Tau Phosphorylation and Sevoflurane Anesthesia

An Association to Postoperative Cognitive Impairment

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ABSTRACT

Background: There is a growing interest in the involvement of anesthetic agents in the etiology of postoperative cognitive dysfunction. Recent animal studies suggest that acute anesthesia induces transient hyperphosphorylation of tau, an effect essentially ascribed to hypothermia. The main aim of the present study was to investigate effects, in normothermic conditions, of acute or repeated exposure to sevoflurane, a halogenated anesthetic agent, on hippocampal tau phosphorylation and spatial memory in adult mice.

Methods: 5 to 6-month-old C57Bl6/J mice were submitted to acute (1 h) or repeated (five exposures of 1h every month) anesthesia using 1.5 or 2.5% sevoflurane, in normothermic conditions. In the acute protocol, animals were sacrificed 1 and 24 h after exposure. In the chronic protocol, spatial memory was evaluated using the Morris water maze following the fourth exposure, and tau phosphorylation evaluated 1 month following the last exposure using bi- and mono-dimensional electrophoresis.

Results: Acute sevoflurane anesthesia in normothermic conditions led to a significant dose-dependent and reversible hippocampal tau phosphorylation, 1 h following the end of exposure ($P < 0.001$). Conversely, repeated anesthesia led to persistent tau hyperphosphorylation and significant memory impairments, as seen in the retention phase of the Morris water maze in sevoflurane-anesthetized animals. These pathologic features may be related to the activation of both Akt and Erk pathways.

Conclusions: The present study demonstrates, in mice, that sevoflurane exposure is associated with increased tau phos-

What We Already Know about This Topic

- Phosphorylation of the protein tau may be important to the pathophysiology of Alzheimer disease and possibly with post-operative cognitive dysfunction
- In animals, anesthesia is associated with increase in tau phosphorylation, but this may have been because of hypothermia

What This Article Tells Us That Is New

- In normothermic mice, sevoflurane anesthesia resulted in an increase in tau phosphorylation, which was transient with a single anesthetic but lasted long after repeated exposures and was associated with memory impairment

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phorylation through specific kinases activation and spatial memory deficits. These data support a correlation between exposures to this anesthetic agent and cognitive decline.

CONSIDERING increasing life span, postoperative cognitive dysfunction (POCD) represents a major public health concern. POCD is often associated with cardiac intervention, occurring in 40% of patients several months after surgery.[1] POCD is also common in adult patients of all ages at hospital discharge after noncardiac surgery (30–40%) and may persist for 3 months in patients aged over 60 yr.[2,3] However, other clinical and epidemiologic studies investigating the prevalence of POCD and dementia following surgery did not show an association with the type of anesthesia or duration of surgery.[4–6] Animal studies suggest that exposure to some halogenated anesthetics increases production of the Alzheimer amyloid peptide and vulnerability to neurodegeneration, but these results are not always supported by clinical data.[7,8] Inflammation and stress responses might also contribute to cognitive decline induced by anesthesia.[9,10] Thus, while etiology of POCD remains unclear so far, anesthetic agents might be involved.[11,12]

Although halogenated agents are thought to have limited effects on POCD,[13,14] sevoflurane anesthesia has been shown to alter exploratory and anxiety-like behavior in animals with a genetically modified cholinergic system.[15] Interestingly, we recently showed cholinergic deficits in a tau transgenic model[16,17] and recent experimental *in vivo* studies showed that exposure to different anesthetic agents induces rapid, massive, and transient hyperphosphorylation of tau.[18–23]

Tau proteins belong to the family of microtubule-associated proteins. They play an important role in the assembly of microtubules, contributing to axonal integrity of the normal mature neuron,[24,25] but also play a role at the dendritic and nuclear levels in neurons.[26,27] Hyperphosphorylated and abnormally phosphorylated forms of tau are the major constituents of intraneuronal paired helical filaments observed in Alzheimer disease and also of other filaments seen in several neurodegenerative disorders referred to as "tauopathies."[24,28,29] Spatiotemporal progression of tau aggregates from entorhinal cortex and hippocampus to isocortical areas[30,31] has been shown correlated with cognitive deficits,[32,33] supporting a pivotal role of tau pathology in Alzheimer disease-related memory impairments. In line with this, accumulation of hyperphosphorylated tau proteins was also found to correlate with memory impairments in several animal models.[21,34–36] One hypothesis for POCD pathogenesis is that anesthesia would lead to tau hyperphosphorylation, thereby initiating or aggravating cognitive decline. However, former studies evaluating relationship between anesthesia and tau were performed using acute paradigms and not always in normothermic conditions.[19,21] The aim of the present study was to determine not only whether acute anesthesia with sevoflurane causes hippocampal tau hyperphosphorylation in normothermic conditions but also whether repeated exposures lead to persistent hippocampal tau hyperphosphorylation and spatial memory deficit in adult mice.

