Energy metabolism in the human brain is not fully understood. Classically, glucose is regarded as the major energy substrate. However, lactate (conventionally a product of anaerobic metabolism) has been proposed to act as an energy source, yet whether this occurs in man is not known. Here we show that the human brain can indeed utilize lactate as an energy source via the tricarboxylic acid cycle. We used a novel combination of $^{13}$C-labelled cerebral microdialysis both to deliver $^{13}$C substrates into the brain and recover $^{13}$C metabolites from the brain, and high-resolution $^{13}$C nuclear magnetic resonance. Microdialysis catheters were placed in the vicinity of focal lesions and in relatively less injured regions of brain, in patients with traumatic brain injury. Infusion with 2-$^{13}$C-acetate or 3-$^{13}$C-lactate produced $^{13}$C signals for glutamate C4, C3 and C2, indicating tricarboxylic acid cycle operation followed by conversion of glutamate to glutamine. This is the first direct demonstration of brain utilization of lactate as an energy source in humans.
Introduction

Controversy exists over how metabolism is coupled between neurons and glia, and how brain metabolism responds to injury or disease. Moreover, much of our current knowledge of brain chemistry is based on animal models or cell cultures and more in vivo evidence in humans is required.

Originally, glucose taken up from the bloodstream was regarded as a universal fuel for brain cells, creating lactate as a waste product. However, more recently, the idea of metabolic trafficking between cell types has evolved (Fig. 1). Notably, Magistretti and Pellerin have described a model in which glucose from the vasculature is metabolized by glial cells to lactate, which is then transported to neurons for use in the tricarboxylic acid (TCA) cycle (Pellerin and Magistretti, 1994; Pellerin et al., 1998; Magistretti et al., 1999; Pellerin et al., 2007). Glutamate, produced by neurons, is taken up by glial cells and converted to glutamine and cycled back to neurons, termed the glutamate–glutamate cycle. Recent results using 13C-labelled acetate, lactate and glucose in rats without brain injury, have suggested that all three substrates can be taken up from the vasculature and used as energy sources—lactate primarily by neurons and acetate by glial cells, while glucose can be utilized by both cell types (Tyson et al., 2003).

The specific high-affinity glial Na+-dependent glutamate transporters GLAST and GLT-1 mediate uptake of glutamate from the synaptic cleft into astrocytes (Danbolt, 2001). Sodium ions (Na+) are co-transported with glutamate. Though the precise stoichiometry is debated, it appears that one molecule of glutamate is imported into the cell along with two or three Na+ and one H+, in exchange for export of one K+ and one OH− (or one HCO3−) (Pellerin and Magistretti, 1994; Danbolt, 2001). This influx of Na+ increases the activity of Na+, K+ ATPase (sodium pump), which in turn stimulates glycolysis, i.e. glucose utilization and lactate production. The Na+, K+ ATPase family consists of many isoenzymes. In rat cerebral cortex, the Na+, K+ ATPase α2 subunit is almost entirely co-localized with GLAST and GLT-1 (Cholet et al., 2002). Glutamate uptake by astrocytes is essential to terminate its effect as a neurotransmitter and to prevent extracellular glutamate from reaching excitotoxic levels (Pellerin and Magistretti, 1994).

Microdialysis is already established in neurocritical care for monitoring patients’ brain chemistry by measurement of endogenous (unlabelled) energy-related molecules (Bhatia and Gupta, 2007; Belli et al., 2008). The microdialysis catheter, whose outer wall at the distal end consists of dialysis membrane, is inserted into the cerebral parenchyma (Supplementary Fig. 1). Molecular exchange by diffusion takes place bi-directionally across the membrane, between the perfusion fluid inside the catheter and the cerebral extracellular space. In conventional microdialysis, a ‘physiological’ artificial solution of salts, termed CNS perfusion fluid, is employed to collect endogenous molecules that diffuse into the catheter from the extracellular space (Tisdall and Smith, 2006). Contrastingly, in the present study, the CNS perfusion fluid was supplemented with a 13C-labelled substrate (lactate, acetate or glucose) that diffused through the microdialysis membrane into the brain’s extracellular space, where it was available for metabolism by cells. Labelled metabolites from the extracellular space then diffused across the microdialysis membrane into the catheter, and the fluid emerging (termed microdialysate) from the catheter was collected in a vial, allowing us to sample brain chemistry and evaluate cerebral metabolism. Nuclear magnetic resonance, is based on animal models or cell cultures and more in vivo evidence in humans is required.

Abbreviations: Ac = acetate; ATP = adenosine triphosphate; C = carbon; CAD = cranial access device; CNS = central nervous system; CoA = coenzyme A; CTO = craniotomy; D2O = deuterium oxide; Glc = glucose; Gln = glutamine; Glt = glutamate; Lac = lactate; NMR = nuclear magnetic resonance; p.p.m. = parts per million; PPP = pentose phosphate pathway; Pyr = pyruvate; ROI = region of interest; TBI = traumatic brain injury; TCA = tricarboxylic acid.
resonance (NMR) spectroscopy of the microdialysates enabled identification of metabolites and determination of the positions of the $^{13}$C-label within the metabolite molecules, thereby shedding light on biochemical pathways.

One aspect of brain chemistry requiring better understanding is that occurring after traumatic brain injury (TBI), a devastating condition affecting thousands of individuals of all ages each year, and the largest single cause of mortality in the age group under 40 years in developed countries. Survivors of severe TBI are left with varying degrees of disability. The primary physical injury caused by impact is followed over the ensuing hours and days by secondary injury mediated by a complex series of biochemical and electrophysiological responses such as spreading depolarizations. The latter are in turn associated with marked changes in glucose and lactate levels in the extracellular fluid of the human brain (Parkin et al., 2005).

