Autophagy attenuates postoperative cognitive dysfunction by up-regulating cystatin C in aged rats undergoing splenectomy

CURRENT STATUS: POSTED

Bi Xing-hua  
Ningbo Medical Center Lihui Easter Hospital

Zhou Long-yuan  
Ningbo Medical Center Lihuili Easter Hospital

Cai Chang  
Ningbo Medical Center Lihuili Easter Hospital

Qi Yong  
Ningbo Medical Center Lihuili Easter Hospital

Yan Li  
liyan894412@163.com  
Ningbo Medical Center Lihuili Easter Hospital

Corresponding Author

ORCID: 0000-0002-1491-7229

DOI: 10.21203/rs.2.12725/v3

SUBJECT AREAS

Cellular & Molecular Neuroscience  Cognitive Neuroscience

KEYWORDS

Cystatin C; Autophagy; Postoperative cognitive dysfunction; Aged rat; Hippocampus; Neuroinflammation.
Abstract

Background: This study aimed to explore whether autophagy can attenuate postoperative cognitive dysfunction (POCD) by up-regulating cystatin C (CysC) in aged rats undergoing splenectomy. Methods: Rats were randomized into four groups (n = 10 per group): normal control (CON), surgery (SUR), surgery + rapamycin (autophagy inducer) at 1.0 mg/kg/d (RAP), and surgery + 3-methyladenine (autophagy inhibitor) at 3.0 mg/kg/d (3-MA). Treatments were carried out for four weeks. Postoperative learning and memory were assessed using the Morris water maze. Hippocampal expression of the autophagy-related proteins ATG5, LC-3B, Beclin1, and p62 as well as Cys C was assayed using Western blotting and real-time polymerase chain reaction. Results: SUR animals showed higher levels of autophagy and higher expression of autophagy proteins and Cys C than CON animals. These levels were even higher in RAP animals, which also showed lower levels of the inflammatory factors IL-1β, IL-6 and TNF-α than the other groups. Learning and memory functions were higher in RAP animals than in the other groups on days 5 and 7. Effects of 3-MA were opposite to those of RAP. Conclusion: Autophagy improves learning and memory in aged rats following splenectomy, which may involve up-regulation of Cys C and attenuation of neuro-inflammation.

Background

Autophagy, a self-degradation progress involving the lysosome system, helps maintain cell homeostasis, e.g. following exposure to injury, and its dysregulation can contribute to many diseases[1]. During autophagy, autophagy gene-related protein complexes induce the conjugation of microtubule-associated protein 1A/1B-light chain 3 (LC3B-I) to phosphatidylethanolamine to form LC3B-II, which helps form autophagosomes [2, 3]. LC3B interacts with sequestosome-1/p62 and sorts cytosolic cargo via a ubiquitin binding
domain [4]. Mammalian target of rapamycin and Beclin1 are also key molecules that support different steps of autophagy [5].

One way to dysregulate autophagy is dysregulation of cystatin C (Cys C). This member of the cysteine protease inhibitor superfamily 2 is expressed in all eukaryotic cells, and it protects cells from inappropriate proteolysis [6]. Deficiency of Cys C in apoE⁻/⁻ mice disrupts autophagy and induces macrophage apoptosis[7]. Conversely, Cys C-mediated activation of autophagy in neurons prevents cerebral vasospasm after subarachnoid hemorrhage and dysfunction of cerebrovascular angiogenesis in Parkinson's disease [7-9]. Mice that do not express the autophagy-related gene Wrd45 in the central nervous system show defective autophagic flux, greatly impaired learning and memory, as well as poor motor coordination [10]. These effects are associated with accumulation of SQSTM1/p62 and ubiquitinated proteins in neurons and swollen axons. Administering propofol to aged rats for 4 h inhibits hippocampal autophagy and significantly impairs their propofol anesthesia-induced cognitive impairment[11].

Many types of surgery, anesthesia and aging-related degenerative disease can result in postoperative cognitive dysfunction (POCD) [12-14]. Using an established rat model of this condition, we examined whether we could attenuate POCD by activating autophagy through Cys C. The results of these experiment may help guide efforts to understand and minimize POCD in patients.

