Differential hippocampal protein expression between normal aged rats and aged rats with postoperative cognitive dysfunction: A proteomic analysis

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Abstract. The aim of the present study was to investigate the differences in the expression of hippocampal proteins between normal control aged rats and aged rats with postoperative cognitive dysfunction (POCD). A total of 24 aged rats were randomly divided into a surgery group (n=12) and a control group (n=12). The rats in the surgery group were treated with 2 h isoflurane anesthesia and splenectomy, while the rats in the control group received 40% oxygen for 2 h without surgery. The cognitive functions of the two groups were examined using a Y-maze test. The protein expression profiles of the hippocampus of six aged rats (three rats with POCD and three from the normal control group) were assessed using two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time of flight mass spectrometry. A total of three differential proteins were further confirmed between the POCD rats and normal rats using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The expression levels of 21 proteins in the rats with POCD were significantly different compared with the normal control rats. These proteins were functionally clustered to synaptic plasticity (three proteins), oxidative stress (four proteins), energy production (six proteins), neuroinflammation (three proteins) and glutamate metabolism (two proteins). In addition, three proteins (fatty acid binding protein 7, brain, glutamate dehydrogenase 1 and glutamine synthetase), associated with astrocytic function, were significantly different in the rats with POCD compared with those in the normal control ($P<0.05$). Similar changes in the mRNA expression levels of the three proteins in the hippocampi of POCD rats were also detected using RT-qPCR. Neuroinflammation, glutamate toxicity and oxidative stress were possibly involved in the pathological mechanism underlying POCD in aged rats. In addition, astrocytes may also be imporant in POCD in aged rats.

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Introduction

Postoperative cognitive dysfunction (POCD) is characterized by a decline of cognitive performance following surgery, particularly following surgery of aged patients (1). It has been identified to occur in 30-80% of aged patients 1 week after surgery and in 10-15% of aged patients 3 months after surgery (2-4). A number of risk factors for POCD have been reported in previous studies, including advanced age, educational level, mental illness, preoperative level of recognition, operative type, and postoperative infection (4,5). However, the pathological mechanisms underlying POCD remain to be elucidated. The toxicity of anesthetics was initially assessed, with simple anesthesia impairing the learning and memory of aged rats (1,6-8) and mice (9). Notably, the duration and type of anesthesia had no significant effects on the incidence of POCD of aged patients at the late stage (>1 week post surgery) (10). This suggested that anesthetics were not important in POCD associated with aged patients. In addition to surgery and anesthesia, another potential mechanism underlying POCD is inflammation induced by surgery. He *et al* (11) and Wang *et al* (12) reported that surgical trauma under general anesthesia causes distinct changes in systemic and central pro-inflammatory cytokines in aged rats at an early stage (<1 week post surgery), corresponding closely to the dysfunction of cognition. Open tibial fractures impair the cognitive function and induce the increase of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) in adult mice (4,13). Inhibiting the signals of TNF-α and IL-1 prior to surgery partly rescued the cognitive dysfunction induced by surgery (4,13). However, a similar incidence of POCD has been detected among aged patients 3 months after surgery, although the extent of the damage induced by surgery was independent of the anaesthetic and the type of surgery (14). This suggested that, in addition to the toxicity of anesthetics and inflammation induced by surgery, other unknown factors also contribute to POCD in aged patients.

The combination of mass spectrometry and two-dimensional gel electrophoresis is a useful method to examine the differential protein expression levels between different treatments. It is usually used to examine novel mechanisms underlying certain diseases (15). The hippocampus is an important structure, closely involved with learning and memory (16,17). During normal aging, the volume of the hippocampus decreases gradually, particularly the dentate gyrus and the CA3 area (18,19). During pathological aging, as observed in Alzheimer's disease, the hippocampal volume decreases more rapidly (18,19). Accordingly, Chen *et al* (20) found that the hippocampal volume in aged patients with POCD is significantly smaller compared with that of aged patients without POCD (20). In addition, the hippocampal volume was negatively correlated with the score of cognitive function of aged patients. These suggested that changes in the volume of the hippocampus were closely involved in the occurrence of POCD in aged patients. In the present study, differences in the protein expression of the hippocampus were compared between aged rats with POCD and normal aged rats.

