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The common inhalation anesthetic isoflurane induces caspase activation and increases A β level *in vivo*

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Abstract

Objective—An estimated 200 million patients worldwide have surgery each year. Anesthesia and surgery have been reported to facilitate emergence of Alzheimer's disease (AD). The commonly used inhalation anesthetic isoflurane has previously been reported to induce apoptosis and to increase levels and aggregation of AD-associated amyloid β -protein (A β) 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, levels of BACE and A β in naïve mice, employing Western blot, immunohistochemistry and RT-PCR.

Results—Here we show for the first time that a clinically relevant isoflurane anesthesia (1.4% isoflurane for two hours) leads to caspase activation and modest increases in levels of the β -site APP-cleaving enzyme (BACE) six hours after anesthesia in mouse brain. Isoflurane anesthesia induces caspase activation, increases levels of BACE and A β up to 24 hours after anesthesia. Isoflurane may increase BACE levels by reducing BACE degradation. Moreover, the A β 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 AD neuropathogenesis and, as such, have implications for use of isoflurane in humans, pending on human study confirmation.

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Introduction

An estimated 200 million patients worldwide undergo surgery each year. Several studies showed the potential association of previous general anesthesia/surgery and development of Alzheimer's disease (AD) 1-2. An earlier study suggested that age of onset of AD was inversely related to anesthesia exposure before age 50 1. A recent study also reported that patients undergoing coronary artery bypass graft surgery under general anesthesia were at increased risk for AD as compared to those having percutaneous transluminal coronary angioplasty under local anesthesia 3.

The excessive accumulation of amyloid β -protein ($A\beta$) in the brain is a major pathological feature of AD [4; reviewed in 5]. $A\beta$ is produced via serial proteolysis of the amyloid precursor protein (APP) by the aspartyl protease β -site APP-cleaving enzyme (BACE) or β -secretase 6, and γ -secretase 7-9. Increasing evidence has revealed a role for caspase activation and apoptotic cell death in AD [Tesco, 2007 #1313]; reviewed in 10].

A recent study showed that an insult from a middle cerebral artery occlusion for two hours in rats caused temporary increases in APP and $A\beta$ staining in brain area near ischemic region, as well as long term (up to 9 months) APP and $A\beta$ deposits in brain area distant from the ischemic region 11. These findings suggest that a transient insult, e.g., ischemia or anesthesia with isoflurane, could lead to secondary and persistent brain injuries. Perioperative factors, including hypocapnia 12 and anesthetics 13-16, 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 $A\beta$ generation 14-16. Our recent *in vitro* studies have shown that isoflurane-induced apoptosis may be dependent on cytosolic calcium levels and can be attenuated by the NMDA receptor antagonist memantine 17, while desflurane only induces caspase-3 activation and enhances $A\beta$ levels under hypoxic condition 18. 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, levels of BACE and $A\beta$ in mouse brain. We also studied effects of inhibition of $A\beta$ aggregation on isoflurane-induced caspase-3 activation in mouse brain.

Experimental Procedures

Mice anesthesia and treatment

The animal protocol was approved by Standing Committee on Animals at Massachusetts General Hospital. C57/BL6 mice (The 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 $37 \pm 0.5^\circ\text{C}$. Mean arterial blood pressure was measured non-invasively 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 (Data not shown). 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 sacrifice. Mice were sacrificed by decapitation two, six, 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, levels of BACE and $A\beta$. For interaction studies, CQ (30mg/kg/day, in 0.05%

carboxymethylcellulose sodium) was given by daily gavage for 7 days 19. Then mice were treated with 1.4% isoflurane for 2 hours, and were sacrificed six hours after the anesthesia.