Materials and Methods

Animals and Anesthesia Procedure

Short-term Effects of Anesthesia. Twenty adult female C57Bl6/J mice (Charles River Laboratories, St Germain sur l'Arbresle, France) aged of 5 months were used. All animals were kept in standard animal cages under conventional housing conditions (12-h light-dark cycle, 22°C), with *ad libitum* access to food and water. Animals were randomly assigned to four groups. Control mice (n = 10) received no treatment but were placed in the same closed plastic room (length = 23 cm, height = 13 cm, width = 12 cm) as anesthetized animals (control group no oxygen, n = 5; control group 100% oxygen, n = 5). Remaining mice (n = 10) were anesthetized by exposition to vapors of sevoflurane (Sevorane®, Abbott, Rungis, France). For this inhalation procedure, mice were placed in the closed plastic room, receiving low-dose or high-dose of sevoflurane (group sevo = 1.5%, n = 5; group SEVO = 2.5%, n = 5) in 100% oxygen. All anesthetized mice were breathing spontaneously, and the body temperature was monitored with a rectal probe and maintained between 36.0° and 37.0°C using a heating pad. All the mice were sacrificed by cervical dislocation immediately after 1 h of anesthesia. Additional mice (n = 3/group) were used to study the reversibility of sevoflurane effects. Three mice received no treatment and six mice were anesthesized with high dose of sevoflurane (2.5%). Three mice per group were then killed 1 h and 24 h after anesthesia, respectively.

Long-term Effects of Repeated Anesthesia. Twenty-three adult female C57Bl6/J mice (Charles River Laboratories) were used. All animals were kept in standard animal cages under conventional housing conditions (12-h light-dark cycle, 22°C), with *ad libitum* access to food and water. Animals were randomly assigned to three groups. Control mice (n = 8) received no treatment. The other mice were anesthetized by either low-dose (1.5%; group sevo; n = 7) or high-dose (2.5%; group SEVO; n = 8) of sevoflurane. From the age of 6 months, animals were submitted every month to 1 h exposure to sevoflurance until the age of 10 months (fig. 1). Anesthesia procedure was the same that described in the first part of the study, except that two mice were placed at the same time in the closed plastic room. Body temperature was monitored with a rectal probe, and maintained between 36.0 and 37.0°C using a heating pad. At the age of 9 months (one week after the fourth anesthesia), spatial learning and memory were tested by Morris water maze. All the mice were sacrificed by cervical dislocation at 11 months (one month after the fifth anesthesia; fig. 1). All protocols followed European animal welfare regulations and were approved by the local ethical committee (approval No. AF 06/2010, granted on March 31, 2010; CEEA Nord-Pas-de-Calais, Lille, France).

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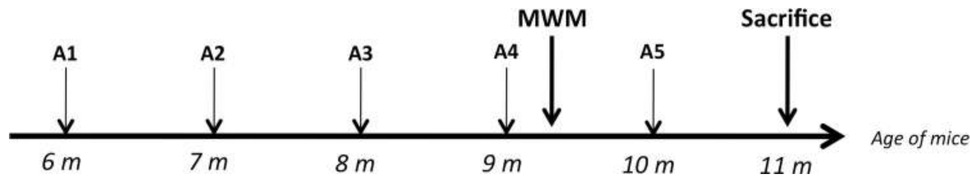

Fig. 1. Experimental protocol for the study of long-term effects of repeated anesthesia by sevoflurane. A = anesthesia; MWM = Morris water maze.

Morris Water Maze Task. Spatial memory abilities were examined in the standard hidden-platform acquisition and retention version of the water maze, adapted for mice.[37] A 152-cm circular pool was filled with water, opacified with nontoxic white paint (Viewpoint, Lyon, France), and kept at 24°C. The pool was located in a room uniformly illuminated by a halogen lamp and equipped with various distal cues. Located inside the pool was a removable, circular (10.5-cm diameter) platform made of transparent plastic and positioned at a fixed position, such that its top surface was 0.5 cm below the water. Four positions around the edge of the tank were arbitrarily designated 1, 2, 3, and 4, dividing the tank into four quadrants (clockwise): target (contained hidden platform), adjacent 1, opposite, and adjacent 2.

