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Brain Development: ^{1}H Magnetic Resonance Spectroscopy of Rat Brain Extracts Compared With Chromatographic Methods

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We compared in vitro ^{1}H magnetic resonance spectroscopy (MRS) measurements of rat brain extracts (rats: 2-56 days old) with chromatographic measurements and in a further step also with results of in vivo MRS. The following substances can be reliably measured in brain extracts by in vitro MRS : N-acetylaspartate (NAA), total creatine (Cr), phosphorylethanoloamine (PE), taurine (Tau), glutamate (Glu), glutamine (Gln), γ-aminobutyrate (GABA) and alanine (Ala). Two different methods of MRS data evaluation compared with chromatographic data on Cr and NAA are shown. During development of the rat from day 2-56 brain concentrations of PE, Tau and Ala decrease, those of NAA, Cr, Glu and Gln increase, while GABA does not change. The developmental patterns of these substances are the same, whether measured by in vitro MRS or by chromatographic methods. Quantification of NAA, Cr, Tau, GABA and PE leads to the same results with both methods, while Glu, Gln and Ala concentrations determined by in vitro MRS are apparently lower than those measured chemically. The NAA/Cr ratios of 7 to 35-day-old rats were determined by in vivo ^{1}H MRS. These results correlate with chromatographic and in vitro data. Using appropriate methods in the in vivo and in vitro MR-technique, the obtained data compare well with the chromatographic results.

KEY WORDS: Brain development; ^{1}H MRS; amino acids; N-acetylaspartate; creatine; rat.

INTRODUCTION

^{1}H magnetic resonance spectroscopy (^{1}H MRS) in vitro and in vivo has become an important tool in studying brain metabolism in normal and pathological conditions. The ^{1}H nucleus has several significant advantages compared to other nuclei: highest sensitivity, 100% natural abundance, and it is found in all metabolites (1).

Intensive analytical work has been focused on the brain, either in extracts or in homogenates (2-8). The development of wide bore magnets allows in vivo ^{1}H MRS of the brain in animals and humans (9-19). Problems not solved yet in all instances are the assignment of peaks in the MR spectrum to single chemical components and the accuracy of quantitative measurements by the MR technique. In vitro ^{1}H MRS has a higher spectral resolution and sensitivity compared to in vivo MRS (2, 15, 20, 21) which is due to both the usually stronger static fields and the smaller and more homogeneous samples - leading to an increased homogeneity of the magnetic field and hence sharper resonance sig-

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nals. This is certainly of some advantage for studying the crowded proton spectra arising from the various metabolites in rat brain extracts. The aim of the in vitro study was, therefore, to resolve, to identify and to determine the concentrations of a few metabolites in rat brain and compare these data with chromatographic results. As age-dependent changes of compounds in the brain are of considerable interest for the investigation of normal and abnormal development, we undertook this investigation in rat brains from day 2 to 56 postnatally.

In vivo ^1H MRS does not allow yet the measurement of absolute concentrations of brain metabolites, and therefore, data are usually given as ratios of the observable and identifiable components. In order to validate such ratios, we compared the NAA/Cr ratios obtained by in vivo MRS with in vitro MRS and chromatographic estimations.

We do not propagate in vitro MRS as an alternative to regular chemical determination methods for amino acids; our study is thought as a first step to monitor brain development in vivo in rats and later also in humans.

EXPERIMENTAL PROCEDURE

Sprague-Dawley rats were housed in a temperature- and light-controlled room (22 °C, 12-hour light/dark cycle). Food (NAFAG, Gossau, Switzerland) and water were supplied *ad libitum*. Newborn rats were grouped in litters of 8 animals, 4 females and 4 males. Weaning was at day 25. At the following time points rats from one litter were sacrificed by decapitation between 1 and 2 p.m.: postnatal day 2, 7, 14, 21, 28, 35, 56. Brains were excised, immediately frozen on dry ice and stored at −80 °C prior to chromatographic or in vitro MR determinations. For all time points 4 animals were measured.

Sodium pentobarbital was from Serva, New York, USA, 3-(trimethylsilyl)propionic acid sodium salt from EGA-Chemie, Steinhausen, W. Germany and tetrabutylammonium phosphate from Sigma, St. Louis, USA. All other substances were from Sigma or Merck, Darmstadt, W. Germany.

