

Sevoflurane Induces Tau Phosphorylation and Glycogen Synthase Kinase 3 β Activation in Young Mice

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ABSTRACT

Background: Children with multiple exposures to anesthesia and surgery may have an increased risk of developing cognitive impairment. Sevoflurane is a commonly used anesthetic in children. Tau phosphorylation contributes to cognitive dysfunction. The authors therefore assessed the effects of sevoflurane on Tau phosphorylation and the underlying mechanisms in young mice.

Methods: Six-day-old wild-type and Tau knockout mice were exposed to sevoflurane. The authors determined the effects of sevoflurane anesthesia on Tau phosphorylation, levels of the kinases and phosphatase related to Tau phosphorylation, interleukin-6 and postsynaptic density protein-95 in hippocampus, and cognitive function in both young wild-type and Tau knockout mice.

Results: Anesthesia with 3% sevoflurane 2 h daily for 3 days induced Tau phosphorylation (257 vs. 100%, $P = 0.0025$, $n = 6$) and enhanced activation of glycogen synthase kinase 3 β , which is the kinase related to Tau phosphorylation in the hippocampus of postnatal day-8 wild-type mice. The sevoflurane anesthesia decreased hippocampus postsynaptic density protein-95 levels and induced cognitive impairment in the postnatal day-31 mice. Glycogen synthase kinase 3 β inhibitor lithium inhibited the sevoflurane-induced glycogen synthase kinase 3 β activation, Tau phosphorylation, increased levels of interleukin-6, and cognitive impairment in the wild-type young mice. Finally, the sevoflurane anesthesia did not induce an increase in interleukin-6 levels, reduction in postsynaptic density protein-95 levels in hippocampus, or cognitive impairment in Tau knockout young mice.

Conclusions: These data suggested that sevoflurane induced Tau phosphorylation, glycogen synthase kinase 3 β activation, increase in interleukin-6 and reduction in postsynaptic density protein-95 levels in hippocampus of young mice, and cognitive impairment in the mice. Future studies will dissect the cascade relation of these effects. (**ANESTHESIOLOGY** 2014; 121:510-27)

CHILDREN who have multiple exposures to anesthesia and surgery at an early age may develop learning disability^{1,2} (reviewed in the study by Sun³). It has also been reported that anesthesia may induce neurotoxicity and neurobehavioral deficits in rodents⁴⁻⁶ and monkeys^{7,8} (reviewed in the study by Sun³). A recent study has shown that anesthesia with 3% sevoflurane 2 h daily for 3, but not 1, days may induce increase in proinflammatory cytokine (e.g., interleukin [IL]-6) in hippocampus of young (6 days old) mice and cognitive impairment in the mice.⁹

Tau protein, one of the microtubule-associated proteins, plays an important role in Alzheimer disease, dementia, and cognitive dysfunction¹⁰⁻¹³ (reviewed in the studies by Small and Duff,¹⁴ Querfurth and LaFerla,¹⁵ Huang and Mucke,¹⁶ and Sheng *et al.*¹⁷). Specifically, abnormal hyperphosphorylation of Tau has been thought to contribute to the

What We Already Know about This Topic

- Anesthetic exposure increases Tau phosphorylation in the brain, and Tau phosphorylation is associated with cognitive dysfunction in certain disease conditions. The underlying mechanism is not known.
- In adult mice, the impact of sevoflurane on brain Tau phosphorylation and on glycogen synthase kinase 3 β , an enzyme that phosphorylates Tau, was evaluated.

What This Article Tells Us That Is New

- Sevoflurane induced Tau phosphorylation and glycogen synthase kinase 3 β activation and led to cognitive impairment 3 weeks after exposure in 6-day-old mice. The simultaneous administration of the glycogen synthase kinase 3 β inhibitor lithium prevented the cognitive impairment.
- Increased Tau phosphorylation may contribute to the anesthesia-induced cognitive impairment in neonatal animals, and glycogen synthase kinase 3 β may serve as a therapeutic target for the prevention of this impairment.

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neuropathogenesis of Alzheimer disease^{13,18,19} and cognitive dysfunction.^{20–23}

Tau phosphorylation is regulated by several protein kinases such as glycogen synthase kinase 3 β (GSK3 β),^{24–27} cyclin-dependent kinase 5 (CDK5),^{25,28} cJun N-terminal kinase (JNK),^{29,30} and extracellular signal-regulated kinase (ERK)³¹ (reviewed in the studies by Wang *et al.*³² and Whittington *et al.*³³). The expression of the kinases peaked postnatally at days 8 to 11 and then returned to low level after 5 weeks.³⁴ Tau phosphorylation homeostasis is maintained through dephosphorylation mediated by protein phosphatase (PP) 2A, PP2B, and PP1.^{35,36} PP2A, PP2B, and PP1 are all involved in the regulation of Tau phosphorylation^{37,38}; however, it has been suggested that PP2A and PP2B are active phosphatases in the adult brain, and that phosphatases in young (6 days old) rats have lesser activity.³⁹ Finally, PP1 is the phosphatase for dephosphorylation of Tau protein at serine-202 (Tau-PS202) and tyrosine 205 (Tau-PT205).⁴⁰ Therefore, we assessed the effects of the sevoflurane anesthesia on the levels of the kinases and PP1 in the hippocampus of young mice.

Anesthetic-induced hypothermia,^{41–43} and anesthetics such as isoflurane,⁴⁴ sevoflurane,⁴⁵ and propofol,⁴⁶ have been reported to induce Tau phosphorylation *in vitro* and in hippocampus of adult mice. Specifically, repeated exposures (once every month for 5 months) of sevoflurane in 5- to 6-month-old mice induced Tau phosphorylation and cognitive impairment in the mice.⁴⁵ However, the effects of the sevoflurane anesthesia (*e.g.*, 3% sevoflurane 2 h daily for 3 days) on Tau phosphorylation in young mice have not been investigated. We therefore set out to study the effects of sevoflurane anesthesia on Tau phosphorylation, its potential up-stream mechanisms, and down-stream consequences in young (6 days old) wild-type (WT) and Tau knockout (KO) mice. The hypothesis in this study was that sevoflurane anesthesia in young mice could cause Tau phosphorylation and activation of GSK3 β , increase in levels of proinflammatory cytokine IL-6, and reduction in levels of postsynaptic density protein-95 (PSD-95), which is a postsynaptic marker,^{47,48} in the hippocampus of young mice, and induce cognitive impairment in the mice. Finally, lithium has been shown to inhibit GSK3 β activity and to reduce Tau phosphorylation⁴⁹; we therefore determined whether lithium would attenuate the sevoflurane anesthesia-induced GSK3 β activation, Tau phosphorylation, increased hippocampus IL-6 levels, and cognitive impairment in the mice.

Materials and Methods

Mice Anesthesia and Treatment

We performed the experiments in accordance with the National Institutes of Health guidelines and regulations. The Massachusetts General Hospital Standing Committee on the Use of Animals in Research and Teaching (Boston, Massachusetts) has approved the animal protocol. We

minimized the number of animals used in the studies. We used both male and female WT mice (C57BL/6J; Jackson Lab, Bar Harbor, ME) and Tau KO mice (B6.129X1-Maptm1Hnd/J; Jackson Lab) in the studies. We randomly assigned the mice into the anesthesia group or the control group. The mice received the sevoflurane at postnatal day (P)6 or from P6 to P8 and then were decapitated for hippocampus harvest at P6, P8, P10, and P31. We used different groups of mice for the behavioral testing from P31 to P35 or P37. The mice received anesthetic sevoflurane (3%) plus 60% oxygen (balanced with nitrogen) as performed in our previous studies.^{9,50} The findings from our previous studies have demonstrated that the 60% oxygen maintains sufficient partial pressure of oxygen levels in the mice during the sevoflurane anesthesia.^{5,9,50} The size of the induction chamber in the current study was 20 \times 20 \times 7 cm. The induction flow rate was 2 l/min for the first 3 min (for the induction) and then 1 l/min (for maintenance). Control groups received 60% oxygen at an identical flow rate in similar chambers. We monitored the anesthetic and oxygen concentrations continuously with a gas analyzer (Ohmeda; GE Healthcare, Tewksbury, MA). The temperature of the anesthetizing chamber was controlled by the DC Temperature Control System (FHC, Bowdoinham, ME), which is a feedback-based system for monitoring and controlling temperature, to maintain the rectal temperature of the mice as 37° \pm 0.5°C. Previous studies^{5,9,50} have shown that anesthesia with 3% sevoflurane for 2 h did not significantly change the values of pH, partial pressure of oxygen, or partial pressure of carbon dioxide as compared with the control group. The eating and drinking of mice between the control group and anesthesia group were assessed by the amount of food and water consumed and the changes in body weight. The general activity between the anesthesia mice and control mice was observed in a blind manner. There was less than 1% mortality rate of mice in the studies. For the intervention studies, we administered lithium (100 mg/kg)⁵¹ to mice through intraperitoneal injection 30 min before each of the 3 days of the sevoflurane anesthesia. All the experiments were performed in a blind manner.

Morris Water Maze

The Morris Water Maze (MWM) studies were performed as described in our previous experiments.⁹ Specifically, the P31 mice were tested in the MWM four trials per day for 5 (P31 to P35) or 7 (P31 to P37) days. Escape latency and platform crossing times were recorded. Mouse body temperature was maintained by active heating as described in the study by Bianchi *et al.*⁵² After every trial, each mouse was placed in a holding cage under a heat lamp for 5 min to dry before returning to its regular cage. The heating lamp was adjacent (2 feet away) to the MWM water tank. Hypothermia has been well reported to induce Tau phosphorylation in mice hippocampus.^{41,43} Therefore, the mice that had been used for the MWM test were not used for the hippocampus harvest. Consequently, the process

of the MWM (mice were put in 20°C water for up to 90 s) would not affect the Tau phosphorylation measured after the sevoflurane anesthesia in the current studies.

Hippocampus Harvest and Protein Level Quantification

Different groups of mice were used for biochemistry studies. At the end of the anesthesia, the mice were killed by decapitation at P6, P8, P10, or P31. The decapitation was performed when the mice were totally recovered from the anesthesia (approximately 1 h after the end of the anesthesia). The hippocampus of each of the mice was harvested and subjected to Western blot analysis. We homogenized the harvested hippocampus on ice using immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.5% Nonidet P-40) plus protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A). The lysates were then collected and centrifuged at 12,000 rpm for 10 min. We used bicinchoninic acid protein assay kit (Pierce, Iselin, NJ) to quantify the amount of protein.

