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

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Research article

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Abstract

B ackground : 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 ubiquitinylated 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 ℃ in air with 70±10% relative humidity before experiments. 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 intraperitonal injection of rapamycin (1.0 mg/kg). The 3-MA group underwent splenectomy, followed by a daily intraperitonal 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 have 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.

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.

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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.

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Tables

Table 1 Primer sequences used to detect target mRNAs

Gene	Primer sequence (5'→3')	Product size (bp)
ATG5	F: AGACCACAACTGAACGGCCT	74
	R: AAGGGTATGCAGCTGTCCATC	
Beclin1	F: TTCAATGCGACCTTCCATAT	256
	R: CAGAACAGTACAACGGCAAC	
p62	F: GTTCCTGAACCCTCTCGTGG	140
	R: ATGGAGCCTCTTACTGGGGT	
Cys C	F: AGCGAGTACAACAAGGGCAGCAAC	247
	R: TTGTCAGGGTGTGTGTGCCTTTCC	
GADPH	F: GGCACAGTCAAGGCTGAGAATG	143
	R: ATGGTGGTGAGA CGCCAGTA	

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

Table 2 Comparison of escape latency and time to cross the original platform in the four groups

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	42.70±2.76[a]	2.44±0.52[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	39.19±2.57[a]	2.37±0.55[a]
	RAP	10	31.15±2.31[b]	3.86±0.43[b]
	3-MA	10	46.45±3.30[abc]	1.83±0.45[abc]
Day 7	CON	10	25.82±3.71	4.52±0.46
	SUR	10	35.09±3.60[a]	3.27±0.50[a]
	RAP	10	26.67±2.77[b]	4.30±0.55[b]
	3-MA	10	39.38±2.36[abc]	2.57±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	448.00	305.90
P		0.000	0.000	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	35.13	51.33	136.80
P		0.000	0.000	0.000	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/LC3B-I	Beclin1	p62	Cys C
CON	10	0.27±0.07	0.55±0.06	0.18±0.04	0.19±0.06	0.13±0.05
SUR	10	0.74±0.06[a]	0.98±0.04[a]	0.72±0.07[a]	0.56±0.07[a]	0.48±0.04[a]
RAP	10	1.02±0.09[ab]	2.01±0.17[ab]	1.04±0.09[ab]	0.91±0.06[ab]	1.01±0.07[ab]
3-MA	10	0.31±0.05[abc]	1.31±0.14[abc]	0.39±0.06[abc]	0.17±0.06[abc]	0.28±0.06[abc]
F		77.99	84.92	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