During the learning procedure, mice were tested during the light phase between 8:00 AM and 6:00 PM. Each mouse was given four swimming trials per day (45-min intertrial interval) for five consecutive days. The start position (1, 2, 3, or 4) was pseudorandomized across trials. A trial consisted of placing the mouse into the water facing the outer edge of the pool in one of the virtual quadrants and allowing it to escape to the submerged platform. A trial terminated when the animal reached the platform, where it was allowed to remain for 15 s. If the animal failed to find the target before 120 s, it was manually guided to the platform, where it was allowed to stay for 15 s. After completion of a trial, mice were removed from the pool and placed back to their home cages, beneath heat lamps in order to reduce the loss of core temperature. Time required for locating the hidden escape platform (escape latency) and traveled distance (path length) were collected using the videotracking system Videotrack (ViewPoint). Swimming speed (*i.e.*, velocity, as a measure of possible motor defects that could interfere with their ability to perform in this task) was also measured. Seventy-two hours following the acquisition phase, a probe trial was conducted. During this probe trial (90 s), the platform was removed and the search pattern of the mice was tracked again. Proportion of time spent in the target quadrant *versus* averaged nontarget quadrants was compared.

Biochemistry. Following sacrifice, hippocampi were quickly dissected out using a coronal acrylic slicer (Delta Microscopies, Ayguesvives, France) at 4°C and immediately stored at −80°C until analysis. Tissue was homogenized in a Tris Buffer pH 7.4, with protease inhibitors (Complete, Mini, EDTA-free). Total protein concentration was determined by the BCA Assay Kit (Pierce, Perbio Sciences, Brebières, France). Total protein extracts were normalized to 1 μg/μl in reducing LDS Sample Buffer (Life Technologies, Invitrogen, Grand Island, NY) and denaturated at 100°C for 10 min. Then, 15 μg of proteins were loaded on 4–12% NuPAGE Novex gels (Invitrogen), and transferred onto nitrocellulose membranes for western blotting. Immunoreactive signals were visualized by chemiluminescence (ECL; Amersham GE Healthcare, Buckinghamshire, United Kindom), and then integrated by LAS 3000 software (Fujifilm Saint Quentin en Yvelines, France), and quantified by Multigauge software (Fujifilm). Four phospho-dependent anti-tau antibodies were used: AD2 (tau pS396/404, BioRad, Marnes la Coquette, France), AT270 (tau pThr181; Innogenetics, Ghent, Belgium), AT100 (aggregated tau pThr212/Ser214; Innogenetics, Ghent, Belgium) and tau-1 (recognize dephosphorylated Ser195/198/199/202; Millipore, Molsheim, France). As total tau antibody, we used tau Cter (homemade well characterized antibody recognizing the 11 amino-acids in C-terminal part of tau[27]) and tau 5 (Invitrogen). The results are presented as the ratio of phospho-tau to total tau, after being normalized by Glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA). Similarly, different kinase antibodies were used against Akt, p-Akt, Erk, p-Erk, pS9-GSK3, p38, and p-p38 (Cell Signaling Technology, Invitrogen), pY216-GSK3ß (Abcam, Cambridge, United Kingdom), and GSK3 (Santa Cruz Biotechnology). For p-Akt, p-Er, pS9-GSK3, and p-38, data are presented as the ratio of phospho-kinase to total kinase, after being normalized by Glyceraldehyde 3-phosphate dehydrogenase.

Two-dimensional Electrophoresis. Samples were precipitated using methanol/chloroform and 150 μg of total proteins were dissolved in 200 μl of two-dimensional electrophoresis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 0.6% pharmalytes). Samples were loaded on immobilized pH gradient strip 3–11 ReadyStrip (Amersham GE) and isoelectrofocused with the Protean IEF cell (Amersham GE) according to the manufacturer's instructions. The strips were then equilibrated in Tris Buffer (25 mM TrisHCl, 20 mM DTT, 10% glycerol, 5% SDS, 0.05% Bromophenol Blue, pH 6.8) and were layered onto a 4–12% BIS-TRIS Polyacrylamide Gel. SDS-PAGE was performed with a Protean II XI Cell (BIORAD) and blotted onto nitrocellulose membranes with Criterion Blotter (BIORAD) as recommended by the manufacturer. Membranes were then incubated overnight with tau Cter antibody.

Statistical Analysis. Data are presented as mean ± SEM. Differences among the groups were determined by Student unpaired two-tailed *t* test, one-way ANOVA analysis fol-

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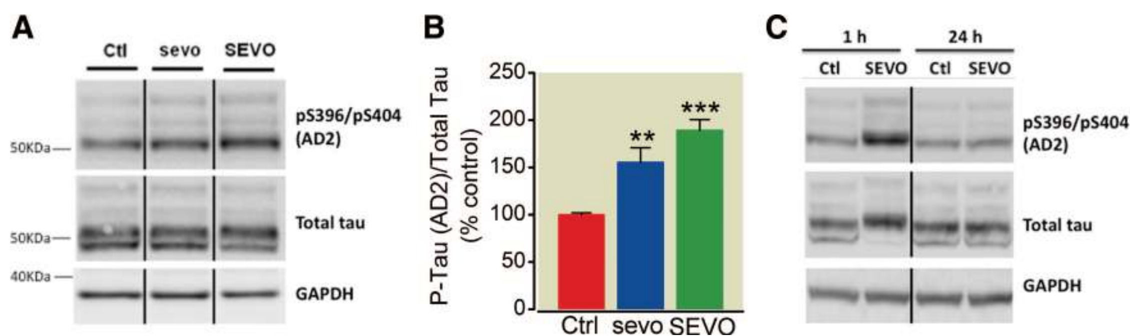