HPLC Determinations of N-Acetylaspartate and Creatine. Brains were homogenized 1:2 in 0.1 M $HClO_4$ by sonication. Homogenates were centrifuged at 44,000 g. Depending on the concentration of each compound the supernatants were diluted in H_2O prior to HPLC analysis.

HPLC parameters for NAA determinations were: Aquapore RP-300 column, 220x4.6 mm, 7 μm particle size (Brownlee Labs, Santa Clara, USA); the mobile phase was adapted according to (22): 0.05% KH_2PO_4, 0.04% tetrabutylammonium phosphate (TBAP), pH 3.7 adjusted with phosporic acid; flow rate 0.75 ml/min. NAA was detected according to Koller at al. (23) at 210 nm (Spectrophotometer UVIKON 720 LC, Kontron, Zürich, Switzerland). For peak quanitification an HP 3380A integrator was used.

HPLC parameters for creatine determinations were: Aquapore RP-300 column, 220x4.6 mm, 7 μm particle size; mobile phase: to 930 ml of a 2:1 (v:v) mixture of 0.02 M citric acid and 0.02 M Na_2PO_4, 0.58 g octylsulfonic acid (final: 2.5 mM), 18.6 mg EDTA (final: 0.05 mM) and 70 ml methanol were added (24). The pH of his solution was between 3.10-3.20; flow rate 0.75 ml/min. Creatine was detected according to Juengling and Kammermeier [22] at 210 nm.

Determination of Brain Amino Acids. For amino acid analysis 30-60 mg brain tissue was homogenized in 0.5 M $HClO_4$ containing norvaline (0.08 mM) as internal standard (1:20, w:v). After centrifugation at 10,000 *g* an aliquot was analyzed using automated ion exchange chromatography (Biotronic LC 7000, Munich, F.R.G.) (25).

Determinaton of Phosphorylethanolamine (PE). PE was measured according to Burri et al. (26). In brief, PE was extracted with 0.1 M $HClO_4$, and separated with TLC. PE was then made visible with ninhydrin. The resulting purple spots were scanned, integrated and compared with standard solutions.

In Vitro MR Spectroscopy. For one measurement brains of 4-8 rats for each time point were pooled. In each pool the number of females and males was the same. Brains were sonicated in 1.5 M $HClO_4$ (1:2, w:v). The homogenates were kept on ice for 15 min. to precipitate proteins. Samples were centrifuged at 44,000 *g*. pH was adjusted to 7.0 by adding 1 M KOH. During this procedure $KClO_4$ was formed which was removed by centrifugation. The supernatants were lyophilized and stored at −20° C. For in vitro MR measurement samples were dissolved in 1 ml deuterated water (deuteration degree 99.5%), and 0.5 ml of a 4 mM solution of sodium 3-(trimethylsilyl)propionate (TSP) dissolved in deuterated water was added as internal standard. The in vitro measurements were carried out on a BRUKER AM 400 spectrometer operating at 9.3 T and equipped with a process controller. The standard one-pulse experiment, slightly modified to preirradiate the strong residual water signal (preirradiation delay 6 s) with a very low decoupling power, was applied. The closest signal of interest (methine proton of NAA) was well outside of the region for which perturbations arising from the irradiation frequency have to be taken into account. The spectral window was 4000 Hz and the acquisition time was 4.1 s corresponding to a digital resolution of 0.244 Hz/point. A relaxation delay of 6 s was introduced, sufficiently long to allow complete relaxation of all protons. 40 scans were accumulated. To prove the assignment of known resonances (2, 20, 21) and to look for accidental signal overlap, a phase sensitive COSY experiment with double quantum filtering and preirradiation of the residual water was applied.

The time responses of the 1D experiment were exponentially weighted (line broadening 5 Hz), Fourier transformed and referenced to the internal standard (TSP)(21). For each of the investigated metabolites the strongest and the best resolved signal (typically the signal of a methyl group) was selected for quantification. Signal areas were determined by using integrals or signal intensities and line widths at half height.

Concentrations for the various metabolites were calculated from a comparison of their signal areas with the signal area of the internal standard TSP, both scaled according to the number of the corresponding protons. According to rather different linewidths measured in the proton spectra of brain extracts of 2 and 56 day-old rats, a strong exponential weighting (LB 30 Hz) was applied to both FIDs prior to Fourier transformation and subtraction of the corresponding spectra. Prior to the calculation of the difference spectrum (Fig. 9) the spectra were scaled to the TSP internal reference.

