

**Supplemental digital content**

**Tau contributes to sevoflurane-induced neurocognitive impairment in neonatal mice**

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**Supplemental Materials and Methods**

**Morris water maze.** In brief, postnatal day 30 or postnatal day 84 mice were tested in the Morris water maze via four trials per day for seven days (postnatal day 30 – 36 or postnatal day 84 – 90). Escape latency was recorded on each trial, and the time of escape latency averaged from the four trials was recorded on each day and used for data analysis by two-way ANOVA. On the last day, the platform was removed, and number of platform crossings of mice was measured. Student's t-test with Bonferroni correction was used to calculate adjusted *P*-values to compare difference in escape latency between mice in the baseline and sevoflurane anesthesia groups during each day of the Morris water maze. We maintained mouse body temperature by using a heating device as described previously.<sup>1</sup> After every trial, each mouse was placed in a holding cage under a heat lamp for 5 minutes to dry and warm the body before being returned to its home cage.

**Brain tissue harvest, lysis, and protein quantification.** Collected brain tissues were used for western blot, ELISA, ATP assay, and Seahorse XFp extracellular flux analyses. We homogenized brain tissues on ice using an immunoprecipitation buffer (Mammalian Protein Extraction Reagent, Cat# 78501, Thermo Scientific, Waltham, MA) plus protease inhibitor cocktail (Cat# 11836170001, Sigma-Aldrich, St. Louis, MO). Lysates were collected and centrifuged for 15 minutes at about 19,000 x g. Total amount of protein was quantified using a bicinchoninic acid protein assay kit (ThermoFisher, Waltham, MA). In the current study, we obtained the data about amounts of Tau, phosphorylated Tau (pTau), PSD-95 and others in both cortex and hippocampus, but more of them in the cortex than hippocampus. Furthermore, Morris water maze can still detect the changes of behaviors associated with the cortex.<sup>2-4</sup> Therefore, we mainly used the data obtained in the cortex to demonstrate the age-dependent effects (e.g., brain Tau amounts) in the mice.

**Reverse transcriptase polymerase chain reaction (RT-PCR).** *Nuak1* mRNA amounts were determined and standardized using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as an internal baseline. Primers of mouse *Nuak1* (ID No., QT01059303) and mouse *GAPDH* (ID No., QT01658692) were purchased from Qiagen. PCR was performed at: 50°C for 30 minutes and 95°C for 15 minutes, followed by 50 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 95°C for 15 seconds, and 55°C for 15 seconds.

**Enzyme-linked immunosorbent assay (ELISA).** Briefly, we added 50 µL of standard or sample and then 50 µL of HRP-conjugate reagent to each well. Wells were incubated for 1 h at room temperature and washed four times with PBS. We added 50 µL of chromogen solution A plus 50 µL of chromogen solution B to each well and incubated for 15 min, halting reactions with 50 µL of stop solution. We determined optical density of each well using a Fluorescence Plate Reader (Medical Device, San Jose, CA) at 450 nm and 570 nm.

**Immunohistochemistry.** For staining, sections were incubated with anti-Tau5 antibody (Cat# ab80579, 1:100, Abcam) and anti-T22 antibody (Cat# ABN454, 1:500, Millipore) overnight at 4°C, followed by immunostaining with Alexa Fluor 594 goat anti-rabbit IgG (1:500, Invitrogen, Carlsbad, CA) and Alexa Fluor 488 goat anti-mouse IgG (1:500, Invitrogen) for 1 h at room temperature in the dark. Sections were incubated with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) in a humidified dark chamber for 10 min, and cover slips were affixed with mounting medium. Slides were analyzed under a fluorescence microscope (Keyence Corporation of America, Itasca, IL). Quantification of immunohistochemical sections (5 slides/mouse) was performed by unbiased, double-blinded stereological counts. The only inclusion criterion was clear observation of cell body under the microscope; exclusion criteria were existence of a dirty background and large amounts of broken cell bodies.

Immunohistochemical photomicrographs were captured at 400X magnification, and arrows in

the photomicrographs indicate cell bodies. Number of Tau5 and T22 positive cells were determined by two-dimensional counting in five random fields per section using Image-Pro Plus 6. Size of counting area in each image was  $287 \mu\text{m} \times 387 \mu\text{m}$ .

