NMR IN BIOMEDICINE
NMR Biomed. 2008; **21**: 396–401
Published online 1 October 2007 in Wiley InterScience
(www.interscience.wiley.com) DOI:10.1002/nbm.1205

Proton T_2 relaxation time of *J*-coupled cerebral metabolites in rat brain at 9.4 T

Lijing Xin, Giulio Gambarota,* Vladimír Mlynárik and Rolf Gruetter

Laboratory of Functional and Metabolic Imaging, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

Received 1 May 2007; Revised 20 June 2007; Accepted 10 July 2007

ABSTRACT: Knowledge of proton T_2 relaxation time of metabolites is essential for proper quantitation of metabolite concentrations in localized proton spectroscopy, especially at moderate to long *TE*s. Although the T_2 relaxation time of singlets, such as that of creatine and *N*-acetylaspartate, has been characterized in several studies, similar information is lacking from coupled spin resonances of cerebral metabolites. In this study, the T_2 relaxation time of coupled spin resonances and singlet resonances of cerebral metabolites was measured in rat brain *in vivo* at 9.4 T. Spectra were acquired at 11 *TE*s using the SPin ECho, full Intensity Acquired Localized (SPECIAL) spectroscopy method. Data analysis was performed in the frequency domain with the LCModel software using simulated *TE*-specific basis sets. The T_2 relaxation times in compounds showing singlet resonances were 113 ± 3 ms (total creatine), 178 ± 29 ms (total choline) and 202 ± 12 ms (*N*-acetylaspartate). The T_2 values of *J*-coupled metabolites ranged from 89 ± 8 ms (glutamate) to 148 ± 14 ms (*myo*-inositol). Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: T_2 relaxation time; coupled spin systems; brain metabolites; proton magnetic resonance localized spectroscopy

INTRODUCTION

^{1}H MRS is an important tool for investigating brain metabolism non-invasively. In animal models, the high spectral resolution and signal-to-noise ratio afforded at high fields allow the detection of a neurochemical profile that includes ~20 metabolites, with ^{1}H MRS performed at very short *TE* (1–2 ms) (1). At short *TE*, no losses due to transverse relaxation occur, and the multiplet resonances from coupled spin systems display virtually no signal modulation induced by the *J*-coupling. As the *TE* increases, a loss of signal intensity occurs in singlets because of transverse relaxation, and in coupled resonances because of transverse relaxation and *J*-coupling effects. As the T_2 of macromolecule resonances is shorter than that of cerebral metabolites, a number of ^{1}H MRS studies are performed at long *TE*, to avoid the confounding effect of macromolecular signals on metabolite quantitation. In addition, ^{1}H MRS editing techniques are typically performed at moderate to long *TE* (2,3) dictated by *J*-evolution, and therefore, for metabolite quantitation, the transverse relaxation effect should be taken into account. This is done by introducing a correction factor which, however, requires knowledge of the T_2 of the metabolites. A working assumption used in previous studies was that T_2 of coupled resonances is similar to that of, e.g., creatine (2,3).

Knowledge of T_2 relaxation time of metabolites is essential not only for absolute quantitation of metabolite concentrations, but also for investigating physiological and pathological processes. Previous MRS studies of T_2 of cerebral metabolites have shown that changes in the cell microenvironment, induced by pathology or naturally occurring during development, result in measurable changes in water and cerebral metabolite T_2 relaxation time (4,5). For example, the increase in T_2 of total choline (tCho) and *N*-acetylaspartate (NAA) observed with growth may reflect the active myelinogenesis processes occurring during brain development (4). Thus T_2 of metabolites could also be used to probe the cell microenvironment so as to provide information on physiological and pathological processes.

Owing to the relative experimental simplicity, most studies to date have focused on measurement of the T_2 of singlets (4–6), with the notable exception of citrate in the prostate (7). To improve metabolite quantitation at moderate to long *TE* and to validate previous approaches based on quantitation by spectral editing, we sought to determine the T_2 for coupled spin resonances. Thus, the aim of this study was to measure the T_2 of coupled spin resonances of cerebral metabolites in rat brain at 9.4 T

**Correspondence to:* G. Gambarota, Swiss Federal Institute of Technology Lausanne (EPFL), SB–LIFMET, CH F0 626, Station 6, CH-1015 Lausanne, Switzerland.
E-mail: giulio.gambarota@epfl.ch
Contract/grant sponsor: Centre d'Imagerie BioMédicale (CIBM) of the UNIL, UNIGE, HUG, CHUV, EPFL, the Leenaards and Jeantet Foundations.
Contract/grant sponsor: SNSF; *contract/grant number:* 3100A0-116220.

