Editing through Multiple Bonds: Threonine Detection

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In in vivo ^{1}H spectroscopy, the signal at 1.32 ppm is usually assigned to lactate. This resonance position is shared with threonine at physiological pH. The similarity of spectral patterns of lactate and threonine renders the separate measurement of either threonine or lactate without and even with editing technically challenging. In this study, the threonine signal was detected using a single-shot multiple-bond editing technique and quantified in vivo in both rat and human brains. A threonine concentration was estimated at 0.8 ± 0.3 mM (mean ± SD, n = 6) in the rat brain and at ~0.33 mM in the human brain. Magn Reson Med 59:245–251, 2008. © 2008 Wiley-Liss, Inc.

Key words: threonine; lactate; TOCSY; editing

In in vivo ^{1}H spectroscopy, the signal at 1.32 ppm is usually assigned to lactate (1,2), but this resonance position is shared with threonine at physiological pH. Lactate and threonine completely overlap at 1.32 ppm with the same chemical shift and the same *J*-splitting. Additionally, the closest *J*-coupled partners of the 1.32-ppm resonance of lactate and threonine resonate at 4.11 and 4.24 ppm, respectively, and they are typically not observed due to weak spectral representation and their close proximity to the water resonance. Another resonance of threonine at 3.58 ppm is overlapped by many other stronger resonances. Due to the above characteristics, measurement of either threonine or lactate alone is very difficult, even with spectral editing.

Presence of threonine in the human brain was reported based on a study using autopsied brains, and the concentration of threonine varied from 0.30 ± 0.09 μmol/(g wet weight) to 0.79 ± 0.37 μmol/(g wet weight) in different regions of the brain (3). The concentration of threonine in the occipital cortex was reported as 0.42 ± 0.16 μmol/(g wet weight). Threonine was also detected in vitro in the tissue of normal rat thymus glands and malignant tumors produced by S49 T-murine lymphoma cells (4), and in vitro in cell cultures of normal glial cells and of intracranial tumors (5) using 2D-COSY (COrrelated SpectroscopY). Variants of the same technique were used to detect threonine in vivo in rat (6) and human (7) brains. Recently, a new *J*-difference editing method was used to quantify the threonine concentration in the human brain by discriminating between the lactate and threonine resonance at 1.32 ppm based on selective refocusing of the *J*-coupled partner at 4.11 ppm and 4.24 ppm (8). The results indicated that threonine (0.56 ± 0.06 mM) was present at slightly higher concentration than lactate (0.47 ± 0.07 mM) in the human occipital cortex (8). This difference editing method required many scans (around 768) to obtain information about both lactate and threonine and was very sensitive to frequency variations because the selective pulses had a bandwidth of 15 Hz. Additionally, the signal at 1.32 ppm could be contaminated with lipids and lead to subtraction artifacts due to subject motion.

A single-shot editing using through-bond polarization transfer (TOtal Correlation SpectroscopY, TOCSY; 9–11) between closest protons has been recently used in in vivo studies to detect γ-aminobutyric acid (GABA) at 3 Tesla (T) in the human brain (12) and to detect the considerably weaker signal of β-glucose under the water peak in rat and human brains and to detect lactate under the lipid resonances in the rat brain (13).

In this study, a single-shot 1D-TOCSY-LASER (13) technique is used to measure the concentration of threonine alone in vivo in both rat and human brains using multiple-bond editing involving spins that are four bonds away from each other. The quantitative measurement of the concentration of threonine in vivo in the rat brain was performed and independently confirmed with brain extracts.

MATERIALS AND METHODS

Molecules

Lactate is an A_3X spin system with three methyl protons and one methine proton (14). The ^{1}H spectrum consists of a doublet at 1.32 ppm corresponding to the methyl group and a quartet at 4.11 ppm corresponding to the methine group. The *J*-coupling between the groups is 6.93 Hz.

Threonine is an A_3MX spin system with five nonlabile protons from a CH_3 and two CH groups (14). The ^{2}CH proton gives a doublet at 3.58 ppm (J_{23}-coupling 4.92 Hz), and the ^{3}CH proton resonates at 4.24 ppm as an eight-line multiplet due to its coupling with the ^{2}CH and $^4CH_3$ protons. The $^4CH_3$ protons give a doublet at 1.32 ppm due to coupling with the ^{3}CH proton (J_{34}-coupling 6.35 Hz).

