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1 **Investigation of metabolic kinetics in different brain regions of awake rats using the [¹H-¹³C]-**

2 **NMR technique**

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26

27 **Abstract:** Energy metabolism and neurotransmission are necessary for sustaining normal life activities.
28 Hence, neurological or psychiatric disorders are always associated with changes in neurotransmitters and
29 energy metabolic states in the brain. Most studies have only focused on the most important
30 neurotransmitters, particularly GABA and Glu, however, other metabolites such as NAA and aspartate
31 which are also very important for cerebral function are rarely investigated, . In this study, most of the
32 metabolic kinetics information of different brain regions was investigated in awake rats using the [¹H-
33 ¹³C]-NMR technique. Briefly, rats (n=8) were infused [1-¹³C] glucose through the tail vein for two
34 minutes. After 20 minutes of glucose metabolism, the animals were sacrificed and the brain tissue was
35 extracted and treated. Utilizing the ¹H observed/¹³C-edited nuclear magnetic resonance (POCE-NMR),
36 the enrichment of neurochemicals was detected which reflected the metabolic changes in different brain
37 regions and the metabolic connections between neurons and glial cells in the brain. The results suggest
38 that the distribution of every metabolite differed from every brain region and the metabolic rate of NAA
39 was relatively low at $8.64 \pm 2.37 \mu\text{mol/g/h}$. In addition, there were some correlations between several
40 ¹³C enriched metabolites, such as Glu₄-Gln₄ ($p=0.062$), Glu₄-GABA₂ ($p<0.01$), Glx₂-Glx₃ ($p<0.001$),
41 Asp₃-NAA₃ ($p<0.001$). This correlativity reflects the signal transmission between astrocytes and neurons,
42 as well as the potential interaction between energy metabolism and neurotransmission. In conclusion, the
43 current study systematically demonstrated the metabolic kinetics in the brain which shed light on brain
44 functions and the mechanisms of various pathophysiological states.

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

47 **1. Introduction**

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

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

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

73 In metabolic flux studies, the infusion of ^{13}C labeled substrates, such as glucose, acetate or ketone
74 bodies is always used in investigations[8]. After the infusion of ^{13}C enriched substrates, the ^{13}C labeled
75 substrates were gradually oxidized to the other ^{13}C labeled metabolites, which could be accurately
76 detected with the NMR method and directly reflect the cerebral metabolic kinetics information[8]. Here,
77 [^1H - ^{13}C] (^1H observed/ ^{13}C -edited) the nuclear magnetic resonance (POCE-NMR) technique is one of the
78 most frequently-used NMR techniques which is an attractive approach for detecting metabolic kinetics.
79 POCE-NMR can help to measure the composition of metabolites, detect changes in the metabolic rate of
80 energy sources and reflect the dynamics of neurotransmitter transmission[8]. By applying this technique,
81 we can detect the ^1H signal which has higher sensitivity than the normal ^{13}C -NMR method, as well as
82 separate hydrogens attached to ^{13}C from those attached to ^{12}C . It has been reported in other studies that
83 there was a 14-fold improvement in sensitivity to detect the CH_3 signal in the rabbit brain using the
84 proton spectroscopy compared to the direct carbon spectroscopy[9]. Thus, the POCE-NMR was applied
85 in the present study.

86 Furthermore, in the field of brain metabolic dynamics, many studies have focused on examining
87 the neurotransmitter circulation between neurons and astrocytes in maintaining neurotransmitter
88 homeostasis, such as Glu-Gln (glutamate-glutamine) cycle and GABA-Glu (γ -aminobutyric acid-
89 glutamate) cycle[10]. However, there are few studies that have investigated the dynamics of other small
90 molecular metabolisms, which may also play a crucial role in brain function. For example, N-
91 acetylaspartate (NAA) has been used as a neuronal biomarker to reflect neuronal function and density
92 [11] and it is implicated in many metabolic processes, such as myelination and oxidative metabolism[12].
93 Thus, the metabolic kinetic information of the NMR detectable metabolites was investigated in the
94 current study.