Brain tissue lysis and protein amount quantification

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

Western blots analysis

The brain tissues were harvested and were subjected to Western blots analyses as described by Xie et al. 14. A caspase-3 antibody (1:1,000 dilution; Cell Signaling Technology, Inc. Danvers, MA) was used to recognize caspase-3 fragment (17-20 kDa) resulting from cleavage at aspartate position 175 and FL-caspase-3 (35 - 40 kDa). Poly-ADP ribose polymerase (PARP) antibody (1:1,000; Cell Signaling Technology) was used to recognize PARP fragment (85 kDa). Rabbit polyclonal anti-BACE-1 antibody (1:1,000, Abcam, Cambridge, MA) was used to detect protein levels of BACE (65 kDa). Antibody anti-β-actin (1:2,000, Sigma) was used to visualize β-actin (42 kDa). Quantification of Western blots was performed as described by Xie et al. 14. Briefly, 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 (e.g., 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. 100% of changes in protein levels in manuscript refer to control levels for the purpose of comparison to 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 of 7.4. Mouse brain tissues were removed and kept at 4 degrees C in paraformaldehyde. For activated caspase-3 staining, sections were cut in paraffin blocks at 5 µm intervals 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 PBS for 5 minutes each. Sections were brought to a boil in 10 mM sodium citrate buffer with pH of 6.0, maintained at a sub-boiling temperature for 10 minutes to unmask antigen, and then put on bench top for another 30 minutes cooling. Sections were quenched for 10 minutes in 3% hydrogen peroxide to block endogenous peroxidase and then incubated for one hour in blocking solution (Goat normal serum, Vector Lab, Burlingame, CA). The sections were then incubated with cleaved caspase-3 antibody (1:100, Cell Signaling Technology Inc.) overnight in 4 degrees C. The sections were washed three times in PBS with 0.1% Tween-20 in room temperature. Then biotinylated secondary antibody (1:200, Vector Lab, Burlingame, CA) and avidin-biotin-peroxidase complex (Vector Lab) were incubated with the sections. The sections were washed with wash buffer PBST. The sections were then incubated in 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 cover slip.

Immunoblot detection of A β

Brain samples were homogenized (150 mM NaCl with protease inhibitor cocktail in 50 mM Tris, pH of 8.0) and centrifuged ($300,000g \times 45$ min), and the supernatant was removed. The pellet was then resuspended by sonication and incubated for 15 min in homogenization buffer containing 1% SDS. Following pelleting of insoluble material ($16,000g \times 15$ min), the SDS-extract was electrophoresed on SDS-PAGE (4-12% Bis-Tris polyacrylamide gel from Invitrogen, Carlsbad, CA), blotted to PVDF membrane and probed with a 1:200 dilution of 6E10 (Signet, Berkeley, CA) (See Supplemental Data).

RT-PCR

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 cDNA and express the amount of cDNA as cycle time (CT, the time when fluorescence from the dye bound to cDNA begins to be detected). The CT then were converted to the amount of mRNA using a standard curve.

Statistics

Data were expressed as mean $\pm$ S.D.. The number of samples varied from 3 to 6, 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 (* or #) were considered statistically significant.

Results

Isoflurane induces caspase activation in mouse brain

Naïve mice were subjected to anesthesia with 1.4% isoflurane for two hours. The mice exhibited no significant effects on blood pressure or blood gas (supplemental data). Next, we assessed effects of isoflurane anesthesia on apoptosis-related caspase-3 activation and Poly ADP ribose polymerase (PARP) cleavage using quantitative Western blots analyses of caspase-3 and PARP cleavage. Anesthesia with 1.4% isoflurane for two hours led to caspase-3 activation as evidenced by increased ratios of cleaved (activated) caspase-3 fragment to full-length (FL)-caspase-3 (Figure 1a), and increased levels of the caspase-generated PARP fragment (Figure 1c). Quantification of these results, based on ratios of caspase-3 fragment to FL-caspase-3 and levels of PARP fragment, revealed that isoflurane anesthesia led to 157% (Figure 1b, $P = 0.016$) and 141% (Figure 1d, $P = 0.021$) increases in cleavages of caspase-3 and PARP, respectively, as compared to control condition. Caspase-3 immunohistochemistry showed that isoflurane anesthesia increased activated caspase-3 positive cells in cerebral cortex of mice as compared to control condition, six hours after anesthesia (Figure 1e). Quantification of the immunohistochemistry sections showed that isoflurane anesthesia led to a 170% increase in activated caspase-3 positive cells as compared to control condition (Figure 1f; $P = 0.011$). These results suggest that isoflurane can induce caspase activation in brain tissues of naïve mice after six hours. However, isoflurane anesthesia did not induce caspase activation at either two or 24 hours after isoflurane anesthesia (data not shown). It is possible that at two hours after anesthesia is too early for isoflurane to induce caspase activation, and that the isoflurane-induced caspase activation does not last very long, e.g., 24 or 48 hours after the anesthesia, owing to rapid clearance of apoptotic cells in mouse brain.