Methods

Animals. Pathogen-free, 20-month-old Sprague-Dawley rats weighing 500-550 g were purchased from the Medical Experimental Animal Center of Guangdong Province, China (certificate no. SCXK-Yue-2015-0002). Rats were fed a normal diet and kept at a room temperature of 23±1 °C in air with 70±10% relative humidity before experiments. The
The experimental protocol was approved by the Animal Care and Use Committee at Lihuili Eastern Hospital of Ningbo Medical Center.

**Reagents.** The autophagy inducer rapamycin (RAP; catalog no. v900930) and autophagy inhibitor 3-methyladenine (3-MA; catalog no. M9281) were purchased from Sigma-Aldrich. TRIzol, primers, reverse transcription and a polymerase chain reaction (PCR) kit were obtained from Takara for real time-quantitative PCR. Primary antibodies against the following proteins were obtained from Cell Signaling Technology: ATG5 (catalog no. 12994), LC3B (3868s), Beclin1 (3495), SQSTM1/p62 (39749), and GAPDH (4970). Horseradish peroxidase-conjugated mouse anti-rabbit secondary antibody (7074) was also purchased from Cell Signaling Technology. Primary antibody against Cys C (catalog no. ab109508) was purchased from Abcam. A Morris water maze was purchased from Beijing Shuolinyuan Science and Technology Company. Tans-Blot® Turbo omnipotent protein transfer system, Mini PROTEIN® Tetracell system and ChemiDoc MP system were both obtained from Bio-Rad biotechnology Co., Ltd, USA. LB940 Functional Enzyme Marker was purchased from Guangzhou Nebula Scientific Instruments Co., Ltd and Mx3000P Fluorescent PCR Amplifier was a product of Agilent Technologies Company (USA).

**Animal group allocations and splenectomy.** This animal model of splenectomy-induced POCD was established as described [15, 16]. Rats were randomized into the follow four groups (10 per group). Control (CON) animals did not undergo any surgery and received a daily intraperitoneal injection of saline. Surgery (SUR) animals underwent splenectomy as described below and received a daily intraperitoneal injection of saline. RAP animals underwent splenectomy, followed by a daily intraperitoneal injection of rapamycin (1.0
mg/kg). The 3-MA group underwent splenectomy, followed by a daily intraperitoneal injection of 3-MA (3.0 mg/kg). The injection volume was the same in all four groups. Intraperitoneal injections began in all groups on postoperative day 3 and continued for four weeks.

For splenectomy, rats were anesthetized intraperitoneally with 80 mg/kg ketamine and 60 mg/kg sodium pentobarbital. During experiments, anesthesia was maintained by injecting one-third of the initial dose of ketamine every 45 min. Rats were placed in a supine position on an adjustable warming pad and superclean bench. A transverse incision 2.0 cm long was made from the lower edge of the left rib in order to open the abdominal cavity, then the spleen was excised. After confirmation of no hemorrhage, the abdominal cavity was stitched and the wound was disinfected with iodophor.

**Collection of samples.** After the four-week treatment period, learning and memory were assessed in a subset of the 10 animals in each group using a Morris water maze. Tests were conducted on postoperative day 1, 3, 5 and 7. Rats were sacrificed by cervical dislocation and hippocampus tissue was collected.

**Morris water maze.** Animals' ability to navigate and explore space was assessed on postoperative day 1, 3, 5, and 7. Tests were conducted four times per day. The platform was placed in the center of the southwest quadrant. Rats facing the water maze wall were randomly positioned at one of four starting positions (southeast, northeast, southwest and northwest) and allowed to swim around the maze until finding the platform. If the rats did not find the platform within 2 min, we assisted them and recorded the latency as 120 s. Rats were allowed to rest on the platform for 15 s, and training was repeated at 30-s intervals. Then the time for rats to swim to the platform (escape latency) was recorded.
using an automatic camera and motion recorder. At 24 h after the last training, the platform was removed to perform space exploration experiments. Rats were placed in the Morris water maze in the northwest quadrant, and the time required to cross the original platform was recorded. Animals who failed to cross the platform within 2 min were recorded as having a crossing time of 120 s.

