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Anesthesia in presymptomatic Alzheimer's disease: a study using the triple-transgenic mouse model

Junxia X. Tang^a, Feras Mardini^a, Breanna M. Caltagarone^a, Sean T. Garrity^a, Rosie Q. Li^a, Shannon L. Bianchi^a, Olubusola Gomes^a, Frank M. Laferla^b, Roderic G. Eckenhoff^a, and Maryellen F. Eckenhoff^{a,*}

^aDepartment of Anesthesiology and Critical Care, University of Pennsylvania School of Medicine, 305 John Morgan, 3620 Hamilton Walk, Philadelphia, PA 19104 USA

^bDepartment of Neurobiology & Behavior, University of California, 1109 Gillespie Neuroscience Facility, Irvine, CA 92697 USA

Abstract

Background—Experimental evidence suggests that anesthetics accelerate symptomatic neurodegenerative disorders like Alzheimer disease (AD). Since AD pathology precedes symptoms, we asked whether anesthetic exposure in the presymptomatic interval accelerates neuropathology and appearance of symptoms.

Methods—Triple transgenic Alzheimer mice exposed to the general anesthetics, halothane or isoflurane, at 2, 4 and 6 months of age, underwent water maze cognitive testing two months afterwards and their brains subsequently analyzed with ELISA, immunoblots, and immunohistochemistry for amyloid and tau pathology and biomarkers.

Results—Learning and memory improved after halothane exposure in the 2 month old group relative to controls, but no changes were noted in any isoflurane group. When gender was examined in all age groups, females exposed to halothane performed better than females exposed to isoflurane or controls. Thus, improvement in the 2 month exposure group is most likely due to a gender effect. Phospho-tau in the hippocampus was significantly increased two months after anesthesia, especially in the 6 month exposure group but changes in amyloid, caspase, microglia or synaptophysin were not detected.

Conclusions—These results indicate that exposure to two different inhalational anesthetics during the presymptomatic period of AD does not accelerate cognitive decline, two months later, and may cause a stress response, marked by hippocampal phosphorylated tau, resulting in preconditioning against the ongoing neuropathology, primarily in female mice.

Keywords

halothane; isoflurane; anesthesia; transgenic mice; preconditioning; gender; amyloid; tau; memory; learning; general anesthetics, triple transgenic mice

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Correspondence: Maryellen F. Eckenhoff, PhD Department of Anesthesiology and Critical Care University of Pennsylvania School of Medicine 305 John Morgan Bldg 3620 Hamilton Walk Philadelphia, PA 19104 Voice: 215-662-3705 FAX: 215-349-5078 maryellen.eckenhoff@uphs.upenn.edu.

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1. Introduction

Although questioned [1], recent studies suggest that elderly patients are at increased risk of dementia following hospitalization [2,3]. An important reason for hospitalization is the need for surgery. It is now well established that elderly patients are at increased risk of cognitive decline after anesthesia and surgery [4-7]. However, it is not yet clear which component of the perioperative experience is responsible, or if it is ultimately reversible. Further, there might be additional vulnerabilities that interact with anesthesia and surgery that not only increase the risk of postoperative cognitive decline (POCD), but also accelerate pre-existing neuropathology. One example is the current concern surrounding anesthesia and Alzheimer disease [8]. Previous retrospective human studies have provided contradictory evidence with regard to the effect of anesthesia and surgery on the risk and progression of Alzheimer's disease, currently the major neurodegenerative disease in the world. Even if an association is found between anesthesia and Alzheimer's disease, it remains unclear whether it is due to the underlying illness or condition that requires surgery, the surgery itself and associated inflammatory processes [9], or the anesthetic management, drugs and physiology that drives the association [10-12].

These features of modern perioperative care are not easily separable in human studies, provoking investigators to study the question in animals. Several studies in old mice and rats have shown that inhaled anesthetic exposure alone, especially isoflurane, can result in cognitive dysfunction long outlasting the *in vivo* residence of the drug [13-15]. Further, isolated anesthetic exposure of older APP_{swe} transgenic mice caused an acceleration of the hallmark lesion (plaque) of Alzheimer disease [15]. In this previous study, no incremental cognitive loss was detected, suggesting that either sufficient time for the additional toxic material to cause cellular or synaptic dysfunction had not transpired, or that they had reached a floor-effect with respect to the rate of cognitive loss. A more recent study in younger APP_{swe} transgenic mice exposed to isoflurane, also showed minimal effects on cognition and neuropathology [16]. Most patients exposed to anesthesia and surgery do not yet have frank, symptomatic Alzheimer disease, so we asked whether exposure to contemporary anesthetics prior to the appearance of cognitive symptoms or during the early stages of mild cognitive impairment (MCI), altered the rate of neuropathology to the point where cognitive loss was accelerated and appeared earlier. Such remote temporal effects of medical care are of concern; detection of the association is difficult unless specifically searched for. In this study, we employed a recently developed triple-transgenic (3xTgAD) mouse model of human AD, which over expresses human amyloid precursor protein (APP_{Swe}), presenilin-1 (PS-1M146V) and tau_{P301L}. Prior characterization suggests close adherence to the human disease progression [17].