Isoflurane increases levels of BACE and A β

We then assessed whether isoflurane elevates BACE and A β levels in mouse brain following anesthesia. Western blot analyses revealed increased levels of BACE (Figure 1g) six hours after isoflurane anesthesia as compared to control condition. Quantification of the Western blots, normalized to β -actin, showed that isoflurane anesthesia led to a 155% (Figure 1f, $P = 0.025$) increase in BACE levels after six hours, as compared to control condition.

We next asked whether isoflurane can increase A β levels subsequent to caspase activation and elevations in BACE levels. For this purpose, we carried out a time course of isoflurane's effects on caspase activation, and levels of BACE and A β . Caspase-3 immunoblotting revealed that anesthesia with 1.4% isoflurane for two hours yielded visible reductions in levels of FL-caspase-3 (Figure 2a) 12 hours after anesthesia; quantification of the results showed that isoflurane anesthesia led to a 23% reduction in FL-caspase-3 levels (Figure 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 21 (Figure 2c and 2d, 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 to control condition after 12 hours (Figure 2e and 2f, a 214% increase, $P = 0.031$). However, we were not able to observe detectable increases in A β levels 12 hours after isoflurane anesthesia (data not shown).

Given that isoflurane induced caspase activation and elevated BACE levels in the absence of detectable increases in A β levels six to 12 hours after isoflurane anesthesia, we next assessed the effects of anesthesia with 1.4% isoflurane for two hours on BACE and A β 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 (Figure 3a) in mouse brain 24 hours after anesthesia; quantification of the results revealed that isoflurane anesthesia led to a 412% increase in BACE levels (Figure 3b, $P = 0.015$) 24 hours after anesthesia. Isoflurane anesthesia also increased A β levels in mouse brain 24 hours after anesthesia (Figure 3c); quantification of these data by Western blot analysis revealed that isoflurane anesthesia caused a 145% increase in A β levels (Figure 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 (MPAC), Clioquinol (CQ), which is known to inhibit A β aggregation 19, 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 19. The mice were then anesthetized with 1.4% isoflurane for 2 hours, and sacrificed six hours after the anesthesia. Pretreatment with CQ prior to isoflurane anesthesia was able to significantly reduce caspase-3 activation (Figure 4a and 4b, 247% versus 137%, $P = 0.031$). These findings suggest that isoflurane may at least partially induce caspase activation via A β aggregation given that A β aggregates have previously been shown to induce apoptosis, and isoflurane can enhance A β oligomerization and potentiate A β -induced cytotoxicity.

Isoflurane reduces GGA-3 levels

Recent study 21 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 two hours decreased GGA-3 levels as

compared to control condition in naïve mice (Figure 5a and 5b). RT-PCR assay showed that isoflurane did not increase BACE mRNA levels (Figure 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 A β generation in human neuroglioma cells 14–16. Here, we set out to determine the *in vivo* relevance of these effects in naïve mice. We have shown for the first time that a clinically relevant concentration of isoflurane can induce caspase activation six and 12 hours after the isoflurane anesthesia, increase level of BACE six, 12 and 24 hours after the isoflurane anesthesia, and finally enhance A β levels 24 hours after the isoflurane anesthesia. These findings suggest that anesthesia with 1.4% isoflurane, analogous to the concentration employed in clinical settings, induces a time-dependent cascade of caspase activation, elevated BACE levels and increased A β levels, most likely due to enhanced BACE cleavage of APP.

Our current 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 A β . 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 A β in mouse brain is likely to be similar to previous described effects of ischemia on caspase activation, BACE stabilization, and A β generation in rodent models 21. This study showed that caspase activation can reduce levels of the golgi associated, gamma adaptin ear containing, ARF binding protein 3 (GGA3), a protein involved in BACE degradation 21. We have found that isoflurane also reduced GGA-3 levels but does not increase BACE mRNA 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 (e.g., 6 hours) following 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 (e.g., 12 hours). Finally, the enhanced β -secretase activity would promote amyloidogenic processing of APP and increase A β levels at later time intervals (e.g., 24 hours).