**Multiplexed quantitative mass spectrometry-based phosphoproteomics.** Whole brain tissues were harvested at the end of sevoflurane anesthesia from postnatal day 8 or postnatal day 62 mice. Brain tissues from each group (n=4/group) were kept on dry ice before being prepared for proteomics measurements. Tissues were re-suspended in lysis buffer [75 mM NaCl, 50 mM HEPES (pH 8.5)], 10 mM sodium pyrophosphate, 10 mM NaF, 10 mM  $\beta$ -glycerophosphate, 10 mM sodium orthovanadate, 1 mM PMSF, 3% SDS, and a complete mammalian protease inhibitor tablet (Roche, Basel, Switzerland). Suspensions were mixed with zirconium oxide beads (1-mM diameter) and lysed on a mini bead beater (Biospec, Bartlesville, OK) four times for 45 s each time, cooling samples on ice in between. Beads were removed, lysate was centrifuged at 15,000 g for 5 min at 4°C, and insoluble debris was discarded. Proteins were reduced, alkylated, precipitated with methanol/chloroform, and digested with LysC and trypsin. Phosphopeptides were enriched using a 4:1 (w/w) ratio of titanium dioxide beads to peptide. Peptides were resuspended in 2 M lactic acid in 50% ACN and added to 1.8 mg of titanium dioxide beads. The mixture was shaken gently for 1 h. Beads were collected by centrifugation and washed three times with 2 M lactic acid in 50% ACN and three times with 50% ACN/0.1% TFA. Phosphopeptides were eluted with 2 x 200  $\mu\text{L}$  of 50 mM  $\text{KH}_2\text{PO}_4$  at pH 10 and acidified with 1% TFA. Eluted phosphopeptides were desalted, lyophilized, and labeled with 2  $\mu\text{L}$  of 10-plex TMT reagents 127n-130c as described above. The combined sample was enriched for phosphotyrosine-containing peptides using phosphotyrosine antibody-conjugated beads (Cell Signaling Technology, Danvers, MA) following the protocol provided by the manufacturer. Unbound peptides (phosphoserine and phosphothreonine peptides) were desalted, lyophilized,

and fractionated through bRPLC using a gradient of 5%–28% Buffer B. A total of 96 fractions were collected and then combined to yield 12 fractions. Bound peptides (phosphotyrosine peptides) were eluted and desalted. All 13 fractions were re-suspended in 5% ACN/5% formic acid and analyzed on an Orbitrap Fusion mass spectrometer using LC-MS2/MS3 for identification and quantification of phosphopeptides.

TMT-labeled peptides were subjected to multiplexed quantitative phosphoproteomics analysis on an Orbitrap Fusion mass spectrometer (Thermo Scientific) coupled to an EASY-nLC 1000 integrated autosampler and HPLC pump system. Peptides were separated over a 100- $\mu$ m inner diameter microcapillary column, packed in-house with 0.5 cm of Magic C4 resin (5  $\mu$ m, 100 Å, Michrom Bioresources, Auburn, CA), followed by 0.5 cm of Maccel C18 resin (3  $\mu$ m, 200 Å, Nest Group, Southborough, MA) and later 29 cm of GP-C18 resin (1.8  $\mu$ m, 120 Å, Sepax Technologies, Newark, DE). Samples were eluted over 165 min at a flow rate of 300 nL/min over a gradient of 6%–25% ACN/0.125% formic acid.

Data analysis was performed on an in-house, SEQUEST-based software platform.<sup>5,6</sup> RAW files were converted into the mzXML format using a modified version of ReAdW.exe. Peptide identification was performed, as reported previously, by searching against a protein sequence database containing all protein sequences in the mouse ORF database (downloaded 01/14/2014), as well as that of known contaminants. For phosphopeptide data, high- and low-resolution spectra were annotated in two separate searches and subsequently combined.