Fig. 2. Acute sevoflurane anesthesia reversibly increases hippocampal tau phosphorylation in 5-month-old C57BL6/J mice in normothermic conditions. (*A*) Following 1 h of anesthesia, mice were sacrificed and their hippocampi analyzed for tau phosphorylation (AD2: phospho-Ser396/404 epitopes) and total expression (tau Cter Ab; Ctl = no anesthesia, sevo = 1.5% sevoflurane, SEVO = 2.5% sevoflurane). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a loading control. (*B*) Densitometric quantification of the AD2 signal over total tau given as mean ± SEM percentage of control group (n = 5 mice per group). (*C*) Reversibility of increased tau phosphorylation. Three mice received no treatment and six mice were anesthetized with high dose of sevoflurane (2.5%). Half were killed 1 h and 24 h after anesthesia, respectively. Short-term increase in tau phosphorylation was found reversible 24 h following anesthesia. ** $P < 0{,}01$, *** $P < 0.001$; comparison *versus* control by ANOVA one-way *post hoc* Newman–Keuls test. Ctl = no anesthesia; Ctrl = no anesthesia; sevo = 1.5% sevoflurane; SEVO = 2.5% sevoflurane.

lowed by Newman-Keuls *post hoc* test or two-way ANOVA using Prism 5 software (Graphpad, La Jolla, CA). Statistical significance was set at $P < 0.05$.

Results

Short-term Effect of Sevoflurane on Hippocampal Tau Phosphorylation in Normothermic Conditions

Tau phosphorylation on phospho-Ser 396/404 was dose-dependently increased 1 h following anesthesia with sevoflurane, whereas total tau levels remained unaltered (figs. 2A, B). Increase reached 55.4 ± 15.5 and 89.3 ± 11.3% of the control at low sevoflurane and high sevoflurane doses, respectively. Short-term increase in tau phosphorylation was found reversible, because it was no more detected 24 h following anesthesia at the highest dose (fig. 2C). Noteworthy was that to avoid any confounding effects related to hypothermia,[12] animal temperature was maintained close to 37°C (37.3° ± 1°C) throughout experiments. Since oxidant injury (*e.g.*, inflammation) can reproduce some changes reported in this first experiment, some control mice were also exposed to 100% oxygen, as done in sevoflurane conditions. Our data indicated that 100% oxygen alone has no effect on tau phosphorylation, ruling out this possibility (data not shown).

Long-term Effect of Sevoflurane on Learning and Memory Performances

In order to determine the long-term consequences of repeated anesthesia, animals were anesthetized each month from 6 to 10 months of age. Leaning and memory performances were determined at 9 months of age and the animal was killed 1 month following the last treatment. Along the treatment period, no mortality was noticed regardless the groups.

The Morris water maze test was performed 1 week following the fourth anesthesia. Treatment did not influence average animal velocity among groups (control: 16.5 ± 2.0 cm/s; low sevoflurane: 17.3 ± 0.9 cm/s; high sevoflurane: 17.4 ± 0.9 cm/s; one-way ANOVA, $P = 0.87$). As shown on figure 3A, following path length calculations, we could not detect significant difference between groups during the learning phase using two-way ANOVA analysis (group: $P = 0.403$; group time: $P = 0.478$) with all animals exhibiting similar performances at day 5 regardless of the treatment. Escape latency calculations gave strictly similar data (not shown). Seventy-two hours following acquisition, a probe trial of 90 s was performed. Although animals from the control group exhibited a significant preference for the target quadrant over averaged nontarget quadrants during the probe trial (47.6 ± 9.2 *vs.* 17.5 ± 3.1%, $P = 0.021$, Student unpaired two-tailed *t* test; fig. 3B), sevoflurane-anesthetized mice did not (low sevoflurane: 29.8 ± 4.1 *vs.* 23.4 ± 1.4%, $P = 0.18$; high sevoflurane: 25.4 ± 2.8 *vs.* 24.9 ± 0.9%, $P = 0.86$; fig. 3B). Probe results were also analyzed by calculating the ratio of time spent in target *versus* opposite quadrants. This ratio was also 4.07 ± 1.31 in the control group, but it was significantly decreased to 1.15 ± 0.26 and 1.13 ± 0.24 in the low sevoflurane ($P = 0.027$) and high sevoflurane ($P = 0.026$) groups, respectively (*vs.* control group using one-way ANOVA analysis).

