), Cho (3.20 ppm) and Cre (3.03 ppm) were estimated through a linear search of their respective concentration weights in the time domain by minimizing the sum of squared residuals between the measured and basis spectra within a ± 0.05 ppm interval of their resonances. In the quantification, the average of the CS of Cho and phosphorylcholine, i.e. 3.20 ppm, was used. To shorten the computation time, concentrations of singlets and the resolved Glu resonance were initially estimated by equalizing the corresponding resonance peak heights in the basis and measured CT-PRESS spectra. A linear search was then conducted in the range of $\pm 30\%$ of the initially estimated concentration. The step size in the linear search was set at 1% of the initially estimated concentration. Using the estimated NAA concentration and T_2 of the coupled NAA resonance at 2.45 ppm, the contribution of NAA to the peak at 2.45 ppm was determined. As demonstrated in the flowchart in Fig. 2, the Gln concentration weight was then estimated again by performing a linear search of concentration weights in the time domain, such that the difference between the basis and measured resonances at 2.45 ± 0.05 ppm was minimized. The linear search started at zero concentration and the step size was set at 100th of the estimated Glu concentration.

To validate the quantification method, 10 spherical phantoms with variable metabolite concentrations were built by mixing metabolites into 50 mM phosphate buffer with 0.1% sodium azide. To reduce metabolite T_1 and T_2 to levels similar to those observed in *in vivo* studies, 0.6 mL/L of gadoteridol (ProHance, Bracco Diagnostics, Princeton, NJ, USA) was added to the buffer. After mixing, the pH of each solution was adjusted to 7.2 by the addition of NaOH. All phantoms contained 10 mM Cre as a concentration reference. Of the 10 phantoms, three calibration phantoms containing only NAA, Glu or Gln at 12.5 mM concentration, in addition to Cre, were built to determine the T_2 values of the NAA singlet at 2.01 ppm, NAA coupled resonances at 2.67 and 2.45 ppm, Glu C4 resonance at 2.35 ppm and Gln C4 resonance at 2.45 ppm. To validate the quantification procedure, the remaining seven phantoms contained the three metabolites at varying concentrations (cf. Table 1). The phantoms were scanned using the CT-PRESS sequence with TR = 7 s to allow for full T_1 relaxation.

As the water-suppressed CT-PRESS data were acquired at 129 TEs ranging from 36.6 to 241.4 ms, the T_2 values of the singlet resonances were estimated by monoexponential fitting of their peak intensities across TEs. Only data with TE > 40 ms were used in the fit to reduce the contributions of macromolecule resonances. Simultaneously, data with TE > 221 ms were excluded because of low SNR. For each t_1 encoding step, only the time domain data from TE onward were used. The data were phase corrected using the residual water signal, apodized with a 5 Hz Gaussian line broadening, and zero filled to 8192 points. After fast Fourier transformation, the peak intensities were calculated by peak integration. With the estimated T_2 values, the concentration weights of the singlets of NAA and Cre were estimated by fitting the basis and measured spectrum at 2.01 ± 0.05 ppm for NAA and 3.03 ± 0.05 ppm for Cre. To determine the T_2 values of the aspartate moiety of NAA at 2.67 and 2.45 ppm, a time-domain basis set $S(t_1, t_2)$ was constructed with the estimated concentration weights of the singlets of NAA and Cre. Using the one-dimensional CT-PRESS spectrum of

the phantom containing only NAA and Cre, T_2 of the resonance from the aspartate moiety of NAA at 2.67 ppm was determined by performing a linear search for a T_2 value such that the basis and measured spectra fitted. Using the same method, T_2 of the resonance from the aspartate moiety of NAA at 2.45 ppm was also determined. To determine T_2 of the Glu resonance at 2.35 ppm, a time-domain basis set $S(t_1, t_2)$ was constructed using the known Glu/Cre concentration ratio of 12.5 mM/10 mM. T_2 of the Glu C4 resonance was then estimated by performing a linear search for a T_2 value that minimized the difference between simulated and measured CT-PRESS for that resonance. T_2 of the Gln C4 resonance was determined in the same way.