By devising the present novel combination of cerebral microdialysis with $^{13}$C metabolic labelling and NMR spectroscopy of the microdialysates, we developed a methodology by which local brain metabolism could be continuously investigated in patients.

Materials and Methods

Patients

The study was approved by the Cambridge Local Research Ethics Committee and assent obtained from each patient’s next of kin. Patients over 16 years with TBI requiring ventilation and intracranial pressure monitoring were eligible for the study. The major exclusion criteria were deranged clotting and/or low platelets, which precluded placement of a microdialysis catheter. All patients were treated in the Neurocritical Care Unit at Addenbrooke’s Hospital, Cambridge, during the period 2007–08. The primary aim of the study was to compare different $^{13}$C-substrates, for which we recruited 14 TBI patients, who were divided into three groups to receive 2-$^{13}$C-acetate (five patients), 3-$^{13}$C-lactate (five patients), or 1-$^{13}$C-glucose (four patients), by infusion via microdialysis catheters. The patients (12 males and 2 females) were aged 17–56 years (average 31.9 years). One further TBI patient (male, 20 years) was recruited and received non-labelled perfusion fluid as per our standard clinical practice, to assess the natural background levels.

NMR spectroscopy

The samples were pooled from the microdialysate collection vials into 3, 4 or 5 mm NMR tubes depending on the volume available and made up to the required depth of solution with deuterium oxide ($D_2O$). $^{13}$C spectra were obtained using a Bruker Avance 500 MHz Spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) equipped with a dual $^1H/^13C$ cryoprobe (CP DUL500/C/H, Bruker BioSpin GmbH). The $^{13}$C spectra were acquired at 125.75 MHz (quarter of the base $^1H$ frequency) using 4096 (4k) scans with a $T_1$ (relaxation delay) of 3 s. Metabolite signals in the brain microdialysates were identified by comparison of their chemical shifts (p.p.m.) to values from NMR databases (BMRB—Biological Magnetic Resonance Bank, University of Wisconsin; NMR Metabolomics Database, University of Linköping; NMRShiftDB) and the literature (Jung et al., 1972; Quirt et al., 1974; London et al., 1978; Cistola et al., 1982), and to those of our own standards and substrates. Unlabelled L-glutamate and L-glutamine standards (from Sigma-Aldrich, Poole, Dorset, UK) were individually dissolved at 10 mM in CNS perfusion fluid with direct visualization of the injured brain and placement in the vicinity of injury (Supplementary Table 1). Cranial access device catheters were oriented perpendicularly into the brain, targetting white matter. Craniotomy catheters were oriented at a shallow angle (i.e. tangential) relative to the brain’s surface, to target grey matter. Catheters were perfused using CMA106 pumps at 0.3 μl/min, with CNS perfusion fluid (CMA Microdialysis AB) composed of NaCl (147 mM), KCl (2.7 mM), CaCl$_2$ (1.2 mM), MgCl$_2$ (0.85 mM) in water, supplemented with 4 mM sodium acetate (2-$^{13}$C), or 4 mM sodium L-lactate (3-$^{13}$C), or 2 mM γ-glucose (1-$^{13}$C). Concentrations of $^{13}$C-labelled substrates were chosen to represent typical endogenous levels seen in brain microdialysates in unlabelled studies, to minimize perturbation (Reinstrup et al., 2000; Hutchinson et al., 2005, 2006; Hutchinson et al., 2007, 2009). All the $^{13}$C-substrates (isotopic enrichment $\geq 98\%$, chemical purity $\geq 98\%$) were obtained from Cambridge Isotope Laboratories (Andover, MA) and were formulated in CNS perfusion fluid by the Manufacturing Unit, Department of Pharmacy, Ipswich Hospital NHS Trust (Ipswich, UK), who then tested them to verify purity, sterility and absence of pyrogenicity, to comply with current regulations, before releasing them for use in patients. Microdialysate collection vials were changed hourly. The samples were then stored at 4°C (or at ~20°C if storage over a weekend was necessary) prior to NMR analysis. Unless stated otherwise, 1 day’s microdialysates (the contents of ~24 vials collected from one catheter) were pooled for NMR spectroscopy. To determine the natural $^{13}$C background spectrum, brain microdialysate (24 h pool, from Patient 15) obtained by perfusion with plain CNS perfusion fluid without labelled substrates, was also analysed by NMR. Catheter placements are coded as follows: CAD A, cranial access device in the opposite hemisphere to the main focal lesion; CAD B, cranial access device in the same hemisphere as the main focal lesion but not in the immediate vicinity; CTO, craniotomy (i.e. in the vicinity of the main focal lesion). In some patients, two catheters were placed in one craniotomy and are coded CTO 1 and CTO 2, the latter being closer to the focal lesion, though not within the lesion itself.
The plain CNS perfusion fluid itself (without $^{13}$C-substrates, prior to infusion) gave no detectable $^{13}$C-signals.

