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Proton Nuclear Magnetic Resonance Spectroscopy Unambiguously Identifies Different Neural Cell Types

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Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy is a noninvasive technique that can provide information on a wide range of metabolites. Marked abnormalities of ^{1}H NMR brain spectra have been reported in patients with neurological disorders, but their neurochemical implications may be difficult to appreciate because NMR data are obtained from heterogeneous tissue regions composed of several cell populations. The purpose of this study was to examine the ^{1}H NMR profile of major neural cell types. This information may be helpful in understanding the metabolic abnormalities detected by ^{1}H NMR spectroscopy.

Extracts of cultured cerebellar granule neurons, cortical astrocytes, oligodendrocyte–type 2 astrocyte (O-2A) progenitor cells, oligodendrocytes, and meningeal cells were analyzed. The purity of the cultured cells was >95% with all the cell lineages, except for neurons (approximately 90%).

Although several constituents (creatine, choline-containing compounds, lactate, acetate, succinate, alanine, glutamate) were ubiquitously detectable with ^{1}H NMR, each cell type had distinctive qualitative and/or quantitative features. Our most unexpected finding was a large amount of *N*-acetylaspartate (NAA) in O-2A progenitors. This compound, consistently detected by ^{1}H NMR *in vivo*, was previously thought to ne present only in neurons. The finding that meningeal cells have an alanine:creatine ratio three to four times higher than astrocytes, neurons, or oligodendrocytes is in agreement with observations that meningiomas express a higher alanine:creatine ratio than gliomas.

The data suggest that each individual cell type has a characteristic metabolic pattern that can be discriminated by ^{1}H NMR, even by looking at only a few metabolites (e.g., NAA, glycine, β-hydroxybutyrate). Some of these features may be useful in interpreting ^{1}H NMR observations, for example, those of patients with brain tumors where there are marked changes in cell population, and in cases where selective neuronal loss or abnormal development can be expected.

[Key words: nuclear magnetic resonance, neurons, astrocytes, oligodendrocytes, oligodendrocyte–type 2 astrocyte progenitors, differentiation, brain development, cell metabolism]

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy can be used to measure a range of cerebral metabolites in both animals and man in a noninvasive manner. The most prominent signals obtained by ^{1}H NMR spectroscopy of living brain are from *N*-acetyl-aspartate (NAA), creatine and phosphocreatine (Cr), and choline-containing compounds (Cho) (Behar et al., 1983; Gadian et al., 1986). Resonances from glutamate (Glu), taurine (Tau), and inositol (Ino) are also detectable in normal brain, whereas glutamine (Gln), GABA, alanine (Ala), and lactate (Lac) can be detected in various pathological states (Rothman et al., 1984; Behar et al., 1985; Bates et al., 1989b). Recent advances have resulted in excellent ^{1}H NMR spectra being recorded from localized regions of the human brain (Frahm et al., 1989; Ross, 1991), and marked abnormalities have been reported in the spectra of patients with cerebral disorders as varied as brain tumors, stroke, inborn genetic abnormalities, and neurodegenerative disorders (Gadian, 1990; Miller, 1991).

At present, the interpretation of changes in ^{1}H NMR spectra of the human brain is complicated because NMR data are obtained from whole tissue regions that include several cell populations distributed heterogeneously. Understanding the neurochemical implications of these metabolic abnormalities requires knowledge of the concentrations of these compounds within the major cell types constituting brain tissue. The purpose of this study was to examine the ^{1}H NMR profile of purified and well-characterized neural cell types. The following cell types were studied: cerebellar granule neurons, cortical astrocytes, oligodendrocyte–type 2 astrocyte (O-2A) progenitor cells, oligodendrocytes, and meningeal cells. Amino acids and NAA data were verified by HPLC analysis. The data suggest that each individual cell type has a characteristic metabolic pattern that can be distinguished by ^{1}H NMR.

Materials and Methods

Preparation of purified brain cell cultures. All cells were grown in Dulbecco's modified Eagle's medium containing 1 gm/liter glucose [DMEM; GIBCO–Bethesda Research Labs (BRL), Paisley, UK]. The culture medium for cortical astrocytes and meningeal cells was supplemented with 10% heat-inactivated fetal calf serum (FCS; Imperial Laboratories), 2 mmol/liter glutamine (Sigma, Poole, UK), and 25 μg/ml gentamicin (Flow Laboratories, Rickmansworth, UK) (DMEM-FCS). O-2A progenitors, oligodendrocytes, and neurons were cultured with chemically defined medium [DMEM-BS: DMEM containing 1 gm/liter glucose and supplemented with 25 μg/ml gentamicin, 2 mmol/liter glutamine, 0.234 IU/ml bovine pancreas insulin, 100 μg/ml human transferrin, 0.0286%

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(v/v) BSA pathocyte (Miles Laboratories, Inc), 0.2 μmol/liter progesterone, 0.1 μmol/liter selenium, and 0.49 μmol/liter 3,3',5-triiodo-L-thyronine (all Sigma) modified from Bottenstein and Sato, 1979]. Cells were cultured at 37°C in a humidified atmosphere containing 7.5% CO_2, using NUNC tissue culture flasks (GIBCO–BRL) coated with poly-L-lysine (PLL; Sigma; 175,000 MW, 13 μg/ml) or PLL-coated glass coverslips (no. 1, 13 mm diameter; Merck Ltd., Poole, UK) for immunocytochemistry. Cells were detached from the culture flask by 10–15 min of incubation at 37°C in Ca^{2+}/Mg^{2+}-free DMEM (DMEM-CMF) containing 0.54 mmol/liter EDTA (Sigma), followed by 5 min incubation with trypsin (300 IU/ml final concentration; bovine pancreas, type III; Sigma). Trypsinization was stopped by adding 1 ml of soybean trypsin inhibitor (SBTI)-DNase [5200 IU/ml soybean trypsin inhibitor, 74 IU/ml bovine pancreas DNase I, and 3.0 mg/ml BSA (fraction V) (all from Sigma)] per 10 ml of cell suspension. Cell suspensions were centrifuged at 500 × *g* for 5 min.

