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Protocol

Rat brain primary neurons immobilized in basement membrane gel threads: an improved method for on-line ^{13}C NMR spectroscopy of live cells

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Abstract

In vivo MRS studies on intact brain reflect the metabolism of all cells present, but do not distinguish between different cell types. NMR studies of immobilized cultured primary cells, such as neurons and astrocytes, are a useful model to monitor the specific differences in metabolism of the various cell types in the brain. The present study shows that primary rat neuronal cells can be cultured in basement membrane gel threads. After 4 days of incubation the threads are filled with viable cells, and represent a population of morphologically differentiated neuronal cells with less than 5% of non-neuronal cells, i.e., astrocytes. These threads were placed into a NMR tube and used for on-line monitoring of neuronal metabolism. Under these conditions cells remained viable and metabolically active for several days. The energy status was monitored by using ^{31}P NMR spectroscopy. To study neuronal glucose metabolism [1-^{13}C]glucose was added to the perfusion medium and 30 min later ^{13}C-labeled metabolites were detectable by ^{13}C NMR spectroscopy. Immobilized neurons synthesized glycolytic products such as [3-^{13}C]lactate and [3-^{13}C]alanine, as well as several tricarboxylic acid cycle products, i.e., [2-^{13}C]glutamate, [3-^{13}C]glutamate, [4-^{13}C]glutamate, [2-^{13}C]aspartate, and [3-^{13}C]aspartate. In summary, ^{31}P and ^{13}C NMR spectra can be recorded from live neuronal cells for up to 24 h using the newly designed procedure described in the present communication.

Themes: Other systems of the CNS

Topics: Brain metabolism and blood flow

Keywords: Neuron; NMR spectroscopy; Metabolism; Basement membrane gel thread; Immobilization; Cell culture

1. Type of research

- Immobilization of primary rat neurons in basement membrane gel threads.
- On-line ^{31}P and ^{13}C NMR spectroscopy on viable neuronal cells.

2. Time required

- 2 h cell preparation and immobilization into basement membrane gel threads.
- 2 h incubation time and 10 min medium exchange.
- 4 d incubation time.
- 30 min transfer of the immobilized cells into the NMR perfusion system.
- NMR measurement: variable times, up to 24 h.

3. Materials

- Basement membrane extract solution (Matrigel EHS solution) was from Serva (Heidelberg, Germany)
- Cell culture media were from Gibco/BRL (Grand Island, NY).
- Polyclonal rabbit antisera against horseradish peroxidase (HRP)-conjugated and fluorescein-isothiocyanate-conjugated (FITC) secondary antibodies were from Sigma (St. Louis, MO).
- Monoclonal antibodies to glial fibrillary acidic protein

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(GFAP) and neurofilament proteins (NF 200 and NF 68) were from Boehringer (Mannheim, Germany).

- [1-^{13}C]Glucose was obtained from Cambridge Isotope Laboratories (Wesel, Germany).

4. Detailed procedure

4.1. Immobilization of primary neuronal cells

(1) Prepare cerebral hemispheres from brains of 16-day-old rat embryos. Take 10 to 12 brains for one preparation and pool them.

(2) To yield single cell suspensions, disrupt soft brain tissue mechanically in 2 ml of Eagle's basal medium (BME) supplemented with 10% fetal calf serum (FCS) using a pasteur pipette. The preparation procedure is described in more detail by Richter-Landsberg [1].

(3) Substitute cell suspension with BME/10% FCS to a volume of 10 ml, and centrifuge at $800 \times g$ for 5 min.

(4) Resuspend cell pellet in 10 ml BME/10% FCS and recentrifuge as above. Resuspend cell pellet gently with 2 ml BME/10% FCS and divide the suspension into four aliquots of 0.5 ml each.