**Mitochondrial metabolism on Seahorse XFp Extracellular Flux Analyzer.** Harvested hippocampus tissues were homogenized 20 times with a plastic tissue grinder (Fisher Scientific) in an Eppendorf tube with 600  $\mu$ L of mitochondrial isolation buffer, which consisted of 70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EGTA, and 0.5% (w/v) fatty acid-free BSA

(pH 7.2 at 37°C). Homogenate was centrifuged at 800 g at 4°C for 10 min. Then, the supernatant was centrifuged at 8,000 x g at 4 ° C for 10 minutes and the pellets were dissolved in mitochondria assay solution (MAS) without BSA. Protein concentrations were measured by BCA assay. MAS was composed of 10 mM sodium succinate, 70 mM sucrose, 220 mM mannitol, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM MgCl<sub>2</sub>, 2 mM HEPES, 1 mM EGTA, and 0.2% (w/v) fatty acid-free BSA (pH 7.2 at 37°C). Remaining supernatant was centrifuged at 8,000 g at 4°C for 10 min, and precipitates were dissolved in MAS plus BSA. After appropriate dilution with MAS plus BSA, mitochondria were seeded into a XFp cell culture miniplate (Seahorse Bioscience, Billerica, MA) at 30 µg /well. The plate was centrifuged at 2,000 g at 4°C for 20 min and incubated in air at 37°C for 10 min. A Seahorse cartridge with detection probes was loaded with adenosine 5'-diphosphate (ADP, 10 mM), oligomycin (2 µM), carbonyl cyanide-4-phenylhydrazone (FCCP, 4 µM), and antimycin A (3 µM) into injection ports A, B, C, and D, respectively. We then transferred mitochondria and these reagents into the Seahorse XFp extracellular flux analyzer. Real-time readings were taken for five stages: stage I, basal level; stage II, addition of ADP to measure ATP production; stage III, addition of oligomycin, a mitochondrial oxidative phosphorylation (OXPHOS) complex 5 inhibitor, to measure protein leakage; stage IV, addition of the mitochondrial OXPHOS complex 4 inhibitor carbonyl cyanide-chlorophenyl hydrazine to measure maximal respiration; and stage V, addition of the mitochondrial OXPHOS complex 3 inhibitor antimycin A to measure nonmitochondrial respiration. Mitochondrial metabolism was determined by measuring oxygen consumption rate calculated and recorded by Seahorse XFp software.

Supplemental Figure 1.

a

**Skin and core temperature of the mice in the baseline and sevoflurane groups at different time point**

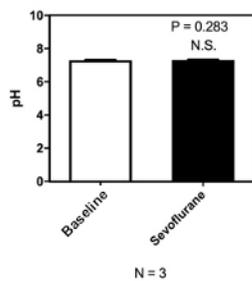
	Baseline skin (°C, N=1)	Baseline core (°C, N=1)	Sevoflurane skin (°C, N=1)	Sevoflurane core (°C, N=1)
0 minute	34.20	36.00	33.90	36.10
10 minute	34.50	36.30	33.80	36.00
20 minute	34.50	36.20	33.30	36.20
30 minute	34.10	36.30	34.10	36.40
40 minute	34.80	36.00	34.20	36.60
50 minute	34.20	36.80	34.50	37.30
60 minute	34.20	37.20	34.40	37.50
70 minute	34.80	37.50	34.30	37.30
80 minute	34.20	36.70	34.60	37.50
90 minute	34.10	36.70	34.00	36.40
100 minute	34.00	36.50	33.90	36.00
110 minute	34.10	36.30	34.00	36.10

b

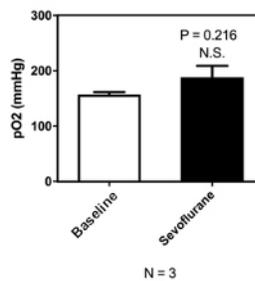
**Comparison of skin temperature between the mice in the baseline and sevoflurane groups at different time point**

	Baseline (°C, N=4)	Sevoflurane (°C, N=4)	P value
0 minute	34.30 ± 0.27	34.58 ± 0.54	> 0.99
10 minute	34.28 ± 0.17	33.90 ± 0.61	> 0.99
20 minute	34.50 ± 0.08	33.65 ± 0.39	> 0.99
30 minute	34.35 ± 0.44	33.75 ± 0.47	> 0.99
40 minute	34.65 ± 0.26	34.20 ± 0.29	> 0.99
50 minute	34.35 ± 0.50	34.45 ± 0.29	> 0.99
60 minute	34.30 ± 0.12	34.55 ± 0.13	> 0.99
70 minute	34.55 ± 0.35	34.80 ± 0.50	> 0.99
80 minute	34.45 ± 0.24	34.75 ± 0.37	> 0.99
90 minute	34.53 ± 0.34	34.08 ± 0.15	> 0.99
100 minute	34.35 ± 0.31	34.20 ± 0.36	> 0.99
110 minute	34.35 ± 0.30	34.33 ± 0.32	> 0.99

