The anesthetic sevoflurane induces tau trafficking from neurons to microglia

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Accumulation and spread of tau in Alzheimer’s disease and other tauopathies occur in a prion-like manner. However, the mechanisms and downstream consequences of tau trafficking remain largely unknown. We hypothesized that tau traffics from neurons to microglia via extracellular vesicles (EVs), leading to IL-6 generation and cognitive impairment. We assessed mice and neurons treated with anesthetics sevoflurane and desflurane, and applied nanobeam-sensor technology, an ultrasensitive method, to measure tau/p-tau amounts. Sevoflurane, but not desflurane, increased tau or p-tau amounts in blood, neuron culture medium, or EVs. Sevoflurane increased p-tau amounts in brain interstitial fluid. Microglia from tau knockout mice took up tau and p-tau when treated with sevoflurane-conditioned neuron culture medium, leading to IL-6 generation. Tau phosphorylation inhibitor lithium and EVs generation inhibitor GW4869 attenuated tau trafficking. GW4869 mitigated sevoflurane-induced cognitive impairment in mice. Thus, tau trafficking could occur from neurons to microglia to generate IL-6, leading to cognitive impairment.
Tau is a microtubule-associated protein that is predominantly found inside neurons and functions in microtubule assembly\textsuperscript{1,2}. Tau phosphorylation, aggregation, and spread\textsuperscript{3-6} [reviewed in refs. 7–9] all contribute to the neuropathogenesis of age-dependent neurodegeneration in aging brain, including Alzheimer’s disease (AD)\textsuperscript{10} [reviewed in ref. 11]. Specifically, tau can spread in brain in a prion-like manner\textsuperscript{12–14} [reviewed in refs. 13,16], and tau trafficking can be regulated by 6-O sulfation on heparan sulfate\textsuperscript{17} and low-density lipoprotein receptor-related protein\textsuperscript{18,19}. However, key aspects of tau trafficking, including the causes, exit from neurons, transportation, and destination still remain largely to be determined.

Microglia can take up and release tau\textsuperscript{20}. Indeed, the microglia obtained from rTg4510 transgenic mice, which overexpress full-length (0N4R) human tau with the P301L frontotemporal dementia mutation in the microglia\textsuperscript{20}, contain human tau although they do not express the human tau gene MAPT\textsuperscript{19}. These data indicate that the tau inside the microglia is from an external source. Additionally, the conditioned culture medium of microglia obtained from rTg4510 mice contains a higher amount of tau and causes more prion-like seeding of tau than the conditioned culture medium of microglia obtained from wild-type littermates\textsuperscript{19,20}. These data indicate that microglia can also release tau. However, it remains unknown whether tau can exit from neurons and then enter microglia. Furthermore, the functional consequences of tau trafficking from neurons to microglia are unclear.

Synthetic tau fibrils\textsuperscript{21}, mouse brain lysates\textsuperscript{22}, and human brain lysates\textsuperscript{23,24}, can all induce the spread of tau in mouse brain tissues. Moreover, synthetic tau fibrils can induce the spread of tau in non-neuronal cells\textsuperscript{25,26} and neurons\textsuperscript{27,28}.

A novel nanobeam-sensor technology, the label-free assay, was used to overcome the existing technical challenges in tau and phosphorylated tau (p-tau) detection\textsuperscript{29,30}. When analytes from the sample bound to the antibodies on the nanobeam-sensor, the spectrum of the nanobeam, as monitored by the laser, shift to the longer wavelength. The nanobeam-sensor is ultrasensitive (up to femtogram/mL), requires a small volume (30 μL)\textsuperscript{29,31}, and thus can measure low concentrations of tau and p-tau.

Sevoflurane and desflurane are among the most commonly used inhalation anesthetics in patients. Our previous studies showed that sevoflurane can induce tau phosphorylation and cognitive impairment in mice\textsuperscript{32}. Therefore, in the present studies, we used sevoflurane and desflurane as clinically relevant tools and employed nanobeam-sensor as the approach to test a hypothesis that tau and/or p-tau can exit from neurons and enter microglia, which increases the generation of interleukin 6 (IL-6), a pro-inflammatory cytokine, leading to cognitive impairment.

Extracellular vesicles (EVs) are secretory vesicles that are abundant in the body and play important roles in cell-to-cell communication. Elevated EVs concentrations are detectable in cerebrospinal fluid of patients with AD, Parkinson’s disease, prion disease, and amyotrophic lateral sclerosis as compared to healthy controls\textsuperscript{33,34}. EVs may contribute to the neurotoxicity of β-amyloid (Aβ) and tau\textsuperscript{35}. However, it is unknown whether tau and p-tau inside neurons can be transported by EVs to extracellular spaces and microglia. Here, we used tools including nanobeam-sensor technology to measure the amounts of tau and p-tau associated with EVs. We also used GW4869\textsuperscript{36–39}, an inhibitor of EVs generation, to assess whether inhibition of EVs generation can attenuate sevoflurane-induced tau trafficking and IL-6 generation in vitro, and cognitive impairment in mice.

Results

Elevation of tau in mouse blood following sevoflurane treatment. AD is characterized by the presence of insoluble tau inclusions within neurons\textsuperscript{40}. We assessed whether tau or p-tau could be released from neurons following the commonly used anesthetic sevoflurane as part of the in vitro confirmation and further in vivo investigations of the finding from our previous in vivo study\textsuperscript{32}. Because it is difficult to measure the amount of tau protein in the blood by conventional methods, we previously developed an innovative ultra-sensitive nanobeam-sensor technology\textsuperscript{29,31,41–43} to accomplish this (Fig. 1a). Our previous study\textsuperscript{44} and present study (Supplemental Fig. 1) demonstrated the specificity and sensitivity of measurement of tau and p-tau by nanobeam-sensor.