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

100

101 **2. Material and methods**

102 ***2.1 Animal preparation***

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

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

110 ***2.2 Animal experiment***

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

116 On the experimental day, animals were anesthetized with 1.5%-2.5% isoflurane mixed with 30%
117 O₂. When rats had no response to a foot pinch under appropriate anesthetic depth, two drops of blood
118 were collected from a needle prick to the tip of the tail to test the blood glucose level using glucose test
119 strips (Yuyue, China) before infusion. Then, PE50 tubing (Instech PA USA) was inserted into the lateral
120 tail vein with a 24-gauge needle to infuse ¹³C labeled glucose and secured with adhesive tape. After that,
121 the rats were allowed to recover for about 15 minutes until they could move freely. The infusion tube
122 was then connected to a swivel (Instech, PA, USA) and the other side of the swivel was connected to the
123 pump (Fusion 100, Chemyx, TX, USA) using PE50 tubing. Finally, [1-¹³C] glucose was pumped at 400-
124 600μL/min (dependent on the animal weight) through the lateral tail vein for two minutes (The dosage
125 was based on the previous infusion protocol[14]), while the rats could move freely in cages.

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

136 **2.3 Tissue extraction**

137 The treatment of brain samples adopted the methanol-ethanol extraction method as previously
138 described. [13] HCl/methanol (80μL, 0.1M) and 400μL of ethanol (60%, *vol/vol*) were added to the tissue
139 and the mixtures were homogenized using Tissuelyser (Tissuelyser II, QIAGEN, German) at a
140 frequency of 20Hz lasting 90s. Then, the mixtures were collected by centrifugation at 14000 g for 15
141 minutes and the supernatant was retained. The above process was repeated twice with 1200 μL of 60%
142 ethanol for adequate extraction. The collected supernatants were lyophilized using the centrifugal drying
143 apparatus (Thermo Scientific 2010, Germany) after removal of organic solvents (methanol and ethanol)
144 in the vacuum environment at 45°C. When the lyophilization was completed, the products were dissolved
145 in phosphate buffer (600μL of D₂O with 0.2 M Na₂HPO₄/NaH₂PO₄, pH 7.2) and the chemical TMSP (3-

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

150 ***2.4 Acquisition of NMR spectrum***

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

160 ***2.5 NMR spectra processing***

161 The NMR data were processed with the commercial software Topspin 2.1 (Bruker Biospin, GmbH,
162 Rheinstetten, Germany) and a homemade software NMRSpec [15], and the steps have been described in
163 detail in our previous study [16]. Here, only a brief description is provided. The FID signals were
164 converted to spectra and the baseline and phase were manually adjusted in Topspin 2.1. Then, the spectral
165 data were automatically loaded to NMRSpec, further pre-processed and analyzed. In NMRSpec, there

166 were several functional blocks, such as spectral alignment, peak picking and peak area integration[15].
167 Each of the functional blocks was automatically completed within a few seconds. Here, the specific
168 regions δ 1.8-4.0 ppm were collected and analyzed. Furthermore, some metabolites were always
169 represented by several peaks, thus it was better to choose a pure signal to avoid the overlap of multiplets,
170 such as Asp₃, Tau₂. However, there were also some specific metabolites without pure signals, such as
171 Glu₃, Gln₄, NAA₂ and so on. Table S1 demonstrates the signal assignment of various metabolites.
172 Therefore, the metabolite concentrations were obtained from the areas of the relative pure signal region,
173 which only represent a part of protons signal in a special position[16]. The selected areas of involved
174 metabolites are listed in Table 1.