Western Blot Analysis

The total Tau was recognized by anti-total Tau antibody (55 kDa, 1:1,000; BD Biosciences, Billerica, MA). Antibody AT8 (55 kDa, 1:1,000; Invitrogen, Carlsbad, CA) was used to detect the levels of Tau phosphorylated at serine-202 (Tau-PS202) and threonine-205 (Tau-PT205). Tau-PS262 antibody (55 kDa, 1:1,000; Invitrogen) was used to detect levels of Tau phosphorylated at serine 262 (Tau-PS262). GSK3β phosphorylated at serine 9, tyrosine 279, tyrosine 216, and total GSK3β were recognized by anti-phospho-GSK3β (Ser9) antibody (46 kDa, 1:1,000; Cell Signaling, Danvers, MA), anti-phospho-GSK3β(Tyr279/Tyr216) antibody (46 kDa, 1:1,000; Millipore, Billerica, MA), and anti-GSK3β antibody (46 kDa, 1:1,000; Invitrogen). Antibodies CDK5 (33 kDa, 1:1,000; Millipore), P35 (35 kDa, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA), P25 (25 kDa, 1:250; Santa Cruz Biotechnology), JNK (46 kDa, 1:500; Abcam, Cambridge, MA), phosphorylated-JNK (46 kDa, 1:1,000; Abcam), ERK (44 kDa, 1:500; Abcam), phosphorylated-ERK (44 kDa, 1:1,000; Abcam), and PP1 (36 kDa, 1:250; Santa Cruz Biotechnology) were used to recognize CDK5, P35, P25, JNK, phosphorylated-JNK, ERK, phosphorylated-ERK, and PP1, respectively. IL-6 antibody (24 kDa, 1:1,000 dilution; Abcam) was used to recognize IL-6. PSD-95 antibody (95 kDa, 1:1,000; Cell Signaling) was used to detect PSD-95. Finally, the antibody to detect nontargeted protein β-actin (42 kDa, 1:5,000; Sigma, St. Louis, MO) was used to control for loading differences in total protein amounts. Western blot quantification was performed as described in the study by Xie *et al.*⁵³ In brief, we used image analysis program Quantity One (Bio-Rad, Hercules, CA) to analyze the signal intensity. We then quantified the Western blots in two steps. First, we used β-actin levels to normalize protein levels (*e.g.*, determining the ratio of AT8 to β-actin amount) and control

for loading differences in the total protein amount. Second, we presented the changes in protein level from the mice undergoing anesthesia as a percentage of those in the mice from control group; 100% changes of protein level refer to control levels for the purpose of comparison with experimental conditions.

Enzyme-linked Immunosorbent Assay Determination of IL-6

We used the mouse IL-6 immunoassay kit (R&D Systems, Minneapolis, MN; Catalog number: M6000B) to determine IL-6 levels in the hippocampus of WT and Tau KO mice at P8. In brief, we coated a monoclonal antibody specific for mouse IL-6 onto microplates. We added 50 µl of standard and samples and then 50 µl of assay diluent RD1-14 to the center of each well. Wells were incubated for 2 h at room temperature and washed five times. Then we added 100 µl of mouse IL-6 conjugate to each well, incubated the wells for another 2 h, and washed the wells three times. Finally, the wells were incubated in 100 µl of substrate solution for 30 min and stopped with stop solution. We determined the optical density of each well using fluorescence reader at 450 nm and corrected at 570 nm.

Statistics

Data in biochemistry changes were expressed as mean ± SD. The data for platform crossing times were not normally distributed and thus were expressed as median and interquartile range. The number of samples varied from 6 to 14 in each group. The number of mice for body temperature measurement was three in each group. We performed a power analysis based on the previous studies⁹ (fig. 1A). Assuming a mean difference of 22 s (55 *vs.* 33) of escape latency in the MWM, an SD of 4 in the control arm, and an SD of 7 in the anesthesia arm, a sample size of 10 per arm will lead to a 90% power to detect a difference in the behavioral changes using a two-sided *t* test with 5% type I error. Interaction between time and group factors in a two-way ANOVA with repeated measurements was used to analyze the difference of learning curves (based on escape latency) between mice in the control group and mice treated with anesthesia in the MWM, which tested a hypothesis that the sevoflurane anesthesia increased escape latency with the assumption that the data were normally distributed based on the previous studies.⁹ *Post hoc* analyses were used to compare the difference in escape latency between the control group and anesthesia group for each day of the MWM, cutoff α was Bonferroni adjusted. The Mann-Whitney test was used to determine the difference between the sevoflurane and control conditions on platform crossing times to test a hypothesis that the sevoflurane anesthesia decreased the platform crossing times with the assumption that the data were not normally distributed based on the previous studies.⁹ There were no missing data for the variables of MWM (escape latency and platform crossing times) during the data analysis. Finally, a Student *t* test was used to

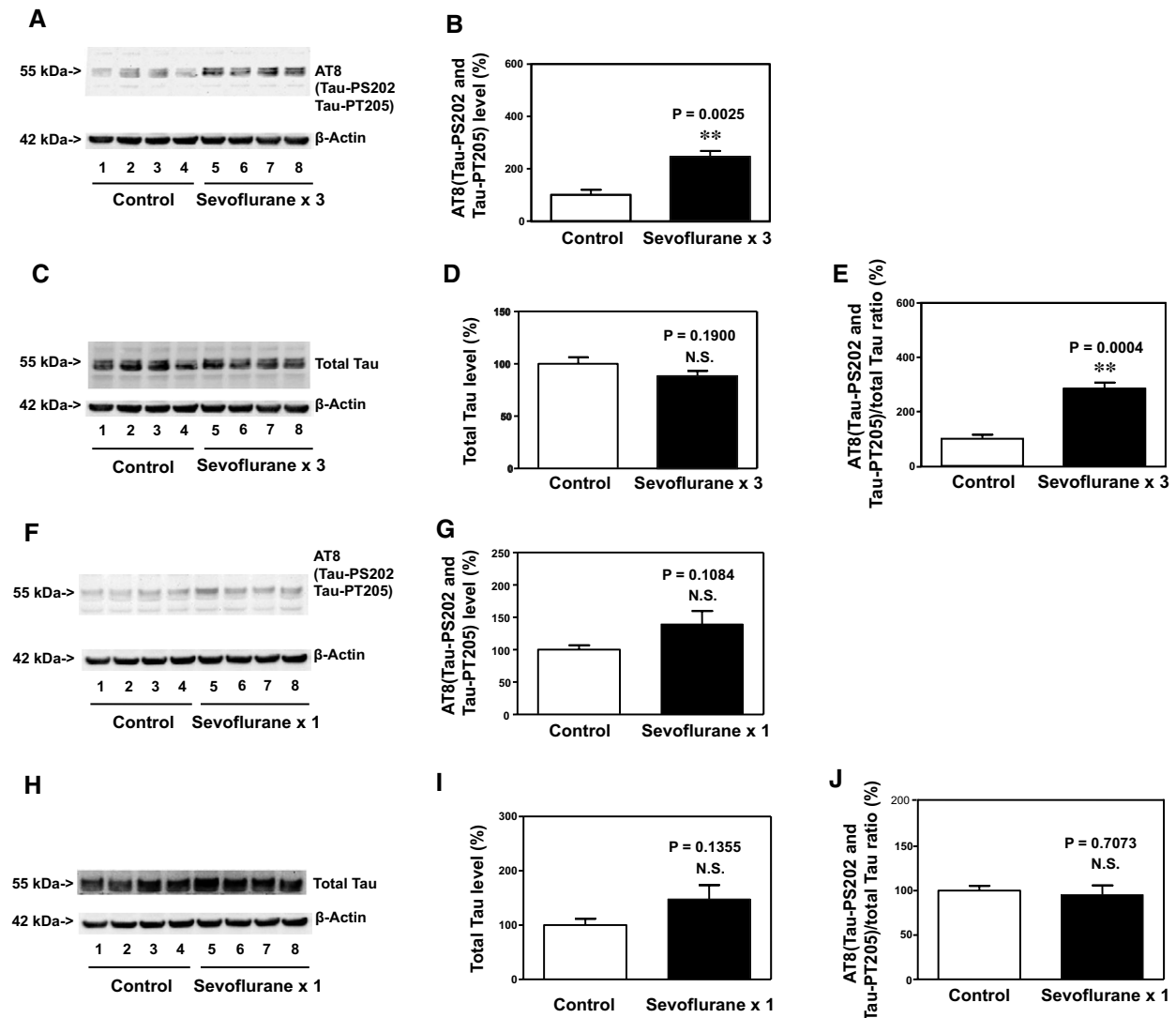


Fig. 1. Anesthesia with 3% sevoflurane 2 h daily for 3 days, but not for 1 day, in P6 wild-type mice induces Tau phosphorylation in hippocampus of the mice. (A) Anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 mice increases the levels of AT8 (detected the levels of Tau-PS202 and Tau-PT205) in the hippocampus of the mice (harvested on P8) as compared with the control condition. There is no significant difference in β -actin levels in the hippocampus of the mice between the sevoflurane anesthesia and control condition. (B) Quantification of the Western blot shows that the sevoflurane anesthesia increases AT8(Tau-PS202 and Tau-PT205) levels in the hippocampus of the mice as compared with the control condition. (C) The sevoflurane anesthesia does not increase the levels of total Tau in the hippocampus of the mice (harvested at P8) as compared with the control condition. There is no significant difference in β -actin levels in hippocampus of the mice between the sevoflurane anesthesia and control condition. (D) Quantification of the Western blot shows that the sevoflurane anesthesia does not increase the levels of total Tau in the hippocampus of the mice as compared with the control condition. (E) Quantification of the Western blots (A and B) shows that the sevoflurane anesthesia increases the ratio of AT8(Tau-PS202 and Tau-PT205)/total Tau in the hippocampus of the mice as compared with the control condition. (F) Anesthesia with 3% sevoflurane 2 h daily for 1 day in P6 wild-type mice does not increase the levels of AT8(Tau-PS202 and Tau-PT205) in the hippocampus of the mice (harvested on P8) as compared with the control condition. There is no significant difference in β -actin levels in the hippocampus of the mice between the sevoflurane anesthesia and control condition. (G) Quantification of the Western blot shows that the sevoflurane anesthesia does not increase AT8(Tau-PS202 and Tau-PT205) levels in the hippocampus of the mice as compared with the control condition. (H) Anesthesia with 3% sevoflurane 2 h daily for 1 day does not increase total Tau levels in hippocampus of the mice (harvested on P8) as compared with control condition. There is no significant difference in β -actin levels in hippocampus of the mice between the sevoflurane anesthesia and control condition. (I) Quantification of the Western blot shows that the sevoflurane anesthesia does not increase the total Tau levels as compared with the control condition. (J) Quantification of the Western blots (F and H) shows that the sevoflurane anesthesia does not increase the ratio of AT8(Tau-PS202 and Tau-PT205)/total Tau in the hippocampus of the mice as compared with the control condition. AT8 = antibody to detect Tau-PS202 and Tau-PT205; N.S. = nonsignificant; P = phosphorylated; Tau-PS202 = Tau phosphorylated at serine-202; Tau-PT205 = Tau phosphorylated at threonine 205. ** $P < 0.01$; N = 6 in each group.

determine the difference between the anesthesia and control groups in the levels of IL-6, total Tau, AT8(Tau-PS202 and Tau-PT205), Tau-PS262, GSK3 β , phosphorylated GSK3 β , and PSD-95, which tested a hypothesis that there was a difference in these measurements between sevoflurane anesthesia and the control condition. The nature of the hypothesis testing was two tailed. *P* values less than 0.05 were considered statistically significant. SAS software (Cary, NC) and Prism 6 software (La Jolla, CA) were used to analyze the data.

Results

Multiple, but Not Single, Exposures of Sevoflurane in Young WT Mice Induced Tau Phosphorylation in Hippocampus of the Mice

Tau phosphorylation may contribute to the cognitive impairment observed in rodents after anesthesia and surgery (reviewed in the study by Whittington *et al.*³³). Anesthesia with 3% sevoflurane 2 h daily for 3 days (multiple exposures of sevoflurane) in P6 WT mice has been reported to induce cognitive impairment in the mice.⁹ We therefore assessed the effects of the multiple exposures of sevoflurane on Tau phosphorylation in hippocampus of the mice.

