The Common Inhalation Anesthetic Isoflurane Induces Caspase Activation and Increases Amyloid β-Protein Level In Vivo

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Objective: An estimated 200 million patients worldwide have surgery each year. Anesthesia and surgery have been reported to facilitate emergence of Alzheimer’s disease. The commonly used inhalation anesthetic isoflurane has previously been reported to induce apoptosis, and to increase levels and aggregation of Alzheimer’s disease–associated amyloid β-protein (Aβ) in cultured cells. However, the in vivo relevance has not been addressed.

Methods: We therefore set out to determine effects of isoflurane on caspase activation and levels of β-site amyloid precursor protein–cleaving enzyme (BACE) and Aβ in naive mice, using Western blot, immunohistochemistry, and reverse transcriptase polymerase chain reaction.

Results: Here we show for the first time that a clinically relevant isoflurane anesthesia (1.4% isoflurane for 2 hours) leads to caspase activation and modest increases in levels of BACE 6 hours after anesthesia in mouse brain. Isoflurane anesthesia induces caspase activation, and increases levels of BACE and Aβ up to 24 hours after anesthesia. Isoflurane may increase BACE levels by reducing BACE degradation. Moreover, the Aβ aggregation inhibitor, clioquinol, was able to attenuate isoflurane-induced caspase-3 activation in vivo.

Interpretation: Given that transient insults to brain may lead to long-term brain damage, these findings suggest that isoflurane may promote Alzheimer’s disease neuropathogenesis and, as such, have implications for use of isoflurane in humans, pending human study confirmation.
months) APP and Aβ deposits in brain area distant from the ischemic region.12 These findings suggest that a transient insult, for example, ischemia or anesthesia with isoflurane, could lead to secondary and persistent brain injuries. Perioperative factors, including hypcapnia13 and anesthetics,14–17 have been reported to potentially contribute to AD neuropathogenesis. Previous in vitro studies have shown that the inhalation anesthetic isoflurane can induce apoptosis, which, in turn, increases BACE activity and Aβ generation.15,17 Our recent in vitro studies have shown that isoflurane-induced apoptosis may be dependent on cytosolic calcium levels and can be attenuated by the N-methyl-D-aspartate receptor antagonist memantine,18 whereas desflurane induces only caspase-3 activation and enhances Aβ levels under hypoxic condition.19 The in vivo relevance of these effects, however, has not yet been determined. We therefore set out to assess effects of isoflurane on caspase activation and levels of BACE and Aβ in mouse brain. We also studied effects of inhibition of Aβ aggregation on isoflurane-induced caspase-3 activation in mouse brain.

Materials and Methods

Mice Anesthesia and Treatment

The animal protocol was approved by Standing Committee on Animals at Massachusetts General Hospital. C57/BL6 mice (Jackson Laboratory, Bar Harbor, ME) were randomly assigned to an anesthesia or control group. Mice randomized to the anesthesia group received 1.4% isoflurane in 100% oxygen for 2 hours in an anesthetizing chamber, whereas the control group received 100% oxygen at an identical flow rate for 2 hours in an identical chamber. The mice breathed spontaneously, and anesthetic and oxygen concentrations were measured continuously (Datex, Tewksbury, MA). Temperature of the anesthetizing chamber was controlled to maintain rectal temperature of the animals at 37 ± 0.5°C. Mean arterial blood pressure was measured noninvasively using a tail cuff (Kent Scientific Corporation, Torrington, CT) in the anesthetized mice. Isoflurane anesthesia did not significantly affect blood pressure and blood gas of mice (see supplemental data). Anesthesia was terminated by discontinuing isoflurane and placing animals in a chamber containing 100% oxygen until 20 minutes after return of righting reflex. They were then returned to individual home cages until death. Mice were euthanized by decapitation, 2, 6, 12, and 24 hours after isoflurane anesthesia. The brain was removed rapidly, and prefrontal cortex was dissected out and frozen in liquid nitrogen for subsequent processing for determinations of caspase activation and levels of BACE and Aβ. For interaction studies, ciprofloxin (CQ; 30mg/kg/day, in 0.05% carboxymethylcellulose sodium) was given by daily gavage for 7 days.20 Then mice were treated with 1.4% isoflurane for 2 hours and killed 6 hours after administration of anesthesia.

