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In vivo nuclear magnetic resonance spectroscopy studies of the relationship between the glutamate–glutamine neurotransmitter cycle and functional neuroenergetics

Douglas L. Rothman[1*], Nicola R. Sibson[2†], Fahmeed Hyder[1], Jun Shen[4], Kevin L. Behar[2] and Robert G. Shulman[3]

Department of [1]Diagnostic Radiology, [2]Neurology and [3]Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT 06520, USA
[4]Division of Medical Physics, Nathan S. Kline Institute for Psychiatric Research, Orangeburg, NY 10962, USA

In this article we review recent studies, primarily from our laboratory, using ^{13}C NMR (nuclear magnetic resonance) to non-invasively measure the rate of the glutamate–glutamine neurotransmitter cycle in the cortex of rats and humans. In the glutamate–glutamine cycle, glutamate released from nerve terminals is taken up by surrounding glial cells and returned to the nerve terminals as glutamine. ^{13}C NMR studies have shown that the rate of the glutamate–glutamine cycle is extremely high in both the rat and human cortex, and that it increases with brain activity in an approximately 1:1 molar ratio with oxidative glucose metabolism. The measured ratio, in combination with proposals based on isolated cell studies by P. J. Magistretti and co-workers, has led to the development of a model in which the majority of brain glucose oxidation is mechanistically coupled to the glutamate–glutamine cycle. This model provides the first testable mechanistic relationship between cortical glucose metabolism and a specific neuronal activity. We review here the experimental evidence for this model as well as implications for blood oxygenation level dependent magnetic resonance imaging and positron emission tomography functional imaging studies of brain function.

Keywords: glutamate–glutamine cycle; *in vivo* ^{13}C NMR; neuroenergetics

1. INTRODUCTION

The application of positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) to localize functional processes is based on observing regional neurophysiological changes when the brain is engaged in mental processes (Posner & Raichle 1994). The regional neurophysiological changes are assumed to reflect the functional activity of the neurons recruited by the mental processes. The neuronal activities of interest are those involved in the communication of information between neurons, such as neurotransmitter release and action potential propagation. Under non-fasting conditions the energy required for these and other neural processes is provided almost exclusively by oxidative glucose metabolism (Siesjo 1978). Functional imaging traces these neural processes by measuring either glucose metabolism or neurophysiological parameters coupled to glucose metabolism (Sokoloff 1991). Depending on the labelled tracer used, PET either directly measures the regional rate of glucose metabolism (CMRglc), or the coupled parameters of the cerebral rate of oxygen metabolism ($CMRO_2$), and the rate of cerebral blood flow (CBF) (Marret *et al.* 1993). The fMRI blood oxygenation level dependent (BOLD) signal is sensitive to both $CMRO_2$ and CBF (Ogawa *et al.* 1998).

A limitation upon the application of functional imaging is that the relationships between neuronal activity and the neuroenergetic changes mapped have been poorly defined. The brain requires energy for many neuronal processes related to function including action potentials, neurotransmitter release and uptake, vesicular recycling and maintenance of membrane potentials (Shephard 1994). In addition, processes not related to short-term function require energy. As recently discussed, the uncertainty in the assignment of the functional imaging signal to specific neuronal processes affects the interpretation of functional imaging studies (Shulman & Rothman 1998).

In vivo nuclear magnetic resonance (NMR) spectroscopy can be used to measure the concentrations and synthesis rates of individual chemical compounds within precisely defined areas in the brain. In particular, *in vivo* ^{13}C NMR has been used to follow the pathways of glucose metabolism. In contrast PET only provides the total regional rate of glucose consumption. Due to the

*Author for correspondence.
†Present address: MRC Biochemical and Clinical Magnetic Resonance Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

sensitivity of the resonance frequency to the chemical environment, ^{13}C NMR allows the non-invasive measurement of the ^{13}C isotopic enrichment of specific carbon positions of glutamate, glutamine and other compounds in living tissue. The natural abundance of the ^{13}C isotope is 1.1% so that in conjunction with the infusion of ^{13}C-enriched substrates the rates of isotopic incorporation may be measured. Substrates labelled with the non-radioactive, stable, ^{13}C isotope have been employed *in vivo* to study metabolic flux, enzyme activity, and metabolic regulation in the living brain of animals and humans (Fitzpatrick *et al.* 1990; Rothman *et al.* 1992; Gruetter *et al.* 1994, 1998; Mason *et al.* 1992, 1995; Lapidot & Gopher 1994; Shen *et al.* 1999*a*).

The focus of this review is the recent use in our laboratory of ^{13}C NMR to non-invasively measure the rate of the glutamate–glutamine neurotransmitter cycle in the cerebral cortex of rats and humans. In the glutamate–glutamine cycle, glutamate released from nerve terminals is taken up by surrounding glial cells (figure 1*a*) and converted to glutamine. Glutamine is then released by the glia and taken up by the neurons, where it is converted back to glutamate, thereby completing the cycle. The ^{13}C NMR experiments have shown that contrary to the traditional understanding of this pathway the rate of the glutamate–glutamine cycle is extremely high in both the rat and human cortex. Furthermore the rate of the cycle increases with brain activity in an approximately 1:1 molar ratio with oxidative glucose metabolism. The measured ratio, interpreted in the light of studies of astrocyte cell cultures by P. J. Magistretti and co-workers, has led to the development of a model in which the majority of brain glucose oxidation is coupled to the glutamate–glutamine cycle (Magistretti *et al.*, this issue). The model provides a testable mechanistic relationship between cortical glucose metabolism and functional neuronal activity.

In this paper, we review the experimental evidence for this model in rat and human cerebral cortex as well as the implications of these findings for the interpretation of BOLD MRI and PET functional imaging studies. Since the key results upon which this model is based have been obtained only within the last five years many questions remain. However, we believe the implications of the results are of sufficient importance for understanding the relationships between neuroenergetics and functional activity to warrant a review at this time.

2. ^{13}C AND ^{15}N NMR MEASUREMENTS OF THE *IN VIVO* RATE OF THE GLUTAMATE–GLUTAMINE CYCLE

Synaptic release of glutamate will deplete the nerve terminal cytosolic precursor pool unless compensated by glutamate reuptake or synthesis. Glial cells have a high capacity for transporting glutamate from the synaptic cleft in order to maintain a low extracellular fluid (ECF) concentration of glutamate (Rothstein *et al.* 1994; Tsacopoulus & Magistretti 1996). *In vivo* and *in vitro* studies indicate that glutamate taken up by glia is converted to glutamine by glutamine synthetase (Meister 1985; Weisinger 1995), an enzyme found exclusively in glia (Martinez-Hernandez *et al.* 1977). Glutamine is released from the glia to the ECF where it is taken up by neurons and converted back to glutamate through the action of phosphate-activated glutaminase (PAG) (Kvamme *et al.* 1985). Based on extensive data from isotopic labelling studies, immunohistochemical staining of cortical cells for specific enzymes, isolated cell and tissue fractionation studies it has been proposed that glutamate (as well as γ-aminobutyric acid (GABA)) taken up by the glia from the synaptic cleft may be returned to the neuron in the form of glutamine (Van den Berg & Garfinkel 1971; Erecinska & Silver 1990; Sonnewald *et al.* 1993; Schousboe *et al.* 1995). The generally accepted model of the glutamate–glutamine neurotransmitter cycle is shown in figure 1*a*.

