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2D COSY ^{1}H NMR: a new tool for studying in situ brain metabolism in the living animal

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2D COSY ^{1}H NMR with surface coil has been used to resolve and assign cerebral metabolites which had previously been detected but could not be resolved or assigned in situ in the living animal by conventional 1D ^{1}H NMR. A wide range of cerebral metabolites, including alanine, *N*-acetyl aspartate, aspartate, choline derivatives, creatine/phosphocreatine pool, GABA, glucose, glutamate/glutamine pool, inositol, lactate and taurine were simultaneously resolved and assigned in situ in the whole animal using the 2D COSY correlation graphs. Global irreversible ischemia caused the appearance and the disappearance of cross-peaks in the 2D COSY ^{1}H NMR map, corresponding to increases in alanine, GABA and lactate and glucose depletion.

Brain metabolism; ^{1}H NMR; 2D COSY NMR; Ischemia

1. INTRODUCTION

The introduction of the radiofrequency surface coil has made NMR a powerful tool for in situ metabolic studies of intact brain in the living animal [1]. The ^{1}H nucleus is the best candidate for such studies because of its natural abundance, its NMR sensitivity (the best of any stable nucleus), and its presence in all cerebral metabolites. However, most of the in vivo NMR studies on brain metabolism published to date have used the ^{31}P nucleus [16]. The ^{1}H nucleus was not used because, first, the ubiquity of water in brain causes NMR dynamic range problems and masks the presence of many interesting cerebral metabolites. This problem may be solved by using a saturation technique to suppress the huge brain water signal [6]. Other methods based on the use of spin-echo techniques, selective and non-selective pulse have also been developed to improve water peak suppression and spatial localization by using surface coils (see [8] and references therein). The second problem which has delayed the use of the ^{1}H nucleus is that the dispersion power of in vivo ^{1}H NMR is lower than that of ^{31}P NMR.

The relative complexity of the in vivo ^{1}H NMR brain spectrum is mainly due to the limited chemical shift range, the presence of numerous cerebral metabolites and the poor in vivo resolution. Consequently, the information on cerebral metabolism in situ obtainable from ^{1}H NMR has been limited either by incomplete assignment of resonances or by difficulties in signal quantification due to partial overlapping of certain relevant resonances. Editing methods may be used to select certain metabolites, but they use selective decoupling or selective excitation pulses so that only one metabolite can be followed at a time [17,20,21]. Hence, in vivo cerebral metabolic studies using ^{1}H NMR have often been restricted to lactate, which provides a strong signal in certain pathological situations [15].

The difficulty in extracting the physiological information from brain ^{1}H NMR spectra is similar to that encountered a few years ago in the first applications of ^{1}H NMR to the structural determination of biopolymers, which can contain many hundreds of atoms. 2D NMR, originally proposed by Jeener [13] and considerably developed over the past decade [5], has been used to solve this problem. The basic principle of this method is the selection and separation of the NMR information in twe two dimensions of a surface. Each peak is characterized by a pair of coordinates in a 2D spectrum instead of the single coordinate used in conventional 1D spectrum. We have applied this approach to the in situ study of brain metabolism in the living animal by ^{1}H NMR using a surface coil.

In the present study, 2D COSY ^{1}H NMR was used to simultaneously detect and resolve in situ a wide range of cerebral metabolites in the living animal. The 1D and 2D methods were first compared on an ex vivo model.

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Abbreviations: 1D, one-dimensional; 2D, two-dimensional; COSY, correlation spectroscopy; TSP, 2,2,3,3 tetradeutero-3-trimethysilyl propionate

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The cross-peaks obtained by a 2D COSY-sequence were then identified by comparing the 2D COSY correlation graphs for each metabolite alone with the experimental data obtained in vitro, ex vivo and in vivo. Finally, the 2D COSY ^{1}H NMR method was applied to monitor in situ the effects of total ischemia on the brain metabolism, in the whole animal.

2. MATERIALS AND METHODS

2.1. *Animal preparation for in vivo ^{1}H NMR*

All in vivo experiments were carried out on 5 male Wistar rats weighing 280–350 g. Two weeks before the experiments, the rats were anesthetized with Ketamine (100 mg kg^{-1} i.p.) and an epoxy tripod was glued directly onto the skull, sealed with dental cement and covered with a plexiglass casing. This tripod was used for positioning and fixing the head and surface coil assembly in the magnet during the NMR experiments. The anesthetized rats were also fitted with cortical screws for electrocorticogram recording. One week before the experiment, the rats were anesthetized with halothane (1% in oxygen) and indwelling cannulae were inserted in the femoral artery and femoral vein for measuring systemic blood pressure, blood sampling and KCl injection. Rats were anesthetized with halothane for control measurements. Body temperature was monitored with a rectal thermometer and was maintained at 37 ± 5°C with a circulating water blanket. Irreversible global ischemia was induced by i.v. injection of a cardioplegic KCl solution.