The resonance spectra of the nanobeam-sensor were recorded in real-time as the blood sample was applied to the nanobeam-sensor, and after the nanobeam-sensor was washed with PBS to eliminate the non-specific absorptions. The spectrum data (Fig. 1b), the Lorentzian-fit resonance real-time data (Fig. 1c), and the analysis of resonance shifts that quantified tau amounts (Fig. 1d) showed that sevoflurane increased the amount of tau in the blood of the mice as compared to control condition. Desflurane, however, did not increase the amount of tau in the blood (Figs. 1e–g). Sevoflurane treatment (Supplemental Fig. 2a) induced tau phosphorylation, as evidenced by increased amounts of p-tau, but not total tau, in neuron lysates (Supplemental Fig. 2b–d). Desflurane (Supplemental Fig. 2a), however, did not induce tau phosphorylation in neuron lysates (Supplemental Fig. 2e–g).

Exit of tau and p-tau from neurons following sevoflurane treatment. To further determine whether tau can exit from neurons, we set up in vitro studies in which neurons harvested from wild-type (WT) mice were treated with sevoflurane (Fig. 2a). Sevoflurane increased p-tau amounts in neuron lysates and lithium, an inhibitor of tau phosphorylation, attenuated this increase [Fig. 2b (Supplemental Fig. 7) and 2c]. More importantly, sevoflurane treatment also increased the amounts of extracellular tau (Fig. 2d), determined by ELISA; and extracellular p-tau (Figs. 2e, f), determined by nanobeam-sensor, in the neuron culture medium. Similarly, lithium inhibited the sevoflurane-induced increase in extracellular tau or p-tau. Interestingly, GW4869, the inhibitor of EVs generation, did not attenuate the sevoflurane-induced increase in extracellular tau or p-tau (Fig. 2g) in the neuron culture medium. Sevoflurane did not increase extracellular LDH amount, which has a similar molecular weight to tau, in the same neuron culture medium (Fig. 2h). These data suggest that LDH release was not altered with sevoflurane, indicating that neuronal plasma membranes and cell viability were not compromised by the sevoflurane treatment. Finally, we showed that sevoflurane increased p-tau amounts in brain interstitial fluid (ISF) of both WT and AD transgenic (Tg) young mice, with greater increases in the AD Tg mice than the WT mice (Fig. 2i).

We repeated these studies employing desflurane (Supplemental Fig. 3a). Desflurane did not increase the amount of extracellular tau (Supplemental Fig. 3b, ELISA studies) or p-tau (Supplemental Fig. 3c, nanobeam-sensor determination) in the neuron culture medium.

Presence of tau and p-tau in EVs following sevoflurane treatment. Next, we determined the effects of sevoflurane or desflurane on the amount of p-tau in EVs (Fig. 3a and Supplemental Fig. 4a). We first showed that sevoflurane increased amounts of Alix, Flo-tillin-2, and CD81, the positive markers of EVs, but not calnexin, the negative marker of EVs\textsuperscript{45} [Fig. 3b (Supplemental Fig. 8)]. Sevoflurane also increased tau amounts in EVs, detected by Western blot using antibody detecting DAKO-tau (Fig. 3b)\textsuperscript{46}.
Next, nanobeam-sensor analysis showed that sevoflurane increased amounts of tau (Fig. 3c) and p-tau (Fig. 3d) in EVs, which were harvested with or without high-salt buffer wash. Nanobeam-sensor analysis showed that sevoflurane increased the amount of extracellular p-tau in neuron culture medium without EVs (after extraction of EVs) and lithium inhibited this increase (Fig. 3e, f). Nanobeam-sensor analysis demonstrated that sevoflurane increased the amount of p-tau in the lysates of EVs harvested from WT neurons, and lithium attenuated this increase (Fig. 3g). By contrast, nanobeam-sensor analysis showed that desflurane did not increase the amount of p-tau in lysates of EVs harvested from WT neurons (Supplemental Fig. 4a, b). Consistently, in neuron culture medium without EVs, GW4869 did not attenuate the sevoflurane-induced increase in the amount of p-tau (Fig. 3h). Electron microscopy (Fig. 3i) and NanoSight (Fig. 3j) showed that sevoflurane increased the count, but not size, of EVs. Western blot showed that sevoflurane increased the amount of Alix and flotillin-2, the markers of EVs, in WT mouse brain tissues (Supplemental Fig. 4c–e).

Finally, we found that sevoflurane treatment induced cognitive impairment (Fig. 4a) in the mice pre-treated with vehicle (Fig. 4b, c), but not in the mice pre-treated with GW4869 (Fig. 4d, e).

Tau and p-tau were taken up by microglia following treatment with sevoflurane-conditioned neuron culture medium. We next asked whether the tau or p-tau exiting from neurons could be taken up by microglia (Fig. 5a and Supplemental Fig. 5a). We treated tau KO microglia, which do not contain any tau or p-tau, with the sevoflurane-conditioned neuron culture medium, which contained tau and p-tau. We first confirmed that the cells used in the study were indeed microglia using flow cytometry (Supplemental Fig. 5b) and immunohistochemistry (Supplemental Fig. 5c). Immunohistochemistry showed the presence of p-tau in tau KO microglia (Fig. 5b, c) following treatment with the sevoflurane-conditioned neuron culture medium. Next, nanobeam-sensor technology demonstrated that the culture medium from neurons treated with sevoflurane plus vehicle increased the amounts of tau (Fig. 5d, e) and p-tau (Fig. 5f, g) in the lysates of tau KO microglia. However, the culture medium from neurons treated with sevoflurane plus lithium caused less increase in the amount of tau (Fig. 5d, e) and p-tau (Fig. 5f, g) in the lysates of tau KO microglia. These data demonstrated that the tau or p-tau exiting from neurons could be taken up by microglia. Finally, we found that the sevoflurane-conditioned neuron culture medium increased the amounts of tau (Fig. 5h) and p-tau (Fig. 5i) in the tau KO microglia, but the conditioned culture medium of neurons treated with sevoflurane plus GW4869 (inhibitor of EVs generation) caused less increases in the amounts of tau (Fig. 5h) and p-tau (Fig. 5i) in the tau KO microglia. Immunohistochemistry also showed that conditioned culture medium of neurons treated with sevoflurane, but not desflurane, might increase the amount of p-tau.
inside tau KO microglia (Supplemental Fig. 5d). Finally, nanobeam-sensor analysis demonstrated that lysates from tau KO microglia did not show increases of p-tau amounts when treated with culture medium from desflurane-treated neurons (Supplemental Fig. 5e).