Materials and methods

Animals and grouping. A total of 24 aged male Sprague-Dawley rats (22 months old) were purchased from the Experimental Animal Nursery of Central South University (Changsha, China). The rats were maintained under temperature-controlled environmental conditions with a 12/12-h light/dark cycle and had *ad libitum* access to food and water. The food consisted of standardized rodent pellets. Ambient conditions remained constant at 23˚C and a relative humidity of 50-60%. All procedures were approved by the Medical Ethics Committee of the Third Xiangya Hospital of Central South University and conformed to the guidelines for animal experiments of Central South University.

A total of 24 rats were handled, according to the flow chart of the study design shown in Fig. 1. The rats were divided randomly into a surgery group (n=12) and a normal control group (n=12). The rats in the surgery group were treated with isoflurane anesthesia for 2 h and splenectomy, while rats in the normal control group received 40% oxygen for 2 h without surgery (1). The cognitive functions of the two groups were examined using a Y-maze test. The upper limit of the 95% confidence interval of the mean training duration of rats in the normal control group in the Y-maze test was used to decide whether the rats in the surgery group had cognitive dysfunction (21). The rats in the surgery group were subdivided into POCD and no-POCD groups, according to previously described methods (21). The protein profile from the hippocampi of six aged rats (three rats with POCD and three rats from the normal control group) were detected using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS).

Y-maze test. The details of the Y-maze assessment (Fig. 2) was described in our previous study (1). Briefly, the voltage for the electric shock in the Y-maze was 50±5 V. The rats were acclimatized to the Y-maze with the light on for 3 min 24 h after treatment. Subsequently, the rats were placed in the stem arm and were administered an electric shock. If the rats arrived at the lit arms within 30 sec, the trial was deemed successful. The same trial was repeated 20 times/day consecutively for 3 days. During the assessment, the following parameters were recorded: The number of correct reactions, which was the total number entering the lit arm of the 20 trials each day; the total reaction duration, which rats spent completing the 20 trials each day and the number of initiative avoidance responses, which was the number of escape responses of the rats within the 5 sec prior to the arms being electrified.

Splenectomy surgery. Splenectomy surgery was performed using the method reported in our previous study (11). Briefly, the rats were initially placed in a closed box with an airflow of 3% isoflurane for 5 min. Anesthesia was then administered through a mask using 2% isoflurane. A 14-gauge catheter was inserted through the glottis. The rats were artificially ventilated, through endotracheal intubation, with room air supplemented with 2 l/min oxygen and 2% isoflurane for 2 h (22). During the anesthesia, the gas concentrations and respiratory rates (RR; breaths per min)were continuously monitored using a multi-function monitor (Datex-Ohmeda, Helsinki, Finland) (Table I). Rectal temperature was maintained at 37±0.5˚C. In addition, heart rate (HR), mean arterial blood pressure (MAP) and pulse oximeter oxygen saturation (SpO2) were measured continuously through a femoral artery catheter (Table I). Under anesthesia, a small incision was made in the upper left quadrant through the skin and muscle wall. The spleen was mobilized, isolated and removed. The wound was infiltrated with 0.25% bupivacaine and then closed with sterile sutures. The rats in the normal control group received 40% oxygen for 2 h in a gas chamber without surgery.