Pulse Sequence: Principle

A 1D-TOCSY-LASER (13) sequence was optimized for detection of the threonine proton bound to ^{2}C (3.58 ppm) by using a multiple-bond coherence transfer. Following a water suppression module, all resonances were excited using a nonselective adiabatic half passage (AHP) pulse (15). A gaussian-shaped 180° pulse was applied at 1.32 ppm with three coherence selective gradient pulses to refocus the source spins (protons bound to the ^{4}C of threonine and the

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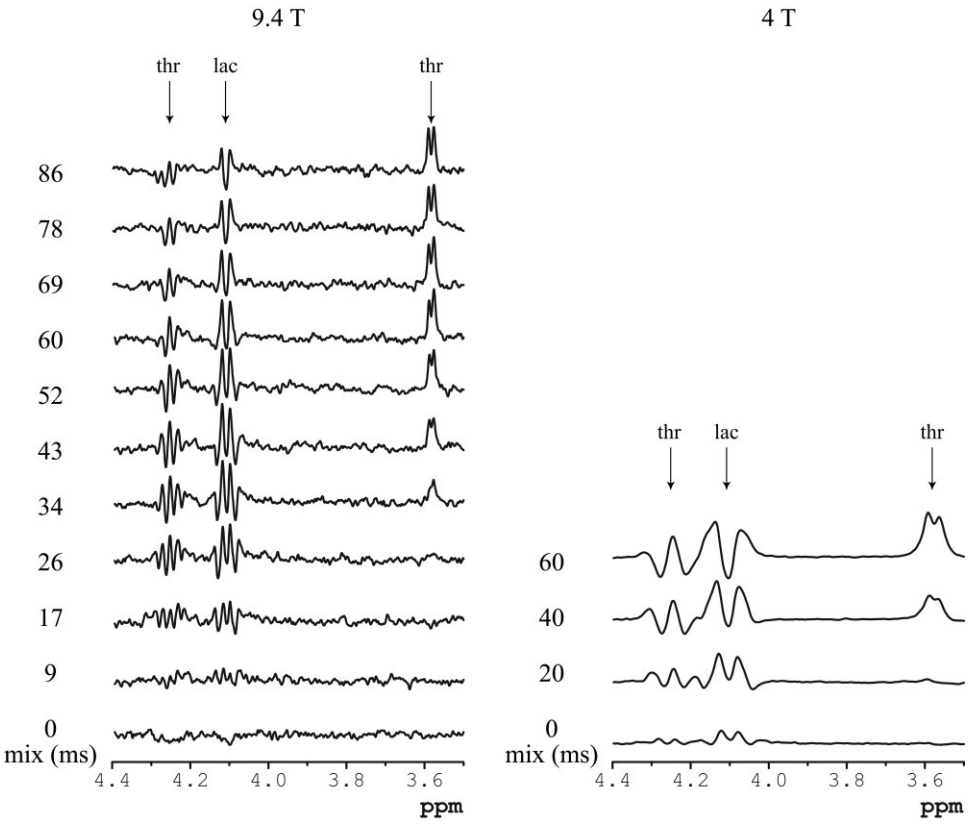

FIG. 1. Series of 1D-TOCSY-LASER spectra used to optimize the mixing time for threonine detection at 9.4 T and 4 T using an equal molar aqueous solution of lactate and threonine with mixing times ranging from 0 to 86 ms at 9.4 T and from 0 to 60 ms at 4 T. Only the region from 3.5 to 4.4 ppm is displayed. TR = 3 s, N_{EX} = 4 at 9.4 T and TR = 4 s, N_{EX} = 16 at 4 T and 2-Hz-line-broadening were used. thr, threonine; lac, lactate

^{3}C of lactate respectively) and to obliterate all the signals outside of the bandwidth of the gaussian-shaped pulse. During the mixing period, polarization transfer through homonuclear *J*-couplings was performed using a scheme reminiscent of selective TOCSY (11). Mixing was optimized using a phantom at 9.4 T and 4 T to maximize signal at 3.58 ppm. The three-dimensional (3D) localization was performed with a LASER sequence (16) immediately after the mixing period.

