

Original Article

Autophagy itself and/or its anti-neuroinflammation action in hippocampus attenuates postoperative cognitive decline in aged rats undergoing splenectomy

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Abstract: It is vulnerable for aged subjects to postoperative cognitive dysfunction and many proof suggested exaggerated neuroinflammation after surgery is the vital cause, but the mechanism needs to be disclosed. Autophagy can inhibit inflammation and itself has protective effect on nervous system. The study aimed to explore whether autophagy itself and/or its anti-neuroinflammation action in hippocampus could attenuate postoperative cognitive decline in rats. Adult or aged rats were subjected to control, anesthesia alone or splenectomy protocol. Postoperative spatial learning and memory was evaluated by Morris water maze. Western blotting was used to determine the expression of interleukin-1 β (IL-1 β) and autophagy specific proteins Beclin 1 and LC3II/I in the hippocampus. In aged rats, there was chronic neuroinflammation, decreased autophagy and cognition preoperatively, and exaggerated neuroinflammation, limited activation of autophagy and significant cognition decline postoperatively, compared with adult rats. The study revealed that autophagy itself or its anti-neuroinflammation action in hippocampus attenuates postoperative cognitive decline in aged rats.

Keywords: Aging, autophagy, cytokines, neuroinflammation, postoperative cognitive dysfunction

Introduction

Postoperative cognitive dysfunction (POCD) is a common postoperative complication especially in aged patients, presenting deteriorating concentration and memory and execution [1, 2]. Many reports suggested that age is an independent and significant dangerous factor for POCD [3]. With aged society coming soon, more and more people are likely to undergo operation, as a result there may be numerous economic and social problems, so it makes vital sense to probe why it has higher incidence of POCD in aged patients.

It is believed that the etiology for POCD is multifactorial, including anesthesia, surgery and patients and other aspects, such as age, lower educational status, preoperative cognitive impairment, anesthesia duration, respiratory complications and second operation [2, 4, 5]. Growing body of evidence showed that there is exaggerated neuroinflammation in aged patients or animals after receiving surgery [6, 7]. It

could be explained by age-specific differences in glial cells. In normal aged, glial cells are primed [8]. Primed glial cells do not secrete appreciable levels of pro-inflammatory cytokines under basal conditions, but they are hyper-responsive to secondary stimuli and can produce an exaggerated and prolonged neuro-inflammatory response when further provoked [8, 9]. Another study suggested that HMGB1, S100B, and RAGE signaling modulate the hippocampal inflammatory response and might play key roles in surgery-induced cognitive decline [10]. Much proof suggested that role of proinflammatory cytokine IL-1 β in POCD resulted from neuroinflammation [6, 11]. A peripheral surgery-induced innate immune response triggers an IL-1 β -mediated inflammatory process in the hippocampus that underlies memory impairment [11, 12], but the exact mechanism needs to be disclosed.

Autophagy (macroautophagy) is a highly conserved mechanism which is essential for the maintenance of cellular homeostasis [13, 14].

It is induced by extracellular and intracellular signals, including oxidative stress, ceramide, and endoplasmic reticulum stress [15]. Its role recently expanded to include unique stand-alone immunological functions and interactions with nearly all parts of the immune system [16]. The deterioration of autophagy during aging reduces the elimination of damaged organelles and results in the accumulation of waste products in cells [17]. So we hypothesized that chronic neuroinflammation develops with aging and declined autophagy activity, which could be exaggerated after receiving operation, for autophagy can not be effectively activated relatively in aged patients compared with adults. We observed effects of age and surgical trauma on autophagy specific proteins Beclin 1 and LC3II/I, and neuroinflammation cytokine IL-1 β , postoperative cognition in aged or adult rats, probed correlation among autophagy and neuroinflammation and postoperative cognition.

Methods

Animals and protocol

All experimental procedures were performed in accordance with the Declaration of the National Institute of Health Guidelines for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Chongqing Medical University.

