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·Original Article·

# Duration-dependent regulation of autophagy by isoflurane exposure in aged rats

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## ABSTRACT

Current evidence suggests a central role for autophagy in many inflammatory brain disorders, including Alzheimer's disease (AD). Furthermore, it is also well accepted that some inhalation anesthetics, such as isoflurane, may cause AD-like neuropathogenesis and resultant postoperative cognitive dysfunction, especially in the elderly population. However, the impact of inhalation anesthetics on autophagic components in the brain remains to be documented. Hence, our objective was to investigate the effects of different durations of isoflurane exposure on hippocampus-dependent learning and hippocampal autophagy in aged rats. Aged Sprague-Dawley rats (20 months old) were randomly exposed to 1.5% isoflurane or 100% oxygen for 1 or 4 h. Animals were then trained in the Morris water maze (4 trials/day for 5 consecutive days). Hippocampal phagophore formation markers, beclin 1 and protein microtubule-associated protein 1 light chain-3B (LC3B), as well as p62, an indicator of autophagic flux, were quantified by western blotting. There was no significant difference in the escape latencies and time spent in the target quadrant, as well as hippocampal expression of beclin 1, LC3B-II, and p62 at 24 h post-anesthesia between the 1-h isoflurane-exposed rats and their controls ($P$ >0.05). Four-hour exposure to isoflurane resulted in spatial learning and memory deficits, as evidenced by prolonged escape latencies on days 4 and 5 post-anesthesia and less time spent in the target quadrant than sham-exposed animals ($P$ <0.05). These events were accompanied by a decline in hippocampal expression of LC3B-I, LC3B-II, and beclin 1 24 h after isoflurane ($P$ <0.01 and $P$ <0.05). Nevertheless, no significant change in p62 expression was found. Further kinetics study of autophagic changes induced by 4 h of isoflurane showed a transient upregulation of LC3B-I, LC3B-II, and beclin 1 at the end of exposure and a subsequent striking decrease within 12–24 h post-anesthesia ($P$ <0.05). Hippocampal p62 peaked at 6 h but subsequently resolved. These results from our pilot *in vivo* study support a duration-dependent relationship between 1.5% isoflurane exposure, and spatial cognitive function as well as hippocampal phagophore formation.

**Keywords:** autophagy; phagophore formation; cognitive dysfunction

## INTRODUCTION

Dementia is a neurodegenerative syndrome in

which there is deterioration in cognition, behavior, and daily living activity[1]. As the population ages, the impact of dementia on the social and clinical burden will increase. Dementia is caused by a variety of diseases and injuries, such as Alzheimer's disease (AD), Parkinson's diseases, multiple sclerosis, and stroke[1, 2]. Recently, surgery and anesthesia have been proposed to increase the incidence of AD[3, 4]. Surgical procedures and administration of anesthesia are associated with a transient or permanent decline in cognitive function, termed postoperative cognitive dysfunction (POCD). POCD delays rehabilitation and increases morbidity and early mortality[5], and also has emerged as a major health concern, especially in the geriatric population[6].

In examining the role of surgery and anesthesia on POCD, it is difficult to discriminate the effects of anesthetics from surgical stress. Nevertheless, increasing numbers of animal studies have indicated that inhalation anesthetics, such as isoflurane, may cause or increase the risk of developing POCD[7, 8]. Specifically, inhalation anesthetics may alter cognitive function *via* NF-κB-dependent neuroinflammation[9, 10], amyloid β (Aβ) accumulation[8, 11-13], tau phosphorylation[13, 14], modified neurotransmission[15] and deregulated calcium homeostasis[16]. Among these, the first three appear to be the shared pathological markers of AD, thereby implying an emerging link between POCD and dementia.

Autophagy is a major catabolic pathway in eukaryotic cells and is a vital pathway for degrading normal and aggregated proteins and altered or unwanted organelles, particularly under stress conditions[17]. Autophagy at an appropriate level not only plays a crucial role in protein quality control, but also acts as a defense response to stress, thus maintaining cellular homeostasis[17]. Previous studies have demonstrated that Aβ is generated in Aβ precursor protein-rich organelles during autophagic turnover[18]. Generally speaking, both increased autophagy induction and defective clearance of Aβ-generating autophagic vacuoles can act together to facilitate Aβ accumulation in AD[19, 20]. In addition, the relationships between autophagy and inflammation, another hallmark of POCD, have also been recently explored in both *in vitro*[21] and *in vivo*[22] models of AD. The results indicated that there is crosstalk between the impairment of autophagy and substantial neuroinflammation in AD.

