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Non-invasive ^{1}H NMR Spectroscopy of the Rat Brain *In Vivo* Using a Short Echo Time STEAM Localization Sequence

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Fully localized proton NMR spectra were obtained from the brains of normal anaesthetized rats *in vivo* using stimulated echo (STEAM) spectroscopy sequences. Investigations were carried out at 2.35 T using a 40 cm bore magnet equipped with an actively shielded gradient system. Localized shimming resulted in water proton linewidths of 6.5–7.8 Hz permitting excellent water suppression. Thus, high-quality proton NMR spectra ($TE = 20$ ms) were acquired within measuring times of 1.5–6.4 min from 64 to 125 μL volumes-of-interest. The spectra show metabolite resonances due to *N*-acetyl aspartate, glutamate, creatine and phosphocreatine, cholines, taurine and inositols. The assignments of strongly spin-coupled resonances were confirmed by comparison with spectra from model solutions obtained under identical experimental conditions to those used *in vivo*. T_1 relaxation times as well as relative metabolite concentrations were evaluated from spectra obtained for repetition times ranging from 900 to 6000 ms. Sequential acquisitions of 1.5 min spectra before, during and after killing the animals exhibited a rapid accumulation of lactate, but did not reveal significant changes in other metabolite levels for several hours *post mortem*.

INTRODUCTION

With the increasing importance of *in vivo* NMR techniques for the non-invasive investigation of tissue metabolites and following on recent advances in proton spectoscopy of the human brain in both normal voluteers and patients, it becomes necessary to update the quality of corresponding spectra in experimental animals. This particularly applies to studies of cerebral metabolites in the rat since it is the most widely used animal for pathological models of human diseases.

Previous animal studies *in vivo* including those of rat brain have suffered from a variety of systematic problems. First, invasive surgical strategies were widely accepted and commonly employed to facilitate the access of the RF coil to the organ of interest as well as to improve the signal-to-noise ratio (SNR). Surface coils were either placed directly on the brain or skull[1–8] or chronically implanted.[9–11] Such procedures may alter the normal physiology and affect the outcome of the investigation to an unknown degree beyond the unavoidable effects of anesthesia. Second, localization by virtue of the B_1 profile of a surface coil alone is not sufficient to properly focus on a particular volume-of-interest (VOI) and to unambiguously exclude signal contamination from surrounding tissues. The loss of spectroscopic 'contrast' by partial volume averaging has the risk of masking metabolic changes in the desired organ. Nevertheless, this strategy was often followed due to its technical simplicity and availability, or because true three-dimensional localization was not considered to improve the biological or medical reasoning. Third, the spectral quality achieved was significantly impaired by poor magnetic field homogeneities causing linewidths of the order of 0.2 ppm, e.g., 20 Hz at 2.35 T. In addition, the access to a variety of metabolites was often hampered by residual eddy currents, insufficient water suppression, T_2 signal attenuation, or complex J modulation of strongly spin-coupled resonances.

The purpose of this paper is to report the application of a state-of-the-art localization method to studies of the normal rat brain *in vivo*, and to present a basis for future proton NMR investigations of cerebral pathologies. At the comparatively low field strength of 2.35 T and based on major technical improvements in the RF and gradient systems,[12] short echo time stimulated echo (STEAM) spectroscopy sequences provide a solution to many of the aforementioned problems. In fact, the present work extends our recent achievements in image-controlled, localized proton NMR spectroscopy of the human brain[13,14] to corresponding studies of the rat brain and further improves on non-invasive studies by others.[15]

MATERIALS AND METHODS

Instrumentation

All measurements were performed at 100 MHz on a Biospec system (Bruker, Karlsruhe, Germany) with a 2.35 T 40 cm bore horizontal magnet. Major modifications of the standard Bruker hardware configuration were necessary. The most important change was made

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Abbreviations used: NAA, *N*-acetyl aspartate; Glu, glutamate; PCr/Cr, phosphocreatine and creatine; Cho, cholines; Tau, taurine; Ins, *myo*-inositols.

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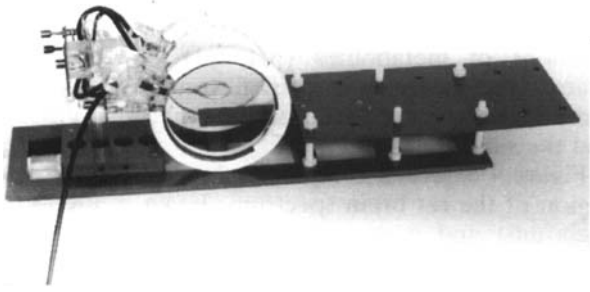


Figure 1. Experimental set-up and RF coil arrangements for fully localized, non-invasive proton NMR spectroscopy of the brain of anaesthetized rats *in vivo*.

with respect to the gradient coils that precluded any meaningful use of gradient-localized spectroscopy techniques due to their eddy current behaviour. The standard gradients were replaced by an actively shielded gradient system (Oxford Instruments, Oxford, UK) driven by an OI 2239 gradient power supply. The present implementation has a clear bore of 23.5 cm and yields a maximum gradient strength of 50 mT/m in each axis with a switching time of 1 ms. No pre-emphasis of gradient waveforms was used.