2. Methods

2.1 Animals

Animal protocols were approved as required by the University of Pennsylvania Institutional Animal Care and Use Committees and all mice were treated in strict accordance to APS/NIH guidelines. Homozygote triple transgenic Alzheimer (3xTgAD) mice [17] were used exclusively. This transgenic AD mouse model develops both plaque and tangle pathology and cognitive dysfunction in an age dependent manner. Genotype was verified from tail biopsies by means of proteinase-K digestion, DNA extraction and PCR. Mice were tagged with BMDS IMI-1000 subcutaneous identification tags (BioMedic Data Systems, Seaford, DE) at weaning. Both male and female mice were used in this study.

2.2 Anesthetic exposure

Groups of mice were anesthetized with humidified 40% O₂ balanced by N₂, with either, a) 1.0 MAC (minimum alveolar concentration) (0.9-1.1%) halothane (2-bromo-2-chloro-1,1,1-trifluoroethane), b) 1.0 MAC (0.9-1.1%) isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) or c) no anesthetic added. Anesthetic vaporizer concentrations were calibrated with a Riken Fi-21 Gas Indicator, and concentrations monitored during exposures with a Datex Engstrom Capnomac gas monitor. Mice were exposed for 5 hours once a week for four weeks for a total of four exposures. Exposures were carried out in acrylic chambers, partially submerged in a 38°C water bath to maintain euthermia. Control mice were placed in similar chambers but not in the water bath. Prior studies have verified that the exposures do not cause changes in vital signs outside a range of normality. The mice breathed spontaneously without intubation and the total gas flow rate was 3 LPM. Chamber CO₂ levels never exceeded 0.1% and rectal temperature was monitored periodically during the anesthetic exposures, and changes were not detected. Body weights were recorded prior to, and after exposure. After exposure, animals fully recovered in 40% oxygen within 30 minutes, then returned to their cages and observed intermittently for 24 hours.

This exposure paradigm was repeated at three different ages: 2 months (isoflurane n=51; halothane n=40; controls n=43), 4 months (isoflurane n=19, halothane n=17, controls n=19), and 6 months (isoflurane n=19, halothane n=19, controls n=20). In addition, three animals from each exposure in the 6m-8m group were euthanized 24h after the last exposure for caspase-3 biochemical studies as described below. In all, 256 mice were examined in this study.

2.3 Behavior

Two months after the first exposure, mice were tested in the Morris Water Maze (MWM) (Fig. 1). The three exposure age groups were designated as age at first exposure-age at start of MWM testing, 2m-4m, 4m-6m, 6m-8m. The water in the 1.5m diameter pool was maintained between 25-29°C and opacified with titanium (IV) dioxide throughout all training and testing to obscure the submerged 15cm diameter platform. After each trial, each mouse was dried and warmed before returning to its cage. The time between trials was at least 30-45 minutes. Mice were tracked with a video camera mounted above the pool that fed to a computer running IMAQ PCI-1407 software. Low performing mice were not excluded from the study.

Cued training (days 1-5, Fig. 1)—During cued training, the mice were trained to escape from the MWM for 5 days with 4 trials per day. The platform was flagged and the mice were allotted 60s to find the submerged platform, and then allowed to remain on the platform for 10-15s. Animals that did not find the platform in 60s were gently guided to it, and allowed to remain for 10-15s prior to removal. The platform location was fixed and starting points were varied. A white curtain surrounded the pool to minimize visual cues for the mice.

Reference memory (days 8-12, Fig. 1)—Two days after the end of cued training, reference memory testing was performed for 5 days with 4 trials per day. The curtain and platform flag were removed and fixed external visual cues were placed on the walls around the pool. The platform was placed in the same location as for the cued training and starting points were varied. After placing the mouse in the pool, the mouse handler remained out of sight of the mouse. The escape latency (the time required to reach the platform) was recorded and the mean for each day calculated for each animal. If an animal did not reach the platform within 60s, it was gently directed to it and the escape latency marked as 60s.