Despite a wealth of evidence from enzyme localization and isolated cell studies the rate of the glutamate–glutamine cycle and its role in brain function have been controversial due to difficulties in performing measurements in the living brain. Since the neurotransmitter glutamate is packaged in vesicles (Maycox 1990; Nicholls & Attwell 1990), controversy has arisen about the amount of glutamate actually involved in the cycle leading to the concept of a small 'transmitter' versus a large 'metabolic' glutamate pool (Peng *et al.* 1993). Supporting the concept that glutamate neurotransmitter flux is a small fraction of total glucose metabolism are findings in isolated cells and non-activated brain slices of a low rate of label incorporation from [1-^{13}C]glucose (Badar-Goffer *et al.* 1992). The concept of a metabolically inactive neurotransmitter pool was brought into question in 1995 when, using ^{13}C NMR, we measured a high rate of glutamine labelling from [1-^{13}C]glucose in the occipitoparietal lobe of human subjects (Gruetter *et al.* 1994). A high rate of glutamine synthesis (V_{gln}) was calculated from this data (Mason *et al.* 1995). If the rapid glutamine synthesis was due to the glutamate–glutamine cycle it would indicate that the previous view of a relatively small fraction of glutamine metabolism being associated with this flux was incorrect.

At the time of the initial ^{13}C NMR study, the rate of the glutamate–glutamine cycle could not be calculated due to the lack of a model for distinguishing isotopic labelling from this cycle from other sources of glutamine labelling. In the traditional view of brain glutamine metabolism the major pathway of synthesis is the removal of cytosolic ammonia produced by metabolism and uptake of plasma ammonia (Cooper & Plum 1987). Net ammonia removal requires *de novo* glutamine synthesis via the anaplerotic pathway in the glia. In addition several other pathways, including the glial tricarboxylic acid (TCA) cycle, have been proposed as being significant precursors for glutamine synthesis (Cooper & Plum 1987; Bader-Goffer *et al.* 1992).

In order to interpret the ^{13}C isotopic labelling data we developed a metabolic model for separating the pathways of glutamine synthesis. The model was tested by comparing the results of *in vivo* ^{13}C and ^{15}N NMR studies, which measured the incorporation of isotope from infused [1-^{13}C]glucose and $^{15}NH_4$ into the glutamate and glutamine pools in rat cortex, at different levels of plasma ammonia. The surprising result of these studies is that the glutamate–glutamine cycle is the major metabolic flux of glutamine synthesis far exceeding *de novo* glutamine synthesis. The metabolic model and results of these NMR studies are described below.

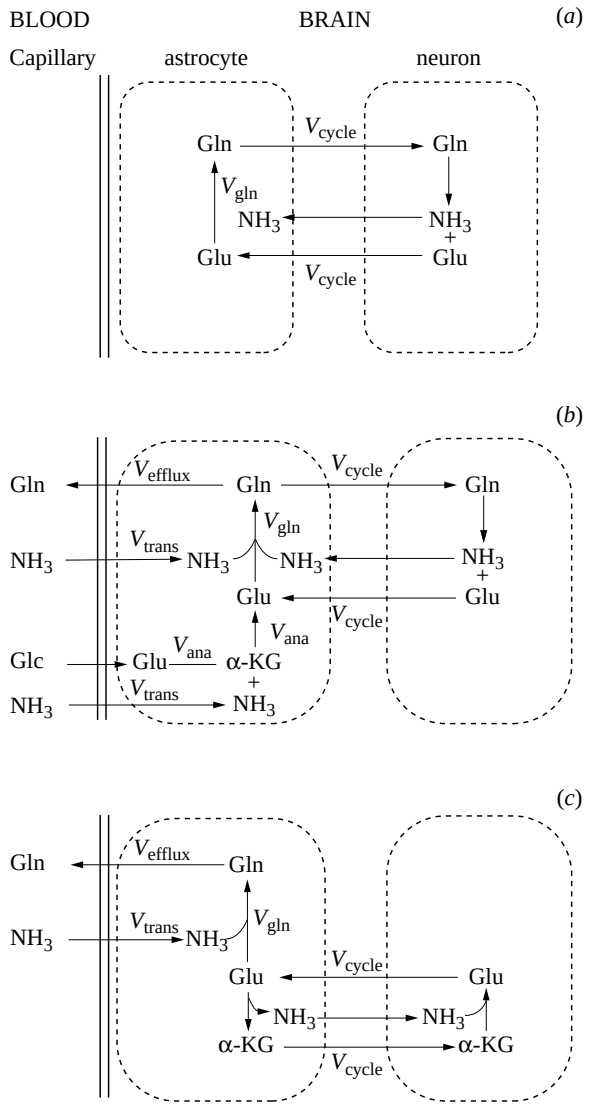

Figure 1. Schematic representations of the glutamate–glutamine cycle between neurons and astrocytes and the detoxification pathway of glutamine synthesis. (*a*) The glutamine–glutamate cycle between neurons and astrocytes. Released neurotransmitter glutamate is transported from the synaptic cleft by surrounding astrocytic end processes. Once in the astrocyte, glutamate is converted to glutamine by glutamine synthetase. Glutamine is released by the astrocyte, transported into the neuron, and converted to glutamate by PAG, which completes the cycle. (*b*) Including the ammonia detoxification (or anaplerotic) pathway of glutamine synthesis. The net rate of glutamine synthesis reflects both neurotransmitter cycling (V_{cycle}) and anaplerosis (V_{ana}). The stoichiometric relationships required by mass balance between the net balance of ammonia and glutamine and V_{ana} are given in equation (2). (*c*). An alternative model for neuronal glutamate repletion in which the astrocyte replenishes lost neuronal glutamate by providing the neuron with α-ketoglutarate (or equivalently other TCA cycle intermediates) (Shank & Campbell 1984; Schousboe *et al.* 1993; Peng *et al.* 1993). α-ketoglutarate is converted back to glutamate by neuronal glutamate dehydrogenase. Glc, glucose; α-KG, α-ketoglutarate: V_{trans}, rate of net ammonia transport into the brain (V_{NH_4} in the text); V_{efflux}, rate of glutamine efflux from the brain; V_{ana}, anaplerotic flux; V_{cycle}, rate of the glutamate–glutamine cycle; V_{gln}, rate of glutamine synthesis. (Reproduced with permission from Sibson *et al.* (1998*b*).)

(a) *Development of a two-compartment metabolic model of glutamine metabolism to separately determine the rate of the glutamate–glutamine cycle and anaplerotic glutamine synthesis*

Glutamine production via glutamine synthetase requires two substrates, glutamate and ammonia. As shown in the flow diagram of figure 1*a*,*b*, glutamine synthesis receives precursor glutamate from both glial uptake of released neurotransmitter glutamate and glial anaplerosis. A mathematical model was developed to interpret isotopic data in order to separate these pathways (Sibson *et al.* 1997; Shen *et al.* 1998, 1999*a*). The model extends previous formulations by imposing mass balance constraints on the brain glutamate and glutamine pools which relate the rate of anaplerotic glutamine synthesis to the net uptake of anaplerotic precursors from the blood. Glutamine efflux is the primary source of nitrogen removal from the brain (Siesjo 1978; Cooper & Plum 1987). Since at steady state the concentration of glutamine remains constant, loss of glutamine by efflux (V_{efflux}) must be compensated for by *de novo* synthesis of glutamine by anaplerosis (V_{ana}). For *de novo* synthesis by anaplerosis, pyruvate derived from glucose is converted by CO_2 fixation (V_{CO_2}) to oxaloacetate by the enzyme pyruvate carboxylase, which is active only in the glia (Wiesinger 1995). Through the action of the TCA cycle, oxaloacetate is converted to α-ketoglutarate. α-ketoglutarate may be converted to glutamate either by ammonia fixation via glia glutamate dehydrogenase or alternatively through transamination with other amino acids. Glial glutamate is then converted to glutamine by glutamine synthetase. Between one and two ammonia molecules are fixed per glutamine molecule synthesized through anaplerosis, depending upon the relative fluxes of NH_4 fixation versus transamination. Applying nitrogen mass balance constraints leads to the relationship $V_{NH_4} = (1 \text{ to } 2)\,V_{efflux}$ at steady state. The additional requirement of carbon mass balance leads to the relationships

$$V_{ana} = V_{efflux} = V_{CO_2} = (1/2 \text{ to } 1)\,V_{NH_4}. \quad (1)$$

Total glutamine synthesis is then related to anaplerotic synthesis for ammonia detoxification (V_{ana}) and the glutamate–glutamine cycle (V_{cycle}) by

$$V_{gln} = V_{cycle} + V_{ana}[= V_{efflux} = V_{CO_2} = (1/2 \text{ to } 1)\,V_{NH_{4s}}]. \quad (2)$$

V_{cycle} may then be derived either from isotopic label analysis or by combining a measurement of total glutamine synthesis with measurement of any of the rates linked by mass balance considerations to anaplerotic glutamine synthesis.

