Regional metabolic patterns of abnormal postoperative behavioral performance in aged mice assessed by 1H-NMR dynamic mapping method

Regional metabolic patterns of postoperative abnormal neurobehavioral performance in aged mice assessed by ^{1}H-NMR dynamic mapping method

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Abstract: Postoperative abnormal neurobehavioral performance (PANP) is a common phenomenon in the early postoperative period. The disturbed homeostatic status of metabolites in the brain after anesthesia and surgery might make a significant contribution to PANP. The dynamic changes of metabolites in different brain regions after anesthesia and surgery, as well as the potential association with PANP is still not well understood. Here, a battery of behavioral tests was used to assess the effects of laparotomy under isoflurane anesthesia in aged mice, and the metabolites in 12 different sub-regions of the brain at different time points were investigated by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopic technique. The abnormal neurobehavioral performance occurred at 6h and/or 9h, and recovered at 24h after anesthesia/surgery. Compared with the control group, the altered metabolites of the model group at 6h were aspartate (Asp) and N-acetyl-aspartate (NAA), and these differences were mainly displayed in the cortex; while significant changes at 9h occurred predominantly in the cortex and hippocampus, and the corresponding metabolites were Asp and glutamate (Glu), these changes returned to baseline level at 24h. The altered metabolic changes could have occurred as a result of the acute PANP, and metabolites of Asp and Glu in the cortex and hippocampus could provide preliminary evidence to our understanding of the PANP process.

***Keywords*:** Postoperative abnormal neurobehavioral performance; ^{1}H-NMR; Metabolites; Aspartate; Glutamate.

Introduction

Emerging evidence indicates that surgery/anesthesia could generate many complications during the perioperative period, such as neurocognitive disorders, found especially in the elderly [1, 2]. Homeostasis of cerebral metabolism is fundamentally essential for neurobehavioral functioning. The disturbance of metabolites in the brain after anesthesia and surgery might contribute significantly to postoperative neurobehavioral disorders. Unfortunately, the dynamic changes of metabolites in the aging brain during the early postoperative period, as well as the potential role of these dynamic changes in the pathophysiology of postoperative abnormal neurobehavioral performance (PANP) in the frail brain remains unknown. Previous reports suggest that several metabolites might serve as biomarkers in neurobehavioral disorders, such as the glutamate (Glu), N-acetyl-aspartate (NAA), creatine (Cr), gamma-aminobutyric acid (GABA), and aspartate (Asp) in human beings and in rodents[3-5]. However, the association between different presentations of PANP and the corresponding metabolites is still not well documented in the aging brain.

As an ionizing radiation-free technique, the *in vivo* magnetic resonance spectroscopy (^{1}H-MRS) could provide tissue chemical information, including NAA, an indicator of viable neuronal tissue density[6]; Cr, an essential molecule in energy homeostasis of the central nervous system (CNS)[7]; choline (Cho), a marker that presents the rate of membrane turnover[8]; Glu, the main primary excitatory neurotransmitter in the CNS; and GABA, the most prevalent inhibitory neurotransmitter in the CNS [9]. However, there are still several limitations of its application, such as lower spectral resolution, limited metabolites, and quantification of the metabolites. The *in-vitro* ^{1}H-NMR ideally resolves these problems by optimizing the homogeneity of the magnetic field and the extracted sample without interference of the macromolecules and lipids. With much higher spectral resolution,

there are more metabolites identified using the *in-vitro* ^{1}H-NMR method [10]. Combined with different quenching metabolism methods, it has been extensively applied in neuroscience studies [5, 11, 12], and the metabolic mapping techniques have been extensively used to explore the variation of metabolites in various brain disorders [13, 14]. Thus, it may present a promising method for analyzing the dynamic metabolic changes in different brain regions following anesthesia and surgery, therefore, shedding light on the basic profile of PANP.

The purpose of this study was to assess the effects of anesthesia/surgery on neurobehavioral performance in aged mice during the early postoperative period and to explore the potential metabolic mechanism *via* a ^{1}H-NMR based dynamic mapping method, which may provide a dynamic map of metabolic information of aging brains following anesthesia/surgery and might be beneficial to our understanding of the postoperative abnormal neurobehavioral performance in the early postoperative period.

Materials and Methods

Animals

The animal experimental protocol was approved by the Animal Care and Use Committee of Peking University (Beijing, China, Certification number: LA201413). Female mice C57Bl/6 (16 months, 20-25g) were purchased from the Experimental Animal Center of Hubei Provincial Academy of Prevention. All animals were housed in plastic cages (four in each cage) and maintained on a 12-h light/dark (lights at 7:00 am-7:00 pm) cycle, with food and water available *ad libitum*. In order to accustom the animal to human interaction and minimize stress, the animals were handled daily for a week before the experimental day, such as grasping the animal, mildly touching its

skin/hair, scratching the animals for about one minute.