Long-term Effect of Sevoflurane on Tau Phosphorylation

Hippocampal tau phosphorylation was then evaluated at 11 months of age, *i.e.*, 1 month following the fifth anesthesia. In a first attempt, we determined the global level of tau phosphorylation by two-dimensional electrophoresis using brain samples from the control and "high-dose" sevoflurane groups. As shown on figure 4, we observed a significant shift of tau isovariants from the basic to the acidic pH range in sevoflurane-treated animals as compared with control mice. We then evaluated several specific phospho-epitopes of tau

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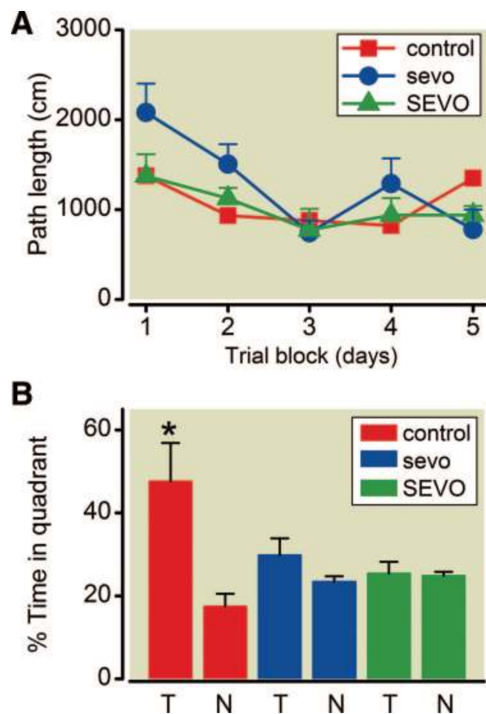

Fig. 3. Impaired spatial memory following repeated anesthesia with sevoflurane. Morris water maze task was realized 1 week following the fourth anesthesia. (*A*) Learning phase of the Morris Water maze task. Performances are given as path length (cm) per day of training. All animals learned similarly following 5 days of training. (*B*) Probe test occurring 72 h following the last day of learning. Results are expressed as a mean ± SEM of the averaged percent of total time spent in target quadrant (T) and nontarget quadrants (N). * $P < 0.05$ *versus* average nontarget quadrants in control group using Student *t* tests. No difference was observed in the sevo and SEVO groups. Ctl = no anesthesia; sevo = 1.5% sevoflurane; SEVO = 2.5% sevoflurane.

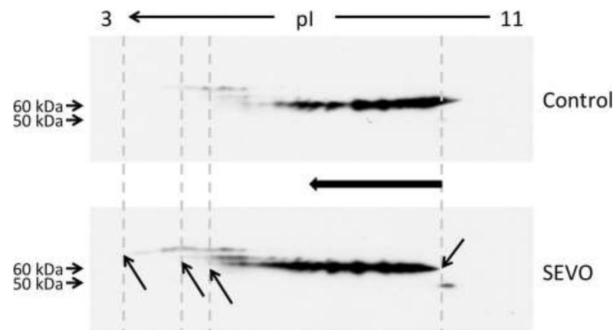

Fig. 4. Analysis of global tau phosphorylation in 11-month-old C57BL6/J mice after repeated sevoflurane anesthesia. One month following the last anesthesia, animals were sacrificed and hippocampal tau phosphorylation analyzed by two-dimensional electrophoresis. Comparison of representative two-dimensional profile of murine tau in controls and repeated high-dose sevoflurane anesthesia group. Data show a global increase in tau phosphorylation in the SEVO group (*arrows*). SEVO = 2.5% sevoflurane.

by immunoblotting. As shown on figure 5, tau phosphorylation on phosphor-Ser396/404 was significantly increased by sevoflurane, reaching 386.3 ± 36.2% of the control in the high sevoflurane group ($P < 0.001$; figs. 5A, B). In addition, in sevoflurane-anesthetized mice, tau phosphorylation on phospho-Thr181 was also strongly increased, whereas tau-1 immunoreactivity (dephosphorylated tau) was decreased (fig. 5A). In accordance with high tau phosphorylation induced by chronic sevoflurane treatment, we observed a significant shift of tau-5 immunoreactive bands toward a higher apparent molecular weight (figs. 5A, C). Nevertheless, there was likely no tau aggregation since AT100 immunoreactivity, signing aggregative tau species, was negative (data not shown).