(b) *^{13}C NMR studies of the glutamate–glutamine cycle in rat cerebral cortex*

To determine V_{gln}, rats were studied under α-chloralose anaesthesia in a 7 T modified Bruker Biospec spectrometer. A small ^{13}C surface coil was used for transmission and reception. The spectroscopic volume was localized primarily to the motor and somatosensory cortices. The rats were infused with [1-^{13}C]glucose and the time-course of label incorporation into the C4 positions of glutamate and glutamine measured. The time-courses

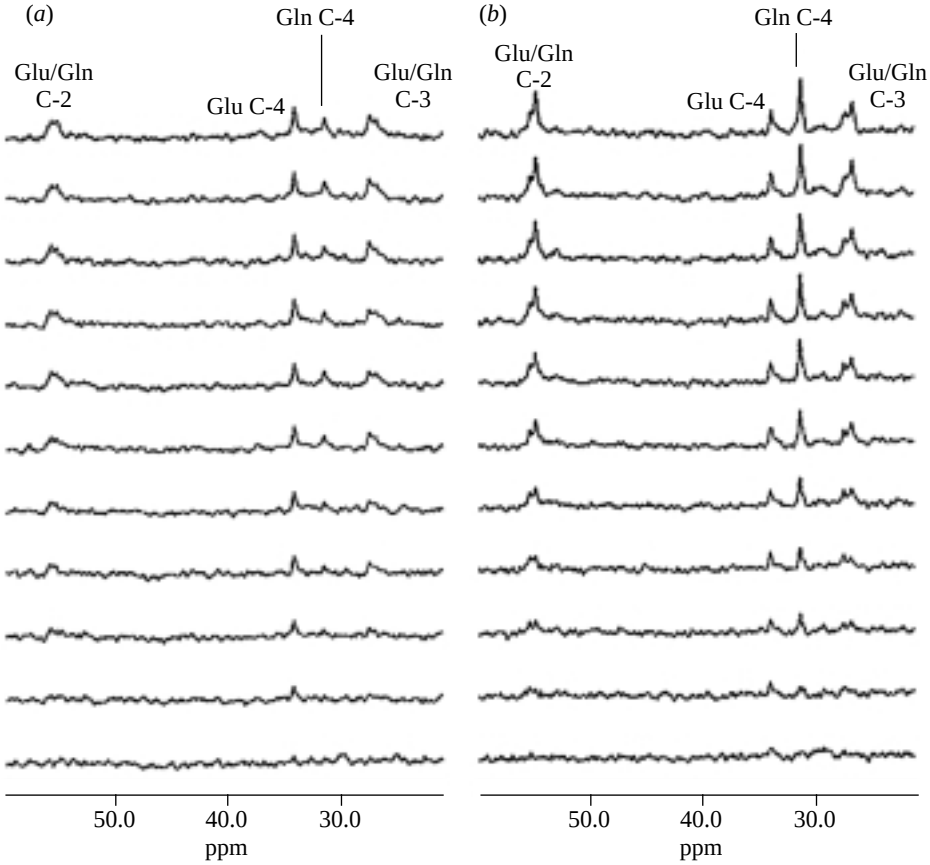

Figure 2. ^{13}C NMR time-courses of glutamate and glutamine labelling in a control (*a*), and hyperammonaemic rat (*b*), demonstrates an increase in glutamine labelling at elevated plasma ammonia. Spectra were obtained using ^{13}C NMR at 7 T of the cortex of the rat during infusion of [1-^{13}C]glucose at two levels of ammonia (adapted from Sibson *et al.* (1997)). The spectra shown were obtained at a time-interval of 10 min. Labelled resonances include [4-^{13}C]glutamate (Glu C-4) and [4-^{13}C]glutamine (Gln C-4). In the hyperammonaemic rat (*b*), the resonance of [4-^{13}C]glutamine is observed to reach a several-fold higher concentration at isotopic steady state and to incorporate the ^{13}C label more rapidly due to the enhanced rate of anaplerosis. (Reproduced with permission from Sibson *et al.* (1997). Copyright 1997 National Academy of Sciences of the USA.)

were fitted using differential equations describing the proposed model of glutamate–glutamine cycling between neurons and glia. The rate of the TCA cycle as measured from label incorporation into glutamate C4 was 0.46 ± 0.12 μmol g^{-1} min^{-1} (mean ± s.d., $n = 5$). Previous studies have shown that label incorporation into the glutamate pool gives an accurate measure of the brain TCA cycle (Mason *et al.* 1992, 1995). The V_{gln} at 0.21 ± 0.04 μmol g^{-1} min^{-1} ($n = 5$), which was nearly half the rate of the TCA cycle (Sibson *et al.* 1997). These results indicate glutamine synthesis is a major metabolic pathway in the rat cortex.

(c) *Comparison of the increase in glutamine synthesis during hyperammonaemia with the predicted stoichiometry with net ammonia and CO_2 uptake, and glutamine efflux*

Elevated plasma ammonia increases the rate of the anaplerotic pathway of glutamine synthesis (Cooper & Plum 1987) in order to remove ammonia from the brain. The metabolic model predicts that under conditions of elevated plasma ammonia the increase in the V_{gln} will be stoichiometrically coupled to the increase in the uptake of the anaplerotic substrates CO_2 and ammonia and the efflux of glutamine from the brain ($\Delta V_{gln} = \Delta V_{ana} = \Delta V_{efflux} = \Delta V_{CO_2} = 1/2 \Delta V_{NH_4}$). To test this prediction, ^{13}C NMR was used to measure glutamine synthesis in rat cerebral cortex under normal and elevated plasma ammonia concentrations. Rats were made hyperammonaemic (0.35 ± 0.08 mM plasma ammonia versus basal levels of $0.05 \pm$ mM) by a primed continuous infusion of ammonia and studied after 4 h of hyperammonaemia to ensure metabolic steady state. The TCA cycle rate was not significantly increased under these conditions relative to the control rats, suggesting that the rate of the glutamate–glutamine neurotransmitter cycle was not substantially altered by this perturbation. V_{gln} under hyperammonaemic conditions increased by 0.11 ± 0.03 μmol g^{-1} min^{-1} relative to the rate under normal plasma ammonia conditions. The increased incorporation of ^{13}C label into glutamine under hyperammonaemic conditions is shown in figure 2 in the comparison between the time-courses of ^{13}C NMR spectra obtained from a rat with normal plasma ammonia with a hyperammonaemic rat.