The mice were treated with 3% sevoflurane anesthesia 2 h daily for 1 day at P6 or for 3 days from P6 to P8. As compared with the control mice, the anesthetized mice did not show significant changes in behavior (*e.g.*, eating, drinking, and general activity) after the anesthesia. There was no significant difference in body temperature ($37.03^{\circ} \pm 0.12^{\circ}\text{C}$ *vs.* $36.93^{\circ} \pm 0.15^{\circ}\text{C}$ at P8, *P* = 0.417, *n* = 3, Student *t* test) and body weight (18.07 ± 2.19 g *vs.* 16.73 ± 2.20 g at P31, *P* = 0.215, *n* = 6, Student *t* test) between the mice in the anesthesia group and the mice in the control group. Finally, there was no significant difference in learning and memory function between the mice that received 60% oxygen and the mice that received 21% oxygen (data not shown).

The hippocampus of each of the mice was harvested and subjected to Western blot analysis at the end of the anesthesia at P8. Immunoblotting of AT8, detected phosphorylated Tau at serine-202 (Tau-PS202) and at tyrosine 205 (Tau-PT205), showed that there was a visible increase in the levels of AT8 fragment in the hippocampus of the mice after the sevoflurane anesthesia (lanes 5 to 8, fig. 1A) as compared with those of the mice after the control condition (lanes 1 to 4, fig. 1A). There was no significant difference in the levels of β -actin in the hippocampus of the sevoflurane-treated mice and the control mice. Quantification of the Western blot, based on the ratio of the levels of AT8 to β -actin, showed that the sevoflurane anesthesia (black bar, fig. 1B) increased AT8 levels as compared with the control condition (white bar, fig. 1B): 257 *versus* 100%, *P* = 0.0025 (Student *t* test). Immunoblotting of total Tau showed that there was no significant difference in the levels of total Tau or β -actin between the sevoflurane anesthesia (lanes 5 to 8, fig. 1C) and control condition (lanes 1 to 4, fig. 1C). Quantification of the Western blot, based on the ratio of total Tau to β -actin,

showed that the sevoflurane anesthesia (black bar, fig. 1D) did not significantly increase the total Tau levels as compared with control condition: 88 *versus* 100%, *P* = 0.1900 (Student *t* test). Finally, Quantification of the Western blot showed that the sevoflurane anesthesia increased the ratio of AT8(Tau-PS202 and Tau-PT205)/total Tau as compared with control condition: 285 *versus* 100%, *P* = 0.0004 (Student *t* test) (fig. 1E). These data suggested that the anesthesia with 3% sevoflurane for 2 h daily for 3 days in 6-day-old WT mice was able to induce Tau phosphorylation in the hippocampus of the mice at P8.

Next, we asked whether a single exposure to sevoflurane anesthesia in young (P6) mice would not induce Tau phosphorylation in the hippocampus of the mice. The mice were treated with 3% sevoflurane anesthesia 2 h daily for 1 day at P6. The hippocampus of each of the mice was harvested at P8. Quantitative Western blot analysis showed that the sevoflurane anesthesia did not significantly affect the levels of AT8(Tau-PS202 and Tau-PT205) (fig. 1, F and G), total Tau (fig. 1, H and I), or the ratio of AT8/total Tau (fig. 1J) in the hippocampus of the mice as compared with control condition.

The hippocampus of each of the mice was harvested at 2 days (P8) after the single exposure of sevoflurane at P6, but at the same day (P8) after the multiple exposures of sevoflurane. To confirm that the Tau phosphorylation was not due to the acute effects of single exposure of sevoflurane rather than the multiple exposures of sevoflurane, we assessed the effects of single sevoflurane exposure on AT8 and total Tau levels at P6 (the same day) and the effects of multiple sevoflurane exposures on AT8 and total Tau levels at P10 (2 days after the last sevoflurane anesthesia exposure). We found that the single exposure of sevoflurane anesthesia did not induce Tau phosphorylation at P6 (fig. 2, A and B), but the multiple exposures of sevoflurane still induced Tau phosphorylation at P10 (fig. 2, C and D) in the hippocampus of mice. Moreover, the multiple exposures of sevoflurane (3% sevoflurane 2 h daily for 3 days) did not increase Tau-PS262 levels in the hippocampus of mice (fig. 2, E and F).

Taken together, these data suggested the selective anesthesia neurotoxicity in young mice that only multiple exposures of anesthetic sevoflurane in young mice were able to induce Tau phosphorylation at serine-202 (Tau-PS202) and tyrosine 205 (Tau-PT205), but not serine 262 (Tau-PS262), in the hippocampus of the mice.

The Effects of the Sevoflurane Anesthesia on the Levels of Kinases and Phosphatase Related to Tau Phosphorylation in the WT Mice

GSK3 β , CDK5 (P25 and P35), JNK, and ERK are the kinases related to Tau phosphorylation (reviewed in the study by Wang *et al.*³²). PP1 is the phosphatase for dephosphorylation of Tau-PS202 and Tau-PT205.⁴⁰ We therefore assessed the effects of the anesthesia with 3% sevoflurane

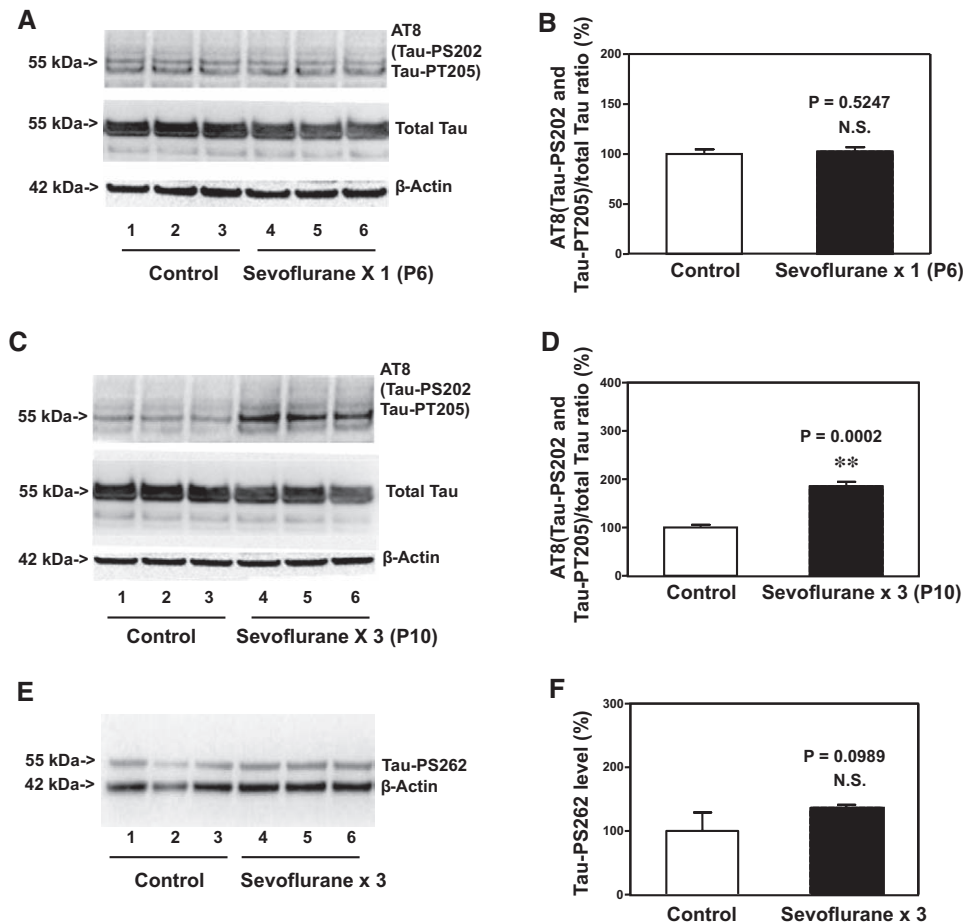


Fig. 2. The effects of the sevoflurane anesthesia on Tau phosphorylation are not dependent on the time of harvest. (A) Anesthesia with 3% sevoflurane 2 h daily for 1 day in P6 wild-type mice does not increase the levels of AT8(Tau-PS202 and Tau-PT205) and total Tau in the hippocampus of the mice (harvested on P6) as compared with the control condition. There is no significant difference in β -actin levels in the hippocampus of the mice between the sevoflurane anesthesia and the control condition. (B) Quantification of the Western blot shows that the sevoflurane anesthesia does not increase the ratio of AT8(Tau-PS202 and Tau-PT205) to total Tau in the hippocampus of the mice as compared with the control condition. (C) Anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 mice increases the levels of AT8(Tau-PS202 and Tau-PT205), but not total Tau, in the hippocampus of the mice (harvested on P10) as compared with the control condition. There is no significant difference in β -actin levels in the hippocampus of the mice between the sevoflurane anesthesia and the control condition. (D) Quantification of the Western blot shows that the sevoflurane anesthesia increases the ratio of AT8(Tau-PS202 and Tau-PT205) to total Tau in the hippocampus of the mice as compared with the control condition. (E) Anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 wild-type mice does not increase the levels of Tau-PS262 in the hippocampus of the mice (harvested on P8) as compared with the control condition. There is no significant difference in β -actin levels in the hippocampus of the mice between the sevoflurane anesthesia and control condition. (F) Quantification of the Western blot shows that the sevoflurane anesthesia does not increase Tau-PS262 levels in the hippocampus of the mice as compared with the control condition. AT8 = antibody to detect Tau-PS202 and Tau-PT205; N.S. = nonsignificant; P = phosphorylated; Tau-PS202 = Tau phosphorylated at serine-202; Tau-PS262 = Tau phosphorylated at serine 262; Tau-PT205 = Tau phosphorylated at threonine 205. ** $P < 0.01$; N = 6 in each group.

for 2 h daily for 3 days on the levels of these kinases and PP1 in the hippocampus of mice.