## Results

Microdialysates from TBI patients’ brains that had been infused via the microdialysis catheter with perfusion fluid containing $^{13}$C-labelled substrates underwent NMR spectroscopy to analyse the $^{13}$C-containing metabolites. Supplementary Table 1 shows patient demography and sampling details. A total of 24 catheters were studied with labelled substrate in 14 TBI patients, plus one unlabelled TBI patient whose catheter was perfused without labelled substrate. NMR results are summarized in Tables 1 and 2 and illustrative examples of spectra are shown in Figs 2 and 3, clearly showing incorporation of label into metabolites. There were also signals corresponding to the $^{13}$C-labelled substrate in each case, at the expected chemical shift (p.p.m.). Spectra obtained prior to infusion into patients for the $^{13}$C-substrates in CNS perfusion fluid demonstrated that background signals from contaminants were negligible. Chemical shift values (p.p.m.) for identifying metabolites were obtained from standards run on our own spectrometer in CNS perfusion fluid (see ‘Materials and methods’ section), as well as from NMR databases (BMRB—Biological Magnetic Resonance Bank, University of Wisconsin; NMR Metabolomics Database, University of Linköping; NMRShiftDB) and from the literature (Jung et al., 1972; Quirt et al., 1978; London et al., 1978; Cistola et al., 1982).

Our analysis focussed on key metabolites in cerebral metabolism, and assignments of the relevant signals above background in the microdialysates are presented in Tables 1 and 2. Several signals not included in Tables 1 and 2 were common to the majority of the catheters, as follows. Signals at 182.4–182.9 p.p.m. for all catheters (except Patient 5 CAD A) were assigned as lactate C1 (standard 182.9 p.p.m.) or other carboxylic carbon. Signals at 160.3–161.4 p.p.m. for all catheters were assigned as either pyruvate C1 (database 161.4 p.p.m.), or, more likely, bicarbonate ($\text{HCO}_3^-$) (literature 161.2 p.p.m.).

**Acetate infusion produces glutamine**

Acetate is a well-described substrate for glial cells. Infusion of 2-$^{13}$C-acetate was performed in a total of 10 catheters, comprising five patients, each with two microdialysis catheters, inserted via a cranial access device or craniotomy. Catheter placements and the $^{13}$C-metabolite results are presented in Table 1. The predominant signal in these spectra was that of the substrate acetate labelled at C2 (23 p.p.m.). The presence of label in glutamine was detected at positions C4, C3 and C2. In one case (Patient 3, CAD A) a small peak was found at 33.46 p.p.m., ascribed to labelling of glutamate at C4. No glutamine or glutamate signals were detected in the 2-$^{13}$C-acetate substrate solution (Fig. 2B) or in the unlabelled brain microdialysate (Patient 15, Fig. 2C). Other $^{13}$C signals in the microdialysates resulting from 2-$^{13}$C-acetate infusion included lactate labelled at C3 and C2 for all of the catheters (Table 1), and glucose C1-6 (see footnote of Table 1), for the majority of the catheters. $^{13}$C-glucose and $^{13}$C-lactate were not contaminants of the 2-$^{13}$C-acetate substrate solution (Fig. 2B). However, the $^{13}$C spectrum of the unlabelled microdialysate (Patient 15) contained signals for glucose and lactate (see below and Fig. 2C), so in the microdialysates resulting from infusion with labelled substrates, glucose and lactate probably contain a

<table>
<thead>
<tr>
<th>Identity</th>
<th>Standard (p.p.m.)</th>
<th>Patient 1 (p.p.m.)</th>
<th>Patient 3 (p.p.m.)</th>
<th>Patient 4 (p.p.m.)</th>
<th>Patient 5 (p.p.m.)</th>
<th>Patient 6 (p.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lac C2</td>
<td>68.52</td>
<td>68.53</td>
<td>68.51</td>
<td>68.53</td>
<td>68.53</td>
<td>68.48</td>
</tr>
<tr>
<td>Gln C2</td>
<td>54.17</td>
<td>54.70</td>
<td></td>
<td>54.53</td>
<td>54.48</td>
<td></td>
</tr>
<tr>
<td>Glt C4</td>
<td>33.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>29.70</td>
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<td>31.12</td>
<td>31.00</td>
<td>30.99</td>
<td>31.07</td>
<td>31.12</td>
</tr>
<tr>
<td>Glt C4</td>
<td>30.25</td>
<td>30.25, 29.66</td>
<td>29.70</td>
<td>29.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln C3</td>
<td>26.21</td>
<td>27.73</td>
<td></td>
<td></td>
<td></td>
<td>27.00</td>
</tr>
<tr>
<td>Ac C2</td>
<td>23.20</td>
<td>23.27</td>
<td>23.25</td>
<td>23.25</td>
<td>23.26</td>
<td>23.26</td>
</tr>
<tr>
<td>Lac C3</td>
<td>20.08</td>
<td>20.10</td>
<td>20.07</td>
<td>20.07</td>
<td>20.10</td>
<td>20.09</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

CAD A and CAD B were contralateral and ipsilateral to the focal lesion, respectively. CTO 2 was closer to the focal lesion than CTO 1, in the same craniotomy. See ‘Materials and methods’ section for more placement details. Dash denotes no signal above background. Standard p.p.m. values were determined in CNS perfusion fluid on our own NMR spectrometer. Given the known dominance of Gln over Glt in brain extracellular concentrations, the 27 and 54 p.p.m. signals in the microdialysates were therefore assigned as Gln C3 and Gln C2, respectively (rather than Glt C3 and Glt C2), particularly as none of them was accompanied by a clear Glt C4 signal at 33–34 p.p.m. Patient 3 (CAD A) showed a clear Glt C4 signal at 33.46 p.p.m., but none at 26–27 p.p.m. or 54 p.p.m. for further explanation, see Discussion. $^{13}$C signals detected but not tabulated included glucose C1 (standard 182.9 p.p.m.), glucose C2 (multiple signals 69–76 p.p.m.), and glucose C6 (61 p.p.m.) for all the above catheters, except for Patient 1 (CTO 2) and Patient 3 (CAD A) whose glucose signatures were negligible.

a Standard chemical shifts for C2 of Gln and Glt (glutamic acid monosodium salt and glutamic acid) were all at around 54 p.p.m.

b Glt C4 signal was at 33.25 p.p.m. for standard glutamic acid monosodium salt.

c Glt C4 was at 30.25 p.p.m. for standard glutamic acid. Small signals at 29.65–30.25 p.p.m., in 7 of the 10 catheters, were not clearly identified but may be due to C4 for differing ionisation states of glutamate or glutamine.

d Standard chemical shifts for C3 of Gln and Glt (glutamic acid monosodium salt and glutamic acid) were all at around 26–27 p.p.m.

e Indicates $^{13}$C-substrate remaining in the microdialysates.
proportion of endogenous material. There were no striking differences in labelling results between the sites of catheter placement.