In Vivo MR spectroscopy. For measurements rats were anaesthetized with sodium pentobarbital. The dosage was between 5 and 50 mg/g body weight depending on the age of the rats. For all time points 4 animals were measured. In vivo MR spectra were obtained on a 2T-GE-CSI spectroscopy-imaging system equipped with 3 G/cm standard gradients. The animal's brain was positioned in the center of the magnet and a double turn 8 mm diameter coil positioned 1-2mm above the skull. The position of the brain was verified using a standard spin echo

imaging technique. Shimming was performed on the FID of the water resonance resulting in a linewidth of 10 to 20 Hz. The flip angle was determined by optimizing the signal strength of the water resonance at 4.8 ppm and the suppression of the surface lipid resonances. Water suppressed proton spectra were recorded using a 11-22 pulse sequence (27) with maximum excitation at 2.5 ppm between the Cr and the NAA resonances. The TE delay was 6 ms and the relaxation delay was 1.5 s. Phase cycling was performed using the Exorcycle scheme. 512 transients were recorded with a digital resolution of 1.0 Hz and a spectral width of 2 KHz.

Data analysis was performed with a resolution enhancement and a convolution difference method (28) to remove broad components. The resolution enhancement consisted of a double exponential multiplication with the function DM (10) provided by the CSI-software. For the convolution difference the resolution enhanced spectrum was linebroadened with 10 Hz and subtracted from the former spectrum. The resulting spectrum was quantified by manually defining a baseline and measuring the peak heights. Reproducibility was checked by having three separate observers analyze the typical sample of data. Their results agreed to within 10 %. After the spectral data were quantified, the ratio NAA/Cr was determined. Since the NAA and Cr had similar linewidths, the above data reduction procedure had little effect on the relative heights of the two peaks.

RESULTS

1) Development Changes and Correlation Between In Vitro MRS and Chromatographic Measurements. A typical in vitro MR spectrum of a brain extract of 21-day-old rats is shown in Figure 1. For each of the investigated metabolites the strongest and best resolved signal was selected. For Cr, NAA and Tau we had the choice of using integrals or intensities and line widths at half height. Integrals would eventually lead to underestimated concentrations for metabolites with signals in highly crowded spectral regions. This effect is shown in Fig. 2. Similar signal area values were obtained with both procedures for the methyl signal of Cr, slightly overlapping with the triplet signal of GABA (γ-CH), and both agreed well with the HPLC data (a). A clear and systematic deviation of the integral based value was observed on the other hand for NAA (b), of which the methyl signal appeared in a highly crowded spectral region. Therefore, we used for Cr, NAA and Tau graphically calculated peak areas, for all other substances peak integrals.

For NAA, Cr and Ala the resonance signal of the methyl group was chosen. PE was visible at 3.98 ppm. Its other resonance at 3.25 ppm was covered by Tau and choline. This was one reason that Tau at 3.26 ppm and choline at 3.22 ppm was not used for quantification. Further, the choline resonance included also signals of phosphorylcholine (PC), glycerophosphorylcholine (GPC) and probably also the signal of the amino-carbon (N-CH_2) of glycerophosphorylethanolamine (GPE). Gln and Glu could be quantified at 2.45 and 2.35 ppm, respectively. However, these resonances were complex (peak multiplets) and, therefore, difficult to evaluate. For GABA quantification we preferred the triplet at 2.30 ppm to the quintet at 1.90 ppm, which contains also a signal from acetate.

Creatine (Figure 2a). Chromatographically measured total creatine increased during development steadily between day 2 and day 28. Cr concentration increased during this time period from 5.30 ± 0.25 μmol/g brain to 9.56 ± 0.92 μmol/g brain. No further changes were found between day 28 and 56. MRS measurements were in good agreement with the HPLC data.

N-Acetylaspartate (Figure 2b). We found a significant increase in brain NAA concentration between day 7 and day 21. During this time period NAA concentrations quadrupled. Between day 21 and day 56 NAA did not change further. Concentrations detemined by in vitro MRS were in good agreement with the HPLC measurements.