Adult (4-5 Months old) and aged (24-25 Months old) Sprague-Dawley male rats were purchased from the Laboratory Animal Centre of the Third Military Medical University. Animals were raised in a temperature-controlled room on a 12-h light and 12-h dark cycle and free to access food and water. After adapting to the environment for one week, adult or aged animals were randomly divided into 3 groups respectively ($n=12$ for each group): anesthesia group, operation group and control group, 6 groups totally. The control group received normal saline to control for possible effects of handling and injection stress. The animals in anesthesia group received intraperitoneal injection of droperidol 500 $\mu\text{g}/\text{kg}$ and fentanyl 20 $\mu\text{g}/\text{kg}$ (Blood gas analysis were monitored and there was no evidence showing cardiopulmonary dysfunction in anesthesia group or operation group, data not shown). The operation group received splenectomy under general anesthesia. Morris water maze (MWM) was used to

evaluate spatial learning and memory function 6 day before and 1 day after operation. When the last MWM test ended, animals were killed by an overdose of pentobarbitone (100 mg/kg intraperitoneal) and their fresh hippocampus were collected and stored at -70°C for western blot tests.

Surgical procedures

Animals were anesthetized satisfactorily by intraperitoneal injection of droperidol 500 $\mu\text{g}/\text{kg}$ and fentanyl 20 $\mu\text{g}/\text{kg}$. A 1-1.5 cm length abdominal incision was made in the upper left quadrant through skin and muscle. The spleen was exposed and the splenic hilum was ligated with 6-0 silk suture then removed. The wound was infiltrated with 0.25% bupivacaine, and closed by suture with 4-0. Animals were recovered and returned to cages and housed individually. Every surgery needed to be finished within 5 minutes and by the same operator.

Cognitive testing

MWM was used to test the animals' cognitive function during the preoperative 6 days. A circular tank with 150 cm in diameter and 30 cm deep was filled with water ($24\sim 26^{\circ}\text{C}$) to a depth of 25 cm. A cylinder platform was located at 1.5 cm below the water surface in the middle of one quadrant constantly in the tank. Animals were placed on the platform for 30 s preceding the start of each trial then released into water facing the wall of tank from one of four randomly assigned release points (E, S, W and N). Animals had to learn to use the distinctive distal visual cues surrounding the tank to navigate a direct path to the hidden platform for 60 s. Animals were remained on the platform for 30 s between trials. If the rat failed to locate the platform within 60 s, it would be guided there and allowed to remain 30 s. Rats were trained for 3 trails each day for 6 consecutive days. After completing 3 consecutive trails, animals were returned to their home cages under a heat lamp for 10 min. 1 day after operation, rats were subjected to a reversal test in which the platform was relocated in the middle of the opposite quadrant of the tank. Swimming process was recorded by a camera and swimming speed, distance, and latency to the platform were analyzed by WMW software (ZH0065; Zhenghua Instruments, China).