Phosphorylated (P)-GSK3 β (ser9) immunoblotting showed that there was a visible reduction in the levels of P-GSK3 β (ser9) (fig. 3A) in the hippocampus of the mice after the sevoflurane anesthesia as compared with control condition. Quantification of the Western blot showed that the sevoflurane anesthesia (black bar, fig. 3B) reduced the levels of P-GSK3 β (ser9) as compared with control

condition: 64 versus 100%, $P = 0.0019$ (Student t test). GSK3 β immunoblotting showed that the anesthesia with 3% sevoflurane for 2 h daily for 3 days did not cause visible reductions in GSK3 β levels as compared with control condition (fig. 3C) in the hippocampus of the mice. Quantification of the Western blot showed that the sevoflurane anesthesia did not decrease GSK3 β levels as compared with control condition (fig. 3D). Quantification of Western blots (fig. 3, A and C) showed that the sevoflurane anesthesia

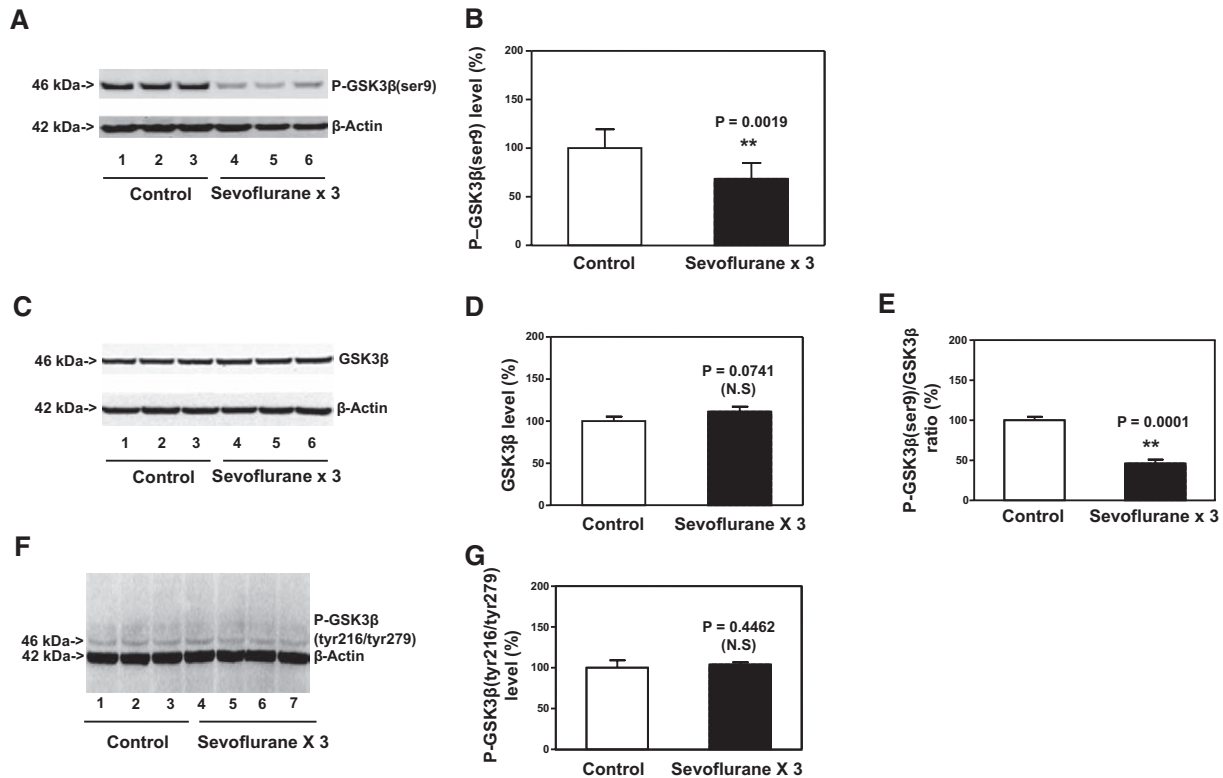


Fig. 3. Anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 wild-type mice decreases P-GSK3β(ser9) levels, but does not alter the levels of GSK3β and P-GSK3β(tyr279/tyr216), in hippocampus of the mice. (A) Anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 mice decreases the levels of P-GSK3β(ser9) in the hippocampus of the mice as compared with the control condition. There is no significant difference in β-actin levels in the hippocampus of the mice between the sevoflurane anesthesia and control condition. (B) Quantification of the Western blot shows that the sevoflurane anesthesia decreases P-GSK3β(ser9) levels as compared with control condition. (C) Anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 mice does not significantly alter the GSK3β levels in the hippocampus of the mice as compared with the control condition. There is no significant difference in β-actin levels in the hippocampus of the mice between the sevoflurane anesthesia and control condition. (D) Quantification of the Western blot shows that the sevoflurane anesthesia does not significantly alter the GSK3β levels as compared with the control condition. (E) Quantification of the Western blot (A and C) shows that the sevoflurane anesthesia decreases the ratio of P-GSK3β(ser9)/GSK3β as compared with the control condition. (F) Anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 mice does not significantly alter the P-GSK3β(tyr279/tyr216) levels in the hippocampus of the mice as compared with the control condition. There is no significant difference in β-actin levels in the hippocampus of the mice between the sevoflurane anesthesia and control condition. (G) Quantification of the Western blot (F) shows that the sevoflurane anesthesia does not significantly alter the P-GSK3β(tyr279/tyr216) levels as compared with the control condition. GSK-3β = glycogen synthase kinase 3β; N.S. = nonsignificant; P = phosphorylated; ser9 = serine 9; tyr216 = tyrosine 216; tyr279 = tyrosine 279. ** $P < 0.01$; $N = 6$ in each group.

(black bar) reduced the ratio of P-GSK3β(ser9)/GSK3β as compared with control condition in the hippocampus of the mice (white bar): 48 versus 100%, $P = 0.0001$ (Student t test; fig. 3E). Finally, the sevoflurane anesthesia did not decrease the levels of P-GSK3β(tyr279/tyr216) as compared with control condition in the hippocampus of the mice (fig. 3, F and G). These data suggested that the anesthesia with 3% sevoflurane 2 h daily for 3 days was able to decrease P-GSK3β(ser9) levels, leading to reductions in the phosphorylation at serine 9 of GSK3β. Reductions in the phosphorylation at serine 9 of GSK3β enhance the activation of GSK3β.^{54–59} Collectively, these findings suggested that the sevoflurane anesthesia could enhance GSK3β activation by decreasing the phosphorylation at serine 9 of

GSK3β, leading to Tau phosphorylation in the hippocampus of the mice.

The sevoflurane anesthesia did not significantly alter the levels of CDK5 (fig. 4, A and B), P25, and P35 (fig. 4, C–E), the ratio of phosphorylated-JNK/JNK (fig. 4, F and G), and the ratio of phosphorylated-ERK/ERK (fig. 4, H and I) in the hippocampus of the mice. Finally, the sevoflurane anesthesia did not significantly alter the levels of PP1 in the hippocampus of the mice (fig. 4, J and K). These data suggested that the anesthesia with 3% sevoflurane 2 h daily for 3 days induced Tau phosphorylation through the activation of GSK3β, but not the changes in CDK5, JNK, ERK, or PP1.

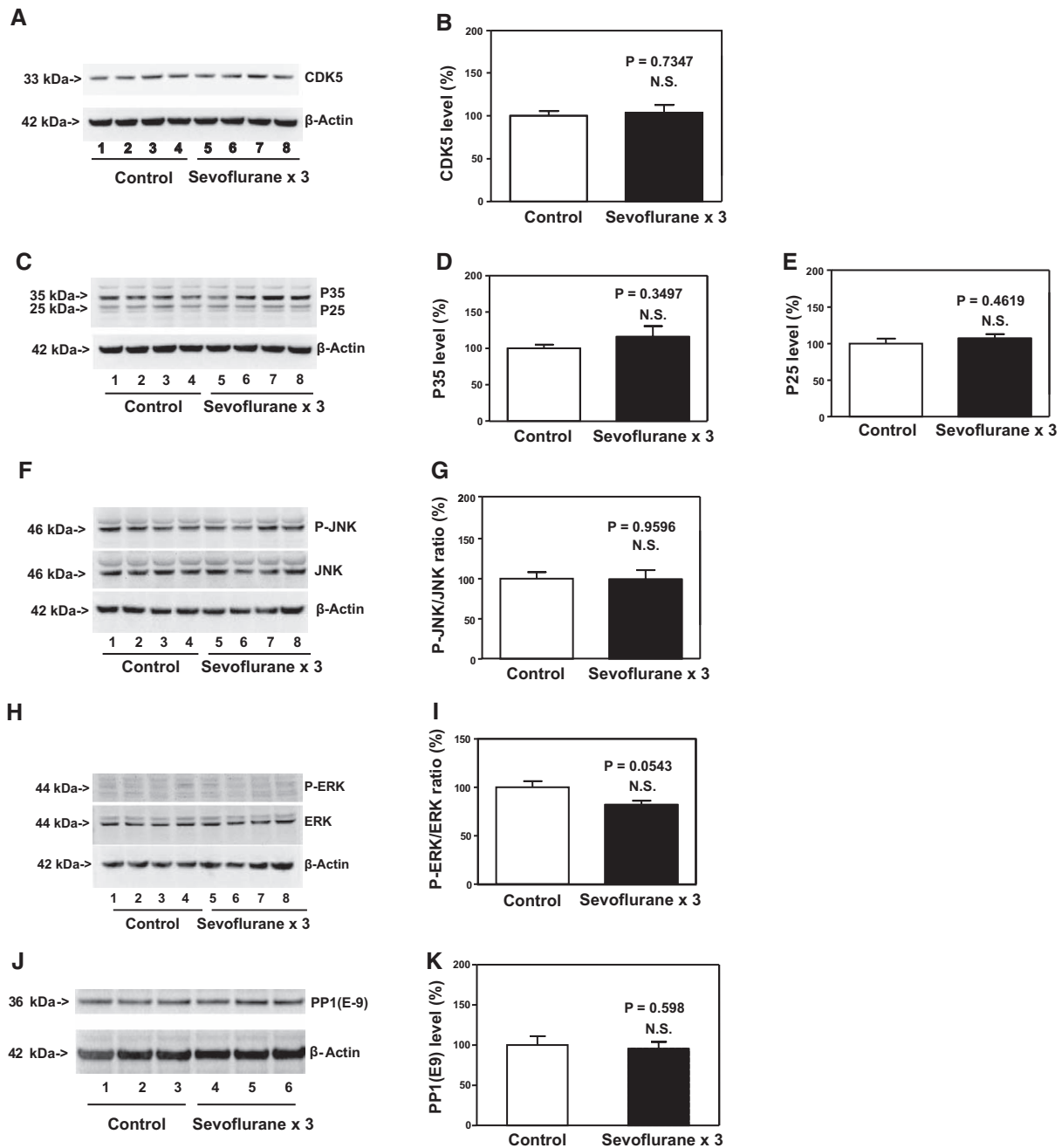


Fig. 4. Anesthesia with 3% sevoflurane 2h daily for 3 days in P6 wild-type mice does not affect the levels of other kinases of Tau phosphorylation and Tau protein phosphatases in hippocampus of the mice. The sevoflurane anesthesia does not significantly alter the levels of cyclin-dependent kinase 5 (CDK5) (A and B), P35 and P25 (C–E), ratio of phosphorylated cJun N-terminal kinase (JNK) to JNK (F and G), ratio of phosphorylated extracellular signal-regulated kinase (ERK) to ERK (H and I), and protein phosphatase 1 (PP1) (J and K) in the hippocampus of the mice as compared with the control condition. There is no significant difference in β -actin levels in hippocampus of the mice between the sevoflurane anesthesia and control condition. GSK3 β = glycogen synthase kinase 3 β ; N.S. = nonsignificant; P = phosphorylated. N = 6 in each group.

