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Investigation of metabolic kinetics in different brain regions of awake rats using the [^{1}H-^{13}C]-NMR technique

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Abstract: Energy metabolism and neurotransmission are necessary for sustaining normal life activities. Hence, neurological or psychiatric disorders are always associated with changes in neurotransmitters and energy metabolic states in the brain. Most studies have only focused on the most important neurotransmitters, particularly GABA and Glu, however, other metabolites such as NAA and aspartate which are also very important for cerebral function are rarely investigated, ,. In this study, most of the metabolic kinetics information of different brain regions was investigated in awake rats using the [^{1}H-^{13}C]-NMR technique. Briefly, rats (n=8) were infused [1-^{13}C] glucose through the tail vein for two minutes. After 20 minutes of glucose metabolism, the animals were sacrificed and the brain tissue was extracted and treated. Utilizing the ^{1}H observed/^{13}C-edited nuclear magnetic resonance (POCE-NMR), the enrichment of neurochemicals was detected which reflected the metabolic changes in different brain regions and the metabolic connections between neurons and glial cells in the brain. The results suggest that the distribution of every metabolite differed from every brain region and the metabolic rate of NAA was relatively low at 8.64 ± 2.37 μmol/g/h. In addition, there were some correlations between several ^{13}C enriched metabolites, such as Glu$_4$-Gln$_4$ (p=0.062), Glu$_4$-GABA$_2$ (p<0.01), Glx$_2$-Glx$_3$ (p<0.001), Asp$_3$-NAA$_3$ (p<0.001). This correlativity reflects the signal transmission between astrocytes and neurons, as well as the potential interaction between energy metabolism and neurotransmission. In conclusion, the current study systematically demonstrated the metabolic kinetics in the brain which shed light on brain functions and the mechanisms of various pathophysiological states.

***Key words**:* Brain, ^{1}H observed/^{13}C-edited, Metabolic kinetics, N-acetylaspartate, Nuclear magnetic resonance.

1. Introduction

Energy metabolism is considered the basis of life which plays an essential role in maintaining normal life activities and metabolic processes[1]. As a high-energy consuming organ, the brain accounts for 20% of glucose consumption, although it comprises only 2% of body weight in adults[2]. The restoration and maintenance of various ionic gradients, as well as the uptake and circulation of neurotransmitters have been gradually regarded as the main reasons for the high energy demand in the brain. In the 1970s, Sokoloff and his partners speculated that the synapse-rich areas of the nervous system consume most of the glucose[2]. Pellerin and his colleagues found close coupling between the glutamatergic neuron activity and glucose metabolism in the cerebral cortex with a stoichiometric ratio of nearly 1:1[3]. These studies indicate that the most generated energy in the brain is used to maintain the functional activity of neurons. Accordingly, the occurrence of various neuropsychiatric diseases is closely related to the state of neurotransmitters and energy metabolism in the brain, in this sense, a bird’s eye view of various metabolic kinetics in different brain regions is very important particularly for brain researchers.

There are many techniques available to reflect the level of energy metabolism coupled with neuronal activity, such as magnetic resonance imaging spectroscopy (MRS)[4], positron emission tomography (PET)[5] and autoradiography[6]. These methods not only provide information about macroscopic cerebral metabolic changes, but also distinguish the changes in markers involved in different metabolic responses and reflect the real-time changes in metabolites. However, the signal to noise ratio is much lower. In contrast with these techniques, the nuclear magnetic resonance spectroscopy

(NMR) approach is not only a noninvasive and stable method, but also provides higher resolution and various information for different chemicals with multiple methods, including ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR, *etc*. ^{1}H-NMR is mainly used for mixture analysis and metabolomics studies; ^{31}P-NMR plays a vital role in detecting ATP generation and pH homeostasis; ^{13}C-NMR is always regarded as a unique technique to reflect the brain metabolic fluxes *in vivo*[7]. Additionally, the chemical shift in all spectra could be used to discriminate specific metabolites.