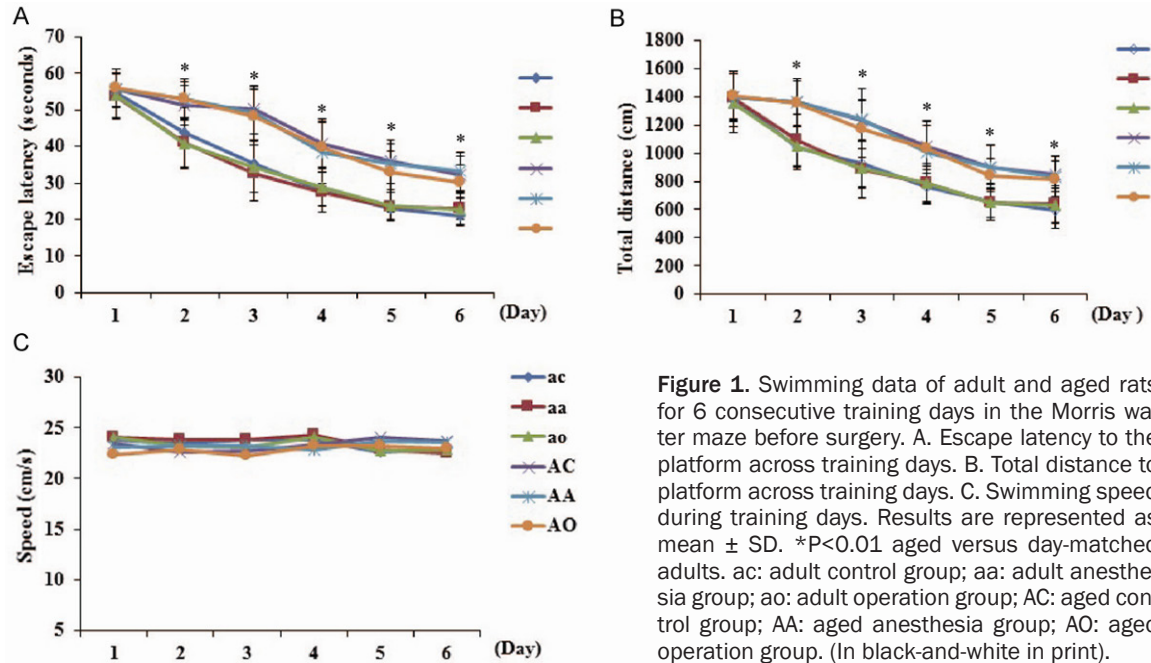


Figure 1. Swimming data of adult and aged rats for 6 consecutive training days in the Morris water maze before surgery. A. Escape latency to the platform across training days. B. Total distance to platform across training days. C. Swimming speed during training days. Results are represented as mean \pm SD. * $P < 0.01$ aged versus day-matched adults. ac: adult control group; aa: adult anesthesia group; ao: adult operation group; AC: aged control group; AA: aged anesthesia group; AO: aged operation group. (In black-and-white in print).

Western blotting

For western blot analysis, animals were killed immediately after MWM test ($n=6$ per group). The hippocampus were homogenized in cold lysis buffer (Beyotime, China) containing protease inhibitors and then centrifuged (12,000 g, 20 min, 4°C) to remove tissue debris. Coomassie brilliant blue was used for quantification of protein in liquid supernatant. Total proteins (50 μ g) were separated in a 10% SDS-PAGE gel and transferred onto the nitrocellulose membrane. The membranes were blocked with 5% skimmed milk and sequentially incubated with the primary antibodies such as goat anti-IL-1 β (1:1000; Santa Cruz), rabbit anti-Beclin 1 (1:1000; Cell Signaling) or rabbit anti-LC3II/I (1:1000; Cell Signaling). Control for protein loading was performed by re-probing membranes with an antibody against GAPDH (CWBIO, China). Membranes were then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (ZSGB-BIO, China) followed visualization of the proteins by using an enhanced chemiluminescence detection kit (WBKLS0100; MILLIPORE). These experiments were repeated twice at least and showed reproducible results. Images were scanned and intensity analysis was carried out using BIO-RAD Gel Doc 2000 (BIO-RAD, USA). Relative expression levels of protein were normalized by

the ratio of target protein (IL-1 β , Beclin 1, LC3II/I) to GAPDH.

Statistical analysis

Two-way repeated-measures ANOVA was used to analyze training behavioral parameters in MWM. A separate two-way ANOVA examined the effects of age (adult or aged) and treatment (control, anesthesia or operation) on spatial learning and memory performance during reversal testing. Data from western blot were analyzed with two-way ANOVA, in which age and treatment were dependent variables. Post hoc Student's t -test was employed when ANOVA showed significance. Pearson's correlation was used to analyze the correlation among MWM parameters and proinflammatory cytokine IL-1 β and autophagic marker protein LC3II/I. A P -value < 0.05 was considered to be statistically significant. All data are presented as means \pm SD.