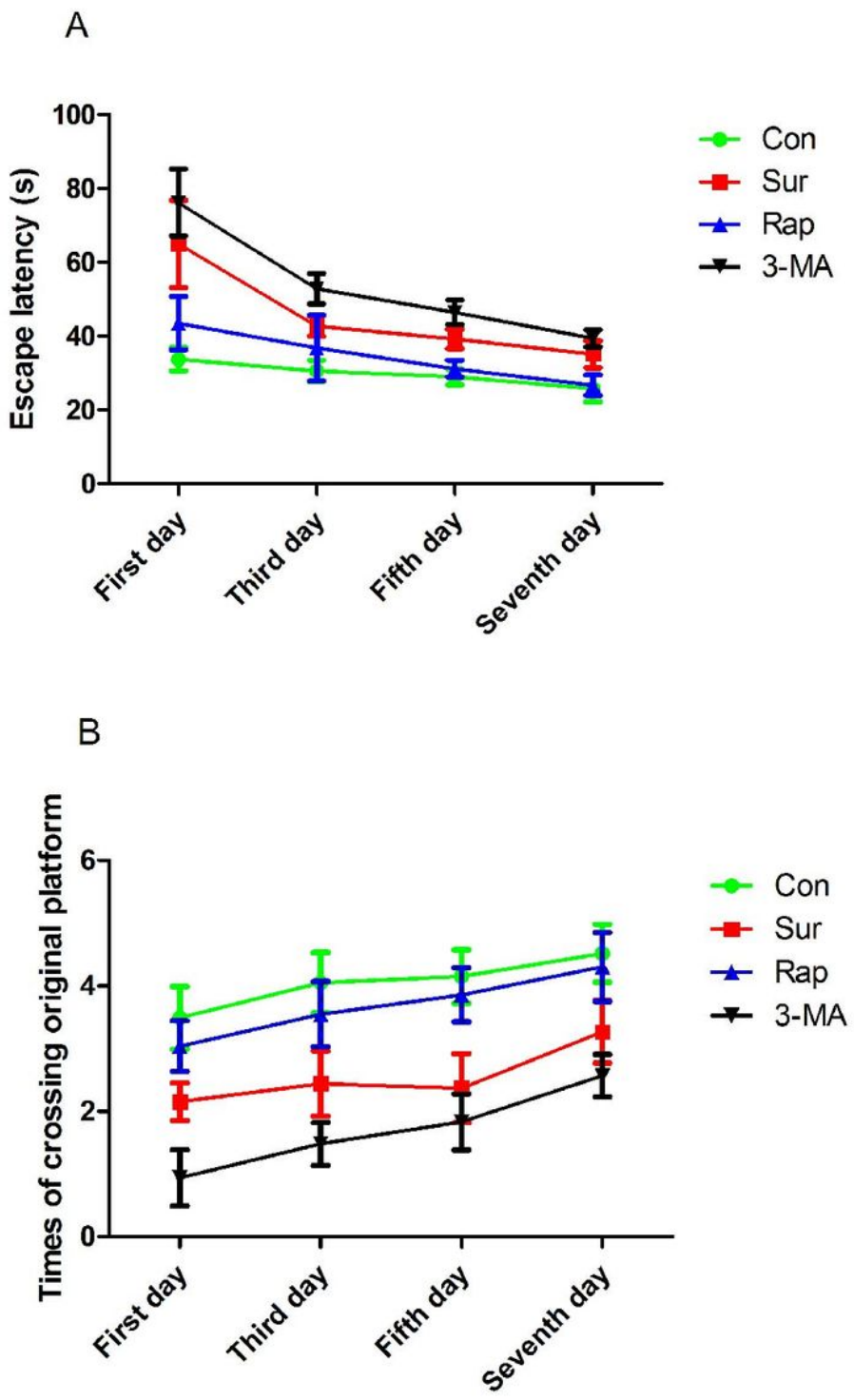

Figure 1

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.

