ORIGINAL ARTICLE

Regional Metabolic Patterns of Abnormal Postoperative Behavioral Performance in Aged Mice Assessed by ^1H -NMR Dynamic Mapping Method

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Abstract Abnormal postoperative neurobehavioral performance (APNP) 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 APNP. The dynamic changes of metabolites in different brain regions after anesthesia and surgery, as well as their potential association with APNP are still not well understood. Here, we used a battery of behavioral tests to assess the effects of laparotomy under isoflurane anesthesia in aged mice, and investigated the metabolites in 12 different sub-regions of

the brain at different time points using proton nuclear magnetic resonance (^1H -NMR) spectroscopy. The abnormal neurobehavioral performance occurred at 6 h and/or 9 h, and recovered at 24 h after anesthesia/surgery. Compared with the control group, the altered metabolite of the model group at 6 h was aspartate (Asp), and the difference was mainly displayed in the cortex; while significant changes at 9 h occurred predominantly in the cortex and hippocampus, and the corresponding metabolites were Asp and glutamate (Glu). All changes returned to baseline at 24 h. The altered metabolic changes could have occurred as a result of the acute APNP, and the metabolites Asp and Glu in the cortex and hippocampus could provide preliminary evidence for understanding the APNP process.

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Introduction

Emerging evidence indicates that surgery/anesthesia can generate many complications such as neurocognitive disorders during the perioperative period, especially in the elderly [1, 2]. Homeostasis of cerebral metabolism is essential for neurobehavioral functioning. 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 changes in the pathophysiology of abnormal postoperative neurobehavioral performance (APNP) in the frail brain remain unknown. Previous reports have suggested that several metabolites serve as biomarkers of neurobehavioral

disorders, such as glutamate (Glu), N-acetyl-aspartate (NAA), creatine, gamma-aminobutyric acid (GABA), and aspartate (Asp) in humans and rodents [3–5]. However, the association between different presentations of APNP and the corresponding metabolites is still not well documented in the aging brain.

As an ionizing radiation-free technique, *in vivo* magnetic resonance spectroscopy (^1H -MRS) can provide information on tissue chemicals, including NAA, an indicator of viable neuronal tissue density [6]; creatine, an essential molecule in energy homeostasis of the central nervous system (CNS) [7]; choline, a marker for the rate of membrane turnover [8]; Glu, the main primary excitatory neurotransmitter in the CNS [9, 10]; and GABA, the most prevalent inhibitory neurotransmitter in the CNS [11, 12]. However, there are still several limitations of its application, such as low spectral resolution, limited metabolites, and quantification of the metabolites. *In-vitro* ^1H -NMR ideally resolves these problems by optimizing the homogeneity of the magnetic field and the extracted sample without the interference of macromolecules and lipids. With much higher spectral resolution, more metabolites can be identified using this method [13]. Combined with different metabolism quenching methods, it has been extensively applied in neuroscience studies [5, 14, 15], and the metabolic mapping techniques have been extensively used to explore the variation of metabolites in various brain disorders [16, 17]. Thus, it presents a promising method for analyzing the dynamic metabolic changes in different brain regions following anesthesia and surgery, shedding light on the basic profile of APNP.

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 metabolic mechanisms *via* an ^1H -NMR-based method to provide a dynamic map of metabolic information of aging brains following anesthesia/surgery and advance our understanding of the APNP in the early postoperative period.

Materials and Methods

Animals

The experimental protocol was approved by the Animal Care and Use Committee of Peking University (Beijing, China, Certification number LA201413). Female C57Bl/6 mice (16 months old, 25–30 g) were purchased from the Experimental Animal Center of Hubei Provincial Academy of Prevention. All animals were housed in plastic cages (4/cage) and maintained on a 12-h light/dark cycle (lights on 07:00–19:00), with food and water available *ad libitum*. In

order to accustom the animals to human interaction and minimize stress, the mice were handled daily for a week before the experimental day, by grasping the animal, mildly touching its skin/hair, and scratching for ~ 1 min.

Anesthesia and Surgery

Mice were randomly assigned into either the surgery plus anesthesia group ($n = 40$, 9–1 each at 0, 6, 9, and 24 h) or the control group ($n = 13$). The experimental procedure was based on previous reports with minor modifications [18, 19]. Specifically, each mouse was initially anesthetized with 1.4%–2.0% isoflurane (in 100% oxygen) in a transparent chamber (RWD Life Science, Shenzhen, China). Fifteen minutes after induction, the mouse was removed from the induction chamber, and a face mask was used to maintain the anesthesia with a 16-gauge 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. At the end of the procedure, EMLA cream (2.5% lidocaine and 2.5% procaine) was applied to the wound area every 8 h to minimize the pain and stress from the surgery. After the surgery, the mouse was returned to the anesthesia chamber for up to 2 h to receive the rest of the 1.4%–2.0% isoflurane in 100% oxygen. The body temperature was maintained with a heating pad during the anesthesia/surgery. The mice in the control group were placed in their home cage with 100% oxygen for 2 h. To minimize the impact of circadian rhythms, anesthesia/surgery began at 08:00 am each day.