Nevertheless, the linkages between autophagy and POCD remain largely unknown, and whether the inhalation anesthetics represented by isoflurane modulate autophagy has never been investigated in the aged brain. Based on the above findings and the similarity between POCD and AD, we therefore preliminarily investigated the effects of exposure to isoflurane for different durations on hippocampus-dependent learning and memory and the autophagy response in the hippocampus of aged rats.

## MATERIALS AND METHODS

### Experimental Animals

Seventy-six 20-month-old male Sprague-Dawley rats weighing 550–650 g were purchased from the Dongchuang Laboratory Animal Center (Changsha, Hunan, China) and housed in standard barrier facilities. They were maintained under a 12-h light/dark cycle (lights on at 07:00) with food and water *ad libitum*. All animals had a recovery period of at least 7 days before experiments. All work was conducted with the approval of Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (Approval No. 20150041).

### Experimental Protocols

To study the effects of different durations of isoflurane exposure on spatial learning and memory, rats were randomly exposed to 1.5% isoflurane or vehicle gas for 1 or 4 h. On day 1 post-anesthesia, 9 rats under each experimental condition were randomly selected and performed the Morris water maze (MWM) task to evaluate hippocampal-dependent spatial learning and memory. In addition, we sought to determine the effects of different durations of isoflurane exposure on the autophagy response in the aged hippocampus. So, the hippocampal expression levels of beclin 1 and protein microtubule-associated protein 1 light chain-3B (LC3B), markers of phagophore formation, and p62, an indicator of autophagic flux, were examined at 24 h after isoflurane using western blotting ($n$ = 4 in each condition). In preliminary studies, we found that the longer duration (4 h) of isoflurane exposure impaired spatial leaning, so we further examined the hippocampal expression levels of beclin 1, LC3B, and p62 at 0, 3, 6, 12, and 24 h after a 4-h isoflurane exposure using western blotting ($n$ = 4 per time-point).

**Isoflurane Exposure**

Isoflurane exposure was performed as previously described[9, 15]. In brief, animals were placed in a transparent anesthetic chamber resting in a temperature-controlled water bath (38°C). In the chamber, rats were exposed to 1.5% isoflurane (Baxter Healthcare, Deerfield, IL) in 100% oxygen carrier gas for 1 or 4 h. The control animals were treated with the same duration of 100% oxygen exposure. Volatile anesthetics at 0.5–1.3 minimum alveolar concentration (MAC, the anesthetic concentration at which 50% of animals do not have a motor response to painful stimuli) is commonly used in clinical practice. The isoflurane was titrated to 1.5% as this represents one MAC in aged rats[23]. Gas composition (isoflurane, oxygen, and carbon dioxide) within the chamber was measured using a gas monitor (Datex-Ohmeda, Louisville, CO). After 1 or 4 h of isoflurane exposure and complete recovery, rats were then returned to their home cages.

**Morris Water Maze Experiments**

As previously described[24] with minor modifications, animals were tested for spatial learning and memory using MWM tests by two investigators blinded to the group allocation. In brief, the rats received four training trials daily for five consecutive days. On each trial, rats were gently placed in a fixed position into the water facing the wall of the maze. Each rat was allowed 120 s to locate the platform submerged ~1 cm below the water surface. The time to reach the platform and swimming speed were recorded. After each trial, the rats were allowed to remain on the platform for 20 s before being removed from the pool. On day six, the platform was removed. Probe trials were conducted to evaluate memory retention. The rats were allowed to swim for 90 s, and the percentage of time spent in the previous platform quadrant was determined.

**Western Blotting**

Hippocampal tissues were homogenized in RIPA buffer (Applygen Technologies Inc., Beijing, China). Protein concentrations were determined using BCA protein assay (Applygen). Sixty micrograms of protein per lane was loaded on 10% SDS-PAGE for separating beclin 1 and p62, and on 15% acrylamide for separating LC3B. After transfer onto nitrocellulose membranes (0.45 μm pore size for beclin 1 and p62 and 0.22 μm for LC3B), the following primary antibodies were used: anti-LC3B (#3868; 1:1 000; CST, Danvers, MA), anti-beclin 1 (#3495; 1:1 000; CST), and anti-p62 (#5114; 1:1 000; CST). Fluorescently-labeled secondary antibodies (1:1 0000; LI-COR Biosciences, Lincoln, NE) were used.