(5) Mix each aliquot individually with 0.5 ml solution

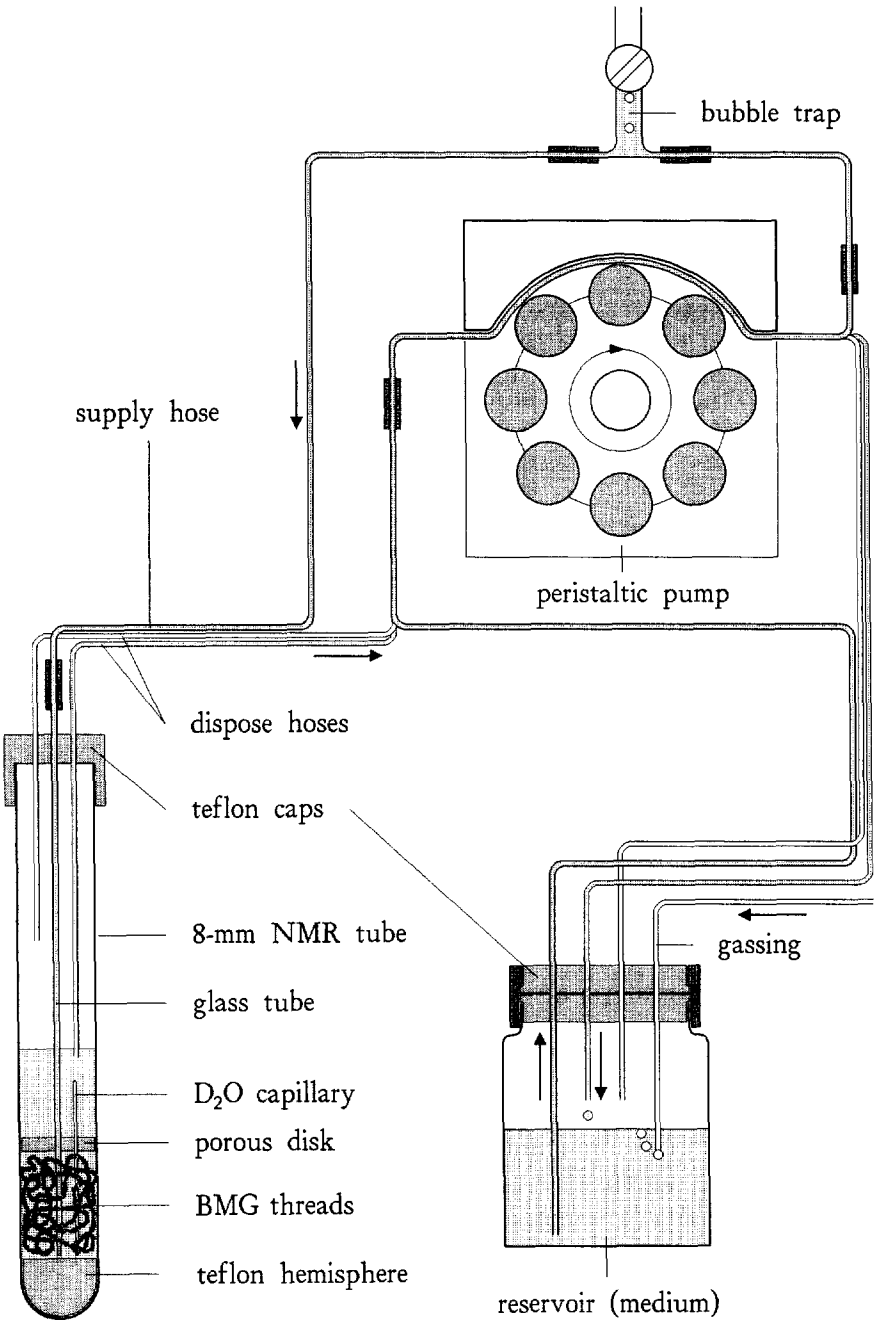

Fig. 1. Schematic representation of the perfusion system used for neuronal cells embedded in basement membrane gel threads.

of basement membrane extract and suspend it carefully by first using a 500 μl and subsequently a 200 μl Eppendorf pipette. To prevent gelation keep the mixture at 4°C using an ice bath.

(6) Draw the cold mixture into a syringe. Immerse syringe in a water bath at 37°C and keep it there for 2 min to let the cell-basement membrane solution mixture gel.

(7) Replace the ordinary syringe cannula by a cannula attached to a sterile Teflon tubing (approximately 1 m length, 0.3 mm i.d.), and extrude threads resulting from the individual samples through the sterile Teflon tubing into 2 plastic culture dishes (15 cm), each prefilled with BME/10% FCS (20 ml).

(8) Incubate cultures for 2 h (37°C and 10% CO_2), remove culture medium and replaced it by BME/0.5% FCS (20 ml). Keep the cultures in an incubator for 4 days.

In the present study cells were viewed and photographed using an Olympus inverted microscope.

4.2. NMR spectroscopy

After 4 days neurons embedded in basement membrane gel threads are pooled and transferred into one 8-mm NMR tube using a 5-ml glass pipette which was cut at the tip to enlarge the opening. Perfusion is carried out at 37°C with BME/0.5% FCS at a flow rate of 1 ml/min. The medium is oxygenated with 95% O_2/5% CO_2 using a perfusion system schematically represented in Fig. 1.

For NMR experiments described in the present study the medium in the reservoir was replaced by DMEM (60 ml) without FCS but containing 5 mM glucose or [1-^{13}C]glucose. D_2O in a sealed capillary was placed in the NMR tube to secure and maintain the lock signal. Shimming was done on the ^1H free induction decay and a line width at half height of 15 Hz could be routinely obtained for the water signal.

^{31}P NMR spectra were recorded on a Bruker AM 360 operating at a frequency of 145.7 MHz. By using a 10-mm ^{1}H/^{31}P dual probe the spectra were obtained within an accumulation time of 30 min, 66° flip angle, 2.5 s repetition time, composite pulse decoupling (CPD) with WALTZ-16 [2], 4500 Hz spectral width, 4 K data size, zero filling to 16 K, and exponential weighting resulting in a 20 Hz line broadening. Phosphocreatine (PCr) was used as reference signal at −2.33 ppm.

^{13}C NMR spectra shown in Figs. 6 and 7 were recorded on a Bruker AMX spectrometer operating at a frequency of 90.5 MHz for ^{13}C measurements. By using a 10-mm ^{1}H/^{13}C dual probe spectra were obtained within an accumulation time of 30 min (Fig. 6) and 60 min (Fig. 7), a 40° flip angle, 1.5 s repetition time, composite pulse decoupling (CPD) with WALTZ-16 [2], 9800 Hz spectral width, 16 K data size, zero filling to 32 K, and exponential weighting resulting in a 5 Hz line broadening. The C-1 carbon signal of β-D-glucose (Glc) was used as reference signal at 96.8 ppm.