### Lactate infusion produces glutamine

To investigate lactate utilization by neurons and glial cells, a 3-13C-lactate infusion was performed in a total of nine catheters, comprising five patients, four of whom had two microdialysis catheters each, and one had a single catheter. Catheter placements (via CAD or CTO) and the 13C-metabolite results are presented in Table 2, and illustrative examples of spectra are shown in Fig. 3A and B. The spectra showed the substrate lactate C3 as the major 13C signal. As seen above with 2-13C-acetate infusion, the 13C metabolite labelling pattern arising from 3-13C-lactate showed glutamine C4, C3 and C2 signals, indicative of lactate utilization via the TCA cycle (see above).

**Table 2 13C-Labelled key metabolites in microdialysates and their chemical shifts (p.p.m.), resulting from perfusion with 3-13C-lactate**

<table>
<thead>
<tr>
<th>Identity</th>
<th>Standard (p.p.m.)</th>
<th>Patient 2 (p.p.m.)</th>
<th>Patient 7 (p.p.m.)</th>
<th>Patient 8 (p.p.m.)</th>
<th>Patient 9 (p.p.m.)</th>
<th>Patient 10 (p.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lac C2</td>
<td>68.52</td>
<td>68.52</td>
<td>68.52</td>
<td>68.53</td>
<td>68.49</td>
<td>68.51</td>
</tr>
<tr>
<td>Gln C2a</td>
<td>54.17</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>54.67</td>
<td>54.55</td>
</tr>
<tr>
<td>Glt C4b</td>
<td>33.25</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>34.15</td>
<td>33.46</td>
</tr>
<tr>
<td>Gln C4</td>
<td>30.80</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>31.04</td>
<td>30.95</td>
</tr>
<tr>
<td>Glt C4c</td>
<td>30.25</td>
<td>30.24</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>29.65</td>
</tr>
<tr>
<td>Ac C2</td>
<td>23.20</td>
<td>23.25</td>
<td>23.26</td>
<td>23.26</td>
<td>23.24</td>
<td>23.24</td>
</tr>
<tr>
<td>Lac C3</td>
<td>20.08</td>
<td>20.09e</td>
<td>20.09e</td>
<td>20.09e</td>
<td>20.11e</td>
<td>20.05e</td>
</tr>
</tbody>
</table>

See Table 1 footnote for explanation of superscript letters a–e, dash and standards. The signals at 33.46 p.p.m. and at 34.15 p.p.m. (Patient 8, CTO) were small but clearly present, and may be due to C4 for differing ionization states of glutamate. Small signals at 29.65 p.p.m. (Patient 10, CTO 2) and 30.24 p.p.m. (Patient 2, CAD A) were not clearly identified, but may be due to C4 for differing ionization states of glutamate or glutamine. Signals at 27.12 p.p.m. and 54.74 p.p.m. in Patient 8 (CTO) may be due to Glt C3 and Glt C2, as they were accompanied by a clear Glt C4 signal at 33-34 p.p.m. Judged by the same criteria as in Table 1, the remaining microdialysates’ 26 and 54 p.p.m. signals in Table 2 were assigned as Gln C3 and Gln C2, respectively (rather than Glt C3 and Glt C2) as they were not accompanied by a clear Glt C4 33-34 p.p.m. signal. For further explanation, see the ‘Discussion’ section. 13C signals detected but not tabulated included glucose (see footnote of Table 1), for all the above catheters. In Patient 10 (CTO 2), a 27.27 p.p.m. signal (smaller than its accompanying 26.42 p.p.m. signal assigned as Gln C3 in the table) may be due to C3 for a different ionization state of glutamine or glutamate. A small signal at 23.23–23.26 p.p.m. assigned as acetate C2 was present in all but one (Patient 8, CAD A) of the catheters. Unidentified signals at 36–51 p.p.m. were sporadic amongst the above catheters.

**Figure 2** Acetate is metabolized to glutamine in the human brain. (A) 13C NMR spectrum of microdialysate from Patient 1 (CTO catheter 1) infused with 2-13C-acetate (4 mM). Plotted in parallel on the same x and y axis scale are: (B) the substrate 2-13C-acetate (4 mM) in CNS perfusion fluid, prior to infusing, and (C) microdialysate from an unlabelled subject (Patient 15), whose microdialysis catheter was perfused with plain CNS-perfusion fluid without labelled substrate. For numerical p.p.m. values of signals see Table 1. The remainder (data not shown) of the spectrum, run as far as 250 p.p.m., showed signals only at 182.5 p.p.m. (lactate C1 or other carboxylate) and 160.9 p.p.m. (bicarbonate, or pyruvate C1).
Also, in one case (Patient 8, CTO) small signals were found at 33.46 and 34.15 p.p.m., ascribed to labelling of glutamate at C4. No glutamine or glutamate signals were present in the substrate solution (3-13C-lactate in CNS perfusion fluid) or in the unlabelled microdialysate (Patient 15). Other 13C signals detected in the microdialysates resulting from 3-13C-lactate infusion included glucose C1-6, and lactate C2 and C1. There appeared to be a preference for glutamine (or glutamate) C4 labelling in the craniotomy sites compared with the cranial access device sites (Table 2), albeit in this small group of patients.