**Immunohistochemical Analysis**

Twenty-four hours after 4-h isoflurane exposure, immunofluorescence staining of hippocampal sections was performed as previously described[25] using an anti-LC3B primary antibody (#3868; 1:200; CST) that detects endogenous levels of total LC3B protein, followed by incubation with Alexa-Fluor 488 conjugated secondary antibody (#ab150077; 1:200; Abcam, Cambridge, UK). Cell nuclei were counterstained with Hoechst 33258 (Invitrogen, Carlsbad, CA). Sections were imaged using a confocal microscope (Olympus FV1000, Tokyo, Japan).

**Statistical Analysis**

Statistics were calculated using SPSS (SPSS Inc., Chicago, IL). All data are expressed as mean ± SEM. Data on escape latency in the MWM tests were assessed with two-way analysis of variance (ANOVA) for repeated measures (treatment condition × day). Data from the kinetics study of autophagic changes induced by 4 h of isoflurane were analyzed using one-way ANOVAs with least significant difference *post hoc* test. Other quantitative data from the two groups were tested by the independent samples *t*-test. Statistical significance was set at $P < 0.05$.

## RESULTS

**Effects of Different Durations of Isoflurane Exposure on Spatial Learning and Memory**

All animals swam normally. There was no significant difference in swimming speeds among different experimental conditions (data not shown). After a 1-h isoflurane exposure, repeated factor (day) ($F = 142.80$, $P < 0.01$), but not group factor (treatment) ($F = 1.13$, $P > 0.05$) significantly affected the escape latency, and no interaction was found ($F = 0.30$, $P > 0.05$). After a 4-h isoflurane exposure, comparison of rats that received isoflurane with their controls revealed a significant effect of days ($F = 42.90$, $P < 0.01$), but a non-significant effect of

groups ($F$ = 1.55, $P$ >0.05) and interaction between groups and days ($F$ = 1.01, $P$ >0.05). The reduced latency over the five daily sessions suggested that all rats were able to learn the task successfully. As shown in Fig. 1B, statistical analyses showed that on days 4 (33.62 ± 5.94 *vs* 17.73 ± 3.29 s, $n$ = 9, $P$ <0.05) and 5 (29.94 ± 4.82 *vs* 17.73 ± 2.73 s, $n$ = 9, $P$ <0.05), the 4-h isoflurane-exposed rats took longer to reach the platform than the sham-exposed rats. In the probe test, the time spent in the target quadrant by rats exposed to 1.5% isoflurane for 4 h was much shorter than that of control rats (22.33 ± 2.79 *vs* 41.10 ± 2.89 s, $n$ = 9, $P$ <0.05; Fig. 1D), thus validating the memory impairments after a 4-h isoflurane exposure. However, there were no significant differences in the time spent in the target quadrant between the rats treated with 1.5% isoflurane and vehicle gas for 1 h (45.67 ± 2.51 *vs* 43.29 ± 2.57 s, $n$ = 9, $P$ > 0.05; Fig. 1C).

### Effects of Different Durations of Isoflurane Exposure on Autophagy

Exposure to isoflurane for only 1 h had no effect on the hippocampal expression levels of LC3B-I, LC3B-II, beclin 1, and p62 at 24 h post-anesthesia compared with their controls (Fig. 2A, $P$ >0.05). Nevertheless, 24 h after a 4-h isoflurane exposure, quantitative analysis of western blots revealed a marked decrease in the expression levels of both LC3B-I and LC3B-II (Fig. 2B), which was consistent with the results from LC3B immunofluorescence staining (Fig. 3). In the hippocampal CA1 region, rats exposed to isoflurane for 4 h, but not 1 h, exhibited decreased staining for LC3B compared with vehicle-treated animals at 24 h after gas exposure. Beclin 1 was also significantly downregulated