In metabolic flux studies, the infusion of ^{13}C labeled substrates, such as glucose, acetate or ketone bodies is always used in investigations[8]. After the infusion of ^{13}C enriched substrates, the ^{13}C labeled substrates were gradually oxidized to the other ^{13}C labeled metabolites, which could be accurately detected with the NMR method and directly reflect the cerebral metabolic kinetics information[8]. Here, [^{1}H-^{13}C] (^{1}H observed/^{13}C-edited) the nuclear magnetic resonance (POCE-NMR) technique is one of the most frequently-used NMR techniques which is an attractive approach for detecting metabolic kinetics. POCE-NMR can help to measure the composition of metabolites, detect changes in the metabolic rate of energy sources and reflect the dynamics of neurotransmitter transmission[8]. By applying this technique, we can detect the ^{1}H signal which has higher sensitivity than the normal ^{13}C-NMR method, as well as separate hydrogens attached to ^{13}C from those attached to ^{12}C. It has been reported in other studies that there was a 14-fold improvement in sensitivity to detect the CH_3 signal in the rabbit brain using the proton spectroscopy compared to the direct carbon spectroscopy[9]. Thus, the POCE-NMR was applied in the present study.

Furthermore, in the field of brain metabolic dynamics, many studies have focused on examining the neurotransmitter circulation between neurons and astrocytes in maintaining neurotransmitter homeostasis, such as Glu-Gln (glutamate-glutamine) cycle and GABA-Glu (γ-aminobutyric acid-glutamate) cycle[10]. However, there are few studies that have investigated the dynamics of other small molecular metabolisms, which may also play a crucial role in brain function. For example, N-acetylaspartate (NAA) has been used as a neuronal biomarker to reflect neuronal function and density [11] and it is implicated in many metabolic processes, such as myelination and oxidative metabolism[12]. Thus, the metabolic kinetic information of the NMR detectable metabolites was investigated in the current study.

Here, [1-^{13}C] glucose was infused in the rats and various metabolites were detected with [^{1}H-^{13}C] NMR technology. The aims of this study were (i) to explore the metabolic kinetics of different brain regions and (ii) to reflect the metabolic cross-talk between neurons and astrocytes through correlations between neurochemicals. This study could provide various metabolic information for various brain regions, which is very important for neuroscience research.

2. Material and methods

2.1 Animal preparation

The experiment was carried out according to protocols approved by the Animal Ethics Committee of Zhongnan Hospital of Wuhan University (Ethics approval number: WP2020-08087). All operations were performed according to the National Institutes of Health Guidelines for the Care and Use of

Laboratory Animals. Adult male Sprague-Dawley rats (2 months old, weighing 230-300g, n=8) used in the current study were obtained from Hubei Center for Disease Control and Prevention (Wuhan, China). The rats were placed in a 12h light-dark cycle with a temperature-controlled environment and food and water available. Every effort was made to reduce any pain in animals and the number of rats used.

2.2 Animal experiment

The ^{13}C enrichment of different carbon positions of metabolites was detected to reflect the metabolic kinetics[13,14] through infusing [1-^{13}C] glucose (Qingdao Tenglong Weibo Technology co., LTD, Qingdao, P.R. China). To obtain higher enrichment of ^{13}C glucose, it is necessary to minimize the endogenous unlabeled glucose. Therefore, all animals were fasted overnight and only had free access to water (16-18 hours) before the experiment.

On the experimental day, animals were anesthetized with 1.5%-2.5% isoflurane mixed with 30% O_2. When rats had no response to a foot pinch under appropriate anesthetic depth, two drops of blood were collected from a needle prick to the tip of the tail to test the blood glucose level using glucose test strips (Yuyue, China) before infusion. Then, PE50 tubing (Instech PA USA) was inserted into the lateral tail vein with a 24-gauge needle to infuse ^{13}C labeled glucose and secured with adhesive tape. After that, the rats were allowed to recover for about 15 minutes until they could move freely. The infusion tube was then connected to a swivel (Instech, PA, USA) and the other side of the swivel was connected to the pump (Fusion 100, Chemyx, TX, USA) using PE50 tubing. Finally, [1-^{13}C] glucose was pumped at 400-600μL/min (dependent on the animal weight) through the lateral tail vein for two minutes (The dosage was based on the previous infusion protocol[14]), while the rats could move freely in cages.

