Research Article

NMR IN BIOMEDICINE

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Direct *in vivo* measurement of glycine and the neurochemical profile in the rat medulla oblongata

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The medulla oblongata (MO) contains a high density of glycinergic synapses and a particularly high concentration of glycine. The aims of this study were to measure directly *in vivo* the neurochemical profile, including glycine, in MO using a spin-echo-based ^{1}H MRS sequence at TE = 2.8 ms and to compare it with three other brain regions (cortex, striatum and hippocampus) in the rat. Glycine was quantified in MO at TE = 2.8 ms with a Cramér–Rao lower bound (CRLB) of approximately 5%. As a result of the relatively low level of glycine in the other three regions, the measurement of glycine was performed at TE = 20 ms, which provides a favorable J-modulation of overlapping myo-inositol resonance. The other 14 metabolites composing the neurochemical profile were quantified *in vivo* in MO with CRLBs below 25%. Absolute concentrations of metabolites in MO, such as glutamate, glutamine, γ-aminobutyrate, taurine and glycine, were in the range of previous *in vitro* quantifications in tissue extracts. Compared with the other regions, MO had a three-fold higher glycine concentration, and was characterised by reduced ($p < 0.001$) concentrations of glutamate (−50 ± 4%), glutamine (–54 ± 3%) and taurine (−78 ± 3%). This study suggests that the functional specialisation of distinct brain regions is reflected in the neurochemical profile.

Keywords: glycine; neurochemical profile; medulla oblongata; NMR spectroscopy

INTRODUCTION

The medulla oblongata (MO) is a part of the brainstem that regulates motor and sensory functions, as well as autonomic processes, such as heart beat, breathing and blood pressure. In MO, glycine (Gly) is present at a particularly high concentration. Gly has a dual role as an inhibitory neurotransmitter, activating Gly receptors, and as a co-agonist for excitatory *N*-methyl-D-aspartate receptors (1). In addition to its roles in neurotransmission and neuromodulation, Gly is the precursor of metabolites such as purines, creatine (Cr) and glutathione (GSH), and may be incorporated in lipids and proteins (2). The enhancement of Gly release in the brainstem has been proposed to be a neuroprotective mechanism against metabolic noxious stimuli, such as hypoxia, hypoglycaemia and ischaemia (3). In addition, lesions of MO are involved in several pathologies, such as hypertension (4) and multiple sclerosis (5). Therefore, it is of interest to investigate *in vivo* MO, with a specific focus on Gly tissue levels.

Localised MRS, as a noninvasive technique of exploring neurochemical information (6,7), is a desirable choice for the *in vivo* measurement of the neurochemical profile, including Gly in MO. As Gly is present at low concentrations in the forebrain and its resonance signal overlaps with the larger resonance of myo-inositol (Ins) in the ^{1}H MR spectrum, *in vivo* detection of Gly by MRS is challenging. In a number of studies, Gly tissue levels have been measured in human brain (8,9). Recently, the direct detection of Gly in rat brain *in vivo* has been reported, using spin-echo excitation at TE = 20 ms (10). To our knowledge, *in vivo*

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Abbreviations used: *Ala, alanine; ANOVA, analysis of variance; Asc, ascorbate; Asp, aspartate; BHB, β-hydroxybutyrate; CO, cortex; Cr, creatine; CRLB, Cramér–Rao lower bound; FAST(EST)MAP, fast, automatic shim technique using echo-planar signal readout for mapping along projections; FID, free induction decay; GABA, γ-aminobutyrate; Glc, glucose; Gln, glutamine; Glu, glutamate; Gly, glycine; GPC, glycerophosphocholine; GSH, glutathione; HI, hippocampus; Ins, myo-inositol; Lac, lactate; MO, medulla oblongata; NAA, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; NT, number of transients; PCho, phosphocholine; PCr, phosphocreatine; PE, phosphoethanolamine; Scyllo, scyllo-inositol; SEM, standard error of the mean; SNR, signal-to-noise ratio; SPECIAL, spin-echo, full-intensity acquired localised; ST, striatum; Tau, taurine; tCr, total creatine; VAPOR, variable power radiofrequency pulses with optimised relaxation delays; VOI, volume of interest.*

measurement of Gly and the neurochemical profile in the rat MO has not been reported to date.