Taurine (Figure 3). Tau decreased during development from 21.89 ± 1.00 μmol/g brain at day 2 to 6.91 ± 1.21 μmol/g brain at day 28. Between day 28 and 56 Tau concentration did not change further. Tau concentrations determined by in vitro MRS were comparable to those chromatographically determined.

GABA (Figure 4). GABA did not change much during development. There was an increase in GABA molarity from 1.53 ± 0.14 μmol/g brain at day 2 to 2.41 ± 0.34 μmol/g brain at day 56. GABA measured by in vitro MRS correlates well with the values measured by HPLC.

PE (Figure 5). By TLC measured brain PE was high in 2- and 7-day-old rats. There was a decrease between day 7 and 21. Between day 21 and 56 no further changes occurred. The in vitro MRS measurements gave similar concentrations for young rats, while for older rats MRS results were slightly higher.

Alanine (Figure 6). During development there was a steep decrease in Ala concentration between day 2 and 7. Between day 7 and 56 Ala concentration was the same for all time points. The developmental patterns were the same with both methods used.

Glutamate (Figure 7) and Glutamine (Figure 8). For both substances we found an increase during development.

The chromatographically determined *developmental patterns* of all substances measured agreed well with the patterns determined by MRS. The correlation coefficients between the chromatographic and the MRS

