Principles of the measurement of neuro-glial metabolism using in vivo $^{13}$C NMR spectroscopy

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This chapter reviews some recent achievements and insights obtained by $^{13}$C NMR in the brain in rats and humans in vivo. The studies discussed include (i) the demonstration that brain glycogen is an important store of glucose equivalents in the brain, providing significant fuel during hypoglycemia; (ii) the demonstration of slow brain glycogen metabolism in non-activated awake brain; (iii) the presence of significant anaplerosis (pyruvate carboxylase activity) in brain in vivo; (iv) the measurement of the energy metabolism of neurons and glia and the metabolic trafficking of glutamate between these two major metabolic compartments; (v) the measurement of a major regulatory metabolic element of oxidative metabolism in the brain, the malate–aspartate shuttle; and (vi) the finding that brain glycogen metabolism is deranged following hypoglycemic episodes,
suggesting an involvement in the hypoglycemia unawareness syndrome clinically observed in diabetes.

1. Introduction

The propagation of electrical impulses between brain cells is accomplished by chemical transmission, achieved by releasing signaling molecules (neurotransmitters) from the presynaptic bouton that interact with receptors on the postsynaptic neuron and thus mediate the transmission of electrical signals from one neuron to the next. It is becoming increasingly clear that normal brain function not only involves the function of neurons on both sides of the synaptic cleft. In addition to the pre- and the postsynaptic neuron, the astroglial compartment has recently gained increased attention by virtue of its importance in maintaining the functionality of the synapse (Schousboe et al., 1993a; Hansson and Ronnback, 1994; Vernadakis, 1996; Magistretti and Pellerin, 1999). Among the many neurotransmitter systems, that of glutamate is probably most abundantly distributed in the central nervous system. The accepted mechanism for the action of glutamate (Shank and Aprison, 1979; Westergaard et al., 1995; Daikhin and Yudkoff, 2000; Lieth et al., 2001), is a prime example for the importance of the interplay of electrical events and metabolism in the action of this important neurotransmitter, as illustrated in the scheme of Fig. 1.

Glutamate uptake from the synaptic cleft is characterized by concurrent electrical events due to electrogenic glutamate transporters which are critical in maintaining a low extracellular glutamate concentration in order to avoid excitotoxicity and to maintain

Fig. 1. Scheme depicting the metabolism of neurotransmitter glutamate, which forms the basis for the concept of the glutamate–glutamine cycle, with the rate VNT. Uptake of glutamate in astrocytes from the synaptic cleft (not shown as a separate step) is followed by conversion into electrophysiologically inactive glutamine.
postsynaptic excitability. Most of the synaptic glutamate is cleared into the astrocytes surrounding the synaptic cleft (Yudkoff et al., 1993; Bergles et al., 1997; Zigmond, 1999; see also the chapter by Schousboe and Waagepetersen). Following uptake into the glial cell, glutamate is converted by glutamine synthetase into the electrophysiologically inactive glutamine, which is transported back to the neuron and converted to glutamate, thereby maintaining the nerve terminal concentration of neurotransmitter glutamate, in a 'glutamate–glutamine cycle' (Yudkoff et al., 1988; Kanamori and Ross, 1995; Westergaard et al., 1995; Brand et al., 1997; Rothman et al., 1999; Daikhin and Yudkoff, 2000). It is therefore quite clear that glutamatergic transmission involves metabolism (through the glutamate–glutamine cycle), as well as energy metabolism of the astroglial compartment due to the requirements for the restoration of the ion balance and for glutamine synthesis (Magistretti and Pellerin, 1996; Attwell and Laughlin, 2001). Indeed, it has been reported that the uptake of Glu into the astrocytes was associated with increased glucose metabolism in the astrocyte (Magistretti et al., 1993; Eriksson et al., 1995), thereby linking stimulated energy metabolism between the astroglial and neuronal compartments during neurotransmission. Because of the mostly neuronal localization of glutamate and the exclusively astroglial localization of glutamine synthesis, the measurement of glutamate to glutamine, uniquely possible using \(^{13}\text{C}\) NMR spectroscopy (Gruetter, 1993; Gruetter et al., 1994; Hassel et al., 1997; Rothman et al., 1999; Chhina et al., 2001; Blum et al., 2002; Gruetter, 2002), in principle could serve as an indicator of the rate of the glutamate–glutamine cycle, thought to reflect the rate of glutamatergic action (Yudkoff et al., 1988; Gruetter et al., 1998a; Rothman et al., 1999). While conceptually very simple in its formulation, the interpretation of the labeling of glutamate and glutamine needs to take into account many additional reactions, whose activity cannot be neglected in vivo, such as the rate of brain oxidative metabolism and pyruvate carboxylase, and, potentially, glycogen metabolism. This chapter deals with the requirements on the modeling that are necessary to understand in order to use in vivo NMR spectroscopy for the assessment of glutamatergic metabolism in the intact brain in vivo, but it does not deal with the technical requirements to implement a successful \(^{13}\text{C}\) NMR spectroscopy program and NMR methodology.

