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REVIEWS

^{13}C Nuclear Magnetic Resonance Investigations of Neurotransmitter Energetics in Brain

Anant Bahadur Patel, Kamal Saba, K.S. Varadarajan and Pravin Kumar Mishra*

Abstract | Glutamate and GABA are the major neurotransmitters in a developed brain. Glucose is the major source of energy in the matured brain under normal physiological conditions. ^{13}C NMR spectroscopy has been used to study neuronal glucose oxidation and neurotransmitter cycle fluxes in rats, mice and human brain. These studies indicate that neurotransmitter energetics is supported by oxidative metabolism.

Although human brain represents only ~2% of the body weight, it accounts for ~20% of total oxygen utilization of the whole body,[1,2] indicating the overwhelming energy demands of the brain. Glutamate and γ-amino butyric (GABA) acid are the major excitatory and inhibitory neurotransmitters, respectively, in the matured mammalian central nervous system. The functioning of these neurotransmitters requires energy in terms of ATP. Glucose metabolism is the major source of energy in matured brain.[3] ^{13}C NMR spectroscopy, together with infusion of ^{13}C labeled substrates, has provided important insight about the energy metabolism in the brain. An energy budget analysis of neurotransmission in the cerebral cortex has indicated that most of the brain energy is utilized for the restoration of ionic gradient across neuronal membrane, action potentials and postsynaptic glutamate receptors.[4,5] In present article, we provide a brief review of ^{13}C NMR approach for the measurement of the energy metabolism in brain.

1 Evidence for Neurometabolism

Initial evidence for neurometabolism was established by studies conducted in mice brain using ^{14}C labeled substrates. The labeling of brain amino acids using ^{14}C labeled glucose or acetate was followed in series of measurements by Van den Berg and colleagues.[6–8] Following observations were made: 1. Incorporation of label into aspartate, glutamate and glutamine from [1-^{14}C]glucose was greater as compared to [2-^{13}C]glucose; 2. Label from [1-^{14}C]/[2-^{14}C]acetate is incorporated to a much larger extent into glutamine than into glutamate. Furthermore, the labeled glutamine is not formed from the glutamate pool, labeled extensively from glucose, but from small glutamate pool. These observations led to proposal for the existence of two distinct TCA cycle for providing energy to brain cells.[9–11] Later, it was discovered that key enzyme, glutamine synthetase and pyruvate carboxylase was compartmentalized in the astroglia.[12] This further consolidated the idea of a glutamate-glutamine neurotransmitter cycle and led to the assignment of the large and small glutamate pools to neurons and glia, respectively.

2 Neurotransmitter Cycle

It has been well established that neuronal astrocytic substrate (glutamate-glutamine) cycle exists in the brain.[13] In this cycle, neurotransmitter glutamate is released from neurons into the synaptic cleft and binds to the receptors (AMPA and NMDA) present on postsynaptic neurons. The binding of neurotransmitter leads to a conformational change in the receptor, which allows the movement of Na^+ into postsynaptic neurons thereby generating action potential. Glutamate is taken up by astrocytes, converted to glutamine by glutamine synthetase[12,14] and returned back to neurons in this synaptic inactive form (Fig. 1). In neurons, glutamine is hydrolyzed to glutamate, re-packaged into vesicles, and is ready for the next release. Similarly, a GABA-glutamine cycle also exists between GABAergic neurons and astrocytes. The net result of the neurotransmitter cycle is to transmit the signal from one neuron to another.

NMR Microimaging and Spectroscopy, CSIR-Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India.
**abpatel@ccmb.res.in*