In addition to validating the quantification of the seven phantoms, this method was applied to re-analyze data from a study in which eight wild-type Wistar rats had been exposed to vaporized ethanol for a total of 24 weeks. As controls, 10 littermates had received only air. MRS was performed prior to ethanol exposure (MRS1) and at weeks 16 (MRS2) and 24 (MRS3) of ethanol exposure. A more detailed description of the experimental procedures can be found in ref. (17).

For *in vivo* rat brain metabolite quantification, the T_2 values of the singlet resonances of NAA, Cho and Cre were first estimated for each individual animal using the same methods as described for phantom quantifications. Paired *t*-tests were performed on the estimated T_2 values of the singlet resonances. No statistically significant difference was found between metabolite T_2 values of control and ethanol-exposed rats at any of the acquisition times, nor between different measurement times for either control or ethanol-exposed rats. Therefore, to reduce variation in the concentration estimate, *in vivo* T_2 values were estimated after averaging the datasets from the baseline, i.e. pre-exposure, acquisitions. The individual datasets were first normalized to tissue water and phase corrected, and then averaged in the time domain. The T_2 values of the NAA, Cho and Cre singlets and T_2 of the NAA resonance at 2.67 ppm were estimated from this averaged dataset using the same methods as described for the calibration phantoms. For quantification, this set of estimated T_2 values was used for all CT-PRESS datasets. T_2 of the NAA resonance at 2.45 ppm cannot be determined from the *in vivo* datasets because of overlap with the Gln C4 resonance. Instead, this T_2 value was estimated by multiplying the estimated T_2 of the coupled NAA resonance at 2.67 ppm by a factor corresponding to the T_2 ratio of the same resonances in the phantom study. Unlike the phantom study, the T_2 values of the Glu and Gln C4 resonances cannot be independently estimated; instead, the *in vivo* T_2 value of the Glu C4 resonance was taken from the literature and set at 125 ms, and the T_2 value of the Gln C4 resonance was also set at 125 ms because of its similar chemical structure to Glu (16).

To determine the dependence and sensitivity of Gln quantification on the T_2 value used in the signal model, two CT-PRESS datasets with Gln concentrations of 10 and 6 mM were simulated, and the NAA, Cho, Cre, mI and Glu concentrations were set at 12.5, 3, 10, 7.5 and 12.5 mM, respectively. The Glu and Gln T_2 values were set at 125 ms in the simulation. Quantification was performed for these two simulated datasets using four Gln T_2 values (100, 125, 150 and 175 ms) to evaluate the Gln concentration estimate at different T_2 values.

The effect of noise on the quantification of Glu and Gln was evaluated in a simulation with the concentrations of NAA, Cho, Cre, mI, Glu and Gln set at 12.5, 3, 10, 7.5, 12.5 and 6 mM, respectively. In this simulation, the *in vivo* metabolite T_2 values were used and white noise was added in the time domain at varying amplitudes to simulate spectra with SNRs of 30, 25, 20 and 15 for the 2.45 ppm resonance. The added noise levels were determined from the standard deviation of a signal-free region at -0.5 to -1.5 ppm. The quantification procedure was performed 20 times for each SNR to determine the standard deviations of the concentration estimates.

RESULTS

The T_2 values from the calibration phantoms were estimated to be 486 ± 13 ms for the Cre singlet, 482 ± 11 ms for the NAA singlet, 135 ± 5 ms for the coupled NAA resonance at 2.67 ppm, 160 ± 6 ms for the coupled NAA resonance at 2.45 ppm, 137 ± 5 ms for the Glu C4 resonance and 133 ± 6 ms for the Gln C4 resonance. With these estimated metabolite T_2 values, quantification was performed on the seven validation phantoms. The measured and fitted one-dimensional diagonal spectra are shown in Fig. 3. Variation in the Glu/Gln/NAA ratio was clearly demonstrated by their resonances at 2.35, 2.45 and 2.01 ppm, respectively. Using the known Cre concentration of 10 mM, the estimated Glu, Gln and NAA concentrations agreed well with the actual concentrations. Table 1 summarizes the actual and estimated concentrations for the seven phantoms.