Enhanced generation of IL-6 in neurons plus microglia following sevoflurane treatment. We assessed the functional consequences of tau trafficking from neurons to microglia by treating neurons, microglia, or neurons plus microglia with sevoflurane (Fig. 6a). ELISA showed that sevoflurane did not increase the amounts of extracellular IL-6 in neurons alone (Fig. 6b) or microglia alone (Fig. 6c). However, sevoflurane significantly increased the amounts of extracellular IL-6 in the culture medium of neurons plus microglia (Fig. 6d).

Inhibition of tau phosphorylation with lithium attenuated the sevoflurane-induced increase in extracellular IL-6 amounts in the culture medium of neurons plus microglia (Fig. 6e). Moreover, the culture medium of WT microglia alone showed the increased extracellular IL-6 amounts when the microglia were treated with the sevoflurane-conditioned neuronal culture medium, and such increase in IL-6 was attenuated by lithium (Fig. 6f) or GW4869 (Fig. 6g). The conditioned EVs specifically extracted from the conditioned medium caused greater increases of extracellular IL-6 amount as compared to the conditioned medium without those extracted EVs (Fig. 6h). Notably, the conditioned culture medium
Fig. 3 Sevoflurane caused the presence of tau and p-tau in EVs. 

**Fig. 3** Sevoflurane caused the presence of tau and p-tau in EVs. 

**a** Experimental design. 

**b** EVs harvested by ExoEasy Maxi kit or ultracentrifuge. 1. Protein detection in EVs by Nanobeam-sensor, 2. EVs markers and tau detection by Western Blot. 

**c** Effects of sevoflurane on the markers of EVs and tau in EVs. 

**d** Amount of tau in EVs with or without treatment of high-salt buffer wash. 

**e** WT neurons culture medium without EVs. 

**f** Quantification of nanobeam-sensor measurement of p-tau in e. 

**g** Nanobeam-sensor measurement of p-tau in the EVs extracted from neurons with and without lithium treatment. 

**h** Nanobeam-sensor measurement of extracellular p-tau amounts in neuron culture medium after extraction of EVs with and without GW4869 treatment. 

**i** Electron microscopy measurement of the count and size of EVs. 

**j** NanoSight analysis of the concentration and size of EVs. Two-way ANOVA and post-hoc analysis with Bonferroni were used to analyze the data presented in Figs. 3c, d, f, g, h, i. The P values of the two-way ANOVA refer to the interaction of group (control condition versus sevoflurane), method (with versus without high-salt buffer wash), or treatment (vehicle versus lithium or GW4869). The P values of post-hoc analysis with Bonferroni refer to the differences in the tau or p-tau amounts between control condition and sevoflurane. P-values of the Student’s t test (Fig. 3i, j) refer to the difference of count, concentration, and size between control condition and sevoflurane. P < 0.05, and ***P < 0.001. N = 3 biologically independent samples in each group in Fig. 3c, d, f, g, h, i (left panel) and Fig. 3j (right panel); N = 5 biologically independent samples in each group in Fig. 3c, j (right panel); N = 7 biologically independent samples in each group of Fig. 3j (left panel); N = 38 EVs in the control group and N = 84 EVs in the sevoflurane group of Fig. 3i (right panel). Error bar indicates standard deviation. P-tau phosphorylated tau, EVs extracellular vesicles, WT wild-type, EM electron microscopy.
from the tau KO neurons treated with sevoflurane did not increase extracellular IL-6 amounts of microglia as compared to control condition (Fig. 6i). Moreover, treatment of WT microglia with exogenous tau increased extracellular IL-6 amounts (Fig. 6j) and the expression of IL-6 mRNA (Fig. 6k).

Activated NF-kB includes the activation of p65, which translocates to the nucleus and binds to the promoter region of multiple genes including cytokine genes to generate IL-6. Thus, we determined the effects of sevoflurane on p65 expression in the nuclei of WT microglia (Supplemental Fig. 6a). Immunohistochemistry showed that treatment with sevoflurane-conditioned neuron culture medium for 30 or 120 min increased the expression of p65 in the nuclei of microglia (Supplemental Fig. 6b–d). Western blot demonstrated that sevoflurane increased the ratio of phosphorylated p65 to p65 (Supplemental Fig. 6e, f) and the ratio of phosphorylated STAT3 and STAT3 (Supplemental Fig. 6g, h) as compared to control condition. These data suggest that sevoflurane could increase IL-6 via, at least partially, activation of NF-kB or STAT3 pathways.

The source data regarding the quantifications of Figs. 1 to 6 and Supplemental Figs. 1 to 6 can be found in the Supplemental data 1 within the Supplemental information of this manuscript.

Discussion
Anesthetics can affect tau pathology and cognitive function. Thus, we used anesthetic sevoflurane and desflurane as clinically relevant tools to study tau trafficking in the present study. The data obtained from in vitro and in vivo experiments suggest that tau can exit from neurons upon phosphorylation, travel through both EVs and non-EVs routes, and then enter microglia, leading to generation of IL-6.