Probe test (days 12, 13, 15, Fig. 1)—Probe tests to determine memory retention were conducted after the last trial of the reference memory testing (Fig 1. day 12), 24h (Fig. 1. day 13), and 60h (Fig. 1. day 15) later. For this test, the platform was removed from the pool and the swim path data for each mouse was recorded for 60s. The starting location was always in the “opposite” quadrant. The percent time spent in each quadrant was measured and the ratios of time in target to opposite were calculated. Swim speeds were recorded for each of the three 60s probe tests and the means calculated.

Spatial working memory (days 15-24)—Immediately after the third probe trial, 10 days of spatial working memory testing began. For these trials, the platform remained hidden. Thus, the mice had to use the visual cues in the room to locate the platform. The animals were allowed 60s to find the platform from varied starting points for each trial, and allowed up to 8 trials per day. In order to advance to a new platform location, the animals had to reach “criterion”, defined as reaching the platform in less than 20s in 3 consecutive trials. If “criterion” was not achieved within 8 trials, then the mouse would begin the next day at the same platform location with 8 more trials to reach criterion. Once the animals reached criterion, they were finished with the testing for that day and began the testing the next day with a new platform location. The number of trials to reach each platform location was recorded. Statistics for all of the behavioral analyses were performed using GraphPad Prism 5 software and are described in the legend for Figure 2.

Euthanasia—On the day following the last day of working memory testing, all animals were briefly anesthetized with a high concentration of halothane, and then perfused via the left ventricle with ice cold phosphate buffered saline (PBS) with simultaneous exsanguination from the right atrium. The time required from loss of consciousness to brain removal was routinely less than 5m. The brains were placed on ice and sagittally hemisected and one side was prepared for immunohistochemistry while the other side was immediately frozen in liquid nitrogen and stored at -80° for biochemical analyses. Thus, the age of the animals at sacrifice was approximately 5, 7 and 9 months.

2.4 Immunohistochemistry

Hemibrains were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Serial coronal $10\mu\text{m}$ sections were cut, deparaffinized, rehydrated with graded alcohols, and washed in distilled water. Antigen retrieval was performed by heating in Antigen Unmasking Solution (Vector Labs, Burlingame, CA) in a decloaking chamber (Biocare Medical, Concord, CA) for 2m. Sections were incubated in 5% hydrogen peroxide to block endogenous peroxidase activity. Using Vectastain MOM kit (Vector Labs, Burlingame, CA), sections were blocked with MOM Mouse IgG Blocking Reagent for 1h. Slides were incubated overnight at 4°C with primary antibody. Antibody 6E10 (1:400; Covance, Dedham, MA) for plaque, amyloid precursor protein (APP), and amyloid beta ($\text{A}\beta$) related peptides, and antibody AT180 (1:500; Thermo Scientific, Waltham, MA) for phosphorylated tau at threonine 231 and serine 235 (pTau231/235) (See Suppl. Table 1 further information on antibodies). Sections were washed in TBS buffer (50mM Tris, 150mM NaCl, pH7.8), incubated in biotinylated anti-mouse IgG (1:1000) for 1h, and incubated with ABC reagent (Avidin-Biotin-horseradish peroxidase Complex, 1:500) for 1h. After a final wash step, sections were stained with DAB (Vector Labs, Burlingame, CA) for 8m and dehydrated with graded alcohols and xylenes.

Quantitative image analysis and statistics—Immunostaining was visualized using an Olympus IX70 Microscope equipped with a SensiCam SVGA High-Speed Cooled Digital Camera (Cooke Corp., Auburn Hills, MI) and IPLab Suite V4.0 Imaging Processing and Analysis software (Biovision Technologies, Exton, PA). Images of three $10\mu\text{m}$ sections

(positioned about 2000 μ m~2200 μ m from frontal pole) through anatomic region of interest (intermediate hippocampal CA1 region) were captured from each animal under 10x magnification. To objectively quantify the APP/ A β and pTau231/235 immunointensity, we chose a reference area in an adjacent low-staining area such as the molecular layer of the dentate gyrus to serve as the baseline of the immunohistochemistry (IHC) staining. The pixel with the largest counts in the histogram of the grayscale image of this reference area was used for thresholding to correct for the signal contamination from tissue holes and artifacts. After thresholding, the average gray value was calculated and represented the intensity of the background (BCK); while the variance (standard deviation, SD) of the grey level distribution of the images was used as an aid to determine which image point should be regarded as significantly stained. The value of BCK+3xSD was then determined as the thresholding value to do segmentation for the region of interest (intermediate hippocampal CA1 region). In other words, the pixels with higher grey values than BCK+3xSD (significantly brighter than the background) were accepted as significantly stained image points. Two values were then calculated, the relative mean intensity (RMI) of the specific staining within the segmented region obtained by subtraction of the BCK, and the percentile areas (Area%) of the significantly stained segmented region within the intermediate hippocampal CA1 region. The average of the values of RMI $\times$ Area%, which we refer to as the staining intensity, from three adjacent sections was then determined for each animal and subsequently analyzed by two-way ANOVA with Bonferroni posttest using GraphPad Prism 5 software.