Anesthesia and surgery treatment

Mice were randomly assigned into either the surgery plus anesthesia group (n=40, 10 animals per time point, including 0h, 6h, 9h and 24h) or the control group (n=10). The experimental procedure was according to previous reports with minor modifications [15, 16]. Specifically, the animal was initially anesthetized with 1.4% isoflurane (in 100% oxygen) in a transparent chamber (RWD Life Science, Shenzhen, China). Fifteen minutes after induction, the mouse was carried out from the induction chamber, and a face mask was used to maintain the anesthesia with a 16-guage sensor monitoring the isoflurane concentration. Then a longitudinal midline incision was made from the xiphoid to the pubic symphysis, cutting through the skin, abdominal muscles and peritoneum. Then, the incision was sutured layer by layer with 5–0 Vicryl thread. At the end of the procedure, EMLA cream (2.5% lidocaine and 2.5% procaine) was applied to the wound area every 8 hours to minimize the pain and stress from the surgery. When the surgery was completed, the mouse was returned to the anesthesia chamber for up to two hours to receive the rest of the isoflurane anesthesia consisting of 1.4% isoflurane in 100% oxygen. The body temperature of the animal was maintained with a heating pad during anesthesia/surgery. The mice in the control group were placed in their home cage with 100% oxygen for two hours. To minimize the impact of circadian rhythms, anesthesia/surgery began at 8 am every day.

Behavior Test

Buried food test, open field test and Y maze were employed to measure the abnormal neurobehavioral performance in different periods after surgery. All animals received these three behavioral tests before the surgery, and the results were set as the baseline for further behavioral

analysis. Then all mice in the control and treated groups received these three tests again at 6, 9, 24 hours after anesthesia/surgery. The protocol of these tests were based on previous studies with slight modifications [15, 17]. For the animals in the treated groups at certain periods (6h, 9h and 24h), the animals were euthanized using the microwave irradiation approach under isoflurane anesthesia just after the behavioral tests at the same time points.

To help habituation, all mice were moved to the behavioral room one hour before the behavior tests. Four mice from each group performed each day, which ensured that all behavior tests were finished within 1 hour, to minimize the impact of circadian rhythms. To avoid the influence of the odor in the equipment, 70% ethanol was used to clean all the equipment after every trial. All the behavioral data were analyzed with the animal tracking system (Smart 3.0, RWD Life science Co., LTD, China). The details of the experimental steps are described in the supplementary material.

Brain Sample Preparation for the NMR Study

To minimize the impact of post-mortem changes of brain metabolites, the mouse was euthanized using the microwave method which was fully described in our previous work [5]. After euthanasia, the mouse brain was removed and dissected into 12 different regions: olfactory bulb (OB), prefrontal cortex (PFC), parietal cortex (PC), occipital cortex (OC), temporal cortex (TC), striatum (STR), hippocampus (HP), thalamus (THA), hypothalamus (HYP), midbrain (MID), medulla-pons (MED-PONs), cerebellum (CE). Separation of the brain regions was according to the Allen brain atlas and previous publications [18, 19], and the method used is also illustrated in the supplemental material (Fig. S1). The tissues were immediately weighed and stored at -80 ^{o}C for further processing.

The protocol for tissue extraction is the same as that mentioned in our previous study [5], which

is briefly described here. HCl/methanol (0.1M, 100 μL) was added to the frozen tissues, and the tissue was homogenized with Tissuelyser for 1.5 minutes at a frequency of 20 Hz (Tissuelyser II, QIAGEN, German). Ice-cold 60% ethanol (800 μL) was further added to tubes and the mixture homogenized again, before being centrifuged at 14,000 g for 10 minutes. The supernatant was then collected. The extraction steps were repeated twice with 800 μL 60% ethanol to extract the left metabolites in the sediment. All the supernatants were collected and desiccated with the centrifugal drying apparatus (Thermo Scientific 2010, Germany) and freezing vacuum dryer (Thermo, Germany). The dried product was preserved for further NMR studies.

The dried product was successively dissolved in 60 μL D_2O (Containing the inner standard, 3-(trimethylsilyl) propionic - 2, 2, 3, 3, d_4 acid sodium salt (120 mg/L, TSP, 269913-1G, Sigma-Aldrich)) and 540 μL phosphate buffer (pH=7.2). The solution was mixed in a high-speed vortex and centrifuged at 14,000 g for 15 min, and the supernatant was withdrawn and transferred to an NMR tube for the NMR study.