Long-term Effect of Sevoflurane on Kinases Activation

To explore which mechanisms are leading to changes observed in sevoflurane conditions, we analyzed activated forms of different kinases including p38, Erk, Akt and GSK3 kinases in the animal group treated with the highest concentration of sevoflurane in the chronic condition. Regarding p38, we did not detect any significant increase in its phosphorylation in the Sevoflurane group (98.8 ± 13.5% in Sevoflurane group *vs.* control; ns using two-tailed unpaired *t* test; fig. 6). Conversely, Akt phosphorylation (and thus activation) was significantly increased in the Sevoflurane group (160.7 ± 13.5% of control; $P = 0.0084$ *vs.* control using two-tailed unpaired *t* test; fig. 6). Increased Akt activity was associated with GSK3 inhibition as seen by increase in its phosphorylation at Ser9 (175.4 ± 21.7% of control; $P = 0.019$ *vs.* control using two-tailed unpaired *t* test; fig. 6) and no significant change for phosphorylation at Y216 (75.9 ± 7.4% of control; ns *vs.* control using two-tailed unpaired *t* test; fig. 6). Finally, Erk kinases were also activated since Erk phosphorylation was increased in the treated group (198.1 ± 26.2% of control; $P = 0.0118$ *vs.* control using two-tailed unpaired *t* test; fig. 6). Altogether, these data suggested that repeated sevoflurane anesthesia leads to activation of Akt and Erk kinases and GSK3 inactivation.

Discussion

In the present study, we have evaluated impact of sevoflurane upon tau phosphorylation with a focus on long-term effects following repeated periods of anesthesia. These results support previous findings showing that different types of anesthesic agents, including isoflurane, promote tau phosphorylation.[18–21] However, effects on tau were mostly ascribed to anesthesia-induced hypothermia rather than anesthesia by itself, through inhibition of phosphatase activity.[19–21] Strikingly, our data indicate that while 1 h of anesthesia with sevoflurane induces a transient and reversible tau hyperphosphorylation, chronic and repeated exposure makes this phenomenon persistent. Our data thus extend previous findings and particularly demonstrate the ability of short- and long-term treatment with sevoflurane to promote tau phosphory-

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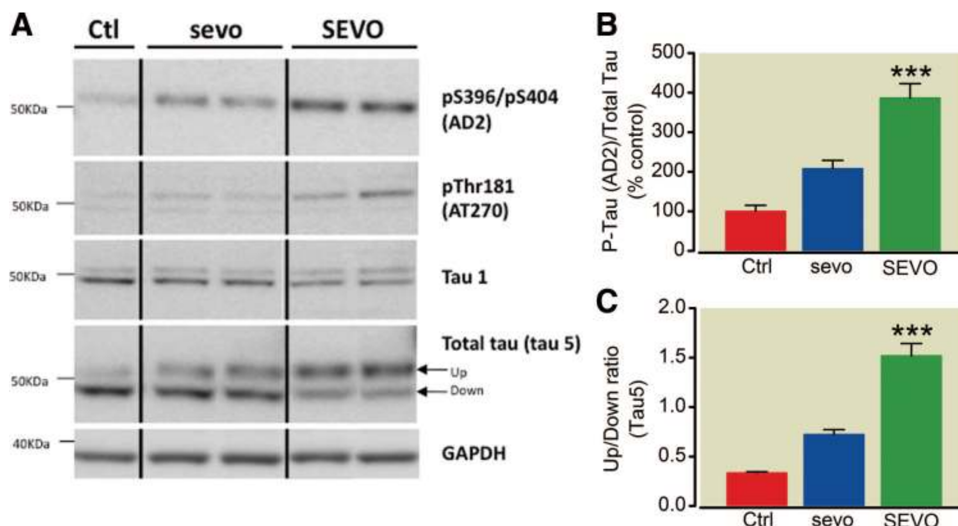

Fig. 5. Repeated sevoflurane anesthesia promotes dose-dependent hippocampal tau phosphorylation in 11-month-old C56Bl6/J mice. (*A*) One month following the last anesthesia, animals were sacrificed and hippocampal tau phosphorylation analyzed by immunoblotting. Immunoblot analysis of tau phosphorylation on phospho-Ser396/Ser404 (AD2), phospho-Thr181 (AT270), tau dephosphorylation using Tau1 antibody or total tau levels using Tau5 antibody. Tau5 antibody detects two bands: the upper band mostly corresponds phosphorylated tau proteins and the lower band mostly corresponds to nonphosphorylated forms. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody is used as a loading control. Tau phosphorylation on phospho-Ser 396/404 and levels of tau phosphorylation on phospho-Thr 181 were increased by sevoflurane, whereas Tau1 immunoreactivity was decreased. In accordance with high tau phosphorylation induced by chronic sevoflurane treatment, we observed a significant shift of Tau5 immunoreactive bands toward a higher apparent molecular weight. (*B*) Densitometric quantification of the phospho-Ser 396/404 tau epitope (AD2). (*C*) Densitometric quantification of the molecular shift visualized by the Tau5 antibody indicated changes in phosphoryltion. *** $P < 0.001$ *versus* control using one-way ANOVA *post hoc* Neuman–Keuls test. Ctrl = no anesthesia; sevo = 1.5% sevoflurane; SEVO = 2.5% sevoflurane.

lation even in normothermic conditions. They also allowed for the identification of transduction signals, including mitogen-activated protein kinase and Akt/GSK3 pathways that may be dysregulated in sevoflurane conditions. These results might be thus more relevant to clinical conditions, since, most of the time, anesthetists manage to keep normal temperature during surgery, even if in some particular situations patients can be exposed to hypothermia in case of long duration, major digestive, and cardiac surgeries.