In this study, we hypothesised that the combination of short-TE spin-echo excitation localisation and high magnetic field strength (9.4 T) would provide a high spectral resolution and signal-to-noise ratio (SNR), which would, in turn, allow a thorough investigation of the neurochemical profile of the medulla. Therefore, the aims of this investigation were to measure *in vivo* the neurochemical profile of rat MO by localised ^{1}H NMR spectroscopy, with particular focus on Gly quantification at TE = 2.8 ms, and to compare the neurochemical profile of MO with that of the hippocampus (HI), striatum (ST) and cortex (CO).

METHODS

Animal preparation

Experiments were performed on Sprague Dawley rats (274 ± 16 g, $n = 6$) under isoflurane anaesthesia (1.5–2% in oxygen). The animals were placed in a homebuilt holder, and the head was fixed by a bite bar and a pair of ear bars. The respiration rate was monitored by a small animal monitor (SA Instruments Inc., Stony Brook, NY, USA) and the body temperature was measured by a rectal thermosensor and maintained at $38.0 \pm 0.5^{\circ}$C by circulating heated water. All animal procedures were performed according to federal guidelines and were approved by the local ethics committee.

MRS

Proton NMR experiments were performed on a Varian INOVA console (Varian, Palo Alto, CA, USA) interfaced to an actively shielded 9.4 T/31 cm magnet (Magnex Scientific, Abingdon, Oxfordshire, UK) with a 12 cm inner diameter, actively shielded gradient (400 mT/m in 120 μs). A homebuilt geometrically decoupled ^{1}H quadrature surface coil (17 mm in diameter) was used as transceiver. Fast automatic shimming employing the fast, automatic shim technique using echo-planar signal readout for mapping along projections [FAST(EST)MAP] (11) was used to adjust all first- and second-order shims.

In vivo proton spectra were acquired using the spin-echo, full-intensity acquired localised (SPECIAL) spectroscopy method, which consists of a 2 ms slice-selective full-passage adiabatic pulse (hyperbolic secant 10) with a bandwidth of 10 kHz ($\gamma B_1/2\pi = 3.7$ kHz) applied on alternate scans, followed by a spin-echo sequence [0.5 ms/90° and 1 ms/180° asymmetric slice-selective pulses with bandwidths of 13.5 kHz, $\gamma B_1/2\pi = 3.35$ kHz and 6.2 kHz, $\gamma B_1/2\pi = 4.95$ kHz, respectively (12)]. Two TEs, i.e. 2.8 and 20 ms, were used for the assessment of the neurochemical profile in all four cerebral regions and for the Gly measurement in CO, HI and ST, respectively. Water suppression was achieved using variable power radiofrequency pulses with optimised relaxation delays (VAPOR) (13) and three outer volume saturation modules were interleaved with water suppression elements (13). Volumes of interest (VOIs) of $4 \times 2 \times 4$, $6 \times 1.5 \times 2$, $3 \times 2.5 \times 2$ and $3 \times 2 \times 2$ mm^3 were located in MO, CO, ST and HI, respectively. The surface coil was shifted to the posterior part of the head for the measurement of MO. Images of rat brain acquired with a fast spin-echo sequence (matrix, 128×128; TE = 40 ms; echo train length, 8; TR = 4 s; slice thickness, 1 mm; 13 slices; 4 averages) were used for the positioning of the VOIs. Flip angles in SPECIAL were adjusted for each VOI. All spectra were acquired with the following acquisition parameters: spectral width, 5 kHz; 4096 complex data points; TR = 4 s. The acquisition at each TE consisted of an array of free induction decays (FIDs) (16 averages for each FID) that were saved separately [CO: number of transients (NT), 240; HI: NT = 240–480; ST: NT = 480–640; MO: NT = 800–960). Spectra acquired without water suppression at the corresponding TE were used as internal reference for the quantification of metabolite concentrations. B_0 shift correction was performed prior to summation of the spectra, and eddy current effects were corrected with the acquired reference water signal.