**Glucose infusion produces lactate**

Infusion of 1-13C-glucose (2 mM) was performed in a total of five catheters (all via CAD), comprising four patients, three of whom had a single catheter each, and one patient who had two catheters. Large 13C signals in these microdialysates’ spectra corresponded to the substrate glucose C1. Also detected were 13C signals for lactate C2 and C3, and glucose C2–C6. In contrast, 13C signals for glutamine and glutamate were negligible.

**Unlabelled microdialysate shows endogenous lactate and glucose**

One subject (Patient 15) received standard CNS perfusion fluid, without labelled substrate. This patient, who had a diffuse injury, and no apparent focal lesion, had a single cranial access device catheter. The plain CNS perfusion fluid prior to infusion gave no detectable 13C-signals. The 13C spectrum of Patient 15’s microdialysate (24-h pool) showed signals for lactate C2 (68.53 p.p.m.) and C3 (20.09 p.p.m.) as the strongest peaks. Other 13C-signals detected were lactate C1 (182.46 p.p.m.), bicarbonate (160.45 p.p.m.), glucose C1 β and α (95.93 p.p.m. and a weak 92.12 p.p.m.), glucose C2–C5 (multiple signals in the range 69–76 p.p.m.), glucose C6 (60.79 p.p.m., 60.63 p.p.m.), and an unidentified small signal at 28.27 p.p.m.

The 13C spectrum of this unlabelled microdialysate is illustrated alongside the labelled spectra in Fig. 2 and 3.

Unlike Patients 1–14 (who were infused with labelled substrates), the microdialysates collected for NMR from the unlabelled Patient 15 were first analysed every hour on a bedside analyser (ISCUS; CMA Microdialysis AB, Solna, Sweden). Average concentrations (±SD) for these vials, which were subsequently pooled for NMR, were glucose 0.67 ± 0.26 mM, lactate 1.77 ± 0.72 mM, pyruvate 73.6 ± 34.4 μM, glutamate 6.7 ± 25.0 μM and glycerol 70.0 ± 189.6 μM.

**Discussion**

This in vivo 13C-labelled microdialysis technique, with laboratory analysis by NMR, for studying cerebral metabolism in humans has proved to be both safe and feasible. The patients received detailed continuous clinical monitoring throughout the 13C-labelled
microdialysate period and beyond, as part of their standard Neurosciences Critical Care Unit routine, and there were no adverse events, nor serious adverse events, related to the infusions. $^{13}$C is a stable isotope and not radioactive. The presence of $^{13}$C-labelled metabolites in the microdialysate emerging from the brain indicates that the $^{13}$C-labelled substrates entered the brain and were taken up by cells and metabolized. Our results from 2-$^{13}$C-acetate and 3-$^{13}$C-lactate infusions are consistent with those (from the same substrates) in animals and cell cultures, where glutamine produced via glutamate emerging from the first turn of the TCA cycle is labelled at C4, with C3 and C2 becoming labelled at subsequent turns (Tyson et al., 2003; Bouzier-Sore et al., 2006). The results of the present study reveal novel evidence in humans for uptake of lactate from the extracellular space by brain cells and its metabolism through the TCA cycle.

**Labelling of glutamine indicates operation of the TCA cycle**

Qualitative similarity is seen for the $^{13}$C-metabolites of 2-$^{13}$C-acetate and 3-$^{13}$C-lactate substrates in our study. These microdialysates' $^{13}$C signals for glutamine (C4, C3 and C2) suggest that the TCA cycle is operating in the regions of interest (ROIs) of the catheters, since glutamine is a product of glutamate that is a spin-off of the TCA cycle. 3-$^{13}$C-lactate substrate can be metabolized by lactate dehydrogenase to pyruvate labelled at C3, and then, by pyruvate dehydrogenase, to acetate labelled at C2, which can enter mitochondria. Incorporation of acetate labelled at C2 into citrate will label α-ketoglutarate (a TCA cycle intermediate), and hence the ensuing spin-off glutamate and glutamine, at C4 on the first turn of the cycle, and at C4 plus C2 or C3 on the second turn (Tyson et al., 2003; Bouzier-Sore et al., 2006). Another route (termed anaplerosis) into the TCA pathway, whereby pyruvate carboxylase converts pyruvate into oxaloacetate, a TCA cycle intermediate, will label glutamate and therefore glutamine at C2 on the first turn of the TCA cycle, and at C2 plus C1 on the second turn (Tyson et al., 2003; Bouzier-Sore et al., 2006). The negligible $^{13}$C signals for glutamine and glutamate in microdialysates resulting from 1-$^{13}$C-glucose infusion might be at least partly due to loss of $^{13}$C via the pentose phosphate pathway (PPP). The PPP is upregulated by cellular stress, e.g. TBI (Bartnik et al., 2007; Dusick et al., 2007). In the first step of the PPP, C1 of glucose is removed as carbon dioxide (Kaibara et al., 1999; Bartnik et al., 2007; Dusick et al., 2007).