The metabolic model allows the measured increase in glutamine synthesis during hyperammonaemia to be directly compared with previous measurements of glutamine efflux and the uptake of anaplerotic precursors. The predicted relationships are expressed quantitatively in equation (2). Studies which have used ^{14}C isotope to

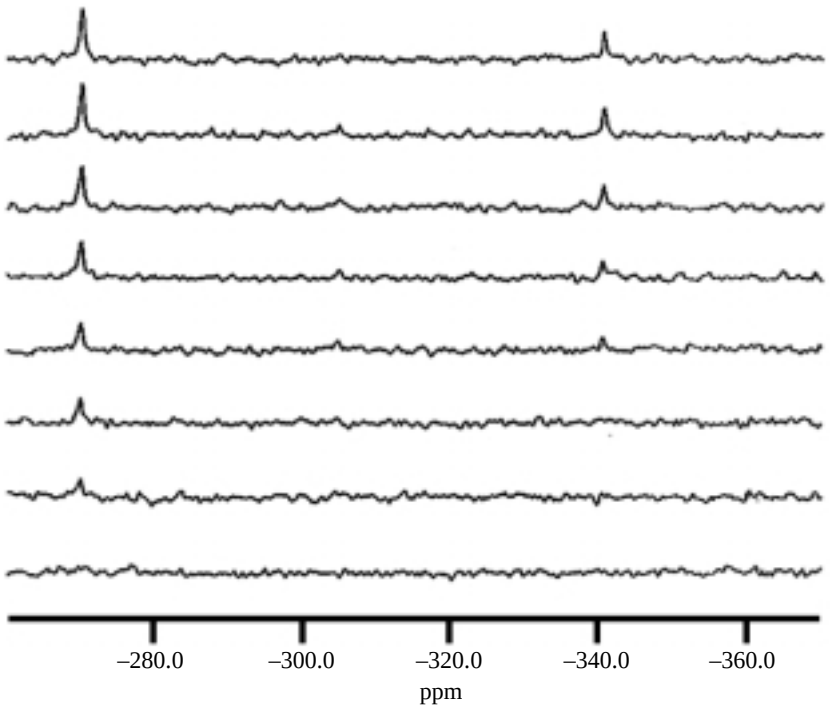

Figure 3. ^{15}NMR time-course of the labelling of glutamate and glutamine from an infusion of $^{15}NH_4$. The downfield resonance of [5-^{15}N] glutamine rises before significant labelling of [2-^{15}N] glutamate and glutamine. (Reproduced with permission from Shen *et al.* (1998).)

measure the increase in V_{CO_2} with hyperammonaemia found a rate of *ca.* 0.12 μmol g^{-1} min^{-1} (Berl *et al.* 1962; Waelsch *et al.* 1964), which is in good agreement with the measured increase in V_{gln}. The rate of glutamine efflux (V_{efflux}) measured under similar conditions was 0.10 μmol g^{-1} min^{-1} (Dejong *et al.* 1992), again in good agreement with the measured V_{gln}. As described below, both aertio-venous difference and direct isotope incorporation measurements of ammonia fixation under hyperammonaemic conditions are also consistent with the predictions of the model. The agreement between the increase in V_{gln} determined by ^{13}C NMR and the increase in anaplerotic substrate use and glutamine efflux predicted by equation (2) provides strong experimental support for the proposed model.

(d) *Determination of the fraction of glutamine synthesis from the glutamate–glutamine neurotransmitter cycle under normal physiological conditions*

To determine the rate of the glutamate–glutamine cycle under physiological conditions we measured V_{gln} by ^{13}C NMR and calculated V_{ana} using equation (2) and previously published measurements of V_{CO_2} and V_{efflux} (Sibson *et al.* 1997). The rate of anaplerotic glutamine synthesis, V_{ana}, was calculated from measurements of glutamine efflux (Hawkins *et al.* 1973; Cooper & Plum 1987) under normal physiological conditions to be 0.00–0.04 μmol g^{-1} min^{-1}. Comparison with our ^{13}C NMR measurement of V_{gln} of 0.21 ±0.04 μmol g^{-1} min^{-1}, yields a V_{cycle} which is 80–90% of the V_{gln}. A similar high percentage of V_{cycle} was calculated using measurements which found the net incorporation of $^{14}CO_2$ into the cerebral cortex to be at most 5–10% of glucose consumption (Cheng 1971).

The conclusion of a low rate of anaplerotic glutamine synthesis under normal physiological conditions is consistent with previous measurements of the total rate of brain anaplerosis (which we propose is almost entirely used for glutamine synthesis). These studies found the incorporation of [2-^{14}C]glucose into glutamate and glutamine to be *ca.* 0.03–0.04 μmol g^{-1} min^{-1} (Van den Berg & Garfinkel 1971; Hawkins *et al.* 1985). A higher fraction of glutamine synthesis from the anaplerotic pathway of 30–50% has been reported in two recent ^{13}C NMR studies using steady-state extract analysis of ^{13}C isotopomers (Lapidot & Gopher 1994; Kunnecke *et al.* 1993). However, in these studies isotopomer formation due to multiple turns of the TCA cycle was neglected leading to a potential overestimate of the fraction from anaplerosis.

(e) *Comparison of the V_{gln} rates determined independently by ^{15}N NMR and ^{13}C NMR*

^{15}N NMR is a useful method for both *in vitro* and *in vivo* study of cerebral glutamate–glutamine metabolism under hyperammonaemic conditions based on the measurement of [5-^{15}N]glutamine and [2-^{15}N]glutamate + glutamine (Kanamori & Ross 1993, 1995). Incorporation of $^{15}NH_4$ into the N5 position of glutamine may be analysed to calculate the flux through glutamine synthetase. In the absence of label exchange the rate of incorporation of labelled ammonia into the N2 position of glutamate + glutamine may be analysed to calculate the rate of glutamate dehydrogenase. The rate of glutamate dehydrogenase (V_{GDH}) determined by $^{15}NH_4$ isotope incorporation under hyperammonaemic conditions is equal to the increase in the rate of anaplerosis.

To obtain an independent measurement of V_{gln} and V_{ana} we have used ^{15}N NMR to measure the rate of $^{15}NH_4$ incorporation into the N5 position of glutamine and the unresolved resonance of N2 glutamate + glutamine (Shen *et al.* 1998). A mathematical analysis based on the model was used to derive V_{gln} from the

NMR measurement of the time-course of [5-^{15}N]glutamine and [2-^{15}N]glutamate + glutamine. As shown in figure 3 the labelling in the first hour was almost exclusively within the N5 position of glutamine, which is consistent with the delayed onset of anaplerosis previously reported under these conditions (Fitzpatrick *et al.* 1989). The low initial rate of label incorporation into N2 glutamate + glutamine is consistent with previous measurements using $^{13}NH_4$ and ^{15}NH4 (Cooper & Plum 1987; Cooper *et al.* 1989; Kanamori & Ross 1993). The low initial rate of anaplerosis allows the rates determined from the ^{15}N NMR study to be compared with the rates measured by ^{13}C NMR under normal physiological conditions. The measured V_{gln} of 0.20 ± 0.06 μmol g^{-1} min^{-1} (mean ± s.d., $n = 6$) from these studies (Shen *et al.* 1998) is in excellent agreement with the results from the ^{13}C NMR measurement of 0.21 ± 0.04 μmol g^{-1} min^{-1} (Sibson *et al.* 1997).

(f) *^{15}N NMR studies to test the relationship in the model between V_{ana} and V_{NH_4}*

To test the relationship of equation (2) between the rate of ammonia uptake in the cerebral cortex (V_{NH_4}) and anaplerotic glutamine synthesis (V_{ana}), total ammonia uptake was calculated from the time-course of the sum of ^{15}N-labelled N5 glutamine and N2 glutamate + glutamine in rat cerebral cortex during infusion of $^{15}NH_4$. These were the only compounds into which appreciable ^{15}N label incorporation was observed, consistent with glutamate and glutamine having the key role in brain ammonia metabolism. The calculated V_{NH_4} from this data was 0.13 ± 0.02 μmol g^{-1} min^{-1} ($n = 6$). Based on the stoichiometric relationship of the model of $1/2\Delta V_{NH_4} = \Delta V_{efflux}$ a rate of anaplerotic glutamine formation of 0.065 ± 0.01 μmol g^{-1} min^{-1} was calculated. From this measurement an increase in the cerebral glutamine pool during the infusion of 0.065 μmol g^{-1} min^{-1} × 180 min = 11.7 μmol g^{-1} glutamine was predicted. This calculation is in excellent agreement with the measured increase in glutamine concentration at the end of the study of 11.1 ± 0.4 μmol g^{-1} (Shen *et al.* 1998). The average rate of anaplerosis calculated over the 3 h period was less than that measured by ^{13}C NMR under steady-state conditions of hyperammonaemia, probably due to the delayed onset of anaplerosis. If a correction is made for the delay of the onset of anaplerosis of approximately 60 min (based on the ^{15}N2 glutamine + glutamate time-course), then the rate of V_{ana} calculated from the study is 0.10 ± 0.02 μmol g^{-1} min^{-1}. This rate is in excellent agreement with the V_{ana} of 0.11 ± 0.03 μmol g^{-1} min^{-1} calculated from the increase in the glutamine synthesis rate determined in the ^{13}C NMR studies.