The Effects of the Sevoflurane Anesthesia on the Levels of Tau, GSK3 β , and PSD-95 in the Hippocampus of WT Mice at P31

Next, we asked whether the sevoflurane anesthesia-induced changes in Tau and GSK3 β levels still occurred at P31. The immunoblotting of AT8(Tau-PS202 and Tau-PT205) and

total Tau showed that the sevoflurane anesthesia did not significantly alter the ratio of AT8(Tau-PS202 and Tau-PT205) to total Tau (fig. 5, A and B), levels of Tau-PS262 (fig. 5, C and D), levels of P-GSK3 β (ser9) (fig. 5, E and F), or GSK3 β levels (fig. 5, G and H), and ratio of P-GSK3 β (ser9) to GSK3 β (fig. 5I) in the hippocampus of mice (harvested

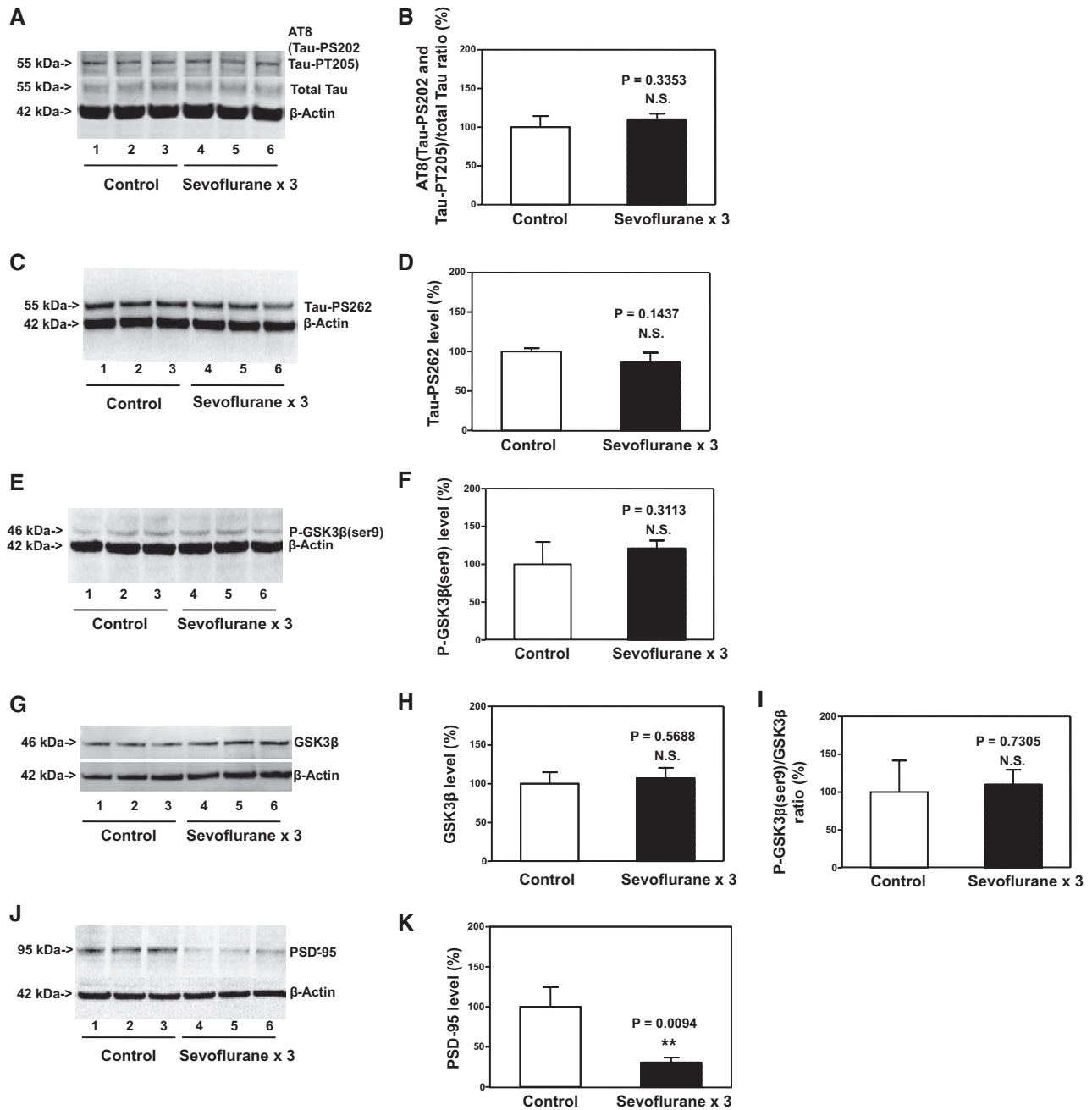


Fig. 5. Anesthesia with 3% sevoflurane 2h daily for 3 days in P6 wild-type mice does not induce Tau phosphorylation or glycogen synthase kinase 3 β (GSK3 β) activation, but decreases postsynaptic density protein-95 (PSD-95) levels in hippocampus of mice at P31. Anesthesia with 3% sevoflurane 2h daily for 3 days in P6 wild-type mice does not significantly affect the levels of AT8(Tau-PS202 and Tau-PT205) and total Tau (A and B), Tau-PS262 (C and D), P-GSK3 β (ser9) (E and F), GSK3 β (G and H), and the ratio of P-GSK3 β (ser9)/GSK3 β (I) in the hippocampus of the mice as compared with the control condition at P31. However, the anesthesia with 3% sevoflurane 2h daily for 3 days at P6 decreases the levels of PSD-95 in the hippocampus of mice as compared with the control condition at P31 (J). There is no significant difference in β -actin levels in the hippocampus of the mice between the sevoflurane anesthesia and control condition. Quantification of the Western blot (J) shows that the sevoflurane anesthesia at P6 decreases the PSD-95 levels in the hippocampus of mice at P31 (K). AT8 = antibody to detect Tau-PS202 and Tau-PT205; N.S. = nonsignificant; P = phosphorylated; ser9 = serine 9; Tau-PS202 = Tau phosphorylated at serine-202; Tau-PT205 = Tau phosphorylated at threonine 205. ** $P < 0.01$; N = 6 in each group.

at P31) as compared with the control condition. However, the sevoflurane anesthesia reduced the levels of PSD-95, a postsynaptic marker,^{47,48} in the hippocampus of the mice (harvested at P31) as compared with the control condition

(fig. 5, J and K): 27 versus 100%, $P = 0.0094$. These data suggested that the sevoflurane anesthesia-induced Tau phosphorylation might lead to cognitive impairment through, at least partially, a synapse-related mechanism.

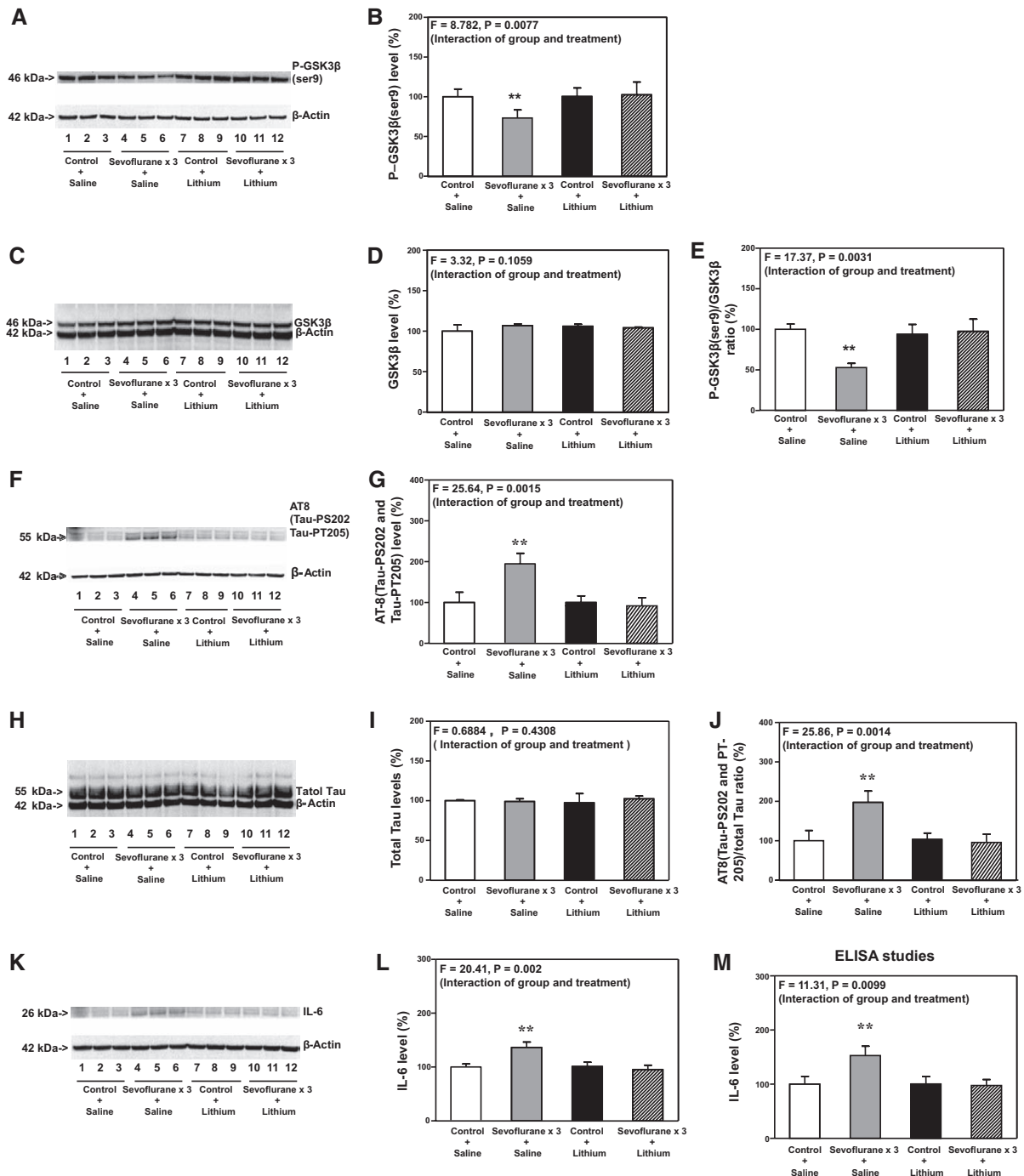


Fig. 6. Lithium attenuates the sevoflurane-induced glycogen synthase kinase 3 β (GSK3 β) activation, Tau phosphorylation, and increase in interleukin-6 (IL-6) in hippocampus of mice. (A) Anesthesia with 3% sevoflurane for 2 h daily for 3 days reduces the levels of P-GSK3 β (ser9) (lanes 4 to 6) as compared with control condition (lanes 1 to 3). Lithium treatment alone (lanes 7 to 9) does not alter the levels of P-GSK3 β (ser9) as compared with control condition, but the lithium treatment (lanes 10 to 12) attenuates the sevoflurane-induced reduction in the P-GSK3 β (ser9) levels (lanes 4 to 6). There is no significant difference in the β -actin levels among these treatments. (B) Quantification of the Western blot shows that lithium (net bar) attenuates the sevoflurane-induced reduction in the P-GSK3 β (ser9) levels (gray bar) ($F = 8.782$, $P = 0.0077$, two-way ANOVA). There is no significant interaction of sevoflurane and lithium on the GSK3 β levels (C and D). (E) The quantification of the Western blots (A and C) shows that lithium attenuates the sevoflurane anesthesia-induced reduction in the ratio of P-GSK3 β (ser9)/GSK3 β (gray bar vs. net bar, $F = 17.37$, $P = 0.0031$, two-way ANOVA). (F) Anesthesia with 3% sevoflurane for 2 h daily for 3 days increases the levels of AT8 (Tau-PS202 and Tau-PT205) (lanes 4 to 6) as compared with control condition (lanes 1 to 3). Lithium treatment alone (lanes 7 to 9) does not alter the levels of AT8 as compared with control condition, but the lithium treatment (lanes 10 to 12) attenuates the sevoflurane-induced increase in the AT8 levels (lanes 4 to 6). There is no significant difference in the

Lithium Attenuated the Sevoflurane Anesthesia–induced GSK3 β Activation, Tau Phosphorylation, Increased Levels of IL-6, and Cognitive Impairment in Young WT Mice

The sevoflurane anesthesia might induce Tau phosphorylation through enhancing the activation of GSK3 β (fig. 3), and lithium has been reported to inhibit Tau phosphorylation by reducing GSK3 β activation.⁴⁹ Therefore, we assessed the effects of lithium on the sevoflurane anesthesia–induced GSK3 β activation, Tau phosphorylation, increased levels of IL-6 in the hippocampus of young WT mice, and cognitive impairment in the mice.