NMR Spectrum Acquirement

^{1}H-NMR spectra were acquired as in previous studies [5, 20]. The extracted samples were measured with a Bruker Avance III 600 MHz NMR spectrometer (298 K) equipped with an inverse cryogenic probe (Bruker BioSpin, Germany). The ^{1}H-NMR spectra were acquired with a standard WATERGATE pulse sequence [21]. The following acquisition parameters were set for every sample: p1 (90° pulse): 8.35 μs; NS (number of scans): 256; Spectral width: 20 ppm; Dummy scans: 8; Number of FID points: 32 k.

NMR Data Processing

All ^{1}H-NMR spectra were processed and analyzed with TOPSPIN (Version 2.1, Bruker

BioSpin) and a home-made software *NMRSpec* [22]. Firstly, the phase correction and baseline distortion were manually completed in TOPSPIN. Then the corrected spectra were imported into *NMRSpec* for spectrum alignment, peaks extraction, spectra integration, and the integration of chemical related peaks. This software has been widely used in several metabolomics studies [5, 23, 24].

The chemical shifts of major amino acids were distributed in the areas of 1.20–4.46 ppm, and thus this gap was extracted for further analysis. First of all, areas of all peaks (area under the curve) in this gap were automatically calculated for further statistical analysis [5]. To compensate for the different concentration, every peak area was normalized to the sum of all the peak areas in this gap of its own spectrum prior to the discriminant analysis [18, 20, 25].

Furthermore, the absolute concentrations (μmol/g wet weight) of the identified metabolites were calculated with the related peak areas in spectrum of the sample, information of the internal standard chemical (TSP, such as concentration, proton number) and weight of specimen. The calculation equation is shown as following:

$$C_{met} = \frac{A_{met}/(R_{met} * N_H)}{A_{TSP}} * (C_{TSP} * V_{TSP}) * 9/Wt \quad (1)$$

Where A_{met} and A_{TSP} are the relative areas of the related peaks of the detected metabolites and TSP, and R_{met} is a constant value for a certain metabolite which was calculated from the ratio between the partial NMR signal of the standard metabolite between the selected regions (almost pure chemical signal) in a real sample and the whole proton signal in the standard spectrum; N_H is the number of protons of the metabolites within the certain area of A_{met}; C_{TSP} and $V_{TSP}$is the concentration and volume of TSP standard solution added in the NMR tube respectively; W_t is the total weight of the wet specimen, and 9 is the number of protons in the TSP.

Statistical Analysis

In the behavioral test, a baseline parameter of every mouse was taken into consideration for abnormal neurobehavioral performance. The relative values of behavior at every time point (6 h, 9 h or 24 h postoperatively) were presented as a percentage compared with its baseline value. The repeated behavioral test of limited duration could influence the performance of mice, thus only the control group and the 24h group, both of which received behavior tests 3 times (6 h, 9 h and 24 h), were used to justify the difference in neurobehavioral performance. Values for behavior performance were compared using the Wilcoxon Mann-Whitney U test.

To discriminate the different metabolic patterns between the control and anesthesia/surgery groups, the orthogonal partial least squares discriminant analysis (OPLS-DA) was applied. There were 12 different brain regions involved. For clarity, the metabolic spectra and statistical analysis of the PFC region were presented as a typical example to show the efficiency of the OPLS-DA method. To determine the significant differences in the corresponding metabolite levels in the whole brain, a one-way analyses of variance (ANOVA), followed by a least significant difference (LSD) post-hoc test were used to analyze the different concentrations of the metabolites in every brain region. The criterion for statistical significance is a probability value of 0.05. All data are presented as means ± SEM.

Results

Anesthesia/surgery increased the latency to find food in a time-associated manner

To assess the effects of anesthesia and surgery on the natural habits of mice, the buried food test was employed in the current study (Fig.1 (A1-A3)). Compared with the control group, the

latency to find food in the anesthesia/surgery group was longer both at 6 hours (114.0%±15.0% *versus* 69.7%±12.8%, Z= -2.098, *p*=0.036, Fig. 1A1) and 9 hours (171.0%±29.6% *versus* 81.2%±12.1%, Z= -2.342, *p*=0.019, Fig. 1A2) after anesthesia/surgery. However, we found no significant difference in the latency at 24 h after the operation when compared with controls (Fig. 1A3). Taken together, these data suggest that the abdominal surgery plus isoflurane anesthesia could undermine the natural ability of mice to find food and this process was time-associated.