Phantom

The 1D-TOCSY-LASER editing scheme was optimized for detection of threonine signal by using an aqueous solution of threonine (9.7 mM) and lactate (9.7 mM). The rate of coherence transfer for the proton resonating at 3.58 ppm was measured at 9.4 T using 11 experiments with a mixing time ranging from 0 to 90 ms in ~9-ms increments and was measured at 4 T using four experiments with a mixing time between 0 and 60 ms in 20-ms increments (Fig. 1).

Animals

All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Six male Spraque-Dawley rats were intubated and ventilated with a 70%:30% N_2O:O_2 mixture and 1.0%–1.5% isoflurane. Body temperature was maintained at 37°C by warm water circulation. Femoral arteries were cannulated for blood sampling. After completion of surgery, animals were placed in a homebuilt holder, and the head position was fixed using ear rods and a bite-bar. Blood gases were measured every 15 min, and physiological parameters (pH = 7.42 ± 0.06, P_{CO_2} = 38 ± 6 mmHg, P_{O_2} > 100 mmHg) were maintained within normal range with few exceptions.

Extracts

At the end of experiments, three rats were killed using a focused microwave fixation device by applying 4 kW focused onto the brain for 1.4 s (Gerling Applied Engineering Inc., Modesto, CA). The brain was dissected out of the skull, immediately placed into liquid nitrogen, and stored at −80°C. Brain tissue was manually reduced to a powder with a mortar and pestle and extracted with 5 mL ice-cold perchloric acid (0.9 M).

Human Subjects

Normal volunteers (n = 4) were studied after giving informed consent according to the procedures approved by the Institutional Review Board of the University of Minnesota Medical School. Subjects were placed on a cushion and wore earplugs to minimize acoustic noise.

In Vivo Spectroscopy: Animal

In vivo animal spectra were acquired on a 9.4 T, 31-cm horizontal bore magnet (Magnex Scientific, Oxford, UK) interfaced with a Varian INOVA console (Varian, Palo Alto, CA). The magnet was equipped with an 11-cm gra-

dient insert capable of reaching 300 mT/m in 500 μs (Magnex Scientific, Oxford, UK). A quadrature 400-MHz ^{1}H radio frequency (RF) surface coil was used to transmit and receive. Localizer T_2-weighted multislice rapid acquisition with relaxation enhancement (RARE) images (17; repetition time [TR] = 5 s; echo time [TE] = 60 ms; echo train length = 8; matrix = 256 × 128; slice thickness = 1 mm; 11 slices) were acquired to select the 6.8 × 4.3 × 6.8 mm^3 volume of interest in the rat brain. Linewidths of 18 Hz for water were obtained after the adjustment of the first- and second-order shims using FAST(EST)MAP (18,19).

In the 1D-TOCSY-LASER sequence, the AHP pulse duration length was 4 ms, and each adiabatic full passage (AFP) pulse in LASER was an offset-independent adiabatic pulse, HS8 (20), using a pulse duration of T_p = 0.0015 s and a frequency sweep amplitude of A = 8.33 kHz (bandwidth [full width half maximum, FWHM]) = 2A). Selective refocusing was performed with either a 2.5-ms (FWHM = 620 Hz) or 4-ms (FWHM = 380 Hz) gaussian-shaped pulse applied at 1.32 ppm. Coherence transfer was performed with a 69-ms-long train of AFP pulses based on hyperbolic tangent and tangent functions (tanh/tan; 15,21) using T_p = 250 μs and A = 80 kHz (γB_1 = 7 kHz, FWHM = 24.3 kHz). The echo time was 37 ms for the LASER module. The echo time for the entire sequence, including both refocusing and coherence transfer, was 114 ms. A repetition time of 3 s was used. All spectra were acquired as a single free induction decay (FID) with 4096 complex points and spectral width of 5 kHz and were frequency corrected using residual N-acetylaspartate (NAA) signal at 2.01 ppm before summation. At the end of experiment, in vivo ultra-short echo-time STEAM spectra (22) were obtained for five of six rats from the same voxel (TR = 5 s; TE = 3 ms; TM = 20 ms; spectral width = 5 kHz; number of acquired complex points = 4096; N_{EX} = 64).