Recent studies 22 have shown that anesthesia with two hours 0.9 - 1.0% isoflurane daily for five days impaired cognition functions in naïve 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 naïve and Tg2576 mice 11 to 14 days following 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 following the isoflurane anesthesia. Moreover, repeated isoflurane exposures may cause preconditioning effects, which can attenuate potential isoflurane-induced neurotoxicity. However, our current results have illustrated that a single clinically relevant isoflurane exposure can induce caspase activation and increase A β levels in naïve mice after only two hours of exposure. Thus, isoflurane-induced caspase activation and A β increase are most likely dose- and time-dependent.

It is also possible that the increase in protein levels of BACE and A β in mouse brain following isoflurane anesthesia could be due to other mechanisms. Velliquette et al. reported

that insulin, 2-deoxyglucose, 3-nitropropionic acid, and kainic acid can induce acute energy inhibition to enhance levels of BACE and A β in wild-type and AD transgenic (Tg2576) mice 23. Isoflurane is a profound cerebral metabolic depressant and decreases glucose utilization in rats 24. Therefore, isoflurane may affect APP processing and increase A β accumulation via energy inhibition. Future studies will be necessary to determine whether isoflurane-induced increase in levels of BACE and A β is dependent on isoflurane-induced changes in glucose utilization or GGA3 levels.

Isoflurane has previously been shown to enhance A β aggregation and potentiate the cytotoxicity of A β 13. It has been reported that oligomeric and fibrillar species of A β are more neurotoxic 25–31. We have previously shown that clioquinol, a metal protein attenuation compound (MPAC) 19, can attenuate isoflurane-induced apoptosis in cultured cells. In the current 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 A β aggregation. Moreover, our results suggest that pretreatment of patients with CQ prior to isoflurane anesthesia may be effective in attenuating isoflurane-induced caspase activation and potential neurotoxicity, pending on further studies.

A recent study by Groen et al. 11 showed that an insult from a two hours occlusion of middle cerebral artery increased levels of APP and A β in axons at corpus callosum and in neurons at the border of the ischemic region. Moreover, this transient insult caused persistent APP and A β deposits in thalamic nuclei (ventroposterior lateral and ventroposterior medial nuclei), which eventually developed to dense plaque-like deposits 9 months after the initial insult 11. This secondary and persistent brain harm could be due to axonal damage of thalamic neurons leading to retrograde degeneration 32; damage from vasogenic edema and some noxious substance 33; or hypometabolism 34–35. Both isoflurane 16 and brain ischemia 21 have been shown to induce caspase activation and apoptosis, which then enhance levels and activities of BACE to facilitate APP processing and to increase A β generation. Thus, we have postulated that the treatment with 1.4% isoflurane for two hours can also induce not only transient injuries (e.g., caspase activation and apoptosis, increases in levels of BACE and A β) but also persistent damage (e.g., APP and A β 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 down-stream consequences of isoflurane-induced apoptosis and A β generation, e.g., determination of the effects of isoflurane-induced apoptosis and A β generation on NMDA 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 inhalational anesthetic isoflurane promotes AD neuropathogenesis.

In conclusion, we have found that isoflurane can induce caspase activation, increase levels of BACE and A β in naïve 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 A β burden, including patients with AD, Down 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, APOE- ϵ 4, that increase A β 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 post-operative cognitive dysfunction and risk for AD.

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Figure 1. Anesthesia with 1.4% isoflurane for two hours induces caspase activation and causes a modest increase in BACE levels six hours after anesthesia