Sevoflurane-induced increase of blood tau amounts demonstrated the potential for tau to exit from neurons (Fig. 1). However, tau can also be released from other organs, including the lungs or other non-neuron cells. Our in vitro studies revealed that sevoflurane, but not desflurane, induced tau phosphorylation at serine 202 and threonine 205 residues in neurons (Supplemental Fig. 2), and simultaneously increased extracellular tau and p-tau amounts in the neuron culture medium (Fig. 2). Sevoflurane also enhanced p-tau amounts in the brain ISF of young mice with greater increase in the AD Tg mice than the WT mice (Fig. 2). These results demonstrated the AD-relevant in vivo findings of tau trafficking following sevoflurane treatment. The tau and p-tau could appear in EVs from WT neurons (Fig. 3) and microglia from tau KO mice (Fig. 5). Importantly, lithium, an inhibitor of tau phosphorylation, and GW4869, an inhibitor of EVs generation, attenuated the sevoflurane-induced increases in p-tau amounts in microglia. GW4869 also attenuated the sevoflurane-induced cognitive impairment (Fig. 4). Taken together, these data suggest tau trafficking occurs through a neuron-EVs-microglia route or neuron-non-EVs-microglia route, which are associated with tau phosphorylation, following certain condition (sevoflurane) since such tau trafficking did not occur upon treatment with desflurane, another commonly used inhalation anesthetic. Importantly, sevoflurane treatment did not cause the exit of LDH, which has a molecular weight similar to that of tau (Fig. 2). These data indicate that the tau exited from neurons was not due to passive transport of cellular contents owing to neuron breakage.

Notably, both tau and p-tau appeared in the neuron culture medium, EVs and microglia (Figs. 2, 3 and 5). These data suggest that both tau and p-tau can exit from neurons. However, the
findings that lithium, the inhibitor of tau phosphorylation, attenuated the sevofoflurane-induced increases in both tau and p-tau in neuron culture medium, EVs and microglia demonstrated that such increases were dependent, at least partially, on tau phosphorylation. Interestingly, anesthetic sevofoflurane, but not desflurane, induced tau trafficking in the present study. The exact reason of such difference is not clear at the present. Compounds with lower chemical bond-dissociation energy levels are more unstable and thus more easily interact with proteins, generating free radicals. Sevofoflurane and desflurane may have different chemical bond-dissociation energy owing to their different molecular structures. Thus, we hypothesized that desflurane could be less toxic (e.g., promoting less or no tau trafficking) due to less generation of free radicals compared with sevofoflurane.

EVs, a heterogeneous group of vesicles released by cells under physiological and pathological conditions, have been classified as ectosomes, microvesicles, microparticles, exosomes, oncosomes, apoptotic bodies, and more based on their origin. Exosomes are released from intracellular compartments known as multi-vesicular bodies, and their biogenesis occurs through endosomal sorting complexes required for transport machinery (ESCRT)-dependent and ESCRT-independent pathways. The ESCRT-independent pathway is regulated by neutral sphingomyelinases. GW4869 is a potent neutral sphingomyelinase inhibitor that can prevent exosome generation and release, allowing the treatment of sevofoflurane-conditioned neuron culture medium with and without GW4869 to mitigate the sevofoflurane-induced changes in tau trafficking and cognitive impairment in mice suggests a role of EVs-associated tau trafficking in AD neuropathogenesis and cognitive function. However, GW4869...
Fig. 6 Sevoflurane increased extracellular IL-6 amounts in the culture medium of neurons plus microglia. a Experimental design. b Extracellular IL-6 amounts in the WT neuron culture medium. c Extracellular IL-6 amounts in the WT microglia culture medium. d Extracellular IL-6 amounts in the culture medium of WT neurons plus WT microglia. e Extracellular IL-6 amounts in the culture medium of WT neurons plus WT microglia with and without lithium treatment. f Extracellular IL-6 amounts in the culture medium of WT microglia treated with sevoflurane-conditioned neuron culture medium with and without lithium. g Extracellular IL-6 amounts in the culture medium of WT microglia treated with sevoflurane-conditioned neuron culture medium with and without GW4869. h Comparison of extracellular IL-6 amounts in the culture medium of WT microglia treated with the conditioned EVs specifically extracted from the conditioned medium versus the same conditioned medium without those extracted EVs. i Effects of sevoflurane conditioned tau KO neuron culture medium on extracellular IL-6 amount of microglia. j Extracellular IL-6 amounts in WT microglia treated with tau protein. k IL-6 mRNA expression in WT microglia treated with tau protein. N = 3 to 8 biologically independent samples in each group as demonstrated in each panel of the figure. Two-way ANOVA and post-hoc analysis with Bonferroni were used to analyze the data in Fig. 6e–g, the P values refer to the interaction of group (control condition and sevoflurane) and treatment (vehicle versus lithium or GW4869) on IL-6 amounts. One-way ANOVA was used to analyze the data in Fig. 6j, k, the P values of one-way ANOVA and the post-hoc analysis with Bonferroni refer to the difference in IL-6 amounts from 0, 100, and 500 pg/ml of tau. Student’s t test was used to analyze the data in Fig. 6b, c, d, h, i, the P values refer to the difference in IL-6 amounts between the control condition versus sevoflurane. *P < 0.05, **P < 0.01, ****P < 0.001. Error bar indicates standard deviation. P-tau phosphorylated tau, KO knockout, EVs extracellular vesicles, WT wild-type.
attenuated the sevoflurane-induced increase in p-tau only in microglia, but not in conditioned medium nor medium without EVs. This was because the p-tau (and/or tau) could exist in different locations: outside and inside of EVs. Thus, the amounts of the p-tau in the conditioned medium were not affected by GW4869, the inhibitor of EVs generation. These data suggest that tau or p-tau associated with EVs may not appear in the neuron culture medium but may appear in the microglia. The conditioned EVs specifically extracted from the conditioned medium were able to increase more extracellular IL-6 amounts as compared to the same conditioned medium but without those extracted EVs (Fig. 6h). These results suggest that the tau and p-tau associated with EVs may have dominant roles on the sevoflurane-induced IL-6 increase in microglia than the tau or p-tau not associated with EVs. Therefore, we hypothesized that the tau or p-tau associated with EVs were most readily internalized by microglia. The future study will use the established system to test this hypothesis, including the identification of the type of tau or p-tau (e.g., free tau or p-tau versus tau or p-tau inside vesicle versus tau or p-tau fragment) that is most readily internalized by microglia. Notably, GW4869 and other compounds reported to prevent EVs release including calpeptin, manumycin A, Y27632, D-pantetheine, and imiparamine, have other effects in addition to their inhibitory effects on the generation and release of EVs.