Results

Aged rats exhibit impaired performance on spatial learning and memory exacerbated by surgical trauma

To determine the effects of surgery on hippocampus-dependent spatial learning and memory, a MWM was used and taking escape latency

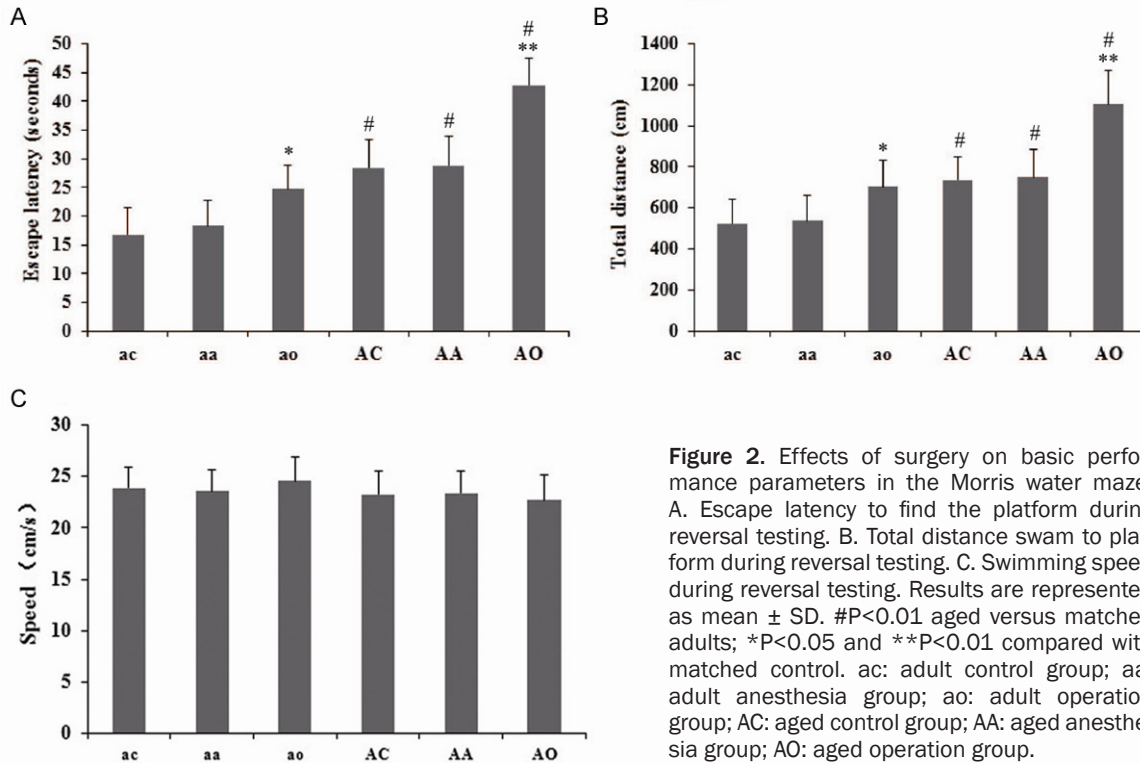


Figure 2. Effects of surgery on basic performance parameters in the Morris water maze. A. Escape latency to find the platform during reversal testing. B. Total distance swam to platform during reversal testing. C. Swimming speed during reversal testing. Results are represented as mean \pm SD. # $P < 0.01$ aged versus matched adults; * $P < 0.05$ and ** $P < 0.01$ compared with matched control. ac: adult control group; aa: adult anesthesia group; ao: adult operation group; AC: aged control group; AA: aged anesthesia group; AO: aged operation group.

(EL) and total distance (TD) as function parameter, in addition, speed was recorded to exclude its disturbance. Analysis of swim data before surgery revealed significant effects of age ($F = 321.643$, $df = 1$, $P < 0.001$; $F = 216.050$, $df = 1$, $P < 0.001$) and day ($F = 254.922$, $df = 5$, $P < 0.001$; $F = 196.504$, $df = 5$, $P < 0.001$) on both EL and TD (Figure 1A and 1B), but not on speed ($F = 2.444$, $df = 1$, $P = 0.119$ and $F = 0.630$, $df = 5$, $P = 0.688$, respectively) (Figure 1C). The lack of effect of speed suggests that the poorer performance of aged rats did not result from lack of motivation or reduced motor ability. The other results indicate that both adult and aged rats showed improvement in spatial learning and memory over time. However, in order to reach the target platform, aged rats swam longer and further.