(g) *Comparison of models of neuronal glutamate repletion from 15N NMR measurements of the fractional enrichment of ^{15}N2 glutamate + glutamine*

The results of the ^{15}N NMR study were used to test the glutamate–glutamine cycle against an alternative proposal for the neuronal repletion. In the alternative model, diagrammed in figure 1*c*, the astrocyte releases α-ketoglutarate to the neuron to replace the carbon skeleton of released glutamate (Shank & Campbell 1984; Shank *et al* 1993). In support of this hypothesis, malate, α-ketoglutarate and citrate are released from glia in cell culture and may be taken up by synaptosomes (Shank 1984; Schousboe *et al.* 1993; Peng *et al.* 1993). This pathway, which is referred to here as the glutamate–α-ketoglutarate cycle, may be distinguished from the glutamate–glutamine cycle based upon the different predictions of ^{15}N labelling of glutamate and glutamine from $^{15}NH_4$. In the glutamate–α-ketoglutarate cycle, one ammonia molecule is incorporated into α-ketoglutarate in the neuron by glutamate dehydrogenase in order to replace each released glutamate molecule, resulting in the production of ^{15}N2 glutamate. In contrast, no ammonia is incorporated into the amino position of glutamate or glutamine in the glutamate–glutamine cycle. Under hyperammonaemic conditions the rate of $^{15}NH_4$ incorporation into the N5 and N2 position of glutamine is the same in the glutamate–α-ketoglutarate cycle since only the anaplerotic pathway of glutamine synthesis is present. In order to distinguish these models the end-point ^{15}N enrichment of the N2 positions of glutamate and glutamine was calculated for each model using the N5 glutamine labelling curve as an input and compared with experimental values. As shown in figure 4, the low fractional enrichment of the ^{15}N2 position of glutamine and glutamate at the end of the study strongly supports the glutamate–glutamine cycle as being the primary pathway of neuronal glutamate repletion.

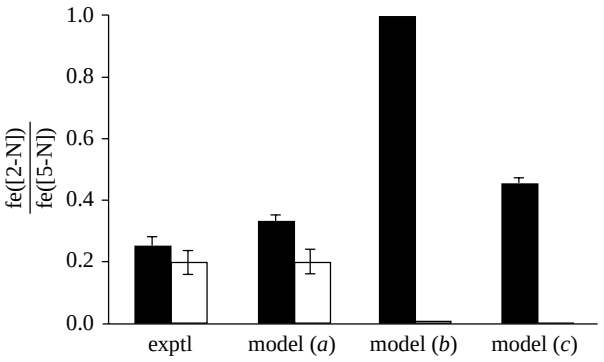

Figure 4. Calculated ^{15}N2:^{15}N5 fractional enrichment (fe) ratios of glutamine (filled bar) and glutamate (open bar) for three models of glial glutamine synthesis. Three models of neuronal glutamate completion were compared with experimental results in which the time-course of [5-^{15}N]glutamine and [2-^{15}N]glutamine and glutamate were measured by ^{15}N NMR in the cortex of a rat infused with $^{15}NH_4$ at 7 T. The measured ratio at the end of the infusion is in excellent agreement with the ratio predicted if the glutamate–glutamine cycle is the major pathway of glutamine synthesis and astrocytic repletion of released neuronal glutamate (model (*a*) in figure 1). If glutamate neurotransmitter repletion took place through the astrocytes providing the neurons with α-ketoglutarate (model (*c*) of figure 1) the rate of anaplerotic and total glutamine synthesis would be similar and the N5:N2 ratio of glutamine would be close to 1.0 as opposed to the measured ratio of 0.25 (model (*b*)). If instead the cycle was internal to the astrocyte the N2:N5 glutamine relative ^{15}N enrichment would be twice as high as measured and no labelling would have been observed in N2 glutamate (model (*c*)).

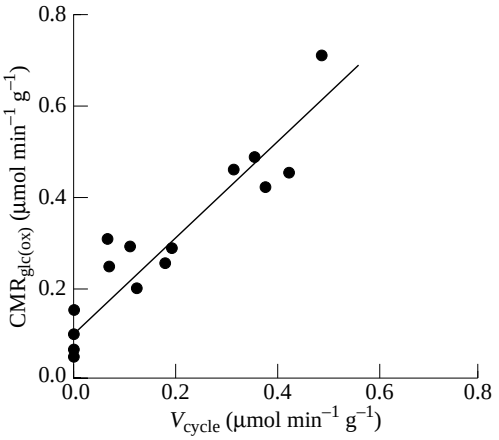

Figure 5. An approximately 1:1 correlation between the rate of oxidative glucose consumption and the rate of the glutamate–glutamine cycle. The rate of glucose oxidation ($CMRglc_{(ox)}$) and the glutamate–glutamine cycle (V_{cycle}) were measured by ^{13}C NMR at 7 T in the rat somatosensory cortex at different levels of cortical activity induced by anaesthesia. A significant positive correlation ($p < 0.001$) was found between $CMRglc_{(ox)}$ and V_{cycle}. The regression line shown is $y = 1.04x + 0.10$ with a Pearson product-moment correlation coefficient, r, of 0.94. (Reproduced with permission from Sibson *et al.* (1998*a*). Copyright 1998 National Academy of Sciences of the USA.)

3. DETERMINATION OF THE *IN VIVO* COUPLING BETWEEN THE RATE OF THE GLUTAMATE–GLUTAMINE NEUROTRANSMITTER CYCLE AND FUNCTIONAL GLUCOSE METABOLISM

In this section we present a description of and evidence for a model in which the glutamate–glutamine cycle directly couples glutamatergic neuronal activity to glucose metabolism. The model is based upon work in cellular systems primarily by P. J. Magistretti and co-workers and our recent findings, using ^{13}C NMR in rat cortex, that the rate of the glutamate–glutamine cycle (i) is in a near 1:1 stoichiometry with glucose oxidation; (ii) increases with increasing brain electrical activity; and (iii) may account for up to 80% of oxidative glucose consumption in the awake resting cortex and an even higher percentage in activated cortex.

(a) *^{13}C NMR studies of the relationship between the glutamate–glutamine cycle and oxidative glucose consumption*

To determine the relationship between the glutamate–glutamine cycle and cortical energetics, ^{13}C NMR was used to measure the rate of glucose oxidation and glutamate–glutamine cycling in rat cortex under conditions of isoelectric EEG induced by high-dose pentobarbital anaesthesia, and at two milder levels of anaesthesia (Sibson *et al.* 1998*a*). Glucose oxidation was determined from the isotopic turnover of [4-^{13}C]glutamate, which has been shown to provide an accurate measure of the brain TCA cycle (Mason *et al.* 1995). Under these conditions the rate of glucose oxidation may be calculated from the rate of the TCA cycle, since glucose is the near exclusive source of substrate for brain energy metabolism (Hyder *et al.* 1996, 1997). Under isoelectric conditions, at which minimal glutamate release takes place, almost no glutamine synthesis was measured, consistent with the ^{13}C NMR measurement of glutamine synthesis primarily reflecting the glutamate–glutamine cycle. Above isoelectricity, the rates of the glutamate–glutamine cycle and glucose oxidation, as assessed from the rate of the TCA cycle determined from the turnover of C4 glutamate (Mason *et al.* 1995), both increased with increasing electrical activity. The results shown in figure 5 indicate an approximately 1:1 relationship between the increase in the glutamate–glutamine cycle and glucose oxidation with brain activity. Under the highest cortical activity studied the glutamate–glutamine cycle rate was approximately 80% of the rate of glucose oxidation.