2.5 Biochemistry

Protein extraction—All ELISAs and western blots, except for caspase-3, used the frozen mice hemibrains from animals that underwent the MWM testing. For caspase-3 western blots, brains from 3 additional mice from each exposure in the 6m-8m group were euthanized 24h after the 4th anesthetic exposure, perfused and frozen as above. Hemibrains were weighed and homogenized in 4X volumes of ice cold PBS (pH 7.4; Invitrogen, Camarillo, CA) supplemented with protease and phosphatase inhibitors (Sigma Aldrich, St. Louis, MO). Homogenates were divided into two aliquots, one for A β extraction, and a second for extraction of other proteins measured in this study. Diethylamine (0.4%) was used to solubilize A β as previously described [18]. Diethylamine allowed for quantification of the low levels of A β found in tissue without plaques (plaque was not observed in any mouse), since other extraction methods dilute the sample excessively. The first aliquot was mixed with 1.5 volumes of PBS, followed by 2.5 volumes of 0.4% diethylamine and 100mM NaCl. The second aliquot was sonicated in 1.5 volumes of concentrated RIPA buffer (50mM Tris, 150mM NaCl, 1.7% NP-40, 0.8% sodium deoxycholate, 0.17% SDS, 1.7mM EDTA and 1.7mM EGTA), centrifuged (16,000g for 90 min at 4°C), and stored at -80°C. The diethylamine extracts were stored after neutralization with 0.1 volumes of 0.5M Tris buffer (pH 6.8). All the procedures were carried out on wet or dry ice as applicable. Protein concentrations were measured using a bicinchoninic (BCA) protein assay (Pierce Thermo Fisher Scientific, Rockford, IL).

ELISA assay—A β ₁₋₄₀, A β ₁₋₄₂ (Invitrogen, Camarillo, CA), total tau (both phosphorylated and nonphosphorylated) (Invitrogen, Camarillo, CA), phosphorylated tau at threonine 181 (pTau181) (Innogenetics, Gent, Belgium), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) (Invitrogen, Camarillo, CA), were quantified. The RIPA extracts were diluted 450 times and 10 times for analyzing total tau and phosphorylated tau, respectively, while diethylamine extracts were loaded onto the ELISA plate directly for analyzing A β ₁₋₄₀ and A β ₁₋₄₂. RIPA extracts were added directly into ELISA plates for IL-6 and TNF- α . A protease inhibitor cocktail with AEBSF (Sigma Aldrich, St. Louis, MO) was added. The standards were prepared using the same diluents as used with the tissue samples in order to

avoid any matrix effect. Plates were read at 450nm and 630nm as the reference wavelength using an Opsys MR microplate reader (Dynex Technologies, Chantilly, VA). The antigen level determined in pg/ml by the ELISA converted to pg/mg of wet brain tissue.

Western blotting—Equal amounts of RIPA-extracted protein (20 µg for synaptophysin and 100 µg for caspase 3, Iba-1, and A8717 antibodies) were subjected to SDS-PAGE using 15% Tris-HCl gels under reducing conditions (Bio-Rad, Hercules, CA), followed by transfer to 0.2 µm polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk in TBST for 1 hour, blots were incubated with primary antibody overnight. Full-length caspase-3 was detected using a caspase-3 antibody (1:200; Cell Signaling Technology, Danvers, MA), APP and CTFs were detected using the anti-APP C-terminal antibody A8717 (1:1000; Sigma, St. Louis, MO), Iba-1 (microglial marker) and synaptophysin were detected using the anti-Iba-1 antibody (1:300; Abcam, Cambridge, MA), and the SY38 antibody (1:10000; Millipore, Billerica, MA), respectively (See Suppl. Table 1 for further information on antibodies). The blots were then incubated in horseradish peroxidase-conjugated secondary antibody. Detection was performed using ECL Plus western blot detection system (Amersham, Piscataway, NJ), and densitometry was performed on film images using Bio-Rad Quantity One software (Bio-Rad, Hercules, CA), with results normalized to β -actin.