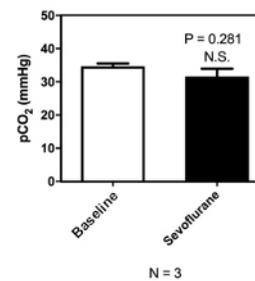
c



d

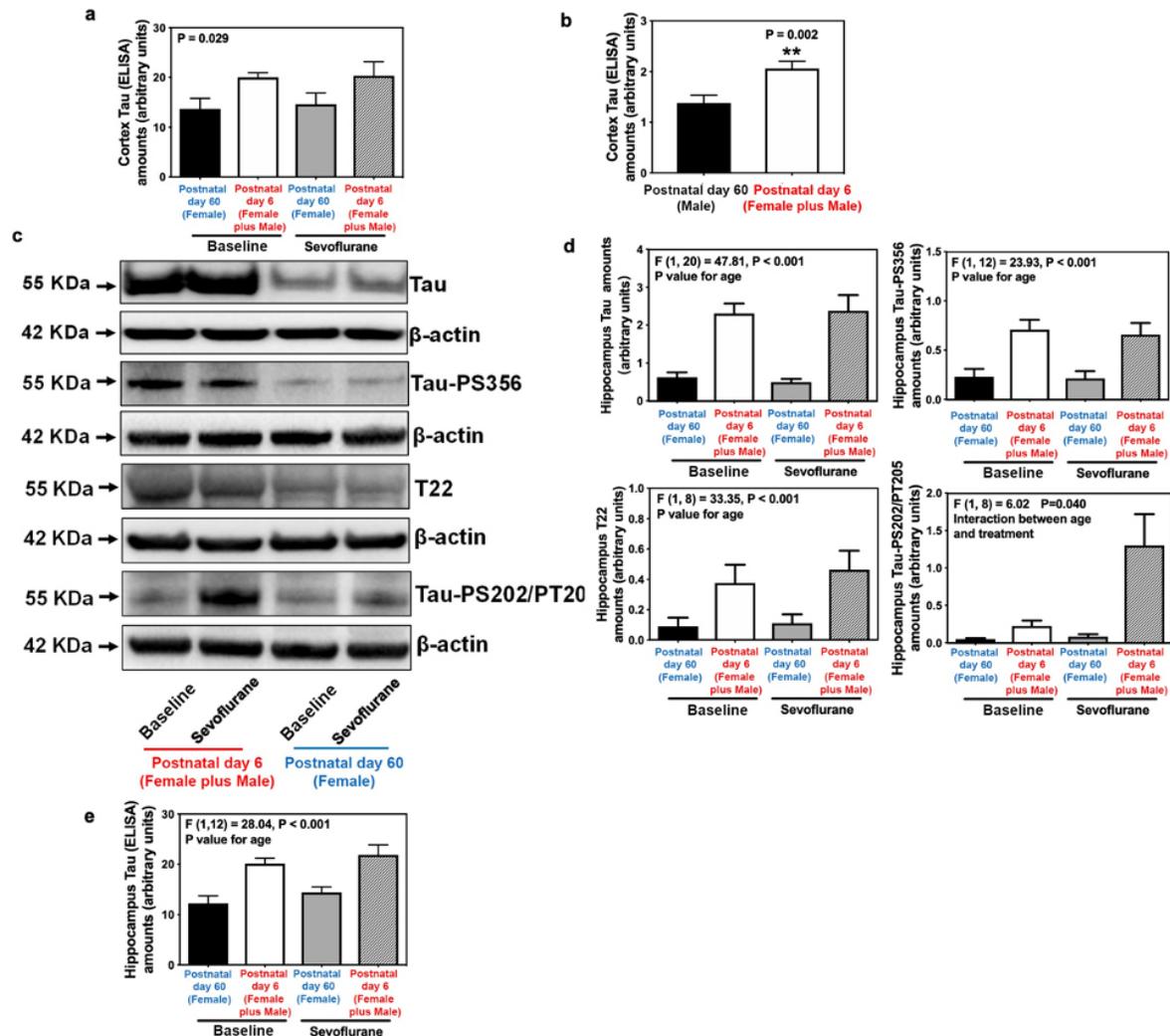


e



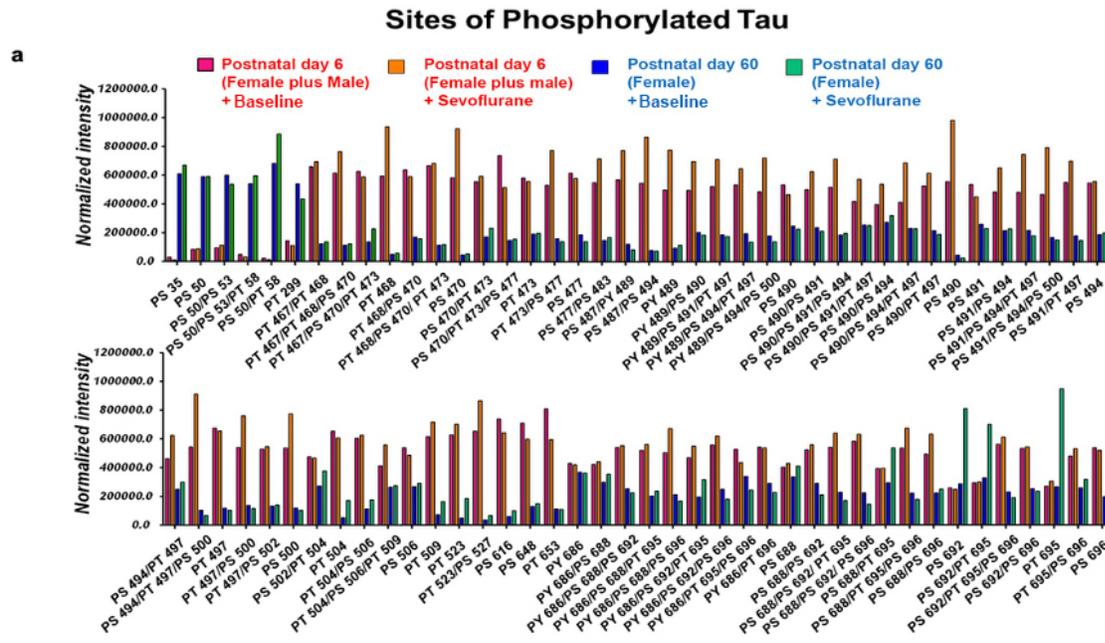
**Supplemental Figure 1. Temperature and blood gas values in neonatal mice.** (a) Skin temperature and core temperature of 6-day-old mice from both sexes (postnatal day 6; neonatal mice from both sexes) from both sexes in baseline and sevoflurane anesthesia groups. (b) Skin temperature of neonatal mice from both sexes in baseline and sevoflurane anesthesia groups (n=4 mice/group). Value was obtained from average of 12 measurements. There was no significant difference in skin temperature between neonatal mice from both sexes in baseline and sevoflurane anesthesia groups. (c) pH value of neonatal mice from both sexes in baseline and sevoflurane anesthesia groups. (d) pO<sub>2</sub> value of neonatal mice from both sexes in baseline and sevoflurane anesthesia groups. (e) pCO<sub>2</sub> value of neonatal mice from both sexes in baseline and sevoflurane anesthesia groups. There were no significant differences in pH, pO<sub>2</sub>, or pCO<sub>2</sub> values between neonatal mice from both sexes in baseline and sevoflurane anesthesia groups. Student's t-test: (b), (c), (d), (e). All data are quantified and expressed as real numbers compared to the baseline (neonatal mice from both sexes in non-anesthetized group).