Behavioral Tests

The buried food test, open field test, and Y maze were used to measure the abnormal neurobehavioral performance in different periods after surgery. All animals received these three tests before surgery, and the results were set as the baseline for further behavioral analysis. Then all mice in the control and experimental groups performed all three tests again at 6, 9, and 24 h after anesthesia/surgery. The protocols of these tests were based on previous studies with slight modifications [18, 20]. The animals in the experimental groups at 6, 9, and 24 h were euthanized using the microwave irradiation approach under isoflurane anesthesia just after the behavioral tests.

To help habituation, all mice were moved to the behavioral testing room 1 h before the tests. Four mice from each group were tested on each day, and all tests were finished within 1 h, to minimize the impact of circadian rhythms. To avoid the influence of odor, all the equipment was cleaned with 70% ethanol after every trial. All the

behavioral data were analyzed with an animal tracking system (Smart 3.0, RWD Life Science Co., Ltd, China). The details of the experimental steps are described in the supplementary Materials and Methods.

Brain Sample Preparation for NMR Study

To minimize the impact of post-mortem changes in brain metabolites, each mouse was euthanized using the microwave method as fully described in our previous work [5]. After euthanasia, the brain was removed and dissected into 12 regions: olfactory bulb (OB), frontal cortex (FC), parietal cortex (PC), occipital cortex (OC), temporal cortex (TC), striatum (STR), hippocampus (HP), thalamus (THA), hypothalamus (HYP), midbrain (MID), medulla-pons (MED-PONS), and cerebellum (CE). Separation of the regions was made according to the Allen Brain Atlas and previous publications [21, 22] (further details in supplemental material; Fig. S1). The tissues were immediately weighed and stored at -80°C for further processing.

The protocol for tissue extraction was the same as that in our previous study [5]. Briefly, HCl/methanol (0.1 mol/L, 100 μL) was added to the frozen tissue and homogenized for 1.5 min at 20 Hz (Tissuelyser II, Qiagen, Germany). Ice-cold 60% ethanol (800 μL) was further added and the mixture homogenized again, before centrifugation at 14,000 g for 10 min. The supernatant was then collected. The extraction steps were repeated twice with 800 μL 60% ethanol to extract the metabolites remaining in the sediment. All the supernatants were collected and desiccated in a centrifugal drying apparatus (Thermo Scientific 2010, Germany) and freezing vacuum dryer (Thermo Scientific). 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 (TSP, 120 mg/L; 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.

Acquisition of NMR Spectra

^1H -NMR spectra were acquired as in previous studies [5, 23]. 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 spectra were acquired with a standard Watergate pulse sequence [24]. The following acquisition parameters were set for every sample: p1 (90° pulse), 8.35 μs ; number of scans, 256; spectral width, 20 ppm;

dummy scans, 8; number of free-induction decay points, 32 K.

NMR Data Processing

All ^1H -NMR spectra were processed and analyzed with TopSpin (Version 2.1, Bruker BioSpin) and a home-made software NMRSpec [25]. First, the phase correction and baseline distortion were manually completed in TopSpin. Then the corrected spectra were imported into NMRSpec for spectrum alignment, peak extraction, spectral integration, and the integration of chemical-related peaks. This software has been used in several metabolomics studies [5, 26, 27].

The chemical shifts of major amino-acids were distributed in the range of 1.20–4.46 ppm, so this gap was extracted for further analysis. First of all, the areas of all peaks (area under the curve) in this gap were automatically calculated for further statistical analysis [5]. To compensate for the different concentrations, each peak area was normalized to the sum of all the peak areas in this gap of its own spectrum prior to the discriminant analysis [21, 23, 28].

Furthermore, the absolute concentrations ($\mu\text{mol/g}$ wet weight) of the identified metabolites were calculated with the related peak areas in spectra from the samples, information on the internal standards (TSP, such as concentration and proton number), and specimen weight. The calculation was as follows:

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

where A_{met} and A_{TSP} are the relative areas of the peaks of the detected metabolites and TSP, and R_{met} is a constant for a specific metabolite calculated as the ratio between the partial NMR signal of the standard metabolite in 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 metabolite within the area A_{met} ; C_{TSP} and V_{TSP} are the concentration and volume of TSP standard solution added to the NMR tube; Wt is the total weight of the wet specimen and 9 is the number of protons in the TSP.

Statistical Analysis

In the behavioral tests, the baseline for each mouse served as the reference for abnormal neurobehavioral performance. The relative values for behavior at each time point (6, 9, and 24 h postoperatively) are presented as a percentage relative to baseline. The repeated behavioral tests of limited duration could influence the performance, thus only the control group and the 24-h group, both of

which received behavioral tests 3 times (6, 9, and 24 h), were used to assess the change in neurobehavioral performance. Values for performance were compared using the Wilcoxon Mann–Whitney U test.

To discriminate the different metabolic patterns among the control and anesthesia/surgery groups, we applied orthogonal partial least-squares discriminant analysis (OPLS-DA). Twelve brain regions were involved. For clarity, the metabolic spectra and statistical analysis of the FC region are 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, we used one-way analyses of variance (ANOVA), followed by Turkey's *post-hoc* test to analyze differences in the concentrations of metabolites in each brain region between the control and different anesthesia/surgery groups. To calculate the main effects of the time-points on the regional metabolites (12 regions), we used two-way ANOVA followed by Turkey's *post-hoc* test. The criterion for statistical significance was set at a probability value of 0.05. All data are presented as the mean $\pm$ SEM.