Anesthesia/surgery decreased the time spent in the center of the open field box in a time-associated manner

The open field test was used to evaluate whether anesthesia/surgery affected the emotion of mice (Fig.2). The anesthesia/surgery decreased the time spent in the center region at both 6 h (39.0%±8.9% *versus* 122.0%±32.6%, Z= -2.075, p=0.038, Fig. 2A1) and 9 h (21.1%±6.9% *versus* 139.0%±49.1%, Z= -2.532, p=0.011, Fig. 2A2) points, but not at the 24 h point (Fig. 2A3), which indicates that the anesthesia/surgery had an adverse effect on the emotion of mice in a time-associated manner. Furthermore, other parameters (e.g. latency to the center and total travel distance) were also analyzed. There was no significant difference between these two groups at any time point (Fig. 2B1-C3), suggesting that anesthesia/surgery did not cause motor dysfunction in this study. Taken together, the results of these behavior tests were consistent with our hypothesis that anesthesia/surgery could cause neurobehavioral disorder in a time-associated manner in aged mice.

Anesthesia/surgery decreased the exploration in the novel arm of the Y maze in a time-associated manner

The spontaneous Y maze test was introduced to evaluate spatial learning and memory in mice

(Fig. 3). Compared with the control group, anesthesia/surgery did shorten the duration in the novel arm at 6 h (81.0%±11.7% *versus* 116.0%±10.0%, Z= -2.203, p= 0.028, Fig. 3A1) after the intervention, but not at 9 h (Fig. 3A2) and 24 h (Fig. 3A3) points. Furthermore, the entries to the novel arm in the anesthesia/surgery group was significantly less than that in the control group at both 6 h (81.7%±4.1% *versus* 105.0%±3.8%, Z= -2.950, p=0.003, Fig. 3B1) and 9 h (84.7%±7.3% *versus* 107.0%±6.4%, Z= -2.061, p= 0.039, Fig. 3B2) points, but not at 24 h point (Fig. 3B3). In addition, the total entries to the three arms and total travel distance were also analyzed. We found no significant difference at any time point (Supplemental material Fig. S2: A1-B3). These results demonstrate that anesthesia/surgery could impair spatial learning and memory in mice in both a time-associated and motor-independent manner.

^{1}H-NMR based metabolic information for the aged brain at different postoperative time points

To obtain the dynamic changes of the concentrations of metabolites that may be related to the abnormal neurobehavioral performance and anesthesia/surgery states, four different time points (0 h, 6 h, 9 h and 24 h after anesthesia/surgery) were selected to analyze the metabolites' information with the ^{1}H-NMR spectra. There were 12 different brain regions involved in the current study, and an example region (PFC) from different time points are illustrated in Fig. 3. As shown in Fig. 4, the average normalization spectra of the different groups were collected, and the basic metabolic information exhibited, including the metabolite name and the related chemical shift. It can be seen that group '0 H' has the minimum spectrum height of aspartate, glutamate; and the maximum spectrum height of alanine. However, without statistical analysis information, it is difficult to compare the significant differences among different groups, therefore, further analysis is necessary.

Metabolic patterns in different brain regions after anesthesia/surgery

The contents of metabolites in the control group were set as the baseline and variations of the metabolites were investigated and identified. The significant changes of metabolites are listed in Table 1. The level of metabolites in every region almost exhibited time-associated patterns (maximum variation at 0 h, returned to normal levels at 24 h). Most of metabolites dramatically decreased in most brain regions (except in the midbrain) after anesthesia/surgery; except for Ala that increased in the 0 H group. The metabolites in most brain regions fully recovered to normal levels (baseline) after 6 h except in the cortex regions. There were eight different brain regions involved (FC, PC, OC, TC, STR, HP, THA and MID) after 9 h, and most metabolites returned to baseline level after 24 h except for OB, FC, TC and CE.

Discriminant analysis between the control and anesthesia/surgery treated groups

The OPLS-DA method was used to select the dominant metabolite changes caused by anesthesia/surgery and to visually discriminate the samples in different groups. There were 12 different brain regions analyzed. Among these brain regions, the metabolites in the PFC region changed during the whole period. It was therefore, selected as an example of the brain regions to illustrate the results of OPLS-DA (Fig. 5). The significant different metabolites corresponding to anesthesia/surgery treatment were screened out with correlation coefficients of the OPLS-DA method ($r>0.4329$, $p<0.05$, $F=19$).