## Supplemental Figure 2.



**Supplemental Figure 2. Tau and Tauopathy in cortex and hippocampus of neonatal and adult mice.** (a) ELISA measurement of Tau amounts in the cortex of neonatal mice from both sexes and female reference group mice in baseline and sevoflurane anesthesia groups ( $n=4$  mice/group). (b) ELISA measurement of Tau in the cortex of neonatal mice from both sexes and male reference group mice in the baseline group ( $n=6$  mice/group). (c) Western blots of amounts of Tau, Tau-PS356, oligomer Tau (T22), and Tau-PS202/PT205 in the hippocampus of 6-day-old mice (postnatal day 6) from both sexes (female plus male mice) and 60-day-old (postnatal day 60) female mice (female reference group mice) in baseline and sevoflurane anesthesia groups.  $\beta$ -actin is a loading control. (d) Summary of western blots of amounts of Tau (left top), T22 (left bottom), Tau-PS356 (right top), and Tau-PS202/PT205 (right bottom) ( $n=6$  mice/group). (e) ELISA measurement of Tau in the hippocampus of neonatal mice from both sexes and female reference mice in baseline and sevoflurane anesthesia groups ( $n=4$  mice/group). All data are quantified and expressed as arbitrary units compared to the baseline group (neonatal mice from both sexes in non-anesthetized group). PS, phosphorylated serine; PT, phosphorylated threonine. Kruskal-Wallis test: (a); Student's t-test: (b); and Two-way ANOVA: (d) and (e). \*\* $P < 0.01$ .

Supplemental Figure 3.