2.2. *Sample preparation for ^{1}H NMR*

Excised rat brains were removed from rat prepared in exactly the same fashion as for the in vivo ischemia experiment. Hemispheres were placed in a 10 mm tube containing 100 mM phosphate buffer in D_2O (pD = 7.2). Perchloric acid brain extracts of brain were prepared by a standard procedure from rat brains frozen in situ in liquid nitrogen [19]. The resulting sample was freeze-dried and redissolved in the same phosphate buffer.

2.3. *NMR measurements*

High resolution ^{1}H spectra were acquired at 400 MHz with a AM-400 widebore spectrometer (Bruker Spectrospin) operating in the pulse Fourier transform mode and interfaced with an ASPECT 3000 computer. The in vitro experiments on standard solutions and perchloric acid extracts were made with a selective 5 mm ^{1}H Bruker probe. The ex vivo experiments on excised ischemic brain were carried out with a laboratory-modified 10 mm Bruker probe. The in vivo experiments were performed with a custom-built probe equipped with a contention cradle designed to maintain the animal vertical within the magnet. In vivo NMR spectra were collected with a single-turn surface coil (1.2 cm diameter) of 1 mm insulated silver-coated copper wire. The magnetic field was optimized by shimming on the strong water signal. The linewidth at half height for in vivo experiments varied from 36 Hz to 44 Hz (0.09 to 0.11 p.p.m.).

In vivo 1D ^{1}H brain spectra with water suppression were obtained with a binomial pulse sequence plus a spin-echo and a composite refocusing pulse cycle according to the Exorcycle scheme [8]. Ex vivo 1D ^{1}H NMR spectra were obtained with a Hahn spin-echo sequence. In vitro spectra were obtained by a simple pulse. The principal acquisition parameters were: digital resolution, 2K points; sweep width, 10 p.p.m.; and repetition rate, 2 s. Spectra were generated from 128 accumulations. The apparent $\pi/2$ pulse was assigned to the pulse width giving the water signal of greatest intensity. The spin-echo delay selected (136 ms) corresponds to the refocusing of the lactate doublet. In vivo, chemical shifts (δ) were expressed relative to the methyl resonance of *N*-acetyl aspartate assigned at 2.023 p.p.m. Ex vivo and in vitro chemical shifts were expressed relative to the reference TSP.

2D ^{1}H COSY spectra were acquired with a SUPER COSY sequence [4,11,14]. The water signal was reduced by spin-lock with a coherent low power composite pulse for presaturation of the water signal during the relaxation delay. The SUPER COSY pulse scheme has two spin-echo delays which are introduced symmetrically with respect to the second $\pi/2$ pulse. The delay may be adjusted for a specific range of scalar coupling constant (J) [12]. This delay was set to 86 ms for 2D COSY ^{1}H spectra of small cerebral metabolites: it corresponds to 2 times 0.3 / J with J = 7Hz (mean scalar coupling value of freely rotating rotors in open chains). The delay also allows further reduction of the remaining water signal and suppression of phospholipid and protein signals by T_2 filtering. Acquisition of 32 free induction decays in the time domain t_2 for each of the 128 increments in the time domain t_1 resulted in a total acquisition time of 1 h 10 min for in vivo experiments. 512 increments were acquired in ex vivo and in vitro experiments. The spectral width in the corresponding frequency domain in F_1 and F_2 dimensions was 10 p.p.m. Before the 2D Fourier transform, the data were multiplied by an unshifted sine bell function in the two dimensions and zero filled to obtain a 1024 × 1024 data point matrix. The spectra were processed in absolute mode and the surfaces were made symmetrical to eliminate the t_1 ridges. The in vivo 2D chemical shifts are expressed relative to the water resonance assigned at 4.70 p.p.m. in the two dimensions.