What are the implications of the present results? Interestingly, some authors previously suggested that tau hyperphoshorylation could reflect a physiologic neuroprotective mechanism in an unfavourable environment, used by the neuron to transiently and reversibly down-regulate its activity when required, not necessarily relating to pathologic effects.[38,39] Arendt *et al.* particularly provided a potentially link between neuronal plasticity and paired helical filaments-like phosphorylation of tau through mitogen-activated protein kinase pathway.[38,40] Indeed, these authors described the formation of highly phosphorylated tau in hippocampus containing a number of paired helical filaments-like epitopes in torpor during hibernation. Paired helical filaments-like phosphorylation of tau was not associated with fibril formation and was paralleled by regression of synaptic contacts. The later were reestablished during arousal, concomitantly with disappearance of paired helical filaments-like tau. During hibernation, overall metabolism is greatly reduced and energy supply and requirements are both low, but still balanced. Homeostatic control is preserved and brain damage does not occur.[41] One cellular mechanism that contributes to this regulated suppression of metabolism is the reversible phosphorylation of enzymes and proteins that limits rates of flux through metabolic pathways. Furthermore, hypothermia is a helpful therapeutic approach for protection of the brain and other organs in patients who have experienced cardiac arrest, and therapeutic hypothermia is actually recommended in such clinical situations by the American Heart Association Guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care.[42] As hibernation-induced hypothermia, anesthesia provides a decreased cerebral metabolism rate. Thus, tau hyperphosphorylation induced by anesthesia could also be a neuroprotective response, not necessarily associated with pathologic effects. Nonetheless, this hypothesis is not supported by memory deficits observed in our long-term study.

Indeed, we found here that persistent hippocampal tau hyperphosphorylation as well as Akt and Erk kinases activation following repeated sevoflurane anesthesia were associated with spatial memory impairments. It is well established that spatial memory assessed using the Morris water maze task is sensitive to hippocampal impairments.[43] Interestingly, previous observations support that hyperphosphorylated forms of tau contribute to cognitive deficits following isoflurane-based anesthesia in 6-month-old rats, without

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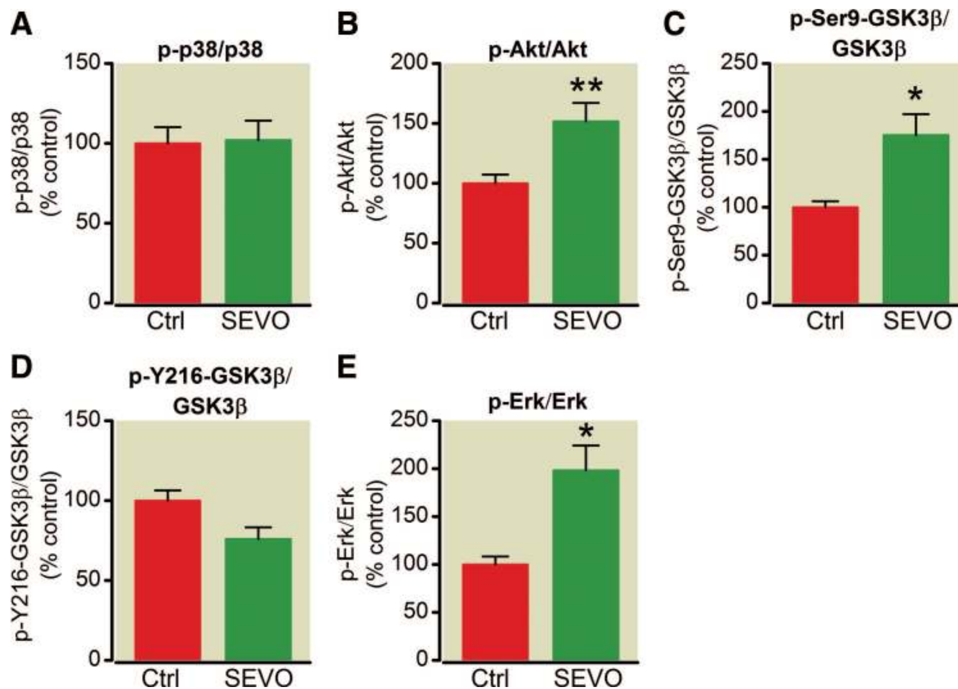