**Assessment of inflammatory response.** Hippocampus tissue was minced using Mayo-Noble scissors and homogenized using a tissue homogenizer. The homogenate was dissolved using Phosphate buffer saline and centrifuged. The supernatant was assayed for inflammatory factors interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α using commercial enzyme-linked immunosorbent assays (ThermoFisher Scientific).

**Analysis of mRNA levels using RT-qPCR.** Total RNA was extracted from hippocampus tissues using TRIzol reagent. Single-stranded cDNAs encoding ATG5, Beclin1, p62 and Cys C were synthesized and then amplified by PCR using specific primers (Table 1). Levels of target mRNAs were quantitated relative to the level of mRNA encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Analysis of protein expression by Western blotting.** Total protein was isolated from hippocampal tissue using ice-cold RIPA lysis buffer with protease inhibitors (Beyotime Institute of Biotechnology, Haimen, China), and protein concentration was determined using the BCA Protein Assay Kit (Pierce). Equal amounts of protein were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Membranes were blocked and then incubated overnight with primary antibodies against ATG5, LC3B, Beclin1, SQSTM1/p62, Cys C and
GADPH. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody to allow chemiluminescent visualization using the ChemiDoc MP system (Bio-Rad).

**Statistical analysis.** Data were analyzed using SPSS 22.0 (IBM, Chicago, IL, USA) and reported as mean ± SD. Inter-group differences were assessed for significance using one-way ANOVA, and pair-wise comparisons were assessed using the LSD and t tests. A two-tailed $P < 0.05$ was defined as statistically significant.

Results

**Autophagy levels correlated positively with postoperative cognitive performance of aged rats undergoing splenectomy**

As expected, surgery by itself compromised postoperative cognitive function: escape latency was significantly longer, and time to cross the original platform shorter, in the SUR and 3-MA groups than in the CON group on days 1, 3, 5 and 7. Similarly compromised function was observed in the RAP group, but only on days 1 and 3, suggesting milder POCD in the presence of induced autophagy. Indeed, escape latency was significantly shorter, and the time to cross the original platform significantly longer, in the RAP group than in the SUR group on days 1, 3, 5 and 7. Treatment with 3-MA led to the opposite effects as RAP: escape latency was significantly shorter, and time to cross the original platform significantly longer, in the RAP group than in the 3-MA group on days 1, 3, 5 and 7 (Table 2 and Figure 1).

**Autophagy levels correlated negatively with neural inflammation in aged rats undergoing splenectomy**

As expected, splenectomy triggered inflammation in our rat model: levels of IL-1β, IL-6
and TNF-α were significantly higher in SUR, RAP and 3-MA groups than in the CON group. Inducing autophagy significantly reduced the levels of all three inflammatory factors: levels were significantly lower in the RAP group than the SUR group. Conversely, inhibiting autophagy exacerbated surgery-induced stress: cytoline levels were significantly higher in the 3-MA group than in the SUR group (Table 3 and Figure 2).

**Autophagy levels correlated positively with expression of autophagy-related proteins and Cys C in aged rats undergoing splenectomy**

Surgery induced the expression of Cys C and of the autophagy-related proteins ATG5, Beclin1 and p62: mRNA levels of these genes were significantly higher in the SUR, RAP and 3-MA groups than in the CON group. Inducing autophagy further increased these levels, whereas inhibiting autophagy significantly reduced them (Table 4 and Figure 3). Similar results were observed at the protein level for Cys C, ATG5, LC3B-II/LC3B-I, Beclin1 and p62 (Table 5 and Figure 4).

**Discussion**

Autophagy is known to mitigate damage due to neurodegenerative disease, but whether it can do the same in POCD is unclear. The present study in a rat model of splenectomy-induced POCD suggests that autophagy increases the expression of Cys C in the hippocampus, which improves postoperative spatial learning and memory. Conversely, inhibiting autophagy reversed splenectomy-induced Cys C up-regulation, further impairing learning and memory.