3. RESULTS

3.1. *Comparison of 1D ^{1}H NMR and 2D COSY ^{1}H NMR in an ex vivo model*

The 1D and 2D COSY ^{1}H NMR methods were compared using excised rat brain. This ex vivo model was used because it provides better resolution and stability than in situ measurements in the living animal and permits the use of a conventional saddle-shape coil. These conditions should help to reduce overlap of the resonance peaks in conventional 1D ^{1}H NMR spectroscopy, facilitating resolution of the maximum number of resonance peaks. Fig. 1 shows a 2D COSY ^{1}H NMR spectrum (A) plus the corresponding 1D ^{1}H NMR spectrum (B) of an excised rat brain; only the aliphatic region of the spectra is shown. This figure illustrates the greater editing power of the 2D COSY spectroscopy over that of 1D conventional spectroscopy. The ex vivo spectra were obtained with a resolution giving a water half-height line-width of 20 Hz (0.05 p.p.m.), which cannot be obtained in vivo. The aliphatic region of the 1D ^{1}H NMR brain spectra of the excised rat brain contains a large amount of information. Many of the resonance peaks have contributions from more than one cerebral metabolite, even at 400 MHz and with the resolution and the stability obtained ex vivo. In contrast, expansion of the NMR information on the COSY surface gives additional information, suppresses or reduces the 1D overlapping of many peaks and reveals certain peaks which had been hidden in the 1D spectra. In the 2D COSY ^{1}H NMR experiment, the information is modulated by the more or less efficient transfer of coherence by the bias of the scalar coupling. The cross-peaks indicate scalar coupling between protons. The NMR signals from protons not coupled to other protons remain on the diagonal peaks. In addition, information on the concentration of a given metabolite can be obtained by volume integration of the cross-peaks [2].

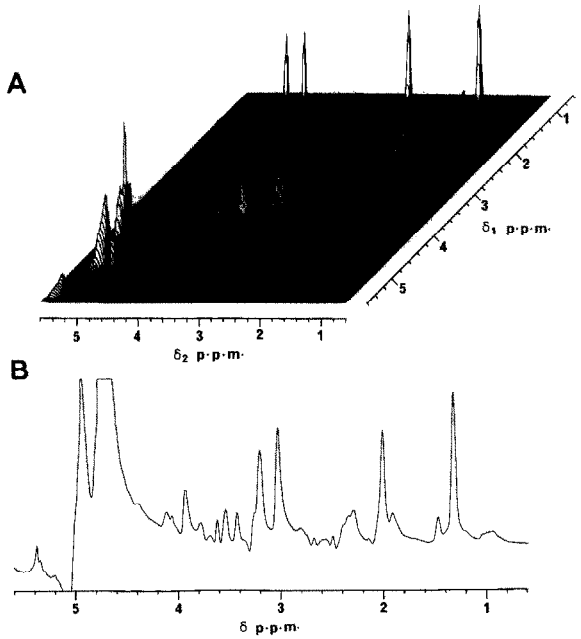

Fig. 1. Stacked plot representation of the 2D COSY ^{1}H NMR spectrum (A) of an excised rat brain and the corresponding 1D ^{1}H NMR spectrum (B).

3.2. *Assignment of the ^{1}H COSY cross-peaks*

The characterization of the cross-peaks by a pair of coordinates in the 2D COSY ^{1}H NMR spectrum allows a more unequivocal assignment of the resonance peaks than in the 1D spectrum. Fig. 2 shows the assignment of the cross-peaks obtained by 2D COSY ^{1}H NMR to different cerebral metabolites. The 2D spectra are presented as contour plots as this simplifies identification of cross-peaks and coordinate measurement. The assignment procedure used for the GABA cross-peaks is given as an example. The GABA peaks are resolved in the in vitro 1D spectrum but partially overlap or are obscured in the ex vivo 1D spectrum. In contrast, the GABA cross-peaks are resolved in the 2D COSY spectra both in vitro and ex vivo. A 2D COSY ^{1}H NMR graph of each pure metabolite in a medium similar to the intracellular medium can be obtained and superimposed on the brain 2D COSY ^{1}H NMR spectra obtained in vitro, ex vivo and in vivo. The pattern of shift correlation is like a 'finger-print' of the molecular skeleton of each cerebral metabolite. It is the graph of correlation which serves to assign the resonance peaks in 2D ^{1}H NMR rather than the chemical shifts the resonance peaks, as in 1D ^{1}H NMR. Even when the geometry of the correlation graph is conserved, the in vitro chemical shifts values of a given metabolite measured in vitro may be modified in vivo within the brain tissue as the consequence of interactions between cellular constituents. In conclusion, the correlation graph in 2D COSY ^{1}H NMR is a more reliable way of resolving the resonance peaks and assigning them to the corresponding cerebral metabolites than the peak position in 1D ^{1}H NMR.