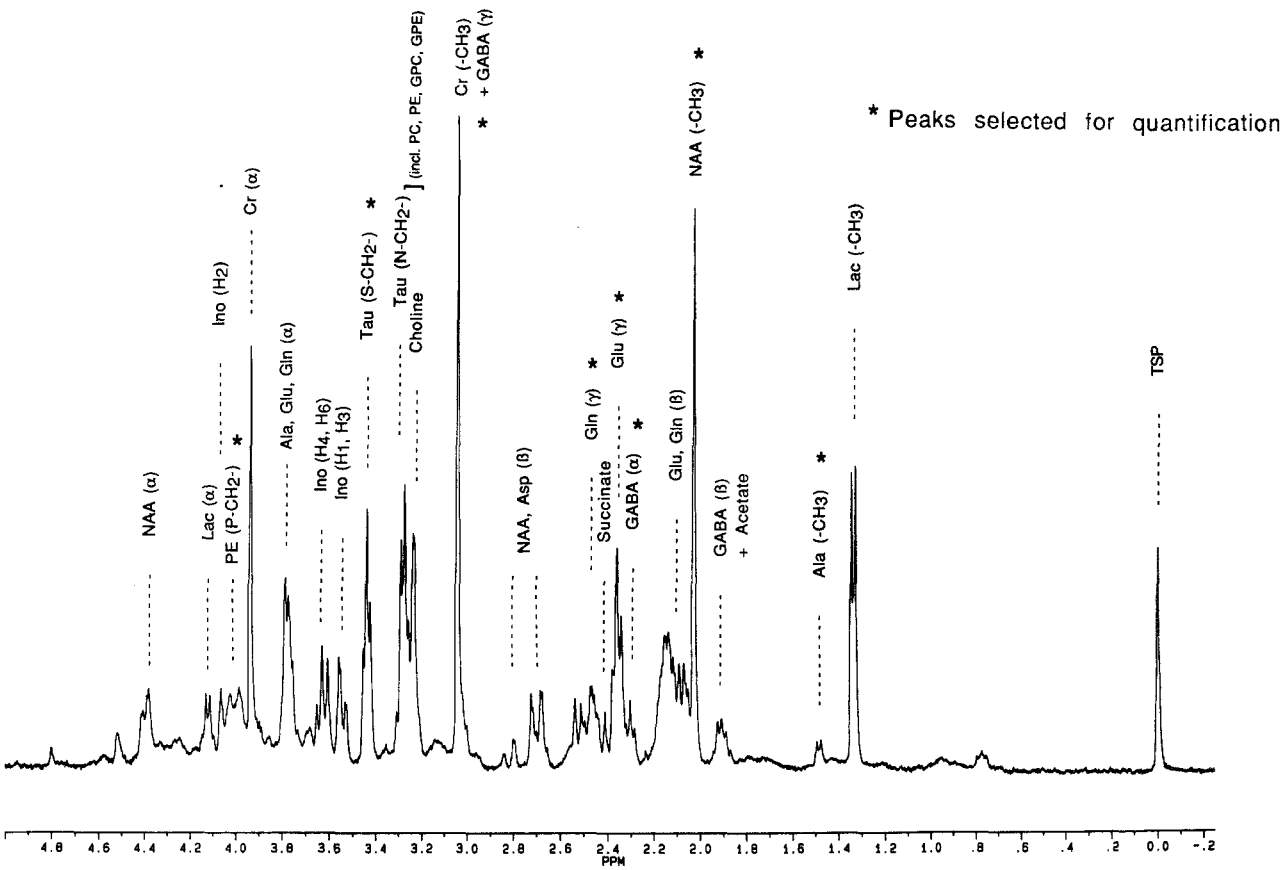


Fig. 1. In vitro ^{1}H MR brain spectrum of 21-day-old rats. Acquisition parameters: see methods, ppm relative to TSP.

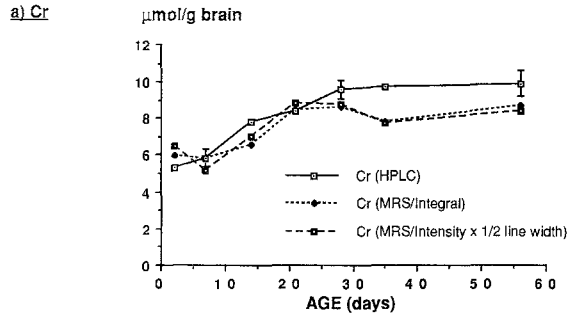
data were: Cr: 0.83, NAA: 0.93, Tau: 0.94, GABA: 0.63, PE: 0.86, Ala: 0.91, Glu: 0.91, Gln: 0.71.

The calculated difference of a spectrum of 56-day-old rats and a spectrum of 2-day-old rats (Figure 9) shows the main changes occurring in the rat brain MR specta during development. There were large increases in Cr, NAA and lactate. Small increases between day 2 and 56 were measured for GABA, Glu, Gln, and inositol, decreases for Tau, PE, Ala, and total choline.

2) Comparison of Preliminary In Vivo MRS Data with HPLC and In Vitro MRS Data. The processed in vivo spectra (Fig. 10) generally show clearly resolved signals from choline/phosphorylcholine (Chol.), creatine/phosphocreatine (Cr), NAA and several less intense resonances. The results show a strong increase of the NAA/Cr ratio. This ratio (Table I) was 0.35 ± 0.02 in 7-day-old rats and increased to 1.07 ± 0.08 in 35-day-old rats. The in vivo results are higher for day 7 ($P < 0.05$) and 35 ($P < 0.001$) than the HPLC results. The developmental patterns of the NAA/Cr ratio of the three different methods were the same.

DISCUSSION

For in vitro MRS quantification we have selected the strongest and best resolved peaks, typically the signal of a methyl group (1, 2, 21, 22). MRS peaks could be evaluated a) by using the integrals calculated by computer (integrals), or b) by graphically calculating peak areas using peak intensities and line widths at half height (areas). For well resolved peaks both methods should give the same results. This is the case for the methyl signal of Cr at 3.04 ppm as shown in Fig. 2a. The Cr signal at 3.04 ppm partially overlaps with a triplet signal of GABA (γ-CH_3). Two shoulders can be seen on the right side of the singulet signal of Cr. The subtraction of the integral value measured for the other GABA triplet observed at 2.3 ppm from the integral measured for the signal at 3.0 ppm is one of the possibilities to obtain the Cr value. However, the signal at 2.3 ppm overlaps partially with other signals and the differentiation and quantification based on integrals is certainly not very accurate. A better method in this case (strong singulet) is the quan-



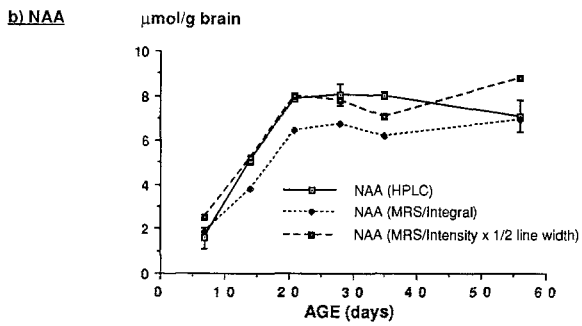


Fig. 2. Two in vitro calculation methods for Cr (a) and NAA (b). Biochemical determinations: n = 4 for all time points, means ± SD; in vitro MRS: pools of 4-8 rat brains.