Cerebellar granule neurons were prepared from 7-d-old Wistar rats using the procedure of Noble et al. (1984) modified according to Ciardo and Meldolesi (1991). Minced cerebella were exposed to mild trypsinization, which was stopped by trituration in SBTI-DNase solution (Noble et al., 1984) supplemented with 13 mg/ml BSA. The suspension was centrifuged, resuspended in DMEM-FCS, and finally dissociated to single cells by triturating the suspension through a 5 ml pipette tip and 23 and 25 gauge hypodermic needles, successively. Cellular aggregates were allowed to sediment before centrifugation of the supernatant through a BSA cushion as described by Ciardo and Meldolesi (1991). The cells were then resuspended in DMEM-FCS and plated at a density of two cerebella per flask (175 cm^2, coated with 50 μg/ml PLL overnight) to remove residual macrophages, glial cells, and meningeal cells. After 15 min incubation at 37°C, neuronal cells were detached by shaking the flasks for 2 min, removed with the medium, and replated on 80 cm^2 flask and coverslips (both coated with 13 μg/ml PLL) (5000 cells per coverslip for immunostaining). After 4 hr, the medium was replaced with DMEM-BS supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) and 50 ng/ml NGF (7S, Boehringer, Mannheim, Germany). The growth factors were subsequently added every second day to improve cell survival. On the second day and every 48 hr thereafter, cultures also received 20 μmol/liter cytosine arabinoside (Sigma) to prevent glial cell proliferation. Cells were harvested for ^1H NMR and HPLC analysis after 5 d in culture.

Purified cortical astrocytes were prepared from cerebral cortices of 1-d-old rats as previously described (McCarthy and de Vellis, 1980; Noble et al., 1984, Wolswijk and Noble, 1989) except that, after selective complement-mediated killing of nonastrocytic cells, the remaining cells were replated on 175 cm^2 flasks (density of 1 × 10^6 cells per flask). Cells were harvested for ^1H NMR and HPLC analysis or immunostaining when a confluent monolayer of flat cells had been formed (6–7 d).

Meningeal cultures were prepared from leptomeninges of newborn or 1-d-old Wistar rats according to Noble et al. (1984). Dissociated cells prepared from five meninges were plated on one flask (80 cm^2) and grown in DMEM-FCS. Once a confluent monolayer of cells had been formed (approximately 6 d), the cells were passaged, diluted in growth medium (1:5), and replated. After two further passages (normally after 11 and 16 d, respectively), the confluent cells were harvested for analysis and immunocytochemistry.

O-2A progenitors were isolated from optic nerves of 7-d-old rats as described previously (Raff et al., 1983b), with some modifications. Optic nerves were dissected from just behind the eyes to the chiasm, minced, and incubated in Leibovitz L-15 medium (Flow Laboratories) supplemented with 25 μg/ml gentamicin (L-15), containing 667 IU/ml collagenase (Worthington Enzymes). After 45 min at 37°C, 3500 IU/ml trypsin in DMEM-CMF was added and incubation was continued for 15 min (37°C). The suspension was centrifuged (1 min, 1500 × *g*), resuspended in DMEM-CMF containing 0.43 mmol/liter EDTA and 6000 IU/ml trypsin, and further incubated for 15 min (37°C). Trypsinization was stopped by addition of an equal volume of SBTI-DNase. The suspension was centrifuged (1 min, 1500 × *g*), resuspended in DMEM-BS, and finally dissociated to single cells by triturating the suspension through a 5 ml pipette tip and 25 and 27 gauge hypodermic needles, successively. Dissociated cells were plated in 25 cm^2 flasks (density of 2 × 10^5 cells per flask) and grown in DMEM-BS with 10 ng/ml of both bFGF and platelet-derived growth factor (PDGF) (Bögler et al., 1990). Recombinant human bFGF and PDGF-AA were a gift of Drs. Larry Coussens and Carlos George-Nascimento (Chiron Corp., CA). After 6–7 d, when a nearly confluent monolayer had formed, cells were passaged, diluted in growth medium to one-third of their original density, and replated onto flasks. Cells were cultured for a further week in DMEM-BS with growth factors before harvesting for ^1H NMR and HPLC analysis or immunostaining.

Oligodendrocytes were derived from O-2A progenitors of the corpus callosum of 7-d-old Wistar rats. The corpus callosum was used instead of the optic nerve to obtain a larger number of O-2A progenitor cells. O-2A progenitors were isolated by fluorescence-activated cell sorting (FACS) of dissociated cells, obtained as described for optic nerve cells. Immunolabeling of O-2A progenitors for FACS was performed with mouse monoclonal antibodies as follows: (1) 30 min incubation at 4°C in 1 ml of L-15 containing either A2B5 (concentrated hybridoma supernatant, diluted 1:50; Eisenbarth et al., 1979) or R-24 (1:100 diluted; a gift of Dr. Lloyd Old), which binds to GD3 ganglioside (Pukel et al., 1982); (2) two washes with 14 ml of L-15 medium; (3) 30 min incubation at 4°C in 1 ml of L-15 containing the secondary fluorescein-conjugated goat anti-mouse IgM or IgG3 antibody (1:100; Southern Biotechnology Associates Inc., Birmingham, AL) to label A2B5 or R-24, respectively; and (4) two final washes with L-15 before resuspension in DMEM-BS (1 × 10^6 cells/ml).

Cell sorting using an FACStar Plus (Becton Dickinson, Mountainview, CA) was performed at a flow rate of 800–1000 cells/sec with phosphate-buffered saline (PBS; GIBCO BRL) as sheath fluid, and an argon laser (100 mW power, 488 nm wavelength). The sorting gates allowed us to select only the brightest fluorescent (antigen-positive) cells and discard small particles showing low-angle light scattering (mostly debris). The purity of sorted fluorescence-tagged cells (approximately one-third of the initial cell population) was assessed by examination of samples with a fluorescent microscope (Zeiss Axiphot microscope equipped with phase contrast, UV epiillumination, and selective filters for fluorescein and rhodamine).

The sorted cells were centrifuged, and cultured as described above for the optic nerve O-2A progenitors, except for the initial cell density, which was slightly higher (3 × 10^5 cells per 25 cm^2 flask). Upon near confluence (5–6 d), cells were passaged, replated on 80 cm^2 flasks (7–8 × 10^5 cells per flask), cultured for 5–6 d, repassaged to remove growth factors attached to the extracellular matrix (Lillien and Raff, 1990), and finally replated at a density of 3–4 × 10^6 per 80 cm^2 flask or 7000–10,000 cells per glass coverslip for subsequent immunostaining. Oligodendrocyte differentiation was promoted by keeping the repassaged cells in DMEM-BS devoid of growth factors for 3–4 d (Raff et al., 1983a,b). Oligodendrocytes were harvested for ^1H NMR and HPLC analysis only when >90% of cells had differentiated into oligodendrocytes (as assessed by analysis of parallel coverslips).

Purity of the cell population. To evaluate the purity of the cell populations, cultures were immunolabeled with various antibodies. Cortical astrocytes and meningeal cells were harvested, plated onto PLL-coated coverslips (7000–10,000 cells per coverslip), and immunolabeled 24 hr later. In order to prevent differentiation, O-2A progenitors were immunolabeled 1 hr after plating (7000–10,000 cells per coverslip; coating was raised to 130 μg/ml). Oligodendrocytes could not be harvested enzymatically (see below), nor could neurons be efficiently replated after passaging. Therefore, purity of these cells was determined in parallel cultures growing on coverslips and immunostained at the time of harvesting.