Off-centre positioning of the VOI is performed by switching the synthesizer frequency for the slice selection RF pulses and subsequently switching back to the centre frequency for data acquisition. This procedure results in a random phase shift with the standard equipment. The corresponding phase instability of the detected signal results in cancellation while signal averaging and artifacts during imaging. Therefore, the synthesizer has been replaced by a synthesizer allowing phase continuous frequency switching (Wavetek 5130A, San Diego, CA, USA). Additionally the synthesizer control word from the spectrometer was double buffered and latched into the synthesizer by a TTL trigger pulse from the pulse programmer in order to achieve precise timing for each frequency switch.

Finally, the home-made coil system comprises a 12 cm Helmholz coil for homogeneous excitation and a coaxial transmission line resonator[16] with a 2 cm diameter loop for signal reception. The coils are rigidly fixed to the sample/animal support as shown in Fig. 1 and can be quickly removed for positioning purposes. The standard RF amplifier has been replaced by a Class A power amplifier (AR1000LP, Amplifier Research, Souderton, PA, USA) controlled by the Bruker RF unit. A maximum power of 1 kW is available in pulsed mode and linear amplification of waveforms is possible up to ca 800 W.

Pulse sequence

The STEAM sequence employed for localized proton NMR spectroscopy of rat brain *in vivo* is essentially the same as that used for human studies. Details are described in Ref. 14. The desired VOI is selected by the intersection of three perpendicular slices in x, y and z that are defined by the three 90° RF pulses of the stimulated echo sequence and the application of corresponding slice selection gradients

$$[\text{CHESS} - \text{Spoil}]_3 - 90°(x) - \text{TE}/2 - 90°(y) - \text{TM} - 90°(z) - \text{TE}/2 - \text{Acq}$$

Suppression of the unwanted water signal is accomplished by three chemical-shift-selective (CHESS) RF pulses and related spoiler gradients preceding the localization pulses. The implementation allows the acquisition of spectra with echo times as short as TE = 20 ms. The middle interval was set at TM = 30 ms.

Selection of 5 mm thick slices was achieved using triangularly apodized sinc-shaped RF pulses of 3 ms duration with two sidelobes applied in the presence of 12 mT/m gradients. The resulting band width of the RF excitation was 510 Hz/mm corresponding to a proton chemical shift uncertainty of 0.2 mm ppm at 2.35 T. In this study the maximum displacement between the outermost metabolite resonances (lactate, N–CH_2 of creatine) was ca 0.5 mm or 10% of the 5 mm linear dimension of the 125 μL VOI. The water suppression pulses were 25.6 ms Gaussian waveforms with a bandwidth of ca 60 Hz (FWHM).

Spectral evaluation

All raw data were acquired with a bandwidth of 1 kHz using 2K data points. The spectra were processed on a VAX 3200 workstation (Digital Equipment, München, Germany) with a MAP 4000 array processor (CSPI, Billerica, MA, USA). Data transfer from the Aspect 3000 of the spectrometer was accomplished via a home-built parallel interface (300 kb/s) connected to a DRV11WA DMA interface in the VAX. Processing consisted of zero filling of the data to 64 K complex points, 2.5 Hz Gaussian multiplication in the time domain, and Fourier transformation followed by zero and first order phase correction. No further resolution enhancement, baseline correction, or other fitting routines have been applied. Relaxation times and relative metabolite concentrations were determined using spectral peak areas as described previously.[17]

Experimental protocol

All studies were performed using Wistar albino rats of 350–420 g body wt. About 20 animals were investigated in 70 studies. The animals were anaesthetized using chloral hydrate at a dosage of 360 mg/kg body wt. Anaesthesia lasted up to 2 h. The rats were placed in a prone position with the receiver coil placed over the skull. No surgery was performed.