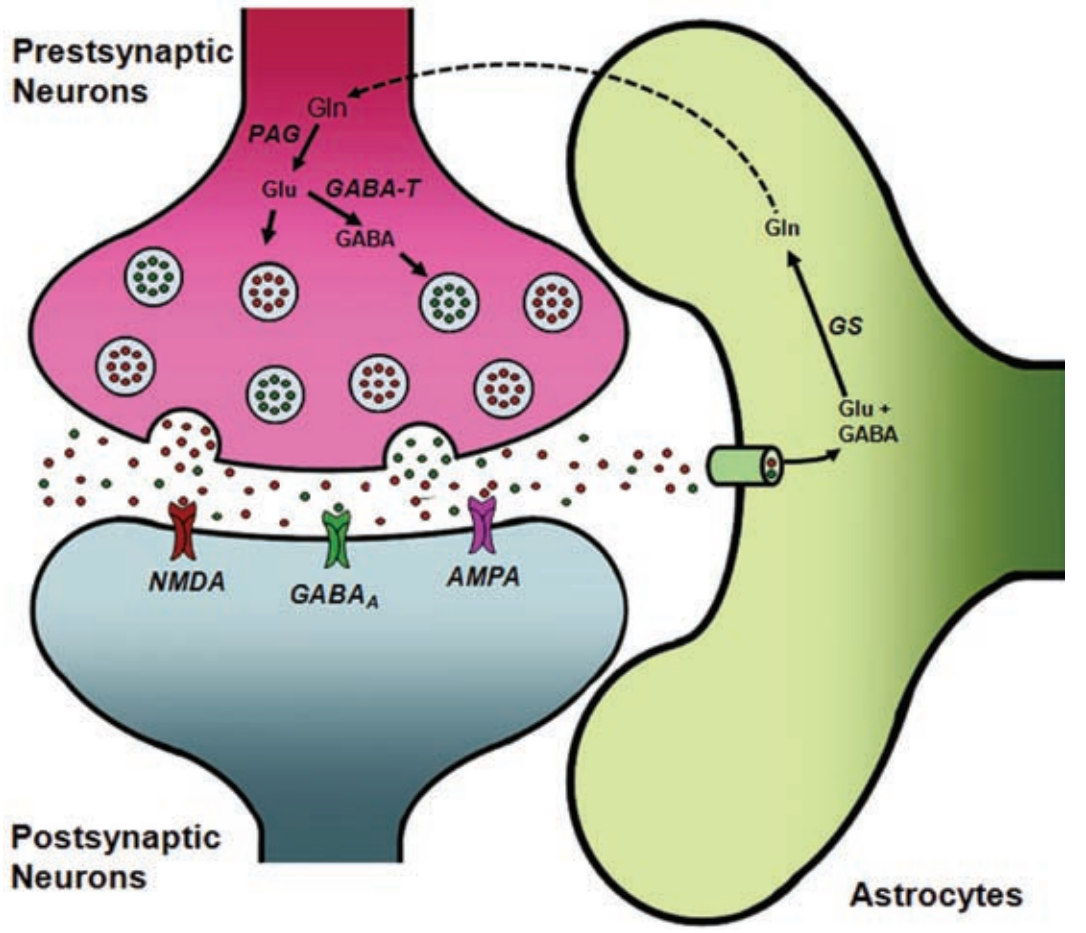

Figure 1: Schematic depiction of neurotransmitter cycle between neurons and astrocytes.

3 Labeling of Brain Amino Acids from ^{13}C Labeled Substrates

^{13}C NMR spectroscopy, together with an infusion of the ^{13}C labeled substrate, has provided insight about the energy metabolism of neural cells.[15–19] The labeling of brain amino acids from different precursors is described below:

3.1 *[1-^{13}C]Glucose and [1,6-$^{13}C_2$]Glucose*

The ^{13}C-labeled glucose is metabolized in the neurons and astroglia to label pyruvate-C3 (Pyr_{C3})/lactate-C3 (Lac_{C3}) by glycolysis and enters the respective TCA cycles through acetyl-CoA-C2 (Fig. 2A). The difference between [1-^{13}C]glucose and [1,6-$^{13}C_2$]glucose is the enrichment of pyruvate-C3, which is doubled in case of [1,6-$^{13}C_2$]glucose as compared to [1-^{13}C]glucose. Acetyl-CoA-C2 condenses with oxaloacetate to yield citrate which is further metabolized, and labels α-ketoglutarate-C4 (α-KG_{C4}), and through fast enzyme-mediated exchange, glutamate-C4 (Glu_{C4}).[15,20,21] In GABAergic neurons, glutamate-C4 is decarboxylated to GABA-C2 by glutamate decarboxylase. Glutamate and GABA released from neurons are transported into astroglia and converted to glutamine-C4 (Gln_{C4}) by glutamine synthetase (Fig. 2A). Gln_{C4} is released from astroglia and transported back to neurons where hydrolysis to Glu_{C4} ($GABA_{C2}$) occurs by phosphate-activated glutaminase. Further metabolism of α-KG_{C4} (Glu_{C4}) in the TCA cycle labels Asp_{C2}, Asp_{C3}, Gln_{C2}, Gln_{C3}, Glu_{C2}, and Glu_{C3}. In addition, carboxylation of Pyr_{C3} in astrocytes yields [3-^{13}C]oxaloacetate, which labels Glu_{C2}/$GABA_{C4}$ and Gln_{C2} by further condensation with acetyl-CoA. [1-^{13}C]/[1,6-$^{13}C_2$]glucose has been used frequently to study neuronal TCA cycle flux and neurotransmitter cycle flux between neurons and astroglia.[20,22–26]