Autophagy is a major pathway for elimination of unfolded proteins and damaged organelles, and its dysregulation in neurons contributes to various neurodegenerative diseases [17][18, 19]. In animal models of Alzheimer's disease, the autophagy inducer rapamycin reduces the accumulation of amyloid beta protein and hyperphosphorylation of
tau protein in hippocampus, inhibiting neuronal apoptosis by improving learning and memory [20]. In rats that suffer POCD as a result of sevoflurane anesthesia or appendectomy, activating autophagy by increasing the expression of light chain 3 II (LC3-II) and Beclin1 as well as decreasing the expression of p62 significantly ameliorates the cognitive dysfunction, and this effect involves AMPK-Sirt1 signaling [21]. Exposing aged rats to propofol inhibits autophagy and significantly impairs cognitive performance, which is substantially improved by pretreating animals with the autophagy activator diaminodiphenyl sulfone [11]. Similarly, we found that giving rats rapamycin enhanced autophagy and ameliorated spatial learning and memory after splenectomy, consistent with studies in aged rats given rapamycin and 3-MA and subjected to exploratory laparotomy under anesthesia with 3% sevoflurane 30 min late, and melamine-treated rats given rapamycin to attenuate impairment of spatial cognition and hippocampal synaptic plasticity [22, 23]. Stresses such as infection, autoimmunity, or traumatic brain injury can trigger neuroinflammation that aggravates brain lesions, resulting in neuron degeneration and synaptic dysfunction [24-26]. We found that levels of inflammatory factors varied inversely with the severity of POCD, and that activating autophagy led to lower cytokine levels, while inhibiting autophagy increased the levels. These results are consistent with previous studies. For example, auricular vagus nerve stimulation of aged rats subjected to surgery reduces postoperative levels of TNF-α, IL-1β and NF-κB and improves postoperative memory based on the Morris water maze [27]. Injecting the agomir miR-181b-5p in the hippocampus of mice before surgery down-regulates TNF-α, IL-1β, and monocyte chemoattractant protein-1, thereby reducing microglial activation and ameliorating hippocampus-dependent memory [28]. In our study, levels of Cys C protein and mRNA correlated positively with autophagy
levels, suggesting that autophagy may activate Cys C expression in hippocampus. Up-regulation of Cys C exerts neuroprotective effects in Alzheimer's disease, amyotrophic lateral sclerosis and subarachnoid hemorrhage by inducing autophagy and angiogenesis in the brain [8, 29, 30].

To our knowledge, this is the first study to explore the role of Cys C and autophagy in a rat model of POCD. We found that inducing autophagy up-regulated Cys C, ameliorating neuroinflammation and postoperative spatial learning and memory ability. Future work should explore how Cys C up-regulation can exert these effects, such as through reduced accumulation of amyloid beta protein, inhibition of tau hyperphosphorylation and activation of angiogenesis in the brain[31]. Future studies should also explore how autophagy up-regulates Cys C. Detailed analyses of these questions may require experiments in vitro and with knockout animals.

Conclusions

Here, multifaceted approach has taken to investigate the autophagy and Cys C in the function of POCD. We have identified autophagy regulation as having positive effects behaviorally and biochemically. Our data demonstrated that inducing autophagy up-regulated Cys C, ameliorating neuroinflammation and postoperative spatial learning and memory ability, which may be a potential target for prevention of POCD.

List Of Abbreviations

POCD, postoperative cognitive dysfunction; CysC, cystatin C; LC3B-I, microtubule-associated protein 1A/1B-light chain 3; Rap, rapamycin; 3-MA, 3-methyladenine; PCR, polymerase chain reaction; ATG, autophagy-related gene; SQSTM1, sequestosome 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor.
Declarations

**Ethics approval**

The study was performed in accordance with the Guidelines for the revised Animals (Scientific Procedures) Act 1986 in the UK and Directive 2010/63/EU in Europe and the Care and Use of Laboratory Animals approved by Ethics and Animal Welfare Committee of the Lihuili Eastern Hospital of Ningbo Medical Center (Ningbo, China, Permission number 2018-022). All possible efforts were undertaken to avoid animal suffering at each stage of the experiments.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The data sets used and analyzed in the current study were available from the corresponding author on reasonable request.

**Conflict of interest**

The authors have no conflict of interests regarding the work.

**Funding**

This work was supported by the Clinical Research Funding Project of Zhejiang Medical Association (2011ZYC-A52) and Ningbo Medical Science and Technology Project (2013A01).