Sevoflurane increased the amounts of positive markers, but not negative marker, of EVs, and the amounts of tau (detected by DAKO-tau antibody) in EVs harvested by either ExoEasy Maxi kit or ultracentrifuge method (Fig. 3). These data suggest that both methods can effectively harvest EVs in the present study. Moreover, high-salt buffer (0.8 M) did not significantly affect the amounts of tau and p-tau associated with EVs (Fig. 3).

We did not lyse blood, brain ISF, and culture medium prior to the measurement of tau or p-tau by the nanobeam technology. Therefore, the tau or p-tau measured in the blood, brain ISF, or culture medium was likely the tau or p-tau that was not in vesicles. Nevertheless, we will use the established system to systemically identify the specific type of tau and p-tau that can be released by anesthetic sevoflurane in the future.

Lithium, the inhibitor of tau phosphorylation, attenuated the sevoflurane-induced increases in extracellular p-tau amount in neuron culture medium, but GW4869 did not attenuate the sevoflurane-induced increases in extracellular p-tau amount in the WT neuron culture medium with (Fig. 2g) or without EVs (Fig. 3h). These data demonstrated that the exit of tau from neurons was dependent, at least partially, on tau phosphorylation, but not totally on EVs generation. There could be other mechanisms of tau release from neurons in addition to EVs.

We used tau KO microglia in the study because the presence of tau or p-tau inside tau KO microglia confirms the uptake of tau or p-tau. The findings that sevoflurane-conditioned (containing increased amounts of tau and p-tau), but not desflurane-conditioned neuron culture medium (not containing increased amounts of tau and p-tau), rendered the presence of tau or p-tau inside the tau KO microglia (Fig. 5 and Supplemental Fig. 5) suggest that the tau or p-tau exited from neurons can enter to microglia and imply that spreading of tau from neurons to microglia is exacerbated by sevoflurane, but not desflurane, treatment.

Sevoflurane only increased the amounts of extracellular IL-6 in the culture medium of neurons plus microglia but not in neurons or microglia alone (Fig. 6). Thus, the combination of neurons and microglia was necessary to generate IL-6, which further supported the hypothesis that tau trafficking occurred from neurons to microglia, leading to IL-6 generation. Moreover, the sevoflurane conditioned culture medium of tau KO neurons did not increase IL-6 amount, further suggesting that the increase in IL-6 amounts was dependent on tau, consistent with the findings in previous studies. Finally, sevoflurane-conditioned neuron culture medium induced activation of NF-kB signaling, which was attenuated by lithium (Supplemental Fig. 6). These data suggest that NF-kB signaling could be one of the underlying mechanisms by which sevoflurane increased IL-6 amounts.

Microglia, the resident immune cells of the brain, play an important role in neuroinflammation and AD neuropathogenesis. Specifically, microglial activation has been shown to promote tauopathy, including tau phosphorylation and aggregation in vitro and in mice. On the other hand, tauopathy also activates microglia. These findings strongly suggest that the interaction of tau and microglia plays a critical role in the propagation of tauopathy throughout the central nervous system. A study by Hopp et al. demonstrated that microglia can uptake, process, and release tau in mice and in vitro. Consistently, the current study indicated that tau or p-tau could move into microglia (Fig. 5 and Supplemental Fig. 5). Moreover, our findings suggest that tau can exit from neurons into the extracellular space via a mechanism associated with tau phosphorylation. The exited tau could “swim” and travel into microglia with the assistance of EVs or non-EVs pathway, leading to generation of IL-6 and cognitive impairment.

Our previous studies and the present study (Supplemental Fig. 1) have shown the specificity and sensitivity of detection of tau and p-tau by using nanobeam technology. However, it is still important to compare our newly established nanobeam technology with other technology in detecting tau and p-tau, including Simoa and mass spectrometry in the future.

In conclusion, using inhalation anesthetics sevoflurane and desflurane as clinically relevant tools and nanobeam-sensor technology as the detection method, we demonstrated that tau or p-tau could exit from neurons to the extracellular space through mechanisms associated with tau phosphorylation. The exited tau or p-tau could travel with the assistance of EVs or non-EVs pathway, and enter microglia, activating the NF-kB signaling pathway and leading to IL-6 generation and cognitive impairment. These data demonstrated a neuron-EVs-microglia or neuron-non-EVs-microglia pathway of tau trafficking, which has functional consequences on IL-6 generation and cognitive impairment. This work will likely lead to more research on anesthesia, tau trafficking, and AD neuropathogenesis.