^{13}C NMR spectra shown in Fig. 8 were recorded on a Bruker DRX 600 spectrometer operating at a frequency of 150.92 MHz for ^{13}C measurements. By using a 8-mm ^{1}H/X/^{31}P triple resonance probe spectra were obtained within an accumulation time of 2 h (Fig. 8, bottom) and 4 h (Fig. 8, top), a 60° flip angle, 1.9 s repetition time, composite pulse decoupling (CPD) with WALTZ-16 [2], 36220 Hz spectral width, 64 K data size, and exponential weighting resulting in a 4 Hz line broadening. The C-1 carbon signal of β-D-glucose (Glc) was used as reference signal at 96.8 ppm.

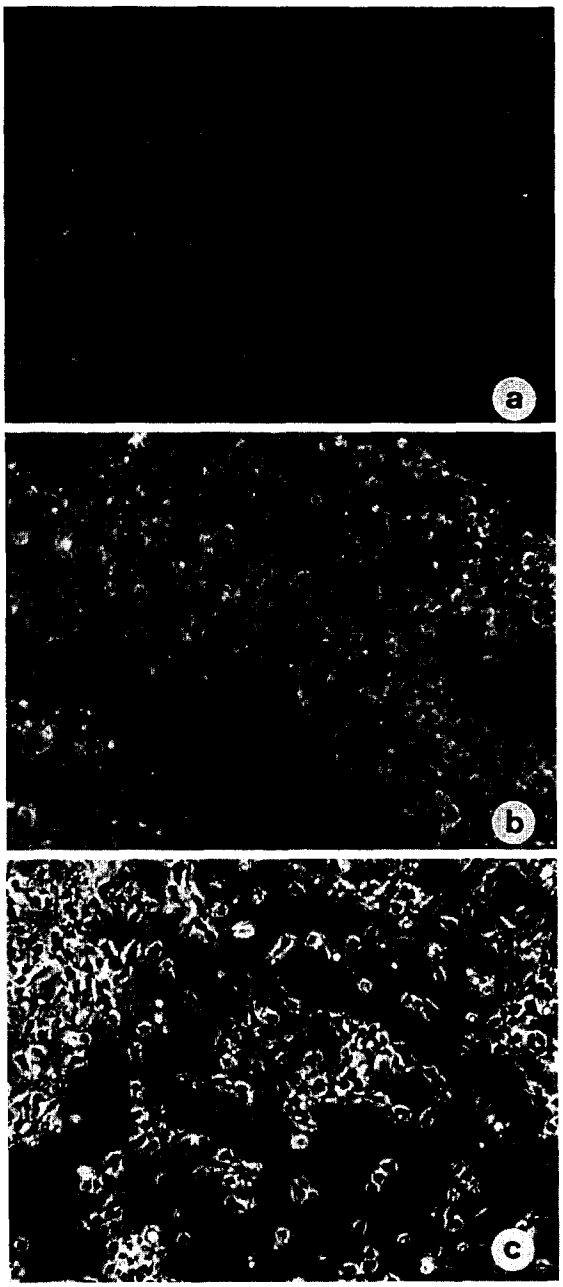

Fig. 2. Phase-contrast micrographs of rat cerebral cells in culture. Neuronal cells in basement membrane gel threads after 2 h (a) and 4 days (b) in vitro are seen. In (c) cells were grown on poly-L-lysine coated cover slips for 4 days. Bars in (a) and (c) represent 25 μm and in (b) 20 μm.