Results

Anesthesia/Surgery Increased the Latency to Find Food in a Time-Associated Manner

To assess the effects of anesthesia/surgery on the natural habits of mice, we used the buried food test (Fig. 1A1–A3). Compared with the control group, the latency to find food in the anesthesia/surgery group was longer at both 6 h ($114.0\% \pm 15.0\%$ versus $69.7\% \pm 12.8\%$, $Z = -2.098$, $P = 0.036$, Fig. 1A1) and 9 h ($171.0\% \pm 29.6\%$ vs $81.2\% \pm 12.1\%$, $Z = -2.342$, $P = 0.019$, Fig. 1A2). 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 suggested that the abdominal surgery plus isoflurane anesthesia undermines

the natural ability of mice to find food and this process is 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 emotional state of mice (Fig. 2). The anesthesia/surgery decreased the time spent in the center region at both 6 h ($39.0\% \pm 8.9\%$ vs $122.0\% \pm 32.6\%$, $Z = -2.075$, $P = 0.038$, Fig. 2A1) and 9 h ($21.1\% \pm 6.9\%$ vs $139.0\% \pm 49.1\%$, $Z = -2.532$, $P = 0.011$, Fig. 2A2), but not at 24 h (Fig. 2A3). This indicates that the anesthesia/surgery had an adverse effect on the emotional state of mice in a time-associated manner. Furthermore, other parameters (latency to the center and total traveling distance) did not show significant difference between the two groups at any time point (Fig. 2B1–C3), suggesting that anesthesia/surgery does not cause motor dysfunction. Taken together, the results of these behavioral tests were consistent with our hypothesis that anesthesia/surgery causes 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 the mice (Fig. 3). Compared with the control group, anesthesia/surgery shortened the time spent in the novel arm at 6 h ($81.0\% \pm 11.7\%$ vs $116.0\% \pm 10.0\%$, $Z = -2.203$, $P = 0.028$, Fig. 3A1) after the intervention, but not at 9 h (Fig. 3A2) and 24 h (Fig. 3A3). Furthermore, the entries to the novel arm in the anesthesia/surgery group were significantly fewer than those in the control group at both 6 h ($81.7\% \pm 4.1\%$ vs $105.0\% \pm 3.8\%$, $Z = -2.950$, $P = 0.003$, Fig. 3B1) and 9 h ($84.7\% \pm 7.3\%$ vs $107.0\% \pm 6.4\%$, $Z = -2.061$,

Fig. 1 Impact at 6, 9, and 24 h of anesthesia/surgery on mouse behaviors assessed by the buried food test. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, Mann–Whitney U test.

Fig. 2 Impact at 6 h, 9 h and 24 h of anesthesia/surgery on mouse behaviors assessed by the open field test. **A1–A3** Time spent in the center. **B1–B3** Latency to the center. **C1–C3** Total traveling distance. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, Mann–Whitney U test.

$P = 0.039$, Fig. 3B2), but not at 24 h (Fig. 3B3). In addition, the total entries to the three arms and total traveling distance showed no significant difference at any time point (Fig. S2). These results demonstrate that anesthesia/surgery impairs spatial learning and memory in mice in both a time-associated and motor-independent manner.

¹H-NMR-Based Metabolic Information for the Aged Brain at Different Postoperative Time Points

To document the dynamic changes in the concentrations of metabolites that may be related to the abnormal neurobehavioral performance and anesthesia/surgery, we selected four time points (0, 6, 9, and 24 h after anesthesia/surgery) to analyze the information on metabolites in the ¹H-NMR spectra [an example region (FC) at different time points is

illustrated in Fig. 4]. The average normalized spectra of the different groups and the basic metabolic information, including the metabolite name and the related chemical shift are shown in Fig. 4. It can be seen that group at 0 h had the minimum spectral heights of Asp and Glu; and the maximum spectral height of alanine (Ala). However, without statistical analysis, it was difficult to compare the significance of differences among the different groups, so 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 were investigated and identified. The significant changes in metabolites are listed in Table 1 and S1. The levels of metabolites in almost every region exhibited time-associated patterns (maximum variation at 0 h, returned to normal levels at 24 h). Most of metabolites

Fig. 3 Impact at 6, 9, and 24 h of anesthesia/surgery on mouse behaviors assessed by the Y-maze test. **A1–A3** Duration in the novel arm. **B1–B3** Entries into the novel arm. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, Mann–Whitney U test.

Fig. 4 Normalized average ¹H-NMR spectra for the frontal cortex samples in the control and model groups at 0, 6, 9, and 24 h after anesthesia/surgery. Horizontal axis, chemical shift of the spectrum. Lower-case, carbon position connected with the hydrogen signal; Lac,

lactate; Myo, myo-inositol; Cre, creatine; Ala, alanine; Glx, glutamine + glutamate; Gly, glycine; Asp, aspartate; EtOH, ethanol; Tau, taurine; GABA, gamma-aminobutyric acid; NAA, N-acetyl aspartate.