2. Key elements of \(^{13}\text{C}\) tracer methodology measured by in vivo \(^{13}\text{C}\) NMR spectroscopy

NMR spectroscopy is a non-destructive method that allows the measurement of signals from several compounds and distinct positions within the molecule. The information content of detecting \(^{13}\text{C}\) label by NMR is illustrated in Fig. 2. While detection of the \(^1\text{H}\) NMR signal of hydrogen nuclei adjacent to \(^{13}\text{C}\) nuclei is clearly most sensitive (Fig. 2B), the detection of label by directly measuring the signal of \(^{13}\text{C}\) provides more information (Fig. 2A). A full review of the methodology involved is beyond the scope of this paper, however, it is important to recognize that many methodological difficulties need to be overcome in measuring \(^{13}\text{C}\) label in vivo and this field is far from being fully developed as illustrated by some examples are provided at the end of this section.
Fig. 2. In vivo NMR detection of $^{13}$C label. (A) illustrates a spectral region depicting the detection of $^{13}$C label for the C3 and C4 region of amino acids in a 400 μl volume of rat brain at 9.4 Tesla. Reproduced from (Choi et al., 2000). (B) shows the spectral region of the $^1$H spectrum covering the $^{13}$C label in all compounds but glucose in a 120 μl volume. Reproduced from Pfeuffer et al. (1998). (NAA - N-acetyl-aspartate; Asp - aspartate; Glu - glutamate; Gln - glutamine; Lac - lactate; Ala - alanine; Crtot - phosphocreatine + creatine).

2.1. ‘Tracer methods’

The administration of a tracer, whether stable or radioactive, and the ability to follow its metabolism non-invasively opens unique opportunities to study the brain in action. When the highly sensitive radiotracers, label in different metabolic pools cannot be distinguished, unless the measurement of the incorporated radioactivity is performed for each compound separately which is only possible using postmortem analysis or if one uses
non-metabolizable analogs, such as deoxyglucose, which is trapped following phosphorylation by hexokinase (Sokoloff et al., 1977).

The fundamental mathematical principle that underlies the modeling of tracer turnover curves is in principle the same, regardless of the type of tracer used: In all cases, the rate of label appearance in the product pool $P$ is given by the sum of metabolic fluxes from any substrate multiplied by the probability that this particular substrate was labeled, $^{13}\Sigma /\Sigma$, resulting in Eq. (1) which is from (Gruetter, 2002):

$$\frac{d^{13}P}{dt}(t) = \sum_i V_{i}^{(in)} \frac{^{13}S_i(t)}{S_i} - \sum_j V_{j}^{(out)} \frac{^{13}P(t)}{P}$$

(1)

For example, the Sokoloff method measures the tissue radioactivity 45 min after administering a measured bolus of labeled glucose, when the radioactivity from non-phosphorylated sources is negligible and when dephosphorylation is not significant. A further extension of the Sokoloff method is the measurement of tissue radioactivity as a function of time, to which a suitable model of the tracer compartments is fitted. The elegance of the Sokoloff method is its operation in the true tracer mode, i.e., when the kinetics of the product buildup do not affect the tracer kinetics nor the biochemical reaction, leading in principle to a simplified mathematical problem, as indicated by the schematic representation of label incorporation (Fig. 3).

Label incorporation into a product from a precursor, such as into glutamate from glucose, is based on the same fundamental mathematical principles as, e.g., the Sokoloff method, yet several quite profound differences must be discussed. In contrast to the radiotracer method, the signal is detected in a naturally occurring compound, which, because of the inherently lower sensitivity of NMR, must be highly concentrated and enriched in order to be measurable, and inherently includes an upper limit of the measurable label incorporation in tissue. These aspects typically lead to ‘tracer’ curves, i.e., label incorporation curves, similar to what is shown in the middle in Fig. 3, with the label in a specific compound (such as the highly concentrated glutamate) reaching a steady-state value after some time.

2.2. Dynamic isotopomer analysis

The true power of modeling label incorporation into tissue pools, as measured by NMR, however, is harnessed by taking into account the ability of NMR to measure the rate of label incorporation not only into different molecules, but also into different positions in a given molecule (Cerdan et al., 1990; Badar-Goffe et al., 1992; Mason et al., 1992; Schousboe et al., 1993b; Shank et al., 1993; Gruetter et al., 1998a), such as the C2, C3 and C4 of glutamate (Fig. 3). The measurement of label incorporation into multiple positions in a molecule in effect is very similar to the measurement of the label distribution in a molecule, traditionally dubbed isotopomer analysis (Malloy et al., 1990; Jeffrey et al., 1991). It has been shown that the time-resolved measurement of label incorporation into the glutamate C3 and C4 is equivalent to the dynamic measurement of the simultaneous, but separate measurement of $[4,^{13}\text{C}]$ glutamate and $[3,4,^{13}\text{C}_2]$ glutamate from the $^{13}\text{C}_2^{13}\text{C}$ singlet and doublet signals (isotopomers) at the C4 position (Jeffrey et al., 1999).
Fig. 3. Measurement of labeling kinetics using radiotracer methods in comparison to in vivo NMR methods. (Top) Radiotracer methods such as the Pulsed Field Gradient method use a non-metabolizable analog, such as deoxyglucose (DG) to measure the accumulation of radioactivity in the target tissues. The additional method of metabolic products (C\textsubscript{i}, C\textsubscript{j}, C\textsubscript{k}, C\textsubscript{l}, C\textsubscript{m}, C\textsubscript{n}) in the tissue is measured by dynamic NMR spectroscopy, which is based on the measurement of label incorporation into multiple positions of the same molecule, leading to measurement of multiple times to courses of label incorporation into the C\textsubscript{i}, C\textsubscript{j}, C\textsubscript{k}, C\textsubscript{l}, C\textsubscript{m}, C\textsubscript{n}.
It is therefore proposed to name the time-resolved measurement of label incorporation into multiple positions of a given molecule ‘dynamic isotopomer analysis’ (bottom in Fig. 3).

When fitting a model of compartmentalized cerebral metabolism to such a model, it is important to recognize that the cost function will be evaluated for all fitted time courses simultaneously. In strictly mathematical terms, this is an extension of the one-dimensional case applicable to the measurement of label incorporation into the glutamate C4 for instance.