Data analysis

Spectral data analysis was performed with LCModel (14), using a basis set containing simulated metabolite spectra and a macromolecular baseline measured by the inversion recovery method (15). As a result of the use of short TE, the effect of an inhomogeneous B_1 within the voxel (approximately 15–30% variation) on the evolution of J-coupled resonances, reported by Snyder *et al.* (16), was considered to be negligible. Therefore, nominal flip angles were used in the simulation of the spectra. For the quantification of short-TE spectra (TE = 2.8 ms), the unsuppressed water signal was used as an internal reference. Regional water content differences were taken into account when assessing the metabolite quantification (17). The Gly concentration in CO, HI and ST was obtained from spectra acquired at TE = 20 ms using the regional total creatine (tCr) concentration obtained at TE = 2.8 ms as the internal reference, assuming similar T_2 relaxation at 9.4 T (18). The spectral basis sets included the following metabolites: alanine (Ala); lactate (Lac); γ-aminobutyrate (GABA); *N*-acetylaspartate (NAA); glutamate (Glu); glutamine (Gln); aspartate (Asp); Cr; phosphocreatine (PCr); glycerophosphocholine (GPC); taurine (Tau); Ins; Gly; GSH; phosphocholine (PCho); ascorbate (Asc); scyllo-inositol (Scyllo); *N*-acetylaspartylglutamate (NAAG); β-hydroxybutyrate (BHB); glucose (Glc); phosphoethanolamine (PE). All results were presented as the mean ± standard error of the mean (SEM), and were compared with two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test.

RESULTS

With the ^{1}H quadrature surface coil (diameter, 17 mm), images acquired with the fast spin-echo sequence showed sufficient coverage of the rat brain, even the ventral part (Fig. 1), to allow volume positioning in MO. The typical linewidth of the water resonance was 10–12 Hz for CO, HI and ST, and 15–17 Hz for MO. *In vivo* proton MR spectra of MO in rat brain were obtained with an SNR (measured for the NAA singlet before applying apodisation, 800–960 averages) of 16 ± 2 ($n = 6$). Compared with the spectra of CO, ST and HI, the spectrum of MO showed a significant reduction in Tau resonances at 3.42 and 3.25 ppm, as well as of Glu and Gln resonances (Fig. 1). In addition, the peak of Ins at approximately 3.55 ppm was unusually high. Using the basis set that did not contain the Gly resonance led to a substantial fitted residual and slight distortion of baseline at 3.55 ppm in the LCModel analysis of *in vivo* NMR spectra obtained from MO (Fig. 2), which indicates the contribution of Gly with a resonance peak at 3.55 ppm. Adding the Gly spectrum to the basis set, this fitted residual was reduced to noise level and the baseline became smooth. The concentration of Gly was