**Authors’ contributions**

Li Yan designed and directed the overall study. Bi Xing-hua carried out experiment, data analysis and drafted the manuscript. Zhou Long-yuan, Cai Chang and Qi Yong revised and approved the manuscript.

**Acknowledgments**

Not applicable.
References

1. Levine B, Kroemer G. Biological Functions of Autophagy Genes: A Disease Perspective. Cell2019 Jan 10;176(1-2):11-42.

2. Zientara-Rytter K, Subramani S. The Roles of Ubiquitin-Binding Protein Shuttles in the Degradative Fate of Ubiquitinated Proteins in the Ubiquitin-Proteasome System and Autophagy. Cells2019 Jan 10;8(1).

3. Zhang Y, Liu G, Dull RO, Schwartz DE, Hu G. Autophagy in pulmonary macrophages mediates lung inflammatory injury via NLRP3 inflammasome activation during mechanical ventilation. American journal of physiology Lung cellular and molecular physiology2014 Jul 15;307(2):L173-85.

4. Jeong SJ, Zhang X, Rodriguez-Velez A, Evans TD, Razani B. p62/SQSTM1 and Selective Autophagy in Cardiometabolic Diseases. Antioxidants & redox signaling2018 Dec 27.

5. Shi B, Ma M, Zheng Y, Pan Y, Lin X. mTOR and Beclin1: Two key autophagy-related molecules and their roles in myocardial ischemia/reperfusion injury. J Cell Physiol2019 Jan 7.

6. Mathews PM, Levy E. Cystatin C in aging and in Alzheimer's disease. Ageing research reviews2016 Dec;32:38-50.

7. Li W, Sultana N, Siraj N, Ward LJ, Pawlik M, Levy E et al. Autophagy dysfunction and regulatory cystatin C in macrophage death of atherosclerosis. Journal of cellular and molecular medicine2016 Sep;20(9):1664-72.

8. Zou J, Chen Z, Wei X, Chen Z, Fu Y, Yang X et al. Cystatin C as a potential therapeutic mediator against Parkinson's disease via VEGF-induced angiogenesis and enhanced neuronal autophagy in neurovascular units. Cell death & disease2017 Jun 1;8(6):e2854.

9. Liu Y, Cai H, Wang Z, Li J, Wang K, Yu Z et al. Induction of autophagy by cystatin C: a
potential mechanism for prevention of cerebral vasospasm after experimental subarachnoid hemorrhage. European journal of medical research 2013 Jul 1;18:21.

10. Zhao YG, Sun L, Miao G, Ji C, Zhao H, Sun H et al. The autophagy gene Wdr45/Wipi4 regulates learning and memory function and axonal homeostasis. Autophagy 2015;11(6):881-90.

11. Yang N, Li L, Li Z, Ni C, Cao Y, Liu T et al. Protective effect of dapsone on cognitive impairment induced by propofol involves hippocampal autophagy. Neurosci Lett 2017 May 10;649:85-92.

12. Skvarc DR, Berk M, Byrne LK, Dean OM, Dodd S, Lewis M et al. Post-Operative Cognitive Dysfunction: An exploration of the inflammatory hypothesis and novel therapies. Neuroscience and biobehavioral reviews 2018 Jan;84:116-33.

13. Lewis M, Maruff P, Silbert B. Statistical and conceptual issues in defining post-operative cognitive dysfunction. Neuroscience and biobehavioral reviews 2004 Jul;28(4):433-40.

14. Luo A, Yan J, Tang X, Zhao Y, Zhou B, Li S. Postoperative cognitive dysfunction in the aged: the collision of neuroinflammaging with perioperative neuroinflammation. Inflammopharmacology 2019 Jan 3.

15. Ning B, Zhang Q, Deng M, Wang N, Fang Y. Endoplasmic Reticulum Stress Induced Autophagy In 6-OHDA-Induced Parkinsonian Rats. Brain research bulletin 2019 Jan 6.

16. Leite Nde C, Montes EG, Fisher SV, Cancian CR, de Oliveira JC, Martins-Pinge MC et al. Splenectomy attenuates obesity and decreases insulin hypersecretion in hypothalamic obese rats. Metabolism: clinical and experimental 2015 Sep;64(9):1122-33.