a. Isoflurane anesthesia (lanes 4 to 7) induces caspase-3 cleavage (activation) by increasing caspase-3 fragment levels and decreasing FL-caspase-3 levels as compared to control condition (lanes 1 to 3) in Western blots. The blot showing the band of caspase-3 fragment only was the same blot with more exposure time in developing the film. *b.* Quantification of the Western blots shows that isoflurane anesthesia (black bar) induces caspase-3 activation, assessed by quantifying ratios of caspase-3 fragment to FL-caspase-3, as compared to control condition (white bar). *c.* Isoflurane anesthesia (lanes 4 to 9) induces PARP cleavage by increasing PARP fragment levels as compared to control condition (lanes 1 to 3) in Western blots. *d.* Quantification of the Western blots shows that isoflurane anesthesia (black bar) induces PARP cleavage compared to control condition (white bar). *e.* Immunohistochemistry reveals cleaved caspase-3 positive cells in frontal cortex. *f.* Quantification of the brain slices shows that isoflurane anesthesia (black bar) induces more cleaved caspase-3 positive cells as compared to control condition (white bar). *g.* Isoflurane anesthesia (lanes 4 to 6) increases BACE levels as compared to control condition (lanes 1 to 3) in Western blots. Synthetic BACE was used as a marker to identify position of BACE in Western blot. *h.* Quantification of the Western blots shows that isoflurane anesthesia (black bar) increases BACE levels as compared to 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 $\pm$ S.D., $n = 3 - 10$ for each experimental group. T-test was used to compare the difference between control condition and isoflurane anesthesia condition, * $p < 0.05$.

Figure 2. Anesthesia with 1.4% isoflurane for two hours induces caspase activation and increases BACE levels 12 hours after anesthesia

a. Isoflurane anesthesia (lanes 4 to 7) decreases FL-caspase-3 levels as compared to control condition (lanes 1 to 3) in Western blots. *b.* Quantification of the Western blots shows that isoflurane anesthesia (black bar) reduces FL-caspase-3 levels as compared to control condition (white bar). *c.* Isoflurane anesthesia (lanes 3 and 4) increases protein levels of APP-N-Caspase-Fragment as compared to 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 to control condition (white bar). *e.* Isoflurane anesthesia (lanes 4 to 7) increases BACE levels compared with control condition (lanes 1 to 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 to 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 $\pm$ S.D., $n = 3 - 10$ for each experimental group. T-test was used to compare difference between control condition and isoflurane anesthesia, * $p < 0.05$.

Figure 3. Anesthesia with 1.4% isoflurane for two hours increases levels of BACE and A β 24 hours after anesthesia

a. Isoflurane anesthesia (lanes 4 to 7) increases BACE levels compared with control condition (lanes 1 to 3) in Western blots. *b.* Quantification of the Western blot shows that isoflurane anesthesia (black bar) increases BACE levels as compared to control condition (white bar). *c.* Isoflurane anesthesia (lanes 4 and 5) increases levels of A β as compared to control condition (lanes 2 and 3) in the Western blot. Synthetic A β was used as a marker to identify position of A β in Western blot. *d.* Quantification of the Western blot shows that isoflurane anesthesia (black bar) increases A β levels as compared to 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 $\pm$ S.D., $n = 3 - 10$ for each experimental group. T-test was used to compare difference between control condition and isoflurane anesthesia, * $p < 0.05$.

Figure 4. Pretreatment (seven days) with clioquinol (CQ) attenuates isoflurane-induced caspase-3 activation

Isoflurane anesthesia (lanes 5 and 6 in Figure 4a; black bar in Figure 4b) induces caspase-3 activation as compared to either control condition (lanes 1 and 2 in Figure 4a; white bar in Figure 4b) or CQ alone (lanes 3 and 4 in Figure 4a; gray bar in Figure 4b). Pretreatment for seven days with CQ (lanes 7 and 8 in Figure 4a; net bar in Figure 4b) attenuates the isoflurane-induced caspase-3 activation (lanes 5 and 6 in Figure 4a; black bar in Figure 4b). There is no significant difference in amounts of β -actin in mouse brain tissue following different treatments. Data are means $\pm$ S.D., $n = 3 - 6$ for each experimental group. T-test was used to compare difference in caspase-3 activation between control condition and isoflurane anesthesia: * $p < 0.05$; and between DMSO treatment and CQ treatment: # $p < 0.05$.

Figure 5. Anesthesia with 1.4% isoflurane for two hours reduces GGA-3 levels without alterations in BACE mRNA levels

a. Isoflurane anesthesia (lanes 4 to 6) reduces GGA-3 levels as compared to control condition (lanes 1 to 3) in Western blots. *b.* Quantification of the Western blots shows that isoflurane anesthesia (black bar) reduces GGA-3 levels as compared to control condition (white bar). *c.* Isoflurane dose not alter BACE mRNA levels as compared to control condition. cDNAs were 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 (inserted figures) using a standard curve of cycle times and mRNA amount (data not shown).