NMR IN BIOMEDICINE

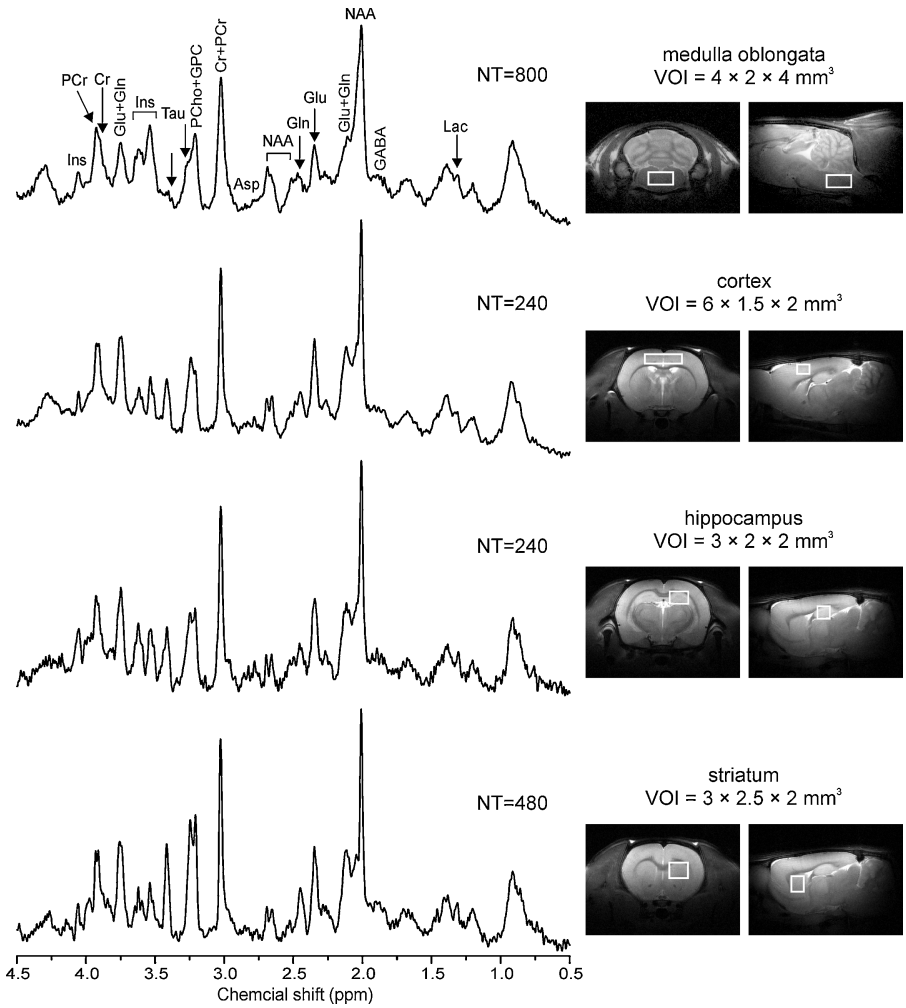

Figure 1. Representative *in vivo* ^{1}H NMR spectra acquired with the spin-echo, full-intensity acquired localised (SPECIAL) sequence at 9.4 T from the volumes of interest (VOIs) located in the medulla oblongata, cortex, hippocampus and striatum [TE = 2.8 ms, TR = 4 s, number of transients (NT) = 240–800]. Fourier transform and Gaussian function weighting (exp[–(t – 0.03)^2/0.12^2]) were applied. No baseline correction or water signal removal was applied. See text for abbreviations.

quantified at 2.9 ± 0.2 μmol/g (n = 6) with CRLB of 5.3 ± 0.1% (Fig. 3a).

A neurochemical profile of 15 metabolites including Asp, Cr, PCr, GABA, Gln, Glu, Ins, Lac, NAA, Tau, Glc, NAAG, PE, Gly and GPC + PCho, was quantified in MO at TE = 2.8 ms with CRLBs lower than 25% (Fig. 3b). In addition, Gly was measured in CO, HI and ST at TE = 20 ms, with CRLBs below 25%, and its concentration was 0.92 ± 0.04 μmol/g (n = 6), 1.04 ± 0.15 μmol/g (n = 5) and 0.95 ± 0.08 μmol/g (n = 6), respectively. These values were, on average, three-fold lower than that in MO ($p < 0.001$, n = 6) (Fig. 3a). Other marked differences were noted in the neurochemical profile of MO compared with the other three brain regions: significantly lower ($p < 0.001$, n = 6) concentrations of Glu (–50 ± 4%), Gln (–54 ± 3%) and Tau (–78 ± 3%) as averaged from CO, HI and ST, respectively. Concentrations of PE were lower in MO, reaching half of the concentration observed in HI (–49 ± 7%, $p < 0.01$, n = 6) and ST (–53 ± 6%, $p < 0.01$, n = 6). The levels of Cr in MO tended to be lower than in the other regions. For instance, compared with HI, the concentration of Cr in MO was 42 ± 2% lower ($p < 0.001$, n = 6). The concentration of NAA in MO was similar to that in ST, but lower than that in CO (–24 ± 4%, $p < 0.001$, n = 6) and HI (–27 ± 4%, $p < 0.001$, n = 6).