All antibodies and staining methods used for cell type identification were as previously described (Noble et al., 1984; Wolswijk and Noble, 1989; Bögler et al., 1990), with minor modifications. Neurons were tested for their positive reaction to the monoclonal antineurofilament antibody RT97 (Wood and Anderton, 1981) and for a negative reaction to antiserum directed against glial fibrillary acidic protein (GFAP; an astrocyte-specific cytoskeletal component; Bignami et al., 1972; Pruss, 1979). Cortical astrocytes were $GFAP^+$ and $A2B5^-$ (Eisenbarth et al., 1979). Meningeal cells were $RAN\text{-}2^+$ (Bartlett et al., 1981) and $GFAP^{ms}$. O-2A progenitors were $A2B5^+$, GC^- (galactocerebroside, an oligodendrocyte-specific glycolipid; Raff et al., 1978; Ranscht et al., 1982), and $GFAP^-$ Oligodendrocytes were GC^+ and negative for labeling with anti-GFAP and A2B5 antibodies.

Cell harvesting and sample preparation. The final medium change always took place 24 hr before cell harvesting. Except for oligodendrocytes, all cells were harvested by trypsinization (see above), washed three times with 25–50 ml of PBS, and extracted with 1–2 ml of ice-cold perchloric acid (PCA; 12% v/v; Merck Ltd.). Oligodendrocytes could not be easily detached from the flask with trypsin or other proteases (J. Urenjak, unpublished observations) and were therefore washed three times with PBS and scraped from the culture flask directly into PCA (2 ml per 175 cm^2 flask). PCA extracts were sonicated (2 × 30

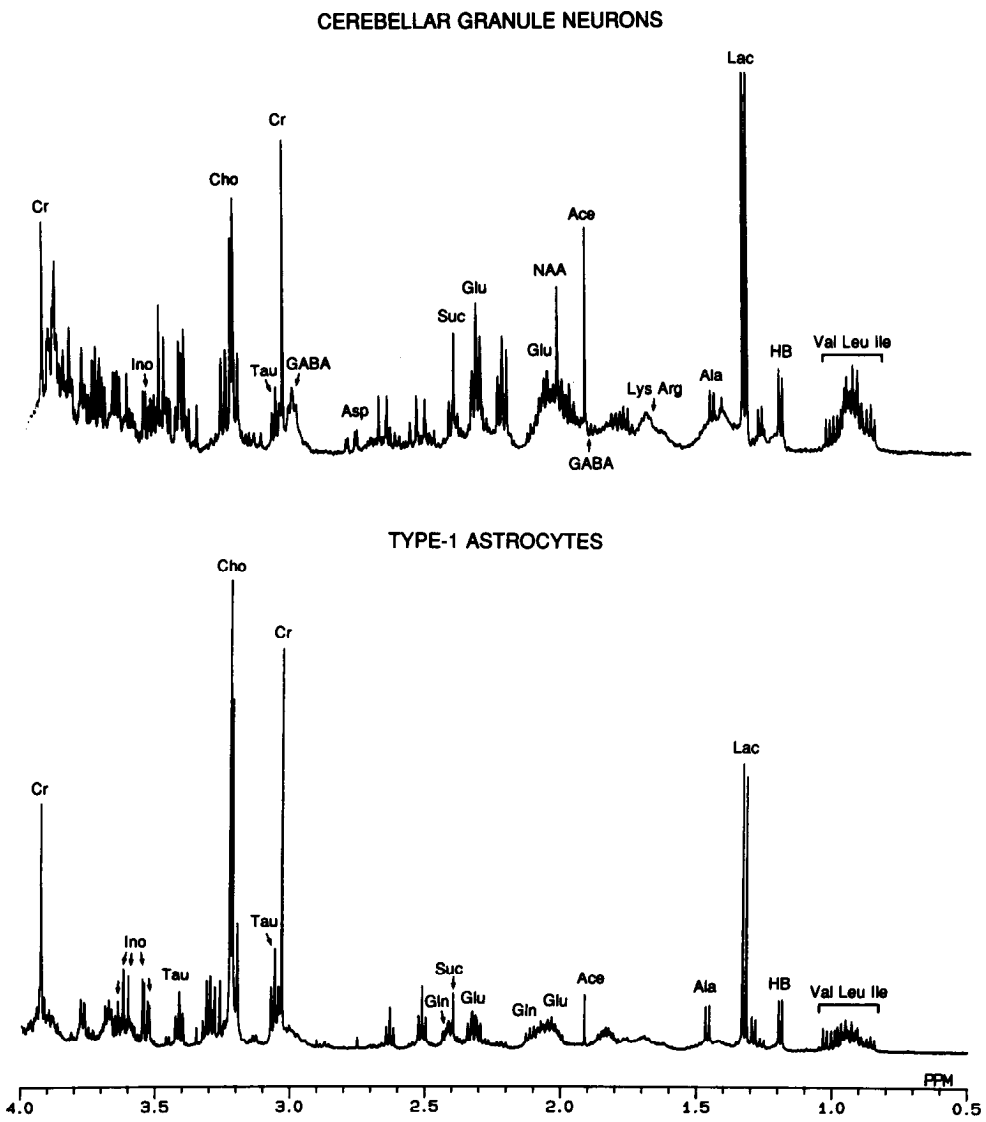

Figure 1. Representative ^{1}H NMR spectra obtained from extracts of cultured cerebellar granule neurons (*top*) and cortical astrocytes (*bottom*). PCA extracts of neurons and astrocytes contained 4.5 and 17.4 mg of protein, respectively. Immunostaining showed that the cell purity was approximately 90% for neurons and >95% for cortical astrocytes. NMR spectroscopic analysis was performed at pH 8.9 with 512 scans recorded at 500 MHz. Spectra, referenced to TSP (0 ppm), are displayed between 0.5 and 4.0 ppm. The amplitude of each peak is proportional to the number of hydrogen atoms resonating at that frequency. Identified signals include, from right to left: valine (*Val*), leucine (*Leu*), and isoleucine (*Ile*), β-hydroxybutyrate (*HB*), lactate (*Lac*), alanine (*Ala*), lysine (*Lys*) and arginine (*Arg*), GABA, acetate (*Ace*), *N*-acetyl-aspartate (*NAA*), glutamate (*Glu*), glutamine (*Gln*), succinate (*Suc*), aspartate (*Asp*), creatine (*Cr*), taurine (*Tau*), choline-containing compounds (*Cho*), and inositol (*Ino*). Note that the ^{1}H spectrum of neurons shows high signals of NAA and of the neuroactive amino acids Glu, GABA, and Asp, while Cho and Cr are more dominant in cortical astrocytes. A quantitative comparison of all the cell types studied is shown in Tables 1 and 2.

sec, 4°C; Soniprep 150, MSE Scientific Instruments, Crawley, UK) and centrifuged at 3000 × *g* for 20 min. The precipitate was used for protein determination. The supernatant was adjusted to pH 8.5–8.9 with 3 mol/liter KOH or saturated $KHCO_3$, separated from precipitated $KClO_4$ by centrifugation (3000 × *g* for 10 min), lyophilized, and stored at −20°C.