The complete study protocol for rat brain spectroscopy comprised: (i) interleaved multi-slice FLASH NMR imaging (256 × 256 pixel resolution, 5 cm field-of-view, $TE = 6$ ms, $TR = 125$ ms, flip angle 70°, five slices, two excitations, total measuring time 64 s) to determine the exact position of the VOI in all three orientations; (ii) localized shimming on the water resonance using the same STEAM sequence as for subsequent acquisitions; (iii) optimization of the water suppression; and (iv) acquisitions using repetition times of 900, 1500, 3000 and 6000 ms. Localized shimming was

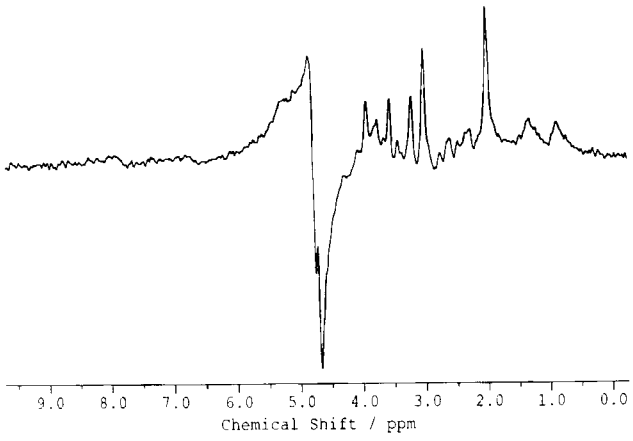


Figure 2. 100 MHz (2.35 T) localized proton NMR spectrum of rat brain *in vivo* ($TR = 1500$ ms, $TE = 20$ ms, $TM = 30$ ms, 256 scans, 6.4 min) acquired from a 125 μL ($5 \times 5 \times 5$ mm^3) VOI. Data processing consisted of zero filling of the data to 64 K complex points, 2.5 Hz Gaussian multiplication in the time domain and Fourier transformation followed by zero and first order phase correction only. The full frequency and signal ranges are shown to demonstrate the accessible water suppression as well as the absence of aromatic proton resonances under the conditions used.

performed using only the linear gradient offsets, while higher order terms were taken from the basic shim set of the magnet.

RESULTS AND DISCUSSION

Spectral quality and assignments

Figure 2 shows a typical proton NMR spectrum from a 125 μL VOI localized in a central position of the brain of an anaesthetized rat. The spectrum demonstrates that excellent water suppression may be achieved independent of the use of a short echo time for the STEAM sequence. In most cases the ratio of the residual water signal to the *N*-acetyl aspartate (NAA) at 2.01 ppm lay between 2 and 12. Localized shimming resulted in water resonance linewidths between 6.5 Hz and 7.8 Hz, i.e., ca 0.07 ppm and a factor of two to three better than previously reported in rats. Major metabolite resonances are due to NAA, glutamate, total creatine, cholines, taurine and inositols. The small signals in the methyl and methylene region of the spectrum at ca 0.9 and 1.3 ppm probably originate from mobile side chains of cytosolic proteins. Similar resonances are observed in proton NMR spectra of human brain at $TE \leq 20$ ms.[13,14] On the other hand, the aromatic part of the spectrum shows very little structure. This is in agreement with human results but contrasts with a previous report on a strong signal at ca 6 ppm in rat brain.[15]

The proton NMR spectrum shown in Fig. 3 originates from a different 125 μL VOI in the same animal. The figure focuses on the aliphatic part of the spectrum and exhibits the same metabolite resonances that are found in normal human brain. While the identification of the CH_2 and CH_3 singlet resonances of NAA, creatines and cholines is straightforward, the assignments of all spin-coupled resonances of metabolites such as glutamate (and glutamine), taurine and inositol were confirmed by studies of model solutions. At the low field strengths commonly in use for *in vivo* spectroscopy the presence of strong coupling and complex *J* modulations leads to ambiguities and signal cancellation. While *J* modulations may be minimized using short echo times, the distortions of the weak coupling lineshape pattern known from high-field NMR may be evaluated by comparing the *in vivo* data with spectra from aqueous solutions of metabolites (pH 7.1) obtained under exactly the same experimental conditions, i.e., the same sequence, the same magnet, the same gradients and the same coils.

Figure 4 depicts a comparison between an extended region of the rat brain spectrum shown in Fig. 3 (top spectrum) and a corresponding spectrum of a 1:1 mixture of NAA and glutamate (bottom spectrum). The middle spectrum of Fig. 4 represents a line broadened version of the bottom spectrum to mimic the linewidth observed *in vivo*. Obviously, the agreement with the top spectrum is striking with respect to both the aspartyl resonances of NAA and the γ-methylene resonances of glutamate. This similarity indicates that the concentrations of NAA and glutamate in the rat cortex must be similar, and that contributions from glutamine seem to be negligible. Moreover, the agreement of the NAA spectrum with what is observed *in vivo* for the lineshape and relative intensities of the aspartyl and methyl resonances emphasizes that the singlet resonance at 2.01 ppm may be entirely attributed to NAA without contamination from other *N*-acetyl moieties.

Interestingly, the relatively low field strength used here is beneficial to the assignment of spin-coupled resonances in contrast to what may be expected from high-field NMR of solutions. This is due to the fact that absolute linewidths of 7 Hz compare favourably to the homonuclear *J* coupling constants of 5–10 Hz observed for protons. It should be noted that this relative linewidth of only 0.07 ppm results in an absolute linewidth of ca 20 Hz at a field strength of 7.0 T completely masking all *J* splittings.