After 20min, the animals were deeply anesthetized with isoflurane. All rats were euthanized with the head-focused microwave irradiation (1 KW，Tangshan Nanosource Microwave Thermal Instrument Manufacturing Co. Ltd., Heibei, PR China). Then about 1mL volume of blood was collected to test the level of blood glucose after infusion and the ^{13}C enrichment of glucose in the plasma. Meanwhile, the brain was divided into 11 regions as described in previous studies[13,14]: frontal cortex (FC), occipital cortex (OC), parietal cortex (PC), temporal cortex (TC), striatum (STR), hippocampus (HP), thalamus (THA), midbrain (MID), hypothalamus (HYP), medulla-pons (MED-PONs) and cerebellum (CE). Blood samples were centrifuged at 10000 *g* for 1 min and 30-40 µL supernatant was collected to detect the ^{13}C enrichment in the plasma glucose. The brain tissue was weighed and immediately frozen at -80℃ for further processing.

2.3 Tissue extraction

The treatment of brain samples adopted the methanol-ethanol extraction method as previously described. [13] HCl/methanol (80µL, 0.1M) and 400µL of ethanol (60%, *vol*/*vol*) were added to the tissue and the mixtures were homogenized using Tissuelyser (Tissuelyser Ⅱ, QIAGEN, German) at a frequency of 20Hz lasting 90s. Then, the mixtures were collected by centrifugation at 14000 *g* for 15 minutes and the supernatant was retained. The above process was repeated twice with 1200 µL of 60% ethanol for adequate extraction. The collected supernatants were lyophilized using the centrifugal drying apparatus (Thermo Scientific 2010, Germany) after removal of organic solvents (methanol and ethanol) in the vacuum environment at 45°C. When the lyophilization was completed, the products were dissolved in phosphate buffer (600µL of D_2O with 0.2 M Na_2HPO_4/NaH_2PO_4, pH 7.2) and the chemical TMSP (3-

(Trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt, 5mM) was selected as the inner standard chemical in the buffer. A high-speed vortex was used to mix the solution fully. The mixtures were then centrifuged at 14000 g for 15 minutes and the supernatant (about 500μL) collected to an NMR tube for further analysis.

2.4 Acquisition of NMR spectrum

All NMR spectra were acquired randomly at 298 K with a BrukerAvance Ⅲ 500 MHz NMR spectrometer (BrukerBiospin, Germany) and were generated with POCE ([^{1}H-^{13}C]-NMR) pulse sequence which is widely used to detect the ^{13}C enrichment in the cerebral extraction[8,13]. The method includes two spin-echo detections, one without a wideband reverse pulse applied at the ^{13}C frequency (total metabolites concentrations,^{12}C+^{13}C) and another with a reverse pulse (the difference in the proton signals connected to ^{12}C and ^{13}C of the metabolite concentrations,^{12}C-^{13}C). Therefore, the subtraction between the two measurements acquires the ^{13}C-labeled metabolites of the spectrum. The whole process used the following parameters: echo time-8 ms; sweep width-20 ppm; repetition time-20s; the number of scans-64; acquisition data-64 K.

2.5 NMR spectra processing

The NMR data were processed with the commercial software Topspin 2.1 (Bruker Biospin, GmbH, Rheinstetten, Germany) and a homemade software NMRSpec [15], and the steps have been described in detail in our previous study [16]. Here, only a brief description is provided. The FID signals were converted to spectra and the baseline and phase were manually adjusted in Topspin 2.1. Then, the spectral data were automatically loaded to NMRSpec, further pre-processed and analyzed. In NMRSpec, there were several functional blocks, such as spectral alignment, peak picking and peak area integration[15]. Each of the functional blocks was automatically completed within a few seconds. Here, the specific regions δ 1.8-4.0 ppm were collected and analyzed. Furthermore, some metabolites were always represented by several peaks, thus it was better to choose a pure signal to avoid the overlap of multiplets, such as Asp_3, Tau_2. However, there were also some specific metabolites without pure signals, such as Glu_3, Gln_4, NAA_2 and so on. Table S1 demonstrates the signal assignment of various metabolites. Therefore, the metabolite concentrations were obtained from the areas of the relative pure signal region, which only represent a part of protons signal in a special position[16]. The selected areas of involved metabolites are listed in Table 1.