In Vivo Spectroscopy: Human

In vivo human spectra were obtained on a 4 T, 90-cm horizontal bore magnet (Oxford Magnet Technology, Oxford, UK) interfaced with a Varian INOVA console (Varian, Palo Alto, CA). The magnet was equipped with a Sonata gradient coil (Siemens, Erlangen, Germany), which was capable of reaching 40 mT/m in 400 μs. A quadrature 169 MHz ^{1}H surface RF coil was used to transmit and receive. Gradient echo (TR = 0.15 s; TE = 17 ms; matrix = 256 × 128; slice thickness = 3 mm) images were acquired to select a 3 cm × 5 cm × 3 cm volume of interest in the visual cortex. Linewidths of 9.9 Hz for water were obtained after the adjustment of the first- and second-order shims using FAST(EST)MAP (18,19).

In the 1D-TOCSY-LASER sequence, the AHP pulse duration was 4 ms, and each AFP pulse in LASER was a hyperbolic secant pulse, HS1 (16,23,24), using T_p = 0.004 s and A = 2.5 kHz. Selective refocusing was performed with either a 10-ms (FWHM = 150 Hz), 12-ms (FWHM = 125 Hz) or 15-ms (FWHM = 100 Hz) gaussian-shaped pulse applied at 1.32 ppm, and the coherence transfer was performed using a 60-ms-long train of tanh/tan pulses (T_p = 1250 μs; A = 16 kHz; γB_1 = 0.7 kHz; FWHM = 2.6 kHz). The echo time was 68 ms for the LASER module and 147 ms for the entire sequence including refocusing and coherence transfer. A repetition time of 4.5 s was used. Each FID was acquired with 2048 complex points using a spectral width of 3 kHz. FIDs were stored separately in memory and were frequency and phase corrected based on the NAA signal at 2.01 ppm before summation.

High-Resolution ^{1}H NMR Spectroscopy

^{1}H NMR spectra of brain extracts (n = 3) were measured at physiological temperature (37°C) under both physiological (pH = 6.8 corresponding to pD = 7.2) and very low (pH = 2.7) pH conditions with a simple pulse-acquired sequence (TR = 24 s; N_{EX} = 32) using a high-resolution 600 MHz Varian INOVA spectrometer (Varian) equipped with a 5-mm probe.

Quantification

In vivo STEAM spectra were analyzed using an LCModel 5.2-3 (25,26; Stephen Provencher, Inc., Oakville, Ontario, Canada), which calculated the best fit of the experimental spectrum as a linear combination of model spectra. The experimentally observed spectra of macromolecules (1) and the spectra of the following 18 metabolites were included in the basis set for LCModel: alanine (Ala), aspartate (Asp), creatine (Cr), GABA, glucose (Glc), glutamate (Glu), glutamine (Gln), glutathione (GSH), glycerophosphorylcholine (GPC), phosphorylcholine (PCho), *myo*-inositol (mIns), lactate (Lac), NAA, N-acetylaspartylglutamate (NAAG), phosphocreatine (PCr), phosphorylethanolamine (PE), *scyllo*-inositol (sIns), and taurine. Quantification was obtained using the unsuppressed water signal measured from the same voxel (assuming 83% brain water content) as internal references. The LCModel fitting was performed over the spectral range from 0.5 to 4.0 ppm.

RESULTS

The change in the resonance pattern and the build-up of signal as a function of mixing time are shown in Figure 1 for an equal molar aqueous solution of threonine and lactate at 9.4 T and 4 T. The threonine resonance of the ^{2}CH proton resonating at 3.58 ppm was easier to detect than the ^{3}CH proton resonating at 4.24 ppm due to simpler spectral pattern. The maximum signal for the ^{2}CH proton was obtained at mixing time of 69 ms at 9.4 T and 60 ms at 4 T.