We did not quantify the percentage enrichments of $^{13}$C label in each position. However, there were no signals for glutamine in the $^{13}$C spectra of either the substrate solutions or the unlabelled brain microdialysate, suggesting that the $^{13}$C-glutamine detected is a metabolite of the $^{13}$C-labelled substrates acetate and lactate rather than being endogenous material or a contaminant of the substrates. $^{13}$C signals for glutamine in the microdialysates resulting from infusion of 1-$^{13}$C-glucose were negligible compared with those resulting from 2-$^{13}$C-acetate or 3-$^{13}$C-lactate. We are unaware of any alternative pathway of glutamine synthesis, other than via the TCA cycle. The production of glutamine (labelled at C4, C3 and C2) from 3-$^{13}$C-lactate substrate thus suggests entry of label, via pyruvate and acetate as intermediates, into mitochondria. Moreover, the results of 2-$^{13}$C-acetate infusion demonstrated feasibility of achieving transport of this molecule from the catheter into the extracellular fluid, across the cell surface membrane, into the cytosol, and then across the mitochondrial membrane into the mitochondrial matrix where it entered the TCA cycle. The finding of $^{13}$C signals for lactate in the microdialysates resulting from infusion of 1-$^{13}$C-glucose is consistent with glycolysis as an energy pathway in injured brains.

Although labelled glutamine was a product of 2-$^{13}$C-acetate and 3-$^{13}$C-lactate substrates, glutamate production was less evident in the present microdialysis study. In contrast, rats that received intravenous infusions of the above $^{13}$C-substrates showed labelling in both glutamate and glutamine, in neocortex tissue extracts (Tyson et al., 2003). The key NMR signal for glutamate synthesis from the above substrates is the appearance of label at C4. Glutamate (and hence its product glutamine) emerging from the first turn of the TCA cycle is labelled at C4, with C3 and C2 becoming labelled at subsequent turns (Tyson et al., 2003; Bouzier-Sore et al., 2006). The NMR behaviour of glutamate C4 is influenced by its proximity to the C5 carboxylate (pKa 4.5). The C4 chemical shift can range from 30 to 35 p.p.m. depending upon glutamate's ionization state, and under 'physiological' conditions C4 is typically 33–34 p.p.m. In contrast, glutamine C4 is around 31 p.p.m. and is less variable as there is no carboxylate at C5. The C3 chemical shifts for glutamate and glutamine (~26–27 p.p.m.) show little difference from each other, as evidenced by standards, and analogously for C2 of glutamate and glutamine (~54 p.p.m.). Thus the only clearly differentially diagnostic signal for glutamate (as opposed to glutamine) is the glutamate C4 signal with a chemical shift of 33–34 p.p.m., if present. Given the known dominance of glutamine over glutamate in brain extracellular concentrations (see below), we have therefore assigned the C3 and C2 signals observed in the labelled microdialysates as glutamate rather than glutamine, except where accompanied, as was only the case for Patient 8 (CTO), by a clear glutamate C4 signal at 33–34 p.p.m., in which case the C3 and C2 may be glutamate, as indicated in the footnote of Table 2.

Glutamate and glutamine are enzymatically inter-convertible. Endogenous glutamine concentrations in human brain microdialysates are substantially higher than those of glutamate (Hutchinson et al., 2002; Samuelsson et al., 2007) (see below and Fig. 1). The majority of glutamate molecules are packaged within intracellular vesicles (Ni et al., 2007). Glutamate is toxic, so extracellular concentrations of glutamate need to be kept low, by means of uptake by astrocytes (Pellerin and Magistretti, 1994; Schousboe and Waagepetersen, 2005). Even so, we originally expected that at least some label would appear in glutamate if glutamine-glutamate cycling were intact, but although microdialysate was pooled from 24h of sampling in each case, labelling of glutamate was lacking, judged by the absence of a clear C4 signal (33–34 p.p.m.) for all but two of the catheters. This might be interpreted as deficiency of glutamine-glutamate cycling in the ROIs of the catheters, or as domination of the cell population...
by astrocytes/glia rather than neurons. The prevailing view of glutamine-glutamate cycling involves trafficking between astrocytes and neurons (Fig. 1). Glutaminase and glutamine synthetase enzymes occur within neurons (Aoki et al., 1991) and astrocytes (Norenberg and Martinez-Hernandez, 1979), respectively. However, glutaminase is not exclusively neuronal and also occurs in glia (Aoki et al., 1991). Astrocytes can inter-convert glutamine and glutamate (Schousboe et al., 1993). Moreover, in some circumstances, astrocytes/glia can release glutamate (Bezzi et al., 1998; Parpura and Haydon, 2000; Angulo et al., 2004; Ni et al., 2007). So, deficiency of $^{13}$C labelling in microdialysate glutamate C4 cannot solely be judged as paucity of neurons in the ROIs. The glutamine–glutamate cycle is not stoichiometric; glutamate can be consumed via the TCA cycle, as well as being metabolized by various other biochemical pathways, in both neurons and astrocytes/glia (McKenna, 2007). Taken together, multiple factors may thus be relevant to the apparent deficiency of clear glutamate C4 signal (33–34 p.p.m.) in the microdialysates.

**Influences on labelling results**

Microdialysis is a focal technique. Catheters were placed either via a cranial access device or directly via a craniotomy. As the cranial access device involves insertion of the microdialysis membrane perpendicular to the brain surface, microdialysate may be collected from white matter (rich in glia) proportionally more than from grey matter (rich in neurons), thus skewing results in favour of glial metabolism. The human cortex is ∼2.5 mm thick, varying from 1 to 4 mm (Fischl and Dale, 2000). To target grey matter, catheters were placed at craniotomy with the microdialysis membrane oriented parallel to the cortex in the first few millimetres. The results for 2-$^{13}$C-acetate substrate showed no obvious differences between the two modes of placement (CAD and CTO). However, for 3-$^{13}$C-lactate substrate there appeared to be a preference for glutamine (or glutamate) C4 labelling in the craniotomy sites compared with cranial access device, consistent with lactate being a neuronal substrate. Our findings, albeit in this small group of patients, thus suggest that the tissue type encountered by the microdialysis catheter may influence the propensity for lactate utilization via the TCA cycle.