P-GSK3 β (ser9) immunoblotting showed that whereas lithium treatment alone (lanes 7 to 9) did not significantly alter the levels of P-GSK3 β (ser9) as compared with the control condition (lanes 1 to 3), the lithium treatment (lanes 10 to 12) reduced the sevoflurane anesthesia–induced reduction in P-GSK3 β (ser9) levels (lanes 4 to 6) in the hippocampus of the young WT mice (fig. 6A). The quantification of the Western blot showed that there was a significant interaction between the group (control *vs.* sevoflurane) and treatment (saline and lithium) ($F = 8.782$, $P = 0.0077$, two-way ANOVA), and the lithium attenuated the sevoflurane anesthesia–induced reduction in P-GSK3 β (ser9) levels in the hippocampus of young WT mice (fig. 6B). The quantitative Western blot analysis (fig. 6, C and D) showed that there was no significant interaction between the group (control *vs.* sevoflurane) and treatment (saline and lithium) ($F = 3.32$, $P = 0.1059$, two-way ANOVA) on the total GSK3 β levels in the

hippocampus of young WT mice. Next, two-way ANOVA showed that there was a significant interaction between the group (control *vs.* sevoflurane) and treatment (saline and lithium) ($F = 17.37$, $P = 0.0031$, two-way ANOVA), and the lithium attenuated the sevoflurane anesthesia–induced reduction in P-GSK3 β (ser9)/GSK3 β ratio in the hippocampus of young WT mice (fig. 6E).

We further found that lithium mitigated the sevoflurane anesthesia–induced increase in the levels of AT8 in the hippocampus of the young WT mice (fig. 6, F and G): $F = 25.64$, $P = 0.0015$, two-way ANOVA. There was no significant interaction between the group (control *vs.* sevoflurane) and treatment (saline and lithium) ($F = 0.6884$, $P = 0.4308$, two-way ANOVA) on the total Tau levels in the hippocampus of young WT mice (fig. 6, H and I). Two-way ANOVA showed that there was a significant interaction between the group (control *vs.* sevoflurane) and treatment (saline and lithium) ($F = 25.86$, $P = 0.0014$, two-way ANOVA) on the ratio of AT8(Tau-PS202 and Tau-PT205)/total Tau, and the lithium attenuated the sevoflurane anesthesia–induced increases in the ratio of AT8(Tau-PS202 and Tau-PT205)/total Tau in the hippocampus of the young WT mice (fig. 6J). Western blot (fig. 6, K and L, $F = 20.41$ and $P = 0.002$, two-way ANOVA) and enzyme-linked immunosorbent assay studies (fig. 6M; $F = 11.31$, $P = 0.0099$, two-way ANOVA) showed that lithium also attenuated the sevoflurane anesthesia–induced increase in IL-6 levels in the hippocampus of the young WT mice.

Finally, two-way ANOVA showed that the sevoflurane anesthesia did not increase the escape latency of MWM from P31 to P35 as compared with control condition with the pretreatment of lithium ($F = 0.4748$, $P = 0.7541$) (fig. 7A). The Mann–Whitney test showed that there was no significant difference in the platform crossing times between the mice after the treatment of control plus lithium and the mice after the treatment of sevoflurane plus lithium tested at P35 (fig. 7B; $P = 0.1187$). In contrast, with the pretreatment of saline, the sevoflurane anesthesia increased the escape latency of MWM (tested from P31 to P35) ($F = 3.096$, $P = 0.0195$, two-way ANOVA) and reduced the platform crossing times (tested at P35) ($P = 0.0208$, Mann–Whitney test) as compared with control condition (fig. 7, C and D). These data suggested that lithium was able to attenuate the sevoflurane anesthesia–induced GSK3 β activation, Tau phosphorylation, increased levels of IL-6 in the hippocampus of young WT mice, and cognitive impairment in the mice.

Multiple Exposures of Sevoflurane in Young Tau KO Mice Induced GSK3 β Activation but Not Increase in IL-6, Reduction in PSD-95, or Cognitive Impairment in the Mice

Next, we further assessed the role of Tau in these effects by using Tau KO mice. The Tau KO mice were treated with 3% sevoflurane anesthesia 2 h daily for 3 days from P6 to P8. The hippocampus of each of the mice was harvested and subjected to Western blot analysis at P8 and P31,

Fig. 6. (Continued) β -actin levels among these treatments. (G) Quantification of the Western blot shows that lithium (*net bar*) attenuates the sevoflurane-induced increase in the AT8(Tau-PS202 and Tau-PT205) levels (*gray bar*) ($F = 25.64$, $P = 0.0015$, two-way ANOVA). There is no significant interaction of sevoflurane and lithium on the total Tau levels (H and I). (J) The quantification of the Western blots (F and H) shows that lithium attenuates the sevoflurane anesthesia–induced increase in the ratio of AT8/total Tau (*gray bar vs. net bar*, $F = 25.86$, $P = 0.0014$, two-way ANOVA). (K) Anesthesia with 3% sevoflurane for 2 h daily for 3 days increases the levels of IL-6 (lanes 4 to 6) as compared with control condition (lanes 1 to 3). Lithium treatment alone (lanes 7 to 9) does not alter the levels of IL-6 as compared with control condition, but the lithium treatment (lanes 10 to 12) attenuates the sevoflurane-induced increase in the IL-6 levels (lanes 4 to 6). There is no significant difference in the β -actin levels among these treatments. (L) Quantification of the Western blot shows that lithium (*net bar*) attenuates the sevoflurane anesthesia–induced increase in the IL-6 levels (*gray bar*) ($F = 20.41$, $P = 0.002$, two-way ANOVA). (M) Enzyme-linked immunosorbent assay (ELISA) studies show that lithium (*net bar*) attenuates the sevoflurane anesthesia–induced increase in the IL-6 levels (*gray bar*) ($F = 11.31$, $P = 0.0099$, two-way ANOVA). AT8 = antibody to detect Tau-PS202 and Tau-PT205; P = phosphorylated; ser9 = serine 9; Tau-PS202 = Tau phosphorylated at serine-202; Tau-PT205 = Tau phosphorylated at threonine 205. ** $P < 0.01$; N = 6 in each group.

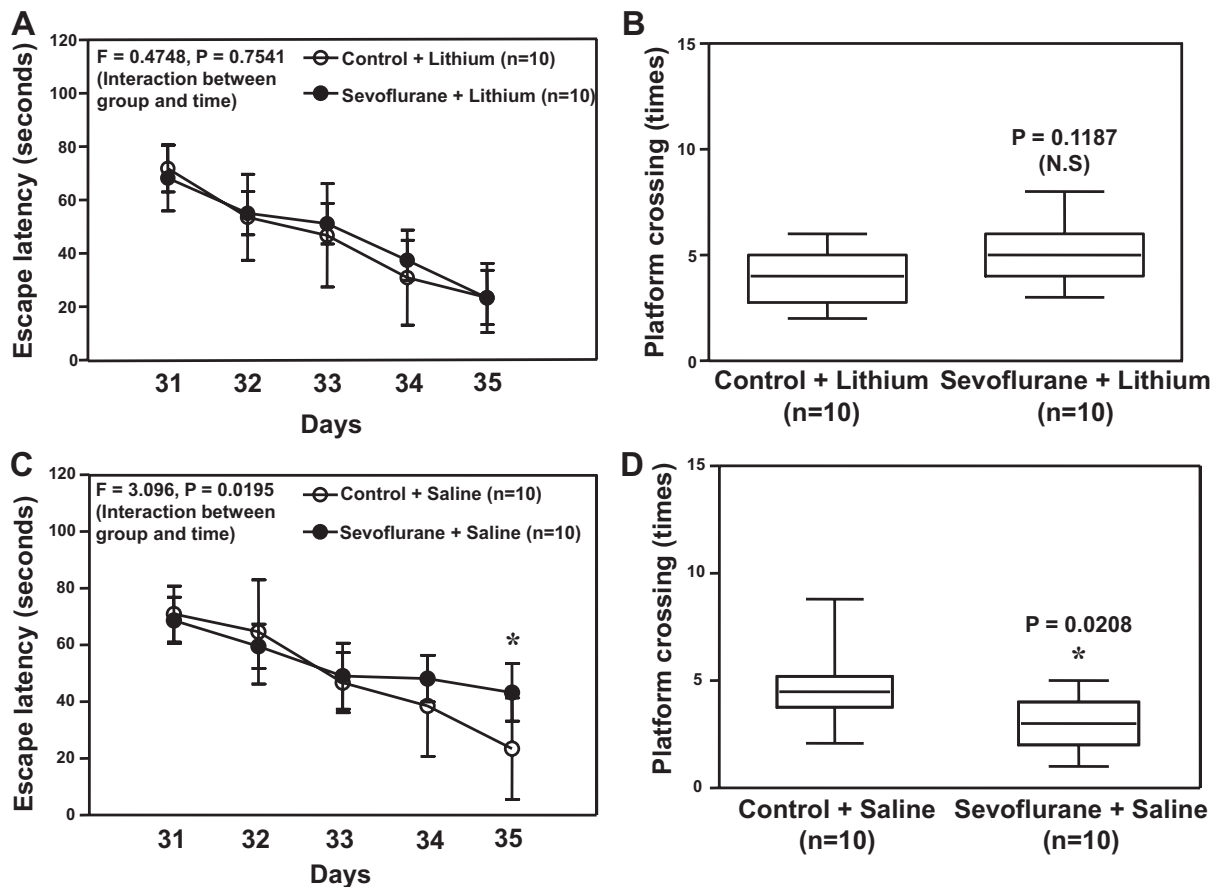


Fig. 7. Lithium mitigates the sevoflurane-induced cognitive impairment in young mice. (A) Two-way ANOVA with repeated-measurement analysis shows that there is no statistically significant interaction of treatment and group based on the escape latency of Morris Water Maze (MWM) between the control plus lithium-treated (N = 10 in each group) and sevoflurane (3% for 2 h daily for 3 days) plus lithium-treated (N = 10 in each group) mice. (B) The Mann-Whitney test shows that there is no significant difference in platform crossing times of mice swimming in the MWM between the control plus lithium (N = 10 in each group) and sevoflurane plus lithium treatments (N = 10 in each group). (C) Two-way ANOVA with repeated-measurement analysis shows that there is statistically significant interaction of treatment and group based on escape latency of MWM between the control plus saline-treated (N = 10 in each group) and sevoflurane (3% for 2h daily for 3 days) plus saline-treated (N = 10 in each group) mice ($F = 3.096$, $P = 0.0195$). The *post hoc* (Bonferroni) test shows that the mice after sevoflurane plus saline treatment has longer escape latency compared with that in the mice after control plus saline treatment at day P35. (D) The Mann-Whitney test shows that the mice after sevoflurane plus saline treatment (N = 10 in each group) have less platform crossing times compared with that in the mice after control plus saline treatment (N = 10 in each group) in the MWM test. N.S. = nonsignificant. $*$ $P < 0.05$.

respectively. Quantitative Western blot analysis showed that the sevoflurane anesthesia reduced P-GSK3 β (ser9) (fig. 8, A and B), but not GSK3 β (fig. 8, C and D) levels, and decreased the ratio of P-GSK3 β (ser9) to GSK3 β in the hippocampus of the mice (fig. 8E) at P8. However, quantitative Western blot analysis (fig. 8, F and G) and enzyme-linked immunosorbent assay studies (fig. 8H) showed that the sevoflurane anesthesia did not increase the levels of IL-6 in the hippocampus of the Tau KO mice at P8. Moreover, the sevoflurane anesthesia did not reduce PSD-95 levels in the hippocampus of the Tau KO mice at P31 (fig. 8, I and J). Finally, the MWM studies showed that the sevoflurane anesthesia did not increase escape latency (fig. 8K) and did not reduce platform crossing times (fig. 8L) as compared with the control condition in the Tau KO mice.