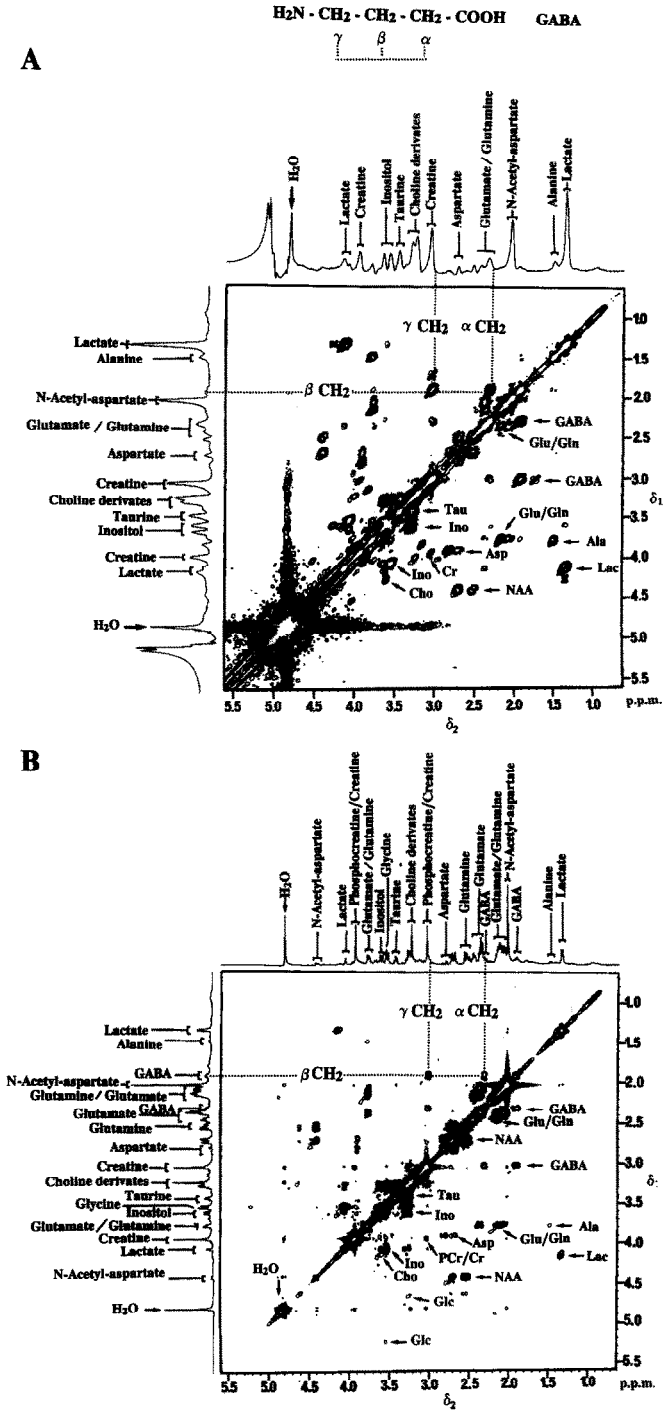

Fig. 2. 1D and 2D COSY ^{1}H NMR spectra of an excised rat brain (A). 1D and 2D COSY ^{1}H NMR spectra of an perchloric brain extract (B). The 2D spectra are presented as contour plots. The broken lines represent the GABA graph of correlation. Assignments of the ^{1}H COSY cross-peaks: alanine (Ala), *N*-acetyl aspartate (NAA), aspartate (Asp), choline derivatives (Cho), creatine (Cr), phosphocreatine (PCr), glucose (Glc), GABA, glutamate (Glu), glutamine (Gln), inositol (Ino), lactate (Lac), and taurine (Tau).

