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

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

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 ^{1}H-NMR, ^{13}C-NMR, ^{31}P-

69   NMR, *etc*. ^{1}H-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   [^{1}H-^{13}C] (^{1}H 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 ^{1}H 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.

4

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 [^{1}H-^{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

5

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 ^{13}C enrichment of different carbon positions of metabolites was detected to reflect the

112   metabolic kinetics[13,14] through infusing [1-^{13}C] glucose (Qingdao Tenglong Weibo Technology co.,

113   LTD, Qingdao, P.R. China). To obtain higher enrichment of ^{13}C 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   O$_2$. 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 ^{13}C 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-^{13}C] 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 ^{13}C 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 ^{13}C

134   enrichment in the plasma glucose. The brain tissue was weighed and immediately frozen at -80℃ 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℃. When the lyophilization was completed, the products were dissolved

145   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-

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 500μL) 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 ([^{1}H-^{13}C]-NMR) pulse

153 sequence which is widely used to detect the ^{13}C enrichment in the cerebral extraction[8,13]. The method

154 includes two spin-echo detections, one without a wideband reverse pulse applied at the ^{13}C frequency

155 (total metabolites concentrations,^{12}C+^{13}C) and another with a reverse pulse (the difference in the proton

156 signals connected to ^{12}C and ^{13}C of the metabolite concentrations,^{12}C-^{13}C). Therefore, the subtraction

157 between the two measurements acquires the ^{13}C-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 Asp_3, Tau_2. However, there were also some specific metabolites without pure signals, such as

171   Glu_3, Gln_4, NAA_2 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   (δ=0) in different types of samples was measured and collected (Fig. 1A). A group of TMSP spectra for

193   the total chemical signal ($^{12}C+^{13}C$, the upper one) and ^{13}C-labeled ($2*^{13}C$, 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}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}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-OG_4 which

204   was formed in the TCA cycle initially interconverted with Glu_4. Glu_4 passed the label to Gln_4 in astrocytes.

205   Then, Gln_4 was transported to GABAergic neurons and glutamatergic neurons to produce $GABA_2$ and

206   Glu_4. 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, NAA_3 was

208   produced by astrocytes, GABAergic neurons and glutamatergic neurons with Asp_3 as the precursor. In

209   the following TCA cycles, Glu_4, Gln_4 and $GABA_2$ passed through the ^{13}C label into the other different

210   carbon positions of these metabolites.

211   *3.3 NMR detection of ^{13}C 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 $[1-^{13}C]$ glucose was detected

215   with the NMR method, which was around $54.65\% \pm 3.52\%$ (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 ^{13}C 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 ($^{12}C+^{13}C$, the red one) and the ^{13}C-related metabolites ($2*^{13}C$, the black one). Clearly,

220   the ^{13}C-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 ^{13}C enrichment of metabolites in different brain regions*

11

225   There were several important neurochemical metabolites as shown in Fig 4. ^{13}C enrichment in

226   different ^{13}C positions of metabolites differed among regions of the rat brain. Interestingly, there were

227   consistent trends in 11 brain regions in different ^{13}C positions of most metabolites (Glu, Glx, GABA and

228   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

229   OC, MID and CE which are depicted in Fig. 4A and Fig. 4B. In addition, Glu_4 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 ^{13}C enrichment in different carbon positions of GABA. Perceptibly, in OC,

232   THA and CE, the labeled carbon enrichment of $GABA_2$ and $GABA_3$ was at a low level. Fig. 4D shows

233   the ^{13}C enrichment of Gln_4 in different brain regions. In Fig 4E, the line graph demonstrates few related

234   trends between the fractional ^{13}C enrichment of NAA_2 and NAA_3. In addition, the average enrichment

235   of NAA_3 was 2.88% $\pm$ 0.79% (n=8). In Fig 4F, the enrichment of labeled Asp_2 and Asp_3 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 Glu_4 and NAA_3 in 11 brain regions (y=0.2053x-2.406, R=0.7193, $p<0.01$).

245   Linear fit of Asp_3 to NAA_3 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). Glx_2 was

247   significantly associated with Glx_3 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 Glu_4 and Gln_4 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 Glu_4 and Gln_4 (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 Glu_4 and $GABA_2$ 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 ^{13}C 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}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*

14

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 NAA_2 and NAA_3.