2.6 Statistical analysis

Data analysis was performed with the GraphPad Prism 8.0 (GraphPad, New York, USA), homemade NMRSpec in MATLAB (R2017b, Mathworks, Inc. 2017) and SPSS 21.0 (IBM, New York, USA). In order to determine the normality of data, the Kolmogorov-Smirnov test was used. We found that the data satisfied the assumption of normal distribution. Most images were obtained in the GraphPad Prism, except for the heat map which was acquired in MATLAB. One-way analysis of variance (ANOVA) was used for comparing the enrichment of types of neurochemicals between 11 brain regions. Correlations between several metabolites were acquired using Pearson’s correlation analysis in SPSS 21.0. $p<0.05$ is regarded as statistically significant. All results are shown as mean ± SEM.

3. Results

3.1 Evaluation of ^{13}C ratio in TMSP

In the current study, the metabolic kinetics of different types of metabolites were detected with the POCE NMR pulse sequence. This method has been widely used to measure changes in cerebral metabolic kinetics of different types of animal models[12,17]. However, it was very important to evaluate the accuracy of this detection method in the current study. As the internal standard chemical, the calculated ^{13}C ratio of TMSP should approach the natural abundance of ^{13}C- 1.1%. The ^{13}C ratio of the CH_3 group ($\delta=0$) in different types of samples was measured and collected (Fig. 1A). A group of TMSP spectra for the total chemical signal ($^{12}C+^{13}C$, the upper one) and ^{13}C-labeled ($2*^{13}C$, the lower one) which were derived from the POCE study are illustrated in Fig. 1A. The ^{13}C ratio for all the samples is 1.09% ± 0.01% (n=88). Furthermore, the ^{13}C ratios for the samples in different brain regions are shown in Fig. 1B, which is also almost consistent with the theoretical value. Thus, the current protocol could be used to detect the ^{13}C enrichment in different types of metabolites.

3.2 Metabolic pathway of metabolites in different types of cells

In astrocytes, GABAergic neurons and glutamatergic neurons, in different positions of each metabolite were gradually labeled with the infusion of [1-^{13}C] glucose. There were two cycles between different types of cells, including the Glu-Gln cycle and the Glu-GABA cycle. Briefly, in the first TCA cycle in cells as illustrated in Fig 2, [1-^{13}C] glucose was carried into cells. It was oxidized to pyruvate and the pyruvate decarboxylated to form acetyl CoA_2. The latter went into the TCA cycle. 2-OG_4 which was formed in the TCA cycle initially interconverted with Glu_4. Glu_4 passed the label to Gln_4 in astrocytes. Then, Gln_4 was transported to GABAergic neurons and glutamatergic neurons to produce $GABA_2$ and

Glu_4. In addition, neurotransmitters, GABA and glutamate were taken up by astrocytes which made up the complete Glu-Gln cycle and Glu-GABA cycle. Notably, after several metabolic reactions, NAA_3 was produced by astrocytes, GABAergic neurons and glutamatergic neurons with Asp_3 as the precursor. In the following TCA cycles, Glu_4, Gln_4 and $GABA_2$ passed through the ^{13}C label into the other different carbon positions of these metabolites.

3.3 NMR detection of ^{13}C signals in different carbon positions of metabolites

After 16-18 hours of fasting, the blood glucose levels were almost similar at 5.03 ± 0.19 mmol/L (n=8). After the infusion was completed, the plasma glucose increased to 13.43 ± 0.65 mmol/L (n=8). Consistent with the blood glucose test results, the enrichment of plasma [1-^{13}C] glucose was detected with the NMR method, which was around 54.65% ± 3.52% (n=8).