Statistical analyses—All biochemical data were analyzed by two-way ANOVA with Bonferroni posttests, except for the western analyses because these measurements were only carried out at one age, 6m-8m, and thus one-way ANOVA with Tukey's multiple comparison test was used. All statistics were done using GraphPad Prism 5 software.

3. Results

3.1 Behavior

Escape Latency—Animals with the lowest escape latency were the better performers in this task. The halothane group performed significantly better than the controls in 4 out of the 5 trial days (Fig. 2A). This effect appeared to be unique to this exposure time period, as no differences between the 4m-6m or 6m-8m exposure groups were noted (Fig. 2B, 2C). However, when ages were combined, the effect persisted; halothane exposure was associated with better performance than controls on 3 out of 5 trials days (Fig. 2D). The literature suggests that 3xTgAD pre-menopausal female mice are more vulnerable than 3xTgAD males [19]. No significant differences were found in gender for each age and exposure group. Thus, when we examined gender effects in the combined ages, we found that the improvement after halothane exposure was limited to females (Fig. 2E). The males exposed to halothane did as well as the controls with a trend for the isoflurane exposed males to perform less well (Fig. 2F). In contrast, control non-exposed females (Fig. 2E) performed significantly worse than the control males (Fig 2F). Therefore, the halothane effect could be interpreted as removing the vulnerability in the female gender.

Probe tests—The ratio of time spent in the target quadrant to the opposite quadrant was calculated for each animal and each probe test for all ages (Suppl. Fig. 1A-F). There were no significant differences between the exposure groups at any ages, except during the second probe trial for the 6m-8m exposure group, where isoflurane did slightly better than the control group (Suppl. Fig. 1C). When all ages were combined, no significant differences were found between the anesthetic exposure groups in the three probe trials (Suppl. Fig. 1D). In addition, a gender effect of age or exposure was not detected (Suppl. Fig 1E,F).

Spatial working memory—The spatial working memory task is a more challenging test for the mice. The platform location changes and the mice must use visual cues in the room

to find and then remember each new location. Animals with the fewest number of trials per platform were the best performers on this task. The 2m-4m halothane exposure group did better than the controls, with a trend for the same effect in the isoflurane group (Fig. 2G). When the data for all ages were combined, the halothane effect persisted (Fig. 2H). As above, this effect is due primarily to the females (Fig. 2I).

Swim speed—In order to determine if any observed differences in behavior could be attributed to differences in motor ability between the groups, swim speeds were calculated during the 60s probe tests since this was a constant swim time for all animals. The 2m-4m halothane exposure group had a significantly higher swim speed than the controls, but no differences in swim speed were found at the other ages (Fig. 2J). When ages were combined, the halothane effect persisted (Fig. 2K). However, no effect of gender could be detected (Fig. 2L).

3.2 Immunohistochemistry and biochemistry

Amyloid beta and caspase-3—3xTgAD mice were free of amyloid plaques at all ages examined in this study (less than 9 months), as revealed by immunohistological staining of the brain with 6E10 antibody (Fig. 3A). 6E10 was cross-reactive to full length APP, soluble APP cleaved at alpha site (sAPP α), c-terminal fragment β (CTF β , C99), and A β (epitope: 1-16). Neurons in the subiculum, cerebral cortex, amygdala, and hippocampal CA1 region were highly stained with 6E10 at all ages. Quantification of the 6E10 intraneuronal immunostaining was carried out in the hippocampal CA1 region and revealed no significant age or anesthetic exposure effects (Fig. 3B). A β levels were also determined by sandwich ELISA assays specifically for human A β ₁₋₄₀ and A β ₁₋₄₂. At the ages examined in this study (less than 9 months), A β ₁₋₄₂ was undetectable, and thus only A β ₁₋₄₀ measurements were reported. As with immunohistochemistry, A β ₁₋₄₀ levels increased significantly with age but were not significantly changed by the either anesthetic (Fig. 3C). We found a significant gender and age effect when we combined all exposure conditions, which has been reported previously in this model [19](Fig. 3D). In addition to measuring A β levels, we examined anesthetic effects on the levels of full length APP, c-terminal fragments (CTFs) C99 and C83 with western blot analysis in the 6m-8m group (only) after the MWM testing, as well as on the apoptotic marker caspase-3 in separate mice from the 6m-8m group immediately after the 4th exposure. No differences were detected between the exposure groups (Fig. 3E, F). Furthermore, we were unable to detect the activated form of caspase-3 resulting from cleavage at the aspartate position 175 at these ages.