Analysis of EL and TD to platform during reversal testing revealed significant effects of age ($F = 145.462$, $df = 1$, $P < 0.001$; $F = 79.369$, $df = 1$, $P < 0.001$), operation ($F = 41.214$, $df = 2$, $P < 0.001$; $F = 33.608$, $df = 2$, $P < 0.001$) and age \times operation interaction ($F = 4.653$, $df = 2$, $P = 0.013$ and $F = 4.063$, $df = 2$, $P = 0.022$, respectively) (Figure 2A and 2B), but no main effect of speed ($F = 2.356$, $df = 1$, $P = 0.130$ and $F = 0.014$, $df = 2$, $P = 0.986$, respectively) (Figure 2C).

Reversal learning reveals whether animals can extinguish their initial learning of the platform's position and acquire a direct path to the new goal position [18]. Analysis of % time in new and old target quadrant also revealed significant effects of age ($F = 458.335$, $df = 1$, $P < 0.001$; $F = 290.304$, $df = 1$, $P < 0.001$), operation ($F = 233.506$, $df = 2$, $P < 0.001$; $F = 248.848$, $df = 2$, $P < 0.001$) and age \times operation interaction ($F = 5.631$, $df = 2$, $P = 0.006$ and $F = 4.627$, $df = 2$, $P = 0.013$, respectively) (Figure 3A and 3B). These results demonstrate that the surgical procedure impaired spatial learning and memory in both adult and aged rats, and induced exacerbated impairment in aged rats. Anesthesia alone did not significantly impair cognitive function.

Surgical trauma elevated pro-inflammatory cytokine IL-1 β levels but enhanced by age

It's believed that the hippocampus is especially sensitive to aging and is involved in the types of cognitive impairment seen in elderly patients following surgical trauma, and cytokine-induced neuroinflammation in hippocampus plays an important role. Therefore hippocampal cytokine protein level of IL-1 β was measured by

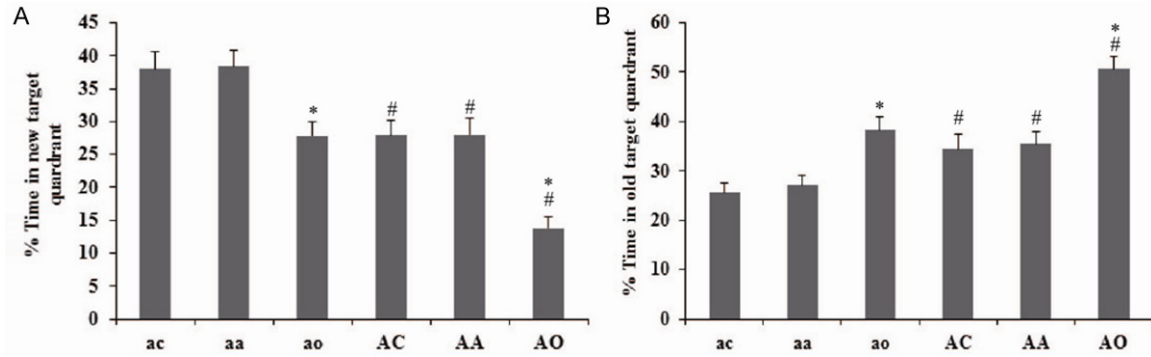


Figure 3. Effects of surgery on performance of adult and aged rats during reversal testing in the Morris water maze. A. Percent time in the new target quadrant. B. Percent time perseverating in the old target quadrant. Results are represented as mean \pm SD. # P <0.01 aged versus matched adults; * P <0.01 compared with matched control. ac: adult control group; aa: adult anesthesia group; ao: adult operation group; AC: aged control group; AA: aged anesthesia group; AO: aged operation group.