Table 1 Metabolite changes in the whole brain classified by region.

	0 h	6 h	9 h	24 h
OB	Myo-14.3%↑ ($P = 0.009$) Glu-39.6%↓ ($P < 0.001$) Ala-76.9%↑ ($P < 0.001$)			
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%↑ ($P = 0.046$)	Asp-16.6%↑ ($P = 0.009$)	
PC	Tau-8.74%↑ ($P = 0.031$) Glu-17.8%↓ ($P < 0.001$) Ala-65.4↑ ($P < 0.001$) GABA-13.4↓ ($P = 0.020$)	Asp-15.8%↑ ($P = 0.011$)	Asp-13.1%↑ ($P = 0.069$)	
OC	Cre-18.6%↓ ($P = 0.039$) Glu-20.1↓ ($P < 0.001$) Ala-67.7↑ ($P < 0.001$)	Asp-15.5%↑ ($P = 0.044$)	Asp-15.8%↑ ($P = 0.053$)	
TC	Asp-19.6%↓ ($P = 0.007$) Glu-14.1%↓ ($P < 0.001$) Ala-76.6%↑ ($P < 0.001$)			
STR	Tau-7.3%↑ ($P = 0.047$) Glu-22.0%↓ ($P < 0.001$) Ala-89.0%↑ ($P < 0.001$)			
HP	Tau-8.8%↑ ($P = 0.027$) Glu-18.2%↓ ($P < 0.001$) Ala-70.6%↑ ($P < 0.001$)		Glu-6.1%↑ ($P = 0.029$)	
THA	Tau-12.0%↑ ($P = 0.045$) Glu-23.9%↓ ($P < 0.001$) Ala-63.7%↑ ($P = 0.005$)			
HYP	Glu-19.1%↓ ($P = 0.007$) Ala-56.0%↑ ($P = 0.003$)			
MID	Ala-71.9%↑ ($P = 0.005$)			
MED	Asp-36.8%↓ ($P < 0.001$)			
CE	Gly-15.9%↓ ($P = 0.030$) Asp-34.0%↓ ($P = 0.003$) Ala-30.2%↑ ($P = 0.029$) Gln-45.9%↓ ($P = 0.015$)			

Variation of metabolites in distinct brain regions at different periods after anesthesia/surgery. Statistically significant differences among groups were assessed by one-way ANOVA followed by Turkey's *post-hoc* multiple comparison test.

dramatically decreased in most regions (except for the MID) after anesthesia/surgery, except for Ala that increased in the 0 h group. The metabolites in most regions fully recovered to normal (baseline) levels after 6 h except in the cortex. Eight regions (FC, PC, OC, TC, STR, HP, THA, and MID) were involved after 9 h, and most metabolites returned to baseline after 24 h except in the OB, FC, TC, and CE.

Discriminant Analysis Between the Control and Anesthesia/Surgery 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. Among the 12 regions, the metabolites in the FC region changed throughout the period. It was therefore selected as an example to illustrate the results of OPLS-DA (Fig. 5). The significantly different metabolites corresponding to anesthesia/surgery treatment were screened out with

Fig. 5 OPLS-DA scores and coefficient-coded loading plots for the models in the frontal cortex at different time points. **A1–D1** OPLS-DA scores at 0, 6, 9, and 24 h after anesthesia/surgery; **A2–D2** Coefficient-coded loading plots at 0, 6, 9, and 24 h after anesthesia/surgery. $t[1]_p$, first predictive component; $t[2]_o$, orthogonal principal component score; 1, Lac; 2, Ala; 3, Glu; 4, Asp; 5, taurine; 6, ethanol + myo-inositol; 7, Ala + Glu + Gln; 8, creatine; 9, N-acetyl Asp; 10, gamma-aminobutyric acid; 11, choline.

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 Glu and Asp accompanied by an increase in Ala. There was also an increase in Asp both at 6 h (Fig. 5B2) and 9 h (Fig. 5C2); but an increase in Glu and a decrease in GABA only occurred at 9 h (Fig. 5C2). In addition, there was a tendency for choline to increase and for Glu to decrease 24 h postoperatively (Fig. 5D2).

Considering the vital function of excitatory amino-acids in the brain, the dynamic changes in Glu and Asp were further investigated (Figs. S3 and S4). Dynamic changes of Glu and Asp in FC are illustrated in Fig. 6A. Our results demonstrated that anesthesia/surgery sharply reduced the level of Asp at 0 h, and there was a gradual reversal of the trend at 6 h and 9 h (blue line), but the level returned close to baseline after 24 h. The other excitatory amino-acid Glu presented a variation tendency similar to Asp (Fig. 6B).