The Gln concentration estimates from the simulation using four different T_2 values for the simulated Gln concentrations of 10.0 and 6.0 mM are as follows: 13.3 mM/8.0 mM for $T_2 = 100$ ms, 10 mM/6 mM for $T_2 = 125$ ms, 8.2 mM/4.9 mM for $T_2 = 150$ ms and 7.1 mM/4.3 mM for $T_2 = 175$ ms. As expected, the quantification showed higher Gln concentration estimates when lower T_2 values were used and lower Gln concentration estimates when higher T_2 values were used. Nevertheless, the estimated concentrations maintained the same ratio of 10 mM/6 mM, i.e. 1.66, at all four T_2 values.

For estimates of Glu and Gln concentrations in the simulation with varying noise levels, the means and standard deviations of the estimated Glu and Gln concentrations are shown in Fig. 4. As expected, the variation of the estimated concentrations decreases with the decrease in noise. At SNR = 20, the ratio of the standard deviation and the mean of the estimated Gln concentration was 20%.

For *in vivo* quantification, the estimated contribution of brain tissue in the voxel ranged from 94% to 100%, the estimated T_2 value of brain tissue water was approximately 70 ms and the T_2' value for B_0 inhomogeneity dephasing ranged from 60 to 160 ms, depending on the quality of shimming performed at acquisition. The T_2 values of the NAA singlet and the coupled NAA resonance at 2.67 ppm were estimated from the averaged CT-PRESS spectra of all animals at baseline. The estimated T_2 value of the NAA singlet was 335 ± 11 ms and T_2 of the coupled NAA resonance at 2.67 ppm was 124 ± 5 ms. The estimated T_2 value of the coupled NAA resonance at 2.45 ppm was set at 148 ms to reflect the same ratio to the T_2 value of the coupled NAA resonance at 2.67 ppm as in the phantom study.

Each *in vivo* CT-PRESS spectrum was normalized to the tissue water estimated from a dataset acquired without water suppression. The CT-PRESS spectra, normalized to the tissue water content, were then averaged for each group at each acquisition time point as shown in Fig. 5a. The ethanol resonance at 1.2 ppm in the spectra of the ethanol group at MRS2 and MRS3 shows that CT-PRESS can detect brain ethanol when present. The spectra from 2.2 to 2.8 ppm are enlarged in Fig. 5b. A larger resonance at 2.45 ppm relative to that at 2.67 ppm can be seen in the spectrum at MRS2 of the ethanol group compared with the control group, indicating increased Gln concentration following 16 weeks of ethanol exposure. The mean and standard error of water-referenced Gln and Glu concentrations using the methods described here are illustrated in Fig. 6 for both control and ethanol-exposed rats. Gln was higher in the ethanol group than in the control group [$t(16) = 3.1$, $p = 0.0076$] at MRS2, i.e. after 16 weeks of ethanol exposure, whereas Glu was higher in the ethanol group than in the control group [$t(16) = 2.6$, $p = 0.0187$] at MRS3, i.e. after 24 weeks of ethanol exposure.