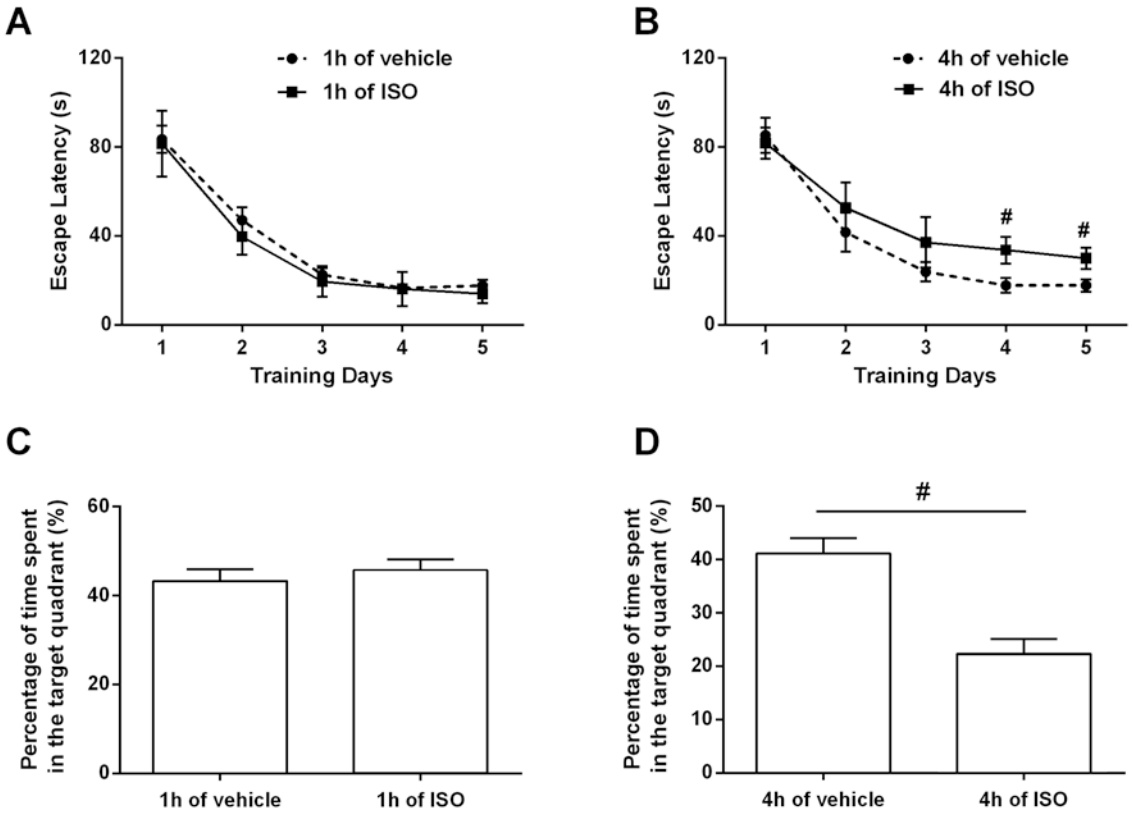


**Fig. 1. Effect of different durations of isoflurane exposure on spatial learning and memory in the Morris water maze test. The diagrams show the escape latency and the percentage of time spent in the previous platform quadrant after exposure to 1.5% isoflurane or vehicle gas for 1 or 4 h. A–B: Acquisition trial demonstrating the latency for rats to reach the platform, measuring spatial information acquisition. C–D: During the probe trial, the time spent in the target quadrant was calculated. Data are mean ± SEM; $^{\#}P$ <0.05 *vs* 4 h of vehicle ($n$ = 9). ISO, isoflurane.**