Tendency of Glutamate and Aspartate to Change in the Whole Brain After Anesthesia/Surgery

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

Collectively, these results suggested that anesthesia/surgery could cause a neurobehavioral disorder in a time-associated manner in aged mice. The anesthesia/surgery also altered the dynamics of some metabolites (Glu, GABA, Ala, Asp, choline, NAA, Gln, and Gly), and the variations in some metabolites (Glu and Asp) were consistent with the dynamic changes of the abnormal

Fig. 6 Normalized average ^1H -NMR spectra of selected metabolites in the frontal cortex at different times after anesthesia/surgery (mean $\pm$ SEM). Horizontal axis, chemical shift of the spectrum. **A** Asp; **B** Glu. Pink, control; red, 0 h; green, 6 h; blue, 9 h; yellow, 24 h.

behavior performance. The changes in metabolites also occurred in a time-associated manner.

Effects of Time and Region on Asp and Glu After Anesthesia/Surgery

To determine whether time and region affected Asp and Glu after anesthesia/surgery, we compared their concentrations in the 12 regions at 0, 6, 9, and 24 h using two-way ANOVA. Both Asp ($F_{\text{time}} = 35.853$, $P < 0.001$; $F_{\text{regions}} = -115.439$, $P < 0.001$) and Glu ($F_{\text{time}} = 91.425$, $P < 0.001$; $F_{\text{regions}} = 98.057$, $P < 0.001$) were associated with time and region during recovery (Tables S2–S5).

Longitudinal Changes in the Metabolites and Behavioral Performance

To investigate the association between the metabolic changes and neurobehavioral abnormalities, we analyzed the correlations between the longitudinal neurobehavioral changes and the significantly altered metabolites (Glu and

Asp). The FC region was selected as it showed the most metabolic changes (Figs. 9, 10). The concentrations of Asp and Glu were positively correlated with the latency to food, especially for Asp (Fig. 9). However, they were negatively correlated with the other behaviors, such as duration in the center, duration in the novel arm, and entries into the novel arm (Figs. 9, 10). Among these relationships, the concentrations of Asp were significantly negatively correlated with the duration ($P = 0.041$) and entries into the novel arm ($P = 0.028$) (Fig. S5).

Discussion

The principal findings of this study were as follows: (1) A series of neurobehavioral changes, including inattention, learning/memory dysfunction, and emotional dysregulation (anxiety), were found in aged mice following surgery under isoflurane anesthesia. (2) Compared with the control group, the altered metabolite in the experimental group at 6 h was Asp, and the differences mainly occurred in the cortex;

Fig. 7 Concentrations of aspartate in distinct brain regions at different time points after anesthesia/surgery. Statistically significant differences between groups were assessed by one-way ANOVA followed by Turkey's *post-hoc* multiple comparison test (* $P < 0.05$, # $P < 0.01$). 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.

while significant changes in Asp and Glu at 9 h occurred predominantly in the cortex and hippocampus. (3) We found correlations between longitudinal changes in the metabolites and behavioral performance. (4) Both Asp and Glu were associated with time and region during recovery.

Application of *In-Vitro* Nuclear Magnetic Resonance Spectroscopy

Two magnetic resonance spectra methods can be used to analyze changes of the metabolites in animal models—*in vivo* MRS and *in vitro* NMR. Generally, the *in vivo* MRS method provides more accurate region-specific information due to its high spatial resolution, ability to longitudinally monitor the changes of metabolites, and reduce the animal numbers in most studies. Moreover, it has direct potential for clinical translation. The most fundamental requirement

Fig. 8 Concentrations of glutamate in distinct brain regions at different periods after anesthesia/surgery. Statistically significant differences among groups were assessed by one-way ANOVA followed by Turkey's *post-hoc* multiple comparison test (* $P < 0.05$, # $P < 0.01$). The full names of the regions are as in Fig. 7.

for ^1H -MRS testing is to keep the animal immobile during the whole data collection process, and there are only two ways to keep an animal immobile—continuous anesthesia or an apparatus to restrict movement. Given that our APNP model was constructed by isoflurane plus surgery, which meant anesthesia was likely an important factor in facilitating changes in behavior and/or metabolites, we did not use ^1H -MRS. The *in-vitro* NMR also involved anesthesia, but the dose and duration were far less than that required for ^1H -MRS. In addition, MRS only tested one specific region in each trial. To screen the regional metabolic patterns during APNP, the mouse brain was divided into 12 regions for NMR measurement.

Abnormal Neurobehavioral Performance in Aged Mice After Anesthesia/Surgery

All the behavioral tests were closely dependent on the movement ability of mice. The results showed that the

Fig. 9 Longitudinal changes in Asp and different kinds of neurobehavioral performance after anesthesia/surgery. Red, metabolite concentrations; blue, different kinds of neurobehavioral performance;

green: metabolite concentrations in the control group. Data are shown as the mean $\pm$ SEM.

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 motor functions. Although there may be other confounders, such as the effect of olfaction on the buried food test [29], each element of intact attention, unsubdued consciousness, normal emotion, and organized thinking was indispensable normal performance.

Our results are not exactly the same as those from a previous study [18], 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 vs 4-month-old mice), which is consistent with the consensus that aging is an independent risk factor for postoperative neurobehavioral disorders [30].

Brain Regions Affected After Anesthesia/Surgery

Our results showed that the most severely-affected regions were all in the four areas of cortex and the HP. These regions play distinct and complementary roles in the processing of normal neurobehavioral function (*e.g.* learning and memory, emotion regulation) [31, 32]. APNP may occur when the FC and HP are simultaneously directly

impaired or their mutual complementary effect is abnormal [33–35].