In order to reflect the concentrations of different metabolites and metabolic kinetics in the brain, the POCE pulse sequence was used to detect the enrichment of ^{13}C in different positions of metabolites in different brain regions. Fig 3 shows a typical POCE NMR spectrum including the total concentrations of the metabolites ($^{12}C+^{13}C$, the red one) and the ^{13}C-related metabolites ($2*^{13}C$, the black one). Clearly, the ^{13}C-NMR provided additional information of various metabolites, such as aspartate (Asp), creatine, GABA, Gln, Glu, Glx (Glu+Gln), glycine (Gly), myo-inositol (Myo), N-acetylaspartate (NAA) and Taurine (Tau). The specific signal assignment of each metabolite was collected in Supplement materials (Table S1).

3.4 ^{13}C enrichment of metabolites in different brain regions

There were several important neurochemical metabolites as shown in Fig 4. ^{13}C enrichment in different ^{13}C positions of metabolites differed among regions of the rat brain. Interestingly, there were consistent trends in 11 brain regions in different ^{13}C positions of most metabolites (Glu, Glx, GABA and Asp). The ^{13}C enrichment of Glu_3 and Glu_4, as well as Glx_2 and Glx_3, were at a relatively low level in OC, MID and CE which are depicted in Fig. 4A and Fig. 4B. In addition, Glu_4 was the most stable molecule as its distribution in every brain region was almost at a similar level, except for PC and MID ($p<0.05$). Fig 4C illustrates ^{13}C enrichment in different carbon positions of GABA. Perceptibly, in OC, THA and CE, the labeled carbon enrichment of $GABA_2$ and $GABA_3$ was at a low level. Fig. 4D shows the ^{13}C enrichment of Gln_4 in different brain regions. In Fig 4E, the line graph demonstrates few related trends between the fractional ^{13}C enrichment of NAA_2 and NAA_3. In addition, the average enrichment of NAA_3 was 2.88% ± 0.79% (n=8). In Fig 4F, the enrichment of labeled Asp_2 and Asp_3 indicates a lower enrichment in OC and CE compared to others.

3.5 Correlation between neurochemicals

Pearson correlation coefficient (r) was analyzed to examine possible relations in detected metabolites. The critical correlation coefficient was the minimum correlation coefficient required to describe the correlation of the variables to be statistically significant. The white dot represents the significant correlation ($p<0.05$).

3.6 Correlativity of several metabolites

The linear correlations of different metabolites were calculated among various brain regions. Fig. 5A displays the correlativity of Glu_4 and NAA_3 in 11 brain regions ($y=0.2053x-2.406$, $R=0.7193$, $p<0.01$).

Linear fit of Asp_3 to NAA_3 enrichment across the different 10 brain regions without MID led to a significant correlation coefficient as portrayed in Fig. 6B ($y=0.097x+0.01$, $R=0.9445$, $p<0.001$). Glx_2 was significantly associated with Glx_3 in all brain regions ($y=0.7894x+2.0778$, $R=0.942$, $p<0.001$, Fig. 5C). Fig. 5D presents the correlations between the Glu_4 and Gln_4 in 9 brain regions (FC, OC, PC, TC, STR, HP, THA, MID, CE) without HYP (the purple) and MED-PONs (the green). Although there was no significant correlation between Glu_4 and Gln_4 ($y=0.5662x+2.6839$, $R=0.6366$, $p=0.062$), there was a tendency of a relationship between the two. A linear correlation between Glu_4 and $GABA_2$ is illustrated in Fig. 5E ($y=2.913x-44.006$, $R=0.7855$, $p<0.01$) (MID was excluded).

4. Discussion

This study systematically reports the metabolic kinetics of neurochemicals with physiological status in 11 regions of the entire brain, detected by the relatively high-sensitivity POCE-NMR method. In addition, the metabolic dynamics information of NAA was also calculated. Furthermore, some significant correlations between several metabolites were found which may serve as a potential indicator for assessing metabolic kinetics. This result should be very valuable for neuroscience research and clinical studies.