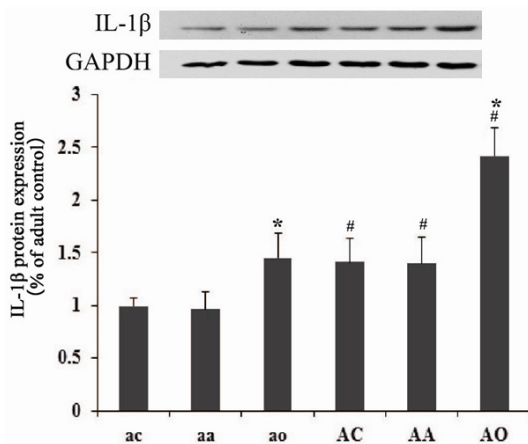


Figure 4. Surgical trauma elevated pro-inflammatory cytokine IL-1 β levels enhanced by age. Hippocampal IL-1 β protein of adult and aged rats were measured 24 hours postoperatively. Bars represent mean \pm SD. # P <0.01 aged versus matched adults; * P <0.01 compared with matched control. ac: adult control group; aa: adult anesthesia group; ao: adult operation group; AC: aged control group; AA: aged anesthesia group; AO: aged operation group.

western blotting 1 day after the surgical procedure in both adult and aged rats. Two-way ANOVA examined the effects of age (adult or aged) and treatment (control, anesthesia or operation) on IL-1 β protein expression. Analysis of hippocampal IL-1 β protein levels revealed a main effect of age (F =73.025, df =1, P <0.001) and operation (F =49.418, df =2, P <0.001) as well as a significant age \times operation interaction (F =6.485, df =2, P =0.005) indicating that surgery induced an increase in IL-1 β in the hippocampus that was enhanced in the aged rats

(**Figure 4**). Significant differences were observed in basal hippocampal IL-1 β , protein levels between adult and aged control rats (t =4.300, df =10, P =0.002). Anesthesia alone did not significantly alter hippocampal IL-1 β levels in adult or aged animals when compared to their age-matched naive controls.

Autophagy in hippocampus was activated by surgical trauma but impaired by age

Autophagy is thought lately as a new anti-inflammation paradigm and protective to neuronal system. Furthermore, the level of autophagy decreases with aging. So in order to evaluate whether autophagy in hippocampus plays an important part in PCOD, specific autophagy related protein Beclin 1 and LC3-II/I was determined by western blotting. Two-way ANOVA examined the effects of age (adult or aged) and treatment (control, anesthesia or surgery) on Beclin 1 and LC3-II/I protein expression. Analysis of Beclin 1 or LC3-II/I protein expression revealed significant effects of age (F =489.820, df =1, P <0.001 and F =126.897, df =1, P <0.001 respectively), operation (F =167.549, df =2, P <0.001 and F =268.191, df =2, P <0.001 respectively) and age \times operation interaction (F =44.869, df =2, P <0.001 and F =6.309, df =2, P =0.005, respectively) (**Figure 5A** and **5B**). These results indicate that surgery induced an increase in autophagy level in the hippocampus and which was attenuated in aged rats. Significant differences were observed for basal hippocampal Beclin 1 or LC3-II/I protein expression between adult and aged rats (t =12.107,

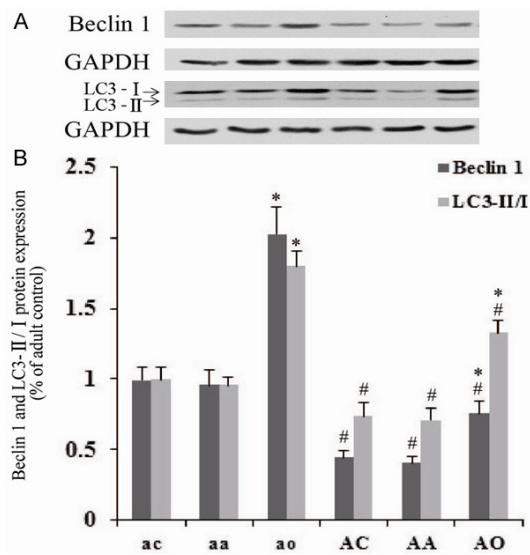


Figure 5. Surgical trauma activated autophagic proteins impaired by age. Hippocampal Beclin 1 and LC3-II/I protein of adult and aged rats were measured 24 hours postoperatively. Bars represent mean \pm SD. # $P < 0.01$ aged versus matched adults; * $P < 0.01$ compared with matched control. ac: adult control group; aa: adult anesthesia group; ao: adult operation group; AC: aged control group; AA: aged anesthesia group; AO: aged operation group.