2-DE and MALDI-TOF-MS. Hippocampal tissue (250 mg) was suspended in 1 ml of 8 M urea, 2 M sulfocarbamide, 20 mM Tris-HCl, 1 mM CHAPS, 40 mM EDTA, 65 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.025 mM RNase, 0.05 mM DNase and 1 mM NaF. The suspension was sonicated for ~30 sec and centrifuged at 150,000 x g for 45 min. The protein concentration in the supernatant was examined using the Coomassie blue method (23). In first dimensional isoelectric focusing, 1,000 μg of each sample was loaded into immobilized pH 3-10 nonlinear gradient strips (18 cm). After 12 h of reswelling at 30 V, voltages of 100, 500, 1,000 and 3,000 V were administered for 1 h each and 8,000 V for 6 h. The second dimensional separation was performed using 12% sodium dodecyl sulfate-polyacrylamide gels (Sangon Biotech Shanghai Co., Ltd, Shanghai, China) (24). Following staining of the gels with colloidal Coomassie blue (25), the gels were scanned using a Umax PowerLook II scanner and Adobe Photoshop 8.0 image software (Adobe Systems Inc., San Jose,. CA, USA), and the images were analyzed using ETTAN ImageMaster 2D Elite 4.01 software (GE Healthcare, Amersham, UK). This software was used for matching and quantitative analysis of the protein spots on the gels. The average gel was constructed as a representative gel for the three hippocampal samples acquired from each group of rats. The average mode of background subtraction was used for normalization of the intensity volume that indicates the protein concentration or the quantity on each spot. The average

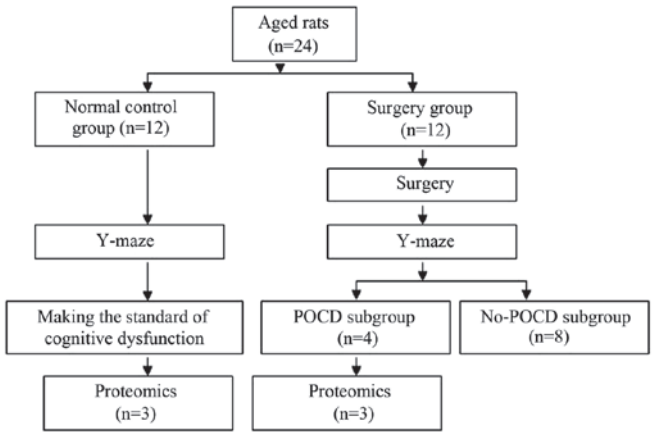

Figure 1. Flow chart detailing the experimental groups and steps performed. POCD, postoperative cognitive dysfunction.

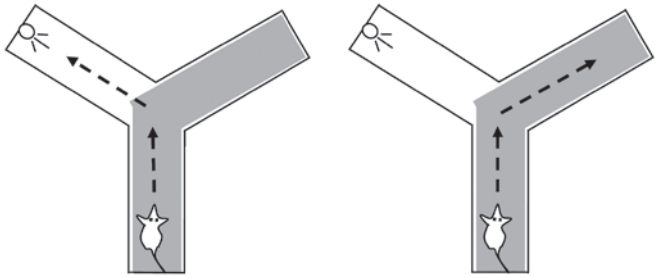

Figure 2. Learning and spatial memory assessment using a Y-maze. Individual cohorts of rats were assessed in the Y-maze apparatus to determine the trial number at which the rat entered into the lit, unshocked arm in 9/10 consecutive trials. The left image indicates a correct reaction and the right image indicates an error reaction.

gel was then used for determination of the existence of differences in the protein expression levels between each group. A value of 2.0 indicated a 2-fold increase and 0.5 indicated a 2-fold reduction (26-29). Biologically relevant differences were defined as changes >2-fold.