4.1 Selection of infusion protocol for ^{13}C labeled glucose

In the study of neurometabolic kinetics, a suitable protocol for energy substance infusion is essential. There are several key factors to consider when selecting a better approach for infusion. First, there are many different approaches that can be used to infuse labeled energy substances in the study of metabolic dynamics, including femoral vein[18], jugular vein[19] and tail vein catheterization[14]. Through tail vein catheterization, animals suffer minimum damage compared to the other methods. Thus, we chose a tail vein to infuse the labeled substance. Second, the animals can be anesthetized or be awake when the labeled energy substance is infused. Some studies choose intravenous infusion while the animals are under anesthesia[10], however, the anesthetic state could influence the metabolic dynamics of experimental animals[20]. Therefore, we chose to infuse the animals while they were awake. There is also another problem with the infusion process. Some studies prefer to infuse ^{13}C glucose into the tail vein slowly for a relatively long period while the rats are awake[13]. However, when the animals can move freely, a longtime infusion can easily cause the needle to drop from the tail vein, which is inconvenient and can result in the loss of animal lives. In this study, the caudal veins of rats were catheterized under the anesthesia state and after recovery to freely move for about 15min. Then, [1-^{13}C] glucose was infused through the caudal vein in two minutes with different rates based on the animal's weight. During the whole procedure of infusion, there was no interference and the operation which ran concurrently, was completed over a short period of time (2min). This method also reflects the metabolic state of the animals in their normal physiological state. Moreover, the rats were quiet and calm without showing any discomfort during the infusion process. If an animal showed pain or struggled, it would immediately be treated with euthanasia. The results of plasma glucose measurements also confirmed the advantages of using this method. Thus, this infusion protocol provides an ideal and effective method for NMR research.

4.2 The distribution of different carbon position of various neurochemicals in the brain

There was a similar trend in the distribution of the enrichment levels among different ^{13}C positions of metabolites in different regions as shown in Fig 4. The various labeled carbon positions of Glu, Glx, GABA and Asp manifested this distribution trend in brain regions, except for NAA_2 and NAA_3. Additionally, Fig 4 also indicates that the enrichment of the metabolites differed among the 11 regions of the rat brain. Previous research has shown that glutamine is the most stably distributed neurochemical in different brain regions, excluding the medulla[21]. However, in this study, Glu_4 was the most homogeneously distributed metabolite of the compounds across regions. Furthermore, it was interesting that $Glu_{3/4}$, $Glx_{2/3}$ and Gln_4 were all at a lower level in the occipital cortex (OC), midbrain (MID) and cerebellum (CE) which illustrates that the number of glutamatergic neurons in these regions maybe relatively less than the others. Additionally, most neurochemicals were at lower levels in the cerebellum, although Sosamma et.al[22] found GABA to be high in the grey layers of the cerebellum. Nevertheless, Wang[14] and Palmi[23] both found lower concentrations of GABA in the cerebellum which is consistent with our result.

4.3 NAA metabolism

NAA has been regarded as a potential neuronal marker and may reflect the volume of neuron cells[24]. Thus, only when there is little or no NAA metabolic activity, the concentration of NAA does not vary thus, enabling it to perform its function. Tyson and Sutherland found that the metabolism of NAA is extremely slow compared to glucose and oxygen metabolism in the brain[25]. In this study, we found a similar characteristic of NAA metabolism. The average enrichment of NAA_3 was 2.88% ± 0.79% after 20 minutes of glucose metabolism. As an abundant amino acid in the adult brain, the concentration of NAA was approximately 10μmol/g[26]. Thus, the metabolic rate of NAA_3 was 0.86 ± 0.23 μmol/g/h. Young and Wolf [26] previously concluded that NAA is produced extremely slowly at 0.6-0.7 μmol/g/h than its precursors lasting longer than 17 hours under anesthesia state. Thus, the metabolic rate of NAA is relatively higher under the free moving state than the anesthesia state. The current study supports the notion that NAA should not be regarded as an energy-buffering store metabolite in the brain because of the slow metabolism. however, it is very important to investigate the changes in NAA for cerebral function.

Although NAA metabolism is slow in the brain, it is crucial to assess the NAA metabolism in order to provide some information about neuronal and mitochondrial functions in neurological disorders[27]. The generation steps of NAA_3 is demonstrated in Fig. 2. In simple terms, [1-^{13}C] glucose leads to acetyl-CoA_2 in glucose metabolism which passes into the TCA cycle. Then OAA_3 passes the label into Asp_3 which is the precursor of $NAA_{3.}$ This is consistent with the current findings about the significant relationship between Asp_3 and NAA_3 among ten different brain regions without MID (Fig. 6B).