Our results are also consistent with recent clinical imaging work. It has been reported that several regions are involved in the pathology of neurobehavioral dysfunction after anesthesia/surgery, including cortex and the HP. These were obtained using diverse technologies such as single-photon emission computed tomography and the xenon-enhanced computed tomography to investigate changes in cerebral blood flow [36, 37], diffusion tensor imaging to analyze neuronal connections [38], and the blood-oxygen-level-dependent signal to measure functional connectivity [39]. Thus, 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, indicating that anesthesia/surgery disturbs the balance of excitatory

Fig. 10 Longitudinal changes in Glu and different kinds of neurobehavioral performance after anesthesia/surgery. Red, metabolite concentrations; blue, different kinds of neurobehavioral performance;

green, metabolite concentrations in the control group. Data are shown as the mean $\pm$ SEM.

and inhibitory functions in the brain. Most of these metabolic alterations had almost disappeared and the neurobehavioral performance returned to normal after 24 h.

The tendency of Asp to be elevated in the cortex at 6 h and 9 h accompanied by evident neurobehavioral disorders is supported by a previous study, in which an increment of Asp was accompanied by impairment of neurobehavioral performance [4]. There are two isomers of Asp, L-Asp and D-Asp. The latter triggers glutamate transmission and is able to activate the N-methyl-D-aspartate receptor [40]; while the former is a critical building block of proteins and can be converted to the latter with the aid of D-Asp racemase [41]. Published data indicate that the application of exogenous D-Asp not only alleviates the cognitive impairment induced by neuropathic pain [42], but also enhances long-term potentiation and improves cognition in aged mice [43]. We therefore concluded that there may be a positive correlation between the content of D-Asp and cognition. However, it has been reported that D-Asp is low and it accounts for $< 1\%$ of the total Asp in adults, while the content of L-Asp is relatively high [44]. 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 cognition are as follows: (1) The effects of D-Asp on cognition might be associated with an individual's age, as reported by a previous study [45]. (2) Excess D-Asp generated D-Asp excitotoxicity as a surplus of the excitatory neurotransmitter Glu. (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 in future studies.

Imbalance of Glu is strongly linked with both acute and chronic neurodegeneration, which can lead to excitotoxicity by disturbing the steady state of calcium, energy balance, and normal neuronal death pathways [46]. In the current study, the excess Glu at 9 h may be excitotoxic and impair cognition-related cerebral regions, such as the HP. This speculation is supported by an animal study, in which increased 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 [47]. This paradoxical evidence might be due to the different species as well as the time-course of the

investigations. In the former study, an acute rat model was investigated over a period < 24 h, while the latter study was a clinical observation for the possible association of Glu and cognitive decline in asthma patients, a common chronic disease of the respiratory system.

Limitations and Perspective

This study aimed to explore the metabolic patterns of APNP associated with surgical treatment; and several potential metabolites were preliminarily screened out using a dynamic mapping technique. Regrettably, we cannot identify the concrete cause (anesthesia or surgery) of APNP due to the lack of an anesthesia-alone group. An ^1H -MRS study has demonstrated that isoflurane anesthesia indeed alters the brain metabolites of mice immediately after anesthesia [48]. But whether (or how) the anesthesia alone affects APNP in the aged brain needs to be explored in a further study.

Conclusions

By means of the ^1H -NMR method, we explored APNP after anesthesia/surgery from a metabolic perspective. Both neurobehavioral performance and the concentrations of metabolites exhibited a parallel pattern in a time-associated manner. The metabolites that fluctuated most in the various cerebral regions were two critical excitatory amino-acids, Asp and Glu. Our results provide a dynamic map of metabolite alterations associated with the neurobehavioral disorders following anesthesia/surgery, which may be conducive to ultimately revealing the metabolic mechanism of postoperative neurobehavioral disorders.

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Conflict of interest The authors declare no competing financial interests.