175 *2.6 Statistical analysis*

176 Data analysis was performed with the GraphPad Prism 8.0 (GraphPad, New York, USA),
177 homemade NMRSpec in MATLAB (R2017b, Mathworks, Inc. 2017) and SPSS 21.0 (IBM, New York,
178 USA). In order to determine the normality of data, the Kolmogorov-Smirnov test was used. We found
179 that the data satisfied the assumption of normal distribution. Most images were obtained in the GraphPad
180 Prism, except for the heat map which was acquired in MATLAB. One-way analysis of variance (ANOVA)
181 was used for comparing the enrichment of types of neurochemicals between 11 brain regions.
182 Correlations between several metabolites were acquired using Pearson's correlation analysis in SPSS
183 21.0. $p < 0.05$ is regarded as statistically significant. All results are shown as mean \pm SEM.

184

185 **3. Results**

186 **3.1 Evaluation of ^{13}C ratio in TMSP**

187 In the current study, the metabolic kinetics of different types of metabolites were detected with the
188 POCE NMR pulse sequence. This method has been widely used to measure changes in cerebral metabolic
189 kinetics of different types of animal models[12,17]. However, it was very important to evaluate the
190 accuracy of this detection method in the current study. As the internal standard chemical, the calculated
191 ^{13}C ratio of TMSP should approach the natural abundance of ^{13}C - 1.1%. The ^{13}C ratio of the CH_3 group
192 ($\delta=0$) in different types of samples was measured and collected (Fig. 1A). A group of TMSP spectra for
193 the total chemical signal ($^{12}\text{C}+^{13}\text{C}$, the upper one) and ^{13}C -labeled ($2*^{13}\text{C}$, the lower one) which were
194 derived from the POCE study are illustrated in Fig. 1A. The ^{13}C ratio for all the samples is $1.09\% \pm 0.01\%$
195 ($n=88$). Furthermore, the ^{13}C ratios for the samples in different brain regions are shown in Fig. 1B, which
196 is also almost consistent with the theoretical value. Thus, the current protocol could be used to detect the
197 ^{13}C enrichment in different types of metabolites.

198 **3.2 Metabolic pathway of metabolites in different types of cells**

199 In astrocytes, GABAergic neurons and glutamatergic neurons, in different positions of each
200 metabolite were gradually labeled with the infusion of $[1-^{13}\text{C}]$ glucose. There were two cycles between
201 different types of cells, including the Glu-Gln cycle and the Glu-GABA cycle. Briefly, in the first TCA
202 cycle in cells as illustrated in Fig 2, $[1-^{13}\text{C}]$ glucose was carried into cells. It was oxidized to pyruvate
203 and the pyruvate decarboxylated to form acetyl CoA_2 . The latter went into the TCA cycle. 2-OG₄ which
204 was formed in the TCA cycle initially interconverted with Glu₄. Glu₄ passed the label to Gln₄ in astrocytes.
205 Then, Gln₄ was transported to GABAergic neurons and glutamatergic neurons to produce GABA₂ and

206 Glu₄. In addition, neurotransmitters, GABA and glutamate were taken up by astrocytes which made up
207 the complete Glu-Gln cycle and Glu-GABA cycle. Notably, after several metabolic reactions, NAA₃ was
208 produced by astrocytes, GABAergic neurons and glutamatergic neurons with Asp₃ as the precursor. In
209 the following TCA cycles, Glu₄, Gln₄ and GABA₂ passed through the ¹³C label into the other different
210 carbon positions of these metabolites.

211 *3.3 NMR detection of ¹³C signals in different carbon positions of metabolites*

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

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

224 *3.4 ¹³C enrichment of metabolites in different brain regions*

225 There were several important neurochemical metabolites as shown in Fig 4. ¹³C enrichment in
226 different ¹³C positions of metabolites differed among regions of the rat brain. Interestingly, there were
227 consistent trends in 11 brain regions in different ¹³C positions of most metabolites (Glu, Glx, GABA and
228 Asp). The ¹³C enrichment of Glu₃ and Glu₄, as well as Glx₂ and Glx₃, were at a relatively low level in
229 OC, MID and CE which are depicted in Fig. 4A and Fig. 4B. In addition, Glu₄ was the most stable
230 molecule as its distribution in every brain region was almost at a similar level, except for PC and MID
231 ($p<0.05$). Fig 4C illustrates ¹³C enrichment in different carbon positions of GABA. Perceptibly, in OC,
232 THA and CE, the labeled carbon enrichment of GABA₂ and GABA₃ was at a low level. Fig. 4D shows
233 the ¹³C enrichment of Gln₄ in different brain regions. In Fig 4E, the line graph demonstrates few related
234 trends between the fractional ¹³C enrichment of NAA₂ and NAA₃. In addition, the average enrichment
235 of NAA₃ was $2.88\% \pm 0.79\%$ ($n=8$). In Fig 4F, the enrichment of labeled Asp₂ and Asp₃ indicates a lower
236 enrichment in OC and CE compared to others.