^{1}H NMR analysis. Lyophilized cell extracts were redissolved in 0.6 ml of deuterium oxide (D_2O; Goss Scientific Instruments Ltd., Ingatestone, UK), and 10 μl of D_2O containing 10 mmol/liter 3-trimethylsilyl-tetradeuterosodium propionate (TSP; Goss Scientific Instruments) was added as a concentration and chemical shift standard for ^{1}H NMR. The pH was adjusted to approximately 8.9 with no more than 10 μl of DCl or NaOD (Aldrich, Gillingham, UK) in D_2O.

^{1}H NMR spectroscopy was performed at 20–22°C with a JEOL 500 spectrometer operating at a frequency of 500 MHz. Fully relaxed spectra were acquired using 45° pulses applied every 5 sec, spectral width of 6002 Hz, and data size of 32,000 points. Preliminary experiments showed that a 5 sec repetition time was long enough for the spectra to be fully relaxed. Gated irradiation was applied between acquisitions to presaturate the residual water peak. Accumulation of 512 scans was required for satisfactory signal:noise ratio. Spectra were Fourier transformed after applying 0.2 Hz exponential multiplication.

Resonance assignments were based on (1) chemical shifts and coupling patterns (Cerdàn et al., 1985; Fan et al., 1986; Sze and Jardetzky, 1990), (2) comparison with spectra of known compounds at the same pH, and (3) in the case of hypotaurine (H-Tau), spiking the samples with the authentic compound.

Metabolite concentrations were calculated after baseline adjustment from the peak height of selected signals relative to the signal height of the internal standard TSP with correction for the number of protons contributing to each signal. Each metabolite was quantified from its best resolved and strongest resonance: β-hydroxybutyrate (HB), CH_3 1.2 ppm (doublet, d); alanine (Ala), CH_3 1.47 ppm (d); *N*-acetyl-aspartate (NAA), CH_3 2.02 ppm (singlet, s); hypotaurine (H-Tau), CH_2 2.65, 3.30 ppm (triplet, t); choline-containing compounds (Cho), $N(CH_3)_3$ 3.21–3.24 ppm (s); creatine (Cr), CH_3 3.04 ppm (s); glycine (Gly), CH_2 3.51 (s); taurine (Tau), CH_2 3.08, 3.42 ppm (t).

HPLC analysis of amino acids, NAA, and organic acids. Separation and quantification of amino acids, NAA, and organic acids were performed by HPLC to verify the NMR data. Analysis of amino acids was performed according to Lindroth and Mopper (1979), with minor modifications. Ten microliters of the NMR samples were diluted 50–1000-fold with distilled water and 20 μl aliquots mixed with 40 μl of reagent containing 0.8 mg/ml *O*-phthaldialdehyde (OPA) (Sigma) and 0.4 M boric acid, pH 9.5. After a reaction time of 2 min, 53 μl of the mixture was injected into a LiChroGraph HPLC system (Merck-Hitachi, Darmstadt, Germany). Separation was achieved with a LiChrospher 100 C-18 column (5 μm; 4 mm i.d., 250 mm length) at 40°C, using a two-step linear gradient of methanol in aqueous potassium phosphate buffer (50 mmol/liter, pH 5.9), starting at 20% methanol, increasing to 35% methanol in 7 min, and finishing at 20 min with 60% methanol. The OPA derivatives were detected with a fluorometer (LiChroGraph F 1050; 340 nm excitation, 450 nm emission). Concentration were calculated from external standards.

Analysis of NAA and organic acids was performed according to Koller et al. (1984). NMR sample aliquots (25–50 μl) were separated by a Polyspher QA HY cation-exchange column (Merck; 6 mm i.d., 300 mm