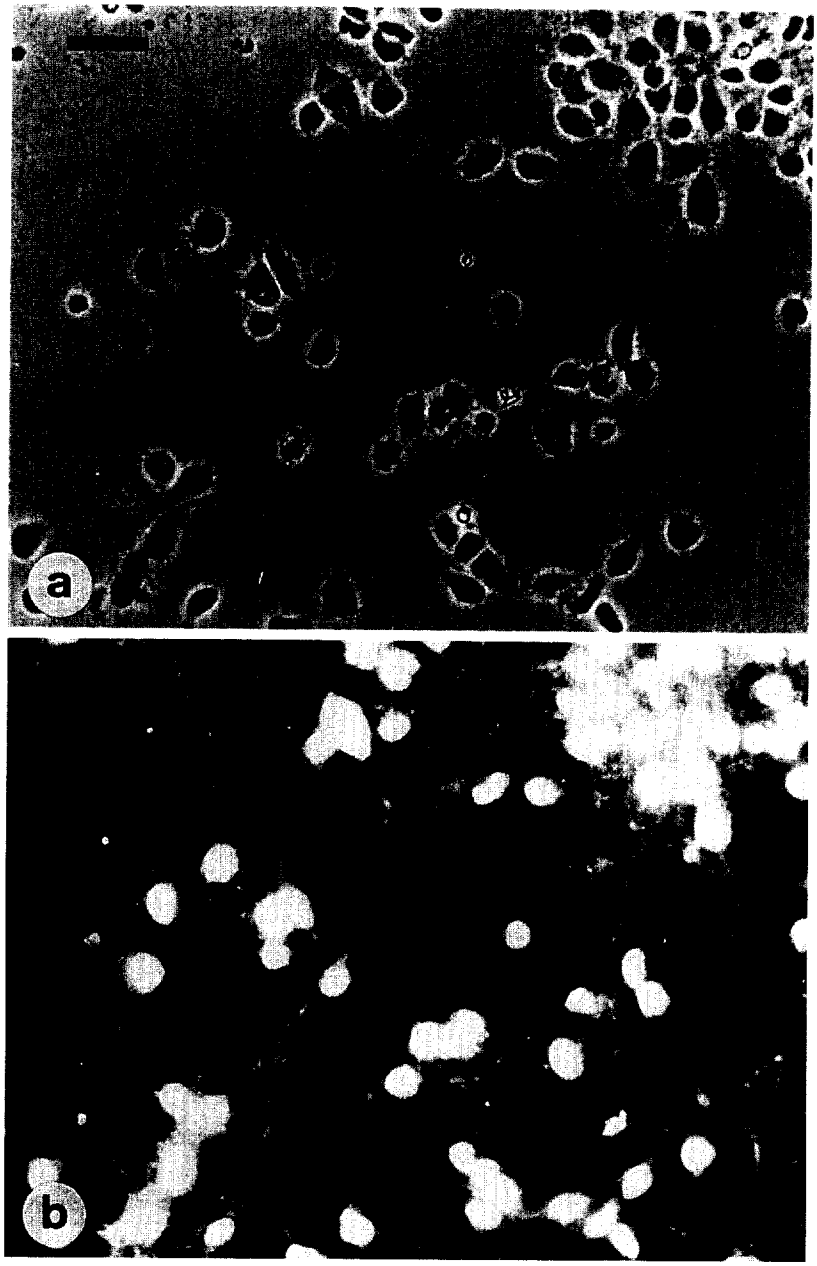

Fig. 3. Indirect immunofluorescent staining of a 7-day-old monolayer culture. Cells were grown on poly-L-lysine coated cover slips for 7 days and staining was carried out with anti-neurofilament antibody (b). (a) Represents the phase contrast image corresponding to (b). Bar represents 25 μm. Neurons and cellular neuritic processes are intensely stained.

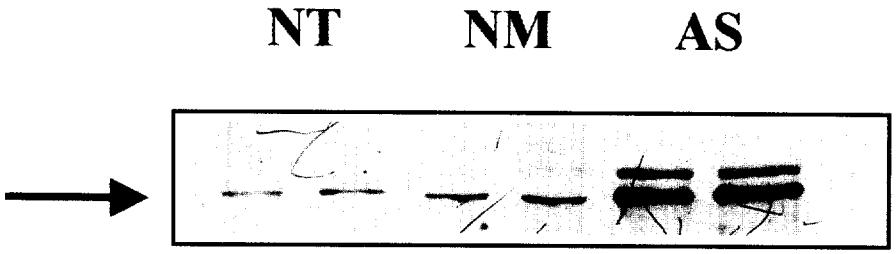

Fig. 4. Immunoblot analysis of cell extracts. Neuronal cells were grown for 7 days either in basement membrane gel threads (NT) or in monolayer cultures (NM). Cell extracts (10 μg of protein each) were separated on SDS-polyacrylamide gels, followed by immunoblot procedure using anti-GFAP antibodies. Additionally, cell extracts from 3 weeks old astrocytes (AS) were prepared and analyzed. The arrow indicates the position of GFAP.

4.3. Monolayer cultures

For monolayer cultures, cells are plated on poly-L-lysine coated culture dishes in BME/10% FCS at a density of 1 embryonic brain/10 cm dish for immunoblot procedure, or 10^5 cells/35 mm dish for indirect immunofluorescence staining. After 2–3 h, culture medium is exchanged to BME/0.5% FCS, and cells are incubated for the indicated times. All cells are kept at 37°C and 10% CO_2. Growth medium is changed once a week.

4.4. Indirect immunofluorescence staining

For fluorescent labeling cells are grown on poly-L-lysine-coated glass cover slips (10^5 cells/35 mm dish). After washing with PBS, cells are fixed with 3% paraformaldehyde for 10 min. Paraformaldehyde-fixed cells are pretreated with 0.1% Triton X-100 (30 min) and then incubated with the first antibodies for 60 min. The antibodies are used at the following dilutions: monoclonal anti-GFAP 1:3, anti-neurofilament 1:3. After washing with PBS cells are incubated with fluorescein-isothiocyanate-conjugated (FITC) goat anti-mouse IgG (1:75) for 45 min, washed with PBS and mounted. Fluorescent labeling is studied using a Zeiss epifluorescence microscope equipped with an automatic camera.

4.5. Western blot analysis

Cellular monolayers or cells grown in basement membrane threads are washed with PBS three times, taken up in sample buffer [3] containing 1% SDS and boiled for 10 min. Protein contents in the samples are determined according to Neuhoff et al. [4]. For immunoblotting, total cellular extracts (10 μg protein/lane) are separated by one-dimensional SDS-PAGE using 10% polyacrylamide gels, and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel/Germany; 0.2 μm) according to Towbin et al. [5]. The blots are washed and incubated with anti-GFAP at a dilution of 1:5000 followed by HRP-conjugated anti-mouse IgG (1:5000), and visualized by the enhanced chemiluminescence (ECL) procedure as described by the manufacturer (Amersham, Braunschweig).

5. Results

5.1. Identification and characterization of neuronal cultures

Two hours after extruding the basement membrane gel thread-cell suspension into the culture dishes, no single cells were observed floating on the culture dishes, and cells had tightly adhered or intruded the basement membrane gel thread (Fig. 2a). During the next 4 days, cells continued to fill the gel threads, which appeared slightly thickened (Fig. 2b). During the same time neuronal cells had formed cellular aggregates and elaborated numerous neuritic extensions in monolayer cultures used as a control (Fig. 2c). Indirect immunofluorescence staining carried out with anti-neurofilament antibodies revealed that cellular aggregates and neuritic processes were prominently stained (Fig. 3). Even after 7 days, cells of astrocytic origin were rarely visible.