DISCUSSION

This study demonstrated the feasibility of using *in vivo* ^{1}H NMR spectroscopy to determine the neurochemical profile in rat MO, composed of 15 metabolites including Gly, the content of which may reflect the high density of glycinergic neurons (19). The low CRLBs of these metabolites (below 25%) indicate the high reliability of the current measurement. This can be ascribed to the high SNR and spectral resolution at 9.4 T, the use of a large quadrature surface coil that covers the ventral part of the rat brain, and the sensitivity improvement using an advanced localised spectroscopic technique at short TE. Water suppression and outer volume suppression sufficiently suppressed unwanted signals, such as lipid signals and large water signals, which were not observed in the spectra of MO despite the location at the brainstem (Fig. 1). An excellent agreement of the concentrations of Glu, Gln,

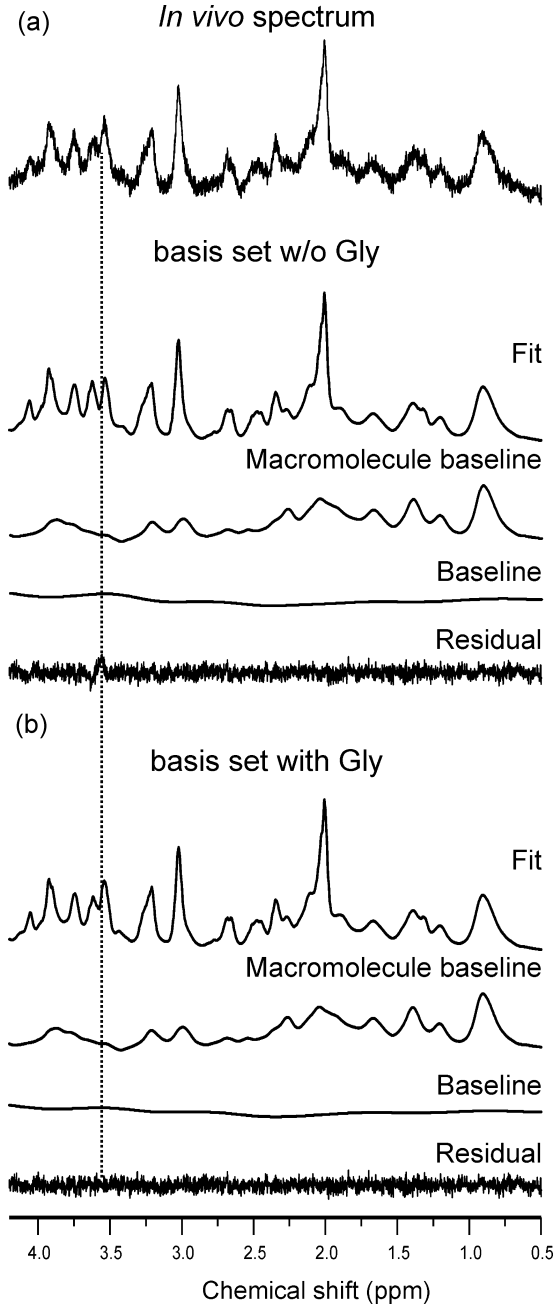

Figure 2. LCModel analysis of *in vivo* spectrum of the rat medulla oblongata (data from the same rat as shown in Fig. 1, no apodisation was applied) using the basis set without (a) and with (b) glycine (Gly). The corresponding fit, macromolecule baseline, baseline and residual are shown.

GABA and Tau in MO was found with previously quantified values in tissue extracts (20).

The measurement in MO using the surface coil was more challenging than that in other brain regions because of its deep location in the posterior part of the brain. However, the SNR obtained was still sufficient for the quantification of 15 metabolites of the neurochemical profile, which can be ascribed to the use of the short TE and high magnetic field. After first- and second-order shimming adjustment, the linewidth of the water signal was 15–17 Hz in MO, which was approximately 5 Hz greater than that in the other regions; this was attributed mainly to the increased magnetic field inhomogeneity because of susceptibility effects induced by the nearby sinuses.

Gly was also measured *in vivo* in MO at TE = 2.8 ms with a high precision, as judged from the low CRLB of 5% (Fig. 3). The concentration of Gly in MO measured in this study is in the range of previous reports using biochemical assays (20), and is somewhat lower than the value published by Aprison *et al.* (21), possibly as a result of the age difference of the rats (22).