MALDI-TOF-MS analysis was performed, as described previously (25-29). The valuable spots in the coomassie blue-stained gels were assessed. The extracts were redissolved in 1 μl extraction buffer and 1 μl matrix solution (α-acyano-4-hydroxycinnamic acid; Sangon Biotech Shanghai Co., Ltd) and targeted onto a MALDI-TOF plate. Following drying the samples completely onto the targeting plate, MALDI-TOF/MS was performed using a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with delay ion extraction. Mass spectra were obtained over a mass range of 800-3,000 Da. For the identification of proteins, peptide mass fingerprinting data were used to search against the Mascot database (http://www.matrixscience.com) at the School of Life (Central South University). Peptide matching and protein identification were performed automatically, as described previously (26-29). A Mascot score >58 was considered to indicate statistical significance (P<0.05).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) for astrocyte-associated proteins. To confirm the differential protein expression based on MALDI-TOF-MS, the mRNA expression levels of the proteins of interest were detected by RT-qPCR. Total RNA from the hippocampus was isolated using the RNeasy plus mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Prior to RT, the total RNA was treated to remove genomic DNA using a DNA-free kit (AM1906; Applied Biosystems). cDNA was synthesized using the Retroscript kit (AM1710; Applied Biosystems). RT-qPCR was performed using Power SYBR Green PCR master mix (Applied Biosystems). The rat β-actin gene was used as a homogenous standard. The running protocol extended to 40 cycles consisting of 95˚C for 15 sec and 60˚C for 1 min using an Applied Biosystems 7500 Fast Real-time PCR system. The primers were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA; Table II).

The relative quantity of the target gene present was calculated based on the expression of β-actin in the endogenous control. The mean cycle threshold (Ct) values and standard deviations were calculated. To calibrate the analysis, the value obtained from the control rats was used. The factor difference was also calculated using the standard relative quantitative method ($2^{-\Delta\Delta CT}$ method) (30,31).

Statistical analysis. All summary data are reported as the mean ± standard error of the mean. The results of the present study were processed using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). The total reaction time in the Y maze was analyzed using a repeated measures analysis of variance (ANOVA) with Bonferroni's post-hoc test. The number of correct reactions and initiative avoidance occurrences were examined using a non-parametric Mann-Whitney U test. The RR, HR and MAP values during surgery were compared using a repeated measures ANOVA with Bonferroni's post-hoc test. The gel electrophoresis and RT-qPCR results were analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Surgery with inhaled anesthesia impairs the cognitive function of aged rats. In the present study, a Y-maze test was used to evaluate cognitive function (1). A decrease of correct reactions and initiative avoidance number, and an increase in total reaction time implied deteriorating cognitive function. The findings demonstrated that the number of correct reactions in the surgery group were 8.90±1.09 and 9.47±2.46 (24 and 48 h, respectively) following surgery, which was significantly lower compared with the control group (P<0.05; Fig. 3A). The number of initiative avoidance occurrences in the surgery group were 1.20±0.59 and 2.07±1.01 (24 h and 48 h, respectively) following surgery, which was also significantly lower compared with the control group (P<0.05; Fig. 3B). These findings demonstrated that surgery with inhaled anesthesia impaired the cognitive functions of aged rats at an early stage.

Surgery with inhaled anesthesia alters the protein profile of the hippocampus in aged rats with POCD. The protein expression profile of the hippocampus was assessed using MALDI-TOF-MS. The levels of 21 proteins in the hippocampus of aged rats with POCD were markedly altered,

Table I. Vital signs of the rats during and after splenectomy surgery.

Group	Index	10 min preoperative	10 min operative	20 min operative	10 min postoperative
Control	RR (bpm)	82.20±3.35	72.06±4.68	71.33±3.29	78.50±2.67
	HR (bpm)	374.83±11.70	359.83±16.49	362.35±20.11	365.00±16.07
	MAP (mmHg)	95.33±10.17	83.83±10.32	85.29±13.25	88.67±7.23
	SpO2 (%)	97.17±1.48	97.50±1.64	97.27±1.06	97.83±1.16
Surgery	RR (bpm)	80.00±4.70	71.50±6.78	73.75±7.69	75.60±8.41
	HR (bpm)	372.57±12.11	348.28±11.33	355.88±13.68	361.78±12.52
	MAP (mmHg)	92.21±8.32	83.21±9.07	85.64±8.33	87.14±8.37
	SpO2 (%)	97.93±1.07	97.78±1.31	97.65±1.08	97.42±1.15

No statistical difference was observed in the RR, MAP and SpO_2 between the rats in control group and surgery group (P>0.05). RR; respiratory rate; HR, heart rate; MAP, mean arterial blood pressure; SpO_2, oxygen saturation; bpm, beats per minute.