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, Glu_4 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 Gln_4 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 NAA_3 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 10μmol/g[26]. Thus, the metabolic rate of NAA_3 was 0.86 ± 0.23 μmol/g/h.

306   Young and Wolf [26] previously concluded that NAA is produced extremely slowly at 0.6-0.7 μmol/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-^{13}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, Glu_4 is the fastest

330   to be labeled and then ^{13}C is transferred to Gln_4 which is exclusively produced in glial cells. The labeled

331   Gln_4 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 Gln_4 and Glu_4 corresponds to the metabolic contact of the Glu-Gln metabolism between

339   glutamatergic neurons and astrocytes in the brain. Therefore, the relationship of Gln_4 and Glu_4 may in

340   part reflect Glu-Gln cycle variation between astrocytes and neurons. However, although there was some

341   correlation between Gln_4 and Glu_4 ($R=0.6366 >0.5$), the p value was higher than 0.05. The reason was

342   probably due to the density of different neurons among brain regions, moreover, Glu_4 is not only involved

343   in the Gln-Glu cycle but is the direct precursor of $GABA_2$ 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 Glx$_2$ and Glx$_3$*

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 Glu$_2$ and Gln$_2$, as well as Glu$_3$ and Gln$_3$, almost had the equal probability of being ^{13}C labeled,

355 respectively. In the present study, there was a significant correlation between Glx$_2$ and Glx$_3$ which is in

356 line with the actual metabolic process. Furthermore, the ^{13}C 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.

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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**

384   [1]   J.W. Błaszczyk, Energy Metabolism Decline in the Aging Brain—Pathogenesis of

385   Neurodegenerative Disorders, Metabolites. 10 (2020) 450.

386   [2]   L. Sokoloff, M. Reivich, C. Kennedy, M.H. Des Rosiers, C.S. Patlak, K.D. Pettigrew, O.

387   Sakurada, M. Shinohara, The [14C]deoxyglucose method for the measurement of local cerebral

388   glucose utilization: theory, procedure, and normal values in the conscious and anesthetized

389   albino rat., J. Neurochem. 28 (1977) 897–916.

390   [3]   L. Pellerin, P.J. Magistretti, Glutamate uptake into astrocytes stimulates aerobic glycolysis: a

391   mechanism coupling neuronal activity to glucose utilization., Proc. Natl. Acad. Sci. U. S. A. 91

392   (1994) 10625–10629.

393   [4]   E. Dhamala, I. Abdelkefi, M. Nguyen, T.J. Hennessy, H. Nadeau, J. Near, Validation of in vivo

394   MRS measures of metabolite concentrations in the human brain., NMR Biomed. 32 (2019)

395   e4058.

396   [5]   E. Shokri-Kojori, D. Tomasi, B. Alipanahi, C.E. Wiers, G.-J. Wang, N.D. Volkow,

397   Correspondence between cerebral glucose metabolism and BOLD reveals relative power and

398   cost in human brain., Nat. Commun. 10 (2019) 690.

399   [6]   L. Sokoloff, Localization of functional activity in the central nervous system by measurement

400   of glucose utilization with radioactive deoxyglucose., J. Cereb. Blood Flow Metab. 1 (1981) 7–

401   36.

402   [7]   J.H.F. Bothwell, J.L. Griffin, An introduction to biological nuclear magnetic resonance

403   spectroscopy, Biol. Rev. 44 (2011) 493–510.

404   [8]   R.A. de Graaf, G.F. Mason, A.B. Patel, K.L. Behar, D.L. Rothman, In vivo 1H-[13 C]-NMR

405   spectroscopy of cerebral metabolism, NMR Biomed. 16 (2003) 339–357.

406   [9]   E.J.J. Novotny, T. Ogino, D.L. Rothman, O.A. Petroff, J.W. Prichard, R.G. Shulman, Direct

407   carbon versus proton heteronuclear editing of 2-13C ethanol in rabbit brain in vivo: a

408   sensitivity comparison., Magn. Reson. Med. 16 (1990) 431–443.

409   [10]   N. Wang, L.-C. Zhao, Y.-Q. Zheng, M.-J. Dong, Y. Su, W.-J. Chen, Z.-L. Hu, Y.-J. Yang, H.-

410   C. Gao, Alteration of interaction between astrocytes and neurons in different stages of diabetes:

411   a nuclear magnetic resonance study using [1-(13)C]glucose and [2-(13)C]acetate., Mol.

