Abstract

Magnetic resonance spectroscopy and imaging provide unique information about the brain to the biochemist and the clinician. In particular, the ability to image metabolites other than water and to get detailed information about dynamic cellular processes (such as blood flow, blood oxygenation and cell swelling) is leading to many new insights into brain function and dysfunction. This review describes the use of old and new NMR techniques which demonstrate that mitochondrial dysfunction plays an important role in the cell death that occurs following an hypoxic-ischaemic insult to the neonatal brain.

Hypoxic-ischaemic damage to the neonatal brain

Decreased oxygen (hypoxia) or reduced blood flow (ischaemia) to the brain is a major cause of perinatal brain injury [1]. The fact that this damage occurs in otherwise healthy babies has led to great interest in developing cerebroprotective strategies. We have previously reviewed the wide range of non-invasive techniques that have been applied to model systems and the human neonate [2]. This review will focus more specifically on the role of NMR spectroscopy and imaging techniques and their application to biochemical questions about the mechanism of cell death in neonatal hypoxia-ischaemia. There are several good books outlining more information about the physical principles and practicalities of making these measurements (e.g. [3]).

Magnetic resonance spectroscopy (MRS)

MRS relies on differences in the magnetic properties of unpaired nuclear spins when in different environments to 'fingerprint' different metabolites [4-6]. Although concentration ratios are frequently cited in the literature, absolute quantification is also possible. Unfortunately the relative insensitivity of MRS means that metabolites at lower than millimolar concentrations are not easy to detect by MRS. However, for those metabolites at high concentrations advances in chemical-shift imaging are allowing detailed metabolite maps to be made.

Many important energy metabolites are readily measured by MRS. Phosphorus MRS allows phosphocreatine (PCr), inorganic phosphate (P_i) and nucleoside triphosphates to be measured. If creatine kinase is present to maintain the creatine/PCr reaction at equilibrium, it is also possible to estimate ADP concentrations [7]. The chemical shift of the P_i peak is pH dependent, allowing measurements of brain pH to be made in vivo [8,9]. Although the first MRS spectra of the neonatal brain were of phosphorus metabolites, proton MRS is now far and away the most common technique employed in animal and patient studies. This allows the detection of lactate, increases in the concentration of which generally indicate a decrease in mitochondrial ATP synthesis and a consequent increase in the glycolytic rate. Almost as importantly, changes in the concentration of the neuronal marker N-acetyl aspartate can pinpoint where and when neuron-specific cell damage is occurring. Of great interest to neuroscientists is the neurotransmitter glutamate, as increases in its concentration have been implicated in excitotoxicity. Unfortunately, distinguishing the glutamate resonances from those of glutamine is not trivial [10]. Furthermore it is unclear how relevant NMR measurements of total cellular glutamate would be to the small, but more important, fraction in the synaptic cleft.

The use of 13C spin labelling makes it possible to measure metabolite fluxes in vivo, either by direct 13C-NMR, or more readily by perturbations of the 13C spins on proton NMR spectra of the relevant metabolites [11]. So, for example, citric acid-cycle flux and glutamine/glutamate metabolism [12] can be estimated non-invasively in
animal models and humans. These techniques yield the same advantages (and potential pitfalls) of traditional $^{14}$C radioactive-labelling studies, but with the clear benefit that human studies do not require the injection of radioactive compounds. Kinetics of individual reactions can sometimes be determined directly without labelling studies using saturation-transfer NMR [7,13]. Measurements of ATP-turnover rates by this method are in many ways more useful than static concentration measurements (which will not change if a rise in ATP demand is compensated for by a rise in ATP synthesis).

Due to changes in the equilibrium populations of hydrogen-bound and monomeric water molecules, the chemical shift of the water resonance varies linearly with temperature in the range 0–40 °C. Therefore it is possible to measure absolute brain temperature by referencing the water resonance to a signal that has a markedly lower temperature dependence. In the brain the methyl resonance of N-acetyl aspartate has been used as a suitable reference enabling brain temperature to be measured in the neonate [14]. This may have important implications for measuring the local temperature variations that accompany brain damage and in monitoring cerebroprotective strategies, such as moderate hypothermia [15].

**Magnetic resonance imaging (MRI)**

MRI techniques represent probably the major non-invasive measurement of brain structure and function [3]. The most common imaging modalities, T1- and T2-weighted images, show few changes until the animal or patient have had considerable levels of brain damage and cell death. However, by judicious use of field gradients and pulse sequences it is possible to generate MRI images that are sensitive to cell function, rather than simply to gross structure.