3.2 *[2-^{13}C]Glucose*

[2-^{13}C]Glucose is metabolized to [2-^{13}C]pyruvate by the glycolytic pathway. Most of the [2-^{13}C]pyruvate enters the TCA cycle through pyruvate dehydrogenase pathways and labels Glu_{C5} via a series of reaction as described in

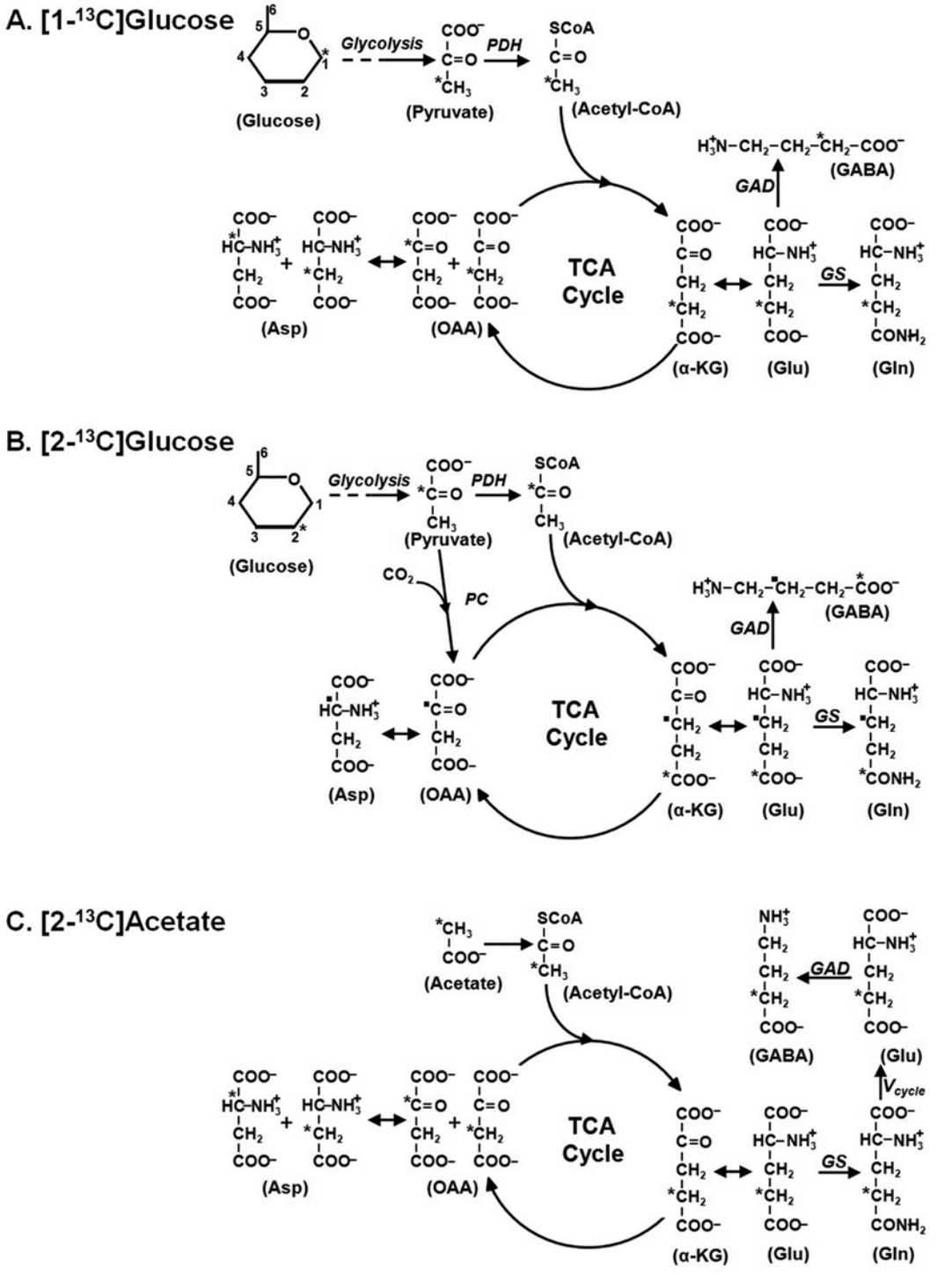

Figure 2: Depiction of ^{13}C labeling of brain metabolites from different precursors.