237 ***3.5 Correlation between neurochemicals***

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

242 ***3.6 Correlativity of several metabolites***

243 The linear correlations of different metabolites were calculated among various brain regions. Fig.
244 5A displays the correlativity of Glu₄ and NAA₃ in 11 brain regions ($y=0.2053x-2.406$, $R=0.7193$, $p<0.01$).

245 Linear fit of Asp₃ to NAA₃ enrichment across the different 10 brain regions without MID led to a
246 significant correlation coefficient as portrayed in Fig. 6B ($y=0.097x+0.01$, $R=0.9445$, $p<0.001$). Glx₂ was
247 significantly associated with Glx₃ in all brain regions ($y=0.7894x+2.0778$, $R=0.942$, $p<0.001$, Fig. 5C).
248 Fig. 5D presents the correlations between the Glu₄ and Gln₄ in 9 brain regions (FC, OC, PC, TC, STR,
249 HP, THA, MID, CE) without HYP (the purple) and MED-PONs (the green). Although there was no
250 significant correlation between Glu₄ and Gln₄ ($y=0.5662x+2.6839$, $R=0.6366$, $p=0.062$), there was a
251 tendency of a relationship between the two. A linear correlation between Glu₄ and GABA₂ is illustrated
252 in Fig. 5E ($y=2.913x-44.006$, $R=0.7855$, $p<0.01$) (MID was excluded).

253

254 **4. Discussion**

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

261 ***4.1 Selection of infusion protocol for ¹³C labeled glucose***

262 In the study of neurometabolic kinetics, a suitable protocol for energy substance infusion is essential.
263 There are several key factors to consider when selecting a better approach for infusion. First, there are
264 many different approaches that can be used to infuse labeled energy substances in the study of metabolic

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

284 *4.2 The distribution of different carbon position of various neurochemicals in the brain*

285 There was a similar trend in the distribution of the enrichment levels among different ^{13}C positions
286 of metabolites in different regions as shown in Fig 4. The various labeled carbon positions of Glu, Glx,
287 GABA and Asp manifested this distribution trend in brain regions, except for NAA₂ and NAA₃.
288 Additionally, Fig 4 also indicates that the enrichment of the metabolites differed among the 11 regions
289 of the rat brain. Previous research has shown that glutamine is the most stably distributed neurochemical
290 in different brain regions, excluding the medulla[21]. However, in this study, Glu₄ was the most
291 homogeneously distributed metabolite of the compounds across regions. Furthermore, it was interesting
292 that Glu_{3/4}, Glx_{2/3} and Gln₄ were all at a lower level in the occipital cortex (OC), midbrain (MID) and
293 cerebellum (CE) which illustrates that the number of glutamatergic neurons in these regions maybe
294 relatively less than the others. Additionally, most neurochemicals were at lower levels in the cerebellum,
295 although Sosamma et.al[22] found GABA to be high in the grey layers of the cerebellum. Nevertheless,
296 Wang[14] and Palmi[23] both found lower concentrations of GABA in the cerebellum which is
297 consistent with our result.