Taken together, these data suggested that the sevoflurane anesthesia-induced increase in IL-6 levels and reduction in PSD-95 levels in hippocampus of young mice and cognitive impairment in the mice were dependent on the sevoflurane anesthesia-induced Tau phosphorylation in the hippocampus of the mice.

Discussion

We found that anesthesia with 3% sevoflurane 2 h daily for 3 days, but not for 1 day, in young WT mice induced Tau phosphorylation at serine-202 and tyrosine 205 (fig. 1), but not at serine 262 (Tau-PS262), in hippocampus of the mice. Moreover, although the multiple exposures of sevoflurane from P6 to P8 still induced Tau phosphorylation at P10 (fig. 2, C and D), the single exposure of sevoflurane

anesthesia at P6 did not induce Tau phosphorylation at P6 (fig. 2, A and B). These findings suggested that the Tau phosphorylation resulted from multiple exposures of sevoflurane but not a single exposure of sevoflurane, which were consistent with the results from the studies by Shen *et al.*⁹ and Le Freche *et al.*⁴⁵

GSK3 β is one of the kinases, the activation of which leads to Tau phosphorylation.^{24–27} Phosphorylation at serine 9 of GSK3 β inhibits the activation of GSK3 β .^{54–59} The current findings that the sevoflurane anesthesia reduced the levels of P-GSK3 β (at serine 9) and the ratio of P-GSK3 β (at serine 9) to GSK3 β (fig. 3, A–E) suggested that the sevoflurane anesthesia in the young WT mice was able to induce GSK3 β activation in the hippocampus of the mice. GSK3 β has been reported to phosphorylate Tau protein at serine-46, threonine-50, threonine-181, serine-184, serine-199, serine-202, threonine-205, threonine-212, serine-214, threonine-217, and threonine-231.^{24–27} Consistently, AT8 antibody detects the phosphorylated Tau levels at serine-202 and threonine-205. The sevoflurane anesthesia did not decrease the levels of P-GSK3 β (tyr216/tyr279) (fig. 3, F and G). These data suggested that the sevoflurane anesthesia might specifically attenuate P-GSK3 β (ser9) levels, leading to activation of GSK3 β , and further studies are pending.

Interestingly, the sevoflurane anesthesia did not induce the activation of CDK5,^{25,28} JNK,^{29,30} or ERK³¹ (fig. 4, A–I), the kinases which may also induce Tau phosphorylation at the sites of serine-202 and threonine-205 (reviewed in the study by Wang *et al.*³²). Finally, the sevoflurane anesthesia did not significantly alter the levels of PP1, which is the phosphatase for Tau phosphorylation (fig. 4, J and K). These findings suggested that the sevoflurane anesthesia could enhance Tau phosphorylation, but not decrease phosphorylated Tau degradation, by inducing activation of specific kinase. Future studies to investigate why the sevoflurane anesthesia only induced activation of GSK3 β would be important to further understand the underlying mechanisms of anesthesia neurotoxicity in developing brain.

The anesthesia with 3% sevoflurane 2h daily for 3 days from P6 to P8 did not induce Tau phosphorylation and GSK3 β activation, but reduced PSD-95 levels, in the hippocampus of mice as compared with control condition at a later time (P31) (fig. 5). These data suggested that the sevoflurane anesthesia-induced synaptic dysfunction could be one of the down-stream consequences of the sevoflurane anesthesia-induced Tau phosphorylation, leading to cognitive impairment. Future studies to test this hypothesis are warranted.

Lithium is an inhibitor of GSK3 β .^{49,60} We found that lithium attenuated the sevoflurane anesthesia-induced GSK3 β activation, Tau phosphorylation, increased levels of IL-6 (fig. 6), and cognitive impairment (fig. 7) in the young mice. IL-6 has been reported to be associated with learning and memory impairment in animals^{61–63} and cognitive

dysfunction in patients.^{64–66} Collectively, these data from the cause–effect relation studies suggested that the sevoflurane anesthesia in the young mice could induce GSK3 β activation, which then caused Tau phosphorylation. The phosphorylation of Tau might increase the hippocampus levels of IL-6, leading to synaptic dysfunction and then cognitive impairment. These findings also suggested that lithium would treat the sevoflurane anesthesia-induced neurotoxicity and neurobehavioral deficits, and further studies are pending. Note that lithium is not a specific inhibitor of GSK3 β activation and has many other effects (reviewed in the study by Carter *et al.*⁶⁷). Thus, future studies may need to include other inhibitors of GSK3 β activation to further test the hypothesis that activation of GSK3 β is one of the up-stream mechanisms by which the sevoflurane anesthesia induces Tau phosphorylation. Moreover, the sevoflurane anesthesia in Tau KO mice still induced GSK3 β activation, but the sevoflurane anesthesia did not induce increases in IL-6 levels, or reduction in PSD-95 levels in the hippocampus of the mice, or cognitive impairment in the mice (fig. 8).

Several studies have suggested the potential association between Tau, neuroinflammation, and synapse. Specifically, enhanced neuroinflammation has been reported in transgenic mice expressing P301L Tau-mutant and Tau-tubulin kinase 1.^{68,69} Lipopolysaccharide-induced information can cause Tau hyperphosphorylation.^{70,71} Moreover, synaptic loss and microglia activation have been reported to occur preceding the appearance of tangle in a P301S Tau transgenic mice, and immunosuppression treatment with FK506 can mitigate the Tau pathology in the mice.⁷² Consistently, our current studies showed that the sevoflurane anesthesia induced Tau phosphorylation, increased IL-6 levels, and reduced postsynaptic marker PSD-95 levels in the hippocampus of young WT mice. These findings will promote more research to investigate the cascade relation of these sevoflurane anesthesia-induced effects, which could shed light on the underlying mechanisms of anesthesia neurotoxicity in the developing brain.

A recent study by Le Freche *et al.*⁴⁵ nicely showed that anesthesia with repeated exposures to sevoflurane in adult mice induced Tau phosphorylation in hippocampus and cognitive impairment in the mice. Consistently, we found that repeated exposures to sevoflurane in young mice induced Tau phosphorylation (fig. 1) and cognitive impairment (fig. 7). However, whereas Le Freche *et al.* found that sevoflurane anesthesia inhibited GSK3 β activation (fig. 5 in the article by Le Freche *et al.*), we found that sevoflurane anesthesia enhanced GSK3 β activation (fig. 3 in the current study). The reason for such difference remains unknown at the present time. Of note, the age of mice (6 to 8 days old *vs.* 5 to 6 months old) and the treatment of sevoflurane anesthesia (3% for 2h daily for 3 days *vs.* 2.5% for 1h monthly for 5 months) were different between the two studies. The studies by Planell *et al.*^{41,43} assessed the effects of anesthesia-induced hypothermia on Tau phosphorylation and found