DISCUSSION

Compared with one-dimensional MRS methods, CT-PRESS optimized for Glu detection offers the advantage of effectively decoupled spectra and improved SNR. As an alternative to the CT-PRESS approach, *J*-resolved spectroscopy with shearing and summation in the reconstruction produces a spectrum with eliminated homonuclear multiplet splitting (11,25,26). For strongly coupled spin systems, the effective decoupling scheme in the CT-PRESS acquisition could lead to additional peaks at the mean CSs of the coupled resonances, with the intensity of these peaks also depending on t_c (27,28). Although Glu and Gln are strongly coupled at 3 T, phantom experiments and simulations using the full density matrix show that these spurious signals can be neglected at the chosen t_c (16). Moreover, the relatively long TEs used in CT-PRESS acquisition sequences result in reduced water relative to metabolite signals, as water T_2 is shorter than metabolite T_2 , and have the added benefit of reducing contributions from macromolecules. Combined with effective water suppression RF pulses, the reconstructed one-dimensional diagonal spectrum has a flat spectral baseline, which simplifies quantification.

The proposed method achieves metabolite quantification by estimating parameters in a physical model to fit the projected one-dimensional magnitude CT-PRESS spectra, instead of fitting the full two-dimensional spectra as when using ProFit (14). For quantification, the concentrations of the singlets, i.e. NAA, Cre, Cho and Glu, are estimated by fitting their resolved individual resonances in the one-dimensional diagonal CT-PRESS spectrum. The concentration of Gln is estimated by fitting the peak at 2.45 ppm with the estimated contribution from NAA. In the phantom experiments, the following parameters can introduce errors in the Gln concentration estimate: (i) T_2 of the NAA singlet; (ii) T_2 of the NAA resonance at 2.45 ppm; (iii) T_2 of the Gln C4 resonance at 2.45 ppm; (iv) T_2 of the Cre singlet; (v) the actual Cre concentration, which is used as an absolute concentration reference. From monoexponential fitting, the NAA singlet T_2 was estimated at 482 ± 11 ms and the Cre singlet T_2 was estimated at 486 ± 13 ms. From a Monte-Carlo simulation of datasets with NAA of 12.5 mM and Gln of 6 mM at an SNR similar to the phantom dataset, the estimated T_2 value of the NAA resonance at 2.45 ppm was 160 ± 6 ms and of the Gln resonance was 133 ± 6 ms. Based on the variation of the Cre peak in all phantoms, the standard deviation of the actual Cre concentration was estimated to be 1.9%. With all these estimated parameters, a Monte-Carlo simulation of Gln quantification was performed for datasets with NAA of 12.5 mM and Gln of 6 mM at an SNR similar to the phantom dataset. In the simulation, a combination of the model parameters with one standard deviation from their mean values was used to calculate worst-case Gln concentration estimates with the most positive and negative biases. For example, a high T_2 value of the NAA resonance at 2.45 ppm (166 ms), high T_2 value of the Gln C4 resonance at 2.45 ppm (139 ms), low T_2 value of the NAA singlet (471 ms), low T_2 value of the Cre singlet (473 ms) and low actual Cre concentration (9.81 mM) result in the lowest Gln concentration estimate. Taking into account the various sources of error, the total Gln quantification error was on the order of 19% or 1.1 mM. Similarly, the total error for Glu was 9% and, for NAA, 4%.

For the *in vivo* data, the measured SNR of the 2.45 ppm resonance from an individual rat spectrum was greater than 15. From the simulations with multiple noise levels shown in Fig. 4, the quantification demonstrated unbiased concentration estimates.

For NAA quantification, T_2 of the NAA singlet was estimated from exponential fitting of its peak area at 2.01 ppm at multiple TEs without removing overlapping resonances from Glu, Gln and *N*-acetylaspartylglutamate (NAAG), because their contributions are small. For NAAG, the T_2 value of its acetyl resonance should be similar to the T_2 value of the NAA singlet, given their similar chemical structure. For the purpose of validation, a CT-PRESS

dataset was simulated from a mixture of NAA (12.5 mM, $T_2 = 335$ ms), NAAG (1.5 mM, $T_2 = 335$ ms), Glu (6 mM, $T_2 = 125$ ms) and Gln (3 mM, $T_2 = 125$ ms). From this dataset, T_2 of the NAA singlet was estimated to be 346 ms. The higher estimated T_2 value of the NAA singlet compared with the T_2 value used in the simulation, i.e. 335 ms, comes mainly from phase changes of Gln resonances at 2.01 ppm, i.e. out of phase with the NAA singlet at early TEs and in phase with the NAA singlet at late TEs. However, the error introduced in the estimated T_2 of the NAA singlet results in an error of its concentration estimate of less than 3%.