298 **4.3 NAA metabolism**

299 NAA has been regarded as a potential neuronal marker and may reflect the volume of neuron
300 cells[24]. Thus, only when there is little or no NAA metabolic activity, the concentration of NAA does
301 not vary thus, enabling it to perform its function. Tyson and Sutherland found that the metabolism of
302 NAA is extremely slow compared to glucose and oxygen metabolism in the brain[25]. In this study, we
303 found a similar characteristic of NAA metabolism. The average enrichment of NAA₃ was $2.88\% \pm 0.79\%$
304 after 20 minutes of glucose metabolism. As an abundant amino acid in the adult brain, the concentration

305 of NAA was approximately $10\mu\text{mol/g}$ [26]. Thus, the metabolic rate of NAA_3 was $0.86 \pm 0.23 \mu\text{mol/g/h}$.
306 Young and Wolf [26] previously concluded that NAA is produced extremely slowly at $0.6\text{-}0.7 \mu\text{mol/g/h}$
307 than its precursors lasting longer than 17 hours under anesthesia state. Thus, the metabolic rate of NAA
308 is relatively higher under the free moving state than the anesthesia state. The current study supports the
309 notion that NAA should not be regarded as an energy-buffering store metabolite in the brain because of
310 the slow metabolism. however, it is very important to investigate the changes in NAA for cerebral
311 function.

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

318

319 ***4.4 Correlation of Glu_4 and Gln_4***

320 In a previous study, it was accepted that glutamatergic neurons depend on glutamine which is
321 synthesized by astrocytes and is regarded as the precursor of glutamate in supplying glutamate[28].
322 Furthermore, Rae[29] reported that through the inhibition of glutamine transport in animal brain tissue,
323 the glutamate neurotransmitter pools were depleted, which showed that the glutamate-glutamine cycle is

324 essential in maintaining neurotransmitter homeostasis. Therefore, it is important to study the metabolic
325 kinetics of glutamate and glutamine in order to assess energy metabolism and neurotransmission.

326 In the current study, *via* specific ^{13}C labeling patterns, substrate flows between astrocyte and
327 neurons in the glutamate/GABA-glutamine cycle and cell-characteristic metabolism are illustrated in Fig
328 2. The balanced cycle between the Glu and Gln which accounts for at least 80% of the glucose
329 consumption in the brain[30] is essential for neuronal function. In the first TCA cycle, Glu₄ is the fastest
330 to be labeled and then ^{13}C is transferred to Gln₄ which is exclusively produced in glial cells. The labeled
331 Gln₄ was sent back to supplement the neurotransmitter pool in glutamate neurons. Then, the released
332 neurotransmitter glutamate was taken in by the synapse of glial cells to reproduce glutamine. These
333 processes form a complete Glu-Gln cycle. The exploration of the relationship between glutamate and
334 glutamine may provide a potential marker to illustrate the metabolic cross-talk between neurons and
335 astrocytes, considering that glutamate serves in neurons but glutamine is located in astrocytes. For
336 example, the accumulation of glutamate in neuronal cells and Glu-Gln cycle disorder play important
337 roles in some diseases related to mental symptoms[31,32]. As depicted in Fig. 6D, the association
338 between Gln₄ and Glu₄ corresponds to the metabolic contact of the Glu-Gln metabolism between
339 glutamatergic neurons and astrocytes in the brain. Therefore, the relationship of Gln₄ and Glu₄ may in
340 part reflect Glu-Gln cycle variation between astrocytes and neurons. However, although there was some
341 correlation between Gln₄ and Glu₄ ($R=0.6366 >0.5$), the p value was higher than 0.05. The reason was
342 probably due to the density of different neurons among brain regions, moreover, Glu₄ is not only involved
343 in the Gln-Glu cycle but is the direct precursor of GABA₂ which may also weakens the association

344 between the two. Garik et al found a significant relationship between Glu and Gln in the cortex and
345 cerebellum[33] which is consistent with our result.