Compared with the control group, the anesthesia/surgery group showed multiple significant changes at 0 h (Fig. 5A2). For instance, there was a decrease in glutamate and aspartate,

accompanied by an increase in alanine. There was also an observable increase in aspartate both at 6 h (Fig. 5B2) and 9 h (Fig. 5C2); but an increase in glutamate and a decrease in GABA were observed only at 9 h (Fig. 5C2). In addition, there was a tendency for choline to increase and for glutamate to decrease 24 h postoperatively (Fig. 5 D2).

Considering the vital function of the brain excitatory amino acid, the dynamic changes in glutamate and aspartate were also further investigated (Fig. S3 and S4). Dynamic changes of Glu and Asp in PFC are illustrated in Fig. 6A. Our results demonstrate that anesthesia/surgery treatment sharply reduced the level of aspartate at 0 h (red line); there was a gradual reversal of the trend at 6 h (green line) and 9 h (blue line), but the level in aspartate returned closely to the baseline (control group, pink line) after 24 h (yellow line). The other excitatory amino acid - glutamate presented a similar variation tendency as the aspartate (Fig. 6B).

The variation tendency in glutamate and aspartate in the whole brain after anesthesia/surgery

To further assess the effects of anesthesia/surgery treatment on glutamate and aspartate in the whole brain, their absolute concentrations were calculated and compared (Fig. 7 and Fig. 8). The effects of anesthesia/surgery on aspartate were mainly reflected in the cortex (FC, PC, OC and TC) at certain time points (0 h, 6 h and 9 h postoperatively) and their variation tendency of aspartate was almost similar. Furthermore, the variation tendency of glutamate caused by anesthesia/surgery mostly occurred in FC, TC, STR, HP and THA (Fig. 8) at certain time points (0 h and 9 h).

Collectively, these results suggest that anesthesia/surgery treatment could cause a neurobehavioral disorder in a time-associated manner in aged mice. The anesthesia/surgery could also alter some metabolites' dynamics (e.g. glutamate, GABA, alanine, aspartate, choline, NAA,

glutamine and glycine), and variations of some metabolites (e.g. glutamate and aspartate) were consistent with the dynamic changes of the abnormal behavior performance in mice with anesthesia/surgery challenge. The alteration of metabolites was also exhibited in a time-associated manner.

Correlation between neurobehavioral and NMR measurements

In order to demonstrate the association between the metabolic changes and behavioral abnormality in the current study, the correlations between the changed behavior and the significantly altered metabolites (Glu and Asp) were analyzed. The region of PFC was selected due to the most metabolic changes in this region. The results are shown in (Fig. 9). The concentrations of the Asp and Glu were positively correlated with the latency to food, especially for ASP. However, they were negatively correlated with the other behaviors, such as duration in center, duration in novel arm, and entries into novel arm. Among these relationships, the concentrations of Asp were significantly negatively correlated with the duration (p=0.041) and entries in novel arm (p=0.028).

Discussion

Application of *in-vitro* nuclear magnetic resonance spectroscopy

There were two magnetic resonance spectra methods that could be used to analyze the changes of the metabolites in animal models – *in vivo* MRS, and *in vitro* NMR. Generally, the *in vivo* MRS method could provide more accurate region-specific information due to its high spatial resolution, ability to longitudinally monitor the changes of metabolites and reduce animal numbers in most studies. Moreover, it has the direct potential for clinical translational value. The most fundamental

requirement for *in-vivo* ^{1}H-MRS test was to keep the animal immobile during the whole data collection process. As we know, there are only two ways to keep the animal immobile, continuous anesthesia or the use of a movement restricted apparatus. Given that the PNAP model in this study was constructed by isoflurane plus surgery, which meant anesthesia could likely be an important factor in facilitating behavior or metabolite changes, thus the *in-vivo* ^{1}H-MRS was not used in this study. The *in-vitro* NMR also involved anesthesia, but the dose and duration of anesthesia were far less than that of the *in-vivo* ^{1}H-MRS. In addition, the *in-vivo* MRS only tested one specific region in every trial. To screen the regional metabolic patterns of PANP, the mouse brain was divided into 12 different regions for NMR measurement, which meant more confounded effects from the continuous administration of anesthesia if the *in-vivo* ^{1}H-MRS was selected.

Abnormal neurobehavioral performance in aged mice after anesthesia/surgery

All the behavioral tests in this study were closely dependent on the movement ability of mice. The present results showed that the locomotor activity was not significantly impaired at any time, suggesting that the abnormal neurobehavioral performance at 6 h or 9 h was not related to the motor function. There may be some other confounders to make a definite conclusion, such as the effect of olfaction to the buried food test [26], however, each element of intact attention, unsubdued consciousness, normal emotion and organized thinking was indispensable for mice to perform normally.