previous section. The label ^{13}C is lost as CO_2 after the metabolism of Glu_{C5} in subsequent turns of the TCA cycle. [2-^{13}C]Pyruvate, the end product of glycolysis, is carboxylated to [2-^{13}C]oxaloacetate via pyruvate carboxylase which is localized in astrocytes (Fig. 2B).[27,28] Metabolism of [2-^{13}C] oxaloacetate via TCA cycle followed by exchange reaction of α-KG with glutamate in subsequent reactions labels Glu_{C3} ($GABA_{C3}$) and Gln_{C3} (Fig. 2B).[29,30] Further metabolism of Glu/α-KG/GABA via TCA cycle pathway labels $GABA_{C4}$, $GABA_{C3}$, Glu_{C2}, Glu_{C3}, Gln_{C2} and Gln_{C3}. [2-^{13}C] Glucose has been used to evaluate the pyruvate carboxylase flux and neurotransmitter cycle.[29,30]

3.3 *[2-^{13}C]Acetate*

The [2-^{13}C]acetate is exclusively transported and metabolized in astroglia.[31] [2-^{13}C]Acetate is converted to AcetyCoA$_{C2}$, which is oxidized by tricarboxylic acid cycle (Fig. 2C). This leads to the transfer of ^{13}C label into α-KG$_{C4}$ (Glu$_{C4}$), which is converted to Gln$_{C4}$ by glutamine synthetase, an enzyme localized into astrocytes.[12] The ^{13}C label is transferred into the neurotransmitters, Glu$_{C4}$ and GABA$_{C2}$, by the glutamate-glutamine and GABA-glutamine neurotransmitter cycling pathway, respectively (Fig. 2C).[32,33] The metabolism of Glu$_{C4}$ and GABA$_{C2}$ in the subsequent turn of the TCA cycle, incorporates the label into Asp$_{C2}$, Asp$_{C3}$, Gln$_{C2}$, Gln$_{C3}$, Glu$_{C2}$, Glu$_{C3}$, GABA$_{C3}$ and GABA$_{C4}$. As acetate is exclusively transported into astrocytes, infusion of [2-^{13}C]acetate, together with ^{13}C NMR spectroscopy, has been used to evaluate the astroglial metabolic flux, and glutamate-glutamine and GABA-glutamine flux in brain.[33–36]

4 ^{13}C NMR Spectroscopy

^{13}C NMR spectroscopy allows the measurement of concentration and rate of synthesis of individual metabolites. The natural abundance of the NMR active ^{13}C isotope is 1.1%, thus allows measurement of kinetics of ^{13}C incorporation into cerebral metabolites from ^{13}C enriched precursors.[20,23,24,37,38] A typical ^{13}C NMR spectrum after infusion of [1,2-^{13}C$_2$]glucose is presented in Figure 3. The labeling of various amino acids at different carbon positions could be easily seen. The dynamic evaluation of labeling provides turnover of metabolites, which could be analyzed to determine the metabolic flux associated with different pathways. The major advantage of ^{13}C NMR spectroscopy is the well resolved resonances with minimal overlap. However, it has inherent low sensitivity. Therefore, ^{13}C NMR based measurements require concentrated samples, which may not be possible under *in vivo* condition due to limited concentration. The signal to noise could be improved by acquiring ^{13}C NMR spectra for longer time. However, it may compromise the time resolution in dynamic ^{13}C NMR spectroscopic study that may affect the kinetic information of the ^{13}C turnover of amino acids.

The sensitivity of ^{13}C detection could be improved drastically by indirect detection and employing a spectral editing approach that utilizes heteronuclear scalar coupling between protons and ^{13}C nuclei.[39,40] This approach, commonly known as proton observed ^{13}C edited (POCE), involves the acquisition of two sets of spin-echo ^{1}H NMR spectra with an alternating 'OFF'/'ON' ^{13}C inversion pulse (Fig. 4). In the absence of RF