Methods

Mice, anesthesia treatment, and brain tissue harvest. The animal protocol was approved by the Standing Committee on Animals at Massachusetts General Hospital, Boston, MA (protocol 2006N000219). Efforts were made to minimize the number of animals used. The manuscript was written according to ARRIVE guidelines. Adult wild-type (WT) mice (C57BL/6J, Strain#: 000664), tau knockout (KO) mice (Strain#: 007251), and AD transgenic (Tg) mice (SXFAD, Strain#: 034848) were purchased from The Jackson Laboratory (Bar Harbor, ME). Female and male WT or AD Tg young mice at postnatal day 6 were obtained by breeding in our lab. Mice were randomly assigned to the following groups: (1) control; (2)
control plus DMSO or saline (vehicle for GW4869 or lithium); (3) control plus GW4869 or lithium; (4) sevoflurane or desflurane; (5) sevoflurane plus DMSO or saline; (6) sevoflurane plus GW4869 or lithium. Mice were anesthetized with 3% sevoflurane or 9% desflurane plus 60% oxygen for 2 h daily on postnatal days 6, 7, and 8, 10, 21, or 6, 8, and 107 (only for mouse brain ISF harvest experiments). Control mice received 60% oxygen at an identical flow rate in identical chambers. We continuously monitored sevoflurane, desflurane, and oxygen concentrations using a gas analyzer (Dash 4000; GE Healthcare, Milwaukee, WI). Anesthesia chamber temperature was monitored and controlled by a feedback-based system with a DC temperature control system (World Precision Instruments Inc, Sarasota, FL), which automatically adjusted to keep the mouse body temperature at 37°C (± 0.5°C) via a warming pad placed under the chamber. For intervention studies, we treated mice with GW4869 (10 mg/kg, dissolved in 10% DMSO and corn oil at 0.45 µg/µL. Cat#: D1692, Sigma, St. Louis, MO)30 through intraperitoneal administration 30 min before each sevoflurane treatment on postnatal days 6, 7, and 8. Mice were decapitated at the end of the sevoflurane or desflurane treatment session on postnatal day 8, and cortex tissues were harvested.

Microdialysis of mouse brain interstitial fluid (ISF). Postnatal day 10 female and male WT or AD Tg mice were used in the studies after the anesthesia with 3% sevoflurane on postnatal day 6, 8, and 10. The ISF was collected by microdialysis50. Specifically, microdialysis probe (Harvard Apparatus, Cambridge, MA) was cannulated into brain (cortex area) of the mice under 3% sevoflurane anesthesia for 10 min on postnatal day 10. On the day of microdialysis, the microdialysis probe was checked to see if any leakage and the membrane of the probe was submerged in 70% ethanol for two seconds. Artificial cerebrospinal fluid (1.3 mM CaCl2, 1.2 mM MgSO4, 3 mM KCl, 0.4 mM KH2PO4, 25 mM NaHCO3, 122 mM NaCl, pH = 7.35) was used for the exchange with ISF. We performed the microdialysis and collected ISF at 1.5 µL per minute for 60 min by using the pump (Harvard Apparatus). The collected ISF was analyzed by nanobeam-sensor for amounts of p-tau (tau-PS202/205).

Morris water maze (MWM). Morris water maze tests were performed on postnatal day 31 to 37, with four trials per day for seven days32,71. Each group had 10 mice. Specifically, we tested the mice in MWM four trials per day from postnatal day 31 to 37 as the reference training. The escape latency was recorded. At the end of the training (on the postnatal day 37), the platform was removed from the pool and the mouse was placed in the opposite quadrant. Each mouse was allowed to swim for 90 s and the numbers the mouse swam to cross the platform area were recorded as platform crossing number.

Mouse blood harvest. After the last sevoflurane or desflurane anesthesia on postnatal day 8, each mouse was removed from the chamber. Anesthesia was maintained with a 1 microliter syringe.

Neuron harvest. Primary neurons were harvested73,74. We euthanized WT mice with the gestation stage of day 15 by using carbon dioxide. We then pull out the embryos and decapitate them in a 100-mm dish of phosphate-buffered saline. We also collected the supernatants from culture medium without EVs to measure the amounts of tau and p-tau. EcoEasy Maxi Kit method: EVs were also isolated using an exoEasy Maxi Kit (Cat#: 76064, QiaGen, Germantown, MD) according to the accompanying protocol. This method can specifically and completely remove the conditioned neuron culture medium (containing tau and p-tau) from EVs. EVs quantity and size were determined by an electron microscope (EM) and NanoSight. The ultracentrifugation method was used for the measurement of the amounts of EVs negative marker Calnexin, and EVs positive markers Alex, Flotillin-2 and CD81, and tau; for the collection of the conditioned culture medium without EVs; for the determination of the amounts of tau and p-tau; for the isolation of the conditioned EVs. The EcoEasy Maxi Kit method was used in the measurement of the amounts of EVs negative marker Calnexin, and EVs positive markers Alex, Flotillin-2 and CD81, and tau; for the isolation of conditioned EVs for the determination of tau and p-tau amounts in EVs, electron microscopy; and for the NanoSight studies determining EVs number and size. EVs were also isolated from 30 million alive neurons. We also collected the supernatants from culture medium without EVs to measure the amounts of tau and p-tau. EcoEasy Maxi Kit method: EVs were also isolated using an exoEasy Maxi Kit (Cat#: 76064, Qiagen, Germantown, MD) according to the accompanying protocol. This method can specifically and completely remove the conditioned neuron culture medium (containing tau and p-tau) from EVs. EVs quantity and size were determined by an electron microscope (EM) and NanoSight. The ultracentrifugation method was used for the measurement of the amounts of EVs negative marker Calnexin, and EVs positive markers Alex, Flotillin-2 and CD81, and tau; for the collection of the conditioned culture medium without EVs; for the determination of the amounts of tau and p-tau; for the isolation of the conditioned EVs. The EcoEasy Maxi Kit method was used in the measurement of the amounts of EVs negative marker Calnexin, and EVs positive markers Alex, Flotillin-2 and CD81, and tau; for the isolation of conditioned EVs for the determination of tau and p-tau amounts in EVs, electron microscopy; and for the NanoSight studies determining EVs number and size. EVs were also isolated from 30 million alive neurons.