Fig. 6. Repeated sevoflurane anesthesia at high concentration promotes kinases activation. Hippocampal phospho-kinases are analyzed by immunoblotting. Densitometric quantifications of the ratio phospho-kinase/kinase are given as mean ± SEM percentage of control group. (*A*) The ratio p-p38/p38 is not increased in the sevoflurane group. (*B*) Akt phosphorylation (and thus activation) is significantly increased in the sevoflurane group. (*C*) The ratio p-S9-GSK3/GSK3 is increased, (*D*) whereas the p-Y-216-GSK3/GSK3 displays no significant change. (*E*) Erk phosphorylation is increased in the treated group of high concentration sevoflurane. * $P < 0.05$ *versus* control; ** $P < 0.01$ *versus* control using two-tailed unpaired *t* test. Ctrl = control group; SEVO = 2.5% sevoflurane.

body temperature maintenance, using the Y-maze test paradigm.[21] In addition, tau hyperphosphorylation was shown to promote memory decline in aged mice using the Morris water maze task even in absence of neurofibrillary degeneration.[36] Tau proteins can be phosphorylated by several kinases (for reviews,[24,25,28,29]). Similarly, molecular mechanisms underlying memory, including long-term potentiation and depression, are regulated through phosphorylation.[44–46] Two main pathways are involved in both processes: mitogen-activated protein kinases and PI3K/Akt/GSK3 kinases. Akt activation leads to GSK3 inactivation, and GSK3 is an essential kinase in long-term potentiation/long-term depression regulation and memory functions.[46] Thus, constitutive Akt activation and subsequent GSK3 inhibition in sevoflurane conditions may lead to long-term depression inhibition and then contribute to cognitive deficits. In line, we have recently demonstrated an impaired hippocampal long-term depression concomitant with spatial memory deficits in a transgenic model of tauopathy associated with GSK3 deregulation (Tariq Ahmed, Ph.D., Detlev Balschun, Ph.D., Rudi D'Hooge, Ph.D., Leuven, Belgium; David Blum, Ph.D., Luc Buee, Ph.D., Lille, France, unpublished data, July 2011).[34] Alternatively, Akt activation could also be considered as a protective event given its downstream inhibitory effect on GSK3 and then on tau phosphorylation and tau pathology development.[47–49] However, Akt is also able to phosphorylate tau proteins.[24,25,28,50] Similarly, on one hand, Erk activation is also involved in pathologic tau phosphorylation.[51–53] On the other hand, it is a key kinase in memory processes.[54–56] It is thus possible that Akt and Erk activations could contribute, in a convergent manner, to both plasticity/memory defects and tau phosphorylation. Dysregulation of this phosphorylation/dephosphorylation balance involved in these key physiologic neuronal pathways may thus be impaired in sevoflurance conditions.

As hyperphosphorylated forms of tau represent a constituent of neurofibrillary lesions, our findings could provide an association between anesthesia and subsequent persistent postoperative cognitive decline, especially observed in patients over 65 yr. Indeed, these patients already exhibit hippocampal neurofibrillary lesions related to age without clinical expression. Our data raise the possibility that repeated anesthesia would further enhance these events. So far, the number of anesthesia needed to seed persistent tau hyperphosphorylation is unknown. However, based on our assumption that cognitive impairment observed in our mice is related to dysregulation of kinase activity balance and tau hyperphosphorylation, we can suggest that it was already present from the fourth anesthesia. Whether anesthesia-induced tau hyperphosphorylation is sufficient to enhance neurofibrillary lesions required for the clinical expression of tauopathies remains to be uncovered. Underlying mechanisms of anesthesia-induced persistent tau hyperphosphorylation and subsequent cognitive deficits remain also unclear

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and may include effect anesthetic agents on central cholinergic system, amyloid-β peptide oligomerisation/deposition, and neuroinflammation.[8,10,11,57] However, these hypothesis were drawn from rodent studies. Several clinical studies failed to provide a link between anesthesia and POCD, obviously because of the difficulty to distinguish between the respective involvement of either anesthesia itself, surgery, perioperative conditions, inflammation, pain, and comorbidities.[8,12,58] For instance, it is clear that sevoflurane increases tau and P-tau levels in the cerebrospinal fluid of surgical patients for at least 48 h and that some surgery, such as coronary artery bypass, also allows for increase in phospho-tau, as detected in cerebrospinal fluid.[59,60] Thus, animal studies provide a tool to circumscribe instrumental factors and adapt these findings to clinical practice, at least in the choice of which anesthetic agents to be used. However, without further studies in higher mammal species, it is not currently possible to establish clear recommendations for clinical care.

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