The glutamate–glutamine cycle measurement described above will also include contributions from the GABA–glutamine cycle (Reubi *et al.* 1978; Sonnewald *et al.* 1993). GABA is the main inhibitory neurotransmitter, and has been measured by *in vivo* ^{1}H and ^{13}C NMR in animals and humans (Rothman *et al.* 1993; Manor *et al.* 1996; Gruetter *et al.* 1994, 1998; Shen *et al.* 1999*b*). Based upon ^{13}C NMR studies under similar conditions the GABA–glutamine cycle may account for as much as 20% of the V_{gln} (Manor *et al.* 1996), if all of the synthesized GABA is released from GABAergic neurons and taken up by the astrocytes. Recently we have developed methods for distinguishing the glutamate–glutamine cycle from the GABA–glutamine cycle and have shown that the glutamate–glutamine cycle is in the order of ten times more rapid in human cortex (Shen *et al.* 1999*a*; see § 3(d)).

(b) *A model for the stoichiometric coupling of the glutamate–glutamine cycle to oxidative glucose metabolism*

Figure 6 shows a model we have proposed in collaboration with P. J. Magistretti and co-workers which provides a mechanistic explanation for the observed ratio of the rates of the glutamate–glutamine cycle to glucose oxidation (Sibson *et al.* 1998*a*,*b*; Magistretti *et al.*, this issue). The model is based upon a proposal of P. J. Magistretti and co-workers that non-oxidative glial glycolysis is coupled to glutamate uptake due to the preference of the glia to use glycolytic ATP to pump out the co-transported Na^{+} ions (Pellerin & Magistretti 1994; Tsacopoulos & Magistretti 1996). The pyruvate and lactate formed by glial glycolysis would then be transported to the neuron where it is oxidized. Prior to the *in vivo* ^{13}C NMR results evidence for the model was primarily from enzyme localization studies and isolated cell studies. Both lines of evidence for localization have been criticized based upon the presence of the enzymes required for glucose transport and glycolysis in the neurons, and the strong dependence of glutamate-stimulated glial glucose metabolism on cell culture conditions (Peng & Hertz 1998).

The ambiguity of the enzyme localization results is not unexpected since it has been shown using metabolic control analysis that the total activity of an enzyme within a metabolic pathway does not determine the flux through the pathway (Fell 1997). Therefore, to determine the rate of a pathway it is necessary to perform measurements in the intact living state. To test the extent that this model is

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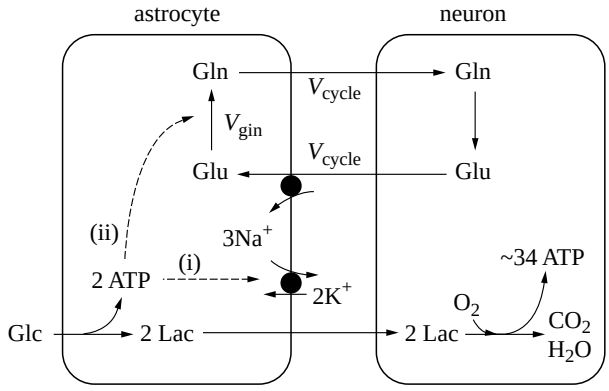

Figure 6. A metabolic model coupling the glutamate–glutamine cycle to oxidative glucose consumption. In this model the two molecules of ATP required by the astrocyte to take up one molecule of glutamate and convert it through glutamine synthetase to glutamine are provided by non-oxidative glycolysis of one molecule of glucose (Glc). The lactate produced by non-oxidative glycolysis is then released from the astrocyte and taken up by the neuron for oxidative glycolysis. Abbreviations: Gln, glutamine; Glu, glutamate; Glc, glucose; Lac, lactate; V_{gln}, rate of glutamine synthesis; V_{cycle}, rate of the glutamate–glutamine cycle. (Reproduced with permission from Sibson *et al.* (1998*a*). Copyright 1998 National Academy of Sciences of the USA.)

supported by the *in vivo* ^{13}C NMR results we calculated the stoichiometric relationship between the glutamate–glutamine cycle and non-oxidative glucose consumption predicted by the model. Glutamate is co-transported into the glia with two or three Na^+ ions, with one K^+ counter-transported (Nichols & Atwell 1990; Danbolt *et al.* 1992; Martin 1995). Transport of three Na^+ out of the glia by the Na^+/K^+-ATPase on the glial end process membrane requires approximately one ATP (Pellerin & Magistretti 1994). Synthesis of glutamine from glutamate through glutamine synthetase requires one ATP molecule per glutamine molecule synthesized (Meister 1985). If the ATP for this process were derived entirely from glycolysis then a 1:1 stoichiometry is predicted between glial non-oxidative glucose consumption and the glutamate–glutamine neurotransmitter cycle. Provided that the lactate formed is released to the neurons for oxidation then this predicted stoichiometry is in excellent agreement with the *in vivo* ^{13}C NMR findings.

(c) *Additional* in vivo *evidence that a large fraction of cortical glucose metabolism is coupled to the glutamate–glutamine cycle*

A prediction of the model is that the majority of glucose oxidation must take place in the synaptic region of glutamatergic neurons. In agreement with this prediction studies using [^{14}C]deoxyglucose autoradiography support the majority of brain glucose uptake being used to support synaptic activity. Increased glucose uptake in response to functional stimulation in peripheral neurons and in cortex is primarily localized in dendritic and nerve terminal cortical layers (where there are associated glial end processes) and not in layers associated with cell bodies (Kadekaro *et al.* 1985; Sokoloff 1991; Kennedy *et al.* 1976).

Glutamatergic neurons make up a large fraction of cortical neurons and account for the majority of glutamate in the cortex. The large glutamate pool which is measured by ^{13}C NMR, which was first identified in ^{14}C tracer studies (Van den Berg & Garfinkel 1971), is believed to correspond to the glutamate pool of glutamatergic neurons (Ottersen *et al.* 1992; Mason *et al.* 1995). Evidence that synaptic glucose uptake is predominantly in glutamatergic neurons, in agreement with the model, is provided by ^{13}C NMR and ^{1}H–^{13}C NMR measurements of glutamate turnover from a [1-^{13}C]glucose precursor (Fitzpatrick *et al.* 1990; Gruetter *et al.* 1994; Mason *et al.* 1992, 1995; Hyder *et al.* 1996, 1997). These studies have shown that over 90% of glucose oxidation in the brain is associated with the large glutamate pool.

The rapid incorporation of ^{13}C label into glutamine by the glutamate–glutamine cycle indicates that the vesicular glutamate pool is rapidly turning over and is in dynamic equilibrium with cytosolic glutamate. This conclusion is in contradiction to the traditional view that the small vesicular pool is metabolically isolated from cellular glutamate metabolism (Bader-Goffer *et al.* 1992; Peng *et al.* 1993; Nicholls & Attwell 1990). However, these studies were performed in cellular and tissue preparations, which have a low rate of synaptic metabolism relative to intact cerebral cortex. In support of this conclusion, Conti & Minelli (1994) showed that inhibition of PAG, which is enriched in nerve terminals (Kvamme 1985) and has been proposed to primarily replete the vesicular pool of glutamate (Peng *et al.* 1993), results in a similar rapid depletion of both synaptic and whole cell glutamate in the cortex of the living rat.