The ^{2}CH resonance was unequivocally and consistently detected at 3.58 ppm in all six in vivo rat studies as judged from the consistent spectral pattern observed at 3.58 ppm, which was similar to the spectral pattern measured in an accordingly line-broadened phantom spectrum (Fig. 2A). The assignment of the resonance was confirmed by the inability to detect it when a train of adiabatic TOCSY pulses was turned off (not shown). The polarization was transferred from protons resonating at 1.32 ppm (lactate and threonine) and protons within the bandwidth of either a 2.5-ms-long or 4-ms-long refocusing pulse, such as *N*-acetylaspartate, glutamate, glutamine, and macromolecules. The signal from lactate at 4.11 ppm was also detected and resembled the signal detected in a solution containing lactate with application of appropriate line-

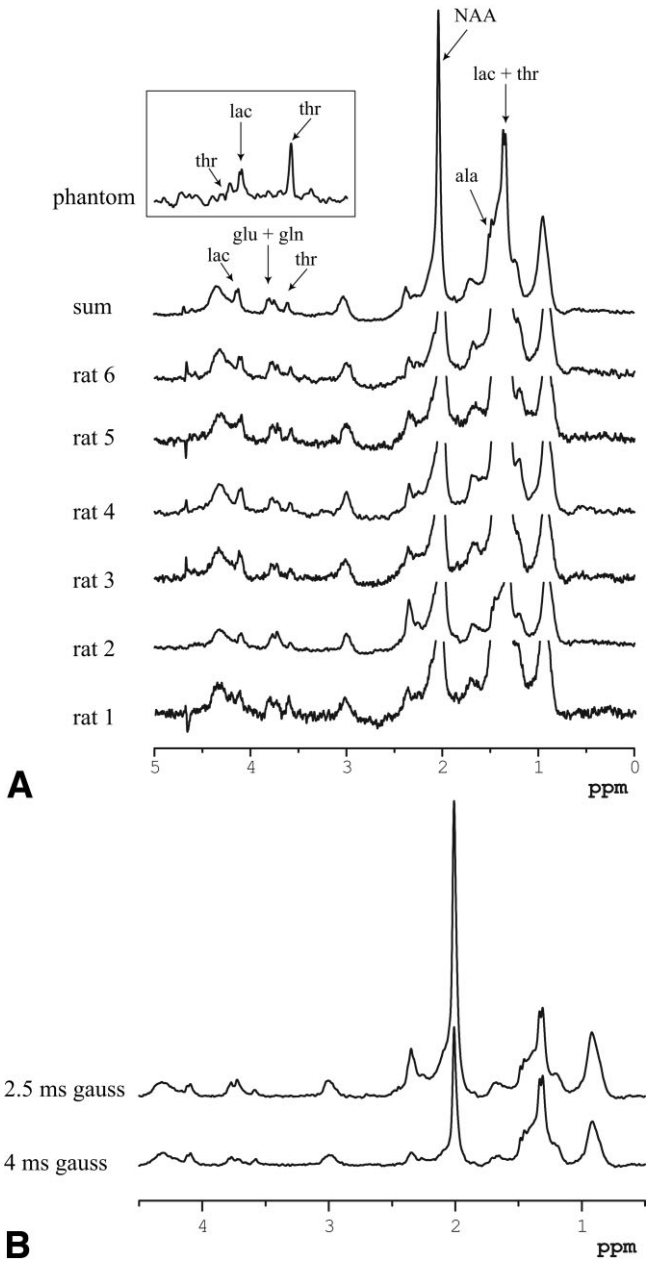

FIG. 2. The consistent detection of threonine in vivo in the rat brains using 1D-TOCSY-LASER. **A:** The spectra from six separate experiments and the sum of all those spectra are presented. Each in vivo spectrum was acquired in approximately 27 min from a 164 μL voxel. The spectra are shown with similar linewidths and with the amplitude adjusted using the residual NAA signal at 2.01 ppm. TR = 3 s, N_{EX} = 512 for each spectrum, mixing time = 69 ms, and a 4-ms gaussian-shaped pulse were used. The inset shows part of the spectrum around the threonine and lactate resonances obtained with a 1D-TOCSY-LASER editing of an equal molar aqueous threonine and lactate solution. The linewidths have been broadened to reflect in vivo linewidths. **B:** The effect of using either a 2.5-ms or a 4-ms gaussian-shaped pulse is shown. NAA, *N*-acetylaspartate; ala, alanine; glu, glutamate; gln, glutamine

broadening, but the other threonine signal at 4.24 ppm could not be resolved from the macromolecules background signal. Additionally, the signal from glutamate and glutamine was observed at 3.76 ppm. As expected, the signal intensity of glutamate and glutamine was reduced when a 4 ms (narrower bandwidth) was used rather than a 2.5-ms gaussian-shaped pulse (Fig. 2B).