Brain Tissue Lysis and Protein Amount Quantification

The harvested brain tissues were homogenized on ice using immunoprecipitation buffer (10mM tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 7.4, 150mM NaCl, 2mM EDTA, 0.5% Nonidet P-40) plus protease inhibitors (1μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstatin A). The lysates were collected, centrifuged at 12,000 rpm for 10 minutes, and quantified for total proteins by BCA protein assay kit (Pierce, Iselin, NJ).

Western Blots Analysis

The brain tissues were harvested and subjected to Western blots analyses as Xie and colleagues15 described. A caspase-3 antibody (1:1,000 dilution; Cell Signaling Technology, Danvers, MA) was used to recognize caspase-3 fragment (17–20kDa) resulting from cleavage at aspartate position 175 and full-length (FL) caspase-3 (35–40kDa). Polyadenosine diphosphate ribose polymerase (PARP) antibody (1:1,000; Cell Signaling Technology) was used to recognize PARP fragment (85kDa). Rabbit polyclonal anti–BACE-1 antibody (1:1,000; Abcam, Cambridge, MA) was used to detect protein levels of BACE (65kDa). Antibody anti-β-actin (1:2,000; Sigma, St. Louis, MO) was used to visualize β-actin (42kDa). Quantification of Western blots was performed as Xie and colleagues15 described. In brief, intensity of signals was analyzed by using a Bio-Rad (Hercules, CA) image program (Quantity One). We quantified Western blots using two steps. First, we used levels of β-actin to normalize (eg, determining ratio of FL caspase-3 amount to β-actin amount) levels of proteins to control for loading differences in total protein amounts. Second, we presented changes in levels of proteins in the mice treated with isoflurane as the percentage of those in the mice treated with controls. One hundred percent of change in protein levels in this article refer to control levels for comparison with experimental conditions.

Immunohistochemistry

Mice were anesthetized with isoflurane briefly and perfused transcardially with heparinized saline followed by 4% paraformaldehyde in 0.1M phosphate buffer with pH 7.4. Mouse brain tissues were removed and kept at 4°C in phosphate-buffered saline for 5 minutes each. The sections were then incubated with primary antibodies at 4°C for 64 hours and washed three times in phosphate-buffered saline for 5 minutes each. Sections were then incubated with secondary antibodies at room temperature for 60 minutes. The slides were then washed in 0.1M phosphate buffer with pH 7.4 for 6 minutes and air dried. The slides were mounted with VectorMount (Vector Laboratories, Burlingame, CA) and were observed using a Leica RM2255 microtome and mounted onto superfrost plus glass slides (Fisher Scientific, Pittsburgh, PA). The sections were then deparaffinized and hydrated in three washes of xylene and two washes of ethanol (100, 95, 80, and 70%) and phosphate-buffered saline for 5 minutes each. The slides were then rehydrated in water for 10 minutes to unmask antigen, and then put on benchtop for another 30 minutes of cooling. Sections were then incubated for 10 minutes in 3% hydrogen peroxide to block endogenous peroxidase and then incubated for 1 hour in blocking solution (goat normal serum; Vector Lab, Burlingame, CA). The sections were then incubated with primary antibodies (1:100; Cell Signaling Technology) overnight in 4°C. The sections were washed three times in phosphate-buffered sa-
line with 0.1% Tween 20 in room temperature. Then biotinylated secondary antibody (1:200; Vector Lab) and avidin-biotin-peroxidase complex (Vector Lab) were incubated with the sections. The sections were washed with wash buffer phosphate-buffered saline with Tween 20. The sections were then incubated in diaminobenzidine tetrahydrochloride (DAB) working solution (DAB Substrate Kit for Peroxidase; Vector Lab) for peroxidase reaction. Finally, the sections were dehydrated through a gradient of ethanol solutions (70–100%) and covered with a coverslip.

**Immunoblot Detection of Amyloid β-Protein**

Brain samples were homogenized (150mM NaCl with protease inhibitor cocktail in 50mM Tris, pH 8.0) and centrifuged (300,000*g for 45 minutes), and the supernatant was removed. The pellet was then resuspended by sonication and incubated for 15 minutes in homogenization buffer containing 1% sodium dodecyl sulfate. After pelleting of insoluble material (16,000*g for 15 minutes), the sodium dodecyl sulfate extract was electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (4–12% Bis-Tris polyacrylamide gel; Invitrogen, Carlsbad, CA), blotted to polyvinyl difluoride membrane, and probed with a 1:200 dilution of 6E10 (Signet, Berkeley, CA) (see supplemental data).