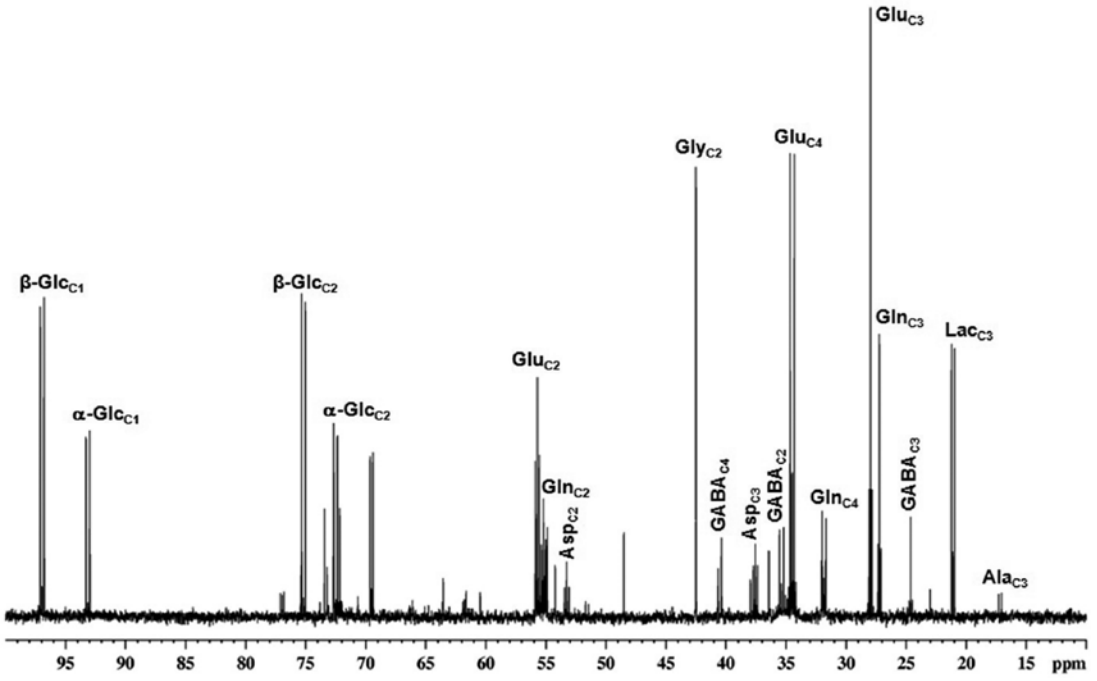

Figure 3: A section of proton decoupled ^{13}C NMR spectrum of mouse cerebral cortex. Mouse was infused with [1,2-^{13}C$_2$]glucose for 90 min. Brain metabolites were extracted using ethanol extract protocol. Abbreviation used are: Ala$_{C3}$, alanine-C3; Asp$_{Ci}$, aspatrate-Ci; GABA$_{Ci}$, γ-aminobutyric acid-Ci; Glc$_{Ci}$, glucose-Ci; Glu$_{Ci}$, glutamate-Ci, Gln$_{Ci}$, glutamine-Ci; Lac$_{C3}$, lactate-C3.

pulses on the Carbon-13 frequency, heteronuclear scalar coupling is refocused and the proton resonances from ^{13}C labeled metabolites appear in phase with the rest of protons (Fig. 4A). When a ^{13}C 180° RF pulse is applied in conjunction with a ^{1}H 180° RF pulse, the phase of the proton magnetization attached with ^{13}C spin evolve due to scalar coupling and become antiphase if the echo time (TE) is set to $1/J_{IS}$ (where J_{IS} is the ^{1}H-^{13}C coupling constant) (Fig. 4B). The subtraction of the two spectra (Figs. 4A and B) will result in the selective observation of protons attached to ^{13}C only (Fig. 4C). In addition to higher detection sensitivity of ^{13}C-labeled metabolites, POCE allows detection of the total (^{12}C + ^{13}C) metabolite pools, leading to a straightforward determination of ^{13}C enrichments.

5 ^{13}C NMR Spectroscopy in Brain

Although the foundation of neurometabolism was laid in late 1960s, the first measurement of flux of glucose to glutamate in the rat brain *in vivo* was carried out in the early 1990 by Yale group.[40,41] In these studies, ^{13}C labeling of brain amino acids, glutamate and glutamine, from [1-^{13}C]glucose was monitored by POCE NMR spectroscopy. It was noted that the incorporation of ^{13}C label into Glu_{C4} and Gln_{C4} increased exponentially, reached to steady state very quickly, while incorporation of label into Glu_{C3}/Gln_{C3} is substantially slow and took much longer to reach the steady state.[22,42] Most importantly, the increase in ^{13}C labeled glutamate and glutamine represented isotopic enrichment and was not due to an increase in total concentration.