LCModel analysis of a representative in vivo STEAM spectrum is shown in Figure 3A. A close match between the LCModel fit and the STEAM spectrum was achieved, as evidenced by the flat residual especially around the region of interest at 1.32 ppm. The peak at 1.32 ppm, which is the sum of lactate and threonine, was quantified with an average Cramer-Rao Lower Bound (CRLB) of 5% resulting in a 3.2 mM concentration. The concentration of lactate is high due to the use of isoflurane as anesthesia.

High-resolution spectra of the rat brain extracts were obtained at physiological (pH = 6.8 corresponding to pD = 7.2) and very low (pH = 2.7) pH to demonstrate the presence of overlapping lactate and threonine signals (Fig. 3B). The overlapping signals of threonine and lactate at 1.32 ppm at pH = 6.8 were completely resolved at pH = 2.7. Threonine and lactate in this particular extract contributed 20% and 80%, respectively, to the signal intensity at 1.32 ppm.

Two different methods were used to independently quantify the threonine concentration. In the first method, the threonine concentration was quantified with respect to residual NAA signal using a 1D-TOCSY-LASER spectra with taking into consideration the efficiency of the TOCSY transfer obtained from a phantom. The concentration of NAA was known from quantified STEAM spectra. The threonine concentration was 0.8 ± 0.3 mM assuming identical T_1 and T_2 for threonine, NAA, and creatine. In the second method, the total (threonine + lactate) signal at 1.32 ppm quantified from the STEAM spectra and the ratio of threonine to lactate obtained from extract were used to quantify the threonine concentration. Using this method, threonine concentration was 0.6 ± 0.2 mM and lactate concentration was 2.7 ± 0.6 mM. The concentrations of threonine obtained by two methods were within standard deviations of each other.

Figure 4A shows the effect of the length of the gaussian-shaped pulse on the ability to detect the threonine signal at 3.58 ppm using a 1D-TOCSY-LASER sequence at 4 T. As was observed at 9.4 T, the narrower the bandwidth of the gaussian-shaped pulse, the smaller the glutamate and glutamine signals were at 3.76 ppm. The glutamate and glutamine resonance at 3.76 ppm under the parameters used for editing at 4 T were in the dispersive mode which was further confirmed using a glutamate phantom at 4 T and by increasing the lengths of the LASER and mixing pulses at 9.4 T to match those at 4 T (not shown).

The ^{2}CH resonance was unequivocally and consistently detected at 3.58 ppm in all four in vivo human studies as judged from the consistent spectral pattern at 3.58 ppm, which was similar to the spectral pattern measured in an accordingly line-broadened phantom spectrum (Fig. 4B). Also, the lactate signal at 4.11 ppm was detected, but the other threonine signal at 4.24 ppm could not be resolved from the macromolecules' background signal. The quantification of threonine proved to be difficult in the individual subjects, but based on the lactate concentration in the summed spectrum and the expected signal recovery (measured on phantom), the concentration of threonine was estimated to be ~0.33 mM.