412   Neurobiol. 51 (2015) 843–852.

413   [11]   J.M.N. Duarte, K.Q. Do, R. Gruetter, Longitudinal neurochemical modifications in the aging

414   mouse brain measured in vivo by 1H magnetic resonance spectroscopy., Neurobiol. Aging. 35

415   (2014) 1660–1668.

416   [12]   C.J. Scavuzzo, C.J. Moulton, R.J. Larsen, The use of magnetic resonance spectroscopy for

417   assessing the effect of diet on cognition., Nutr. Neurosci. 21 (2018) 1–15.

418   [13]   L. Wu, Z. Niu, X. Hu, H. Liu, S. Li, L. Chen, D. Zheng, Z. Liu, T. Liu, F. Xu, A. Manyande, J.

419   Wang, H. Xia, Regional cerebral metabolic levels and turnover in awake rats after acute or

420   chronic spinal cord injury., FASEB J. 34 (2020) 10547–10559.

421   [14]   J. Wang, L. Jiang, Y. Jiang, X. Ma, G.M.I. Chowdhury, G.F. Mason, Regional metabolite

422   levels and turnover in the awake rat brain under the influence of nicotine., J. Neurochem. 113

423   (2010) 1447–1458.

424   [15]   Y. Liu, J. Cheng, H. Liu, Y. Deng, J. Wang, F. Xu, NMRSpec: An integrated software package

425   for processing and analyzing one dimensional nuclear magnetic resonance spectra, Chemom.

426   Intell. Lab. Syst. 162 (2017) 142–148.

427   [16]   T. Liu, Z. He, X. Tian, G.M. Kamal, Z. Li, Z. Liu, H. Liu, F. Xu, J. Wang, H. Xiang, Specific

428   patterns of spinal metabolites underlying α-Me-5-HT-evoked pruritus compared with histamine

429   and capsaicin assessed by proton nuclear magnetic resonance spectroscopy., Biochim. Biophys.

430   Acta. Mol. Basis Dis. 1863 (2017) 1222–1230.

431   [17]   S.J. Calvert, S. Reynolds, M.N. Paley, S.J. Walters, A.A. Pacey, Probing human sperm

432   metabolism using 13C-magnetic resonance spectroscopy., Mol. Hum. Reprod. 25 (2019) 30–

433   41.

434   [18]   L. Xin, B. Lanz, H. Lei, R. Gruetter, Assessment of metabolic fluxes in the mouse brain in vivo

435   using 1H-[13C] NMR spectroscopy at 14.1 Tesla., J. Cereb. Blood Flow Metab. 35 (2015)

436   759–765.

437 [19] H. Zheng, Y. Zheng, D. Wang, A. Cai, Q. Lin, L. Zhao, M. Chen, M. Deng, X. Ye, H. Gao,

438   Analysis of neuron-astrocyte metabolic cooperation in the brain of db/db mice with cognitive

439   decline using 13C NMR spectroscopy., J. Cereb. Blood Flow Metab. 37 (2017) 332–343.

440 [20] S.-L. Peng, H. Chiu, C.-Y. Wu, C.-W. Huang, Y.-H. Chung, C.-T. Shih, W.-C. Shen, The

441   effect of caffeine on cerebral metabolism during alpha-chloralose anesthesia differs from

442   isoflurane anesthesia in the rat brain., Psychopharmacology (Berl). 236 (2019) 1749–1757.

443 [21] K.I. Pogodaev, V. V Logunov, Dynamics of the formation and bonding of ammonia in rat brain

444   tissue under the influence of acoustic stimulation inducing convulsions., Biull. Eksp. Biol.

445   Med. 66 (1968) 46–49.

446 [22] S.J. Berger, J.C. Carter, O.H. Lowry, The distribution of glycine, GABA, glutamate and

447   aspartate in rabbit spinal cord, cerebellum and hippocampus., J. Neurochem. 28 (1977) 149–

448   158.

449 [23] M. Palmi, S. Brooke, A.D. Smith, J.P. Bolam, GABA-like immunoreactivity in different

450   cellular populations of cerebellar cortex of rats before and after treatment with amino-oxyacetic

451   acid., Brain Res. 543 (1991) 277–286.

452 [24] D.J. Meyerhoff, S. MacKay, L. Bachman, N. Poole, W.P. Dillon, M.W. Weiner, G. Fein,

453   Reduced brain N-acetylaspartate suggests neuronal loss in cognitively impaired human

454   immunodeficiency virus-seropositive individuals: in vivo 1H magnetic resonance spectroscopic

455   imaging., Neurology. 43 (1993) 509–515.