Abbreviations used: CRLB, Cramér–Rao lower bound; tCho, total choline; tCr, total creatine; GABA, γ-aminobutyric acid; NAA, N-acetylaspartate; SPECIAL, spin echo, full intensity acquired localized.

using a localization sequence based on spin echo [SPin ECho, full Intensity Acquired Localized (SPECIAL) spectroscopy] (8) in combination with frequency domain analysis of the spectra (LCModel) based on simulated basis sets.

MATERIALS AND METHODS

Animal preparation

All animal preparation procedures were in accordance with local and federal guidelines and were approved by the local ethics committee. Experiments were performed on five healthy Sprague–Dawley rats (318 ± 64 g, mean $\pm$ SD). During the experiment, the animals were anesthetized with 1.5% isoflurane, and the body temperature was maintained at 37.5°C with circulating heated water.

MRS

^{1}H MR experiments were performed on a Varian INOVA console (Varian, Palo Alto, CA, USA) connected to an actively shielded 9.4 T/31 cm magnet (Magnex Scientific, Abingdon, Oxon, UK) with actively shielded gradients (400 mT/m in 120 μs, 12 cm inner diameter). A home-built 14 mm diameter ^{1}H quadrature surface coil was used for radio frequency transmission and signal reception. *In vivo* spectra were acquired at 11 echo times ($TE = 2.8$, 20, 40, 60, 80, 110, 130, 150, 170, 200, and 300 ms) using a localization sequence (SPECIAL) that retains full signal sensitivity by combining a one-dimensional ISIS technique with a slice-selective spin echo sequence (8). Briefly, voxel selection is based on a 2 ms slice-selective full-passage adiabatic pulse applied on alternate scans (resulting in an add–subtract scheme), followed by a spin echo sequence consisting of 0.5 ms 90° and 180° asymmetric slice-selective pulses with a CYCLOPS phase cycling scheme (9). A $2.5 \times 5 \times 5\,\text{mm}^3$ voxel was centered in the hippocampus of the rat brain. Echo-planar imaging-based FASTMAP (10) was used to shim a volume of $3.5 \times 6 \times 6\,\text{mm}^3$ centered on the measured volume of interest. After first and second order shimming, the typical linewidth of water resonance at $TE = 2$ ms was 12–14 Hz. Water suppression was achieved with VAPOR (11). All spectra were acquired under the following acquisition parameters: spectral width of 5 kHz, 4096 complex data points, and *TR* of 4 s. The total scan time per rat was ~2 h. Acquisition at each *TE* (~11 min) consisted of 10 free induction decays (16 averages each), which were saved separately. Fourier transformation and B_0 shift correction were performed before summation of the 10 spectra. A phantom containing 50 mM glutamate and 50 mM creatine, and pH-balanced (pH = 7.0), was prepared to measure the *in vitro* T_2 of glutamate and creatine. During the measurements, the temperature of the phantom was kept at ~37°C. Spectra were acquired at the same *TE*s as in the *in vivo* measurements. The volume of interest was $3 \times 5 \times 5\,\text{mm}^3$, and 64 averages were accumulated for each spectrum.