In this context it may be confusing that some models seem to be governed by many more differential equations than parameters that are fitted or number of time courses that are measured. However, in practice, even with so-called simpler models involving only a few explicit differential equations, many more differential equations are in fact involved. In those cases they do not enter the model explicitly because it has been assumed that the small pool size compared to the metabolic rate leads to a negligible effect on the measured metabolic rate. In other words, in the case of a series of chemical reactions involving at least three pools of metabolites, when assuming that the second pool is small compared to the third pool and compared to metabolic flux, the labeling rate of the third pool is not likely to be substantially affected whether that of the second pool was explicitly calculated or not. For metabolic branching points, such as 2-oxoglutarate, however, it is necessary to explicitly include the calculation of the rate of labeling of 2-oxoglutarate, even though the pool size of 2-oxoglutarate itself is unlikely to affect the labeling curve. Hence, the argument that the number of differential equations is too large needs to take into account whether this implies more reactions with a significant impact on the labeling curves or just a more explicit mathematical formulation of reality.

Lastly, an important difference between NMR measurements using isotopes and radiotracer methods lies in the fact that the degree of isotopic labeling in the NMR studies typically is very high, such that small fluctuations of the isotopic enrichment of the precursor pool are not likely to affect the outcome of the analysis.

In any of these methodologies it is clear that the rate of label incorporated as a function of time can in principle be related to the metabolic rate and thus allows measuring absolute metabolic fluxes. Another interesting approach consists in measuring the relative amount of label in different molecules or even different positions between different molecules when metabolic steady-state has been achieved (Gruetter et al., 1998a; Gruetter et al., 2001; Bluml et al., 2002; Lebon et al., 2002), and some relative fluxes can be derived using equations such as the following:

$$\frac{^{13}P}{P} = \frac{\sum_{j} ^{V_{j}^{(in)}}}{\sum_{j} ^{^{13}S_{j}}} \frac{^{13}S_{n}}{S_{n}}$$

Eq. (2) is derived from Eq. (1) assuming steady-state and only one substrate $S_n$ leading to label incorporation into the product $P$. Such steady-state analysis can lead to a simplified analysis and has also been used for the measurement of differential labeling in the C4 following acetate labeling (Lebon et al., 2002). In the case of acetate labeling, the scrambling of label into many molecules needs to be taken into account. For example, in
the case of the glutamate–glutamine cycle, not only are there four metabolic pools to be considered (glutamate and glutamine in the neuronal and glial compartments) that can be labeled, but also the magnitude of the fluxes between the mitochondrial Krebs cycle intermediate and the cytosolic glutamate, $V_i$, is expected to have an effect on the calculated relative metabolic rates. That the derived labeling can depend on this exchange can be appreciated from the following 'Gedankenexperiment': Consider a case where the glutamate does not have a significant exchange with 2-oxoglutarate in the neuron (small $V_i$). In this case the relative labeling of neuronal glutamate ($^{13}$Glu$^{n}$)/Glu($^{n}$) will be identical to that of glial glutamine. However, as the exchange rate increases, so does the contribution of unlabeled carbon from the neuronal Krebs cycle to neuronal glutamate, leading to increasingly different labeling in glutamate relative to glutamine, which may very well affect the interpretation of the relative quantitative magnitude of the glutamate–glutamine cycle. Therefore, for the calculation of relative rates of the glutamate–glutamine cycle, at least 6 pools into which label is accumulated, need to be considered even for the case of labeling from acetate or [2-$^{13}$C] glucose.

### 2.3. $^{13}$C NMR methodological aspects

Unfortunately, the technical development of $^{13}$C NMR spectroscopy in vivo has been limited to a handful sites worldwide (Gruetter et al., 1998a; Bluml, 1999; Rothman et al., 1999; Chen et al., 2001; Chhina et al., 2001; Henry et al., 2002) and largely requires further development. The technical underpinnings of $^{13}$C NMR in vivo is not the focus of this chapter, but shall be briefly illustrated by reviewing a few key advances in this field.

In 1992, two important advances in MR methodology were introduced to in vivo $^{13}$C NMR spectroscopy: First, the use of automated shimming (i.e., in vivo optimization of the main static magnetic field, $B_0$, such that it becomes largely independent of the spatial coordinates) dramatically improved sensitivity by narrowing linewidths by almost an order of magnitude compared to what was reported at the time (Gruetter and Boesch, 1992; Gruetter, 1993). Second, the introduction of three-dimensional localization allowed for well-defined volumes to be measured (Gruetter et al., 1992a,b). Two improvements were immediately realizable, namely (i) the complete elimination of the intense scalp lipid signals from the extra-cerebral tissue, which overlap with numerous signals from amino acids, and (ii) the collection of signals from a well-defined volume, which together with excellent shimming improved the spectral resolution. The increases in sensitivity were demonstrated with the rather surprising observation that natural abundance signals from brain metabolites can be observed in vivo, such as those from myo-inositol (Gruetter et al., 1992b). These methodological advances lead to the landmark discovery that labeling of glutamine can be detected in the in vivo brain (Gruetter, 1993; Gruetter et al., 1994), which is now recognized as a window to study cerebral metabolic compartmentation (Sonnwald et al., 1994; Bachelard, 1998; Cruz and Cerdan, 1999; Magistretti et al., 1999; Rothman et al., 1999) and provides a unique window on the brain. Localization has also proven critical in the ability of NMR to detect and measure the signals of brain glycogen, because the several-fold higher than the glucose, and does not eliminate any limitations for in vivo studies (Oz et al., 2000).