**Reverse Transcriptase Polymerase Chain Reaction**

We extracted RNA as described in the protocol of Qiagen Rneasy mini kit (Valencia, CA). We determined RNA concentration using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). We designed and obtained primers of BACE from Qiagen. We used SYBR Green I fluorescent dye (Sigma, St. Louis, MO) to detect amount of complementary DNA and express the amount of complementary DNA as cycle time (the time when fluorescence from the dye bound to complementary DNA begins to be detected). The cycle times then were converted to the amount of messenger RNA using a standard curve.

**Statistics**

Data were expressed as means ± standard deviation. The number of samples varied from three to ten, and the samples were normally distributed. We used a two-tailed *t* test to compare differences between experimental groups. *p* values less than 0.05 were considered statistically significant.

**Results**

**Isoflurane Induces Caspase Activation in Mouse Brain**

Naive mice were subjected to anesthesia with 1.4% isoflurane for 2 hours. The mice exhibited no significant effects on blood pressure or blood gas (see supplemental data). Next, we assessed effects of isoflurane anesthesia on apoptosis-related caspase-3 activation and PARP cleavage using Western blot analyses of caspase-3 and PARP cleavage. Anesthesia with 1.4% isoflurane for 2 hours led to caspase-3 activation as evidenced by increased ratios of cleaved (activated) caspase-3 fragment to FL caspase-3 (Fig 1A) and increased levels of the caspase-generated PARP fragment (see Fig 1C). Quantification of these results, based on ratios of caspase-3 fragment to FL caspase-3 and levels of PARP fragment, demonstrated that isoflurane anesthesia led to 157% (see Fig 1B; *p* = 0.016) and 141% (see Fig 1D; *p* = 0.021) increases in cleavages of caspase-3 and PARP, respectively, as compared with control condition. Caspase-3 immunohistochemistry showed that isoflurane anesthesia increased activated caspase-3–positive cells in cerebral cortex of mice as compared with control condition, 6 hours after anesthesia (see Fig 1E). Quantification of the immunohistochemistry sections showed that isoflurane anesthesia led to a 170% increase in activated caspase-3–positive cells as compared with control condition (see Fig 1F; *p* = 0.011). These results suggest that isoflurane can induce caspase activation in brain tissues of naive mice after 6 hours. However, isoflurane anesthesia did not induce caspase activation at either 2 or 24 hours after isoflurane anesthesia (data not shown). It is possible that at 2 hours after anesthesia is too early for isoflurane to induce caspase activation, and that the isoflurane-induced caspase activation does not last long, for example, 24 or 48 hours after the anesthesia, because of rapid clearance of apoptotic cells in mouse brain.

**Isoflurane Increases Levels of β-Site Amyloid Precursor Protein–Cleaving Enzyme and Amyloid β-Protein**

We then assessed whether isoflurane increases BACE and Aβ levels in mouse brain after anesthesia. Western blot analyses demonstrated increased levels of BACE (see Fig 1G) 6 hours after isoflurane anesthesia as compared with control condition. Quantification of the Western blots, normalized to β-actin, showed that isoflurane anesthesia led to a 155% (see Fig 1H; *p* = 0.025) increase in BACE levels after 6 hours, as compared with control condition.

We next asked whether isoflurane can increase Aβ levels subsequent to caspase activation and increases in BACE levels. For this purpose, we conducted a time course of the effects of isoflurane on caspase activation and levels of BACE and Aβ. Caspase-3 immunoblotting demonstrated that anesthesia with 1.4% isoflurane for 2 hours yielded visible reductions in levels of FL caspase-3 (Fig 2A) 12 hours after anesthesia; quantification of the results showed that isoflurane anesthesia led to a 23% reduction in FL caspase-3 levels (see Fig 2B; *p* = 0.047). Whereas we did not detect cleaved caspase-3 fragment in mouse brain, we were able to show that isoflurane anesthesia increased levels of caspase-cleaved APP N-terminal fragment (see Figs 2C, D; a 133% increase; *p* = 0.030) 12 hours after isoflurane anesthesia. These results suggest that 12 hours after anesthesia, isoflurane can still cause caspase activation, but to only a moderate degree. Isoflurane
anesthesia also significantly increased BACE levels as compared with control condition after 12 hours (see Figs 2E, F; a 214% increase; \( p < 0.031 \)). However, we were not able to observe detectable increases in \( \text{A}\beta \) levels 12 hours after isoflurane anesthesia (data not shown).