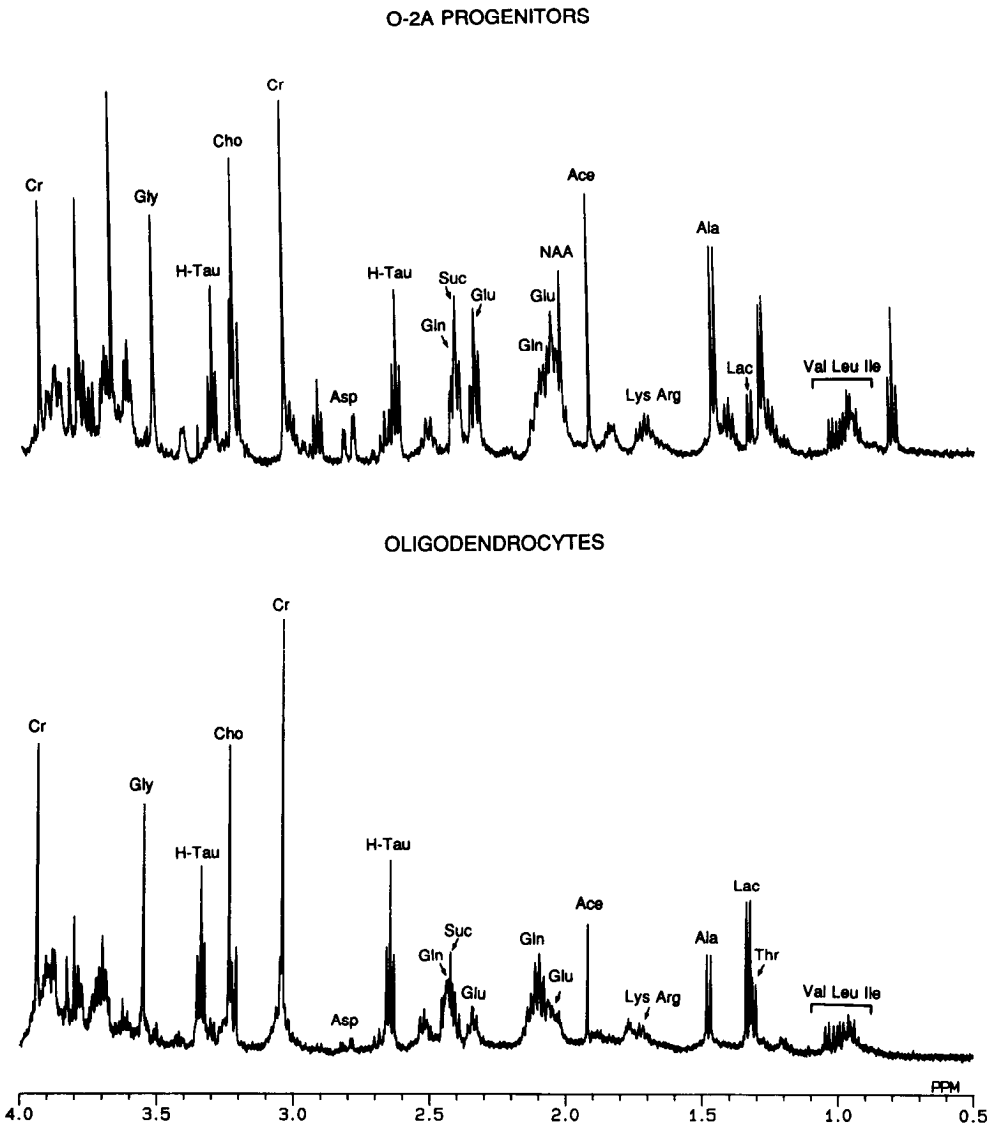

Figure 2. Representative ^{1}H NMR spectra obtained from extracts of cultured O-2A progenitors (*top*) and oligodendrocytes (*bottom*). PCA extracts of O-2A progenitors and oligodendrocytes contained 1.0 and 1.5 mg of protein, respectively. Immunostaining showed that the cell purity was >95% for both cell types. Parameters for NMR spectroscopy were as described in Figure 1. Identified resonances include those from valine (*Val*), leucine (*Leu*), and isoleucine (*Ile*), threonine (*Thr*), lactate (*Lac*), alanine (*Ala*), lysine (*Lys*) and arginine (*Arg*), acetate (*Ace*), *N*-acetyl-aspartate (*NAA*), glutamate (*Glu*), glutamine (*Gln*), succinate (*Suc*), hypotaurine (*H-Tau*), aspartate (*Asp*), creatine (*Cr*), choline-containing compounds (*Cho*), and glycine (*Gly*). Note the high levels of NAA, Ala, Glu, and Asp in the O-2A progenitor spectrum. A quantitative comparison of all the cell types studied is shown in Tables 1 and 2.

length), at 55°C, with 5 mmol/liter H_2SO_4 as mobile phase flowing at 0.8 ml/min. NAA, succinate (Suc), and lactate were detected with a UV detector (210 nm; LiChroGraph, L-4000, Merck). The NAA concentration was calculated from external standards.

Protein determination. The PCA precipitate was dissolved in 0.5–2 ml 1 mol/liter KOH and incubated for 1 hr at 37°C, and the protein concentrations were determined with the bicinchoninic acid method (BCA Kit, Pierce, Rockford, IL; Smith et al., 1985), with BSA as standard.

Data treatment and statistical analysis. Results are expressed as nmol/mg of protein and averaged as mean ± SEM. Some of the data are also given as concentration ratios, which can be readily determined from the NMR spectra alone. These ratios are particularly relevant to NMR spectroscopy *in vivo*, where data are commonly expressed in terms of metabolite ratios rather than as absolute concentrations. Statistical analysis was performed using the Student's *t* test. Differences were considered significant when $p < 0.05$.

Results

Cell purity and protein content of the cell extracts

The purity of the cell cultures was >95% with all the cell lineages, except for neurons whose purity was approximately 90%.

^{1}H NMR spectra had a satisfactory signal:noise ratio only when cell extracts contained more than 0.5–1 mg of protein, which corresponded to 5×10^6 to 1×10^7 cells. In this study, which comprised five or six independent preparations for each cell type, extracts for NMR and HPLC analysis contained 2.4 ± 0.8 mg protein for neuronal cells, 11.9 ± 3.9 mg protein for cortical astrocytes, 0.8 ± 0.1 mg protein for O-2A progenitors, 0.7 ± 0.3 mg protein for oligodendrocytes, and 12.0 ± 3.5 mg protein for meningeal cells.

Comparison of NMR with HPLC

There was good agreement between ^{1}H NMR and HPLC data for the amino acids Ala, Tau, and Gly for all cell types studied (data not shown). Precise quantification of NAA by NMR spectroscopy was made difficult by the glutamate and glutamine multiplets at around 2.06 ppm (Figs. 1, 2). However, when concentrations of NAA higher than 9 nmol/mg of protein were measured with HPLC, ^{1}H NMR of the corresponding extract resolved a well-defined peak at 2.02 ppm (pH 8.9), together with smaller resonance intensities from the β-protons [H_{β}' 2.48, 2.52 ppm (doublet of doublets, dd), H_{β} 2.66, 2.72 ppm (dd)], confirming the assignment of the 2.02 ppm singlet to NAA.