df=10, $P < 0.001$; $t = 5.103$, df=10, $P < 0.001$, respectively). Anesthesia alone did not significantly alter hippocampal autophagic protein expression in adult or aged animals when compared to their age-matched naive controls.

Correlation analyses

Correlation analyses among behavioral markers EL and proinflammatory cytokine protein IL-1 β and autophagic marker protein LC3-II/I were carried out for both adult and aged rats. Significant positive correlations were found between hippocampal pro-inflammatory cytokine protein IL-1 β and EL in MWM ($r = 0.705$, $P < 0.001$; $r = 0.930$, $P < 0.001$). A positive correlation was observed between autophagy marker LC3-II/I and EL in MWM ($r = 0.745$, $P < 0.001$; $r = 0.845$, $P < 0.001$). There was positive correlation between autophagy marker LC3-II/I and IL-1 β ($r = 0.748$, $P < 0.001$; $r = 0.936$, $P < 0.001$).

Discussion

Aged rats have worse performance preoperatively in MWM compared to adult ones. Postoperative spatial learning and memory decline

in both adult and aged rats, and which in aged rats impair more seriously. Aged rats have hippocampal neuroinflammation while there is no any intervention. Neuroinflammation is triggered by surgical trauma, furthermore aged rats have exaggerated neuroinflammation after receiving splenectomy. Aged rats have lower level of basic autophagy in hippocampus which can be activated by surgical trauma, however, the extent is also less than those in adult rats.

It is usually believed that anesthesia is one of the important factors resulting in POCD, especially in developing or aged brain [19, 20]. However, between general anesthesia and local anesthesia, there weren't significant difference in incidence of POCD but correlated with anesthesia duration [2]. In the present study, operation duration was limited within 5 minutes to reduce anesthesia duration maximally and no significant effect of anesthesia on spatial learning and memory was found, partly because animals are exposed to anesthetics in a short time. Effect of anesthesia on neuroinflammation in hippocampus was not found as well in this study. These results were in accordance with Wan et al. [21]. There was no effect of anesthesia on hippocampal autophagy in adult or aged rats in this study. Intravenous anesthesia using propofol could inhibit autophagy activated by myocardial ischemia and reperfusion injury in rats [22]. But the researchers didn't observe the effect of anesthesia alone on autophagy in animals. Because there was no surgery alone group in this study for it is not in accordance with ethics if animals received operation alone but no anesthesia, so it's still unclear whether autophagy activation resulting from surgery could be inhibited by neuroleptic anesthesia.

Much evidence indicates that a significantly elevated expression of pro-inflammatory cytokines, particularly in the hippocampus, results in impairments in long-term potentiation (LTP) [23, 24] and performance deficits in hippocampal-mediated cognitive tests [25]. There was higher incidence of POCD especially in some surgery having more severe trauma, such as cardiac surgery [26]. However, another study suggested that minor surgery leads to an exaggerated neuroinflammatory response in aged mice but does not result in significantly impaired performance in the MWM [27]. In this study,

there was neuroinflammation and cognitive deficits in both adult and aged rats after surgery. It is speculated that these different results are due to different level of surgical trauma.

The pro-survival function of autophagy has been suggested at the cellular and organismal level in different contexts, including during endoplasmic reticulum stress, nutrient and growth factor deprivation, microbial infection, development, and diseases characterized by the accumulation of protein aggregates [28, 29]. In this study, autophagy was activated by surgery in both adult and aged rats, but activated level of autophagy in aged rats was lower than that in adults.