Given that isoflurane induced caspase activation and increased BACE levels in the absence of detectable increases in \( \text{A}\beta \) levels 6 to 12 hours after isoflurane anesthesia, we next assessed the effects of anesthesia with 1.4% isoflurane for 2 hours on BACE and \( \text{A}\beta \) levels 24 hours after anesthesia. Isoflurane anesthesia no longer induced caspase activation in mouse brain 24 hours after anesthesia (data not shown). However, isoflurane anesthesia robustly increased BACE levels (Fig 3A) in mouse brain 24 hours after anesthesia;
Fig 2. Anesthesia with 1.4% isoflurane for 2 hours induces caspase activation and increases β-site amyloid precursor protein (APP)–cleaving enzyme (BACE) levels 12 hours after anesthesia. (A) Isoflurane anesthesia (lanes 4–7) decreases full-length (FL) caspase-3 levels as compared with control condition (lanes 1–3) in Western blots. (B) Quantification of the Western blots shows that isoflurane anesthesia (black bar) reduces FL caspase-3 levels as compared with control condition (white bar). (C) Isoflurane anesthesia (lanes 3 and 4) increases protein levels of APP-N-caspase fragment as compared with control condition (lanes 1 and 2) in Western blots. (D) Quantification of the Western blots shows that isoflurane anesthesia (black bar) increases ratio of APP-N-caspase fragment to FL APP as compared with control condition (white bar). (E) Isoflurane anesthesia (lanes 4–7) increases BACE levels compared with control condition (lanes 1–3) in Western blots. Synthetic BACE was used as a marker to identify position of BACE in Western blot. (F) Quantification of the Western blot shows that isoflurane anesthesia (black bar) increases BACE levels as compared with control condition (white bar). There is no significant difference in amounts of β-actin in control condition- or isoflurane-treated mouse brain tissue. Data are means ± standard deviation. n = 3 to 10 for each experimental group. t test was used to compare difference between control condition and isoflurane anesthesia, *p < 0.05.
quantification of the results showed that isoflurane anesthesia led to a 412% increase in BACE levels (see Fig 3B; \( p = 0.015 \)) 24 hours after anesthesia. Isoflurane anesthesia also increased Aβ levels in mouse brain 24 hours after anesthesia (see Fig 3C); quantification of these data by Western blot analysis showed that isoflurane anesthesia caused a 145% increase in Aβ levels (see Fig 3D; \( p = 0.023 \)) in mouse brain 24 hours after anesthesia.

**Clioquinol Attenuates Isoflurane-Induced Caspase-3 Activation**

We next tested whether the metal protein attenuation compound, clioquinol (CQ), which is known to inhibit Aβ aggregation, \(^{20}\) could reduce isoflurane-induced caspase-3 activation in mouse brain. CQ (30mg/kg/day, in 0.05% carboxymethylcellulose sodium) was given by daily gavage for 7 days. \(^{20}\) The mice were then anesthetized with 1.4% isoflurane for 2 hours and euthanized 6 hours after the anesthesia. Pretreatment with CQ before isoflurane anesthesia was able to significantly reduce caspase-3 activation (Figs 4A, B; 247 vs 137%; \( p = 0.031 \)). These findings suggest that isoflurane may at least partially induce caspase activation via Aβ aggregation given that Aβ aggregates have previously been shown to induce apoptosis, and isoflurane can enhance Aβ oligomerization and potentiate Aβ-induced cytotoxicity. \(^{13}\)
Isoflurane Reduces GGA-3 Levels

A recent study10 showed that ischemia-induced caspase activation can reduce levels of the Golgi-associated, gamma adaptin ear–containing, ARF-binding protein 3 (GGA-3), a protein involved in BACE degradation, leading to accumulation of BACE. Given isoflurane can enhance BACE levels, we asked whether isoflurane can reduce GGA-3 levels. We were able to show that anesthesia with 1.4% isoflurane for 2 hours decreased GGA-3 levels as compared with control condition in naive mice (Figs 5A, B). Reverse transcriptase polymerase chain reaction assay showed that isoflurane did not increase BACE messenger RNA levels (see Fig 5C). Collectively, these results suggest that isoflurane may increase BACE levels by reducing BACE degradation, rather than by increasing BACE generation.