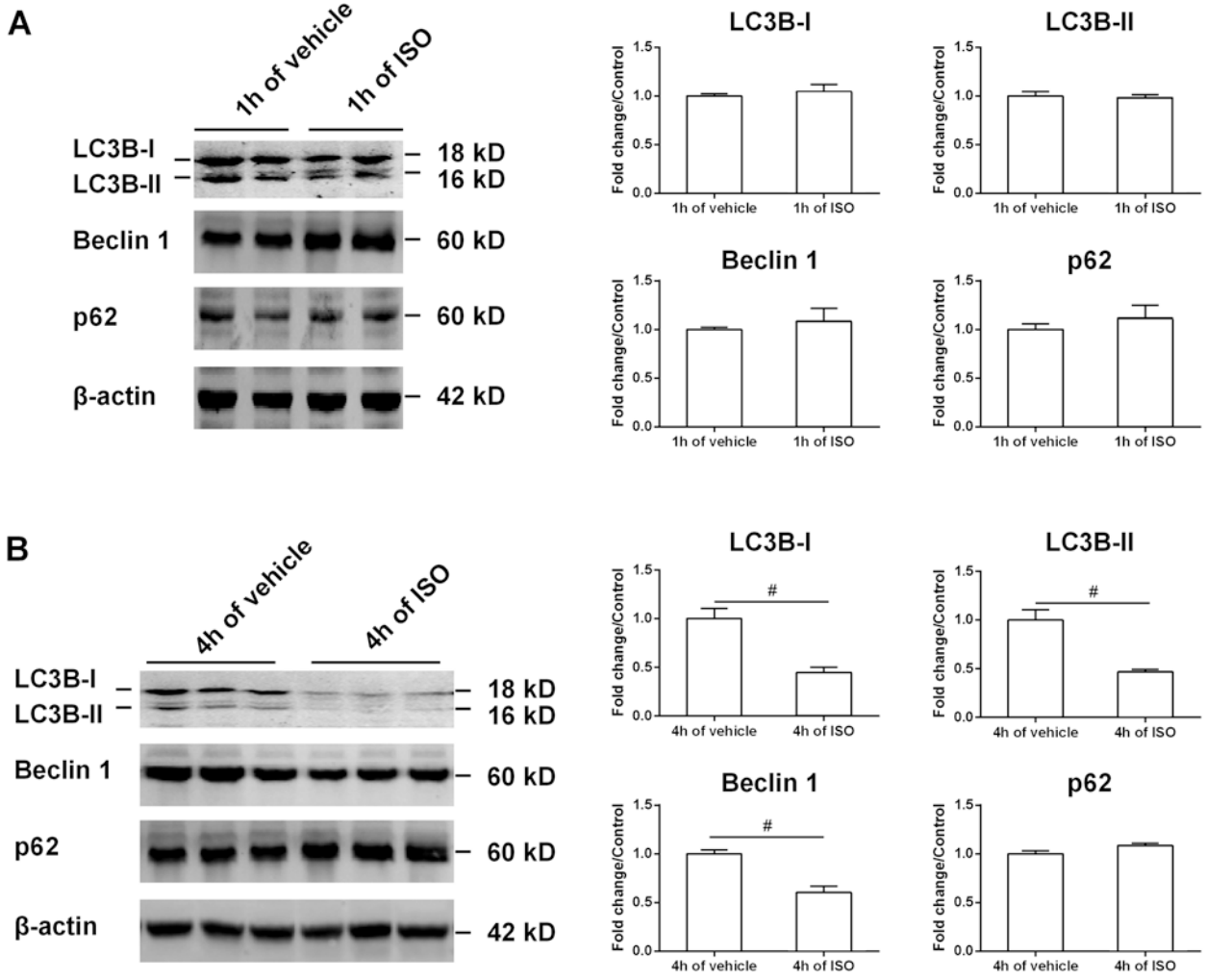


**Fig. 2. Effect of different durations of isoflurane exposure on the protein levels of autophagy markers in the hippocampus of aged rats at 24 h post-anesthesia. Left panels in A and B: western blots showing expression of LC3B and beclin 1, phagophore formation markers, and p62, an indicator of autophagic flux, in the hippocampus of aged rats after exposure to 1.5% isoflurane or vehicle gas for 1 h (A) or 4 h (B). Right panels in A and B: semi-quantitative data showing protein expression levels of LC3B-I, LC3B-II, beclin 1, and p62. Values are mean ± SEM of protein densities normalized to β-actin; $^{\#}P$ <0.05 *vs* 4 h of vehicle ($n$ = 4). ISO, isoflurane.**

in rats after a 4-h isoflurane exposure compared with controls (Fig. 2B, $P$ <0.01). No difference was found in the expression of p62 (Fig. 2B, $P$ >0.05).

### Kinetics of Autophagy Response Induced by 4 Hours of Isoflurane

Since exposure to isoflurane for 4 h, but not 1 h, impaired spatial learning and memory, we therefore further examined the dynamics of the hippocampal expression levels of beclin 1, LC3B, and p62 after a 4-h isoflurane exposure. The expression of both LC3B-I and LC3B-II significantly increased, first at the end of exposure, and then decreased within 12 to 24 h in rats exposed for 4 h compared with vehicle gas (Fig. 4C and D, $P$ <0.05). Similarly, compared with controls, beclin 1 expression increased immediately after isoflurane exposure and remarkably decreased 12 and 24 h after isoflurane (Fig. 4E, $P$ <0.05). p62 peaked at 6 h but subsequently resolved at 12 to 24 h after isoflurane (Fig. 4F, $P$ <0.05), suggesting that the defect in autophagic flux is temporary.