Data analysis

Measurements of T_2 relaxation time of singlet resonances are relatively simple because of the absence of *J*-modulation effects on the signal intensity. For resonances of coupled spin systems, the *J*-modulation strongly affects both the spectral area (i.e. the signal intensity) and the spectral lineshape. Thus, to fit the spectral shape of coupled spin resonances at $TE \gg 0$, it is necessary to take into account the effect of *J*-modulation on the lineshape (7). A further complication in the assessment of the T_2 relaxation time of coupled spin resonances arises from the spectral overlap in the ^{1}H spectrum. To overcome these difficulties, the LCModel (12) software was used to analyze spectra at different echo times. A series of metabolite basis sets of LCModel for different echo times were created by quantum mechanics simulations, based on the density-matrix formalism (13), using published values of *J*-coupling constants and chemical shifts (14). In the basis sets, T_2 relaxation was not included; furthermore, each metabolite was considered as one component, and thus the T_2 reported here is the mean T_2 of all proton groups in the molecule. Additional basis sets with separate NAA acetyl and aspartate moieties, and the CH_3 group and CH_2 group of total creatine (tCr), were prepared to obtain the T_2 of different groups in the same molecule. The 'apparent' concentration of each metabolite obtained after LCModel analysis decreased with increasing *TE* due to T_2 relaxation. The Cramér–Rao lower bound (CRLB) was provided by LCModel as a measure of the reliability of the apparent metabolite concentration quantitation. The mean apparent concentrations were obtained from five rats, and then the T_2 relaxation time was calculated by performing a mono-exponential fit of these apparent concentrations as a function of *TE* using the curve-fitting tool of Matlab (Version 7.1; The MathWorks, Inc., Natick, MA, USA) combined with the Trust–Region algorithm. For each fit, the coefficient of determination (R^2) was evaluated to assess the quality of the fit, which was also visually inspected. T_2 values of glutamate and creatine in the phantom were calculated by the same method.

RESULTS

The *J*-modulation of the coupled resonance signals was adequately mimicked by the density-matrix spin simulations, as demonstrated by the good agreement between

DOI: 10.1002/nbm

simulated and *in vitro* spectra of glutamate and creatine (Fig. 1), and between the fit and *in vivo* spectra at all echo times (Fig. 2). At $TE = 2.8$ ms, singlets and coupled resonances were in pure absorption mode. At longer *TE*, there was a decrease in the signal intensity, but no change in the spectral lineshape, of singlets (notably, the NAA CH_3 protons at 2.01 ppm, the tCr CH_3 protons at 3.03 ppm, and CH_2 protons at 3.92 ppm). On the other hand, both spectral area (i.e. signal intensity) and spectral lineshape of coupled spin resonances changed with *TE*. With increasing *TE*, the *J*-modulation of the signal intensity was particularly evident for the coupled resonances of glutamate, *myo*-inositol, and the aspartate moiety of NAA. Already at *TE* of 20 ms, a substantial signal modulation was observed in strongly coupled spin systems, such as the AB spin system of the NAA aspartate moiety at 2.48 and 2.67 ppm. For $TE = 40$–60 ms (i.e. $TE \sim 1/2J$), the typical antiphase pattern of outer bands was observed in the triplet of the ^{4}CH and ^{6}CH proton of *myo*-inositol at 3.61 ppm, and in the multiplet of the glutamate CH_2 protons at 2.34 ppm (Fig. 2).