Immunoblot analysis of cell extracts from 5-day-old cultures using anti glial fibrillary acidic protein (GFAP) antibodies, did not reveal GFAP immunoreactivity (not shown), while in extracts of 7-day-old cultures a faint GFAP-immunoreactive band was detectable (Fig. 4). Thus, as expected, during culture maturation, astrocytes develop and proliferate. However, at the time when cells were used for NMR experiments, astrocytic contamination was negligible.

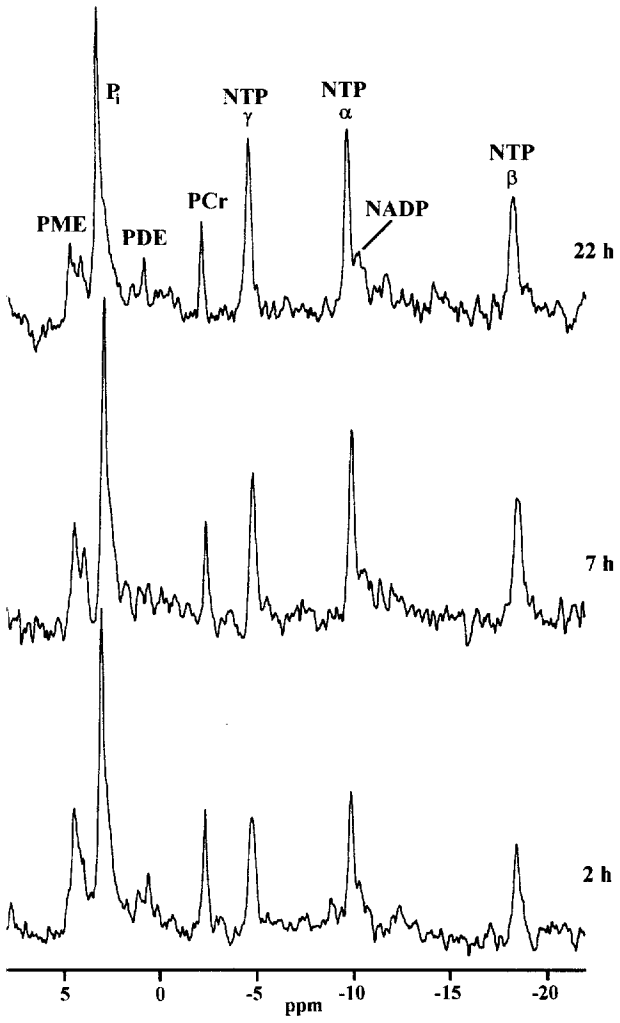

Fig. 5. ^{31}P NMR spectra of primary neurons embedded in basement membrane gel threads. Spectra were obtained on a Bruker AM-360 NMR spectrometer after 2, 7, and 22 h perfusion (acquisition time 30 min). Assignments: NADP, nicotinamide adenine dinucleotide phosphate; NTP, nucleoside triphosphate; PCr, phosphocreatine; PDE, phosphodiester; Pi, inorganic phosphate; PME, phosphomonoester.

5.2. NMR spectroscopy

Before starting ^{13}C NMR experiments, ^{31}P NMR spectra were recorded to monitor the energy status of the cells in the NMR tube. The spectra show signals of energy rich phosphates as nucleoside triphosphates (NTP) and phosphocreatine (PCr) representing normal energy charge even after 22 h perfusion (Fig. 5).

During incubation with normal medium containing unlabeled glucose no natural abundance signals of metabolites were detectable at a field strength of 8.4 T (Fig. 6, bottom). If the medium was subsequently replaced by DMEM without FCS but with 5 mM [1-^{13}C]glucose, glutamate (Glu) ^{13}C-labeled in position C-4 was identified in spectra of primary neurons already 30 min after the start of perfusion (Fig. 6). Glutamate is not only a neurotransmitter in glutamatergic neurons, but also serves as a precursor for several other amino acids, protein biosynthesis and further metabolites. With a concentration of approximately 12 mM glutamate is the most abundant amino acid in the central nervous system.

While the ^{13}C label in position C-4 of glutamate, synthesized via pyruvate dehydrogenase activity, is already detectable after 30-60 min the ^{13}C label in position C-3 and C-2 is detectable only after 3 h (Fig. 6). [2-^{13}C]glutamate and [3-^{13}C]glutamate can be synthesized either via pyruvate dehydrogenase activity after at least 1.5 turns of the TCA cycle or, more likely, via malic enzyme and/or pyruvate carboxylase activity [6]. Additionally, neurons synthesized labeled lactate ^{13}C-enriched in position C-3, which appeared in the spectra at a later point of time than [4-^{13}C]glutamate (Fig. 5).

Whereas the intensity of the glutamate signals (C-2, C-3 and C-4) reached a steady state after several hours, the lactate signal (C-3) increased continuously during the time of observation (up to 10 h) (Fig. 7). Lactate was not restricted to the intracellular space, but primarily exported into the medium. Since the perfusion system is a closed system, intra- and extracellular metabolites were recorded simultaneously, and the slow increase of the lactate signal reflects a steady accumulation in the medium. Due to the efficient buffer system, the pH remained constant, even under these conditions, as can be demonstrated by ^{31}P NMR spectroscopy (data not shown).