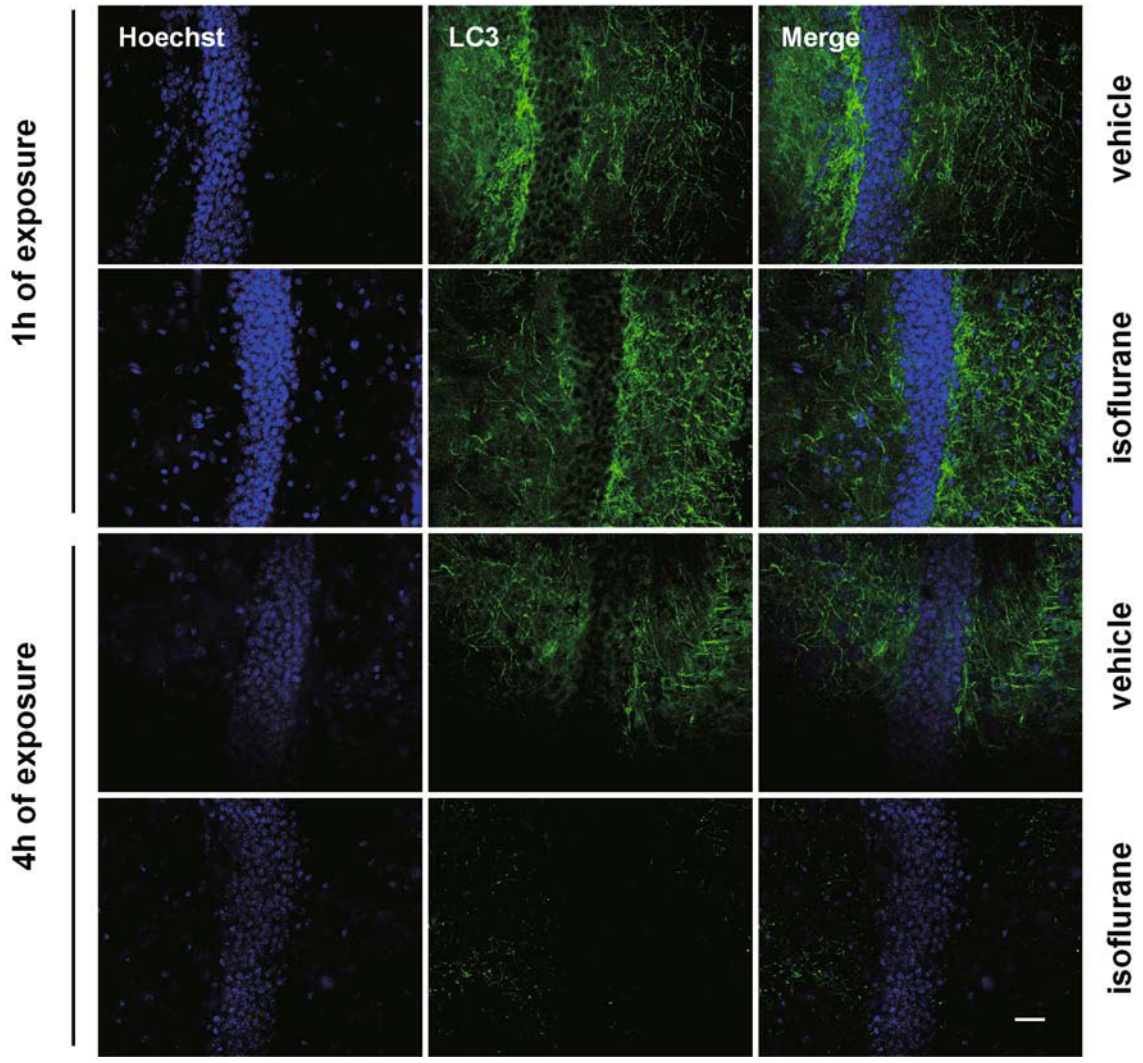


**Fig. 3. Representative confocal images of LC3 staining in the hippocampus of vehicle and isoflurane-treated animals. (A) Exposure to isoflurane for 1 h did not alter the expression of LC3 in the CA1 region. (B) Rats exposed to 4 h of isoflurane exhibited significantly decreased staining of LC3 compared with vehicle-treated animals in the CA1 region at 24 h after gas exposure (*n* = 4/group; scale bar, 50 μm).**