Differences in glutamate signal between patients might be at least partly due to spreading depolarizations. The latter have been demonstrated in TBI patients by use of subdural strip electrodes (Strong et al., 2002). Spreading depolarizations can propagate from lesion sites to the more healthy surrounding tissue, so might be able to reach microdialysis catheters even if they are not in close proximity to a focal lesion. Glutamate release during spreading depolarizations, even in healthy tissue, has been shown in rats (Fabricius et al., 1993). Spreading depolarizations were also associated with marked changes in glucose and lactate levels in the extracellular fluid of the human brain (Parkin et al., 2005). Moreover, clusters of prolonged spreading depolarizations were associated with lesion progression, in patients with subarachnoid haemorrhage (Dreier et al., 2006). Monitoring with subdural strip electrodes was not attempted in the present study, but could be a useful adjunct to $^{13}$C-labelled microdialysis studies in the future.

The influence of catheter placement in injured versus ‘non-injured’ sites cannot be definitively answered by the present study. All of the craniotomy catheters, which were inherently perpendicular, were therefore targeted on white matter. In those patients with two cranial access device catheters (A and B, in different hemispheres), or two craniotomy catheters (1 and 2, within the same craniotomy), there was little difference within-patient for the NMR results. This suggests that there may be too small a pathophysiological differential at the cellular level between CTO 1 and 2 sites to impact significantly on the chemistry, or likewise between CAD A and B, especially with such a small number of patients. Future comparison of white matter chemistry in peri–lesional versus ‘non-injured’ sites would be achievable by comparing a perpendicularly inserted craniotomy catheter with a cranial access device catheter. Also, in future it might be possible to compare grey matter chemistry in peri–lesional versus ‘non-injured’ sites by placing the catheters tangentially via decompressive craniectomy, since the extent of bone removal required to relieve intracranial pressure is potentially large enough to encompass both injured and relatively ‘non-injured’ brain.

Sedation can lower brain energy metabolism (Archer et al., 1990). All our patients were under propofol sedation throughout microdialysis, except Patient 10, who was allowed to wake and was extubated before removal of the microdialysis catheters. Even so, there was no clear $^{13}$C-glutamate labelling (evidenced by absence of C4 signal at 33–34 p.p.m.) in this subject’s microdialysates, although $^{13}$C-glutamine C4 was detected.

Microdialysis is selective for the extracellular pool. Brain extracellular endogenous levels of glutamate are usually very low (∼1–20 μM), whereas extracellular glutamate levels are much higher (∼400–1000 μM) (Hutchinson et al., 2002; Samuelsson et al., 2007). Normally, the vast majority of glutamate molecules are intracellular (Ni et al., 2007). During ischaemia, extracellular glutamate can rise to ∼100–200 μM, as the glutamate—glutamine cycle is energy demanding, but even so, the extracellular glutamine/glutamate ratio is usually much greater than one (Hutchinson et al., 2002; Samuelsson et al., 2007). In future, in vivo magnetic resonance spectroscopy (MRS) could be teamed with $^{13}$Clabelled microdialysis, to measure intra- and extra-cellular molecules.

Infusion of the $^{13}$C-substrates might in theory perturb the endogenous brain chemistry, although concentrations of $^{13}$C-substrates were representative of levels found in previous unlabelled microdialysis studies (Reinstrup et al., 2000; Hutchinson et al., 2005; Tisdall and Smith, 2006; Hutchinson et al., 2007, 2009). Glucose, lactate, pyruvate, glutamate and/or glycerol in microdialysates are routinely measured hourly for severe TBI patients using a bedside analyser, also performed for this study’s patients after (and, for the unlabelled patient, during) the 24 h collection period for NMR. If endogenous levels were measured prior to the labelling period then the $^{13}$C-substrate concentration could be individually tailored to each patient, although logistically this would be more complicated.
Microdialysis is a continuous technique with inherently very low flow rates. To have sufficient volume for $^{13}$C-NMR necessitated pooling a whole day’s microdialysate collection and topping up with $\text{D}_2\text{O}$ in the NMR tube. The NMR cryoprobe we used possesses greater sensitivity than older technology. Very recently, NMR micro-cryoprobones have become commercially available, for smaller samples, enabling detailed time-course measurements of metabolic $^{13}$C-labelling in future.

Strengths of NMR are that it identifies the position of labelled atom within the metabolite molecule, very little sample manipulation is required, it is non-destructive, and it is prospective, not requiring prior selection of analytes to seek. Our $^{13}$C-labelled microdialysis technique can be used safely for severe TBI patients without interrupting their standard medical care. The use of appropriate substrates can potentially separate different metabolic pools, and other $^{13}$C-labelled substrates could be used if desired. To our knowledge, we are the first to perform $^{13}$C-labelled microdialysis in the human brain. A $^{1-13}$C-glucose microdialysis study has been carried out in human adipose tissue, with microdialysate analysis by gas chromatography–mass spectrometry (GC–MS) (Gustafsson et al., 2007). However, sample preparation for GC–MS was much lengthier than for NMR, and moreover GC–MS did not reveal the position of $^{13}$C within the metabolite molecules, unlike NMR.