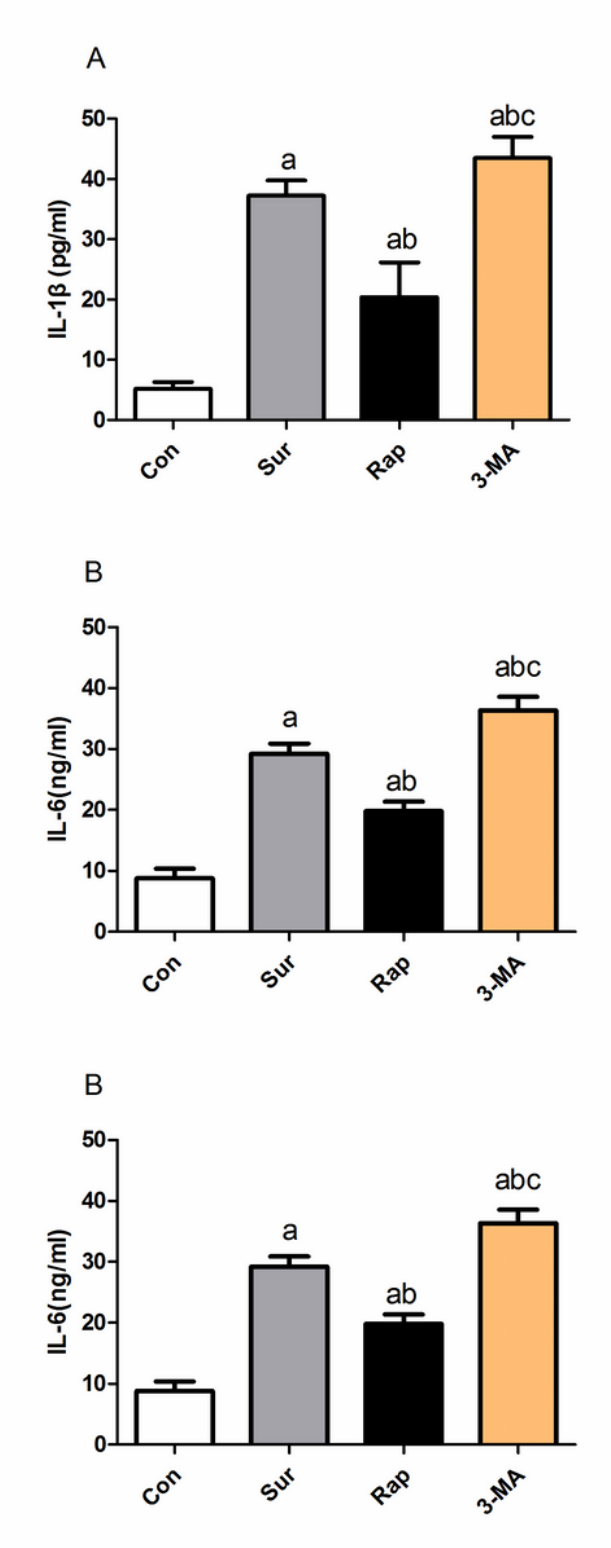

Figure 2

evels 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.

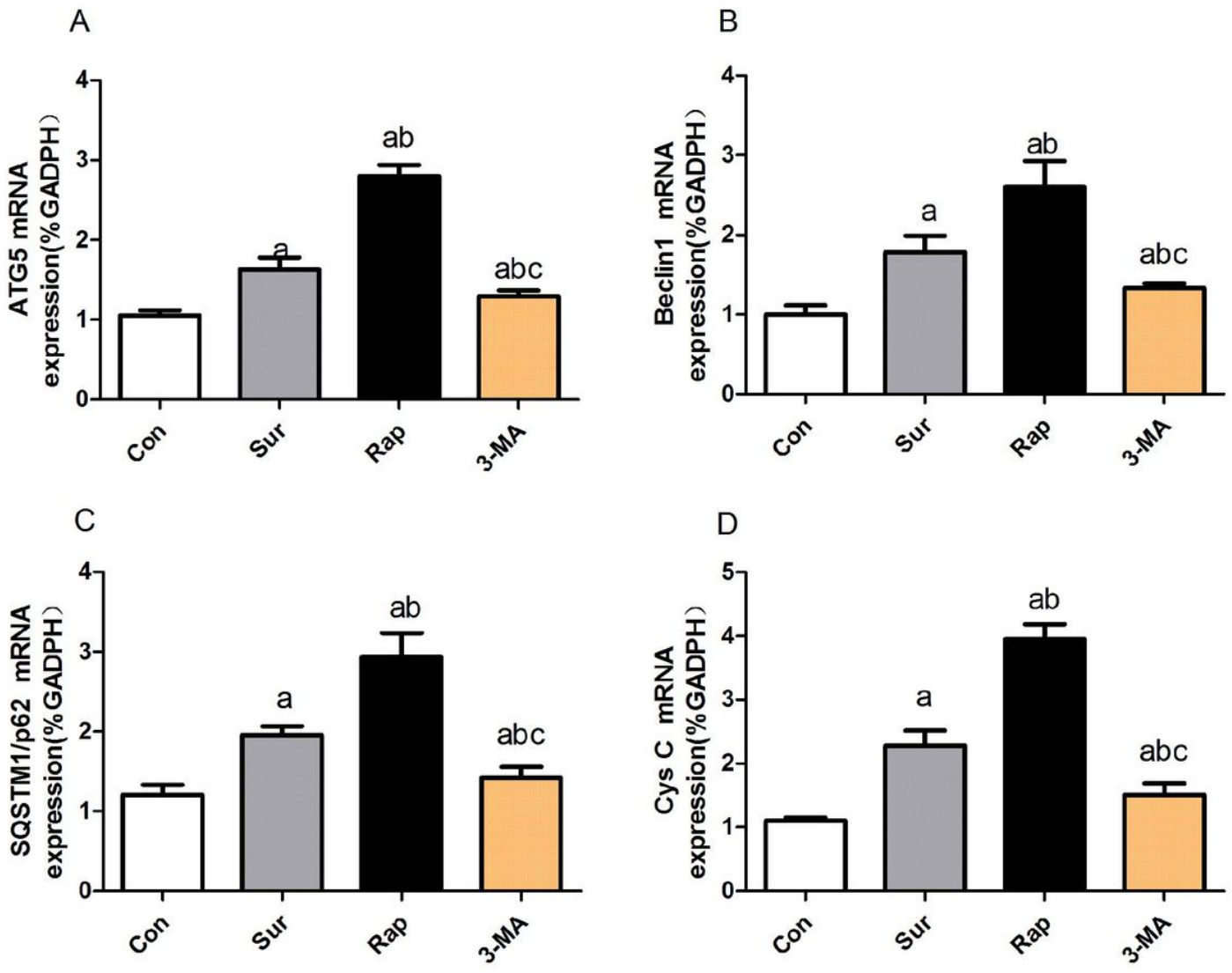

Figure 3

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.