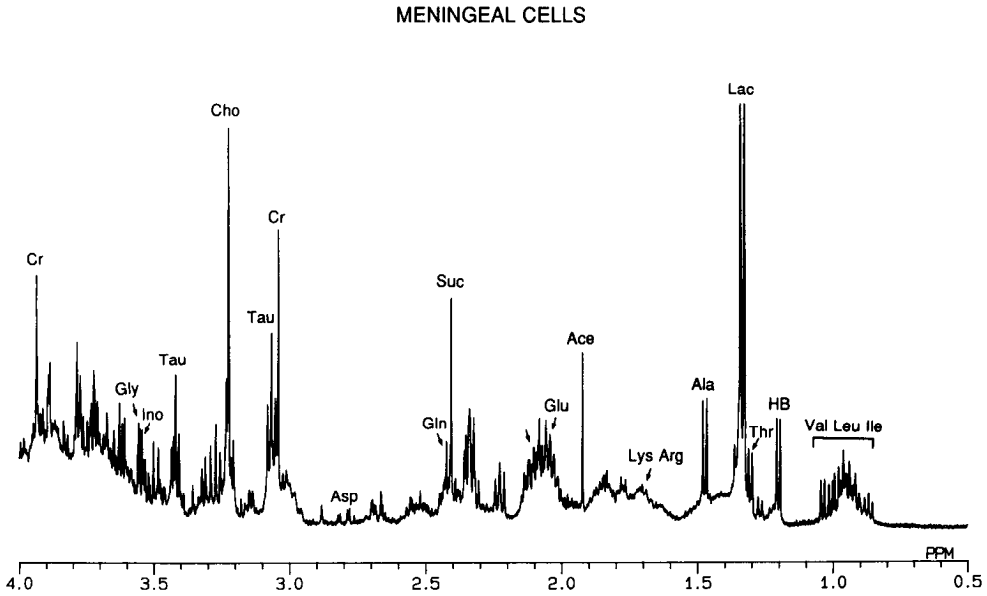

Figure 3. Representative ^{1}H NMR spectra obtained from extract of cultured meningeal cells. PCA extracts of meningeal cells contained 18.8 mg of protein. Immunostaining showed that the cell purity was >95%. Parameters for NMR spectroscopy were as described in Figure 1. Identified resonances include those from valine (*Val*), leucine (*Leu*), and isoleucine (*Ile*), β-hydroxybutyrate (*HB*), threonine (*Thr*), lactate (*Lac*), alanine (*Ala*), lysine (*Lys*) and arginine (*Arg*), acetate (*Ace*), glutamate (*Glu*), succinate (*Suc*), glutamine (*Gln*) aspartate (*Asp*), creatine (*Cr*), taurine (*Tau*), choline-containing compounds (*Cho*), and inositol (*Ino*). A notable feature of this spectrum is a relatively large Suc signal. A quantitative comparison of all the cell types studied is shown in Tables 1 and 2.

Creatine and choline-containing compounds—prominent constituents in all the ^{1}H NMR spectra

Typical spectra obtained from extracts of brain cell types are shown in Figures 1–3. The spectra include signals from a wide range of metabolites, with many qualitative and quantitative differences between different cell types. A large number of resonances with considerable overlap are present in the methylene region (2–3 ppm). The methine region (3.2–4.0 ppm) is also complex, especially for the cerebellar granule neurons and meningeal cells.

Two signals were prominent with all the cell types studied. They corresponded to Cr (peaks at 3.04 ppm and 3.94 ppm), with a contribution from phosphocreatine emerging as a small shoulder (Fig. 2, bottom spectrum), and Cho partially resolved at 3.22–3.25 ppm. The singlets relating to the Cho were tentatively assigned to choline (3.21 ppm), phosphorylcholine (3.23 ppm), and glycerophosphorylcholine (3.24 ppm), although other metabolites including carnitine could also have contributed to these signals.

The averaged Cr and Cho contents expressed as nmol/mg protein are given in Table 1. For both Cr and Cho, concentration/mg protein declined in the following descending order: oligodendrocytes, cortical astrocytes, O-2A progenitor cells, cerebellar granule neurons, and meningeal cells. Oligodendrocytes had a particularly high Cr content in comparison to the other cells (Table 1). As a consequence of this feature, oligodendrocytes had the lowest Cho:Cr ratio (0.51 ± 0.05). This ratio was significantly different ($p < 0.05$) from that of O-2A progenitors, which had the highest Cho:Cr ratio (1.01 ± 0.16), and from cortical astrocytes (0.85 ± 0.07). However, the oligodendrocyte Cho:Cr ratio was not significantly different from that of neurons (0.61 ± 0.04) or meningeal cells (0.77 ± 0.19).

Common qualitative features present in all cell lines

In addition to Cho and Cr, ^{1}H NMR spectroscopy consistently detected Lac (doublet at 1.32 ppm), acetate (Ace; singlet at 1.92 ppm), and Suc (singlet at 2.41 ppm) in all the cultures examined (Figs. 1–3). The Lac content was highly dependent on the speed with which the cells were harvested and extracted. The ketone body HB (doublet at 1.2 ppm) was also conspicuous in neurons, cortical astrocytes, and meningeal cells (Figs. 1, 3, Table 1) but was barely detectable in O-2A progenitors or oligodendrocytes (Fig. 2, Table 1).

Two amino acids were prominent in all spectra (Figs. 1–3): Ala (doublet at 1.47 ppm) and Glu (multiplets at 2.1 and 2.34 ppm). In addition, all non–O-2A cells examined contained Tau (triplet at 3.08 and 3.42 ppm), while O-2A progenitors and oligodendrocytes contained H-Tau (triplet at 2.65 and 3.30 ppm). Several overlapping resonances near 1 ppm were also present in all spectra; these are assigned to the amino acids valine (Val), leucine (Leu), and isoleucine (Ile) (Figs. 1–3).

Table 1. Concentration of HB, NAA, Cr, and Cho in different neural cell types

	Neurons	Astro.	O-2A Pro.	Oligo.	Mening.
HB	24.8 ± 5.3	19.9 ± 4.5	+	+	24.7 ± 5.1
NAA	12.4 ± 1.2	ND	23.4 ± 2.8	ND	ND
Cr	33.5 ± 3.2	64.9 ± 10.7	51.2 ± 10.3	122.2 ± 8.6	18.5 ± 4.0
Cho	20.9 ± 3.1	53.0 ± 6.4	45.7 ± 2.5	62.4 ± 6.7	11.0 ± 0.8

Concentrations, expressed as nmol/mg protein (mean ± SEM), were determined by ^{1}H NMR from five or six independent experiments. Neurons: cerebellar granule neurons; Astro., cortical astrocytes; O-2A Pro., O-2A progenitor; Oligo., oligodendrocytes; Mening., meningeal cells. ND indicates that the corresponding metabolite was not detectable with either NMR or HPLC. + indicates that the corresponding metabolite was detectable, but not quantifiable, with NMR.

Table 2. Comparative amino acid analysis of different neural cell types

	Neurons	Astro.	O-2A Pro.	Oligo.	Mening.
Ala	14.7 ± 2.9	8.1 ± 1.4	59.1 ± 9.1	34.5 ± 5.2	17.9 ± 4.1
Arg	12.2 ± 3.2	5.1 ± 0.5	18.3 ± 1.5	26.0 ± 9.8	5.8 ± 1.3
GABA	9.8 ± 2.0	0.9 ± 0.3	5.2 ± 2.3	0.6 ± 0.3	2.3 ± 1.0
Glu	87.6 ± 20.3	20.3 ± 4.2	177.7 ± 53.9	74.4 ± 20.6	62.5 ± 14.3
Gln	33.0 ± 8.3	54.6 ± 13.5	128.2 ± 13.1	205.0 ± 42.5	30.3 ± 6.9
Asp	25.9 ± 2.8	3.5 ± 0.9	84.4 ± 24.6	36.0 ± 19.9	14.8 ± 4.2
Tau	24.9 ± 5.9	70.9 ± 5.6	39.8 ± 13.6	45.5 ± 10.5	37.8 ± 5.7
Gly	15.2 ± 3.0	8.8 ± 1.3	76.8 ± 2.9	85.7 ± 16.8	17.9 ± 6.0

Concentrations, expressed as nmol/mg protein (mean ± SEM), were determined by ^{1}H NMR and HPLC from five or six independent experiments. Ala, alanine; Arg, arginine; GABA; Glu, glutamate; Gln, glutamine; Asp, aspartate; Tau, taurine; Gly, glycine. Neurons, cerebellar granule neurons; Astro., cortical astrocytes; O-2A Pro., O-2A progenitor; Oligo., oligodendrocytes; Mening., meningeal cells.