3.3. *Application of 2D COSY ^{1}H NMR to global irreversible ischemia*

The real power of 2D COSY ^{1}H NMR is best seen in the in vivo studies, where most of the metabolic infor-

mation provided by ^{1}H NMR is unusable by standard 1D NMR because of lack of resolution. Surface-coil 2D COSY ^{1}H NMR provides simultaneous in situ resolution and assignment of several cerebral metabolites including: alanine, *N*-acetyl aspartate, choline derivates, creatine/phosphocreatine pool, GABA, glucose, glutamate/glutamine pool, inositol, lactate and taurine in the whole animal (see Fig. 3). The in vivo 1D and 2D COSY ^{1}H NMR brain spectra recorded before and 30 min after irreversible cardiac arrest in the same animal illustrate the effect of global irreversible ischemia on the ^{1}H NMR brain spectra. In the 1D spectrum, ischemia results in the emergence of a lactate peak and a small decrease in the *N*-acetyl aspartate resonance peak with respect to the stable creatine/phosphocreatine pool peak. The 2D COSY ^{1}H spectrum confirms the marked increase in lactate which is univocally characterised by its correlation graph. The 2D spectrum also reveals the increases in analine and GABA after ischemia by the appearance of new cross-peaks and the depletion of glucose after ischemia by the disappearance of the αD glucose cross-peak. All 5 rats studied provided similar results.

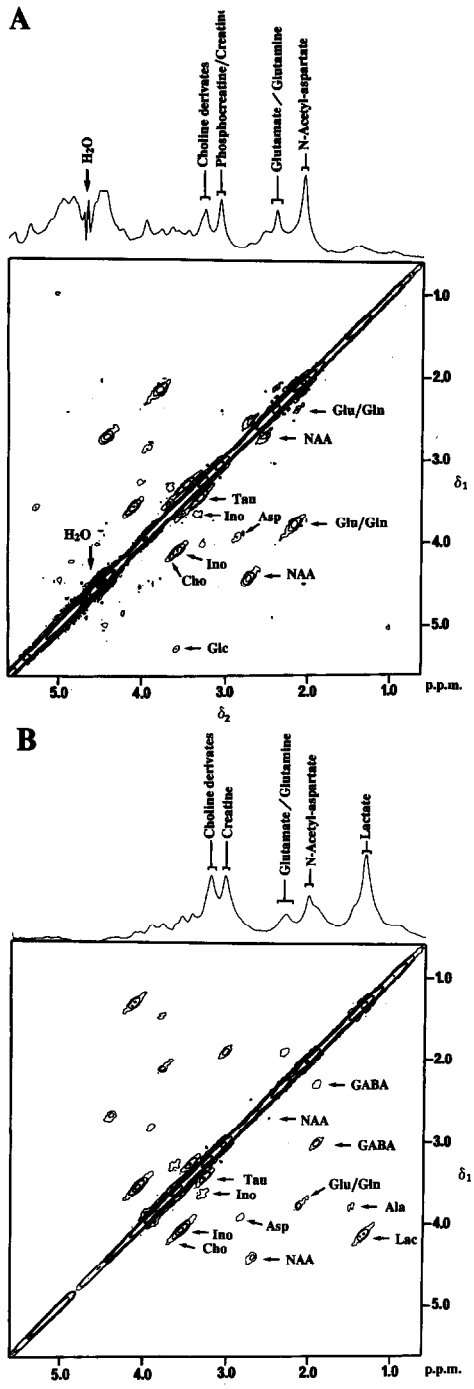

Fig. 3. 1D and 2D COSY ^{1}H NMR spectra of an intact rat brain obtained with a surface coil before (A) and after total irreversible ischemia (B). Assignments: alanine (Ala), *N*-acetyl aspartate (NAA), aspartate (Asp), choline derivatives (Cho), creatine (Cr)/phosphocreatine pool (PCr), GABA, glucose (Glc), glutamate (Glu)/glutamine (Gln) pool, inositol (Ino), lactate (Lac) and taurine (Tau).