Localization

As in human spectroscopy animal studies require certainty about the spatial origin of tissue metabolites. In the past most problems were associated with the sup-

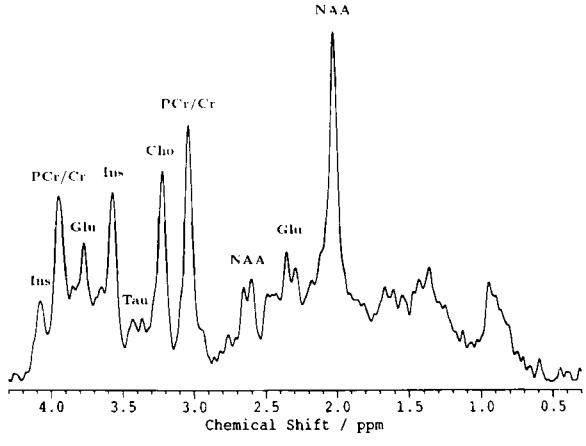


Figure 3. 100 MHz (2.35 T) localized proton NMR spectrum of rat brain *in vivo* ($TR = 1500$ ms, $TE = 20$ ms, $TM = 30$ ms, 256 scans, 6.4 min) acquired from a centrally located 125 μL VOI ($5 \times 5 \times 5$ mm^3). Resonances are due to NAA, glutamate (Glu), phosphocreatine and creatine (PCr/Cr), cholines (Cho), taurine (Tau) and inositols (Ins).

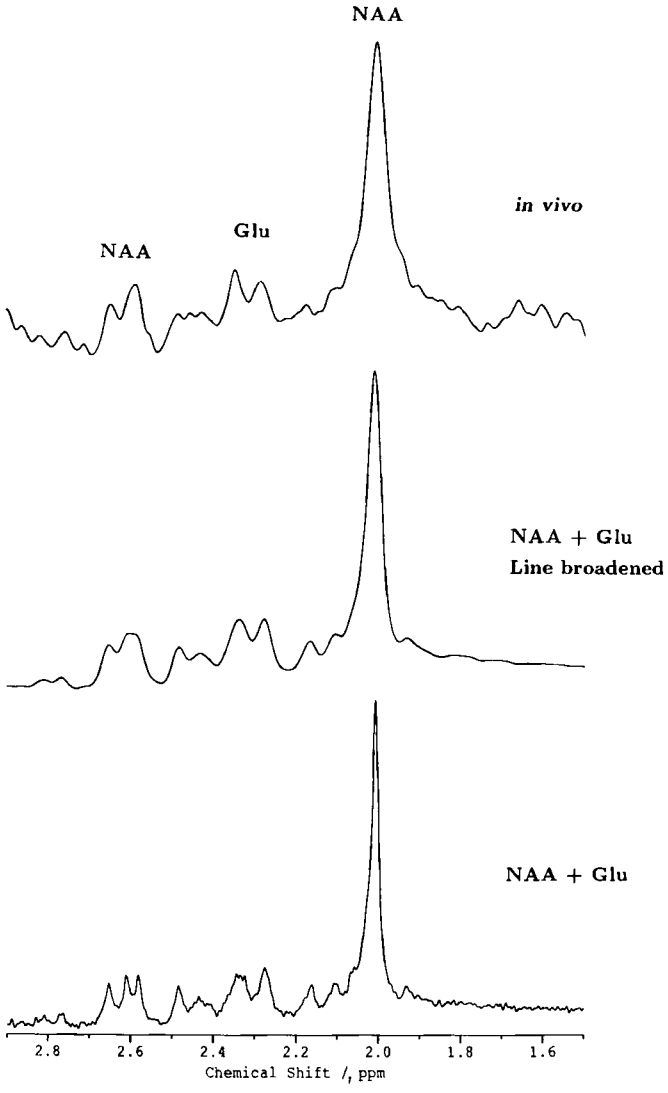
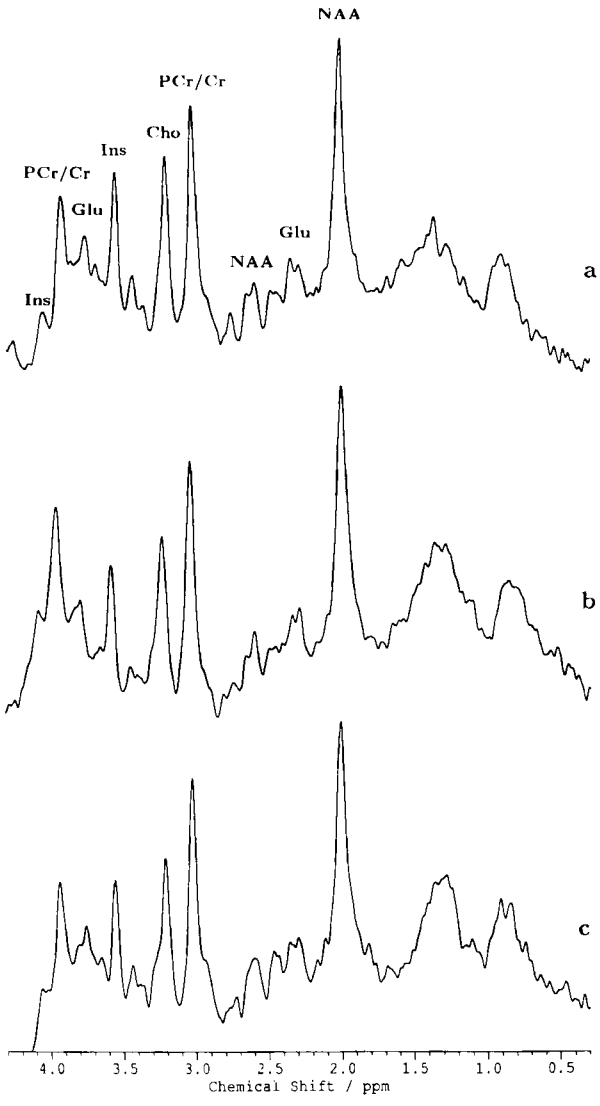