Table II. Primers used for reverse transcription quantitative polymerase chain reaction.

Gene	Primer	Amplicon length(bp)
Glutamate dehydrogenase	Forward: GCTCTGGACTCTTCCCAACA	115
	Reverse: AAATGCCACACGCCTACTTC	
Glutamine synthetase	Forward: CGCTCTTCGTCTCGTTCTC	119
	Reverse: CTGCTTGATGCCTTTGTTCA	
Fatty acid binding protein 7, brain	Forward: GAAGGGCAAGGATGGTAGATG	133
	Reverse: ACCGTTGGTTTGGTCACATT	
β-actin	Forward: GGAAATCGTGCGTGACATTA	181
	Reverse: GAAGGAAGGCTGGAAGAGAG	

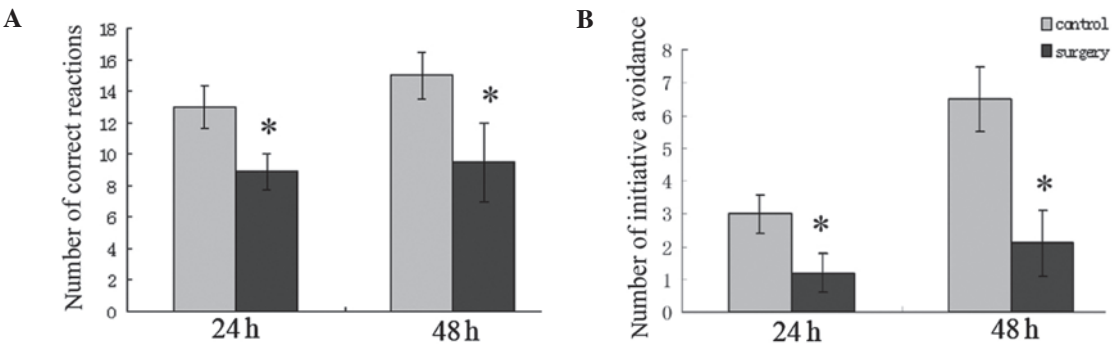

Figure 3. Surgery impairs the cognitive functions of aged rats during the first 2 days postoperatively. In the Y-maze test, the number of (A) correct reactions and (B) initiative avoidances were used to demonstrate cognitive function. Compared with the control group, the number of correct reactions in the surgery group increased and the number of initiative avoidances decreased 24 and 48 h after isoflurane anesthesia (*P<0.05, vs. control).

compared with the normal control. These proteins included dynamin-1, superoxide dismutase, glutamine synthetase and coronin-1A (Table III, Fig. 4). Further functional clustering revealed that three proteins were closely involved in synaptic plasticity, four in oxidative stress, six in energy production three in neuroinflammation and two in glutamate metabolism. The protein expression levels were downregulated, with regard to synaptic plasticity, oxidative stress, energy production and glutamate metabolism; however, the protein expression levels associated with neuroinflammation were upregulated in the POCD rats.

Astrocytes are the predominant type of cell in the hippocampus, with important roles in supporting neurons. Thus, from the identified differentially expressed proteins, three proteins associated with astrocytes were selected [fatty acid binding protein 7, brain (FABP7) (32), glutamate dehydrogenase 1 (GLUD1) (33) and glutamine synthetase (GS) (34)] to confirm the reliability of the data from the MALDI-TOF-MS assessment. It was identified that the mRNAs of FABP-7 and GLUD increased in aged rats with POCD and the mRNAs of GS decreased, closely corresponding to the changes observed in the MALDI-TOF-MS data (P<0.05; Fig. 5).

Table III. Differential protein expression between the control group and postoperative cognitive dysfunction group.