Additional metabolic information can be obtained using an NMR spectrometer operating at even higher field strength (16.1 T). Already 2 h after [1-^{13}C]glucose was provided to the neuronal cells, labeled lactate and all three isotopomers of glutamate were detected (Fig. 8, bottom). Four hours later, not only the ^{13}C enrichment in lactate and

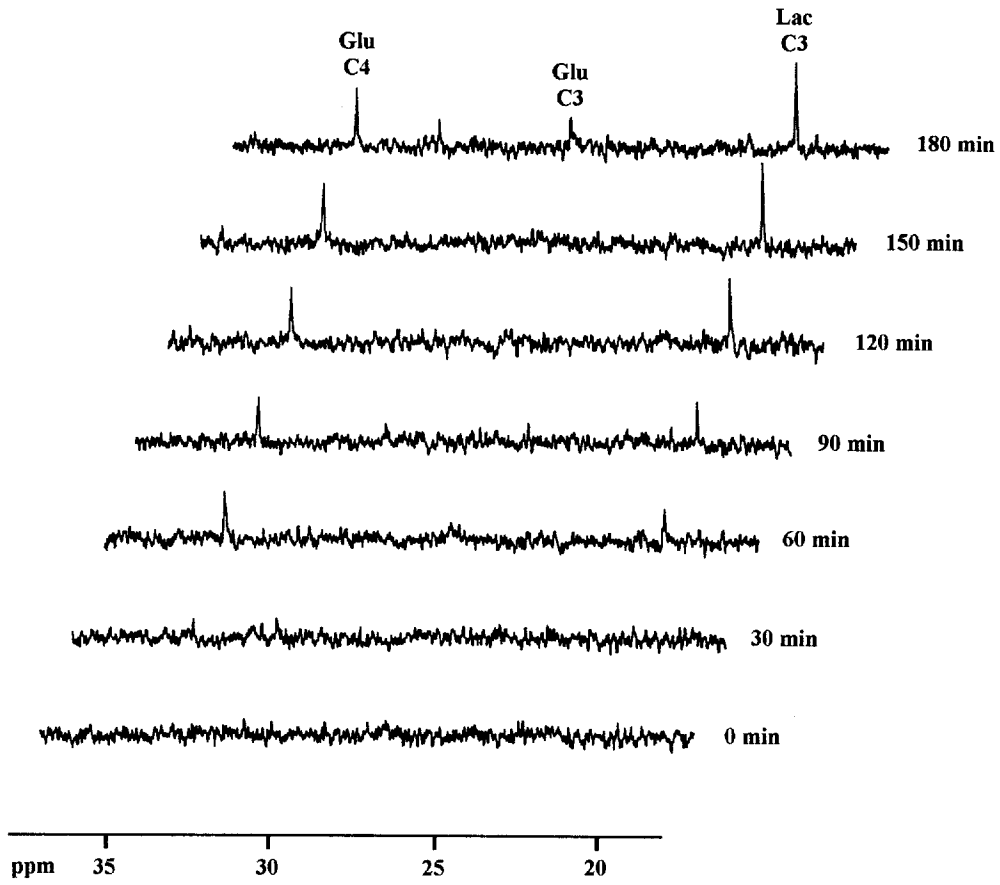

Fig. 6. ^{13}C NMR spectra of primary neurons embedded in basement membrane gel threads. Spectra were obtained on a Bruker AMX-360 NMR spectrometer after incubation with 5 mM [1-^{13}C]glucose for 3 h (acquisition time 30 min). Assignments: Glu, glutamate, Lac, lactate.