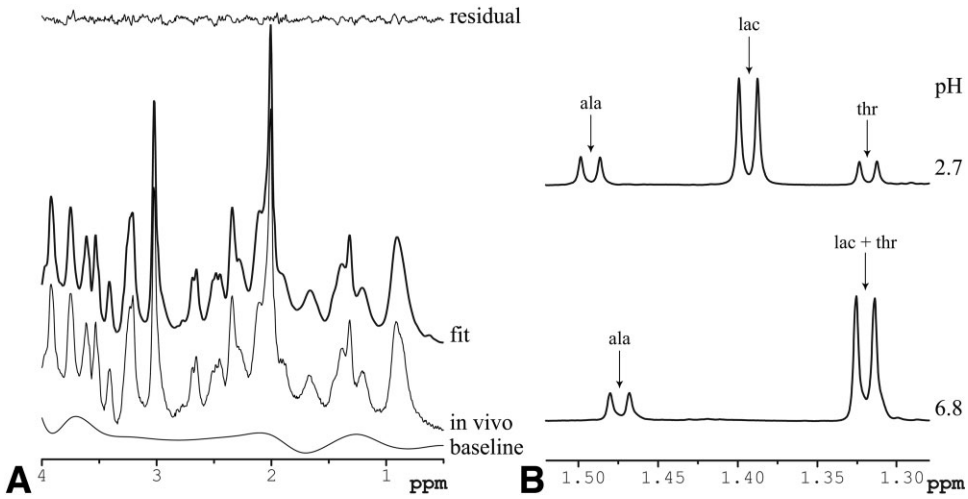

FIG. 3. **A:** LCModel quantification of the representative STEAM spectrum acquired from the same voxel that 1D-TOCSY-LASER spectrum. TR = 5 s, TE = 3 ms, TM = 20 ms, N_{EX} = 64, no line-broadening. **B:** High-resolution spectra of the whole rat brain extract at pH = 6.8 and pH = 2.7 obtained with a pulse-acquired sequence using a 600 MHz spectrometer. TR = 24 s, N_{EX} = 32, line-broadening = 0.5 Hz

DISCUSSION

In this study, the threonine signal was unequivocally detected in vivo in the rat brain and in the human brain using a single-shot 1D-TOCSY-LASER method. In the case of threonine, a multiple-bond polarization transfer across four bonds was used, and the signal at 3.58 ppm was detected. The polarization was transferred across four bonds. The polarization from the 1.32 ppm peak was transferred initially to the 4.24 ppm peak, which was subsequently transferred to the 3.58 ppm peak. The detection of the threonine signal at 4.24 ppm was not possible due to the dispersion of the threonine signal and its overlap with the intense signal from macromolecules at 4.23 ppm and 4.30 ppm. The 1D-TOCSY-LASER data are consistent with the connectivity assignments for the macromolecule resonances, M1 (1.24 ppm), M2 (1.43 ppm), and M3 (1.72 ppm), which resonated within the bandwidth of the selective gaussian-shaped pulse and were connected to the resonances at 4.23 ppm, 4.30 ppm (M10), and 3.00 ppm (M7), respectively (27,28).

The concentration of threonine detected in vivo in the rat brain using the 1D-TOCSY-LASER technique (0.8 ± 0.3 mM) was, within experimental error, the same as the concentration of threonine obtained from extracts (0.6 ± 0.2 mM). The concentration of threonine obtained using NAA signal as an internal reference could be influenced by the editing efficiency of the residual NAA signal especially when narrow-banded refocusing pulses are used to minimize glutamate and glutamine signal at 3.76 ppm. The concentration of threonine from extracts, which was obtained by quantification of the 1.32 ppm peak in an ultrashort echo time STEAM spectrum, could be influenced by