Recently, threonine, which has one doublet close to the Gly resonance peak at 3.55 ppm, was quantified *in vivo* in rat brain (23) and human brain (24). Additional LCModel analysis using the basis set including threonine was performed to investigate the effect on the assessment of Gly (data not shown). However, the resonance peaks of threonine and Lac at 1.32 ppm are undistinguishable for LCModel, which tends to assign the whole peak to threonine, leading to strong overestimation of threonine and underestimation of Lac concentration. Consequently, the Gly concentration would also be systematically underestimated. The overestimated concentration of threonine was between 1.1 and 2.6 mM, which leads to a 9–21% alteration in Gly level. As the concentration of threonine should be 0.6 ± 0.2 mM (23), its effect on the quantification of Gly is expected to be less than 9%.

In CO, ST and HI, Gly cannot be reliably quantified at TE = 2.8 ms because of its lower regional concentration (approximately 1 mM) and the overlap with the strong resonances of Ins. Therefore, the measurement of the Gly concentration in CO, HI and ST was performed at TE = 20 ms, where the signal contribution from Ins was minimised as described previously (10).

When assessing the level of Gly from the spectra acquired at TE = 20 ms, T_2 relaxation of Gly was assumed to be similar to that of tCr. According to previously published T_2 values of metabolites at 9.4 T (18), T_2 of singlets ranged from 113 ms (tCr) to 202 ms (NAA). Even when assuming that T_2 of Gly is close to the longest T_2, i.e. that of NAA, the Gly concentration in these three regions (i.e. CO, ST and HI) would be 8% lower than the values calculated using the assumption of a similar T_2 to tCr, which is a quantitatively unimportant difference.

Relative to CO, ST and HI, Gly in MO showed a three-fold higher concentration (Fig. 3a), which is in accordance with the reported Gly regional distribution in rat brain (20,22). The high concentration of Gly in MO is consistent with its neurotransmitter function in this region. Gly has a dual role as a co-agonist for excitatory glutamatergic neurotransmission, by binding *N*-methyl-D-aspartate receptors, and as an inhibitory neurotransmitter, by activating strychnine-sensitive Gly receptors, which are particularly concentrated in MO (1). Defects in glycinergic neurotransmission may result, for example, in motor disorders, such as that observed in hyperekplexia (25), pain perception (26) or idiopathic generalised epilepsies (27). Thus, the concentration of Gly may eventually become a marker of these pathological conditions.

Among the differences in the neurochemical profile of MO compared with that of the other regions, some are of particular interest, as they may be related to the function of Gly, e.g. the Tau concentration in MO was particularly low. In the central nervous system, Tau not only has an important osmoregulatory function, but is also a modulator of Gly and GABA receptors (28). As an agonist of glycinergic and GABAergic neurotransmission systems, which are abundant in MO, the Tau concentration is likely to be tightly regulated. Interestingly, the other osmolyte, Ins, was found in MO at similar concentrations to that in other regions, namely CO and ST. Another marked characteristic of MO was the low