In the present study, quantification was not attempted, other than noting whether $^{13}$C signals were strong or weak. A future refinement would be to acquire the $^{13}$C-spectra quantitatively with full spin relaxation and without nuclear Overhauser enhancement, though would take significantly longer. Percentage $^{13}$C enrichments at individual positions within the metabolite molecules could be measured by spin-echo difference NMR spectroscopy (Tyson et al., 2003). A limitation to measuring low incorporations of $^{13}$C label in metabolites is the natural abundance (non-enriched) background of $^{13}$C, which constitutes 1.1% of carbon atoms. Double labelling (on adjacent atoms) could be used to track specific bonds within a metabolic pathway. Chemical shift (p.p.m.) can be affected by variables such as solvent, pH, ionization, temperature, etc., and whether the molecule is free or bound (e.g. in an ester or peptide). Identification by $^{13}$C-chemical shift can be complicated by slight differences in p.p.m. values. 2D NMR techniques can provide more information to help resolve ambiguity.

Previous studies have shown that 1-$^{13}$C-glucose or 2-$^{13}$C-acetate intravenous infusions resulted in labelled glutamate and glutamine in the brain tissue of healthy volunteers, demonstrated by in vivo MRS (Bluml et al., 2002; Lebon et al., 2002). However, no labelled infusions of lactate appear to have been previously tested in humans with direct measurements in brain, nor direct infusion into the cerebral parenchyma. In healthy volunteers, intravenous infusions of unlabelled lactate produced an increase of lactate levels in human brain tissue, measured by MRS (Dager et al., 1992), and lowered the cerebral metabolic rate of glucose, measured by FDG–PET, suggesting preferential utilization of lactate (Smith et al., 2003). Moreover, the normal human brain can import endogenous extra-cranial exercise-generated lactate from the bloodstream, judged by arterial–jugular venous difference (AJVD) (Ide et al., 2000; Larsen et al., 2008). An AJVD study with intravenous 1-$^{13}$C-lactate infusion in healthy volunteers demonstrated slight net export of lactate from brain at rest prior to infusion, shifting to slight net import of lactate into brain on infusion at rest, and increasing net import with infusion plus exercise (van Hall et al., 2009). Lactate import into brain was accompanied by a decrease in net glucose uptake, and $^{13}$CO$_2$ was detected in blood, indicating lactate utilization. An AJVD study of TBI patients has demonstrated brain uptake of endogenous blood-borne lactate, with an association between more efficient lactate uptake and better outcome (Glenn et al., 2003, 2005).

To our knowledge, the results of the present study are the first in vivo demonstration in humans that brain cells can take up labelled lactate from the extracellular space and process it via the TCA cycle, evidenced by the finding of labelled glutamine as a metabolite in the microdialysates. Our results are compatible with the astrocyte-neuron lactate shuttle hypothesis, whereby astrocytes metabolize glucose by glycolysis to form lactate, which is then exported into the extracellular space and taken up by neurons to use as fuel (Pellerin and Magistretti, 1994; Pellerin et al., 1998; Magistretti et al., 1999; Pellerin et al., 2007).

The brain’s ability to utilize lactate as an energy source may be an evolutionary adaptation to minimize the risk of energy shortfall when demand for rapid energy generation entails abundant lactate production by glycolysis. The orientation of the catheters appeared to influence the results of 3-$^{13}$C-lactate infusion, with more labelling of glutamine (or glutamate) being apparent for the catheters targeted on grey matter rather than on white.

Microdialysis is primarily a research technique. It is increasing our understanding of the pathophysiology of brain injury. However, we are now using it (specifically the lactate/pyruvate ratio) to assist in the management of patients on an individual basis. The findings of the present study, i.e. that the human brain utilizes lactate via the TCA cycle, contribute to our knowledge and raise the interesting issue as to whether there is an ideal flux of lactate to optimize astrocyte-neuronal interaction and energy metabolism. High extracellular lactate levels are generally associated with poor outcomes following TBI (Goodman et al., 1999). Low extracellular lactate levels, with better outcomes, might be because astrocytic glycolysis-derived lactate is being efficiently taken up by neurons and utilized via the TCA cycle. Moreover, in an AJVD clinical study of TBI, high endogenous arterial lactate concentrations were associated with poorer outcome, while better outcome was associated with higher rate of lactate uptake by brain relative to arterial lactate concentration, i.e. higher lactate extraction fraction (Glenn et al., 2003, 2005). Beneficial effects of intravenous lactate infusions post-TBI have been reported in rats (Holloway et al., 2007) and in a small-scale patient study (Ichai et al., 2009). This might be partly due to lactate acting as an energy fuel for neurons, assuming that they have an adequate oxygen supply and functioning mitochondria. Astrocytes might constitute a potential primary site of simultaneous uptake of blood-borne lactate and production of lactate by glycolysis, followed in both instances by release of
lactate for neuronal utilization (van Hall et al., 2009). A better understanding of lactate’s function in human brain may assist in guiding patient care, and the combination of 13C-labelling with microdialysis may be able to help address this.

Conclusion

Here we have shown that 13C-labelled microdialysis for studying human brain chemistry is safe and feasible, and that metabolism in the injured human brain can be interrogated using 13C-labelled substrates. There is much scope for future refinement of the technique and extension to other substrates, including labelled analogues of naturally occurring molecules or pharmaceutical agents, to explore brain chemistry further. More research is needed on human cerebral metabolism before we can understand the interactions between brain cells after injury. This technique is a potentially useful tool in that undertaking. The results of the present study shed light on the biosynthetic pathways involved in energy metabolism in the human brain, and include the first direct demonstration that the human brain can utilize lactate as an energy source via the TCA cycle.

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Supplementary material

Supplementary material is available at Brain online.

References