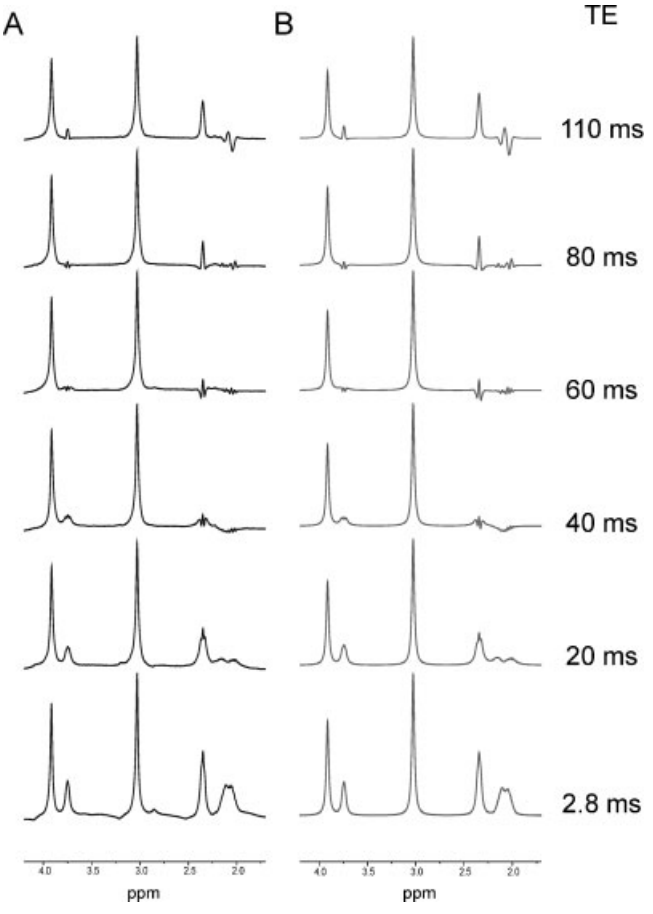

Figure 1. (A) *In vitro* ^{1}H MR spectra of glutamate and creatine measured using SPECIAL at *TE* ranging from 2.8 to 110 ms (volume of interest $= 3 \times 5 \times 5$ mm^3, $TR = 4$ s, 64 scans, broadened to 12 Hz linewidth of the creatine methyl signal). Because of the longer T_1 of the creatine methyl group, $TR = 4$ s caused greater saturation of the CH_3 signal than of the CH_2 signal. (B) Simulated spectra at the same *TE*s. Signal intensities are scaled using the *in vitro* methyl signal of creatine as a reference. T_2 relaxation effect is not included.

When *TE* approached values of 110–130 ms (i.e. $TE \sim 1/J$), most of the resonances had a positive phase because of the *J*-induced rephasing of the signal intensity, illustrated with, e.g., the glutamate resonances at 2.34 ppm and at 2.03 and 2.12 ppm (Fig. 2). Macromolecular resonance signals decayed within the noise level after 60–80 ms because of their short T_2 relaxation time, and thus the long-*TE* spectra were characterized by a flat baseline.

The signal intensity of singlets (data not shown) decreased monotonically and exponentially with *TE*. In contrast with the behavior of singlets, the evolution of the coupled spin resonances such as glutamate, *myo*-inositol, and taurine was more complex (Fig. 3), displaying a steep and substantial signal loss already at *TE* of 20 ms, mostly due to *J*-induced signal dephasing. At longer *TE*, there was a first signal intensity minimum at $TE \sim 60$ ms and a relative maximum at ~ 110 ms. At even longer *TE*, the signal intensity decayed further because of T_2 losses and further *J*-evolution. The apparent concentrations decreased with increasing *TE* because of T_2 relaxation in a nearly mono-exponential fashion (Fig. 3).

To evaluate the accuracy of the metabolite quantitation, data analysis of the CRLBs, obtained from LCModel analysis, was performed. Up to *TE* of 130 ms, the CRLBs of coupled resonances of highly concentrated metabolites such as glutamate, *myo*-inositol, and taurine were below 10%. For metabolites such as glutamine and γ-aminobutyric acid (GABA), which are present at lower concentration and also suffer from large overlap with other resonances, the CRLBs were below 20% at most *TE*s up to 130 ms. Finally, for *TE*s up to 200 ms, the CRLBs of tCr and NAA were below 5%, and the CRLB of tCho was below 10%. Overall, the high spectral resolution and signal-to-noise ratio afforded at 9.4 T allowed the detection of a neurochemical profile (13 metabolites) at $TE = 110$ ms with CRLBs $< 20\%$.

In vitro, the T_2 values of glutamate, creatine CH_3, and creatine CH_2 were 190 ± 20 ms ($R^2 = 0.9457$), 376 ± 19 ms ($R^2 = 0.9862$), and 354 ± 9.5 ms ($R^2 = 0.9963$), respectively. In rat brain, the T_2 relaxation time of the singlet resonances ranged from 113 ± 3 ms (tCr) to 202 ± 12 ms (NAA) (Table 1). The T_2 of macromolecules was 19 ± 0.4 ms ($R^2 = 0.9992$). The T_2 of coupled metabolites ranged from 89 ± 8 ms (glutamate) to 148 ± 14 ms (*myo*-inositol). The average R^2 was above 0.96 for glutamate, *myo*-inositol, taurine, and phosphorylethanolamine, and in the range 0.78–0.90 for GABA, glutamine, glucose, and glutathione. With the additional basis sets, separate T_2 values for the NAA methyl (202 ± 9 ms) and aspartyl resonances (131 ± 15 ms) and creatine methyl (115 ± 3 ms) and methylene resonances (101 ± 2 ms) were obtained.