Tau—Total tau was measured using sandwich ELISA. There was an overall significant increase in total tau with age and treatment but no differences were found between anesthetic exposure and controls at any age with the 2-way ANOVA (Fig. 4A). Phosphorylated tau (pTau181) showed neither an age or exposure effect (Fig. 4B) with ELISAs. The ratios of phosphorylated tau to total tau were calculated for each animal and the ratios averaged, but no significant anesthetic effects were found (data not shown). Like pTau181 [20], pTau231/235 has also been commonly used as a biomarker of Alzheimer's disease (for review, see [21]) and is visualized immunohistochemically with the AT180 antibody. AT180 immunoreactive neurons were found in the subiculum, cerebral cortex, and amygdala but were found in highest numbers in the CA1 region of the hippocampus (Fig. 4C). Although the AT180 epitope is associated with pathologic tau, it cannot distinguish between different oligomeric forms of tau. Quantitative analysis of the AT180 staining in the hippocampal CA1 region revealed a significant increase in pTau231/235 with exposure and age. The significant anesthetic exposure effect was limited to the 6m-8m age group (Fig. 4D). No statistically significant gender differences were detected for total or phosphorylated tau levels at any of the ages examined.

Synaptic and inflammatory markers—To assess whether inflammation was promoted by anesthetic exposure, we measured levels of the microglia marker ionized calcium-binding adaptor molecule 1 (Iba-1) and the pro-inflammatory cytokines TNF-alpha and interleukin-6 (IL-6), only in the 6m-8m group of mice. To determine if synaptic dysfunction was occurring after exposure to anesthetics, levels of synaptophysin were also measured in the 6m-8m group of mice. Western blot analysis found no differences in the levels of synaptophysin or Iba-1 (Fig. 5A,B) and likewise, quantitation of sandwich ELISAs showed no significant changes in levels of IL-6 or TNF-alpha in any exposure group (Fig. 5C).

4. Discussion

This study had as its main goal the evaluation of whether anesthetic exposure alters the course of presymptomatic neuropathology in the setting of genetic AD vulnerability. In previous work, we found that anesthetic exposure, especially halothane, enhanced amyloidopathy in the symptomatic Tg2576 mouse, but produced no incremental cognitive effect [15]. We speculated that a floor-effect in terms of cognitive decline explains these findings, and that anesthetic exposure at an earlier stage of the neuropathology may accelerate the subsequent cognitive decline. This question, addressed in this study, better reflects the clinical situation as well, because the vast majority of surgical patients are either asymptomatic or show only MCI, but many would be predicted to have slowly progressive Alzheimer-like neuropathology. To the extent that animal models are predictive of human consequences, this study provides some comfort that anesthetic exposure in this pre-symptomatic interval is well-tolerated and does not appear to produce early decrements in cognition, at least for 2 months afterward. Since cognitive consequences of the combined transgenes in this mouse model are generally not detected until about 10-12 months of age, it remains a remote possibility that symptom acceleration does occur and could have been detected had we waited longer.

In contrast to cognitive decline, halothane exposure resulted in improvements in memory and learning as revealed by our water maze measurements. This might have been predicted from other studies that have demonstrated no change or subtle improvements after anesthetic exposure in young adult and aged wild-type animals [22-24] but improvement has not been demonstrated in the setting of a vulnerable brain, as in this study. Two features of this improvement bear discussion. First, it was only observed after halothane exposure, and not isoflurane. This is somewhat perplexing, as our previous study in the Tg2576 model found that halothane, and not isoflurane, provoked greater amyloidopathy in the aged brain [15], and might therefore be considered as more neurotoxic, not less. However, in the younger more plastic brain [7], a greater neurotoxic stress may produce a greater stress response, resulting in a greater protective, or preconditioning response. Indeed, we and others have demonstrated that these drugs produce an upregulation of heat shock and other stress-related proteins [25-32]. If this interpretation is true, it is perhaps surprising that only halothane produces this effect, as isoflurane has also been shown by others to evoke a robust stress and preconditioning response [33-38]. This might indicate that in the AD-vulnerable brain, halothane is more provocative than isoflurane because of its stronger interactions with the ongoing amyloidopathy [10,15], which itself elicits a stress response [39]. This is of particular interest since millions of aging people worldwide have previously been exposed to this drug, which is still used in non-western countries today.