17. Li Y, Zhou D, Ren Y, Zhang Z, Guo X, Ma M et al. Mir223 restrains autophagy and promotes CNS inflammation by targeting ATG16L1. Autophagy 2018 Sep 13:1-15.
18. Moloudizargari M, Asghari MH, Ghobadi E, Fallah M, Rasouli S, Abdollahi M. 
Autophagy, its mechanisms and regulation: Implications in neurodegenerative 
diseases. Ageing research reviews 2017 Nov;40:64-74.

19. Menzies FM, Fleming A, Caricasole A, Bento CF, Andrews SP, Ashkenazi A et al. 
Autophagy and Neurodegeneration: Pathogenic Mechanisms and Therapeutic 
Opportunities. Neuron 2017 Mar 8;93(5):1015-34.

20. Zare-Shahabadi A, Masliah E, Johnson GV, Rezaei N. Autophagy in Alzheimer's 
disease. Reviews in the neurosciences 2015;26(4):385-95.

21. Yan WJ, Wang DB, Ren DQ, Wang LK, Hu ZY, Ma YB et al. AMPKalpha1 overexpression 
improves postoperative cognitive dysfunction in aged rats through AMPK-Sirt1 and 
autophagy signaling. J Cell Biochem 2019 Feb 18.

22. Zhang Q, Yuan TB, Yang SH, Li YN, Wang XL, Wang QY. [Relationship between 
autophagy and apoptosis during postoperative cognitive dysfunction in aged rats].
Chinese Journal of Anesthesiology 2018;38(2):159-62.

23. Fu J, Wang H, Gao J, Yu M, Wang R, Yang Z et al. Rapamycin Effectively Impedes 
Melamine-Induced Impairments of Cognition and Synaptic Plasticity in Wistar Rats.
Mol Neurobiol 2017 Mar;54(2):819-32.

24. De Felice FG, Ferreira ST. Inflammation, defective insulin signaling, and 
mitochondrial dysfunction as common molecular denominators connecting type 2 
diabetes to Alzheimer disease. Diabetes 2014 Jul;63(7):2262-72.

25. Bettcher BM, Kramer JH. Inflammation and clinical presentation in neurodegenerative 
disease: a volatile relationship. Neurocase 2013 Apr;19(2):182-200.

26. Bostanciklioglu M. An update on the interactions between Alzheimer's disease, 
autophagy and inflammation. Gene 2019 Apr 25;705:157-66.

27. Cai L, Lu K, Chen X, Huang JY, Zhang BP, Zhang H. Auricular vagus nerve stimulation
protects against postoperative cognitive dysfunction by attenuating neuroinflammation and neurodegeneration in aged rats. Neurosci Lett 2019 Mar 21;703:104-10.

28. Lu Y, Xu X, Dong R, Sun L, Chen L, Zhang Z et al. MicroRNA-181b-5p attenuates early postoperative cognitive dysfunction by suppressing hippocampal neuroinflammation in mice. Cytokine 2019 Apr 16;120:41-53.

29. Liu Y, Li J, Wang Z, Yu Z, Chen G. Attenuation of early brain injury and learning deficits following experimental subarachnoid hemorrhage secondary to Cystatin C: possible involvement of the autophagy pathway. Mol Neurobiol 2014 Apr;49(2):1043-54.

30. Watanabe S, Hayakawa T, Wakasugi K, Yamanaka K. Cystatin C protects neuronal cells against mutant copper-zinc superoxide dismutase-mediated toxicity. Cell death & disease 2014 Oct 30;5:e1497.

31. Shen Y, Ye B, Chen P, Wang Q, Fan C, Shu Y et al. Cognitive Decline, Dementia, Alzheimer's Disease and Presbycusis: Examination of the Possible Molecular Mechanism. Front Neurosci 2018;12:394.

Tables

Table 1. Primer sequences used to detect target mRNAs

| Gene | Primer sequence (5'→3') | Product size (bp) |
|------|-------------------------|------------------|
| ATG5 | F: AGACCACAACCTGAACGGCCT  
      | R: AAGGCTATGCAGCTGTCCATC  | 74               |
| Beclin1 | F: TTCAATGCGACCTTCCATAT  
       | R: CAGAACATGACAAACGGCAAC  | 256              |
| p62  | F: GTTCTTGAACCCCTCTGGTGG  
      | R: ATGGAGCCTCTTACTGGGTG   | 140              |
| Cys C | F: AGCGAGTACAACAGGGCAACAC  
      | R: TTGTCAAGGGGTGTGTGCTTTGT  | 247              |
| GADPH | F: GGCACAGTCAAGGGCTGAGATAG  
      | R: ATGGTGGTGAAGCCAGTA      | 143              |

Note: F, forward primer; R, reverse primer.