Our results are not completely the same as those from a previous study[15], in which there was no significant difference in the latency to eat food at 6 h and the time spent in the center at 9 h. This discrepancy is probably due to the difference in research subjects (16 month-old *versus* 4 month-old mice), which is consistent with previous consensus that aging is an independent risk factor for

postoperative neurobehavioral disorders[27].

Brain regions affected after anesthesia/surgery

Our results showed that the most severely affected regions are all in the four areas of the cerebral cortex and the HP. These regions are the most indispensable regions in the brain and play distinct and complementary roles in the process of normal neurobehavioral function (e.g. learning and memory, emotion regulation) [28, 29]. The PANP may occur simultaneously when any of the FC, TC and HP is directly impaired or the mutual complementary effect is abnormal [30-32].

Our results are also consistent with recent clinical imaging work. It was reported that several regions are involved in the pathology of neurobehavioral dysfunction after anesthesia/surgery, including the cortex, HP, THA and CE. These were obtained using diverse technologies, such as the single photon emission computed tomography (SPECT) and the xenon-enhanced computed tomography to investigate the cerebral blood flow changes [33, 34], the diffusion tensor imaging (DTI) to analyze the neuronal connection [35], and the blood-oxygen-level-dependent (BOLD) signal to measure functional connectivity [36]. Thus, cerebral regions of the cortex and HP could play key roles in the pathology of neurobehavioral disorders after anesthesia/surgery.

Metabolic variation after anesthesia/surgery

Many metabolites in the whole brain fluctuated significantly following anesthesia/surgery, including Myo, Asp, Glu, Gln, GABA, Tau, and Ala. The general tendency was that the concentration of excitatory amino acids decreased (*e.g.* Asp and Glu) and the concentration of inhibitory amino acids (e.g. GABA and Tau) increased, which indicates that anesthesia/surgery may disturb the balance of excitatory and inhibitory function in the brain. Most of these metabolic

alterations had almost disappeared and the neurobehavioral performance returned to normal after 24 h.

The elevated tendency of Asp in the cortex at 6 h and 9 h accompanied with evident neurobehavioral disorders in this study is supported by a previous study, in which the increment of Asp was accompanied by impairment of neurobehavioral performance [4]. There are two isomers of Asp, L-Asp and D-Asp. The latter can trigger glutamate transmission and is able to activate the N-methyl-D-aspartate (NMDA) receptor [37]; while the former is a critical building block of proteins and can be converted to the latter with the aid of D-Asp racemase [38]. There are published data indicating that the application of exogenous D-Asp could not only alleviate the cognition impairment induced by the stimulus of neuropathic pain [39], but also enhanced the long term potential (LTP) and improved cognition in aged mice [40]. We, therefore, concluded that there may be a positive correlation between the content of D-Asp and cognition. However, it is reported that the D-Asp decreased and its content accounted for less than 1% of the total Asp in adults, while the content of L-Asp increased relatively [41]. This suggests that the metabolic changes detected in our study were mainly L-Asp, and there might have been more D-Asp due to conversion from the L type. The possible explanations of the increased Asp and the decline in mice cognition are as follows: (1) the effects of D-Asp on cognition might be associated with the individual age, which was confirmed by a previous study [42]; (2) excess D-Asp generated the “D-Asp excitotoxicity” as the surplus of excitatory neurotransmitter glutamate; (3) the increase of Asp was a result of compensatory feedback from the decreased D-asp. Nevertheless, the exact content of D-Asp needs to be determined by future studies.

Imbalance of Glu is strongly linked with both acute and chronic neurodegeneration, which

could lead to the phenomenon of excitotoxicity through disturbing the steady state of calcium, the balance of energy, the normality of neuronal death pathways, and so forth [43]. In the current study, the excess Glu at 9 h may possess excitotoxicity in the brain and impair the cognitive related cerebral regions, such as FC and HP. This speculation is supported by an animal study, in which increment of Glu was associated with cognitive dysfunction [4]. However, Kroll *et al* reported that reduced Glu might also be related to the impairment of cognition in asthma patients [44]. The paradoxical evidence might be due to the different species as well as the time-course observed. In the former study, an acute animal model with rats was investigated and the observation time-course was less than 24 hours; while the latter study was a clinical observation for the possible association of Glu and cognition decline in asthma patients, a common chronic disease of the respiratory system.

Limitations and perspective

This study aimed to explore the metabolic patterns of the PANP associated with surgical treatment; and several potential metabolites were preliminarily screened out through the dynamic mapping technique. Regrettably, we cannot elaborate the concrete cause (anesthesia or surgery) of PANP due to the lack of an anesthesia alone group. A ^{1}H-MRS study demonstrated that isoflurane anesthesia indeed altered the brain metabolites of mice immediately after the anesthesia[45]. But whether (or how) the anesthesia alone affected the PANP in aged brain needs to be explored in a further study.