The other intriguing feature of the cognitive improvement is that the protective effect was confined almost entirely to the female gender. It is well known that females have a greater incidence of Alzheimer's disease [40], a finding also described in the 3xTgAD model [19]. In accordance, we found here that unexposed control 3xTgAD female mice perform significantly less well than male controls, and this difference may be due to increased levels

of intraneuronal hippocampal A β in the females [41]. However, A β is not likely to be responsible for the female cognitive changes observed here since halothane exposure was associated with cognitive improvement, without a significant change in A β levels. The swim speed for the halothane exposed mice was greater than controls, though not significantly different than the isoflurane exposed animals and not significantly different between males and females and thus cannot be attributed to the observed halothane effect on females. The specific mechanism for the female vulnerability remains unclear, but it seems that halothane selectively reverses it, since halothane produced no change in male performance.

As might be predicted from the absence of cognitive deterioration, anesthetic exposure results in few changes in the biochemical markers of neuropathology. Whereas our previous study in the Tg2576 model noted that halothane caused an increase in plaque load [15] we could detect neither plaque nor any changes in 6E10 immunoreactive material in the anesthetic exposed 3xTgAD model up to 8 months of age. It may be that the amyloid beta concentration has not increased to the point where anesthetic-enhanced oligomerization, a concentration-dependent effect [42], can be detected. Our results do make less likely, however, the notion that amyloid beta production is increased due to anesthetic exposure, as this should have been detected by our ELISAs at these ages.

On the other hand, the immunohistochemical analyses detected an increase in hippocampal phosphorylated tau after exposure to both anesthetics. It is of interest that this was not detected in our ELISAs, which may be due to dilution of these regional effects, or to a more specific effect on the AT180 (pTau213/235) epitope. Acute increases in phosphorylated and insoluble tau have been reported before, but attributed largely to the hypothermia which accompanies unsupported anesthesia [43]. In our exposures, temperature was closely monitored and hypothermia was rigorously avoided. Further, because the temperature effect on tau was rapidly reversed [43], and because we detected this change in tau months later, hypothermia as a cause is effectively ruled out. Moreover, anesthetic-induced changes in tau have been noted in animals where hypothermia was unlikely [44,45]. These robust changes in hippocampal tau likely reflect anesthetic triggering of a persistent stress, the implications of which in terms of AD pathogenesis or symptoms are not yet clear.

In this study, the ages of the 3xTgAD mice exposed to anesthetics (2-6 months) and behavioral testing (4-8 months), represent early points in the pathogenesis of AD. Although others have demonstrated mild cognitive effects with this same model at 4 months of age [46], we could not show any age related change in cognitive performance up to 8 months. Thus, we consider this a model of presymptomatic AD, although the latest time point (6m-8m) could be considered early MCI. Regardless, it is a model of the vulnerable brain which better reflects the situation in most of our operative patients. However, we cannot rule out an anesthetic effect later in the pathogenesis.

In summary, early exposure to general anesthetics does not accelerate the appearance of AD-like symptoms within 2 months after exposure in the 3xTgAD mouse model. Halothane conferred protection in female mice, but both anesthetics enhanced hippocampal phospho-tau, a potentially detrimental interaction with the underlying, slowly progressive neuropathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Summary of the time-line for the 3xTgAD behavioral testing paradigm.

Figure 2.

Morris water maze behavioral testing quantitation. (A-C). Significant improvement in learning was found in the halothane exposed mice compared to control exposed mice in the 2m-4m group (* $P < 0.05$, ** $P < 0.01$). The isoflurane group was not different from controls at this age. (D) When all three age groups were combined, the significant improvement in escape latency after halothane exposure compared to controls was maintained (* $P < 0.05$, ** $P < 0.01$). (E,F) Gender differences were examined in the combined ages, and a similar significant improvement in escape latency after halothane exposure compared to controls was found in the females (* $P < 0.05$, *** $P < 0.001$) (E) but not males (F). In the males, the isoflurane group performed slightly less well than control or halothane exposures in one out of the 5 days (* $P < 0.05$). (G-I) Spatial working memory. (G) Significant memory improvement was seen after halothane exposure in the 2m-4m group (** $P < 0.01$; 2m-4m ISO $n = 51$, HAL $n = 40$, CON $n = 43$; 4m-6m ISO $n = 19$, HAL $n = 17$, CON $n = 19$; 6m-8m ISO $n = 19$, HAL $n = 19$, CON $n = 20$). (H) Analysis of the data for the combined age groups showed a significant improvement in the halothane exposed animals (* $P < 0.05$). (I) Significant memory improvement was found in the female 3xTgAD mice exposed to halothane ($P < 0.01$; females ISO $n = 43$, HAL $n = 42$, CON $n = 46$; males ISO $n = 46$, HAL $n = 34$, CON $n = 36$). (J-L) Swim speeds were measured during the three 60s probe tests and a significant improvement was found in the halothane exposed mice in the 2m-4m group (* $P < 0.05$; 2m-4m ISO $n = 51$, HAL $n = 40$, CON $n = 43$; 4m-6m ISO $n = 19$, HAL $n = 17$, CON $n = 19$; 6m-8m ISO $n = 19$, HAL $n = 19$, CON $n = 20$). (K) the data for the combined age groups showed that overall halothane mice had faster swim speeds than the isoflurane or control mice (*** $P < 0.0001$). (L) No gender effect was found on swim speeds after anesthetic exposure (females ISO $n = 43$, HAL $n = 42$, CON $n = 46$; males ISO $n = 46$, HAL $n = 34$, CON $n = 36$). For all bar graphs, light grey = isoflurane, white = halothane and black = controls, and for all graphs the means and standard error bars are shown. Statistical analyses for all of the above graphs were performed using 1