Protein symbol	Protein name	Number of spots in gel	Changes in expression (POCD, vs. control)	Protein function
DNM1	Dynamin-1	2	↓	Synaptic plasticity
DHPRP2	Dihydropyrimidinase-related protein 2	3	↓	Synaptic plasticity
GABA-T	GABA transaminase	19	↓	Modulating neuronal plasticity
SOD1	Superoxide dismutase (Cu-Zn)	8	↓	Antioxidant protective role
GSTP1	Glutathione S-transferase P	12	↓	Antioxidant protective role
PRDX2	Peroxiredoxin-2	5	↑	Antioxidant protective role
ADR	Aldose reductase	14	↑	Oxidative stress
DLST	Dihydrolipoyllysine-residue succinyltransferase	4	↓	Energy production in mitochondria
ATP5D	ATP synthase δ chain	7	↓	Energy production in mitochondria
NDUFA10	NADH dehydrogenase1 α subcomplex subunit 10	13	↓	Energy production in mitochondria
PGAM1	Phosphoglycerate mutase 1	11	↓	Energy production in mitochondria
TKT	Transketolase	20	↓	Energy production and neurogenesis
PK-M1/M2	Pyruvate kinase isozymes M1/M2	21	↓	Energy production in mitochondria
CORO1A	Coronin-1A	1	↑	Neuroinflammation marker
GMFB	Glia maturation factor b	6	↓	Modulating neuroinflammation
CRYAB	α crystallin B chain	11	↑	Modulating neuroinflammation
FABP7	Fatty acid-binding protein,	9	↑	Regulation of astrocyte function
GS	Glutamine synthetase	17	↓	Modulating extracellular glutamate level
GLUD1	Glutamate dehydrogenase	18	↑	Modulating extracellular glutamate level and energy production of mitochondria
CCT2	T-complex protein 1 subunit β	23	↑	Protein folding
EF-Tu	Elongation factor Tu	16	↓	Modulating translation of DNA

Expression factors were calculated, as described in the Materials and methods. Spot number of the corresponding reference gel, the full name and the function are provided. ↑ indicates increased expression, ↓ indicates decreased expression.

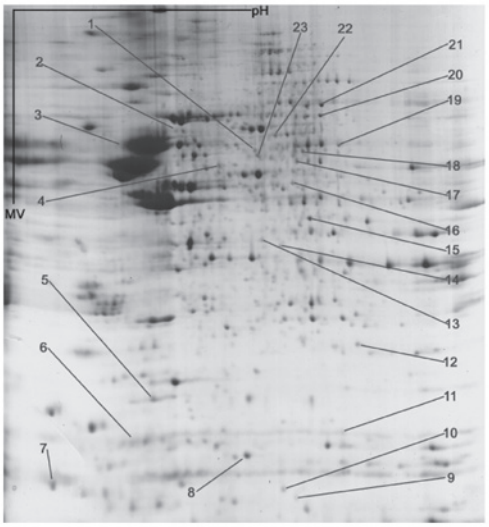

Figure 4. Representative image of two-dimensional gel electrophoresis. Protein separation was performed with immobilized non-linear pH 3-10 gradient gel strips and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 12.5% matrix followed by Coomassie blue staining. Spot numbers were provided for all the spot-cut proteins, which were identified using a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer.