4. DISCUSSION

In vivo ^{1}H NMR can be used to detect a large number of cerebral metabolites. The difficulty lies in extracting the information from the crowded in vivo conventional 1D ^{1}H NMR brain spectrum. Earlier studies using conventional 1D ^{1}H NMR assigned some of the resonance peaks in vivo by comparing them with the better-resolved ex vivo spectra of excised brain and in vitro spectra of brain perchloric acid extracts [6]. But, many of the resonance peaks obtained in vivo include contributions from more than one cerebral metabolite, as has been shown by more detailed indirect studies on excised brains ex vivo [3] and by in vitro studies on brain extracts [9]. These studies allowed refinement of the assignments of certain metabolites and new assignments for cerebral metabolites. The assignments made by 2D COSY ^{1}H NMR on excised brain or perchloric acid extracts confirm the assignments made previously, and thus validate the method used. But the great advantage of our method is best-seen in the in situ studies of brain metabolism in the whole living animal, where conventional 1D ^{1}H NMR is of limited use. In the in vivo 1D ^{1}H NMR brain spectra certain peaks overlap (e.g. *N*-acetyl aspartate, glutamate/glutamine pool between 2 and 3 p.p.m. and creatine/creatine phosphate pool, choline derivatives, inositol and taurine between 3 and 4 p.p.m.. Some other peaks are completely obscured (e.g. aspartate, GABA between 2 and 3 p.p.m. or glucose between 3 and 4 p.p.m.). Consequently, the metabolic information obtained so far from in vivo ^{1}H NMR spectroscopy has been limited either by incomplete assignment of the resonance peaks or by difficulties encountered in peak integration due to partial overlapping of certain relevant resonances. These problems are potentially solved by the editing power of the 2D COSY ^{1}H NMR.

The data presented here demonstrate that 2D COSY

^{1}H NMR can be used in vivo with a surface coil to simultaneously assign and resolve several cerebral metabolites in situ, in the whole animal; these metabolites include those not assigned or resolved by 1D ^{1}H NMR spectroscopy. 2D COSY ^{1}H NMR has only been used previously for studies on living tissue with conventional probes employing saddle-shaped coils producing uniform radiofrequency fields, such as those on surgically prepared kidney [7], excised muscle [2] and on slices of neonatal brain [11]. To our knowledge, these results represent the first reported use of the 2D COSY ^{1}H NMR method to study in situ brain metabolism in the living animal. The SUPERCOSY sequence used here has two advantages over the standard COSY sequence, because it uses two spin-echo delays one on either side of the second COSY sequence pulse. First, these delays improve suppression of water and lipids. Second, they reduce the problems encountered when using the standard COSY sequence in low digitization in vivo conditions. In these conditions, the multiplets within the cross-peaks have an antiphase character and can cause mutual cancellation of those peaks during signal acquisition. SUPERCOSY pulse scheme corrects this drawback [12,14] for selected J values of the ^{1}H-^{1}H scalar coupling constants. The importance of 2D COSY ^{1}H NMR lies in its resolving power and in the fact that spin-spin correlation cross-peaks can be obtained simultaneously and without the selective excitation required by the 1D editing ^{1}H NMR method [17,20,21]. Furthermore, metabolite levels are measured in situ, thereby avoiding the potential artefacts that can arise from the tissue freezing and extraction procedures used in classical invasive biochemical methods [19].

We have demonstrated that 2D COSY ^{1}H NMR spectroscopy can be used for pathological studies of brain metabolism (e.g. ischemia). We have qualitatively followed the more marked metabolic changes (appearance/disappearance of cross-peaks) accompanying energy failure following total ischemia induced by cardiac arrest. These changes involved anaerobic metabolism (lactate, glucose) and alterations in the levels of the amino-acid alanine and the neurotransmitter GABA. Improvements are presently being made in resolution time (e.g. reduction of preparation delay), evaluation of the effect of relaxation times (e.g. spin-echo effects), identification of new metabolites and quantification (cross-peak volume integration) of all metabolites. 2D COSY ^{1}H NMR spectroscopy can be used to simultaneously follow the time courses of the levels of several cerebral metabolites. More significantly, 2D COSY ^{1}H NMR spectroscopy provides a method for repeated, atraumatic, semi-dynamic in situ mapping of several key cerebral metabolites in the living animal. 2D COSY ^{1}H NMR spectroscopy is therefore suitable for in vivo studies of the relationship between energy metabolism (glucose, lactate) and the metabolism of some amino acid neurotransmitters and their derivatives (GABA, glutamate, glutamine, aspartate, etc.) 2D COSY ^{1}H NMR also gives access to the cerebral contents of metabolites implicated in maturation and adaptation to hypoxia (taurine) and cellular communication (inositol) [9,18]. Cerebral metabolism in physiological and pathological situations can only be clearly described by non-invasive studies of metabolic transformations as they take place in their own environment. Recently, in vitro experiments in phantom solutions of lactate and ethanol have demonstrated that the acquisition of localized 2D COSY ^{1}H NMR spectra with a surface coil is feasible [10].

In conclusion, the above data indicate that 2D COSY ^{1}H NMR is a promising tool with which to expand the field of in situ NMR studies on cerebral metabolism in the living animal.

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