References

1. Pinho C, Cruz S, Santos A, Abelha FJ. Postoperative delirium: age and low functional reserve as independent risk factors. *J Clin Anesth* 2016, 33: 507–513.
2. Pendlebury ST, Lovett NG, Smith SC, Dutta N, Bendon C, Lloyd-Lavery A, *et al.* Observational, longitudinal study of delirium in consecutive unselected acute medical admissions: age-specific rates and associated factors, mortality and re-admission. *BMJ Open* 2015, 5: e007808.
3. Allaili N, Valabregue R, Auerbach EJ, Guillemot V, Yahia-Cherif L, Bardinet E, *et al.* Single-voxel H-1 spectroscopy in the human hippocampus at 3 T using the LASER sequence: characterization of neurochemical profile and reproducibility. *NMR Biomed* 2015, 28: 1209–1217.
4. Cui B, Wu MG, She XJ, Liu HT. Impulse noise exposure in rats causes cognitive deficits and changes in hippocampal neurotransmitter signaling and tau phosphorylation. *Brain Res* 2012, 1427: 35–43.
5. Liu TT, He ZG, Tian XB, Kamal GM, Li ZX, Liu ZY, *et al.* Specific patterns of spinal metabolites underlying alpha-Me-5-HT-evoked pruritus compared with histamine and capsaicin assessed by proton nuclear magnetic resonance spectroscopy. *Biochim Biophys Acta-Mol Basis Dis* 2017, 1863: 1222–1230.
6. Meyerhoff DJ, MacKay S, Bachman L, Poole N, Dillon WP, Weiner MW, *et al.* Reduced brain N-acetylaspartate suggests neuronal loss in cognitively impaired human immunodeficiency virus-seropositive individuals: *in vivo* ^1H magnetic resonance spectroscopic imaging. *Neurology* 1993, 43: 509–515.
7. Andres RH, Ducray AD, Schlattner U, Wallimann T, Widmer HR. Functions and effects of creatine in the central nervous system. *Brain Res Bull* 2008, 76: 329–343.
8. Boulanger Y, Labelle M, Khiat A. Role of phospholipase A(2) on the variations of the choline signal intensity observed by ^1H magnetic resonance spectroscopy in brain diseases. *Brain Res Brain Res Rev* 2000, 33: 380–389.
9. Niciu MJ, Kelmendi B, Sanacora G. Overview of glutamatergic neurotransmission in the nervous system. *Pharmacol Biochem Behav* 2012, 100: 656–664.
10. Wang XF, Zhao TY, Su RB, Wu N, Li J. Agmatine prevents adaptation of the hippocampal glutamate system in chronic morphine-treated rats. *Neurosci Bull* 2016, 32: 523–530.
11. Maddock RJ, Buonocore MH. MR spectroscopic studies of the brain in psychiatric disorders. *Curr Top Behav Neurosci* 2012, 11: 199–251.
12. Shi J, Li Q, Wen T. Dendritic cell factor 1-knockout results in visual deficit through the GABA system in mouse primary visual cortex. *Neurosci Bull* 2018, 34: 465–475.
13. Govindaraju V, Young K, Maudsley AA. Proton NMR chemical shifts and coupling constants for brain metabolites. *NMR Biomed* 2000, 13: 129–153.
14. Wang J, Du H, Jiang L, Ma X, de Graaf RA, Behar KL, *et al.* Oxidation of ethanol in the rat brain and effects associated with chronic ethanol exposure. *Proc Natl Acad Sci U S A* 2013, 110: 14444–14449.
15. Gonzalez-Riano C, Garcia A, Barbas C. Metabolomics studies in brain tissue: a review. *J Pharm Biomed Anal* 2016, 130: 141–168.
16. Clausen MR, Edelenbos M, Bertram HC. Mapping the variation of the carrot metabolome using H-1 NMR spectroscopy and consensus PCA. *J Agric Food Chem* 2014, 62: 4392–4398.
17. Peng JN, Patil SM, Keire DA, Chen K. Chemical structure and composition of major glycans covalently linked to therapeutic monoclonal antibodies by middle-down nuclear magnetic resonance. *Anal Chem* 2018, 90: 11016–11024.
18. Peng M, Zhang C, Dong Y, Zhang Y, Nakazawa H, Kaneki M, *et al.* Battery of behavioral tests in mice to study postoperative delirium. *Sci Rep* 2016, 6: 29874.
19. Yang SM, Gu CP, Mandeville ET, Dong YL, Esposito E, Zhang YY, *et al.* Anesthesia and surgery impair blood-brain barrier and cognitive function in mice. *Front Immunol* 2017, 8: 902.
20. Nathan BP, Yost J, Litherland MT, Struble RG, Switzer PV. Olfactory function in apoE knockout mice. *Behav Brain Res* 2004, 150: 1–7.