Figure 4. 100 MHz (2.35 T) localized proton NMR spectra (125 μL VOI, *TR* = 3000 ms, *TE* = 20 ms, *TM* = 30 ms, 256 scans, 6.4 min) used for metabolite assignments of NAA and glutamate. The top spectrum represents the 1.5–2.9 ppm spectral range of the *in vivo* rat brain spectrum shown in Fig. 3. The bottom spectrum was acquired under identical conditions using a model solution containing a 1 : 1 concentration mixture of NAA and glutamate. The middle spectrum is a line broadened version of the bottom spectrum using a 2.5 Hz Gaussian multiplication in the time domain to mimic the *in vivo* resolution.

pression of lipid signals from subcutaneous fatty tissues. The absence of such contamination in STEAM-localized spectra is further supported by the results shown in Figs 5 and 6 showing data from different locations and VOI sizes. Obviously, adequate spatial selection of the target VOI eliminates the need for additional means such as frequency-selective spin-echo refocusing and/or spatial presaturation to attenuate outside signals. According to the results of Fig. 5 the VOI may be freely positioned inside the rat brain without compromising either linewidth or water suppression. Each spectrum was acquired within 6.4 min (*TR* 1500 ms, 256 scans) using the full optimization protocol for the positions indicated in Fig. 5(d). The method opens a reliable way to the investigation of more focal alterations of rat brain metabolites, e.g., in only one hemisphere, as is often required in tumor or stroke models.



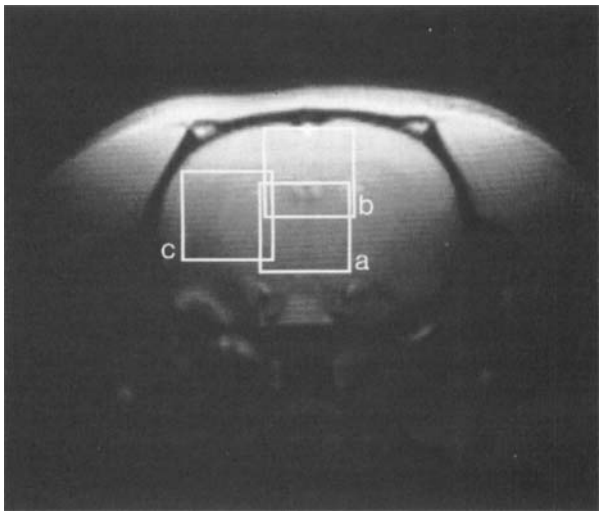


Figure 5. 100 MHz (2.35 T) localized proton NMR spectra ((a)–(c)) of rat brain *in vivo* (125 μL, *TR* = 1500 ms, *TE* = 20 ms, *TM* = 30 ms, 256 scans, 6.4 min) acquired from three different VOI indicated on the FLASH image shown in (d). Localized shimming and water suppression was performed for each volume independently. Resonances are due to NAA, Glu, PCr/Cr, Cho and Ins.