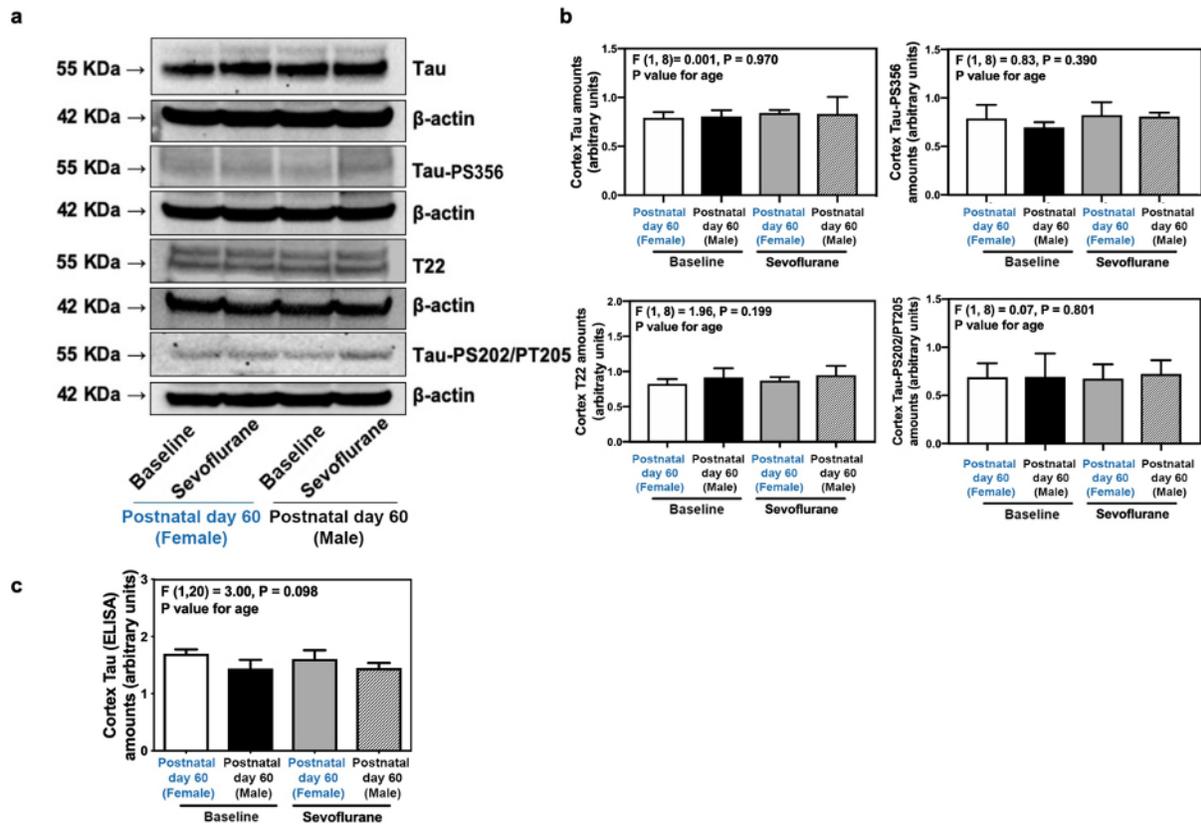


b

	Postnatal day 6 (Female plus Male)		Postnatal day 60 (Female)	
	Baseline	Sevoflurane	Baseline	Sevoflurane
Total sites of phosphorylated Tau	80	80	80	80
The number of sites with normalized intensity unit > 200000	74/80	74/80	43/80	36/80
The number of sites with normalized intensity unit > 400000	70/80	70/80	6/80	11/80
The number of sites with normalized intensity unit > 600000	16/80	42/80	2/80	5/80
The number of sites with normalized intensity unit > 800000	1/80	6/80	0/80	3/80
The number of sites with higher normalized intensity compared with control group		42/80		11/80

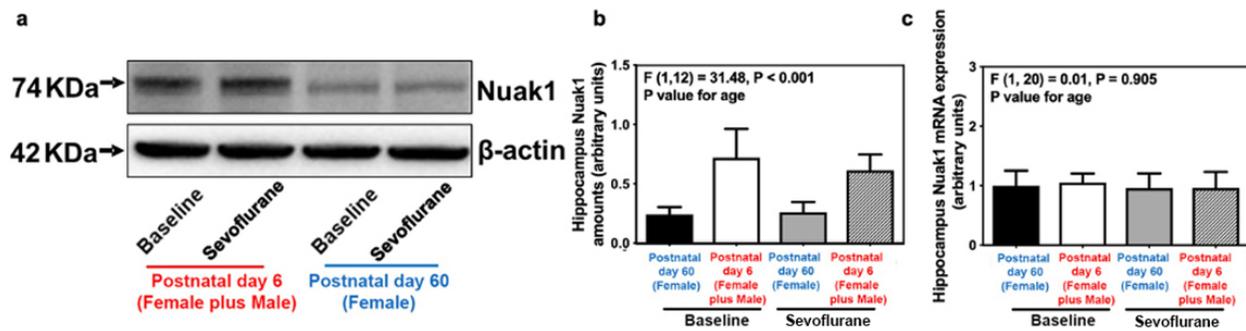
**Supplemental Figure 3. Tau phosphorylation in neonatal and adult mice.** (a) Quantitative mass spectrometry data demonstrating phosphorylated sites of Tau in the cortex of 6-day-old mice (postnatal day 6) from both sexes (female plus male mice) and 60-day-old (postnatal day 60) female mice (female reference group mice) (n=4 mice/group) in baseline and sevoflurane anesthesia groups. (b) Data summarizing differences in phosphorylated Tau sites and amounts in the cortex of neonatal and female reference group mice.