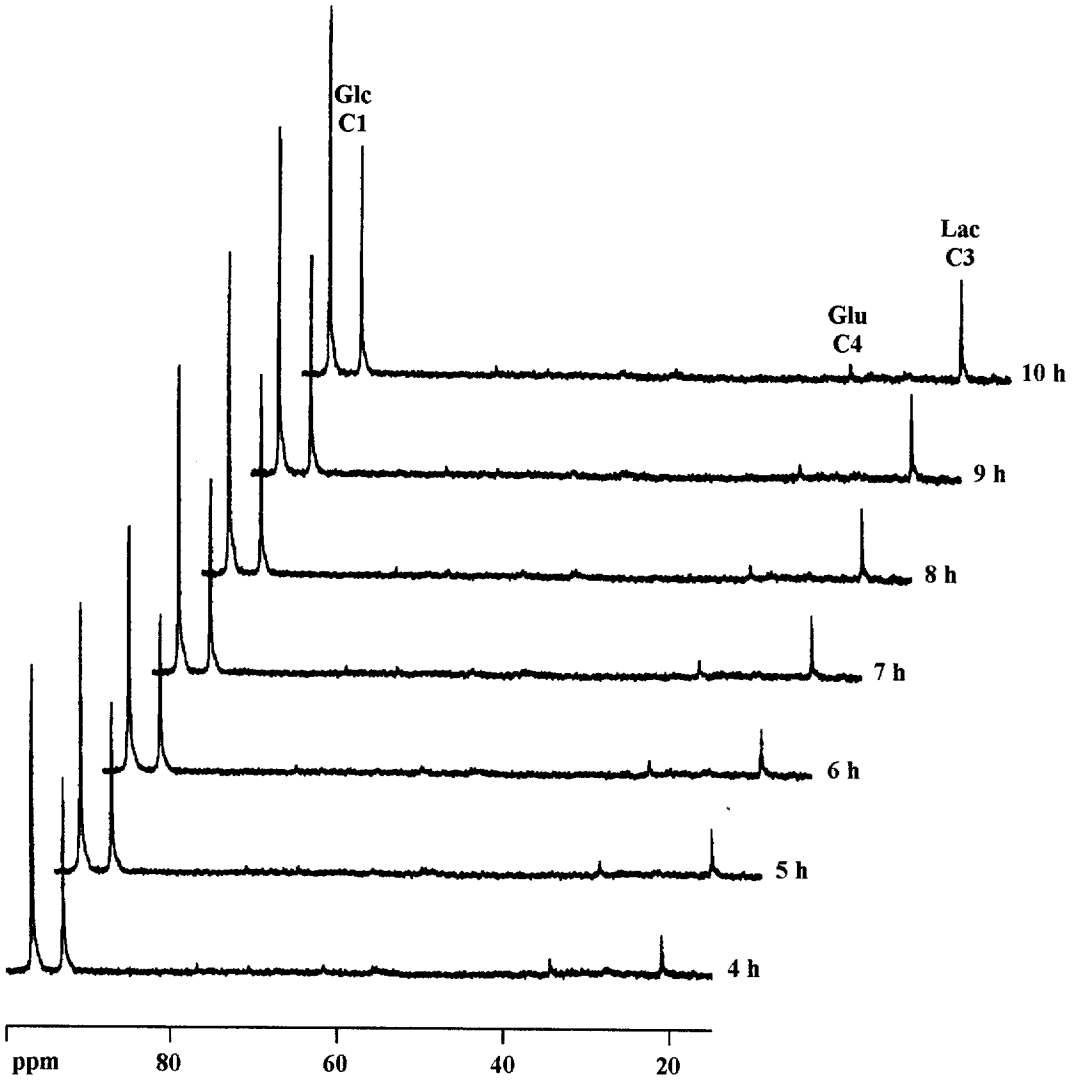

Fig. 7. ^{13}C NMR spectra of primary neurons embedded in basement membrane gel threads. Spectra were obtained on a Bruker AMX-360 NMR spectrometer after incubation with 5 mM [1-^{13}C]glucose for 10 h (acquisition time 60 min). Assignments: Glc, glucose; Glu, glutamate, Lac, lactate.

glutamate was increased, but also several other labeled metabolites, i.e., alanine, aspartate, and glutamine were observed (Fig. 8, top).

6. Discussion

Various approaches have been carried out to provide metabolic information from the brain. With ^{1}H NMR spectroscopy rather large volumes (typically 1 ml in humans and several l in rats) can be recorded non-invasively in vivo. ^{13}C NMR spectroscopy requires even larger volumes, but allows to monitor metabolic fluxes, if ^{13}C-labeled metabolites are injected. Both spectra acquisition modes have in common, that the selected tissue region contains a variety of metabolically different cell types and do not allow the differentiation of neuronal and glial cell metabolism. If brain slices are used, the same cellular heterogeneity has to be considered. However, cellular interactions in brain slices occur and can be taken into account.

Cell culture systems of neuronal or glial cells provide an excellent means to evaluate the metabolism of individual cell types of the brain. In the present study immobilized primary neuronal cultures were established for on-line monitoring of neuronal metabolism in viable neurons by ^{31}P and ^{13}C NMR spectroscopy. Neuronal cells were obtained from the brains of embryonic rats as described before [1] and immediately after preparation, cells were incorporated into basement membrane gel threads, providing an excellent support for three-dimensional growth of viable neuronal cells. When single cell suspensions of the cerebral hemispheres were seeded on poly-L-lysine coated culture dishes, under low serum growth conditions, during the first week in vitro these monolayer cultures represent a pure neuronal population and non-neuronal cell contaminations were negligible. These homogeneous cell cultures have been successfully used to study the potential of

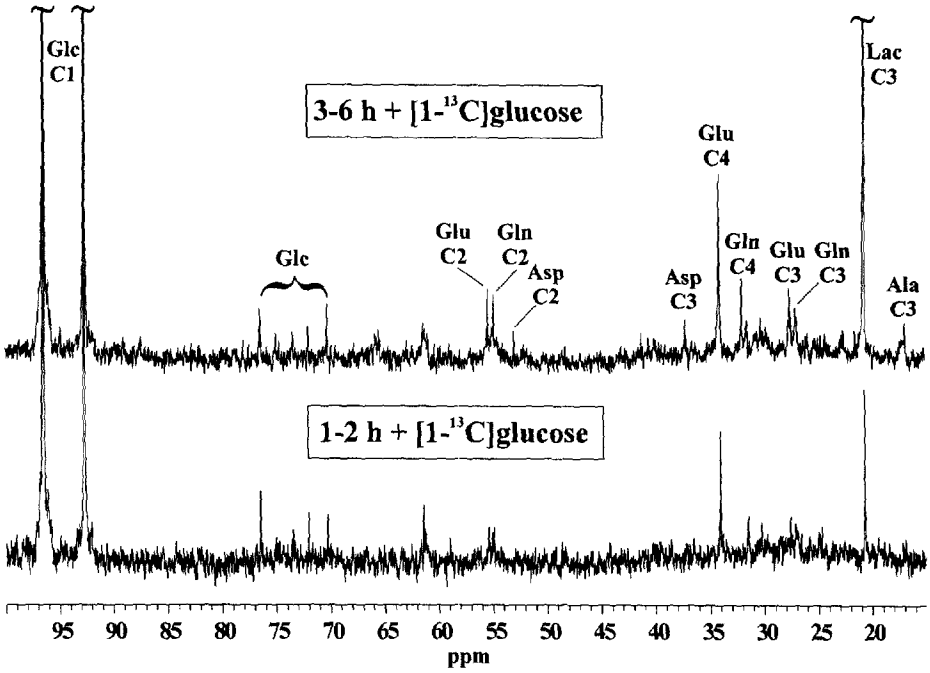

Fig. 8. ^{13}C NMR spectra of primary neurons embedded in basement membrane gel threads. Spectra were obtained on a Bruker DRX-600 NMR spectrometer after incubation with 5 mM [1-^{13}C]glucose for 2 h (bottom) (acquisition time first two 2 h of incubation) and 6 h (top) (acquisition time last 4 h of incubation). Assignments: Ala, alanine; Asp, aspartate, Glc, glucose; Gln, glutamine; Glu, glutamate, Lac, lactate.