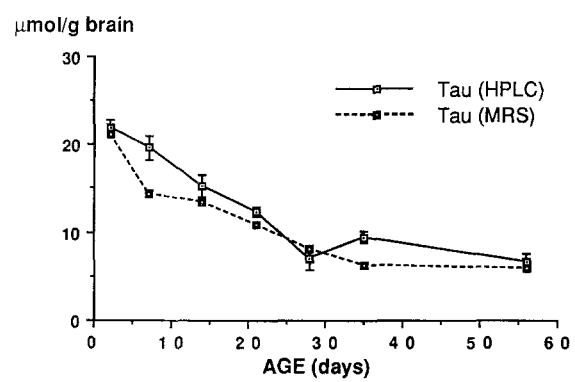


Fig. 3. Brain development: Taurine. Biochemical determinations: n = 4 for all time points, means ± SD; in vitro MRS: pools of 4-8 rat brains.

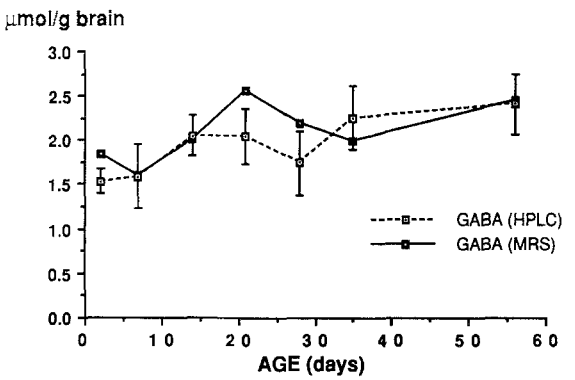


Fig. 4. Brain development: GABA

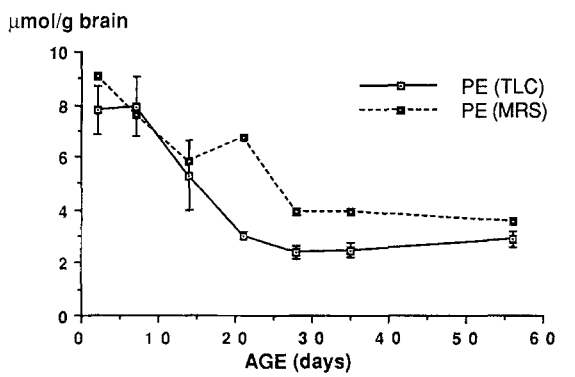


Fig. 5. Brain development: Phosphorylethanolamine

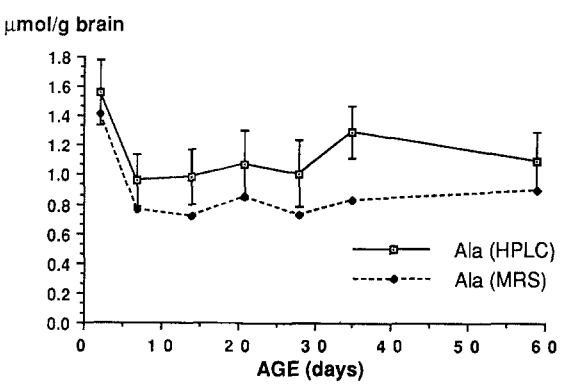


Fig. 6. Brain development: Alanine

tification based on signal intensities (calculated from the line heights and line widths). The intensity of the Cr signal is increased by one of the outer signals of the GABA triplet by at most 6%.

In the case of the methyl signal of NAA (Figure 2b) which is not well resolved from the β-protons of Glu and Gln, the integrals are smaller than the areas. Using the intergral of a peak in a crowded spectral region probably leads to an underestimated concentration because part of the peak at the peak's base may be excluded from the quantification. For small peaks like Glu, Gln, GABA,