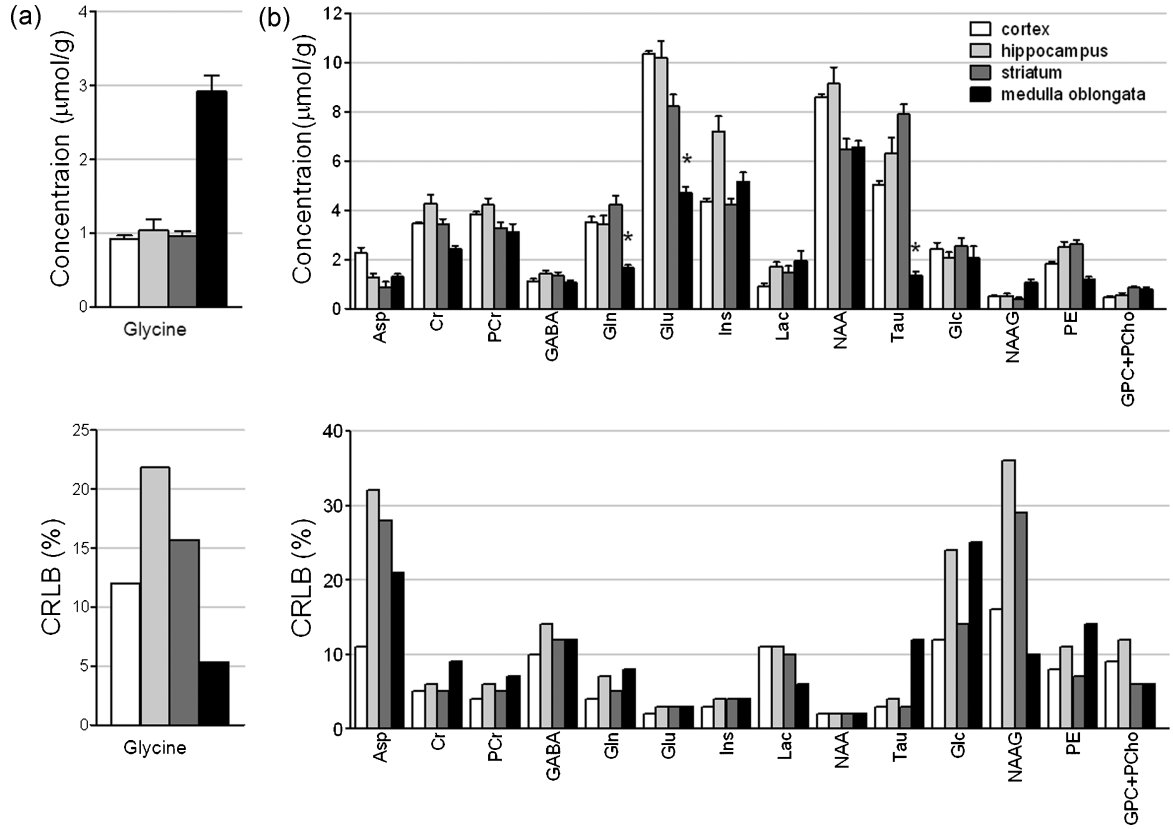

Figure 3. Glycine (a) and other metabolite (b) concentrations and the corresponding Cramér–Rao lower bounds (CRLBs) given by LCModel in the cortex, hippocampus, striatum and medulla oblongata (MO). Data shown as the mean ± SEM of six different rats. The asterisk indicates the concentrations of metabolites in MO that are significantly different from those in the other three regions ($p < 0.001$, two-way ANOVA followed by Bonferroni's multiple comparison test). See text for abbreviations.

concentrations of Glu and Gln when compared with those in other regions (Fig. 3b), being closely related to the prominence of inhibitory systems in MO, and thus perhaps reflecting a lower density of excitatory glutamatergic neurons.

Recently, the volume of MO measured by MRI has been suggested to correlate with the degree of dysfunction in multiple sclerosis, which is a neurodegenerative disorder leading to atrophy of the brain and spinal cord and resulting in motor impairment (5). Hence, the direct monitoring of the neurochemical profile of MO by *in vivo* ^{1}H NMR spectroscopy may thus provide a complementary investigation of multiple sclerosis-associated neurodegeneration affecting this brain area.

A few MRS studies, performed in human MO at 1.5 or 3 T (29–31), have reported exclusively the measurement of three major metabolites, i.e. tCr, NAA and total choline, because of the inferior spectral quality caused by susceptibility effects from the surrounding environment, the low SNR and spectral resolution at low magnetic field strength, and signal loss caused by spectroscopic methods performed at long TE. With an increase in the magnetic field strength, such as at 7 T, the spectral resolution and SNR can be improved, allowing the assessment of a larger number of metabolites in human MO.

CONCLUSION

We conclude that a neurochemical profile composed of 15 metabolites, including Gly, can be reliably quantified in rat MO by *in vivo* ^{1}H NMR spectroscopy at TE = 2.8 ms at 9.4 T. The ability to measure the neurochemical profile may be useful to investigate MO dysfunction, such as motor impairment in multiple sclerosis. We further conclude that the functional specialisation of distinct brain regions is reflected by specific patterns of metabolite concentrations.

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