By following the flow of ^{13}C label into different metabolites from precursors (glucose, acetate, etc.), ^{13}C NMR spectroscopy provides turnover of different brain metabolites.[20,23,24,33,37,38,42] These data, in combination with knowledge of compartmentalized glutamate metabolism, allowed the development of a quantitative, mathematical model of cerebral metabolism.[24,37,42,43] These models allow determination of the individual rate of glucose oxidation in glutamatergic neurons and GABAergic neurons. Additionally, it yields the rate of neurotransmitter cycle.[33,37,42] The rates of glucose oxidation measured by ^{13}C NMR

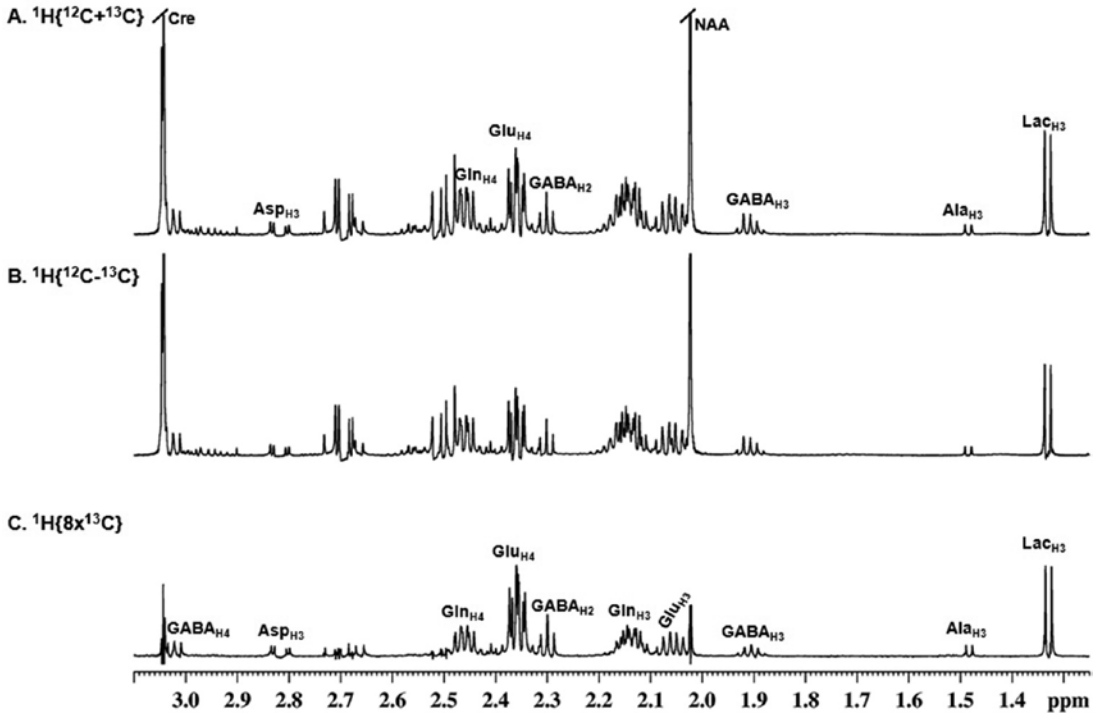

Figure 4: A section of ^{1}H-[^{13}C]-NMR spectrum of mouse cortical extract. Mouse was infused with [1-^{13}C]glucose for 90 min. Brain metabolites were extracted using ethanol extract protocol. (A) ^{1}H[^{12}C + ^{13}C] represents total concentration of metabolites; (B) ^{1}H[^{12}C – ^{13}C] spectrum depicts signal intensity of unlabeled minus labeled molecules; (C) The ^{1}H[8 × ^{13}C] spectrum was obtained by subtracting spectrum (B) from spectrum (A), and represents the concentration of ^{13}C labeled molecule. The abbreviations used are: Ala_{H3}, alanine-C3H; Asp_{H3}, aspatrate-C3H; $GABA_{Hi}$, γ-aminobutyric acid-CiH; Glu_{Hi}, glutamate-CiH, Gln_{Hi}, glutamine-CiH; Lac_{H3}, lactate-C3H.

spectroscopy have been in excellent agreement with that obtained by arteriole-venous difference and positron emission tomography.[17] However, NMR is unique in allowing assessment of the mitochondrial energetics of specific cell types and neuronal-astroglial neurotransmitter cycles.