Neuron culture medium supernatants were carefully collected after sevoflurane or desflurane treatment with or without lithium (0.5 mM) or GW4869 (10 μM) treatment. Ultra centrifugation: EVs were directly isolated from the supernatants through differential centrifugation. The neuron culture medium was first centrifuged at 300 × g for 10 min at 4 °C to remove free cells, then at 3000 × g for 20 min at 4 °C to remove cellular debris, and finally at 10,000 × g for 30 min at 4 °C to remove free organelles. Supernatant was transferred to a clean quick-seal ultracentrifuge tube and ultracentrifuged at 100,000 × g for 2 h at 4 °C (70 Ti Beckman rotor, Beckman Coulter, Jersey City, NJ) to attain an EVs-enriched pellet. We measured EVs positive markers Alex, Flotillin-2 and CD81, and EVs negative markers using an exoEasy Maxi Kit (Cat#: 76064, Qiagen, Germantown, MD) according to the accompanying protocol. This method can specifically and completely remove the conditioned neuron culture medium (containing tau and p-tau) from EVs. EVs quantity and size were determined by an electron microscope (EM) and NanoSight. The ultracentrifugation method was used for the measurement of the amounts of EVs negative marker Calnexin, and EVs positive markers Alex, Flotillin-2 and CD81, and tau; for the collection of the conditioned culture medium without EVs; for the determination of the amounts of tau and p-tau; for the isolation of the conditioned EVs. The EcoEasy Maxi Kit method was used in the measurement of the amounts of EVs negative marker Calnexin, and EVs positive markers Alex, Flotillin-2 and CD81, and tau; for the isolation of conditioned EVs for the determination of tau and p-tau amounts in EVs, electron microscopy; and for the NanoSight studies determining EVs number and size. EVs were also isolated from 30 million alive neurons.
Brain tissue, neuron, microglia, and EVs lysis, and protein quantification. Harvested brain tissues were homogenized on ice using a lysis buffer including immunoprecipitation buffer (Mammalian Protein Extraction Kit, Cat#: 78501, Thermo Scientific) plus protease inhibitor cocktail (Cat#: 1183617001, Sigma). The neurons treated with anesthetics were detergent-extracted on ice using the same lysis buffer and lysates were collected with a cell scraper. Microglia were treated with sevoflurane-, desflurane-, or control-conditioned neuron culture medium. After removing the conditioned culture medium and washing the samples twice with 1 x PBS, the microglia were incubated in 0.05% trypsin-EDTA (Cat#: 25300-054, ThermoFisher) for one minute to remove the possible binding of tau to the cell surface. The trypsin was quickly removed, and the microglia were washed two more times and then detached from the extracellular matrix on ice in the extracellular volume of the lysis buffer. Specifically, we re-suspended the isolated EVs (obtained from the same amounts of neurons) with equal volumes of lysis buffer, and waited 10 min to allow complete lysis for the detection of Alix, Flotillin-2, CD81, Calnexin and tau by Western blot, and tau and p-tau by neobeam-sensor. Lysates were collected and centrifuged for 15 min at about 19,000 x g. The total amount of protein was quantified using the Pierce protein assay kit (Cat#: 34224, ThermoFisher).

Reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was harvested and isolated from microglia after tau protein treatment. Real-time RT-PCR was performed using the QuantiTec SYBR green real-time PCR kit (Cat#: 204243, Qiagen) to detect the amounts of IL-6 mRNA. The catalog number of the IL-6 primers was PTQ0098875 (Qiagen).

Western blot. Total tau was detected using the anti-tau 5 antibody (Cat#: ab80579, 55 kDa, 1:1000, Abcam). Tau-PS202/PT205 antibody (AT8) (Cat#: MN1020, 55 kDa, 1:200, Agilent technologies, Santa Clara, CA) were used to detect Alix, Flotillin-2, CD81, Calnexin and tau by Western blot, and tau and p-tau by neobeam-sensor. Lysates were collected and centrifuged for 15 min at about 19,000 x g. The total amount of protein was quantified using the Pierce protein assay kit (Cat#: 34224, ThermoFisher).

Lactate dehydrogenase (LDH) assay. Amounts of LDH released into the cell culture medium were used to detect cell death by measuring the integrity of the cellular membrane. Extracellular LDH amounts were assessed using a cytotoxicity detection kit (Cat#: 11644793001, Sigma) according to the manufacturer’s instructions.

Enzyme-linked immunosorbent assay (ELISA). Tau ELISA. We used the mouse tau immunoassay ELISA kit (Cat#: KM8701, ThermoFisher) to determine extra-cellular tau amounts in the neuron culture medium after sevoflurane or desflurane treatment with or without lithium. Briefly, we added 50 µL of standard diluent buffer and 50 µL of standard or sample to each well. Plates were mixed and incubated for 2 h at room temperature and washed four times with 1 x wash buffer. We added 100 µL of 1 X tau biotin conjugate solution to each well and incubated for one hour, then washed four times with 1 x wash buffer. After incubation of the substrate solution for 30 min, the reaction was stopped with 100 µL stoppping buffer per well. We determined the optical density of each well using a SpectraMax i3x Multi-Mode microplate reader (Medical Device, San Jose, CA) at 450 nm. IL-6 ELISA. The mouse IL-6 immunoassay kit (Cat#: M6600B, R&D Systems, Rochester, MN) was used to determine the amount of IL-6 in the culture medium of neurons, microglia, neurons plus microglia, and the medium of microglia treated with sevoflurane- or desflurane-conditioned culture medium or neuron EVs. Briefly, a monoclonal antibody specific for mouse IL-6 was coated onto microplates. We added 50 µL of assay diluent RD1-14, and then added 50 µL of standard or samples to the center of each well. Wells were mixed and incubated for 2 h at room temperature followed five times with wash buffer. Then, 100 µL of mouse IL-6 conjugate was added to each well and incubated for another 2 h and the mixtures were washed five times. After the wells were incubated in 100 µL of substrate solution in a dark room for 30 min, the reaction was stopped with the stop solution. Determination of the optical density of each well was set at 450 nm and corrected at 570 nm.