For the phantom studies, the T_2 value of the C4 resonance of Glu was estimated to be at 137 ms and that of Gln at 133 ms. For *in vivo* quantification, the T_2 values of Glu and Gln were set to the same value, 125 ms, because of similarities in their chemical structures. The actual *in vivo* T_2 values of Glu and Gln C4 resonances could differ from this value, leading to an over- or under-estimation of their concentrations, i.e. having a T_2 value higher than the actual value would cause the estimated concentration to be lower than the actual concentration, and vice versa. Nevertheless, as demonstrated in the simulated Gln estimation with four different T_2 values, when differentiating Gln concentrations between control and manipulated animals, the accuracy of T_2 is not critical, provided that the Gln T_2 relaxation times are not different for the comparison groups. In addition to the assumptions for the *in vivo* T_2 values of Glu and Gln, the *in vivo* T_2 value of the coupled NAA resonance at 2.45 ppm is estimated from the T_2 value of the NAA resonance at 2.67 ppm by assuming the same T_2 ratio as in the phantom. As a result of the overlap with the Gln C4 resonance, an error in T_2 of the coupled resonance of NAA at 2.45 ppm will affect the Gln estimation. For example, for an actual concentration of NAA of 12.5 mM with T_2 of the coupled NAA resonance at 2.45 ppm of 160 ms and concentration of Gln of 8 mM with T_2 of 133 ms, overestimation of the T_2 value of the coupled NAA resonance at 2.45 ppm by 10% leads to a 12% underestimation of the Gln concentration. Similarly, a 10% underestimation of the T_2 value of the coupled NAA resonance at 2.45 ppm corresponds to a 12% overestimation of the Gln concentration.

In phantom studies, the data were acquired with $TR = 7$ s to allow for full T_1 relaxation. For *in vivo* studies, TR of CT-PRESS was 2 s. Because the *in vivo* T_1 relaxation times for Cho, Cre and NAA are on the order of 1 s, the *in vivo* one-dimensional diagonal spectra are T_1 weighted (29,30). Therefore, the difference in estimated metabolite concentrations between control and ethanol-exposed rats may be caused by T_1 differences between the groups.

Glu and Gln have traditionally been studied as the combined resonance, Glx, using MRS. A variety of neuropsychiatric disorders, including migraine, depression, bipolar disorder, epilepsy, schizophrenia, Alzheimer's disease and human immunodeficiency virus infection, are associated with brain changes in Glx (31–37). Measurements in terms of Glx, however, blur the complex relationship between Glu and Gln, subtle changes of which may have important implications for brain functioning. Thus, the separation of Glu from Gln in spectroscopic data acquired in humans and in *in vivo* animal models is a significant advance in the ability to interrogate the metabolic underpinnings of several neuropathological disorders.

The current analysis shows that Gln was elevated at week 16 and Glu was elevated at week 24 in ethanol-exposed rats. Increases in brain Gln have been reported in patients with hepatic encephalopathy on postmortem examination (38). Evidence for mild liver damage in the ethanol group (17) and, perhaps, elevations in blood ammonia with ethanol exposure (39) suggests an explanation for elevated Gln: liver pathology impairs the major organ for ammonia elimination via the urea cycle, and the consequent increase in blood ammonia levels can lead to elevated brain ammonia (40). The mechanism of brain ammonia

detoxification is the formation of Gln from Glu by the enzyme Gln synthetase (40). With prolonged ethanol exposure, the levels of Gln synthetase may be compromised (41), leading, instead, to a build-up in the levels of Glu (42), as observed after 24 weeks of ethanol exposure.