DOI: 10.1002/nbm

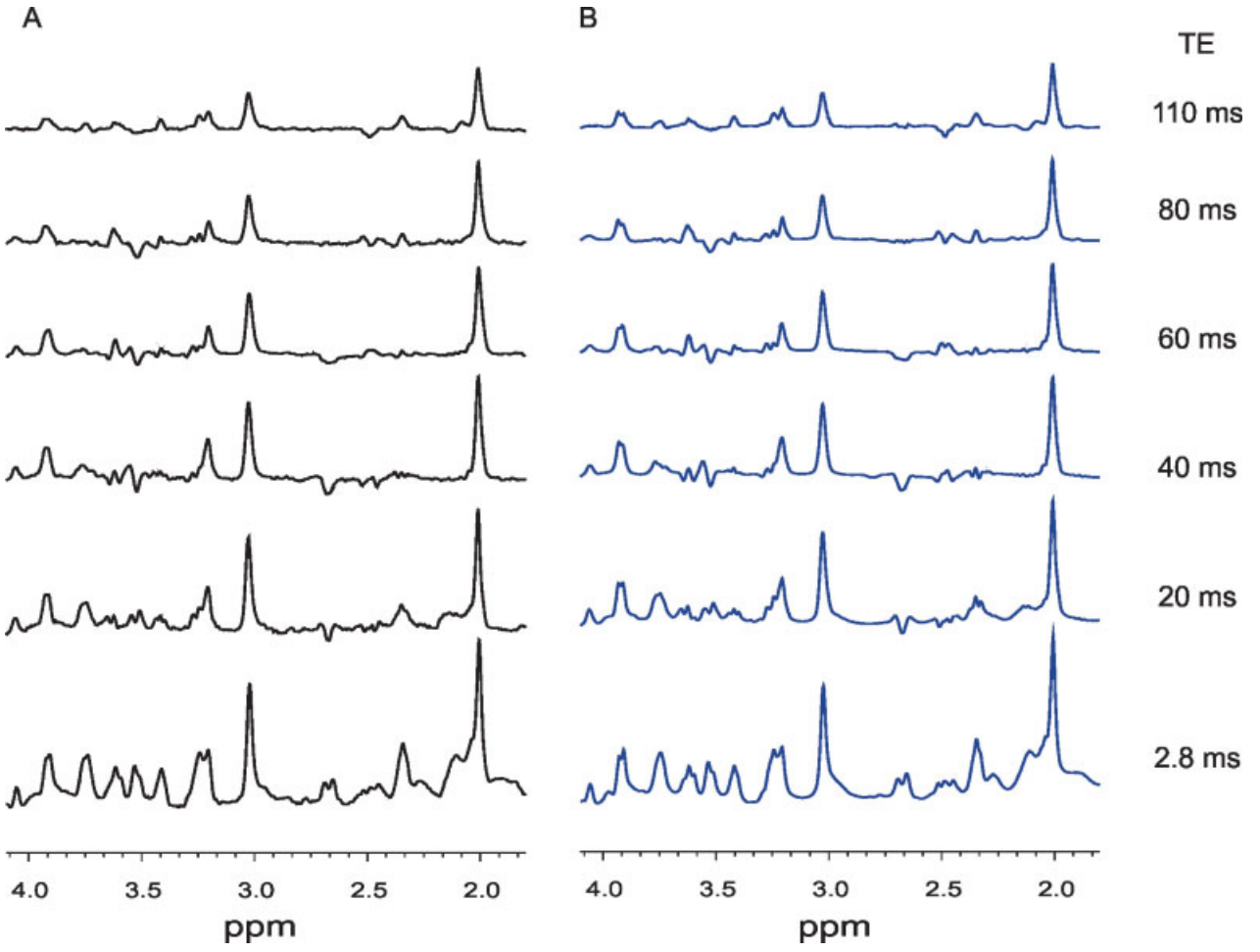

Figure 2. (A) *In vivo* water-suppressed ^{1}H MR spectra of the rat brain measured using SPECIAL at *TE* ranging from 2.8 to 110 ms (volume of interest = $2.5 \times 5 \times 5$ mm^3, $TR = 4$ s, 160 scans, Gaussian function weighting: $gf = 0.12$; no baseline correction, eddy current correction, or post-processing water removal were performed). (B) The LCModel fit of the corresponding spectrum.

DISCUSSION

In this study, the T_2 relaxation time of coupled spin resonances of cerebral metabolites was measured in rat brain *in vivo* at 9.4 T, benefiting from a high spectral resolution and sensitivity which allowed the measurement of 13 metabolites even at $TE = 110$ ms.