### 3. Glial metabolism

The brain requires glucose for normal brain function. In the brain, it the largest energy source, and glycogen has been studied in detail (Lehninger et al., 1997). However, the metabolism of glycogenolysis and gluconeogenesis in the brain has been understudied (Gruetter et al., 2004). A new method for its evaluation has been developed by biochemical demonstration of a new method for its evaluation, as shall be described.

#### 3.1. Brain glycogen metabolism

Brain glycogen metabolism has been studied, when using an in vivo NMR technique demonstrated to be effective in vivo (Gruetter et al., 1999). The study of the metabolism of glycogen in brain, was transferred from tissue to in vivo, with the synthesis measured in glycogen. In the first study, we showed that glycogen synthesis could be measured and was demonstrated to be responsive to changes in regulated plasma glucose levels. The results showed a hyperglycemic rise in brain glycogen, consistent with the expected rise in normal glucose levels. The study also showed that the glycogen content of the brain was inversely related to the level of glucose in the blood, suggesting a hyperglycemic rise in brain glycogen.

It is quite possible that the role for brain glycogen is to maintain a constant level of brain glucose content in brain glycogen in neurons (Swanson et al., 1991; Gruetter et al., 1999).
several-fold higher concentration of muscle glycogen requires dedicated efforts to eliminate any non-cerebral source of glycogen signal (Choi et al., 1999; Choi et al., 2000; Oz et al., 2003).

3. Glial metabolism I: brain glycogen

The brain relies on a continuous supply of glucose from the blood for maintaining normal brain function, yet the brain maintains a significant level of brain glycogen making it the largest endogenous carbohydrate reserve in the brain. The concentration of brain glycogen has been estimated at a few μmol/g (Choi and Gruetter, 2003) and references therein, however, several recent studies have suggested that due to the rapid postmortem glycogenolysis (Lowry et al., 1964; Swanson et al., 1989; Choi et al., 1999), as well as glycogen breakdown during the assay itself, these brain glycogen concentrations may have been underestimated (Cruz and Dienel, 2002; Kong et al., 2002). The problems with the biochemical determination of brain glycogen clearly point to the need of a non-invasive method for its measurement. In vivo NMR spectroscopy has unique capabilities in that regard, as shall be illustrated below.

3.1. Brain glycogen, an endogenous store of fuel

Brain glycogen metabolism and concentrations obviously can be measured by NMR when using suitable methodology (Choi et al., 1999, 2000). Pulse-chase experiments demonstrated that glycogen breakdown in the α-chloralose anesthetized rat was very slow with a turnover rate on the order of 0.5 μmol/g/h during glucose infusions (Choi et al., 1999). The study further precluded label turnover as the only mechanism by which label was transferred to the brain glycogen pool and it was concluded that net brain glycogen synthesis must have occurred (Choi et al., 1999). In a follow-up study, the effect of insulin was measured by measuring the effect of hyperinsulinemia on label incorporation at a controlled plasma glucose level fixed close to normoglycemia (Choi et al., 2003). The results showed that in vivo at mild hyperglycemia, plasma insulin has a profound effect on brain glycogen metabolism and leads to a net accumulation. These findings were in agreement with data from cell cultures showing an effect of high insulin concentrations on culture glycogen concentrations (Nelson et al., 1968; Dringen and Hamprecht, 1992; Sorg and Magistretti, 1992; Swanson and Choi, 1993). The study extends previous studies suggesting an effect of insulin on brain glycogen content at supraphysiologic hyperglycemia (Daniel et al., 1977).

It is quite plausible that brain glycogen may serve as a reservoir of glucosyl units that are mobilized whenever demand for glucose is in excess of supply and such a neuroprotective role for brain glycogen can be implied from, e.g., the mechanism of glutamate neurotransmission (Fig. 4), where glycogen is able to provide energy during hypoglycemia to maintain a low extracellular glutamate concentration. Such a neuroprotective role for brain glycogen has been suggested on the basis of preloaded astrocytes in co-culture with neurons (Swanson and Choi, 1993) and for axonal survival during glucodeprivation (Wender et al., 2000). A recent study demonstrated that degradation of brain glycogen
initiated by hypoglycemia started when the brain glucose concentration approached zero, which is the point at which glucose transport became rate-limiting for metabolism (Choi et al., 2003). Interestingly, at this point cerebral blood flow (CBF) was increased abruptly (Fig. 5A), indicating an attempt by the brain to increase supply, by decreasing the arteriovenous gradient for glucose (Choi et al., 2001). The textbook literature implied that brain glycogen must be a limited storage form for glucose due to its low content and, thus, the role of brain glycogen as a glucose reservoir has been generally dismissed in the literature. Nonetheless, during hypoglycemia, glycogen need only account for part of the deficit in glucose supply and hence can survive longer periods of sustained hypoglycemia. Indeed, a preliminary estimate indicated conservatively that brain glycogenolysis accounted for a majority of the deficit in glucose supply, supporting the quantitative importance of brain glycogen in hypoglycemia (Choi et al., 2003). Measurements of brain glycogen during hypoglycemia indicated that brain glycogen degradation occurred at a rate during hypoglycemia that resulted in brain glycogen concentrations to be substantial even after 2 h of moderate hypoglycemia (Fig. 5B). It is therefore likely that the brain tries to defend itself against moderate hypoglycemia by using brain glycogen and by increasing CBF and that these defenses are triggered by the point at which glucose transport becomes rate-limiting for metabolism, or by the point at which the brain glucose concentrations become rate-limiting for metabolism.