21. Wang J, Zeng HL, Du H, Liu Z, Cheng J, Liu T, *et al.* Evaluation of metabolites extraction strategies for identifying different brain regions and their relationship with alcohol preference and gender difference using NMR metabolomics. *Talanta* 2018, 179: 369–376.
22. Wang J, Du H, Ma X, Pittman B, Castracane L, Li TK, *et al.* Metabolic products of [2-(13) C]ethanol in the rat brain after chronic ethanol exposure. *J Neurochem* 2013, 127: 353–364.
23. Du H, Fu J, Wang S, Liu H, Zeng Y, Yang J, *et al.* ¹H-NMR metabolomics analysis of nutritional components from two kinds of freshwater fish brain extracts. *RSC Adv* 2018, 8: 19470–19478.
24. Liu ML, Mao XA, Ye CH, Huang H, Nicholson JK, Lindon JC. Improved WATERGATE pulse sequences for solvent suppression in NMR spectroscopy. *J Magn Reson* 1998, 132: 125–129.
25. Liu Y, Cheng J, Liu HL, Deng YH, Wang J, Xu FQ. NMRSpec: an integrated software package for processing and analyzing one dimensional nuclear magnetic resonance spectra. *Chemom Intell Lab Syst* 2017, 162: 142–148.
26. Kamal GM, Wang XH, Yuan B, Wang J, Sun P, Zhang X, *et al.* Compositional differences among Chinese soy sauce types studied by C-13 NMR spectroscopy coupled with multivariate statistical analysis. *Talanta* 2016, 158: 89–99.
27. Kamal GM, Yuan B, Hussain AI, Wang J, Jiang B, Zhang X, *et al.* C-13-NMR-based metabolomic profiling of typical Asian soy sauces. *Molecules* 2016, 21: 1168.
28. Zhang LM, Wang LM, Hu YL, Liu ZG, Tian Y, Wu XC, *et al.* Selective metabolic effects of gold nanorods on normal and cancer cells and their application in anticancer drug screening. *Biomaterials* 2013, 34: 7117–7126.
29. Lehmkuhl AM, Dirr ER, Fleming SM. Olfactory assays for mouse models of neurodegenerative disease. *J Vis Exp* 2014, 90: e51804.
30. Maldonado JR. Delirium pathophysiology: an updated hypothesis of the etiology of acute brain failure. *Int J Geriatr Psychiatry* 2017, 33: 1428–1457.
31. Frank LM, Brown EN, Wilson M. Trajectory encoding in the hippocampus and entorhinal cortex. *Neuron* 2000, 27: 169–178.
32. Bannerman DM, Rawlins JNP, McHugh SB, Deacon RMJ, Yee BK, Bast T, *et al.* Regional dissociations within the hippocampus—memory and anxiety. *Neurosci Biobehav Rev* 2004, 28: 273–283.
33. Chang RYK, Nouwens AS, Dodd PR, Etheridge N. The synaptic proteome in Alzheimer's disease. *Alzheimers Dement* 2013, 9: 499–511.
34. Kang MG, Byun K, Kim JH, Park NH, Heinsen H, Ravid R, *et al.* Proteogenomics of the human hippocampus: the road ahead. *BBA-Proteins Proteom* 2015, 1854: 788–797.
35. Focking M, Lopez LM, English JA, Dicker P, Wolff A, Brindley E, *et al.* Proteomic and genomic evidence implicates the postsynaptic density in schizophrenia. *Mol Psychiatr* 2015, 20: 424–432.
36. Fong TG, Bogardus ST, Daftary A, Auerbach E, Blumenfeld H, Modur S, *et al.* Cerebral perfusion changes in older delirious patients using 99mTc HMPAO SPECT. *J Gerontol A-Biol Sci Med Sci* 2006, 61: 1294–1299.
37. Yokota H, Ogawa S, Kurokawa A, Yamamoto Y. Regional cerebral blood flow in delirium patients. *Psychiatr Clin Neurosci* 2003, 57: 337–339.
38. Cavallari M, Dai WY, Guttmann CRG, Meier DS, Ngo LH, Hsieh TT, *et al.* Neural substrates of vulnerability to postsurgical delirium as revealed by presurgical diffusion MRI. *Brain* 2016, 139: 1282–1294.
39. Choi SH, Lee H, Chung TS, Park KM, Jung YC, Kim SI, *et al.* Neural network functional connectivity during and after an episode of delirium. *Am J Psychiatry* 2012, 169: 498–507.
40. D'Aniello G, Tolino A, D'Aniello A, Errico F, Fisher GH, Di Fiore MM. The role of D-aspartic acid and N-methyl-D-aspartic acid in the regulation of prolactin release. *Endocrinology* 2000, 141: 3862–3870.
41. D'Aniello S, Somorjai I, Garcia-Fernandez J, Topo E, D'Aniello A. D-aspartic acid is a novel endogenous neurotransmitter. *FASEB J* 2011, 25: 1014–1027.
42. Palazzo E, Luongo L, Guida F, Marabese I, Romano R, Iannotta M, *et al.* D-aspartate drinking solution alleviates pain and cognitive impairment in neuropathic mice. *Amino Acids* 2016, 48: 1553–1567.
43. Errico F, Nistico R, Napolitano F, Mazzola C, Astone D, Pisapia T, *et al.* Increased D-aspartate brain content rescues hippocampal age-related synaptic plasticity deterioration of mice. *Neurobiol Aging* 2011, 32: 2229–2243.
44. Dunlop DS, Neidle A, Mchale D, Dunlop DM, Lajtha A. The presence of free D-aspartic acid in rodents and man. *Biochem Biophys Res Commun* 1986, 141: 27–32.
45. Errico F, Nistico R, Napolitano F, Oliva AB, Romano R, Barbieri F, *et al.* Persistent increase of D-aspartate in D-aspartate oxidase mutant mice induces a precocious hippocampal age-dependent synaptic plasticity and spatial memory decay. *Neurobiol Aging* 2011, 32: 2061–2074.
46. Benarroch EE. Glutamate transporters diversity, function, and involvement in neurologic disease. *Neurology* 2010, 74: 259–264.
47. Kroll JL, Steele AM, Pinkham AE, Choi C, Khan DA, Patel SV, *et al.* Hippocampal metabolites in asthma and their implications for cognitive function. *Neuroimage Clin* 2018, 19: 213–221.
48. Boretius S, Tammer R, Michaelis T, Brockmoller J, Frahm J. Halogenated volatile anesthetics alter brain metabolism as revealed by proton magnetic resonance spectroscopy of mice *in vivo*. *Neuroimage* 2013, 69: 244–255.