Figure 6 demonstrates the influence of the VOI size on the measuring time, SNR and spectral resolution. Spectra were acquired from a 216 μL ($6 \times 6 \times 6$ mm^3),

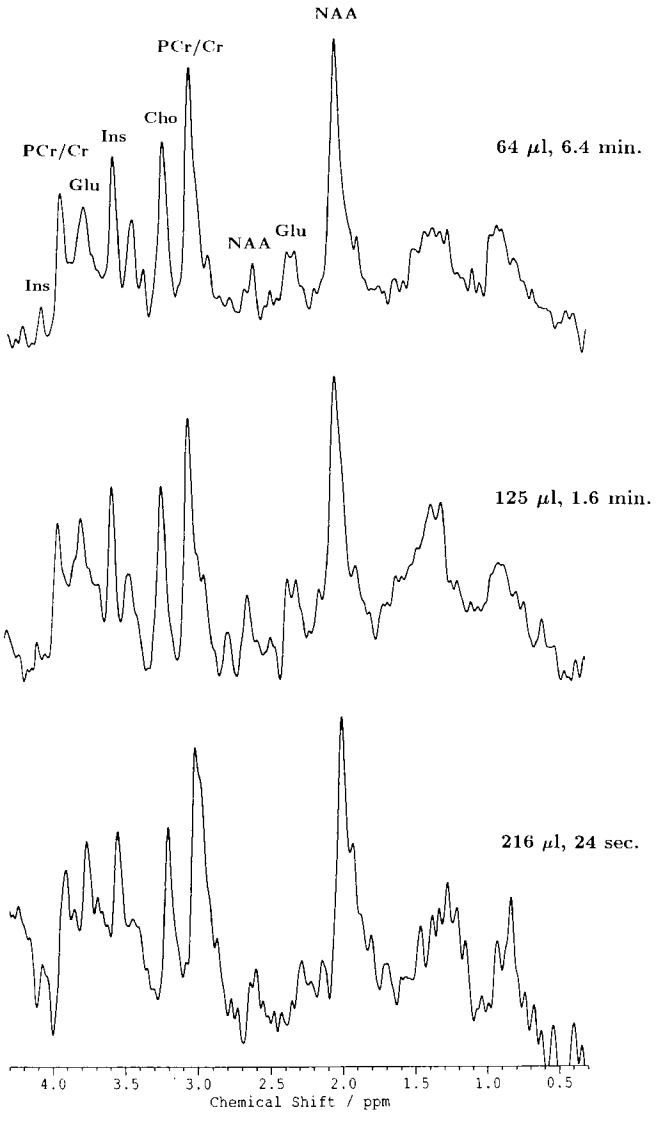


Figure 6. 100 MHz (2.35 T) localized proton NMR spectra of the rat brain *in vivo* ($TR = 1500$ ms, $TE = 20$ ms, $TM = 30$ ms) demonstrating the influence of the size of the VOI on the measuring time, SNR and spectral resolution. The spectra were acquired from three centrally located and concentric VOIs: 64 μL using 256 scans (top); 125 μL using 64 scans (middle); and 216 μL using 16 scans (bottom). The corresponding linewidths are 6.8, 7.8 and 9.8 Hz after independent shimming of each VOI. Resonances are due to NAA, Glu, PCr/Cr, Cho and Ins.

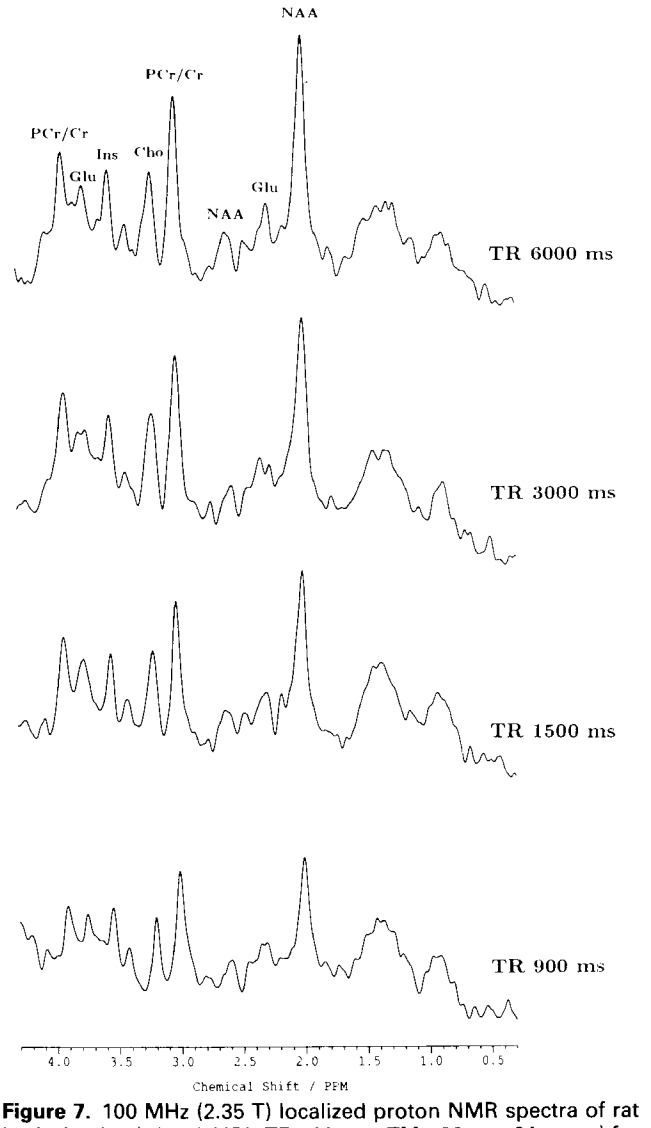


Figure 7. 100 MHz (2.35 T) localized proton NMR spectra of rat brain *in vivo* (125 μL VOI, $TE = 20$ ms, $TM = 30$ ms, 64 scans) for different repetition times ranging from $TR = 900$ (bottom) to 6000 ms (top).