346 *4.5 Correlation of Glx₂ and Glx₃*

347 The molecular structure of Glu is similar to that of Gln which results in a similar magnetic
348 resonance spectrum. In order to avoid the spectral assignment confusion of Glu and Gln, the term ‘Glx’
349 has been used to represent the superposition of Glu and Gln enrichment (Glx=Glu+Gln). It has been
350 widely reported that the increase or decrease in levels of Glx was found in some cerebral tumors[34,35].
351 For example, the level of Glx in oligodendrogliomas was higher compared with white matter which can
352 differentiate these tumors from others[34]. Thus, it may be regarded as a metabolic marker for diagnosing
353 and differentiating different types of brain tumors. Starting with the following TCA cycle proceeding,
354 Glu₂ and Gln₂, as well as Glu₃ and Gln₃, almost had the equal probability of being ¹³C labeled,
355 respectively. In the present study, there was a significant correlation between Glx₂ and Glx₃ which is in
356 line with the actual metabolic process. Furthermore, the ¹³C enrichment for these two metabolites was
357 almost similar, which also supports their generation pathways.

358 **5. Conclusion**

359 In conclusion, we detected the enrichment of various metabolic molecules to reflect the metabolic
360 kinetics of different metabolites among brain regions. We also found a slow metabolic rate of NAA,
361 implying that NAA is not considered to provide energy buffering for energy metabolism in the brain.
362 Furthermore, the significant correlations between some metabolites reflect the possible cross-talk
363 between astrocytes and neuron cells, indicating the close connection between energy metabolism and

364 neurotransmission. This study provided some explanations of neurological or psychiatric disorders and
365 systematically explored the metabolic kinetics of some neurochemicals, which plays an important role
366 in the study of brain function and the mechanisms of some neurological or psychiatric disorders.

367

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374

375 **7. Author contribution statement**

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

382

383 **8. References**

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491 **Figure legends**

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

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

501 Fig. 3: Examples of NMR spectra for total metabolites ($^{12}\text{C}+^{13}\text{C}$, the red one) and ^{13}C -labeled metabolites
502 ($2*^{13}\text{C}$, the black one) from the occipital cortex. *Note: Asp: aspartate; Cre: creatine; GABA: γ -*
503 *aminobutyric acid; Gln: glutamate; Glu: glutamate; Glx: glutamine+glutamate; Gly: Glycine; Myo:*
504 *myo-Inositol; NAA: N-acetylaspartate; Tau: Taurine; Subscript number: ^{13}C labelled positions in*
505 *different metabolites.*

506 Fig. 4: The ^{13}C enrichment in different positions of metabolites in different samples of 11 brain regions.
507 *Note: Subscript number: ^{13}C labelled positions in different metabolites. (A) ^{13}C enrichment of Glu₂*
508 *and Glu₃; (B) ^{13}C enrichment of Glx₂ and Glx₃; (C) ^{13}C enrichment of GABA₂, GABA₃ and GABA₄;*
509 *(D) ^{13}C enrichment of Gln₄; (E) ^{13}C enrichment of NAA₂ and NAA₃; (F) ^{13}C enrichment of Asp₂ and*
510 *Asp₃.*

511 Fig. 5: Pearson correlation analysis between ^{13}C enrichment in different kinds of metabolites (Glu₄, Glu₃,
512 NAA₂, NAA₃, Glx₂, Glx₃, GABA₂, GABA₃, GABA₄, Gln₄, Asp₃, Asp₂). *Note: The correlation*
513 *coefficient (r) was calculated from the linear correlation between the ^{13}C enrichments data for*
514 *different metabolites in all the brain regions in the cross location of the figure; The white dot*
515 *represents the significant correlation ($p < 0.05$).*

516 Fig. 6: The linear correlations between ^{13}C enrichment in different kinds of metabolites: NAA₃-Glu₄ (A),
517 Asp₃-NAA₃ (B), Glx₂-Glx₃ (C), Glu₄-Gln₄ (D), Glu₄-GABA₂ (E). *Note: The linear trendlines are*
518 *represented with the blue dot lines and calculated from the selected blue dots; X and Y-axis represent*
519 *the ^{13}C enrichment ratios for different metabolites. Subscript number: ^{13}C labeled positions in different*
520 *metabolites; Non blue dots: Outliers from the other brain regions.*

521