Conclusions

By means of the ^{1}H-NMR method, this study explored the PANP after anesthesia/surgery from a metabolic perspective. Both neurobehavioral performance and concentrations of metabolites

exhibit a parallel pattern in a time-associated manner. The most fluctuated metabolites in the various cerebral regions are two critical excitatory amino acids, aspartate and glutamate. The current study may provide a dynamic map of metabolite alterations associated with the neurobehavioral disorders following anesthesia/surgery, which may be conducive to ultimately reveal the metabolic mechanism of neurobehavioral disorders postoperatively.

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Author Contributions

T. Liu, J. Wang, H. Xiang, F. Xu and X. Guo designed the research; T. Liu, Z. Li, J. He, N. Yang, and J. Wang conducted the experiment; T. Liu, X. Tian, D. Han, Y. Li, H. Liu, J. Wang, and H. Xiang analyzed the data; T. Liu, J. Wang, H. Xiang, A. Manyande and X. Guo wrote the manuscript, A. Manyande reviewed & proofread. All authors have reviewed and approved the final manuscript.

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Figure legends:

Fig. 1. The impact of time-associated anesthesia/surgery (6 h, 9 h and 24 h after anesthesia/surgery) on animal behaviors assessed by the Buried food test and the Y maze test. Data is represented by means ± SEM. *: $p< 0.05$, **: $p < 0.01$, Mann-Whitney u test.

Fig. 2. The impact of time-associated anesthesia/surgery (6 h, 9 h and 24 h after anesthesia/surgery) on animal behaviors assessed by the Open field test. (A1-A3) Time spent in the center; (B1-B3) Latency to the center; (C1-C3) Total travel distance. Data is represented by means ± SEM. *: $p< 0.05$, **: $p < 0.01$, Mann-Whitney u test.

Fig. 3. The impact of time-associated anesthesia/surgery (6 h, 9 h and 24 h after anesthesia/surgery) on animal behaviors assessed by the Y maze test. (A1-A3) Duration in the novel arm; (B1-B3) Entries in the novel arm. Data is represented by means ± SEM. *: $p< 0.05$, **: $p < 0.01$, Mann-Whitney u test.

Fig. 4. The normalized average ^{1}H–NMR spectra for the frontal cortex samples in the control and model groups at different time points (0 h, 6 h, 9 h, and 24 h after the anesthesia/surgery). The horizontal axis represents the chemical shift of ^{1}H–NMR spectrum. *Note: Lowercase, the carbon position connected with the hydrogen signal; Lac, lactate; Myo, myo-institol; Cre, creatine; Ala, alanine; Glx, glutamine + glutamate; Gly, glycine; Asp, asparte; EtoH, ethanol; Tau, taurine; GABA, gamma-aminobutyric acid; NAA, N-aceytl aspartate.*

Fig. 5. OPLS-DA scores and Coefficient-coded loadings plot the models in the frontal cortex at different time points. (A1-D1) OPLS-DA scores at 0h, 6h, 9h, and 24h after anesthesia/surgery; (A2-D2) Coefficient-coded loadings plot the models at 0h, 6h, 9h, and 24h after the anesthesia/surgery. *Note: t[1]p, first predictive component; t[2]o, Orthogonal principal*

component score; 1, lactate; 2, alanine; 3, glutamate; 4, aspartate; 5, taurine; 6, ethonal + myo-institol ; 7, alanine + glutamate + glutamine ; 8,cretine; 9, N-aceytl aspartate; 10, gamma-aminobutyric acid; 11, choline.

Fig. 6. The normalized average ^{1}H–NMR spectra of selected metabolites in the frontal cortex at different periods after anesthesia/surgery (means ± SEM). The horizontal axis represents the chemical shift of ^{1}H–NMR spectrum. (A) Aspartate; (B) Glutamate. *Note: Pink background, control; Red background, 0h; Green background, 6h; Blue background, 9h; Yellow background, 24h.*

Fig. 7. Concentrations of aspartate in distinct brain regions at different time points after anesthesia/surgery. Statistical significant differences between groups were assessed by one-way ANOVA followed by LSD post-hoc multiple comparison test ($^{*}p < 0.05$, $^{\#}p < 0.01$). *Note: FC, Frontal Cortex; PC, Parietal Cortex; OC, Occipital Cortex; TC, Temporal Cortex; STR, Striatum; HP, Hippocampus; THA, Thalamus; HYP, Hypothalamus; OB, Olfactory Bulb ; MID, Midbrain; MED-PONs, Medulla-Pons; CE, Cerebellum.*

Fig. 8. Concentrations of glutamate in distinct brain regions at different periods after anesthesia/surgery. Statistical significant differences among groups were assessed by one-way ANOVA followed by LSD post-hoc multiple comparison test ($^{*}p < 0.05$, $^{\#}p < 0.01$). *The full names of the regions are the same as Fig. 6.*

Fig. 9. Correlations of aspartate and glutamate in PFC at different periods after anesthesia/surgery.