Discussion

We have previously shown that the commonly used inhalation anesthetic isoflurane can induce cellular apoptosis and increase Aβ generation in human neuroglioma cells.15–17 Here, we set out to determine the in vivo relevance of these effects in naive mice. We have shown for the first time that a clinically relevant concentration of isoflurane can induce caspase activation 6 and 12 hours after the isoflurane anesthesia, increase level of BACE 6, 12, and 24 hours after the isoflurane anesthesia, and finally enhance Aβ levels 24 hours after the isoflurane anesthesia. These findings suggest that anesthesia with 1.4% isoflurane, analogous to the concentration used in clinical settings, induces a time-dependent cascade of caspase activation, increased BACE levels, and increased Aβ levels, most likely because of enhanced BACE cleavage of APP.

Our findings are the first in vivo studies illustrating that isoflurane can yield a time-dependent effect in inducing apoptosis and enhancing levels of both BACE and Aβ. All previously reported studies on these key features of Alzheimer’s pathology, including our own, have been in vitro studies. This is the first confirmation study that the phenomena are relevant to the brains of living animals.

The mechanism by which isoflurane enhances levels of BACE and Aβ in mouse brain is likely to be similar to previously described effects of ischemia on caspase activation, BACE stabilization, and Aβ generation in rodent models.10 This study showed that caspase activation can reduce levels of the GGA-3, a protein involved in BACE degradation.10 We have found that isoflurane also reduced GGA-3 levels but does not increase BACE messenger RNA levels. Thus, according to the ischemia model, we postulate that isoflurane anesthesia initially induces caspase activation, which would reduce GGA-3 levels at relatively short time intervals (eg, 6 hours) after anesthesia. Reduced GGA-3 levels would then lead to attenuated BACE degradation, leading to accumulation of BACE and increased β-secretase activity at later time intervals (eg, 12 hours). Finally, the enhanced β-secretase activity would promote amyloidogenic processing of APP and increase Aβ levels at later time intervals (eg, 24 hours).

Recent studies22 have shown that anesthesia with 2 hours of 0.9 to 1.0% isoflurane daily for 5 days impaired cognition functions in naive mice; however, it did not further enhance cognition decline in Tg2576 mice. In addition, isoflurane anesthesia did not increase brain plaque density or activated caspase-3–positive cells (per immunohistochemistry analysis) in brain tissue of both naive and Tg2576 mice 11 to 14 days after the anesthesia. Given rapid clearance of apoptotic markers from brain tissue, it was likely too late to detect caspase-3 activation 11 to 14 days after the isoflurane anesthesia.
rane anesthesia. Moreover, repeated isoflurane exposures may cause preconditioning effects, which can attenuate potential isoflurane-induced neurotoxicity. However, our results have illustrated that a single clinically relevant isoflurane exposure can induce caspase activation and increase Aβ levels in naive mice after only 2 hours of exposure. Thus, isoflurane-induced caspase activation and Aβ increase are most likely dose and time dependent.

It is also possible that the increase in protein levels of BACE and Aβ in mouse brain after isoflurane anesthesia could be caused by other mechanisms. Velliquette and colleagues reported that insulin, 2-deoxyglucose, 3-nitropropionic acid, and kainic acid can induce acute energy inhibition to enhance levels of BACE and Aβ in wild-type and AD transgenic (Tg2576) mice. Isoflurane is a profound cerebral metabolic depressant and decreases glucose utilization in rats. Therefore, isoflurane may affect APP processing and increase Aβ accumulation via energy inhibition.