Flow cytometry. Isolated microglia were treated with 0.25% trypsin-EDTA and collected for flow cytometry. Cells were counted and 10⁶ cells in 45 µL PBS were treated with 5 µL mouse Fc block (anti-CD16/CD32 antibody in the form of 2.4G2, Cat#: 553142, BD Biosciences, San Jose, CA) for 10 min at room temperature to inhibit nonspecific binding. Cells were stained with fluorochrome-conjugated anti-CD11b-PE antibody (Cat#: 101208, BioLegend, Dedham, MA) and Toll-like receptor 4 (TLR4)-FITC antibody (Cat#: ab45126, Abcam) for 30 min in the dark at room temperature. We washed cells twice with 4 mL PBS and re-suspended them in 0.5 mL PBS. A total of 10⁷ cells were acquired per sample using a LSRFortessa X-20 cell analyzer (BD Biosciences), and data were analyzed using FlowJo software (BD Biosciences).

Immunohistochemistry. Tau KO microglia were treated with sevoflurane- or desflurane-conditioned neuron culture medium. At the end of treatment, microglia seeded onto FluoroDishes were washed with 0.05% trypsin followed by PBS. We fixed the microglia with 4% paraformaldehyde in 0.1 M PBS at pH 7.4 for 15 min on ice for 30 min or 1 h. We then incubated with biotinylated goat anti-mouse AT8 (Cat# MN1020, 1:500, ThermoFisher, ZOO), mouse anti-IBAI (Cat# 019-19741, Fujifilm Wako, Richmond, VA) antibodies overnight at 4°C, followed by streptavidin-conjugated Alexa Fluor 594 (Cat# S32356, 1:500, ThermoFisher), Alexa Fluor 488 donkey anti-rabbit IgG (Cat#: A21206, 1:500, ThermoFisher), and Alexa Fluor 546 goat anti-rabbit IgG (Cat#: A11010, 1:500, ThermoFisher) antibodies for 1 h at room temperature in the dark. Microglia were analyzed in FluoroShield mounting medium with DAPI (Cat#: ab104139, Abcam) under a 10 X objective of a KEYENCE BZ-9000E all-in-one fluorescence microscope (Keyence Corporation of America, Itasca, IL) for IBA1 study. To detect p-tau in brain tissue, Tau KO microglia, we employed a Nikon Eclipse Ti e microscope (Nikon, Melville, NY) with 20X and 60X objective lenses and photographs were taken.

NF-kB activation. WT microglia seeded in FluoroDishes were treated with sevoflurane-conditioned neuron culture medium with or without lithium for 30 min or 120 min. Microglia were fixed with 4% paraformaldehyde and stained overnight with phospho-p65 antibody (pS546, Cat#: sc-109, 1:200, Santa Cruz), followed by Alexa Fluor 546 goat anti-rabbit IgG (1:500, ThermoFisher). Quantification of immuno-histochemical dishes (9–14 fields in each treatment group) was performed by unbiased, double-blinded stereological counts. The only inclusion criterion was clear microscopic observation of the cell body; exclusion criteria included a dirty background and broken cell bodies. Immunohistochemical photomicrographs were captured at 200 X magnification with a confocal microscope. Percentage of NF-kB p65 positive cells was determined per random field. Moreover, the ratios of phosphorylated p65 to p65 and phosphorylated STAT3 to STAT3 were also assessed to detect potential NF-kB activation.

Transmission Electron Microscope (EM) studies of EVs. EVs samples were fixed in 1% with 2% glutaraldehyde (v/v; Sigma Aldrich) for 30 min. A fixed sample of 5 µL was pipetted onto a 400-mesh copper grid and incubated for 10 min. Images were acquired through the Tecnai F20 (FEI) microscope system (ThermoFisher Scientific). Images were obtained from the average of two 700 mini-second acquisitions with 2048 × 2048 resolution and Imaging Solutions software (Olympus, Tokyo, Japan). To find a magnification showing as many EVs as possible on a single image with sufficient detail to distinguish EVs morphological features, we evaluated images at 100,000X, 50,000X, and 32,000X magnifications. Higher magnification images appeared to have better background quality. We defined EVs diameters of ≥20 nm with sufficient contrast for picture selection.

Statistics and reproducibility. Based on our previous studies, we determined that 10 mice per group for behavioral studies, and 3-9 samples per group for biochemistry studies including nanobeam-sensor, Western blot, ELISA, LDH assay, NanoSight, RT-PCR, EM, and immunohistochemistry would provide sufficient statistical power. We present data from biochemistry studies and Morris water maze escape latency as mean ± SD; and platform crossing numbers from the Morris water maze are presented as median and interquartile range. Interaction between time and group factors was determined using two-way ANOVA with repeated measures to analyze the difference in platform crossing number between the control and sevoflurane treatment group. There were no missing data for variables of the Morris water maze test (escape latency and platform crossing number). Two-way
ANOVA and post-hoc analysis with Bonferroni were used to determine the interaction and difference between groups (e.g., control versus sevoﬂurane) and treatments (e.g., vehicle versus lithium). Student’s t test was used to determine the difference in two-group comparison when data passed a normality test. One-way ANOVA and post-hoc analysis with Bonferroni were used to determine the differences between groups. P < 0.05 was considered statistically signiﬁcant, and signiﬁcance testing was two-tailed. Adjusted Bonferroni correction P-values were calculated by dividing real P-values by experiment size, and adjusted P-values were reported in the manuscript. The sample sizes were from 3 to 10 in each group in the present study. The behavioral study to determine the effects of sevoﬂurane on cognitive function of the mice in MWM was replicated three times by repeating the same anesthesia and using the same Morris water maze protocol. The study to determine EVs makers (N = 1 in each group) was replicated three times by repeating the same sevoﬂurane treatment and using the same Western blot analysis. Statistical analysis was conducted using GraphPad Prism software (version 8) and SPSS statistics software (version 21.0).

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Author contributions
Y.D., F.L., L.H., and F.F. performed the experiments. Y.D., F.L., Y.Z., Q.Q., and Z.X. created the concept, designed the experiment, and wrote the manuscript. G.Y. and R.T. provided critical comments for the studies and manuscript.

Competing interests
Dr. Zhongxiong Xie provides consulting services to Shanghai 9th and 10th hospital, Baxter (invited speaker), and Novartis. All other authors declare no competing interests.

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