Spin echoes are usually used to measure T2-weighted images. However, reversing an applied field gradient can readily generate a gradient echo. Unlike a spin echo, a gradient echo is sensitive to changes in the local magnetic-field inhomogeneity (in NMR parlance it is T2*-weighted, not T2-weighted). Although this might be considered a disadvantage, one of the major generators of magnetic-field inhomogeneities are the local fields associated with the paramagnetic deoxyhaemoglobin molecule. These can stretch well beyond the red blood cell and into the surrounding tissue. Therefore T2*-weighted images are sensitive to changes in the deoxyhaemoglobin concentration [16]. With appropriate calibrations, absolute regional measures of deoxyhaemoglobin concentration can be made [17]. This is the basis of blood-oxygenation-level-dependent MRI (BOLD MRI). Whereas one might think that changes in deoxyhaemoglobin concentrations would be the most useful in studies of hypoxic-ischaemic brain damage, this is not the case. The signal change is small and absolute quantitation is difficult. Therefore the best sensitivity is obtained using difference images obtained over a brief timescale. This makes BOLD MRI mainly suitable for short-term stimulus-response studies, rather than long-term measurements of brain damage. Of course, this is exactly what is required for functional activation studies of the brain (i.e. for determining where in the brain stimuli are processed) and this is the major use of this technique [18].

More dynamic measurements can be made directly by using arterial spin tagging to provide quantitative measurements of cerebral blood flow. This is done by inverting spins in the arterial blood supply in the neck and watching as these spins perturb the observed signal from brain water [19]. This has proved useful in defining areas of reduced blood flow during an hypoxic-ischaemic insult or in regions of the brain where blood flow is restricted subsequently (as happens in the adult brain following a stroke [20]). However, a feature of neonatal hypoxia-ischaemia is that there is, if anything, overperfusion subsequent to the insult. In this case the secondary energy failure and cell death is apparently not caused by reduced cerebral blood flow and reagents that reduce this excess blood flow do not necessarily reduce cell death [21].

Applying pulsed magnetic-field gradients during a standard spin-echo experiment allows an image to be measured that is sensitive to the diffusion of water (diffusion-weighted imaging or DWI). Diffusion of water between the gradient pulses will prevent the later gradients refocusing these spins optimally, and result in a decreased signal intensity. The size of this decrease is proportional to the diffusion rate of the water [22]. DWI has proved to be a very useful early marker of damage in animal models, enabling testing of cerebroprotective strategies [23,24]. DWI images are obtainable in the human neonatal brain [25,26] and are now regularly, if not routinely, acquired in adult stroke patients [27]. The contrast in the DWI image probably occurs as a result of cell swelling due to a failure of

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extracellular water has to take have been suggested water. This cell swelling results in an increase in the water concentration inside the cell and a squashing of the extracellular space available for water diffusion. Both the decrease in the concentration of the rapidly diffusing extracellular water and the more tortuous path the remaining extracellular water has to take have been suggested to cause the observed drop in the water diffusion rate. However, it is also possible that changes inside the cell lead to a direct decrease in the rate of diffusion of intracellular water.

**Use of NMR spectroscopy and imaging to demonstrate mitochondrial dysfunction during delayed energy failure**

Most babies suspected of having suffered critically impaired gas exchange to the brain have phosphorus MRS spectra identical to control babies of similar gestational age [30]. However, during the subsequent 1–3 days they undergo a significant decrease in their PCr/Pi ratio (and in severe cases a fall in ATP levels as well). The extent of this decrease correlates with the long-term neurological outcome for the patient [31]. Our explanation for these effects has been that a primary mitochondrial energy failure occurs in utero that leads directly to a delayed mitochondrial dysfunction 1–3 days later. The in utero event is due to the lack of oxygen, but the secondary event is caused by inhibition of mitochondrial energy metabolism (e.g. by nitric oxide or related compounds) as blood flow and oxygenation remain high following the insult [2].