125 μL ($5 \times 5 \times 5$ mm^3) and 64 μL ($4 \times 4 \times 4$ mm^3) VOI within measuring times of 24 s (16 scans), 1.6 min (64 scans) and 6.4 min (256 scans), respectively. The obvious improvement in quality with reduction of the VOI from 216 to 64 μL is due to an improvement of the local field homogeneity. In fact, the linewidths of the water resonance decreased from 9.8 (216 μL) to 6.8 Hz (64 μL). This variation in magnetic field homogeneity may not only be explained by inherent tissue susceptibility differences but also arise from the specific structure of the rat skull. The large air-filled cavities associated with the ears cause rather striking susceptibility-induced signal losses in coronal FLASH images even at comparatively short gradient echo times when cutting through the auditory meatus. In general, better homogeneity values may be achieved using similar VOI sizes but larger animals such as cats.[18]

T_1 relaxation times and metabolite concentrations

Spin-latice relaxation times T_1 of metabolite resonances were evaluated from series of spectra obtained with different repetition times. Figure 7 shows a set of *in vivo* rat brain spectra that were sequentially acquired from the same location with repetition times of 900, 1500, 3000 and 6000 ms ($TE = 20$ ms, 125 μL VOI, 64 scans). The T_1 relaxation times from eight different studies are summarized in Table 1. The values for NAA and cholines are close to those reported[17] for white matter in human brain at 1.5 T but slightly smaller than those at 2.0 T.[19] The T_1 value for phosphocreatine/creatine is significantly smaller (35%) than the human values at 1.5 and 2.0 T, while the inositol T_1 relaxation time is significantly longer (40%).

Relative concentrations of the major metabolites were determined using fully relaxed spectra obtained at $TR = 6000$ ms. The numbers are arbitrarily normalized to 10 for the concentraton of total creatine. If a concentration of 10 mM is taken as an internal reference for total creatine,[20] then the values in Table 1 may be read as absolute concentrations. Thus, the relative concentration of NAA in the rat brain is 35% lower than in

white matter of the adult human brain as determined by *in vivo* proton NMR spectroscopy.[17,19] However, an absolute concentration of 12 mM is higher than values of 4.5–9.5 mM obtained by biochemical analysis.[21] All other metabolites are rather similar in rat and human brain. This behaviour is not unexpected in view of the high degree of structural similarity and neuronal organization in mammals. The concentration of glutamate may be estimated to be the same as that of NAA according to the findings reported in Fig. 4. It is worth noting that an absolute concentration of 12 mM for glutamate is in agreement with literature values.[22] The absence of a resonance from lactic acid, e.g., in the bottom spectrum of Fig. 8, indicates that the concentration of cerebral lactate in the brain of anaesthetized rats is probably <1 mM.

Post-mortem changes

A large number of proton NMR spectroscopy studies of rat brain have concentrated on the feasibility of directly investigating the metabolic changes following an ischaemic insult, in particular the increase of lactic acid. Figure 8 shows a sequential series of rat brain spectra exhibiting the rapid increase of lactate after the death of an animal by administration of potassium cyanide. The spectra were acquired with a time resolution of 1.5 min ($TR = 1500$ ms, 60 scans) for a continuous period of at least 30 min before and several hours after death. It is worth noting that since the spectra were acquired fully localized within the brain there was no requirement for spectral editing to remove lipid contamination in the region of the lactate methyl signal at 1.33 ppm. The lactate methyl signal increases rapidly after death, reaching a plateau within ca 12–15 min. This time course is similar to that observed *in vivo* during the initial phase of hypoxia caused by reduction of oxygen supply.[1] No significant changes were observed for other metabolite resonances including NAA.[23–25] This finding again emphasizes the remarkable differences that exist between global ischaemia in an animal model (or metabolic alterations *post mortem*) and the situation encountered in a focal ischaemia in a stroke patient.[26]