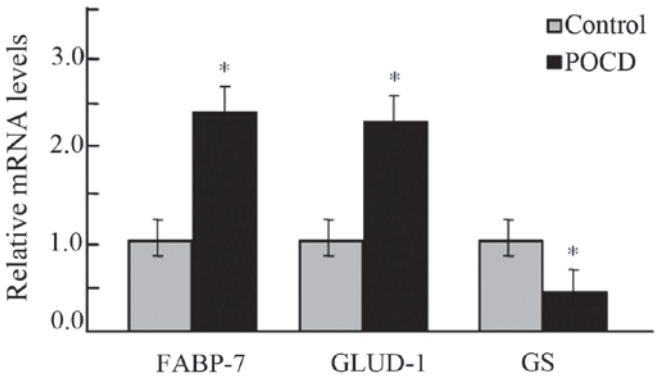

Figure 5. Comparison of the gene expression levels of FABP7, GLUD1 and GS in the hippocampus. Values are expressed as the mean ± standard error of the mean of three rats. *P<0.05, vs. control. The graph was constructed using Igor Pro 4.09A software. FABP7, fatty acid binding protein 7, brain, GLUD-1, glutamate dehydrogenase 1; GS, glutamine synthetase; POCD, postoperative cognitive dysfunction.

Discussion

In the present study, the protein profile of the hippocampi in aged rats with POCD and normal aged rats were screened. A total of 21 proteins were significantly altered in aged rats with POCD compared with normal aged rats. These proteins were functionally clustered in association with synaptic plasticity, oxidative stress, energy production, glutamate metabolism and neuroinflammation. These data provided important indications that the hippocampus is functionally important in the POCD of aged rats.

POCD is usually detected among aged patients following surgery. It was initially reported by Bedford in 1955 (35). In the present study, the cognitive function of aged rats was initially assessed with or without surgery using a Y-maze. The number of correct reactions and the number of initiative avoidance occurrences in the surgery group were significantly lower compared with the control group (P<0.05). These results suggested that surgery with inhaled anesthesia impaired the cognitive function of aged rats at an early stage. Based on these findings, changes in the protein expression profile of the hippocampi of aged rats with POCD was assessed. Compared with the normal control, 14 proteins increased in the aged rats with POCD and seven proteins decreased (Table II). PubMed (http:// http://www.ncbi.nlm.nih.gov/pubmed/) was used to search for useful information using key words. Among the 21 proteins, three proteins, DNM1 (36-38), DHPRP2 (39) and GABA-T (40,41) were involved in synaptic plasticity and their expression were decreased in aged rats with POCD. A total of four proteins, SOD1 (42,43), glutathione S-transferase P (44,45), peroxiredoxin 2 (46) and ADR (47) were associated with oxidative stress. In addition, six proteins, dihydrolipoyllysine-residue succinyltransferase (48), ATP synthase δ chain (49), NADH dehydrogenase 1α (50), phosphoglycerate mutase 1 (51), transketolase (52) and pyruvate kinase isozymes M1/M2 (53), were involved in energy production and their expression levels were decreased in the aged rats with POCD. A total of three proteins, coronin-1A (54), glia maturation factor b (55), α crystallin B chain (56) were involved in neuroinflammation. These findings suggested that surgery induced neuronal damage in aged rats with POCD. Neuroinflammation and oxidative stress were important in neuronal damage and the data were consistent with previously reported results (4,11-13,20,57). Chen *et al* (20) found that aged patients with POCD had a smaller volume hippocampus and the size of the hippocampus was negatively correlated with the cognitive function of aged patients following surgery. It has been found that surgery induced increased neuroinflammation in mice and aged rats (4,11,12), corresponding to cognitive dysfunction. In addition, An *et al* (57) found that surgical trauma induced oxidative stress in rats with POCD (57). Notably, the expression of FABP7 (32), a modulator of astrocyte function, was higher in aged rats with POCD. At the same time, GS (58) and GLUD1 (33,59-61), which are two astrocytic enzymes modulating the level of extracellular glutamate, were also changed in aged rats with POCD. These findings suggested that astrocytes are important in the mechanism underlying POCD, and astrocytes may offer a novel direction in the investigation of POCD.

The present study did not distinguish the effect of surgery in POCD from the anaesthetic agent, as our previous study demonstrated that anesthesia and surgery can result in POCD. It has been confirmed that surgery, rather than anesthetic agents lead to long-term changes in exploratory behavior (62). It is disputed whether the mechanism underlying POCD is associated with surgery and/or anesthesia, however, it is considered to be a multi-factorial process. Further investigation is required to discriminate each component of the entire process.

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