There is significant clinical and experimental evidence that inflammation within the central nervous system increases with age. Some microarray studies demonstrate that there is an overall increase in inflammatory and pro-oxidant genes in the brain of older rodents compared with adults [30]. Moreover, there are increased protein levels of several inflammatory cytokines, including IL-1 β and IL-6, in the brain of aged rodents [30, 31]. A modest increase in the inflammatory profile of the CNS in ageing is associated with deficits in neuronal plasticity and cognition. There are age-associated memory impairments in the reversal task of the MWM [32]. Another research reported that aged mice tested in the MWM had learning/acquisition impairments compared with adult mice [27]. Lowering CNS inflammation by treating with the anti-inflammatory agent luteolin resulted in better performance in the MWM [32]. Moreover, in both the contextual fear conditioning test and radial arm maze, aged mice showed memory impairments when compared with adult mice [33].

About autophagy, multiple studies demonstrated that autophagy-related (Atg) proteins or other proteins required for autophagy induction, have reduced expression in aged tissues and that autophagy diminishes with aging. With normal human brain aging, Atg5, Atg7, and Beclin 1 are downregulated [34], and Sirtuin1 is downregulated in insulin resistance and metabolic syndrome [35], and ULK1, Beclin1, and LC3 are downregulated in osteoarthritis [36]. Defective autophagy has been extensively linked to aging and the development of age-related neurodegeneration [37]. In addition, proteins

aggregation owing to defects in autophagic activity and the loss of the basal autophagy play a vital role in neurodegenerative diseases such as AD and Parkinson's diseases showing declined cognitive function.

The current study found that autophagy marker correlates positively with neuroinflammation cytokines or spatial learning and memory. Recent developments reveal a crucial role for the autophagy pathway and proteins in immunity and inflammation [38]. Nakahira K et al. [39] found that autophagic proteins regulate NALP3-dependent inflammation by preserving mitochondrial integrity. Another study also found that autophagy can regulate NLRP3 inflammasome negatively. Lacking autophagy-related 16-like 1 (Atg16L1) could activate NLRP3 and produce high amounts of the inflammatory cytokines IL-1 β and IL-18 in vivo and in vitro [40]. Furthermore, autophagy controls the production of IL-1 β by targeting pro-IL-1 β for lysosomal degradation and by regulating activation of the NLRP3 inflammasome. The current study can't suggest whether autophagy could protect cognitive function through inhibiting neuroinflammation or not.

On the other hand, autophagy itself could have protective effects on neural function. Autophagy is necessary for the clearance of aggregate-prone proteins that are toxic, especially for neurons [41]. Synapse development and plasticity is known the basis of learning and memory function and neuronal autophagy plays an important role in synapse development. A recent study demonstrates that decreasing or increasing autophagy results in corresponding effects on synapse size [42]. They found that neuronal autophagy positively regulates synaptic development in the *Drosophila* neuromuscular junction. In addition, studies indicate that autophagy may play an important role in neurodegenerative diseases such as AD [43]. One probable contributor to autophagy deficiency in AD appears to be Beclin 1, whose expression is strongly reduced in the brains of AD patients to levels that would be predicted to impair autophagosome synthesis [44]. Decreasing Beclin 1 levels in AD transgenic mice reduces neuronal autophagy, disrupt lysosomes, promote fibrillogenic beta amyloid (A β) accumulation, and enhance neurodegeneration. Conversely, increasing Beclin 1 expression results in diminished amyloid pathology in these AD

transgenic mice [45]. POCD may have the same pathological basis as Alzheimer disease (AD) [46, 47].

Conclusion

In conclusion, the results of this study indicate that, with aging, low level of autophagy and neuroinflammation in the hippocampus may play a significant role in disrupting normal cognitive function. Surgical trauma resulted in inappropriately activation of autophagy and exaggerated neuroinflammatory response and greater cognitive impairments in aged rats. Activating autophagy appropriately and/or inhibiting neuroinflammation triggered by surgical trauma may be a promising strategy for POCD prevention.

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Disclosure of conflict of interest

None.

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