Differential amino acid composition

The concentrations of selected amino acids, determined by ^{1}H NMR (or HPLC when the amino acid was not detectable or quantifiable with NMR), are presented in Table 2. In addition to Ala, Glu, and Tau/H-Tau (see above), several other amino acids were detectable with ^{1}H NMR, but not in all the cells studied. The resonance from GABA (quintet at 1.9 ppm and triplet 3.0 at ppm) was only detectable in neuronal cells (Fig. 1). The signal for aspartate (Asp; doublet of doublet at 2.79 ppm) was especially high in O-2A progenitors, detectable in cerebellar granule neurons, oligodendrocytes, and meningeal cells, but undetectable in cortical astrocytes (Figs. 1–3). The multiplets at 2.13 and 2.41 ppm, assigned to Gln, were prominent in all the cell types studied except for cerebellar granule neurons. The glycine singlet (Gly, 3.51 ppm) was a consistent feature of the O-2A lineage spectra (Fig. 2). It was identifiable in the meningeal cells (Fig. 3) but remained undetectable in cortical astrocytes (Fig. 1). The detection of Gly in neuronal extracts was impaired by the complexity of the methine region in these spectra (3.2–4 ppm).

Metabolite markers of different neural cell types

A metabolite or metabolite ratio was considered as a marker for some cell types when its concentration or value within these cells was at least close to one order of magnitude higher than in the other cells (see Tables 3, 4). The best example was that of NAA, present in high concentration in neurons and O-2A progenitors (12.3 ± 2.6 nmol/mg protein and 23.4 ± 2.7 nmol/mg protein, respectively) (Tables 1, 3) but absent in the other cells. Note that, although the highest NAA concentration was found in O-2A progenitors, this compound was undetectable once these cells became mature oligodendrocytes. The presence or absence of NAA clearly discriminated the spectra of differentiated glial cells from those of neurons or O-2A progenitors (Tables 1, 3). Another "specific" metabolite was GABA, present in much higher concentration in cerebellar granule neurons (9.8 ± 1.8 nmol/mg protein) than in glial cells (cortical astrocytes, 0.9 ± 0.3 nmol/mg protein; oligodendrocytes, 0.6 ± 0.2 nmol/mg protein) (Table 2). The resonances of HB, which were clearly identifiable in neurons but barely detectable in O-2A progenitors (Figs. 1, 2; Table 1), also provide useful markers. Finally, the

Table 3. Discrimination of various neural cell types using metabolite concentrations and metabolite ratios

Cell type	Neurons	Astro.	O-2A Pro.	Oligo.	Mening.
Neurons	—	NAA Asp/Cr	HB	NAA, HB	NAA
Astro	Cho/Asp Cho/Glu	—	HB, Tau Cho/Asp	HB, Tau	Cho/Asp Cho/Glu
O-2A Pro.	H-Tau	NAA H-Tau Gly/Cr Glu/Cr	—	NAA	NAA H-Tau
Oligo.	H-Tau Gln	H-Tau Gly	—	—	H-Tau Cr
Mening.	Suc	Suc Asp/Cr	HB	HB	—

This matrix outlines which metabolite(s) and/or metabolite ratio(s) may be used to distinguish a given cell type. At each intersection point of a row and a column, the metabolite or ratio can be used to distinguish the cell type in that row from the cell type in that column. The metabolite or metabolite ratio is always at least one order of magnitude higher for the cell type given by the row. Neurons, cerebellar granule neurons; Astro., cortical astrocytes; O-2A Pro., O-2A progenitors, Oligo., oligodendrocytes; Mening., meningeal cells. All the metabolite concentrations are presented in Table 1 and 2 except for Suc (see spectra, Figs. 1–3). The metabolite ratios are assembled in Table 4.

Table 4. Cell type "specific" metabolite ratios

Ratio	Neurons	Astro.	O-2A Pro.	Oligo.	Mening.
Cho:Asp	*0.87 ± 0.19*	*16.1 ± 3.4*	*0.72 ± 0.17*	*3.7 ± 1.4*	*1.1 ± 0.37*
Cho:Glu	*0.27 ± 0.04*	*2.6 ± 0.32*	0.35 ± 0.09	0.99 ± 0.15	*0.2 ± 0.04*
Cho:Gly	1.6 ± 0.3	*5.7 ± 0.33*	*0.6 ± 0.06*	0.81 ± 0.09	1.01 ± 0.38
Asp:Cr	0.79 ± 0.1	*0.06 ± 0.01*	*2.1 ± 0.95*	*0.28 ± 0.14*	0.81 ± 0.13
Glu:Cr	2.8 ± 0.76	*0.36 ± 0.08*	*4.4 ± 2.1*	0.63 ± 0.19	3.9 ± 0.67
Gly:Cr	0.48 ± 0.12	*0.15 ± 0.02*	*1.8 ± 0.38*	0.72 ± 0.13	0.91 ± 0.14

A metabolite ratio was considered "specific" (in italic) when its value for some cell types was at least close to one order of magnitude different than with some other cell types, but not all the other cell types. Neurons, cerebellar granule neurons; Astro., cortical astrocytes; O-2A Pro., O-2A progenitors, Oligo., oligodendrocytes; Mening., meningeal cells. Cho, Choline-containing compounds; Asp, aspartate; Glu, glutamate; Gly, glycine; Cr, creatine. All the absolute metabolite concentrations are presented in Tables 1 and 2.

concentration of glycine was much higher in oligodendrocytes (85.7 ± 16.8 nmol/mg protein) and O-2A cells (76.8 ± 2.9 nmol/mg protein) (Fig. 2, Table 2) than in cortical astrocytes (8.8 ± 1.3 nmol/mg protein) (Tables 2, 3), and could be used to discriminate O-2A lineage cells from cortical astrocytes.

One of the most striking differences between the metabolite ratios observed in different cell types was the high ratio of Cho to amino acids observed for cortical astrocytes (Table 4). The Cho:Asp ratio was 15–22-fold higher in astrocytes than in the other cells, except for oligodendrocytes, for which only a four-fold difference was seen. Similarly, the Cho:Glu ratio was much higher in astrocytes than in neuronal and meningeal cells, and Cho:Gly ratio appeared potentially useful in differentiation of astrocytes and O-2A progenitors. A similar picture could be drawn for amino acids (Asp, Glu, Gly) to Cr ratios (Tables 3, 4). One example is that of Glu:Cr, which was approximately 15 times higher in O-2A progenitors than in astrocytes, even though both Glu and Cr were present in all ^{1}H NMR spectra.