456 [25] R.L. Tyson, G.R. Sutherland, Labeling of N-acetylaspartate and N-acetylaspartylglutamate in

457   rat neocortex, hippocampus and cerebellum from [1-13C]glucose., Neurosci. Lett. 251 (1998)

458   181–184.

459 [26] I.-Y. Choi, R. Gruetter, Dynamic or inert metabolism? Turnover of N-acetyl aspartate and

460   glutathione from D-[1-13C]glucose in the rat brain in vivo., J. Neurochem. 91 (2004) 778–787.

461 [27] G. Oz, C.D. Nelson, D.M. Koski, P.-G. Henry, M. Marjanska, D.K. Deelchand, R. Shanley,

462   L.E. Eberly, H.T. Orr, H.B. Clark, Noninvasive detection of presymptomatic and progressive

463   neurodegeneration in a mouse model of spinocerebellar ataxia type 1., J. Neurosci. 30 (2010)

464   3831–3838.

465   [28]   A. Schousboe, Role of astrocytes in the maintenance and modulation of glutamatergic and

466   GABAergic neurotransmission., Neurochem. Res. 28 (2003) 347–352.

467   [29]   C. Rae, N. Hare, W.A. Bubb, S.R. McEwan, A. Bröer, J.A. McQuillan, V.J. Balcar, A.D.

468   Conigrave, S. Bröer, Inhibition of glutamine transport depletes glutamate and GABA

469   neurotransmitter pools: further evidence for metabolic compartmentation., J. Neurochem. 85

470   (2003) 503–514.

471   [30]   N.R. Sibson, A. Dhankhar, G.F. Mason, D.L. Rothman, K.L. Behar, R.G. Shulman,

472   Stoichiometric coupling of brain glucose metabolism and glutamatergic neuronal activity.,

473   Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 316–321.

474   [31]   C. Yüksel, D. Öngür, Magnetic resonance spectroscopy studies of glutamate-related

475   abnormalities in mood disorders., Biol. Psychiatry. 68 (2010) 785–794.

476   [32]   S. Moriguchi, A. Takamiya, Y. Noda, N. Horita, M. Wada, S. Tsugawa, E. Plitman, Y. Sano,

477   R. Tarumi, M. ElSalhy, N. Katayama, K. Ogyu, T. Miyazaki, T. Kishimoto, A. Graff-Guerrero,

478   J.H. Meyer, D.M. Blumberger, Z.J. Daskalakis, M. Mimura, S. Nakajima, Glutamatergic

479   neurometabolite levels in major depressive disorder: a systematic review and meta-analysis of

480   proton magnetic resonance spectroscopy studies., Mol. Psychiatry. 24 (2019) 952–964.

481   [33]   G. V Mkrtchyan, A. Graf, L. Trofimova, A. Ksenofontov, L. Baratova, V. Bunik, Positive

482   correlation between rat brain glutamate concentrations and mitochondrial 2-oxoglutarate

483   dehydrogenase activity., Anal. Biochem. 552 (2018) 100–109.

484   [34]   M. Rijpkema, J. Schuuring, Y. van der Meulen, M. van der Graaf, H. Bernsen, R. Boerman, A.

485   van der Kogel, A. Heerschap, Characterization of oligodendrogliomas using short echo time

486   1H MR spectroscopic imaging., NMR Biomed. 16 (2003) 12–18.

487   [35]   S. Chawla, L. Oleaga, S. Wang, J. Krejza, R.L. Wolf, J.H. Woo, D.M. O'Rourke, K.D. Judy,

488   M.S. Grady, E.R. Melhem, H. Poptani, Role of proton magnetic resonance spectroscopy in

489   differentiating oligodendrogliomas from astrocytomas., J. Neuroimaging. 20 (2010) 3–8.

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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.

511   Fig. 5: Pearson correlation analysis between ^{13}C enrichment in different kinds of metabolites (Glu$_4$, Glu$_3$,

512   NAA$_2$, NAA$_3$, Glx$_2$, Glx$_3$, GABA$_2$, GABA$_3$, GABA$_4$, Gln$_4$, Asp$_3$, Asp$_2$). *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: NAA$_3$-Glu$_4$ (A),

517   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*

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