6 Measurements of the Rate of Neuronal TCA Cycle

The mathematical modeling of ^{13}C turnover of amino acids from glucose/acetate provides metabolic rates in a region of brain. Initially, one compartment model was used for the analyses of measured NMR data.[41,44] However, with the improvement in the quality of NMR spectra (due to superior pulse sequences and higher field magnet), ^{13}C labeling of amino acids at different carbon positions could be monitored precisely. The turnover data of amino acids at multi-carbon positions led to the development of multi-compartments mathematical models, two compartments[20,23,24,33,45] and three compartments,[37,42] to estimate the metabolic fluxes of different cell types (glutamatergic neurons, GABAergic neurons, astroglia). In addition to neuronal TCA cycle flux, these models also provide an estimate of mass flow between neurons and astrocytes, commonly known as neurotransmitter cycling flux. The rate of the neuronal TCA cycle (glucose oxidation) has been determined in several studies using ^{13}C-{^{1}H}-NMR and ^{1}H-{^{13}C}-NMR measurements of cortical glutamate turnover from a [1-^{13}C]glucose in animals[20,22,37,43] and human.[24,38,46] The total glucose oxidation measured by ^{13}C NMR spectroscopy in the rat and human brain is associated with the large glutamate pool, and is believed to reflect the rate of glutamatergic neurons. A large fraction of cortical synapses is glutamatergic and the high electrical activity of glutamatergic pyramidal cells may explain why such a large fraction of total glucose oxidation is associated with glutamatergic neurons.[5]

7 Neurotransmitter Cycle is a Major Metabolic Pathway for Glutamine Synthesis

Although the pathways of astroglial glutamate uptake and cycling were well established from cellular studies, their physiological importance was controversial prior to *in vivo* NMR studies. Because the neurotransmitter glutamate is packaged in vesicles,[47,48] the concept arose of a small, non-metabolic 'transmitter' pool, which did not interact with the large 'metabolic' pool. Initial studies which brought this concept into question was the observation of a high rate of glutamine labeling from [1-^{13}C]glucose using ^{13}C NMR.[49] To test if this rapid labeling was due to the glutamate-glutamine cycle, series of ^{13}C NMR studies were performed in rats to determine whether the glutamine was synthesized primarily from released neuronal glutamate or for ammonia detoxification as previously believed.[50] The cerebral Tricarboxylic Acid (TCA) cycle rate and the rate of glutamine synthesis were measured in rats *in vivo* under normal physiological and hyperammonemic conditions using ^{13}C NMR spectroscopy.[51] The rate of glutamine synthesis under hyperammonemic condition was significantly higher than that in the control rats. This study indicated that the glutamate-glutamine cycle accounts for major fraction (>80%) of glutamine synthesis, and most importantly has a metabolic rate similar to neuronal glucose oxidation.[26] Similar results were found in rat cerebral cortex by using $^{14}CO_2$ as a label precursor[52] and ^{13}C NMR studies.[20,21,26,37,38,43]

8 Measurements of Astroglial TCA Cycle Flux

Traditionally, brain activity is linked to neuronal function. Therefore, most of studies were devoted to understand the metabolic activity of neurons under different stimuli, and very little effort was made to understand the astroglial metabolic activity. As depicted in Figure 1, astroglia play important role in clearance of neurotransmitters from the synapse and further processing to complete the neurotransmitter cycling. Therefore, it is prudent to investigate metabolic activity of astrocytes. The astroglia activity could be monitored by using [2-^{13}C]acetate, an astroglial specific substrate.[31] The [2-^{13}C]acetate transport and metabolic measurement carried out under halothane in rat cerebral cortex suggested that distribution space for acetate in brain is small, indicative of large brain volume is excluded for acetate.[33] Moreover, this study also indicated that astroglia contribute to ~21% of oxidative metabolism of the brain. The astroglial metabolic flux measured under α-chloralose, a deeper anesthetic than halothane, accounts for the 38% of the oxidative metabolism of rat cerebral cortex.[34] The increased contribution of astroglia to the total metabolic activity may be due to the reduced activity of neurons under α-chloralose condition. In human, the astroglial TCA cycle flux is estimated to be ~15% of total energy requirement of the cerebral cortex.[35] Further studies are needed to understand energetics of astrocytes under different brain activity.

9 Relationship Between the Rate of Neurotransmitter Cycle and the Neuronal TCA Cycle Flux

To determine the relationship between the neurotransmitter cycle and cerebral cortex mitochondrial energetics, ^{13}C NMR study was carried out to measure the rate of neuronal TCA cycle and the neurotransmitter cycle, simultaneously, in rat cerebral cortex with graded anesthesia.[20,26] From these measurements, the rates of tricarboxylic acid (TCA) cycle and glutamate-glutamine neurotransmitter cycling between neurons and astrocytes were calculated. The glutamate-neurotransmitter cycling provides a quantitative measure of glutamatergic neuronal activity. The relationship between the neuronal TCA cycle and glutamate-neurotransmitter cycle was evaluated by measuring the rates of the TCA cycle and glutamine synthesis over a wide range of synaptic activity in the rat cerebral cortex. With increasing electrical activity, the rate of neurotransmitter cycle and neuronal mitochondrial TCA cycle flux increased proportionately with a near 1:2 slope[16] (Fig. 5), indicating neurotransmitter energetics is supported by neuronal oxidative glucose metabolism. Although these analyses were carried out using two compartment metabolic model, the results obtained using three compartment metabolic models[21,42,53] lie very close to the line predicted by the previous study. These data indicate that the majority of cortical energy production supports functional (synaptic) glutamatergic neuronal activity. Secondly, brain activation studies, which map cortical oxidative glucose metabolism, provide a quantitative measure of synaptic glutamate release. These findings are consistent with the high rate of the neurotransmitter cycle found in NMR studies of human cerebral cortex.[24,35,38]