Because brain glycogen is an insulin-sensitive glucose reservoir it is interesting to explore whether brain glycogen metabolism is deranged following hypoglycemia. Indeed,
Fig. 5. Effect of hypoglycemia on cerebral blood flow, brain glucose concentrations and brain glycogen metabolism as measured by NMR. (A) Measurements of brain glucose concentration (left scale) as a function of plasma glucose concentration. The solid line is the fit of the reversible Michaelis–Menten model to the eu- and hyperglycemic brain glucose (open circles). The open squares indicate brain glucose measurements below 4.5 mM plasma glucose. When brain glucose approaches zero (dotted vertical line), the measurement of cerebral blood flow (solid triangles, right scale) indicate CBF values above the 95% confidence interval (shaded area) and this is also the point where brain glycogenolysis started (arrow in A and vertical dotted line in B). (B) Measurement of the effect of insulin-induced hypoglycemia at 4 h on brain glycogen metabolism and glucose concentrations. When the brain glucose concentrations (open squares) approached zero, brain glycogenolysis started (dotted vertical line) at a rate that sustained brain glycogen concentrations (solid circles) for at least 2 h. Restoration of brain glucose concentrations at 7 h typically resulted in a brain glycogen rebound (supercompensation). Modified from (Choi et al., 2001) and (Choi et al., 2003).
data suggest that brain glycogen concentrations following hypoglycemia increase substantially above normal (Fig. 5B) leading to increased neuroprotection. It is therefore reasonable to conclude that brain glycogen metabolism may play a role in the development of defective recognition of hypoglycemia (hypoglycemia unawareness) by the brain as proposed recently (Choi et al., 2003).

3.2. Human brain glycogen metabolism in vivo

The studies mentioned in Section 3.1 have been performed in anesthetized animals and, consequently, the legitimate question arises as to whether the slow brain glycogen turnover observed also translates to the awake brain. This is of interest, since several studies suggesting an involvement of brain glycogen in brain activation have done these measurements in the conscious animal (Swanson, 1992; Diener et al., 2002). Our results thus far suggest that brain glycogen is only utilized when supply is insufficient to cover demands in metabolism, possibly only when brain glucose concentrations become so low that they significantly limit the rate of glucose phosphorylation. Some of the reported increases in brain glucose metabolism observed during focal activation imply increased usage of carbohydrates other than blood glucose because the reported increases (Hyder et al., 1997) exceed the transport capacity of the blood-brain barrier by several-fold (Choi et al., 2001). One such source of glucose equivalents is brain glycogen, which is present in sizable amounts in brain (Sagar et al., 1987; Choi et al., 1999; Cruz and Diener, 2002; Kong et al., 2002). It is possible that parts of the glycogen molecule may undergo rather rapid metabolism. However, in line with our results in the α-chloralose anesthetized rat brain (Choi et al., 1999), we found that metabolism of bulk brain glycogen was also very slow in the awake rat brain, with a turnover time on the order of that of NAA (Choi and Gruetter, 2003) and a total brain glycogen concentration of ~3 μmol/g wet weight in line with the literature.

However, the important question arises how these measurements relate to human brain glycogen metabolism, which has never been measured in vivo. The question remained as to whether in the conscious human, brain glycogen metabolism is also slow. We have adapted our previously developed methods to the measurement of brain glycogen in humans and measured the rate of label incorporation into brain glycogen during administration of [1-13C] glucose in humans (Oz et al., 2003). The results indicated a much slower rate of label incorporation in the human than in the rat (Fig. 6) with a turnover rate of approximately 0.15 μmol/g/h. Such a slow rate of turnover certainly does not suggest an involvement of brain glycogen metabolism in the background activity of the conscious human brain. Nonetheless, it does not preclude the activation of the reservoir in conditions of extreme metabolic demand. Instead, it favors the overall influence of the sleep–wake cycle on brain glycogen metabolism as reported (Kong et al., 2002), and supported by altered gene expression (Petit et al., 2002): For example, it is quite conceivable that small bursts of brain activity will lead to transient mismatches in glucose supply and demand causing, e.g., small decreases in brain glycogen that can accrue over time during the day. Such a slow rate of turnover of glycogen in humans suggests that turnover of the glucosyl units in brain glycogen may require days and that altered brain glycogen concentrations (such as a superorder of a waking is consistent with hypoglycemia unawareness and the pathogenesis of i

4. Glial metabolism

Because of the activation-deactivation question whether brain has become the late 1980s such that CBF that exceed provides the unique and lactate, both of
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![Graph showing the relationship between time and [1-13C]Glycogen concentration](image)

**Fig. 6.** Measurement of the label incorporation into human brain glycogen C1. The solid squares represent the measurement of [13C] label in glycogen C1 following administration of 80 g of 1-13C glucose in three humans begun at t = 0 min. The solid line is the result of a linear regression of the measurements in the first 5 h of the study and indicates a rate of label incorporation consistent with a very slow glycogen turnover rate on the order of 0.1–0.2 µmol/g/h. From Oz et al. (2003).

(such as a super-compensation following a hypoglycemic episode) may require time on the order of a week to be restored to normal. This time scale of brain glycogen metabolism is consistent with the time scale it takes to revert the syndrome of hypoglycemia unawareness and is consistent with the proposed involvement of brain glycogen in the pathogenesis of impaired recognition of hypoglycemia.