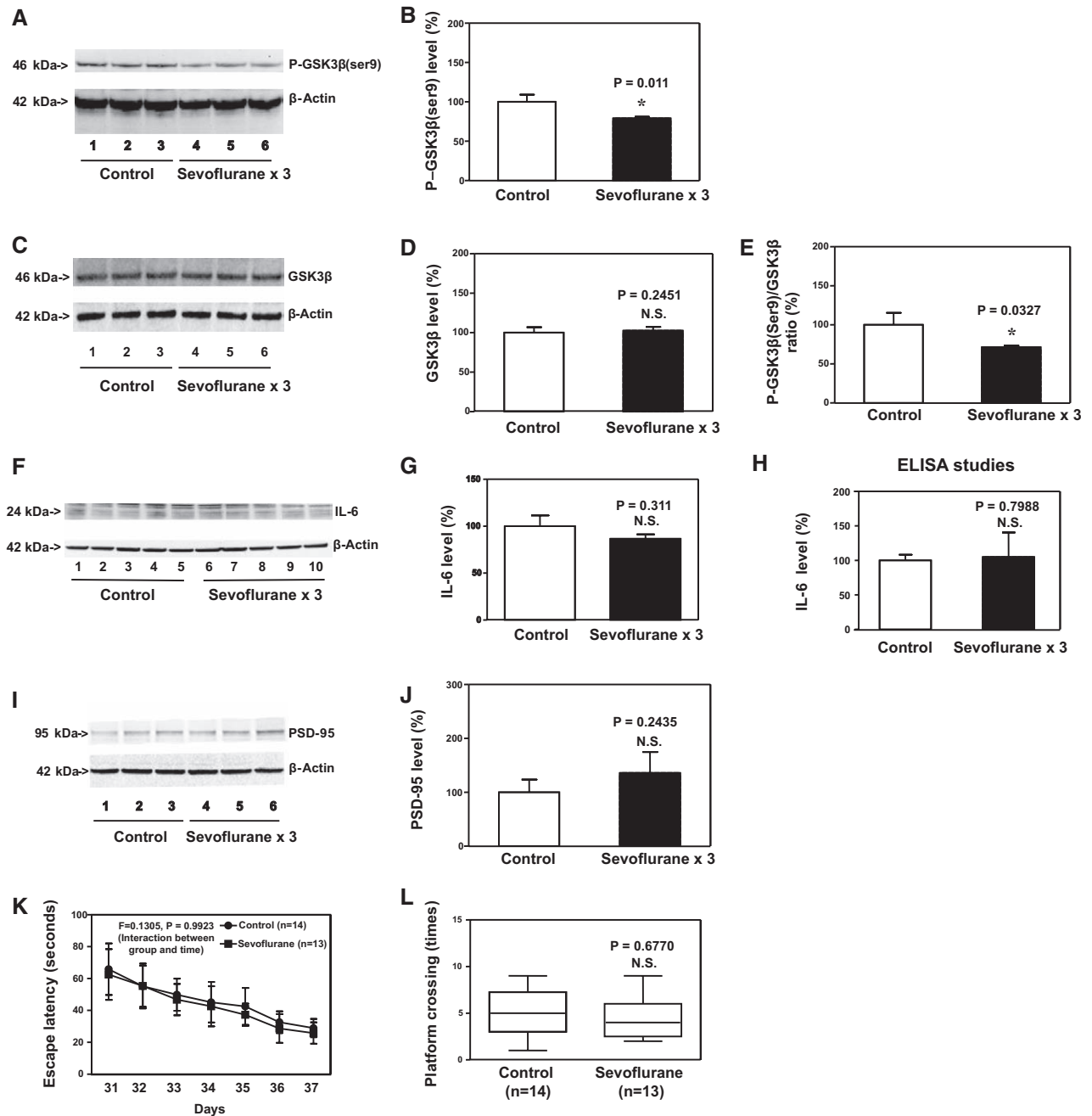


Fig. 8. Anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 Tau knockout (KO) mice induces glycogen synthase kinase 3 β (GSK3 β) activation. The sevoflurane anesthesia induces neither increases in interleukin-6 (IL-6) levels in hippocampus of the mice nor cognitive impairment in the mice. (A) The anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 Tau KO mice decreases P-GSK3 β (ser9) levels as compared with the control condition in the hippocampus of the mice (harvested on P8). There is no significant difference in β -actin levels in the hippocampus of the mice between the sevoflurane anesthesia and control condition. (B) Quantification of the Western blot (A) shows that the sevoflurane anesthesia decreases P-GSK3 β (ser9) levels as compared with control condition. (C) The anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 Tau KO mice does not significantly alter GSK3 β levels as compared with control condition in hippocampus of the mice (harvested on P8). There is no significant difference in β -actin levels in the hippocampus of the mice between the sevoflurane anesthesia and control condition. (D) Quantification of the Western blot (C) shows that the sevoflurane anesthesia does not significantly alter GSK3 β levels as compared with control condition. (E) Quantification of the Western blots (A and C) shows that the sevoflurane anesthesia decreases the ratio of P-GSK3 β (ser9) to GSK3 β in hippocampus of the mice. (F) The sevoflurane anesthesia does not increase the IL-6 levels in the hippocampus of the Tau KO mice as compared with the control condition. There is no significant difference in β -actin levels in hippocampus of the mice between the sevoflurane anesthesia and control condition. (G) Quantification of the Western blot shows that the sevoflurane anesthesia does not increase the IL-6 levels in the hippocampus of the Tau KO mice. (H) ELISA studies show that the sevoflurane anesthesia does not increase the IL-6 levels in the hippocampus of the Tau KO mice. (I) The sevoflurane anesthesia does not increase the PSD-95 levels in the hippocampus of the Tau KO mice as compared with the control condition. There is no significant difference in β -actin levels in hippocampus of the mice between the sevoflurane anesthesia and control condition. (J) Quantification of the Western blot shows that the sevoflurane anesthesia does not increase the PSD-95 levels in the hippocampus of the Tau KO mice. (K) The sevoflurane anesthesia does not increase the escape latency in the hippocampus of the Tau KO mice as compared with the control condition. There is no significant difference in escape latency between the sevoflurane anesthesia and control condition. (L) The sevoflurane anesthesia does not increase the platform crossing in the hippocampus of the Tau KO mice as compared with the control condition. There is no significant difference in platform crossing between the sevoflurane anesthesia and control condition.

that the hypothermia did not affect GSK3 β activation. Run *et al.*⁷³ used ether or sodium pentobarbital as the anesthetics in their studies to determine the effects of anesthesia on Tau phosphorylation in adult mice and found that the anesthesia decreased GSK3 β activation. The difference in the anesthesia and age of mice between these studies and our current experiments could cause the different findings in the GSK3 β activation observed.

Moreover, in an *in vitro* study, Zhang *et al.*⁷⁴ found that treatment with 4.1% sevoflurane for 6 h in mouse neural progenitor cells increased the levels of GSK3 β (fig. 3 in the article by Zhang *et al.*). The studies by Zhang *et al.*, however, did not measure the effects of sevoflurane on the levels of P-GSK3 β (ser9). Taken together, we postulate that sevoflurane may have a dose- and time-dependent dual effect (inhibition and enhancement) on the activation of the kinases related to Tau phosphorylation.

Wan *et al.*⁷⁵ found that 14-month-old mice with partial hepatectomy under anesthesia with 10% chloral hydrate and intradermal injections of 0.25% bupivacaine induced Tau phosphorylation. Sevoflurane (in the study by Le Freche *et al.*⁴⁵ and in the current study), isoflurane,⁴⁴ and propofol,⁴⁶ the commonly used anesthetics, have been shown to induce Tau phosphorylation. Therefore, it is important to determine whether these anesthetics can potentiate the surgery-induced Tau phosphorylation.

Fig. 8. (Continued) as compared with the control condition. (H) Enzyme-linked immunosorbent assay (ELISA) studies show that the sevoflurane anesthesia does not increase IL-6 levels in the hippocampus of the Tau KO mice (harvested on P8) as compared with the control condition. (I) The sevoflurane anesthesia does not decrease the postsynaptic density protein-95 (PSD-95) levels in the hippocampus of the Tau KO mice (harvested at P31) as compared with the control condition. There is no significant difference in β -actin levels in hippocampus of the mice between the sevoflurane anesthesia and control condition. (J) Quantification of the Western blot shows that the sevoflurane anesthesia does not decrease the PSD-95 levels in the hippocampus of the Tau KO mice (harvested at P31) as compared with the control condition. (K) Anesthesia with 3% sevoflurane 2 h daily for 3 days in P6 Tau KO mice does not increase the escape latency of mice swimming in the Morris Water Maze (MWM) as compared with the control condition (tested from P31 to P37). Two-way ANOVA with repeated-measurement analysis shows that there is no statistically significant interaction of treatment and group based on escape latency of MWM between mice following the control condition and mice following the sevoflurane anesthesia (3% for 2 h daily for 3 days) in the MWM (control: N = 14, sevoflurane: N = 13). (L) Anesthesia with 3% sevoflurane 2 h daily for 3 days in the P6 Tau KO mice does not reduce the platform crossing times of mice swimming in the MWM as compared with the control condition tested at P37 (control: N = 14, sevoflurane: N = 13; $P = 0.6770$). N.S. = nonsignificant; P = phosphorylated; ser9 = serine 9. * $P < 0.05$; N = 6 in each group (biochemistry studies); N = 13–14 in each group (behavioral studies).

The studies have several limitations. First, we did not compare the effects of sevoflurane and other inhalation anesthetics isoflurane and desflurane on Tau phosphorylation and cognitive impairment in the studies. This is mainly because multiple exposures of desflurane have been shown not to induce cognitive impairment in young mice⁹ and isoflurane is not often used in children. Second, we only used Western blot analysis to determine the protein levels of GSK3 β , P-GSK3 β (ser9), and P-GSK3 β (tyr216/tyr279), as well as the other kinases and PP1. This is mainly because we do not have the facilities to use the isotope to measure the activation of these kinases and phosphatase. However, the reduction in P-GSK3 β (ser9) has been well recognized as the indication of enhanced GSK3 β activation.^{54–59} Moreover, GSK3 β activation inhibitor lithium attenuated the sevoflurane anesthesia-induced Tau phosphorylation, which further suggested that sevoflurane could induce Tau phosphorylation *via* GSK3 β activation.

In conclusion, we found that anesthesia with 3% sevoflurane for 2 h daily for 3 days induced hippocampus GSK3 β activation, Tau phosphorylation at serine-202 and tyrosine 205, increase in IL-6 levels, and reduction in PSD-95 levels, as well as cognitive impairment in young WT mice. Lithium, an inhibitor of GSK3 β , attenuated these sevoflurane anesthesia-induced effects. The sevoflurane anesthesia did not induce increase in hippocampus IL-6 levels, or reduction in PSD-95 levels, or cognitive impairment in the young Tau KO mice. Note that there is currently no satisfactory way to extrapolate these findings in animals to clinical practice. The clinical relevance of the current findings remains to be further determined. However, these findings have illustrated the role of Tau phosphorylation in the anesthesia neurotoxicity in the developing brain and would promote more studies to investigate the underlying mechanisms and targeted interventions of the anesthesia neurotoxicity in the developing brain, ultimately leading to safer anesthesia care and better postoperative outcomes for children who could be vulnerable to brain damage.

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Competing Interests

The authors declare no competing interests.

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Pain Medicine, Massachusetts General Hospital and Harvard Medical School, 149 13th Street, Room 4310, Charlestown, Massachusetts 02129. zxie@mgh.harvard.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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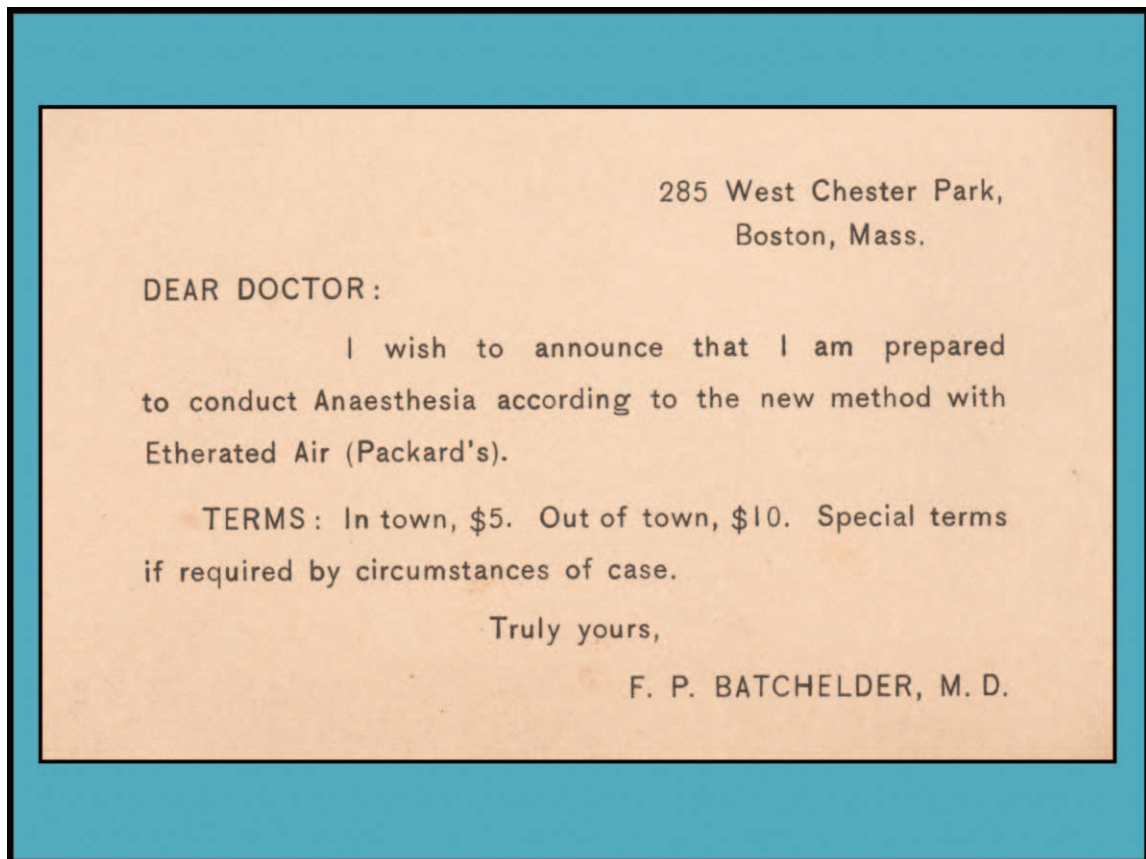
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Frederick P. Batchelder, M.D. (1868–1942), was a Bostonian homeopath who chose to specialize in delivering anesthetics. His trade card (*above*) sidesteps the ethics of “physician advertising” by announcing to fellow physicians that Dr. Batchelder was “prepared to conduct Anaesthesia according to the new method with Etherated Air (Packard’s).” By referring to the “new method” (an inhaler) designed ca. 1885 by his fellow Bostonian homeopath Horace Packard, M.D., Dr. Batchelder has helped us date his otherwise dateless trade card. (Copyright © the American Society of Anesthesiologists, Inc.)

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