## DISCUSSION

### Longer but not Shorter Isoflurane Exposure Inhibits Phagophore Formation

Alteration of autophagy is a central feature common to various brain diseases such as AD, Pakinson's disease, and Huntington's disease[26], all of which are accompanied by cognitive dysfunction. However, the relationship between autophagy and isoflurane-induced acute cognitive dysfunction remains unknown. The current study therefore aimed at determining whether isoflurane could modulate the autophagic process in the aged brain. Here, we first found that isoflurane influenced autophagy in the hippocampus of aged rats and spatial cognitive function in a time-dependent manner. Specifically, exposure to 1.5% isoflurane for 4 h impaired spatial learning and memory, accompanied by impaired autophagy following a transient activation; however, exposure for only 1 h did not affect cognition and autophagy.

Strictly speaking, the direct effects of isoflurane on

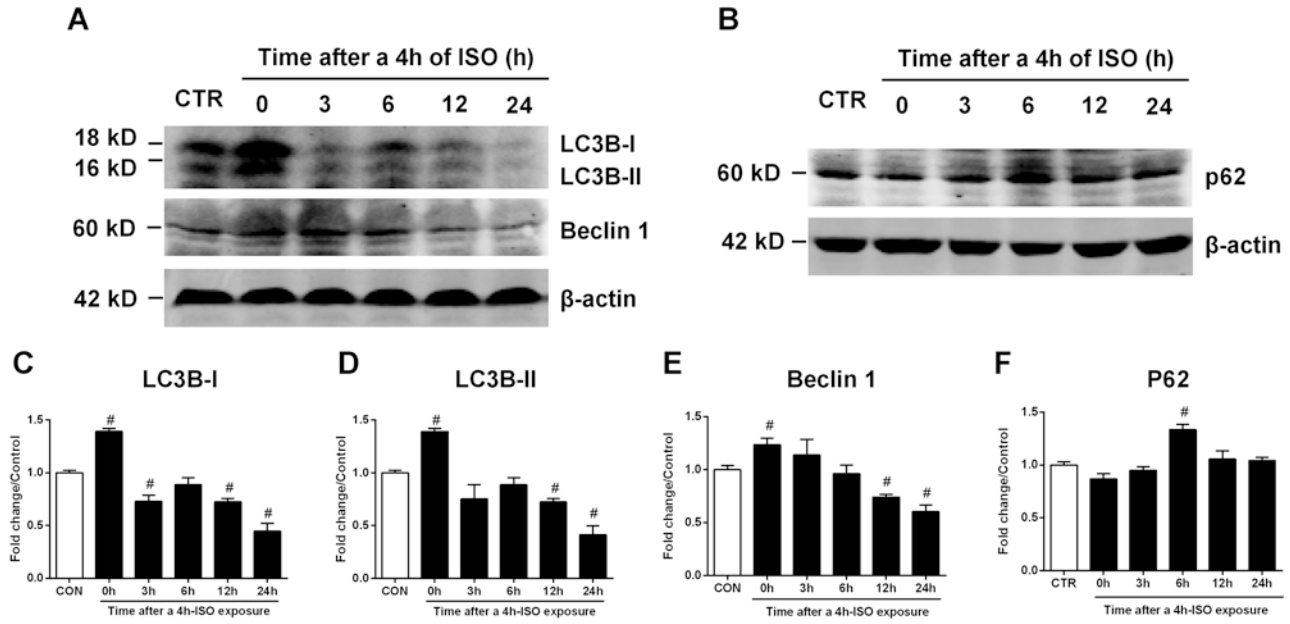


**Fig. 4. Kinetics of autophagy induced by 4 h of isoflurane exposure. A–B: Western blots showing expression of autophagic components LC3B, beclin 1, and p62 in the hippocampus of aged rats after exposure to 1.5% isoflurane or vehicle gas for 4 h. C–F: Semi-quantitative data showing protein expression levels of LC3B-I (C), LC3B-II (D), beclin 1 (E), and p62 (F). Values are mean ± SEM of protein densities normalized to β-actin; $^{\#}P$ <0.05 *vs* 4 h of vehicle ($n$ = 4). ISO, isoflurane.**