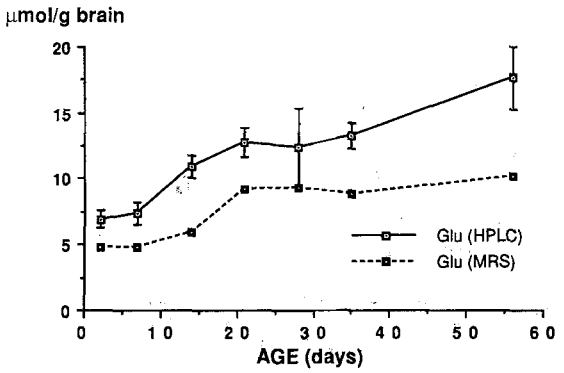


Fig. 7. Brain development: Glutamate

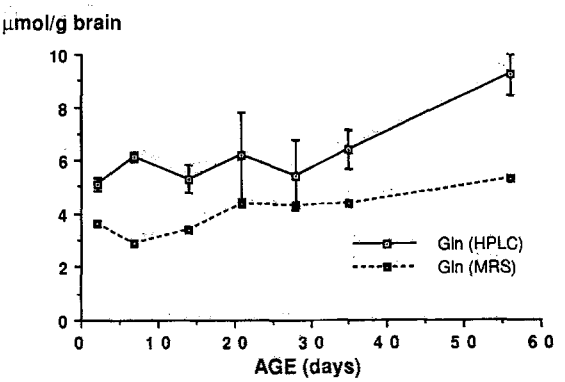


Fig. 8. Brain development: Glutamine

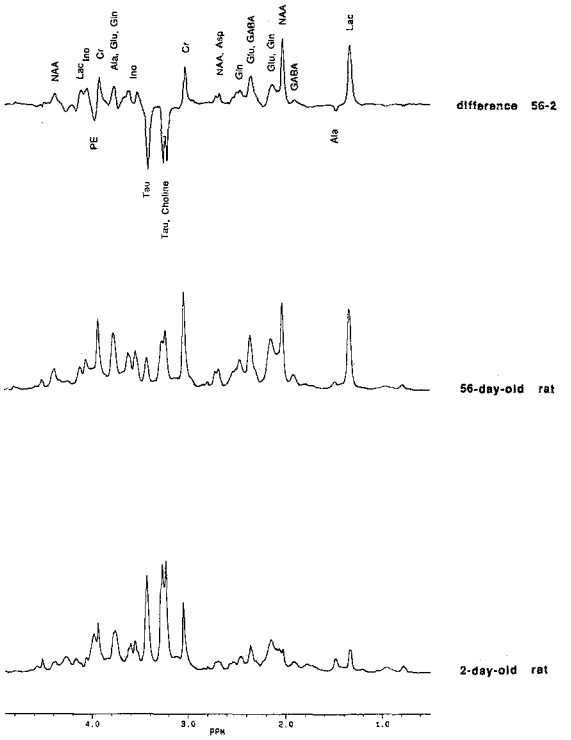


Fig. 9. Difference between the in vitro brain spectra of 56 day-old and 2-day-old rats. Prior to the calculation of the difference spectrum both spectra were scaled to the TSP internal reference. Acquisition parameters: see methods, LB = 5, ppm relative to TSP.

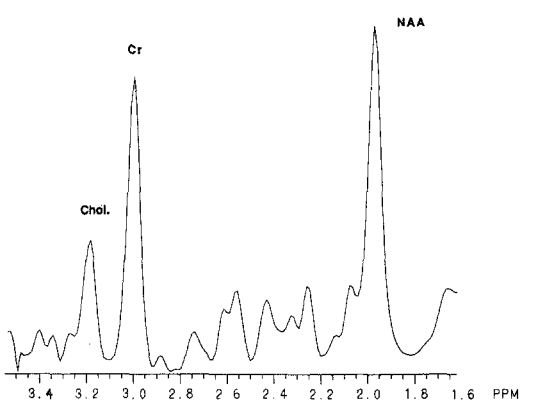


Fig. 10. In vivo ^{1}H MR brain spectrum of a 35-day-old rat. Acquisition parameters: see methods. The spectrum is referenced to the water peak.

PE and Ala and also for complex peaks (Glu, Gln, PE) the graphic measurements of areas could not be used. We had to use integrals. This may be the reason why Glu, Gln and Ala are apparently underestimated by in vitro MRS. MRS results of Cr, NAA, Tau and GABA are in excellent agreement with the chromatographic results. Our chromatographic results are in good agreement with the results of other groups (20, 29-32).

We found also an increase with age in lactate concentration. However, lactate may depend on the freezing time of the brain which is longer in large brains (day 56) than in small brains (day 2). Therefore, the lactate concentrations in our brain extracts are not comparable to the in vivo situation.