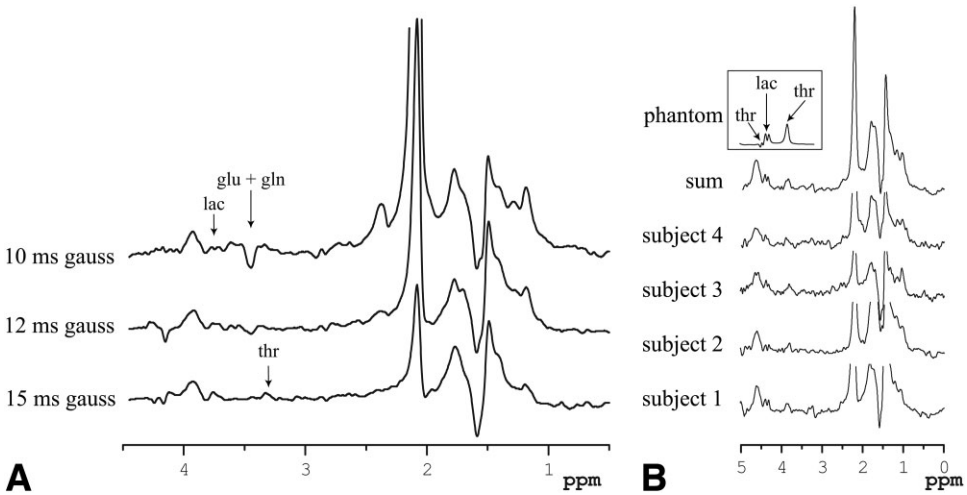

FIG. 4. **A:** The effect of using a 10-, 12-, or a 15-ms gaussian-shaped refocusing pulse on the ability to detect threonine signal at 4 T. TR = 4 s, N_{EX} = 256, line-broadening = 5 Hz. **B:** The consistent detection of threonine in vivo in the human brain using 1D-TOCSY-LASER. Each spectrum was acquired in approximately 20 min from a 45-mL voxel placed in the occipital lobe. Spectra are shown with similar linewidths and with the amplitude adjusted using macromolecules signal at 4.35 ppm. TR = 4.5 s, N_{EX} = 256, mixing time = 60 ms, and a 15-ms gaussian-shaped pulse were used. The inset shows part of the spectrum around the threonine and lactate resonances obtained with the 1D-TOCSY-LASER editing of an equal molar aqueous threonine and lactate solution. The linewidths have been broadened to reflect the in vivo linewidths.

the slight buildup of lactate during the process of microwave fixation. The concentration of threonine was significantly lower than the concentration of lactate which was in agreement with normal physiology under isoflurane anesthesia (1).

The threonine signal was also detected in vivo in the human brain using the 1D-TOCSY-LASER method and the concentration of threonine was estimated to be ~0.33 mM, which was approximately two times lower than the concentration of lactate. Of interest, the estimated lactate concentration was substantially below 1 mM, which is in excellent agreement with the 0.5 mM lactate concentration estimated from ^{13}C turnover studies (29). The efficiency of the detection of the threonine signal in the human brain at 4 T was not optimal due to the inability of performing scan to scan frequency correction using NAA signal and due to SAR limitations, which required either using a shorter mixing time or making the mixing pulses sub-adiabatic. The second option was used to obtain the human data at 4 T.

Measurement of threonine in the human brain was recently reported using a difference *J*-editing technique and yielded estimates of threonine concentrations with a standard deviation of 0.06 mM (precision of 10%) (8). The standard deviation of threonine measurement using 1D-TOCSY-LASER in the present work in the rat brain was 0.2 mM (30%) and is consistent with the typical 2% Cramer-Rao lower bounds obtained when measuring much more strongly represented NAA signal at TE = 2 ms, amounting to an absolute error of ~0.2 mM. Although based on the precision *J*-editing may be preferable to 1D-TOCSY-LASER editing for measurement of threonine, the use of single-shot detection may still be advantageous in situations in which movement cannot be avoided because 1D-TOCSY-LASER is less sensitive to frequency shifts (pulses in (8) are very narrow). In that case, sufficient precision can be obtained by more averaging at the expense of total acquisition time.

It is worth noting that, although implementation of the technique was similar to our previous study (13), the present work demonstrates for the first time the feasibility of in vivo editing through *multiple* C–C bonds, which cannot typically be achieved with *J*-editing in vivo due to the low value of long range *J*-couplings. Editing of threonine using this method demonstrates the feasibility of this approach, which may prove useful for detection of compounds that cannot be edited using conventional editing.

The detection of threonine and quantification of threonine in vivo in rat and human brain shine a new light on the previous studies related to the lactate detection and on the assignment of the peak at 1.32 ppm. In previously published work that introduced the 1D-TOCSY-LASER technique (13), the selective refocusing pulse placed at 4.11 ppm included lactate at 4.11 ppm (100% of signal refocused) and threonine at 4.24 ppm (93% of the signal refocused). During polarization transfer, the signal buildup at 1.32 ppm came from both lactate and threonine. In postmortem spectra, the majority of the signal came from lactate. The contribution of threonine to the signal at 1.32 ppm can be reduced using a more narrowband selective refocusing pulse, for example, when 20-ms gaussian-shape pulse is used only 23% of threonine signal is refocused. In the studies investigating metabolism in the activated human visual cortex at 7 T (2,30), the peak at 1.32 ppm should be assigned to both lactate and threonine. However, there is no evidence of change of threonine concentration under stimulation in the difference spectrum (30), nor is there a known biochemical mechanism linking functional activation with threonine biosynthesis.

In conclusion, this study established that the threonine can be reproducibly detected in vivo in both rat and human brains by using 1D-TOCSY-LASER. Approximately one-third of the signal at 1.32 ppm belongs to threonine and two thirds of the signal at 1.32 ppm belongs to lactate in the human brain. To correctly quantify lactate concentration using 1.32 ppm peak, the concentration of threonine has to be taken into consideration.

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