Table 2. Comparison of escape latency and time to cross the original platform in the four
| Testing day | Group | n | Escape latency (s) | Time to cross original platform (s) |
|-------------|-------|---|-------------------|-------------------------------------|
| Day 1*      | CON   | 10 | 33.72±3.20        | 3.49±0.50                           |
|             | SUR   | 10 | 64.98±11.83\(^a\) | 2.15±0.30\(^a\)                     |
|             | RAP   | 10 | 43.46±7.23\(^ab\) | 3.04±0.40\(^ab\)                    |
|             | 3-MA  | 10 | 76.15±9.05\(^abc\)| 0.94±0.45\(^abc\)                   |
| Day 3       | CON   | 10 | 30.54±2.88        | 4.05±0.48                           |
|             | SUR   | 10 | 64.98±11.83\(^a\) | 2.15±0.30\(^a\)                     |
|             | RAP   | 10 | 36.78±8.93\(^ab\) | 3.55±0.40\(^ab\)                    |
|             | 3-MA  | 10 | 52.81±4.11\(^abc\)| 1.48±0.34\(^abc\)                   |
| Day 5       | CON   | 10 | 28.94±2.19        | 4.15±0.43                           |
|             | SUR   | 10 | 64.98±11.83\(^a\) | 2.15±0.30\(^a\)                     |
|             | RAP   | 10 | 36.78±8.93\(^ab\) | 3.55±0.40\(^ab\)                    |
|             | 3-MA  | 10 | 52.81±4.11\(^abc\)| 1.48±0.34\(^abc\)                   |
| Day 7       | CON   | 10 | 28.94±2.19        | 4.15±0.43                           |
|             | SUR   | 10 | 64.98±11.83\(^a\) | 2.15±0.30\(^a\)                     |
|             | RAP   | 10 | 36.78±8.93\(^ab\) | 3.55±0.40\(^ab\)                    |
|             | 3-MA  | 10 | 52.81±4.11\(^abc\)| 1.48±0.34\(^abc\)                   |

*Postoperative day.

Note: \(^a\) P < 0.05 vs. CON group \(^b\) P < 0.05 vs. SUR group \(^c\) P < 0.05 vs. 3-MA group.

**Table 3. Comparison of levels of inflammatory cytokines IL-1β, IL-6 and TNF-α in the four groups**

| Group | n | IL-1β (pg/ml) | IL-6 (ng/ml) | TNF-α (ng/ml) |
|-------|---|---------------|--------------|---------------|
| CON   | 10 | 5.15±1.17     | 8.82±1.55    | 14.02±3.09    |
| SUR   | 10 | 37.21±2.57\(^a\) | 29.17±1.73\(^a\) | 40.98±3.53\(^a\) |
| RAP   | 10 | 20.38±5.78\(^ab\) | 19.80±1.55\(^ab\) | 20.84±3.08\(^ab\) |
| 3-MA  | 10 | 43.46±3.50\(^abc\) | 36.35±2.22\(^abc\) | 61.13±5.26\(^abc\) |

\[ F = 222.60 \]
\[ P = 0.000 \]

Note: \(^a\) P < 0.05 vs. CON group \(^b\) P < 0.05 vs. SUR group \(^c\) P < 0.05 vs. 3-MA group.