To our knowledge this is the first time that PE in rat brain extracts has been identified by ^{1}H MRS. The resonances of PE were established by measuring PE standards together with TSP and by the analysis of a 2D COSY spectrum. The triplet of the proton pair close to the amino group of PE can be found at 3.25 ppm. In brain extracts this triplet is covered by the choline/Tau peaks in the same spectral region. The resonance of the proton pair neighbouring the phosphoric acid group is at 4.00 ppm. In brain extracts this resonance can be used for quantification. However, it is unclear if the quite complex peaks at 4.00 ppm are only from PE.

As a standard for in vitro MRS quantification we

Table I. Brain Development: NAA/Cr Ratio

	NAA/Cr		
AGE	HPLC	MRS in vitro	MRS in vivo
7	0.27 ± 0.04	0.32	0.35 ± 0.02
14	0.65 ± 0.04	0.58	0.76 ± 0.20
21	0.93 ± 0.02	0.76	1.00 ± 0.21
28	0.84 ± 0.06	0.78	1.08 ± 0.23
35	0.82 ± 0.01	0.79	1.07 ± 0.08

Means ± SD; n = 4; MRS in vitro: pools of 4-8 brains

used TSP (20). An interesting fact was that TSP had to be added just before the MRS measurements. If TSP was added to the samples before the extraction procedure and was taken through the whole sample preparation the results came out wrong. This may be because part of the TSP is lost during the extraction procedure.

To compare the chromatographic developmental pattern and the MRS developmental pattern for one substance the correlation coefficient of the two data sets can be used. The correlation coefficient does not say anything about how well HPLC and MRS results agree. Examples: Even though the MRS Ala values are underestimated, the developmental patterns for Ala for both methods look quite similar. The correlation factor is 0.91. In contrast, GABA concentrations determined by both methods are the same. The developmental patterns are not in all parts parallel. The correlation coefficient is only 0.63. To summarize, it can be said that *the correlations of the developmental patterns* of MRS and chromatographic measurements are good for Cr, NAA, Tau, PE, Ala and Glu.

For the in vivo MRS we used a very small surface coil with a diameter of only 8 mm. The sensitive volume of such a coil reaches at most 4 mm deep into the head of the rat. This distance is well within brain tissue, also in the smallest rats, therefore, signals from the lower skull could not influence our measurements. However, signals from the surface tissue can be received from the sides of the rat's head. These signals are reduced by the convolution difference method we used. In young animals of less than 14 days old the quantification of the NAA resonance was more difficult due to its low intensity as compared to the Cr signal and due to residual lipid signals. These spectral contaminations could only be suppressed to a certain degree with the chosen localization scheme. The NAA peak at about 2.0 ppm is partially overlapped by the resonances of the β-protons of Gln/Glu at 2.1 ppm. However, also the Glu/Gln signal, which is a broad signal in vivo, is reduced by the convolution difference method we used. The creatine resonance includes the signal of the γ-protons of GABA. This signal influences Cr peak heights not more than 6%, according to our in vitro calculations. Such overlap problems may be the reason why in 7-day-old and 35-day-old rats the NAA/Cr ratios measured by in vivo MRS are significantly higher than measured by HPLC. In general, the NAA/Cr ratios determined in vivo agree well with the same ratios of in vivo MRS and HPLC. Therefore, if brain Cr concentration is known, NAA concentration *in vivo* can be calculated, and the results should be within 25% of the "correct" chemical value.

The results emphasize the usefullness of MR in vivo measurements to determine relative levels of abundant metabolites in the developing brain. With more sophisticated localization methods (33) using self shielded switchable field gradients, the spectral quality may be greatly improved. This will allow one to distinguish more spectral resonances, while providing a substantially improved spatial localization. It will then also be possible to monitor the regional distribution of selected metabolites during brain development.

Conclusions: a) Measurements of brain substances by in vitro MRS and chromatographic methods lead to the same results. Differences may result for small and complex peaks because of difficulties in MRS quantification. b) The developmental patterns of in vitro MRS and chromatographic data correlate with correlation coefficients between 0.63 and 0.94. c) Brain NAA/Cr ratios estimated in vivo correlate well with the aequivalent in vitro MRS and chromatographic measurements.

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