Both these criticisms can be addressed using NMR spectroscopy. Animal models of neonatal hypoxia-ischaemia have proved useful, especially if used in tandem with patient studies [34]. We have focused on the < 1-day-old neonatal pig, as at birth the brain development is similar to that of the human infant. Also the size of the neonatal pig is similar to that of the human, allowing the same level of intensive care and the same non-invasive methodologies to be applied as to a human infant. Using an animal model we can track the temporal relationships between the observed parameters far more closely than with a patient. Thus the piglet is monitored continuously in the bore of a 7T magnet, allowing MRI and MRS to be performed on the same animals at similar times. The insult is obtained by dropping the inspired oxygen fraction (FIO2) to 0.12 and occluding the carotid arteries [35]. At the end of the insult, the animals are resuscitated by releasing the carotid occluders and raising the FiO2 to normalize arterial haemoglobin-saturation levels. During the insult there is a fall in ATP and PCr/Pi (Figure 1). What is significant is that within a couple of hours post-resuscitation these levels are back to normal. Thus the animal model is now at the stage where we would see a human infant suspected of having impaired intrapartum gas exchange, i.e. its brain appears energetically normal. However, during the next 2 days the MRS shows exactly the same rise in lactate and fall in PCr/Pi and ATP as seen in the babies. This is consistent with the idea that the delayed energy failure we see in the patients is a result of an acute lack of oxygen delivery in utero. The final piece of the jigsaw is the discovery that the extent of the secondary damage we see in the animal model is directly proportional to how much their ATP is decreased during the primary insult [35], i.e. a mild insult results in a small drop in PCr/Pi, at 48 h, but a severe insult results in a large drop in PCr/Pi, This demonstrates the clear causal link between a primary hypoxic-ischaemic insult and a delayed energy failure.

The ability to make multiple measurements from the same animal is one of the advantages of NMR spectroscopy over more invasive tissue-metabolite measurements. This allows us to make a detailed analysis of the temporal relationship between the fall in PCr and the decline in ATP levels; the resultant data answer the second criticism and demonstrate that a true 'energy failure' is occurring in the secondary phase. The creatine kinase equilibrium maintains constant cerebral ATP levels during the early phases of the primary
Figure 1
Effect of a hypoxic-ischaemic insult on brain energetics
Changes in ATP concentration and PCr/Pi, ratio in a neonatal pig brain, during and 2 days subsequent to a hypoxic-ischaemic insult. Note the logarithmic scale for time. For further details see text.

Figure 2
Relationship of ATP with PCr/Pi, ratio during primary and secondary energy failures
Data calculated from Figure 1 for n = 6 piglets.
There is a dramatic decrease in the apparent water white matter and the damage occurs in the lateral general, cortical grey matter is affected prior to basal ganglia. In this, the animal model mimics the hypoxia-ischaemia DWI has been the most useful. grey matter prior to the medial region decrease in the latter. diffusion, simultaneous with the fall in PCr/Pi. In way to test this hypothesis would be via saturation-availability using imaging techniques. In neonatal neonate during delayed energy failure the rate of ATP turnover in the former case and a oxidative ATP synthesis. An interesting alternative explanation, namely that excessive ATP consumption during post-ischaemic fits leads to the cell damage, rather than a primary failure in ATP production, has recently been advanced. Although this model seems inconsistent with the increased cerebral oxygenation observed during the secondary energy failure, the simplest way to test this hypothesis would be via saturation-transfer NMR, which would yield an increase in the rate of ATP turnover in the former case and a decrease in the latter.

One of the main advantages of NMR techniques is the temporal and spatial discrimination available using imaging techniques. In neonatal hypoxia-ischaemia DWI has been the most useful. There is a dramatic decrease in the apparent water diffusion, simultaneous with the fall in PCr/Pi. In general, cortical grey matter is affected prior to white matter and the damage occurs in the lateral grey matter prior to the medial region. In some animals, however, damage is first seen in the basal ganglia. In this, the animal model mimics the heterogeneous effects observed in the human neonate, where some infants show more effects on higher brain function (cortical grey-matter damage), whereas others have motor co-ordination dysfunction (basal ganglia damage). Despite the considerable technical difficulties several groups are now measuring DWI images of the neonatal human brain and it will be interesting to see if the same pattern of damage is observed as in the animal models [26,42].

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Adipocyte studies: systems for investigating effects of growth hormone and other chronically acting hormones
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Abstract
Adipose tissue is very amenable to study in vitro. Collagenase digestion yields free adipocytes which usually respond well to acute stimulation/inhibition by hormones and other factors. Chronic effects of hormones are best studied using explants of adipose tissue which, from some species (e.g. sheep), can be maintained in culture for up to a week without loss of function. Alternatively, pre-adipocytes can be readily isolated from adipose tissue and induced to proliferate and differentiate in culture, while various adipocyte-like cell-lines have been established, which can be used for chronic studies. Use of these various systems for investigating the mechanisms of action of growth hormone are described.

Introduction
Survival away from immediate supplies of food requires a store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy’. Survival away from immediate supplies of food requires a store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy’. Poikilotherms, such as fish and reptiles, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but in addition they have evolved a specialist store of ‘energy'.