neuronal cell metabolism in cellular extracts and the dynamic properties of exogenously added substrates, such as ^{13}C-labeled glucose or acetate [6,7]. Here we show that immobilizing neuronal cells in basement membrane gel threads is a suitable means to ensure neuronal viability and provides cellular support and mechanical protection. This system allows on-line NMR monitoring over a period of at least 24 h.

Various systems have been established to enable three-dimensional growth of mammalian cells, such as beads [8], gels [9,10] and hollow fibers [11]. Beads or microcarriers are available in a large variety of organic materials [12]. Their major drawback with respect to neuronal growth is that neuronal cells growing on the surfaces of beads are easily mechanically damaged during processes of transfer to different tubes as well as by shear stress during superfusion within the NMR tube. Furthermore, cells are growing only two dimensionally on the bead surface and thus the sensitive volume of the NMR probe is not efficiently used. To some extent porous microcarriers may overcome this disadvantage, but in this system medium exchange is rather inefficient. Hollow fibers allow an excellent superfusion, but are much less suitable as a support for adherently growing cells. We have previously shown that basement membrane gel threads are enabling the immobilization of glial tumor cells [13]. These threads consist mainly of collagen type IV and laminin. Neuronal cells preferentially attach to and grow on laminin. Neurons growing three-dimensionally in basement membrane gel threads, are mechanically well protected and very efficiently supplied with the nutrient medium, as demonstrated by a normal and constant energy charge which was detected by ^{31}P NMR spectroscopy for more than 24 h of perfusion (Fig. 5). Growing of the cultured neurons inside the three-dimensional structure of the basement membrane gel uses the sensitive volume much better than growing on microcarrier beads [14] and therefore, a much higher signal to noise ratio is yielded with NMR spectroscopy.

Already 30 min after perfusion with [1-^{13}C]glucose was started, intracellular metabolites such as glutamate and lactate were detected with a Bruker AM 360 MHz spectrometer. Using a Bruker DRX 600 MHz spectrometer, time dependent changes in the labeling pattern of alanine, aspartate, glutamate, glutamine, and lactate could be detected. The comparison of the spectra received after 1–2 h (Fig. 8, bottom) and after 3–6 h (Fig. 8, top) perfusion time confirms the de novo synthesis of glutamate not only via pyruvate dehydrogenase but also via malic enzyme and/or pyruvate carboxylase activity (observed from labeled C-2 and C-3 glutamate) by neurons. These results are confirmed by a previous study when [2-^{13}C]pyruvate was used as substrate for neuronal metabolism [6]. In that

study it was demonstrated by detecting a specific isotopomer pattern of glutamate, that primary rat neurons in culture show some activity of the anaplerotic way of pyruvate carboxylase or malic enzyme [6], but the two pathways cannot be distinguished in this kind of whole cell studies.

As shown before, embryonic neurons in culture also show some glutamine synthetase activity [7]. The observed GS activity in fetal neurons is in agreement with former observations in cell culture work [15,16], but was not observed in the adult brain based on immunocytochemical studies [17]. Whether this reflects the plasticity of fetal neurons or the different gene expression pattern in fetal neurons, because of missing astrocyte factors or the different culture conditions per se, remains unanswered and needs further clarification.

Thus, the present data demonstrate that rat cerebral neurons can be efficiently embedded into basement membrane gel threads and can be monitored during an extended period of time by multinuclear magnetic resonance spectroscopy. This system represents an excellent tool to follow up the energy status and/or neuronal metabolism through glycolysis and the tricarboxylic acid cycle under control conditions or during pathological constraints. Investigating the specific differences in the metabolism of various cell types in the brain on a cellular level can help to derive more diagnostic benefit from in vivo MRS and may provide a detailed insight into the metabolism of pathological situations in the brain.

7. Quick procedures

- Prepare cerebral hemispheres from the brains of 16-day-old rat embryos.
- Centrifuge cell suspension and resuspend twice.
- Resuspend cell pellet gently with 2 ml BME/10% FCS and divide into four samples of 500 μl each.
- Mix each sample with 500 μl solution of basement membrane extract (4°C).
- Drawn cold mixture into a syringe, that is immersed in a water bath at 37°C for 2 min.
- Extrude threads through a sterile Teflon tubing (ca. 1 m length, 0.3 mm i.d.) into plastic culture dishes (15 cm) filled with BME/10% FCS (20 ml).
- 2 h incubation (37°C and 10% CO_2).
- Remove the culture medium and replace by BME/0.5% FCS (20 ml).
- Incubation of the immobilized neurons for 4–5 days (37°C and 10% CO_2).
- NMR measurement of different isotopes, i. e. ^{13}C,^{31}P.

8. Essential literature references

Essential references: Refs. [1,12,13].

Acknowledgements

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