or 2-way ANOVAs, or repeated measures ANOVAs as appropriate, with Bonferroni posttests.

Figure 3.

The effect of anesthetic exposure on the accumulation and processing of A β in 3xTg-AD mice. (A) A representative example of 6E10 immunostaining in the 3xTgAD hippocampal CA1 region from the 4m-6m group. Intracellular 6E10 staining (brown) was observed at all ages but no plaques were found. 6E10 is cross-reactive to APP, sAPP α , CTF β and A β . (B) No significant interaction was found in immunostaining intensity in the hippocampal CA1 region for age and anesthetic condition (3 animals per exposure condition and 3 sections per animal). (C) Levels of soluble A β from DEA-extracted homogenates using ELISA and reported in pg of A β /mg of wet tissue weight. No significant anesthetic effect was found in A β ₁₋₄₀ levels at any age. As expected, A β ₁₋₄₀ levels significantly increased with age ($P=0.02$) and A β ₁₋₄₂ was not detectable at these ages ($n=3$ for each column). (D) When gender differences were examined in combined treatment groups, there were significant gender ($P=0.025$) and age ($P<.004$) differences in A β ₁₋₄₀ levels (females $n=4, 5, 4$ and males $n=5, 4, 5$ at ages 2m-4m, 4m-6m and 6m-8m respectively). (E, F) Western blot analysis of RIPA-extracted homogenized hemibrains from the 6m-8m group of mice revealed no significant difference in the expression of APP, C99 and C83 fragments after anesthetic exposure using anti-APP C-terminal A8717 antibody ($n=3$ for each exposure condition). No significant difference in full length caspase-3 activation were found in RIPA-extracted homogenized hemibrains from 6 month old mice immediately euthanized after the 4th anesthetic exposure using caspase-3 antibody ($n=3$ for each exposure condition). For all the above graphs, 1 and 2-way ANOVAs with post-hoc Bonferroni or Tukey tests as appropriate were used for comparisons. Means and standard error bars indicated for each column.

Figure 4.

The effect of anesthetic exposure on the levels of total and phosphorylated tau in 3xTgAD mice. (A) Quantitative ELISA results showed an overall significant age ($P=0.0001$) and exposure effect ($P=0.017$) in total tau levels but no significant differences were observed between anesthetic exposures and controls within age groups by 2-way ANOVA ($n=3$ animals per column). (B) No differences across age or anesthetic exposure was detected in the phosphorylated tau 181 epitope by ELISA ($n=3$ animals for each column). (C) Representative immunohistochemical staining of phosphorylated tau using AT180 antibody in the hippocampal CA1 region from the 6m-8m group. (D) Quantitative AT180 immunostaining in the hippocampal CA1 region showed a significant increase in phosphorylated tau (Thr231 and Ser235) in mice exposed to isoflurane and halothane in the 6m-8m group ($*P<0.05$), and an overall significant age ($P=0.0002$) and exposure effect ($P=0.007$) ($n=3$ sections per animal and 3 animals per column) was noted. For each graph, means and standard errors shown, and 2-way ANOVA with Bonferroni posttests were used.

Figure 5.

The effect of anesthetic exposure on the levels of synaptic and inflammation markers in 3xTgAD mice. (A,B) Western blot analysis of RIPA-extracted homogenized whole brain hemispheres from the 6m-8m group of mice showed no significant difference in the expression of synaptophysin or Iba-1 in the anesthetic exposed groups. (C) Levels of TNF-alpha and IL-6 were not affected by exposure to anesthetics as determined by ELISA. Means and standard errors shown for each graph, and 1-way ANOVA with Tukey's multiple comparison test used for each marker, and n=3 for each exposure group.