Although the T_2 measurement of singlets is comparatively straightforward, measurement of T_2 of coupled and especially strongly coupled spin resonances needs to take into account the complex *J*-modulation. In this study, this was accomplished by using the LCModel and *TE*-specific basis sets to fit coupled spin resonances at different *TE*s. The LCModel enabled a robust and rigorous data analysis based on prior knowledge. The use of simulated spectra as prior knowledge of spectral evolution at different *TE* for complex spin systems is consistent with a previous study, where the T_2 relaxation time of citrate in the prostate was measured (7). Simulated spectral patterns were validated by comparison with the *in vitro* measurement (Fig. 1), and the spectral appearance of the fit using the simulated basis set for LCModel analysis closely matched that observed *in vivo* at all *TE*s (Fig. 2).

The T_2 values of the singlets measured in this study (NAA, tCr, and tCho) were, within experimental error, comparable to those reported by others in rat (5) and mouse (4) brain at 9.4 T. Another study reported a longer T_2 for the methyl singlet of, e.g., tCr (171.1 ± 3.9 ms) and NAA (294.3 ± 5.1 ms) (6) using the LASER-type localization method (15). The longer T_2 can be explained by the use of a CPMG-like sequence, which provides a T_2 closer to the intrinsic T_2 (16).

In vivo, the T_2 values of individual groups from creatine and NAA indicate that the methyl moiety has a longer T_2 than CH_2 and CH groups, probably because of the faster rotation of the methyl group. It is of interest to note that the aspartyl NAA T_2 is comparable to that of most coupled spin resonances measured here, making aspartyl NAA a suitable internal concentration reference in experiments where its signal is co-edited, such as in the case of glutathione detection (2).