Fig 5. Anesthesia with 1.4% isoflurane for 2 hours reduces Golgi-associated, gamma adaptin ear-containing ARF-binding protein 3 (GGA-3) levels without alterations in β-site amyloid precursor protein (APP)-cleaving enzyme (BACE) messenger RNA (mRNA) levels. (A) Isoflurane anesthesia (lanes 4–6) reduces GGA-3 levels as compared with control condition (lanes 1–3) in Western blots. (B) Quantification of the Western blots shows that isoflurane anesthesia (black bar) reduces GGA-3 levels as compared with control condition (white bar). (C) Isoflurane dose not alter BACE mRNA levels as compared with control condition. Complementary DNA was detected by a SYBR Green I fluorescent dye and were represented as cycle time (the time when amounts of fluorescence begin to be measured). The cycle times were then converted to mRNA amount (inset) using a standard curve of cycle times and mRNA amount (data not shown). PCR = polymerase chain reaction.
Future studies will be necessary to determine whether isoflurane-induced increase in levels of BACE and Aβ is dependent on isoflurane-induced changes in glucose utilization or GGA-3 levels.

Isoflurane has previously been shown to enhance Aβ aggregation and potentiate the cytotoxicity of Aβ. It has been reported that oligomeric and fibrillar species of Aβ are more neurotoxic. We have previously shown that clioquinol, a MPAC, can attenuate isoflurane-induced apoptosis in cultured cells. In our experiments, we found that CQ was able to attenuate the 1.4% isoflurane-induced caspase-3 activation in mice. Collectively, these findings suggest that isoflurane-induced apoptosis can be potentiated by Aβ aggregation. Moreover, our results suggest that pre-treatment of patients with CQ before isoflurane anesthesia may be effective in attenuating isoflurane-induced caspase activation and potential neurotoxicity, pending further studies.

van Groen and coworkers study showed that an insult from a 2-hour occlusion of the middle cerebral artery increased levels of APP and Aβ in axons at corpus callosum and in neurons at the border of the ischemic region. Moreover, this transient insult caused persistent APP and Aβ deposits in thalamic nuclei (ventroposterior lateral and ventroposterior medial nuclei), which eventually developed to dense plaquelike deposits 9 months after the initial insult. This secondary and persistent brain harm could be caused by axonal damage of thalamic neurons leading to retrograde degeneration, damage from vasogenic edema and some noxious substance, or hypometabolism. Both isoflurane and brain ischemia have been shown to induce caspase activation and apoptosis, which then enhance levels and activities of BACE to facilitate APP processing and to increase Aβ generation. Thus, we have postulated that the treatment with 1.4% isoflurane for 2 hours can also induce not only transient injuries (eg, caspase activation and apoptosis, increases in levels of BACE and Aβ) but also persistent damage (eg, APP and Aβ deposits) in brains. The future studies will include determining long-term effects of isoflurane on AD neuropathogenesis in mouse brain tissue to test this hypothesis. The future studies should also include assessing the downstream consequences of isoflurane-induced apoptosis and Aβ generation, for example, determination of the effects of isoflurane-induced apoptosis and Aβ generation on N-methyl-D-aspartate receptor endocytosis, mitochondrial abnormalities, and free radical production.

Even though our in vitro and limited in vivo studies together with the findings from other studies suggest that isoflurane may affect AD neuropathogenesis, it is necessary to perform further determination of the in vivo relevance of these effects, especially confirmation studies in humans, before we can conclude that the inhalation anesthetic isoflurane promotes AD neuropathogenesis.

In conclusion, we have found that isoflurane can induce caspase activation and increase levels of BACE and Aβ in naive mice, which may lead to secondary and persistent brain damage. These findings raise novel concerns regarding the use of isoflurane, a commonly used anesthetic, in individuals with increased Aβ burden, including patients with AD, Down’s syndrome, and β-amyloid angiopathy. A similar concern may also apply to unaffected carriers of APP or presenilin gene mutations, and the late-onset AD risk factor, apolipoprotein E ε4, which increase Aβ accumulation in the brain. In addition, these findings may shed light on the mechanism by which anesthesia increases risk for postoperative cognitive dysfunction, a dementia associated with surgery and anesthesia. These studies should ultimately facilitate design of safer anesthetics and provision of better anesthesia care to patients, especially senior patients, who are particularly susceptible to the incidence of postoperative cognitive dysfunction and risk for AD.

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