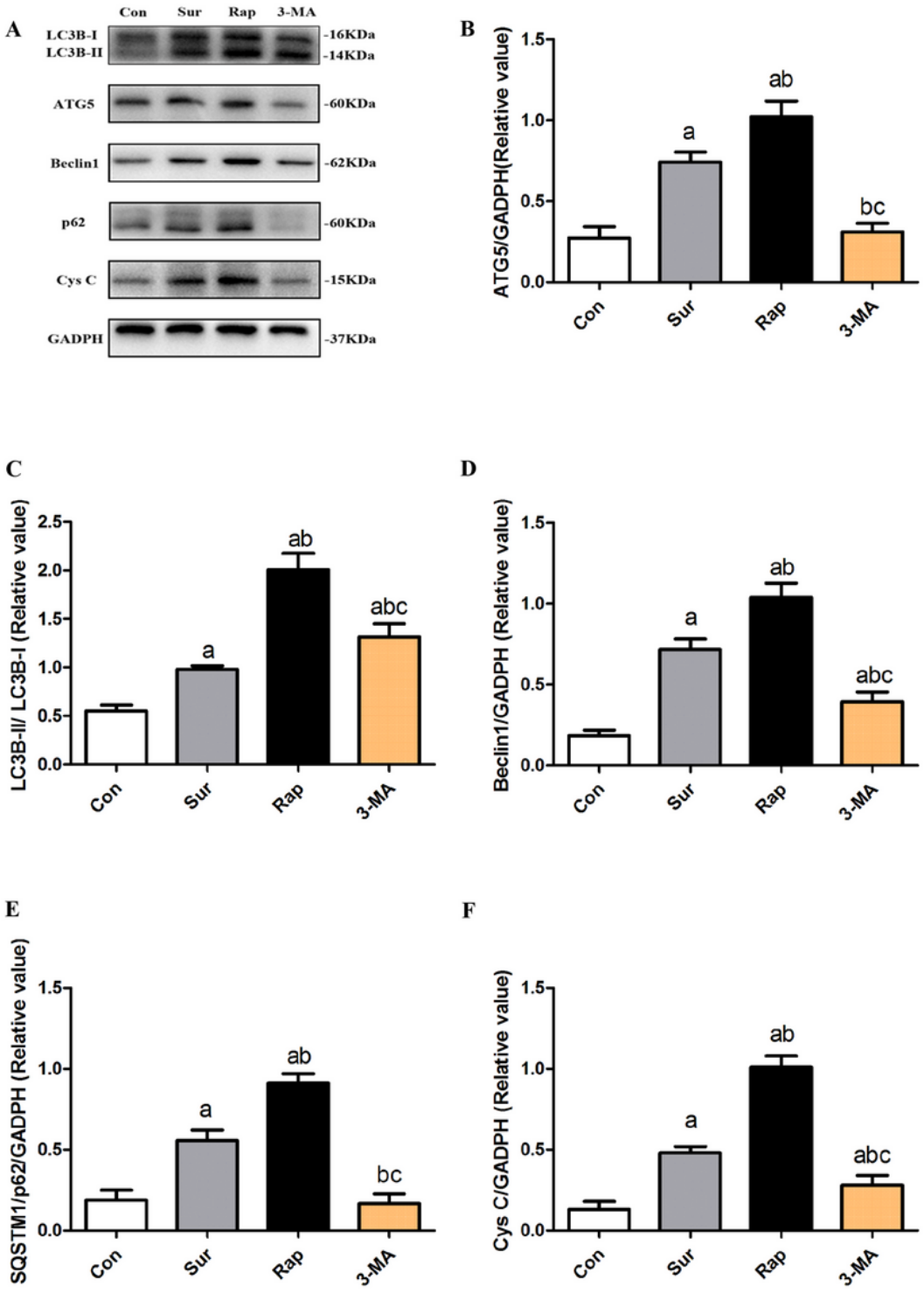

Figure 4

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

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- NC3RsARRIVEGuidelinesChecklist2014.docx