DOI: 10.1002/nbm

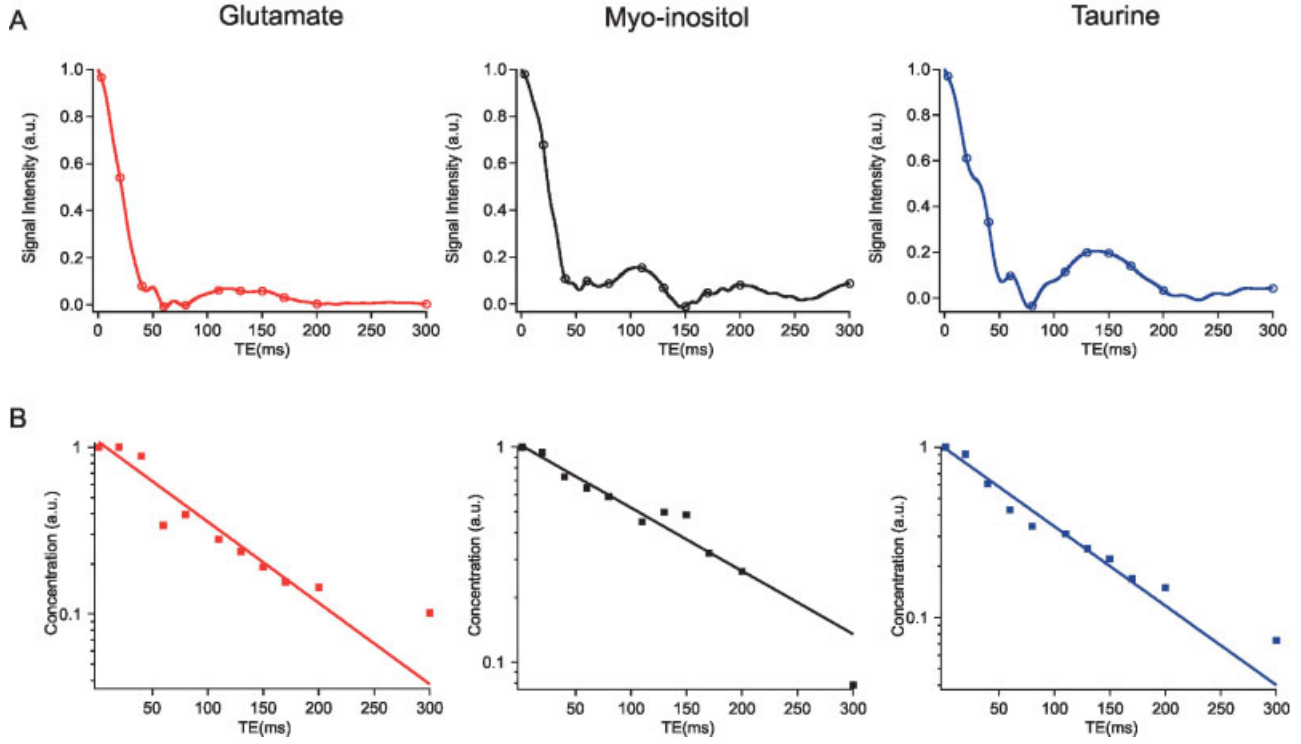

Figure 3. (A) Graphs of the simulated effect of *TE* on total multiplet intensities of glutamate, *myo*-inositol, and taurine. Data points indicate the simulated signal intensities at each *TE* in the *in vivo* measurement. Relative maxima occur at *TE* values in the range 110–130 ms ($TE \sim 1/J$), and first minima at *TE* values ~60 ms ($TE \sim 1/2J$). (B) Semilogarithmic plot of the normalized apparent concentration of glutamate, *myo*-inositol, and taurine as a function of *TE* derived using LCModel analysis. An exponential fit yielded $T_2 = 89 \pm 8$ ms with $R^2 = 0.976$ for glutamate, $T_2 = 148 \pm 14$ ms with $R^2 = 0.961$ for *myo*-inositol, and $T_2 = 93 \pm 9$ ms with $R^2 = 0.966$ for taurine.

In virtually all editing techniques, a *TE* of at least 60 ms (i.e. ~1/2J) is used, and therefore the potential effects of T_2 relaxation have to be considered in the quantitation. Typically, quantitation has been performed by comparing signal intensity of the edited resonance with that of a singlet measured at the same *TE* (2). With the notable exception of *myo*-inositol, the T_2 relaxation times of coupled metabolites were within 25% of that of creatine. Note that, for the frequently edited spin systems such as GABA and glutathione, T_2 was within 10% of that of creatine, supporting the assumption of similar T_2 used in previous editing studies (2,3,17,18).

Table 1. Proton T_2 relaxation times of cerebral metabolites in rat brain *in vivo* at 9.4 T (SD was obtained from the exponential fitting procedure)

Metabolite	T_2 (ms)	SD (ms)	R^2
NAA	202	12	0.9843
Creatine	114	3	0.9973
Phosphocreatine	113	5	0.9921
tCr	113	3	0.9978
tCho	178	29	0.8694
Glutamate	89	8	0.9764
Taurine	93	9	0.9658
myo-Inositol	148	14	0.9608
Phosphorylethanolamine	96	12	0.9415
Glutamine	116	29	0.9019
Glucose	104	21	0.9071
GABA	105	23	0.8572
Glutathione	106	23	0.7852

As expected, the T_2 value of glutamate measured in the phantom was longer than that measured *in vivo* in rat brain. This finding is consistent with the fact that the T_2 relaxation time of metabolites is likely to be sensitive to the surrounding microenvironment. The method presented here can be used to investigate T_2 changes for a larger number of cerebral metabolites, as T_2 changes have been postulated to reflect microenvironment variations induced by pathology or naturally occurring during development (4,5).

As spectral overlap is more severe at lower fields, spectral editing methods are often used at 1.5 and 3 T. Accurate quantitation of GABA and glutathione, for example, by editing methods would benefit from such a T_2 assessment at these field strengths. The methodology used in this study can be extended to these field strengths and to human studies, to the extent that deconvolution methods such as LCModel allow assessment of individual metabolites. On the other hand, measurement of the T_2 relaxation time of coupled spin resonances is likely to be more challenging at the field strength of clinical

DOI: 10.1002/nbm

scanners because of the lower spectral resolution, as well as the limited signal-to-noise ratio and *J*-modulation effects. Given the general trend observed for singlets to have longer T_2 values at lower field (6), the T_2 values of coupled resonances of cerebral metabolites at lower field are likely to be longer than those measured in the present study.

We conclude from this study that it is feasible to measure the T_2 relaxation time of coupled spin resonances of metabolites, provided that the signal modulation due to *J*-coupling is properly taken into account by spectral simulation.

Acknowledgements

This study was supported by Centre d'Imagerie BioMédicale (CIBM) of the UNIL, UNIGE, HUG, CHUV, EPFL, the Leenaards and Jeantet Foundations, and SNSF grant 3100A0-116220.