Discussion

We have used ^{1}H NMR spectroscopy and HPLC analysis to compare the patterns of metabolite expression in purified neurons, cortical astrocytes, meningeal cells, O-2A progenitors, and oligodendrocytes. We have found that each of these cell types expresses a unique metabolite spectrum that can be reproducibly observed in different experiments. To our knowledge, this work provides the first demonstration that ^{1}H NMR analysis can provide a method for unambiguously distinguishing between all of the major cell types of a complex tissue.

One of the important considerations in undertaking studies such as the present ones concerns the reproducibility of observations in different experiments. It is well known that harvesting time, composition of the culture medium, and method of extraction can influence the metabolite composition of cultured cells (Drummond and Phillips, 1977; Patel and Hunt, 1985). However, the methods we utilized (see Materials and Methods) were sufficiently exact so as to yield essentially identical results in independent assays of populations grown at different times. Of particular importance in this respect was the observation that purified cortical astrocytes exhibited a similar metabolite spectrum whether grown in the presence of 10% FCS or in chemically defined medium containing growth factors (Urenjak, Williams, Gadian, and Noble, unpublished observations). It was critical, however, that cultures were fed with fresh medium at similar times before harvesting, as prolonged growth in the absence of medium changes was associated with reductions in the levels of several metabolites.

What is the relationship between our *in vitro* observations and the metabolite spectra expressed by individual cell types *in vivo*? In most respects, it is not yet possible to answer this question, as analysis of intact tissue can only provide information on the average levels of compounds expressed in a heterogeneous population of cells and does not yet provide any information relevant to understanding the cell type specific distribution of metabolites. At present, however, we note that the overall amino acid composition we observed in purified cortical astrocytes and neurons was close to that observed in the brains of 2–3-week-old rats (Banay-Schwartz et al., 1979; Burri et al., 1990) and that, except for oligodendrocytes, the total Cr concentration seen in all cell types studied was within the same range as that found in the rat brain *in vivo* (Lolley et al., 1961; Bates et al., 1989a; for information on neuron:glia ratio, see Brizzee et al., 1964).

One of the wholly unexpected observations we have made concerns the presence of large amounts of NAA in O-2A progenitors (see also Urenjak et al., 1992). It has previously been thought that this amino acid derivative, which represents one of the major compounds detectable by ^{1}H NMR *in vivo*, was only found in neurons. This assumption has had a number of practical consequences, not least of which is the paradox offered by the disorder of Canavan's disease. This autosomal recessive leukodystrophy is characterized by spongy degeneration of the white matter beginning in the first years of life, elevated levels of NAA in the brain and cerebrospinal fluid, and a deficiency in the enzyme asparto-acylase (which cleaves NAA to aspartate and Ace) (Matalon et al., 1989; Austin et al., 1991). It has been difficult to reconcile the putatively neuron-specific location of NAA with the clinical evidence of myelin degeneration and the preservation of relatively normal gray matter during the early stages of disease expression. However, the finding that NAA is expressed at high levels by a cell in the lineage responsible for producing the myelinating oligodendrocytes raises the possibility that defects in the metabolism of this compound would have metabolic consequences for these cells. Consistent with such a hypothesis are observations suggesting that NAA may contribute to lipid synthesis by providing an important source of acetyl groups (D'Adamo and Yatsu, 1966; Burri et al., 1991). Further experimentation is required to determine whether NAA is not detectable in oligodendrocytes because it is not synthesized in these cells, or because it is very rapidly cleaved such that its steady state concentration is very low.

Another example of the potential relevance of our analyses to clinical problems concerns the similarity between the metabolite spectrum expressed by meningeal cells in our studies and meningiomas *in vivo*. The finding that meningeal cells have an Ala:Cr ration three to four times higher than astrocytes, neurons, or oligodendrocytes is in striking agreement with observations that meningiomas also express a higher Ala:Cr ratio than do gliomas (Gill et al., 1990). This difference between meningiomas and other CNS tumors has led to the suggestions that this may be a useful criterion for noninvasive identification of meningiomas. The finding that there are several metabolite ratios that might prove useful in recognizing individual cell types (Tables 3, 4) raises questions about whether it will be possible to extend our present studies to development of noninvasive means of discriminating between different types of glial tumors. Such a possibility is now under investigation.

The concentrations of some metabolites detected in the cells are quite different to their concentration *in vivo*. For example, ^{1}H NMR analysis revealed unexpectedly high levels of the ketone body HB (β-hydroxybutyrate) in neurons, cortical astrocytes, and meningeal cells. The adult brain in physiological situations derives all its energy from glucose, but during the postnatal period, HB can provide an important alternative to glucose as an energy and carbon source (Williamson, 1982; Vicario et al., 1991). Ketone body utilization is pronounced during periods of prolonged hypoglycemia and ketosis, such as during early development (resulting from high-fat content of maternal milk) and following starvation of adult animals. Physiologically, HB is synthesized only in the liver and would not normally be synthesized by brain cells. Since there was no HB or acetoacetate in the medium, the presence of HB in the extracts indicated that it must have been synthesized by the cells. Further experiments are required to determine whether these results may be of relevance to development *in vivo*.

Another surprising aspect of the metabolite spectrum observed from O-2A progenitors and oligodendrocytes was their unique expression of high amounts of H-Tau, which was not detectable in any other cell type examined. H-Tau levels as high as 35 μmol/mg protein would not be anticipated since its content in brain tissue is very low (0.03 μmol/gm wet weight) (Perry et al., 1981). H-Tau is the immediate precursor of Tau and is thought to share the same physiological functions, for example, neuromodulation and brain tissue osmoregulation (van Gelder, 1983; Holopainen et al., 1985). The reason for this preferential synthesis of H-Tau by O-2A lineage cells is not known.

In conclusion, ^{1}H NMR can be used to analyze simultaneously most of the extractable, hydrogen-containing compounds in the cell at concentrations in excess of about 100 μM. Although less sensitive than other forms of analysis such as HPLC and gas chromatography–mass spectometry, no preselection of compounds or derivatization is required. The technique is therefore unrivaled for producing a metabolite profile, and would lend itself to investigations of effects of a wide range of biochemical interventions, as metabolite concentrations. These could include such metabolic challenges as hypoxia, hypoglycemia, and hyperammonemia as well as exposure to drugs, toxic agents, and biological response modifiers.

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