4. Glial metabolism II: the glutamate–glutamine cycle

Because of the ever increasing importance of functional MRI, a mechanism of which is the activation-dependent change in the venous concentration of deoxyhemoglobin, the question whether there is tight coupling between glucose and oxygen consumption in the brain has become of paramount importance. The landmark study by Fox and Raichle in the late 1980s suggested that there are indeed large increases in glucose metabolism and CBF that exceed the changes in oxygen metabolism (Fox et al., 1988). In principle, NMR provides the unique capability to measure cerebral concentration changes of brain glucose and lactate, both of which are key components in addressing this question, and increases in
lactate and glucose concentration have been reported (Prichard et al., 1991; Sappey-Marinier et al., 1992; Frahm et al., 1996). In addition, from the incorporation of label from a suitable precursor such as glucose, into glutamate, the cerebral oxygen consumption could be computed. A majority of studies in the brain have focused on measuring glutamate turnover (Rothman et al., 1992; Hyder et al., 1997; Lukkarinen et al., 1997; Pan et al., 2000), which was motivated by the fact that glutamate turnover is linked to the metabolism of the Krebs cycle (Mason et al., 1992; Chatham et al., 1995; Mason et al., 1995; Yu et al., 1997; Gruetter et al., 1998a, 2001; Cruz and Cerdan, 1999), and that the glutamate C4 resonance, which is labeled in the first turn of the Krebs cycle, presents a readily detectable NMR signal due to the high concentration of glutamate. Using this methodology, one such study compared the rate of label incorporation and found a significant difference between the activated and the resting visual cortex, indicating that the cerebral oxygen consumption increased at most by 30%, which is approximately half of the blood flow increase measured using this stimulation paradigm (Chen et al., 2001). This study supported the idea that oxygen consumption increases are less than the associated blood flow increases, leading to a net decrease in deoxy-hemoglobin content during focal activation, which forms the basis of blood-oxygen-level-dependent functional MRI (Ogawa et al., 1998).

Perhaps the major advantage of in vivo NMR is not to provide neuroscientists with an alternative alternative method to measure CMRO₂ and CMRglc (although this may be very useful as indicated above), but to shed light on metabolic processes not accessible by any other method, one of which (glycogen) was addressed above and some of which will be discussed below.

4.1. Glutamate turnover: neuronal oxygen metabolism and the malate–aspartate shuttle

The measurement of cerebral oxygen consumption from turnover of glutamate (as referred to in the previous paragraph) assumes a direct stoichiometric relationship between that measurement and the rate of oxygen consumption. Unfortunately, this relationship is not directly inferred, as the brain is intricately compartmentalized, which shall be discussed further below. In addition, most of the glutamate signal that is observed is in the cytosol, whereas the labeling occurs in the mitochondrion and hence label has to be transported across the charged inner mitochondrial membrane (Fig. 7), which has been shown to be the rate limiting step in many tissues, such as the heart (Chatham et al., 1995; Yu et al., 1997; Sherry et al., 1998) and the liver (Garcia-Martin et al., 2002).

Initially it was thought that the exchange between 2-oxoglutarate and glutamate, Vᵣ, is very fast in the brain in vivo. However, many studies in heart, liver and muscle have indicated the opposite in these tissues (Chatham et al., 1995; Yu et al., 1997; Sherry et al., 1998; Garcia-Martin et al., 2002). More recent evidence now suggests that in the brain Vᵣ is on the order of the flux through pyruvate dehydrogenase, VᵣDH (Gruetter et al., 2001; Choi et al., 2002), which may vary in pathologic conditions (Henry et al., 2002). The observation that Vᵣ was comparable to the flux through pyruvate dehydrogenase implied that the malate–aspartate shuttle may be a major mechanism mediating the exchange of lactate and glucose in the Krebs cycle. As a result, glutamate-glutamine exchange is not only limited to the blood–brain barrier, but occurs also within most brain compartments within the brain, including the astrocytic matrix (Martinez-Hernandez et al., 1997).
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Fig. 7. Measurement of oxidative glucose consumption from the flow of label from glucose to glutamate. The label in glucose (or any other precursor, such as pyruvate or acetyl-CoA for that matter) is metabolized in the mitochondrion and then transferred to glutamate, most of which is in the cytosol. The flow of label into glutamate thus is in principle a combined effect of Krebs cycle flux ($V_{PDH}$) and label exchange across the mitochondrial membrane ($V_x$).

The exchange of label across the mitochondrial membrane (LaNoue and Tischler, 1974; Yu et al., 1997; Gruetter, 2002). The assumption that $V_x$ is very fast will affect the modeling results depending on the pool sizes that participate in this exchange (Gruetter et al., 2001; Gruetter, 2002).

Furthermore, it is important to recognize that because of the mostly neuronal localization of glutamate, the measurement of glutamate turnover alone, as done in several previous studies (Rothman et al., 1999; Pan et al., 2000; Sibson et al., 2001), mainly measures neuronal metabolism. The astroglial compartment does contain significant oxidative capacity for metabolism, as pointed out recently (Gruetter et al., 2001), despite some previous assumptions to the contrary (Sibson et al., 1998). This shall be discussed in Section 4.2.

4.2. Glutamine turnover: the hallmark of glial metabolism

It is well known that brain metabolism is characterized by at least two major compartments with a large neuronal and a small glial glutamate pool associated with the Krebs cycle. As pointed out previously, these two pools are metabolically linked by the glutamate—glutamine cycle. The compartmentation of brain metabolism is based on that of several enzymes. In addition to those of glycogen (see above), glutamine synthetase (Martinez-Hernandez et al., 1976) and pyruvate carboxylase (Shank et al., 1985) are almost exclusively in the glial compartment, as summarized in more detail elsewhere.
(Bachelard, 1998; Cruz and Cerdan, 1999; Gruetter et al., 2001; Gruetter, 2002). Compartmentation furthermore extends to metabolites, such as glutamate (neuronal) and glutamine (glial) (Ottersen et al., 1992; Shulpiakov et al., 1997), as well as to mitochondria and other systems (Schousboe et al., 1993b).