REFERENCES

1. Pfeuffer J, Tkac I, Provencher SW, Gruetter R. Toward an *in vivo* neurochemical profile: quantification of 18 metabolites in short-echo-time ^{1}H NMR spectra of the rat brain. *J. Magn. Reson.* 1999; **141**: 104–120.
2. Terpstra M, Henry PG, Gruetter R. Measurement of reduced glutathione (GSH) in human brain using LCModel analysis of difference-edited spectra. *Magn. Reson. Med.* 2003; **50**: 19–23.
3. Terpstra M, Ugurbil K, Gruetter R. Direct *in vivo* measurement of human cerebral GABA concentration using MEGA-editing at 7 Tesla. *Magn. Reson. Med.* 2002; **47**: 1009–1012.
4. Larvaron P, Bielicki G, Boespflug-Tanguy O, Renou JP. Proton MRS of early post-natal mouse brain modifications *in vivo*. *NMR Biomed.* 2006; **19**: 180–187.
5. Lei H, Zhang Y, Zhu XH, Chen W. Changes in the proton T_2 relaxation times of cerebral water and metabolites during forebrain ischemia in rat at 9.4 T. *Magn. Reson. Med.* 2003; **49**: 979–984.
6. de Graaf RA, Brown PB, McIntyre S, Nixon TW, Behar KL, Rothman DL. High magnetic field water and metabolite proton T_1 and T_2 relaxation in rat brain *in vivo*. *Magn. Reson. Med.* 2006; **56**: 386–394.
7. Scheenen TW, Gambarota G, Weiland E, Klomp DW, Futterer JJ, Barentsz JO, Heerschap A. Optimal timing for *in vivo* ^{1}H-MR spectroscopic imaging of the human prostate at 3T. *Magn. Reson. Med.* 2005; **53**: 1268–1274.
8. Mlynarik V, Gambarota G, Frenkel H, Gruetter R. Localized short-echo-time proton MR spectroscopy with full signal-intensity acquisition. *Magn. Reson. Med.* 2006; **56**: 965–970.
9. Hoult DI, Richards RE. Critical factors in design of sensitive high-resolution nuclear magnetic-resonance spectrometers. *Proc. Roy. Soc. Lond. A Math. Phys. Eng. Sci.* 1975; **344**: 311–340.
10. Gruetter R, Tkac I. Field mapping without reference scan using asymmetric echo-planar techniques. *Magn. Reson. Med.* 2000; **43**: 319–323.
11. Tkac I, Starcuk Z, Choi IY, Gruetter R. *In vivo* ^{1}H NMR spectroscopy of rat brain at 1 ms echo time. *Magn. Reson. Med.* 1999; **41**: 649–656.
12. Provencher SW. Estimation of metabolite concentrations from localized *in vivo* proton NMR spectra. *Magn. Reson. Med.* 1993; **30**: 672–679.
13. Mulkern R, Bowers J. Density matrix calculations of AB spectra from multipulse sequences: quantum mechanics meets *in vivo* spectroscopy. *Concepts in Magnetic Resonance* 1994; **6**: 1–23.
14. Govindaraju V, Young K, Maudsley AA. Proton NMR chemical shifts and coupling constants for brain metabolites. *NMR Biomed.* 2000; **13**: 129–153.
15. Garwood M, DelaBarre L. The return of the frequency sweep: designing adiabatic pulses for contemporary NMR. *J. Magn. Reson.* 2001; **153**: 155–177.
16. Michaeli S, Garwood M, Zhu XH, DelaBarre L, Andersen P, Adriany G, Merkle H, Ugurbil K, Chen W. Proton T_2 relaxation study of water, *N*-acetylaspartate, and creatine in human brain using Hahn and Carr-Purcell spin echoes at 4T and 7T. *Magn. Reson. Med.* 2002; **47**: 629–633.
17. Terpstra M, Vaughan TJ, Ugurbil K, Lim KO, Schulz SC, Gruetter R. Validation of glutathione quantitation from STEAM spectra against edited ^{1}H NMR spectroscopy at 4T:application to schizophrenia. *Magma* 2005; **18**: 276–282.
18. Rothman DL, Petroff OA, Behar KL, Mattson RH. Localized ^{1}H NMR measurements of gamma-aminobutyric acid in human brain *in vivo*. *Proc. Natl. Acad. Sci. USA* 1993; **90**: 5662–5666.

DOI: 10.1002/nbm