**Table 4. Comparison of mRNA levels of Cys C and autophagy-related genes in the four groups**

| Group | n | ATG5 | Beclin1 | p62 | Cys C |
|-------|---|------|---------|-----|-------|
| CON   | 10 | 1.05±0.07 | 1.00±0.11 | 1.21±0.13 | 1.11±0.05 |
| SUR   | 10 | 1.63±0.15\(^a\) | 1.79±0.20\(^a\) | 1.95±0.11\(^a\) | 2.28±0.24\(^a\) |
| RAP   | 10 | 2.80±0.13\(^ab\) | 2.60±0.33\(^ab\) | 2.93±0.30\(^ab\) | 3.95±0.24\(^ab\) |
| 3-MA  | 10 | 1.30±0.07\(^abc\) | 1.33±0.06\(^abc\) | 1.42±0.14\(^abc\) | 1.57±0.15\(^abc\) |

\[ F = 146.50 \]
\[ P = 0.000 \]

*Levels are expressed relative to the level of GAPDH mRNA.

Note: \(^a\) P < 0.05 vs. CON group \(^b\) P < 0.05 vs. SUR group \(^c\) P < 0.05 vs. 3-MA group.
Table 5. Comparison of levels of Cys C and autophagy-related proteins in the four groups*

| Group | n  | ATG5      | LC3B-II   | Beclin1   | p62       | Cys C     |
|-------|----|-----------|-----------|-----------|-----------|-----------|
| CON   | 10 | 0.27±0.07 | 0.21±0.05 | 0.18±0.04 | 0.19±0.06 | 0.13±0.0  |
| SUR   | 10 | 0.74±0.06a| 0.44±0.06a| 0.72±0.07a| 0.56±0.07a| 0.48±0.0c |
| RAP   | 10 | 1.02±0.09ab| 1.14±0.14ab| 1.04±0.09ab| 0.91±0.06ab| 1.01±0.07 |
| 3-MA  | 10 | 0.31±0.05abc| 0.39±0.04abc| 0.39±0.06abc| 0.17±0.06abc| 0.28±0.06 |

| F     |    | 77.99    | 74.82     | 103.00    | 101.00    | 139.60    |
| P     |    | 0.000    | 0.000     | 0.000     | 0.000     | 0.000     |

*Levels are expressed as ratios with respect to the level of GAPDH protein.

Note: a $P < 0.05$ vs. CON group b $P < 0.05$ vs. SUR group c $P < 0.05$ vs. 3-MA group.

Figures
Figure 1

Postoperative cognitive performance in aged rats without splenectomy (CON) or with splenectomy and treatment with saline (SUR), the autophagy activator rapamycin (RAP) or the autophagy inhibitor 3-methyladenine (3-MA). (A) Escape latency in positional navigation experiments. (B) Time to cross the original platform in space exploration experiments. Experiments were performed in triplicate. a P < 0.05 vs. CON group; b P < 0.05 vs. SUR group; c P < 0.05 vs. 3-MA group.
Figure 2

Levels of the inflammatory cytokines (A) IL-1β, (B) IL-6 and (C) TNF-α in the hippocampus of aged rats without splenectomy (CON) or with splenectomy and treatment with saline (SUR), the autophagy activator rapamycin (RAP) or the autophagy inhibitor 3-methyladenine (3-MA). Experiments were performed in triplicate. a P < 0.05 vs. CON group; b P < 0.05 vs. SUR group; c P < 0.05 vs. 3-MA group.
Figure 3

Levels of mRNAs encoding (A) ATG5, (B) Beclin1, (C) SQSTM/p62 and (D) Cys C in aged rats without splenectomy (CON) or with splenectomy and treatment with saline (SUR), the autophagy activator rapamycin (RAP) or the autophagy inhibitor 3-methyladenine (3-MA). Experiments were performed in triplicate. a P < 0.05 vs. CON group; b P < 0.05 vs. SUR group; c P < 0.05 vs. 3-MA group.
Figure 4
Protein levels of LC3B, ATG5, Beclin1, SQSTM/p62 and Cys C in aged rats without splenectomy (CON) or with splenectomy and treatment with saline (SUR), the autophagy activator rapamycin (RAP) or the autophagy inhibitor 3-methyladenine (3-MA). (A) Representative Western blot results. (B-F) Quantitation of Western blots. Experiments were performed in triplicate. a P < 0.05 vs. CON group; b P < 0.05 vs. SUR group; c P < 0.05 vs. 3-MA group.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

Cys C.tif
ATG5.tif
GADPH.tif
p62.tif
Data.pzf
NC3Rs ARRIVE Guidelines Checklist 2014.docx
Beclin-1.tif
LC3B.tif