It is of interest to note that the exclusively glial localization of glutamine synthetase implies that the observation of glutamine synthesis in vivo, first achieved in human brain (Gruetter, 1993; Gruetter et al., 1994), is a direct manifestation of glial metabolism, whereas the observation of label incorporation into glutamate implies a mainly neuronal event. Clearly, glutamine synthesis can be measured non-invasively by NMR, as shown in Fig. 2, and therefore demonstrates the ability of NMR to study cerebral compartmentation non-invasively in intact brain.

The mechanism of inactivation of glutamate by uptake into the astroglial compartment implies a much more active role for astrocytes than is conventionally assumed, due to the imperative involvement of glial energy metabolism (Eriksson et al., 1995; Magistretti and Pellerin, 1996; Silver and Erecinska, 1997). The neuron-astrocyte triad thus has to be considered the functional unit intimately involved in achieving chemical transmission (Magistretti et al., 1993, 1999; Tsacopoulos and Magistretti, 1996). The link between astrocytes and neurons is generally accepted from a metabolic as well as from a neurophysiological standpoint (Bergles et al., 1997), yet differences exist as to the precise coupling and the specific energetics involved.

The simplest scheme for measuring glutamate neurotransmission in vivo is shown in Fig. 8. This model assumes very rapid exchange $V_t$ and negligible glial Krebs cycle rate, as well as negligible anaerobiosis. Based on this simple and elegant scheme, it was proposed that the rate of glutamate/glutamine inter-conversion (the glutamate–glutamine cycle), identified in the scheme in Fig. 8 by $V_{NT}^{app}$, is equal to the glucose consumption rate (Sibson et al., 1998; Rothman, 2001; Shulman et al., 2001a,b; Shen and Rothman, 2002; Rothman et al., 2003). This elegant, but perhaps oversimplified model assumed that the two ATP produced by glycolysis were almost completely consumed by glutamine synthesis and restoration of the ion balance through the Na/K ATPase with a negligible oxidative metabolism in the glial compartment. Under these circumstances it was postulated that glucose metabolism must be directly linked to glutamate neurotransmission with a 1:1 stoichiometry. The proposal put forth by Shulman and coworkers (Sibson et al., 1998; Rothman et al., 1999), that the glial ATP production needed to maintain neuronal glutamate is solely provided by glycolysis pathway is intriguing as it emphasizes the coupling between neurons and glia at the level of energy metabolism. However, only a few percent of pyruvate molecules need to be diverted to the Krebs cycle to generate as many ATP as are formed in the absence of oxidative metabolism of glucose in the astrocytes.

Furthermore, a recent study (Choi et al., 2002) measured brain glucose and glycogen metabolism in deep pentobarbital anesthesia under conditions similar to what was used (Sibson et al., 1998) and what had been shown to result in isoelectric coma (Contreras et al., 1999). In that study it was shown that the brain glucose concentration changed only slightly despite a drastic reduction in electrical activity and that a substantial gradient in brain glucose concentration relative to that in plasma persisted, as illustrated in Fig. 9.
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Fig. 8. Proposed link between glucose metabolism and glutamate neurotransmission. Adapted according to Sibson et al. (1998). The proposed stoichiometric coupling between glucose metabolism and glutamate neurotransmission is based on the assumption that all of the glucose is metabolized in the glia and that the two ATP produced are consumed by the need to restore ion potential following glutamate uptake and by glutamine synthetase. Oxidative metabolism of glucose and anaplerosis (pyruvate carboxylation) are neglected in this simple, yet elegant model.

In addition, that study indicated that when using the simplified scheme shown in Fig. 8 (Choi et al., 2002), similar metabolic rates as those reported by (Sibson et al., 1998) were obtained, however, the rate of label incorporation into glutamate C4 and C3 was inconsistent with that observed (dashed line in Fig. 10). Thus, the study reiterated the importance of minimizing the number of assumptions made in the modeling, which was also emphasized by two other independent studies (Gruetter et al., 2001; Henry et al., 2002). One additional assumption of the study by Sibson et al. (1998) was that the magnitude of glutamine synthesis not related to neurotransmitter cycling was constant over a large range of electrical activity. A surprising observation of our study was that under deep pentobarbital anesthesia, astrocyte metabolism was as significant as was neuronal metabolism with approximately equal magnitude per volume brain tissue. This observation is consistent with results from previous studies in culture, suggesting an effect of barbiturates on neuronal metabolism that is different in magnitude from that on astrocytes (Yu et al., 1983; Hertz et al., 1986; Swanson and Seid, 1998; Qu et al., 2000).

Oxygen consumption has been reported to increase in cultured astrocytes when exposed to extracellular glutamate (Eriksson et al., 1995) and large increases in oxygen consumption have been reported in brain during functional activity (Hyder et al., 1996, 1997), which support the idea that oxygen metabolism in astrocytes is stimulated during focal...
Fig. 9. Effect of pentobarbital anesthesia on brain glucose content in the rat brain. Shown is a comparison of the brain glucose concentration between light α-chloralose anesthesia and deep pentobarbital anesthesia. Modeling of brain glucose transport according to previous studies (Gruetter et al., 1998b; Choi et al., 2001; Seaquist et al., 2001) indicated a decreased rate of glucose metabolism (CMRglu) relative to the apparent maximal rate of glucose transport (Tmax). Even under deep pentobarbital anesthesia, brain glucose concentrations were significantly lower than expected if glucose metabolism was abolished (as indicated by the dashed line).

Fig. 10. Effect of the exchange rate between 2-oxoglutarate and glutamate, V_e, on the relative labeling of glutamate C3 and C4 during deep pentobarbital anesthesia. When assuming V_e = 0.57 μmol/g/min and fitting to the measured label incorporated into the C4 of glutamate only, an oxidative glucose consumption rate similar to that reported by Sibson et al. (1998) was obtained (V_for = 0.15 μmol/g/min), however, the label incorporation into the C3 of glutamate relative to that into the C4 (solid squares) was not very well reproduced (dashed line). In contrast using the scheme in (Gruetter et al., 1998; Gruetter et al., 2001; Gruetter, 2002), lead to a much better approximation (solid line), indicating that V_e is on the order of V_for also in deep pentobarbital anesthesia, and thus brain activity dependent.