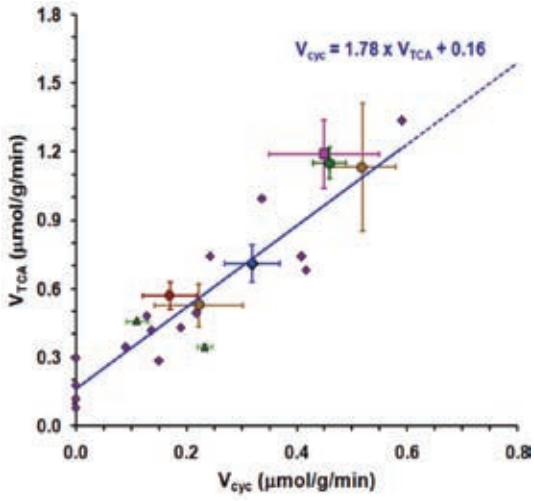

Figure 5: Correlation between tricarboxylic acid cycle (V_{TCA}) and neurotransmitter cycling flux (V_{cyc}) in rat, human and mouse cortex. The metabolic fluxes were obtained by modeling of ^{13}C turnover of amino acids form [1-^{13}C]/[1,6-$^{13}C_2$]glucose by two or three compartment metabolic model. The data used are—red color diamond (Sibson et al., 1998);[26] blue color diamond (Shen et al., 1999);[38] green color circle (Tiwari et al., 2013);[21] green color triangle (Duarte et al., 2011, 2013);[42,45] pink color square (Oz et al., 2004);[43] red color circle (Gruetter et al., 2001);[24] yellow color circle (Patel et al., 2004).[20]

10 Conclusion

^{13}C NMR spectroscopy in conjunction with infusion of ^{13}C labeled substrates provides turnover of brain metabolites at specific carbon position. The appropriate modeling of these data yield quantitative estimate for the brain activity. This approach might be useful to understand the brain pathology under different cerebral disorders.

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Anant Bahadur Patel 1971. M.Sc. (1993) in Chemistry from Banaras Hindu University, Varanasi and Ph.D. (1999) from the Chemical Physics Group, Tata Institute of Fundamental Research, Mumbai. He was a Postdoctoral Research Associate (1999–2003) and Associate Research Scientist (2003–2006) at MRRC, Yale University, USA. Dr. Patel, after returning to India in 2006, has set up a state-of-the-art NMR Microimaging and Spectroscopy laboratory at the CSIR-CCMB, and is carrying out research in the field Brain Energy Metabolism in Neurological Disorders. His research interest includes development and application of the state of art stable ^{13}C isotope methods to study different pathways in brain. He has authored more than 45 research articles in peer reviewed journal.

Kamal Saba 1986. M.Sc. (2011) in Biotechnology from Brindavan College, Bangalore University, Bangalore. Currently working as Project Junior Research Fellow at the CSIR-CCMB, Hyderabad. His research interests is to investigate the energetics of neurotransmission using ^{13}C NMR spectroscopy together with infusion of stable ^{13}C substrates in genetic and chemical models of Alzheimer's disease in mice.

K.S. Varadarajan 1983. B.Sc. (2004) and M.C.A. (2008) from Osmania University. Currently working as Technical Assistant Gr III(2) in NMR Microimaging and Spectroscopy Facility at the CSIR-CCMB. His research interest is to understand neurotransmitter energetics in genetic and chemical models of Parkinson's disease.

Pravin K. Mishra 1983. B.Sc. (2007) Chemistry (Hons.), Banaras Hindu University and M.Sc. (2009) in Biochemistry from Dr. R.M.L. Awadh Uni. Faizabad. Currently pursuing Ph.D. under supervision of Dr. Anant B. Patel at CCMB, Hyderabad since 2010. His current research interest is to explore the energetics of glutamatergic and GABAergic neurotransmission in depression using ^{13}C NMR spectroscopy in conjunction with infusion of stable ^{13}C labeled substrates.