autophagy have been described in only two studies. At the peripheral level, Kashiwagi *et al.* reported that several anesthetics including isoflurane (1.5% for 2 h) lead to autophagy upregulation in skeletal muscle[27]. In the brain, Sheng *et al.* found that isoflurane preconditioning (2% for 0.5 h) up-regulates LC3 in primary cortical neurons at 24 h post-anesthesia, and that 1% isoflurane exposure for 3 h induces autophagy activation 24 h later in the cortex and striatum, but not in the hippocampus of adult mice[28]. On the contrary, in the present study, we found that exposure to 1.5% isoflurane for 4 h induced a decrease in autophagy at 24 h post-anesthesia, as evidenced by decreased LC3B and beclin 1, phagophore markers in the early stage of autophagy. This discrepancy could be due to the age of the animals as there is an age-dependent deficit of autophagy[29]. Furthermore, the protein level of p62, an autophagic flux marker, was not significantly changed, suggesting that a 4-h isoflurane exposure mainly inhibits phagophore formation.

Here, we did not find any of the autophagy markers (LC3B, beclin 1, and p62) to be significantly changed 24 h after a 1-h isoflurane exposure. These results indicate that a short isoflurane exposure may not be sufficient to induce autophagy in the aged hippocampus. These events were accompanied by unchanged spatial learning and memory in the MWM tests.

### Longer Isoflurane Exposure Induces Suppression of Autophagy Following Brief Activation

The kinetics study of autophagic changes induced by 4 h of isoflurane revealed that it suppressed autophagy following a transient activation, as evidenced by increased LC3B-II and beclin 1 immediately after isoflurane for 4 h, followed by decreased levels 12 h later. Taking the time-course pattern of the autophagy response into consideration, our results suggested that the whole process occurs in the aged hippocampus within 6 h after isoflurane. Support for this time-window comes from the increased p62 level at 6 h, which would be a consequence of the decreased autophagic flux that would result in inhibition of the degradation of p62 by lysosomes. Our results also suggested that the effect of modulating autophagy by isoflurane is context-dependent, and the level and duration of modulation should be fully considered. Since the

polyubiquitin-binding protein p62/SQSTM1 is degraded by autophagy[30], further investigation of ubiquitination levels may reinforce this hypothesis.

### Impaired Hippocampus-dependent Spatial Learning and Memory after Longer but not Shorter Isoflurane Exposure

Volatile anesthetics have long been considered neuroprotective. Nevertheless, available evidence has also suggested that there are growing concerns about potential neurotoxicity. Logically, it is easy to understand how volatile anesthetics can provide both neuroprotection and neurotoxicity. The most popular theory in this field is that almost all general anesthetics have dual effects, depending on the concentration and duration[32, 33]. Short exposure to general anesthetics can provide neuroprotection at low concentrations but become lethal stress factors at high concentrations for prolonged durations[32]. In accordance with this, we found that exposure to isoflurane for a longer duration (4 h) impaired hippocampus-dependent spatial learning and memory in aged rats. Nevertheless, we did not find any cognition-improving effects in the aged rats after a short (1 h) exposure. This does not deny the possibility of neuroprotection since there are conflicting findings in the literature regarding the effect of isoflurane on cognition. Two research groups reported improved cognitive performance in adult mice after exposure to 1 MAC isoflurane for 2 h[34,35]. In contrast, impaired cognitive performance after the same dose and duration of isoflurane has been reported in adult rats (4 months)[36] and in aged rats (18–20 months)[37]. Different methodologies, including animal age, strain, anesthetic carrier gas, outcome measurement time, or behavioral method may have contributed to these different findings.

### Summary

In conclusion, although time-dependent spatial cognitive changes concomitant with different autophagic responses were found in aged rats, our results did not establish an *in vivo* link between central autophagy and cognitive dysfunction in the aged hippocampus after isoflurane challenge. Further research is warranted to determine the functional relationship between impaired autophagy and cognitive dysfunction, as well as the crosstalk between autophagy, neuroinflammation, and Aβ accumulation in the aged brain induced by isoflurane.

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