(d) *Neuroenergetic requirements of other processes in the cerebral cortex*

The relationship found between glucose oxidation and the glutamate–glutamine cycle above basal energetic requirements appears to leave no room for other energy-consuming processes in the cortex. For example, what about the energy required for axonal action potentials, and other neurotransmitter systems such as dopamine and serotonin? Within the error of the ^{13}C NMR measurement 10–15% of cortical glucose oxidation may support functional neuroenergetic processes not related to glutamatergic and GABAergic neurons. An additional 15–20% of total glucose oxidation is available to provide energy for processes not related to communication between neurons such as protein and membrane biosynthesis and turnover. However, even with these contributions subtracted the *in vivo* ^{13}C NMR results support the majority of cerebral energy consumption taking place in glutamatergic and GABAergic neurons. This conclusion is consistent with cell staining studies which have shown that the vast majority of synapses and neurons in the cortex are either glutamatergic or GABAergic (Shephard 1994).

(e) *Other potential coupling mechanisms between the glutamate–glutamine cycle and glucose oxidation*

Although the ratio between the glutamate–glutamine cycle and glucose oxidation supports a direct mechanistic coupling with astrocytic glucose uptake and non-oxidative glycolysis there are other potential explanations of these

results. Neurons have the full complement of enzymes needed to perform glycolysis. Several comprehensive reviews of the evidence from molecular and cellular studies supporting astrocytic localization of glucose uptake related to functional neuroenergetics have been published by P. J. Magistretti and co-workers (for example, see Tsacopoulos & Magistretti 1996) and will not be duplicated here. However, *in vivo* fluxes may not be conclusively inferred from *in vitro* studies of enzyme distribution, isolated cells and enzyme kinetics (Fell 1997). For example, the high rate of the glutamate–glutamine cycle found in the *in vivo* NMR studies was not predicted from *in vitro* studies. Therefore, to confirm the proposed coupling of the glutamate–glutamine cycle to glucose oxidation it will be necessary to devise strategies for distinguishing glial glucose uptake from neuronal uptake in the intact cerebral cortex.

4. *IN VIVO* ^{13}C NMR STUDIES OF THE GLUTAMATE–GLUTAMINE CYCLE IN HUMAN BRAIN

With recent improvements in the capability of performing *in vivo* ^{13}C NMR studies of humans there have been several studies performed on human brain during infusions of [1-^{13}C]glucose (Gruetter *et al.* 1992, 1994, 1998; Mason *et al.* 1995; Shen *et al.* 1999*a*) which have measured glutamate and glutamine labelling. These studies are reviewed below with particular emphasis on a recent study in which the rate of the glutamate–glutamine cycle was quantified using the model developed and validated in the rat (Shen *et al.* 1999*a*).

(a) *Measurement of V_{cycle} in the resting occipitoparietal cortex of human brain*

In 1994 we first demonstrated that *in vivo* ^{13}C NMR may be used to measure the rate of glutamine labelling (Gruetter *et al.* 1994; Mason *et al.* 1995) from [1-^{13}C]glucose in human occipitoparietal cortex. These and subsequent studies (Gruetter *et al.* 1998) showed clearly that glutamine is labelled rapidly from [1-^{13}C]glucose. However, the rate of the glutamate–glutamine cycle was not uniquely determined from these first experiments due to the inability to distinguish the glutamate–glutamine cycle from other sources of glutamine labelling. To assess whether such a high level of glutamatergic activity is present in human cortex we determined the rates of the TCA cycle, glutamine synthesis, and the glutamate–glutamine cycle in the human occipitoparietal lobe (Shen *et al.* 1999*a*).

A time-course of the rapid labelling of [4-^{13}C]glutamine and [4-^{13}C]glutamate from [1-^{13}C]glucose is shown in figure 7. The time-course is the summed data from six subjects. A best fit of the metabolic model is plotted through the data. A lag is clearly shown in the labelling of [4-^{13}C]glutamine relative to [4-^{13}C]glutamate, which is consistent with the large neuronal glutamate pool being the main precursor for glutamine synthesis. The combination of the metabolic model and improved NMR sensitivity allowed the rate of the glutamate–glutamine cycle, and anaplerotic glutamine synthesis to be calculated from the ^{13}C NMR data. The analysis gave a total TCA cycle rate of 0.77 ± 0.05 μmol g^{-1} min^{-1} (mean ± s.d., $n = 6$), a glutamate–glutamine cycle rate of

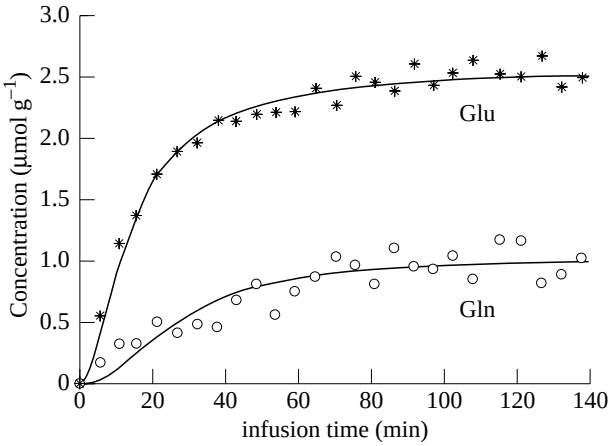

Figure 7. *In vivo* ^{13}C NMR time-course of human occipitoparietal lobe. The time-course of the summed ^{13}C NMR data of six subjects for the concentrations of [4-^{13}C]glutamate and [4-^{13}C]glutamine and the best fit of the two-compartment model. At time zero, an intravenous infusion of [1-^{13}C]glucose was started. The model is shown to provide an excellent fit to the data. The labelling of [4-^{13}C]glutamine is clearly seen to lag behind the labelling of [4-^{13}C]glutamate, consistent with neuronal glutamate being the main precursor for glutamine synthesis via the glutamate–glutamine cycle. Glutamate: stars; glutamine: open circles. (Data from Shen *et al.* (1999*b*).)

0.32 ± 0.04 μmol g^{-1} min^{-1} (mean ± s.d., $n = 6$), and a glucose oxidation rate of 0.39 ± 0.03 μmol g^{-1} min^{-1} (mean ± s.d., $n = 6$). In agreement with studies in rat cortex the glutamate–glutamine cycle is a major metabolic flux in the resting human brain with a rate approximately 80% of glucose oxidation.

In a recent 4 T study of Gruetter *et al.* (1998), a similar rapid labelling of glutamate and glutamine from infused [1-^{13}C]glucose was measured by ^{13}C NMR, although the labelling of [4-^{13}C]glutamine was less well determined than in the 2.1 T study. A high rate of the glutamate–glutamine cycle was reported using a two-compartment model in two subjects, similar to that of the study of Shen *et al.* (1999*a*). Based on deconvolution of the overlapped [2-^{13}C]glutamate and [2-^{13}C]glutamine resonances, *ca.* 50% of glutamine synthesis was calculated to be from the anaplerotic pathway. Such a high rate of anaplerotic glutamine synthesis is inconsistent with previous studies which have found a very low rate of CO_2 fixation and glutamine efflux from the human brain (Cheng 1971; Siesjo 1978). The high rate of anaplerosis may be due to errors in deconvoluting the [2-^{13}C]glutamine resonance from the overlapping more intense resonances of [2-^{13}C]glutamate. However, despite the disagreement on the fraction of glutamine synthesis from anaplerosis, both studies support a high rate of glutamate–glutamine neurotransmitter cycle in human cortex.