4.3. Anaplerosis of brain glutamate

Astrocytes thus serve as the sole contributor to the uptake of glutamate and its transport into neurons. However, early studies in the brain (Gruetter et al., 1998a) showed that the inequality of the bound state of glutamate on the relative rate of exchange (deduced from Eq. 1) could be taken into account and thus the inequality of 13C labeling of pyruvate carboxylase (Koss, 2001). Therefore, clearance of glial glutamate, an important process.

One reaction that can be used to explain the different positions of the label the C2 more easily is the reaction of pyruvate carboxylase (Gruetter et al., 1998a; Gruetter and Gopher, 1999; Gruetter, 2002), which incorporates in a rate of A uptake and conversion of pyruvate into glucose.
activation. Glucose metabolism at rest is likely to be oxidative in glia, as judged from the well-known incorporation of acetate label into glutamine (Van den Berg, 1973; Hassel et al., 1997; Waniek and Martin, 1998; Dienel et al., 1999), and proposed from $^{13}$C studies in vivo by NMR (Brand et al., 1997; Hassel et al., 1997; Blumi et al., 2002; Lebon et al., 2002). The previously put forth argument that the majority of glial metabolism in resting brain is probably oxidative (Gruetter, 2002) thus appears valid and there is now general consensus that glial energy metabolism has a significant oxidative component on the order of 0.2–0.1 of that in the glutamatergic compartment, which we were the first to show in vivo (Gruetter et al., 2001).

The presence of dominant oxidative metabolism in the astrocyte does not disprove the hypothesis that lactate produced in astrocytes is also a fuel for oxidative metabolism in neurons. The results obtained first by our laboratory and then confirmed by others suggest that in the human brain approximately one sixth of the ATP production from glucose measured by NMR is in the glial compartment. This leaves at least five sixth of the lactate for export to neurons, if assuming the extreme case that phosphorylation of glucose is an exclusively glial process.

4.3. Anaplerosis and the astroglial TCA cycle

Astrocytes thus clearly have oxygen metabolism at rest and during activation. Assuming, as implied by the scheme in Fig. 8, that the glutamate–glutamine cycle is the sole contributor to flux through glutamine synthetase, the labeling of the carbon backbone of glutamate and glutamine must be identical at isotopic and metabolic steady-state. However, early studies in rat brain extracts (Lapidot and Gopher, 1994), and in human brain (Gruetter et al., 1998a, 2001) have reported that this is not the case. In this context, the inequality of the label distribution between brain glutamine and glutamate does depend on the relative rate of the glutamate–glutamine cycle relative to other reactions as can be deduced from Eq. (2). Furthermore, all potential contributions of label must be taken into account and thus the effect of a variable $V_i$ must be accounted for. We demonstrated that the inequality of label in glutamate and glutamine implied significant contribution of pyruvate carboxylase to the flux through glutamine synthetase (Gruetter et al., 1998a, 2001). Therefore, other metabolic reactions must contribute substantially to the labeling of glial glutamate, and eventually glial glutamine.

One reaction that can lead to a differential labeling of glutamine and glutamate at the different positions of the molecule is the glial enzyme pyruvate carboxylase, which can label the C2 more than the C3 when administering glucose labeled at the 1 and/or 6 position. Pyruvate carboxylase activity is significant in vivo (Lapidot and Gopher, 1994; Gruetter et al., 1998a, 2001; Shen et al., 1999). Although differences exist as to the magnitude of the flux through pyruvate carboxylase, the relative amount of label incorporation into glutamine differs from that into glutamate (Martin et al., 1993; Lapidot and Gopher, 1994; Gruetter et al., 1998a, 2001). As pointed out previously (Gruetter, 2002), even the lowest reported value of 0.04 µmol/g/min (Shen et al., 1999) results in a rate of ATP generation that amounts to ~ 2/3 of the ATP needed for glutamate uptake and conversion to glutamine. A recent study measured the labeling of glutamate
and glutamine from 2-13C glucose and concluded that in the rat brain pyruvate carboxylase contributes approximately 30% to the flux of glutamine synthetase (Sibson et al., 2001). In this context it is important to note that using 2-13C glucose labels the C3 of glutamate directly through pyruvate carboxylase and indirectly through the pentose phosphate shunt.

The fact that pyruvate carboxylase activity is now accepted as a substantial and significant metabolic flux in astrocytes and that astrocytes thus have substantial oxidative energy metabolism calls into question the underlying mechanism for the proposed 1:1 stoichiometric relationship between glutamate neurotransmission and oxidative glucose metabolism (summarized in Fig. 8), but it does not rule out the proposed predominantly astrocytic location of incremental glucose metabolism during activation as suggested (Magistretti and Pellerin, 1996), which remains an intriguing hypothesis. In fact, the observation that during hypoglycemia, astrocytic glycogen accounts for a majority of the metabolic deficit (see above) implicitly supports the presence of this mechanism.

5. Concluding remarks

The new non-invasive method 13C NMR has come a long way: Increases in sensitivity and methodology have paved the way for many new measurements that are now feasible in the live and intact brain, leading to unique insights in anaplerosis, glial and neuronal energy metabolism, metabolic trafficking, brain glycogen metabolism and the regulation of oxidative energy metabolism. It is concluded that considerable care must be exercised when attempting to interpret and model the measured rates of label incorporation.

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