Table.1. Metabolites changed in the whole brain classified by regions

	0H	6H	9H	24H
OB	**Myo**-14.3% ↑ (*P*=0.001) **Asp**-22.3% ↓ (*P*=0.016) **Glu**-39.6% ↓ (*P*<0.001) **Ala**-76.9% ↑ (*P*<0.001)			**Gln**-20.6% ↓ (*P*=0.017)
FC	**Asp**-22.2% ↓ (*P*<0.001) **Glu**-20.2% ↓ (*P*<0.001) **Ala**-89.9% ↑ (*P*<0.001) **Lac**-56% ↑ (*P*<0.001)	**Asp**-13.0% ↑ (*P*=0.006) **NAA**-6.29% ↑ (*P*=0.025)	**Asp**-16.6% ↑ (*P*=0.001) **Glu**-6.39% ↑ (*P*=0.017)	**Cho**-32.6% ↑ (*P*=0.024) **Gln**-25.6% ↓ (*P*=0.012)
PC	**Tau**-8.74% ↑ (*P*=0.004) **Asp**-11.7 ↓ (*P*=0.035) **Glu**-17.8% ↓ (*P*<0.001) **Ala**-65.4 ↑ (*P*<0.001) **GABA**-13.4 ↓ (*P*=0.002)	**Asp**-15.8% ↑ (*P*=0.001)	**Asp**-13.1% ↑ (*P*=0.010)	
OC	**Cre**-18.6% ↓ (*P*=0.005) **Asp**-16.2% ↓ (*P*=0.013) **Glu**-20.1 ↓ (*P*<0.001) **Ala**-67.7 ↑ (*P*<0.001)	**Asp**-15.5% ↑ (*P*=0.006)	**Asp**-15.8% ↑ (*P*=0.007)	
TC	**Asp**-19.6% ↓ (*P*=0.001) **Glu**-14.1% ↓ (*P*<0.001) **Ala**-76.6% ↑ (*P*<0.001)	**Cho**-30.6% ↓ (*P*=0.020) **Asp**-11.6% ↑ (*P*=0.018) **NAA**-8.7% ↑ (*P*=0.014)	**Glu**-6.7% ↑ (*P*=0.027)	**Glu**-6.7% ↓ (*P*=0.021)
STR	**Tau**-7.3% ↑ (*P*=0.006) **Glu**-22.0% ↓ (*P*<0.001) **Ala**-89.0% ↑ (*P*<0.001)		**Glu**-8.0% ↑ (*P*=0.011)	
HP	**Tau**-8.8% ↑ (*P*=0.003) **Glu**-18.2% ↓ (*P*<0.001) **Ala**-70.6% ↑ (*P*<0.001)		**Asp**-15.8% ↑ (*P*=0.020) **Glu**-6.1% ↑ (*P*=0.004)	
THA	**Tau**-12.0% ↑ (*P*=0.006) **Glu**-23.9% ↓ (*P*<0.001) **Ala**-63.7% ↑ (*P*<0.001)		**Glu**-8.2% ↑ (*P*=0.039)	
HYP	**Glu**-19.1% ↓ (*P*=0.001) **Ala**-56.0% ↑ (*P*<0.001) **GABA**-29.8% ↑ (*P*=0.009)			
MID	**Ala**-71.9% ↑ (*P*=0.001)		**Cho**-12.5% ↓ (*P*=0.027)	
MED	**Asp**-36.8% ↓ (*P*<0.001) **Ala**-23.2% ↑ (*P*=0.032)			
CE	**Gly**-15.9% ↓ (*P*=0.004) **Asp**-34.0% ↓ (*P*<0.001) **Ala**-30.2% ↑ (*P*=0.004) **Gln**-45.9% ↓ (*P*=0.002)	**Asp**-18.3% ↑ (*P*=0.023)		**Gln**-30.8% ↓ (*P*=0.015)

Note: Variation of metabolites in distinct brain regions at different periods after anesthesia/surgery. Statistical significant differences among groups were assessed by one-way ANOVA followed by LSD post-hoc multiple comparison test.