4.4 Correlation of Glu_4 and Gln_4

In a previous study, it was accepted that glutamatergic neurons depend on glutamine which is synthesized by astrocytes and is regarded as the precursor of glutamate in supplying glutamate[28]. Furthermore, Rae[29] reported that through the inhibition of glutamine transport in animal brain tissue, the glutamate neurotransmitter pools were depleted, which showed that the glutamate-glutamine cycle is essential in maintaining neurotransmitter homeostasis. Therefore, it is important to study the metabolic kinetics of glutamate and glutamine in order to assess energy metabolism and neurotransmission.

In the current study, *via* specific ^{13}C labeling patterns, substrate flows between astrocyte and neurons in the glutamate/GABA-glutamine cycle and cell-characteristic metabolism are illustrated in Fig 2. The balanced cycle between the Glu and Gln which accounts for at least 80% of the glucose consumption in the brain[30] is essential for neuronal function. In the first TCA cycle, Glu_4 is the fastest to be labeled and then ^{13}C is transferred to Gln_4 which is exclusively produced in glial cells. The labeled Gln_4 was sent back to supplement the neurotransmitter pool in glutamate neurons. Then, the released neurotransmitter glutamate was taken in by the synapse of glial cells to reproduce glutamine. These processes form a complete Glu-Gln cycle. The exploration of the relationship between glutamate and glutamine may provide a potential marker to illustrate the metabolic cross-talk between neurons and astrocytes, considering that glutamate serves in neurons but glutamine is located in astrocytes. For example, the accumulation of glutamate in neuronal cells and Glu-Gln cycle disorder play important roles in some diseases related to mental symptoms[31,32]. As depicted in Fig. 6D, the association between Gln_4 and Glu_4 corresponds to the metabolic contact of the Glu-Gln metabolism between glutamatergic neurons and astrocytes in the brain. Therefore, the relationship of Gln_4 and Glu_4 may in part reflect Glu-Gln cycle variation between astrocytes and neurons. However, although there was some correlation between Gln_4 and Glu_4 (R=0.6366 >0.5), the p value was higher than 0.05. The reason was probably due to the density of different neurons among brain regions, moreover, Glu_4 is not only involved in the Gln-Glu cycle but is the direct precursor of $GABA_2$ which may also weakens the association between the two. Garik et al found a significant relationship between Glu and Gln in the cortex and cerebellum[33] which is consistent with our result.

4.5 Correlation of Glx_2 and Glx_3

The molecular structure of Glu is similar to that of Gln which results in a similar magnetic resonance spectrum. In order to avoid the spectral assignment confusion of Glu and Gln, the term 'Glx' has been used to represent the superposition of Glu and Gln enrichment (Glx=Glu+Gln). It has been widely reported that the increase or decrease in levels of Glx was found in some cerebral tumors[34,35]. For example, the level of Glx in oligodendrogliomas was higher compared with white matter which can differentiate these tumors from others[34]. Thus, it may be regarded as a metabolic marker for diagnosing and differentiating different types of brain tumors. Starting with the following TCA cycle proceeding, Glu_2 and Gln_2, as well as Glu_3 and Gln_3, almost had the equal probability of being ^{13}C labeled, respectively. In the present study, there was a significant correlation between Glx_2 and Glx_3 which is in line with the actual metabolic process. Furthermore, the ^{13}C enrichment for these two metabolites was almost similar, which also supports their generation pathways.

5. Conclusion

In conclusion, we detected the enrichment of various metabolic molecules to reflect the metabolic kinetics of different metabolites among brain regions. We also found a slow metabolic rate of NAA, implying that NAA is not considered to provide energy buffering for energy metabolism in the brain. Furthermore, the significant correlations between some metabolites reflect the possible cross-talk between astrocytes and neuron cells, indicating the close connection between energy metabolism and neurotransmission. This study provided some explanations of neurological or psychiatric disorders and systematically explored the metabolic kinetics of some neurochemicals, which plays an important role in the study of brain function and the mechanisms of some neurological or psychiatric disorders.