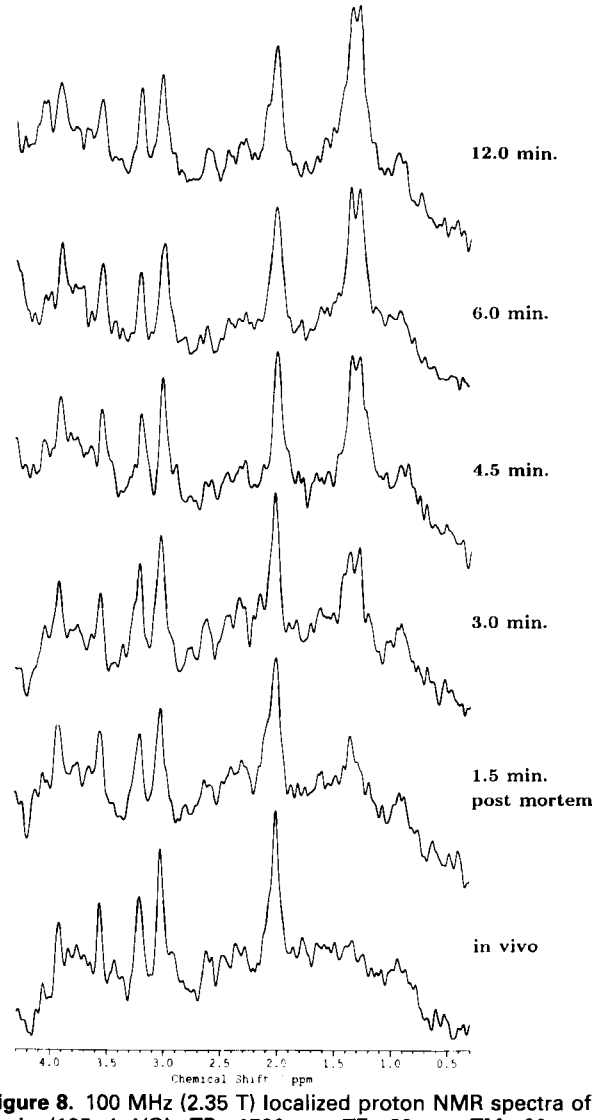


Figure 8. 100 MHz (2.35 T) localized proton NMR spectra of rat brain (125 μL VOI, $TR = 1500$ ms, $TE = 20$ ms, $TM = 30$ ms, 64 scans) showing the immediate *post mortem* accumulation of lactate. The spectra were acquired continuously using sequential 1.5 min time intervals before, during and after killing the animal by administration of potassium cyanide.

Table 1. Spin-lattice (T_1) relaxation times (2.35 T) and relative metabolite concentrations of cerebral metabolites detected in localized proton NMR spectra of rat brain *in vivo*

Compound	δ (ppm)	T_1 (ms)	Rel. concn. (mM)
NAA	2.01	1520	12
Glu	2.28	—	12[a]
Cr/PCr	3.03	1150	10
Cho	3.22	1050	2.1
Ins	3.56	1800	7.4

The relaxation times represent mean values for $n = 8$, with SD ±20%. Chemical shifts δ are referenced to NAA (2.01 ppm). Relative metabolite concentrations were obtained from fully relaxed spectra ($TR = 6000$ ms). The values are normalized to total creatine and may be read as absolute concentrations assuming 10.0 mM for creatine as an internal reference.

[a] The concentration of glutamate is an estimate by comparing the intensity and lineshape of its methylene signals with the NAA methyl signal (cf. Fig. 4).

CONCLUSIONS

Fully localized proton NMR spectra of the rat brain *in vivo* were obtained following the same non-invasive strategy of image-controlled spectroscopy that has been previously applied to studies of human volunteers and patients. The approach is based on a single voxel STEAM spectroscopy sequence implemented on an animal imaging/spectroscopy system equipped with actively shielded gradients. Spectra with linewidths of ca 7 Hz (2.35 T) were acquired from 64 to 125 μL VOIs within measuring times of 1.5–6.4 min. Metabolite resonances due to NAA, glutamate, creatines, cholines, taurine and inositols have been identified. The results are comparable to those obtained for human

brain *in vivo*. In fact, the use of similar field strengths not only makes such comparisons more meaningful, but is also beneficial to the evaluation of metabolites with spin-coupled resonances due to the achievable absolute linewidths.

In general, non-invasive techniques in the form of image-selected three-dimensional localization are considered to be a prerequisite for more reliable physiological and biochemical investigations of anaesthetized animals. The spectroscopic results of the normal rat brain presented here are intended to define the bottom line for future pathological applications exploiting the power of *in vivo* NMR spectroscopy to detect metabolic alterations in the living animal.

Acknowledgements

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Note added in proof: The strong resonance at 3.43 ppm in Figs 5, 6 and 7 has now been assigned to glucose (M. L. Gyngell, T. Michaelis, D. Hörstermann, H. Bruhn, W. Hänicke, K. D. Merboldt and J. Frahm. Cerebral glucose is detectable by localized proton NMR spectroscopy in normal rat brain *in vivo*. *Magn. Reson. Med.* in press).

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