(b) *Estimate of the GABA–glutamine cycle in resting human cortex*

As described above, the *in vivo* ^{13}C NMR measurement may potentially be extended to study the rate of GABA synthesis and the GABA–glutamine cycle. However, the ability to directly measure GABA synthesis at 2.1 T is limited by the [2-^{13}C]GABA resonance being overlapped

at 2.1 T by the isotopomer sideband of [4-^{13}C]glutamate. Due to the entry of GABA into the glial TCA cycle at the level of succinate, the labelling kinetics of [4-^{13}C]glutamine derived from GABA are indistinguishable from the label entering through anaplerosis. From our data the maximum estimate of the rate of the GABA–glutamine cycle, obtained by assuming that V_{ana} is entirely due to GABA, would be 11% of the V_{gln}. In the 4 T study of Gruetter *et al.* (1998) the C2 resonance of GABA was resolved, which indicates that direct quantification of the GABA–glutamine cycle will be possible at higher-field NMR systems.

5. IMPLICATIONS OF NMR STUDIES OF THE GLUTAMATE–GLUTAMINE CYCLE FOR THE INTERPRETATION OF FUNCTIONAL IMAGING STUDIES

The measurement of the glutamate–glutamine cycle has implications for connecting models of brain function at the macroscopic level, as studied by functional imaging, with neurobiology studies at the level of synapses and networks of neurons. In this section it is shown that the relationship between the glutamate–glutamine cycle and glucose oxidation may be used to calibrate the PET and fMRI signals in terms of neurotransmitter cycling. Some implications of this quantification are explored.

(a) *The relationship between the glutamate–glutamine cycle and the PET and BOLD MRI functional imaging signal*

The relationship between functional imaging and the glutamate–glutamine cycle is through the tight coupling between glutamate–glutamine cycling and the cerebral metabolic rate of glucose oxidation ($CMRglc_{(ox)}$) in the rat. The BOLD and PET functional imaging signal measures either CMRglc or neurophysiological parameters ($CMRO_2$ or CBF) coupled to glucose metabolism. The similarity in the ratio of V_{cycle} to $CMRglc_{(ox)}$ in human cerebral cortex with the rat (Gruetter *et al.* 1998; Shen *et al.* 1999*a*) supports that the approximately 1:1 molar relationship between CMRglc and the glutamate–glutamine cycle established in the rat holds for the awake resting human brain. Although data are needed to establish the exact stoichiometry during changes of activity, it is reasonable to extrapolate a positive (and possibly stoichiometric) relationship between changes in $CMRglc_{(ox)}$ and V_{cycle} during activation. In this case functional imaging measurements can be interpreted to reflect changes in the rate of the glutamate–glutamine (and to a lesser extent GABA–glutamine) neurotransmitter cycle (Shulman & Rothman 1998).

(b) *Implications of the high rate of V_{cycle} in the non-stimulated cerebral cortex for the quantification of functional neuronal activity*

Despite the lack of stimulation of the occipital and parietal cortices in the ^{13}C NMR studies of human subjects a high rate of the glutamate–glutamine cycle was measured. The high neurotransmitter activity in the non-stimulated brain brings into question the standard cognitive psychology model of the working brain often used to interpret functional imaging data (Posner & Raichle 1994). In simplified terms this model represents the brain as a network of computational modules or mental processes, each with some degree of functional and anatomical segregation (Pinker 1998). The goal of the functional imaging study is to determine the function and anatomical localization of these modules. To achieve this goal subjects are given tasks to perform while being scanned. The degree of involvement of the mental processes or modules supported by a region in the performance of the task is determined by the increment in the magnitude of the imaging signal relative to the signal when the subject is in a resting state. An implicit assumption in this analysis is that the size of the increment of the signal is proportional to the neuronal activity recruited by these mental processes. This assumption would not be valid if instead the entire neuronal activity in the region, which the ^{13}C NMR quantification of V_{cycle} shows is much larger than the increment, was recruited.

The potential confound presented by the high level of non-stimulated neuronal activity may be illustrated through a simple example. Consider a hypothetical experiment in which a subject performs two cognitive tasks. In one task an increment in the functional imaging signal in the frontal lobe of 1% over the resting signal is induced, while the second task induces an increment of 2%. In the standard interpretation, the mental processes used in performing the second task recruit twice the functional neuronal activity needed to perform the first task. However, if the large underlying neuronal activity (which accounts for approximately 80% of the resting signal) was also recruited in performing both tasks, then the differences in the functional neuronal activity between tasks would be minimal (81% versus 82% of the baseline signal).

(c) *Estimation of the fraction of neuronal activity required to support functional processes*

The question of what fraction of total neuronal activity is required to support sensory function was recently addressed in a reanalysis of the functional imaging signals (CMRglc and $CMRO_2$) measured in the sensory cortex of animals exposed to sensory stimulation under awake and anaesthetized conditions (Shulman *et al.* 1999) in which the baseline CMRglc was reduced. Based on the standard paradigm, in which the energy required for the neuronal activity induced by the stimulus is given by the increment in signal from the awake non-stimulated state, a constant increment of signal during stimulation would be predicted independent of whether the animal was anaesthetized or awake. In contrast, if the majority of neuronal activity is required for sensory processing the total signal during stimulation, as opposed to the increment, would be independent of whether the animal was awake or anaesthetized. Results from a large number of studies indicated that the same level of cortical activity was reached during stimulation, independent of the degree of suppression of resting CMRglc by the anaesthesia (Shulman *et al.* 1999). This finding was interpreted as supporting the view that during stimulation the majority of neuronal activity in the sensory regions is involved in supporting sensory function.

(d) *Estimation of the neuronal activity recruited by internally generated mental processes*

The high rate of the glutamate–glutamine cycle found in the unstimulated occipitoparietal cortex, which is considerably higher than the increments predicted during stimulation, raises the question of what is the function of this internally generated neuronal activity. One possibility is that this neuronal activity is recruited by internally generated mental processes, similar to the mental processes induced by experimentally guided tasks and stimuli. Evidence for this possibility are the recent findings of negative signals in both fMRI and PET studies of cognitive processes (Raichle 1998). These changes have been considered paradoxical (Raichle 1998) in the standard interpretation, in which the energy supporting mental processes is represented solely by the increment in signal. In this view a negative change would imply mental processes requiring less energy than the inactive resting state of the brain. However, the negative signal is no longer paradoxical if the high level of neuronal activity in the resting state derives from internally generated mental processes (Shulman & Rothman 1998). In this case, the negative difference signal may be simply explained by the mental processes involved in the task requiring less neuronal activity than the similar internal mental processes of the resting subject.

(e) *Implications for future studies of brain function*

On a cellular level the information transferred by glutamate neurotransmission is dauntingly complex and depends upon myriad factors including synaptic release frequency and quantity, the types of neurons involved and their connectivities, vesicular versus non-vesicular glutamate release, receptor sensitivity, and co-released neuropeptides. The ^{13}C NMR measurement averages over these complexities and provides a bulk measure of glutamate release. However, this bulk measurement provides an important boundary condition for the development of realistic neuronal models of brain function. Although the findings of the relationship between the glutamate–glutamine cycle and brain glucose metabolism are recent, and many aspects of the coupling mechanisms remain to be established, they have significant potential implications for advancing our understanding of brain function. Several implications for the present interpretation of functional imaging studies were presented in a recent article by Shulman & Rothman (1998). We briefly suggest some future directions below.

(i) The signal obtained from functional imaging experiments, insofar as they can be converted to CMRglc or $CMRO_2$, can potentially measure the specific neuronal activity of the glutamate–glutamine cycle. This provides an incentive for present attempts to relate the BOLD signal to cerebral oxygen consumption as well as studies to understand better the mechanisms coupling glucose metabolism to neurotransmitter cycling.

(ii) The magnitude of regional glucose metabolism, not only its increment, links functional imaging to neuronal activity. Experimental paradigms need to be developed which allow determination of the total magnitude of neuronal activity recruited by specific processes.

(iii) Functional imaging, taking advantage of its potential to provide localized quantitative measurements of neuronal activity, should serve as a basis for developing new paradigms for understanding the neurobiological basis of complex brain functions.

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