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Abbreviations used

Cho	choline
Cre	creatine
CS	chemical shift
CT-PRESS	constant-time point-resolved spectroscopy
Gln	glutamine
Glu	glutamate
Glx	combined resonance of glutamate and glutamine
LCModel	linear combination of model spectra
mI	myo-inositol
NAA	N-acetylaspartate
NAAG	N-acetylaspartylglutamate
RF	radiofrequency
SNR	signal-to-noise 'ratio
STEAM	stimulated echo acquisition mode

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Figure 1.
Timing of the constant-time point-resolved spectroscopy (CT-PRESS) sequence.

Figure 2.

Flowchart of the glutamine (Gln) quantification procedure. Cho, choline; Cre, creatine; CT-PRESS, constant-time point-resolved spectroscopy; Glu, glutamate; mI, myo-inositol; NAA, *N*-acetylaspartate; 1D, one-dimensional; 2D, two-dimensional.

Figure 3.

One-dimensional diagonal constant-time point-resolved spectra from phantoms with glutamate/glutamine/*N*-acetylaspartate (Glu/Gln/NAA) concentration ratios of 0 mM/0 mM/12.5 mM (a), 12.5 mM/0 mM/12.5 mM (b), 0 mM/12.5 mM/12.5 mM (c), 12.5 mM/6 mM/12.5 mM (d), 12.5 mM/12.5 mM/6 mM (e), 12.5 mM/12.5 mM/12.5 mM (f) and 6 mM/12.5 mM/12.5 mM (g). The measured spectra are plotted as full black lines and the fitted spectra are plotted as broken red lines. Cre, creatine; Glx, combined glutamate and glutamine.

Figure 4. Means and standard deviations of the estimated glutamate (Glu) and glutamine (Gln) concentrations for signal-to-noise ratios (SNR s) of 30, 25, 20 and 15. The concentrations of Glu and Gln used in the simulation were 12.5 and 6 mM.

Figure 5.

(a) Constant-time point-resolved spectra from control and ethanol (EtOH)-exposed rats. MRS was performed prior to EtOH exposure (MRS1) and at weeks 16 (MRS2) and 24 (MRS3) of exposure. (b) Enlarged spectra from 2.2–2.8 ppm from (a). Each *in vivo* constant-time point-resolved spectrum was normalized to the tissue water estimated from a dataset acquired without water suppression. The constant-time point-resolved spectra, normalized by the tissue water content, were then averaged for each group at each acquisition time point. Cho, choline; Cre, creatine; Gln, glutamine; Glu, glutamate; Glx, combined resonance of glutamate and glutamine; mI, myo-inositol; NAA, *N*-acetylaspartate; Tau, taurine.

Figure 6. Quantified glutamine (Gln) (a) and glutamate (Glu) (b) from control and ethanol (EtOH)-treated rats. MRS was performed prior to EtOH exposure (MRS1) and at weeks 16 (MRS2) and 24 (MRS3) of exposure.

Actual and estimated glutamate (Glu), glutamine (Gln) and *N*-acetylaspartate (NAA) concentrations for seven validation phantoms with known concentrations

Table 1

	Phantom a	Phantom b	Phantom c	Phantom d	Phantom e	Phantom f	Phantom g
Actual Glu concentration (mM)	0.0	12.5	0	12.5	12.5	12.5	6.0
Estimated Glu concentration (mM)	0.1	13.1	0.1	13.2	13.4	12.9	6.1
Actual Gln concentration (mM)	0.0	0.0	12.5	6.0	12.5	12.5	12.5
Estimated Gln concentration (mM)	0.0	0.3	12.7	6.1	13.6	12.1	11.3
Actual NAA concentration (mM)	12.5	12.5	12.5	12.5	6.0	12.5	12.5
Estimated NAA concentration (mM)	12.6	12.8	12.8	12.7	6.2	12.7	12.8