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7. Author contribution statement

Meimei Guo: Conceptualization, Methodology, Data curation, Writing-original draft; Yuanyuan Fang: Methodology, Formal analysis; Jinpiao Zhu: Methodology, Formal analysis; Chang chen: Investigation; Zongze Zhang: Investigation, Visualization; Xuebi Tian:Software, Investigation; Hongbing Xiang: Visualization, Software; Anne Manyande, Mojtaba Ehsanifar and Ahmad Jonidi Jafari: Writing-review & editing; Jie Wang: Software, Data curation, Writing-review & editing; Mian Peng: Visualization, Project administration, Funding acquisition.

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Figure legends

Fig. 1: Examples of NMR spectra of TMSP and calculated natural ^{13}C enrichments of TMSP in the samples of different brain regions. (A) Examples of NMR spectra for total TMSP (^{12}C+^{13}C, the red one) and ^{13}C-labeled TMSP (2*^{13}C, the black one). (B) The natural ^{13}C enrichments (Calculated from the ratio of ^{13}C/^{13}C+^{12}C) of TMSP in all the samples from 11 different brain regions. *Note: TMSP: 3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt.*

Fig. 2: Schematic diagram of ^{13}C labeling of metabolites from [1-^{13}C] glucose in the first TCA circle between astrocytes, GABAergic neurons and glutamatergic neurons. *Note: TCA circle: tricarboxylic acid cycle; Glc: glucose; Pyr: pyruvate; Acetyl-CoA: Acetyl coenzyme A; 2-OG: 2-oxoglutarate; OAA: oxaloacetate; Subscript number:* ^{13}C *labelled positions in different metabolites.*

Fig. 3: Examples of NMR spectra for total metabolites (^{12}C+^{13}C, the red one) and ^{13}C-labeled metabolites (2*^{13}C, the black one) from the occipital cortex. *Note: Asp: aspartate; Cre: creatine; GABA: γ-aminobutyric acid; Gln: glutamate; Glu: glutamate; Glx: glutamine+glutamate; Gly: Glycine; Myo: myo-Inositol; NAA: N-acetylaspartate; Tau: Taurine; Subscript number:* ^{13}C *labelled positions in different metabolites.*

Fig. 4: The ^{13}C enrichment in different positions of metabolites in different samples of 11 brain regions. *Note: Subscript number:* ^{13}C *labelled positions in different metabolites. (A)* ^{13}C *enrichment of* Glu_2 *and* Glu_3*; (B)* ^{13}C *enrichment of* Glx_2 *and* Glx_3*; (C)* ^{13}C *enrichment of* $GABA_2$, $GABA_3$ *and* $GABA_4$*; (D)* ^{13}C *enrichment of* Gln_4*; (E)* ^{13}C *enrichment of* NAA_2 *and* NAA_3*; (F)* ^{13}C *enrichment of* Asp_2 *and* Asp_3*.*

Fig. 5: Pearson correlation analysis between ^{13}C enrichment in different kinds of metabolites (Glu_4, Glu_3, NAA_2, NAA_3, Glx_2, Glx_3, $GABA_2$, $GABA_3$, $GABA_4$, Gln_4, Asp_3, Asp_2). *Note: The correlation coefficient (r) was calculated from the linear correlation between the ^{13}C enrichments data for different metabolites in all the brain regions in the cross location of the figure; The white dot represents the significant correlation ($p<0.05$).*

Fig. 6: The linear correlations between ^{13}C enrichment in different kinds of metabolites: NAA_3-Glu_4 (A), Asp_3-NAA_3 (B), Glx_2-Glx_3 (C), Glu_4-Gln_4 (D), Glu_4-$GABA_2$ (E). *Note: The linear trendlines are represented with the blue dot lines and calculated from the selected blue dots; X and Y-axis represent the ^{13}C enrichment ratios for different metabolites. Subscript number: ^{13}C labeled positions in different metabolites; Non blue dots: Outliers from the other brain regions.*