## Supplemental Figure 4.



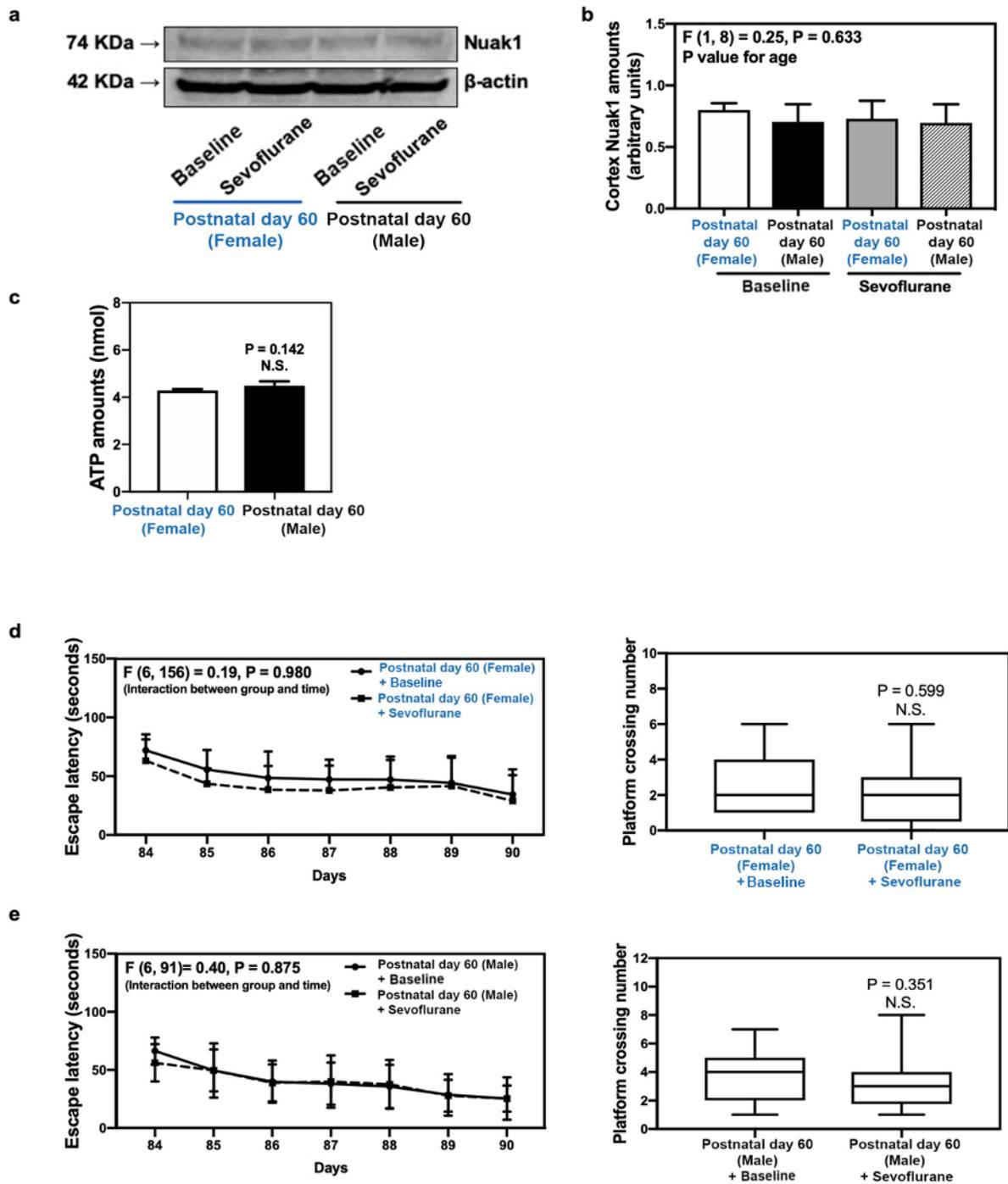
**Supplemental Figure 4. Brain Tau amounts and Tau metabolism in female and male 60-day-old (postnatal day 60) mice.** (a) Western blots of brain amount of Tau, Tau-PS356, oligomer Tau (T22), and Tau-PS202/PT205 in the cortex of female and male postnatal day 60 mice in baseline and sevoflurane anesthesia groups.  $\beta$ -actin is a loading control. (b) Summary of western blots of amounts of Tau (left top), T22 (left bottom), Tau-PS356 (right top), and Tau-PS202/PT205 (right bottom) ( $n=6$  mice/group). (c) ELISA measurement of Tau in the cortex of female and male postnatal day 60 mice in baseline and sevoflurane anesthesia groups ( $n=6$  mice/group). All data are quantified and expressed as arbitrary units compared to the baseline group (female adult mice in non-anesthetized group). PS, phosphorylated serine; PT, phosphorylated threonine. Two-way ANOVA: (b) and (c). \*\* $P<0.01$ ; \*\*\* $P<0.001$ .

## Supplemental Figure 5.



**Supplemental Figure 5. Nuak1 amount in hippocampus of neonatal and adult mice.** (a) Western blots of amounts of Nuak1 in the hippocampus of 6-day-old mice (postnatal day 6) from both sexes (female plus male mice) and 60-day-old (postnatal day 60) female mice (female reference group) in baseline and sevoflurane anesthesia groups.  $\beta$ -actin is a loading control. (b) Summary of western blots of Nuak1 amounts in the hippocampus of neonatal mice from both sexes and female reference mice in baseline and sevoflurane anesthesia groups. (c) mRNA expression of *Nuak1* in the hippocampus of neonatal mice from both sexes and female reference mice in baseline and sevoflurane anesthesia groups. All data are quantified and expressed as arbitrary units compared to the baseline group (neonatal mice from both sexes in non-anesthetized group). Two-way ANOVA: (b) and (c).

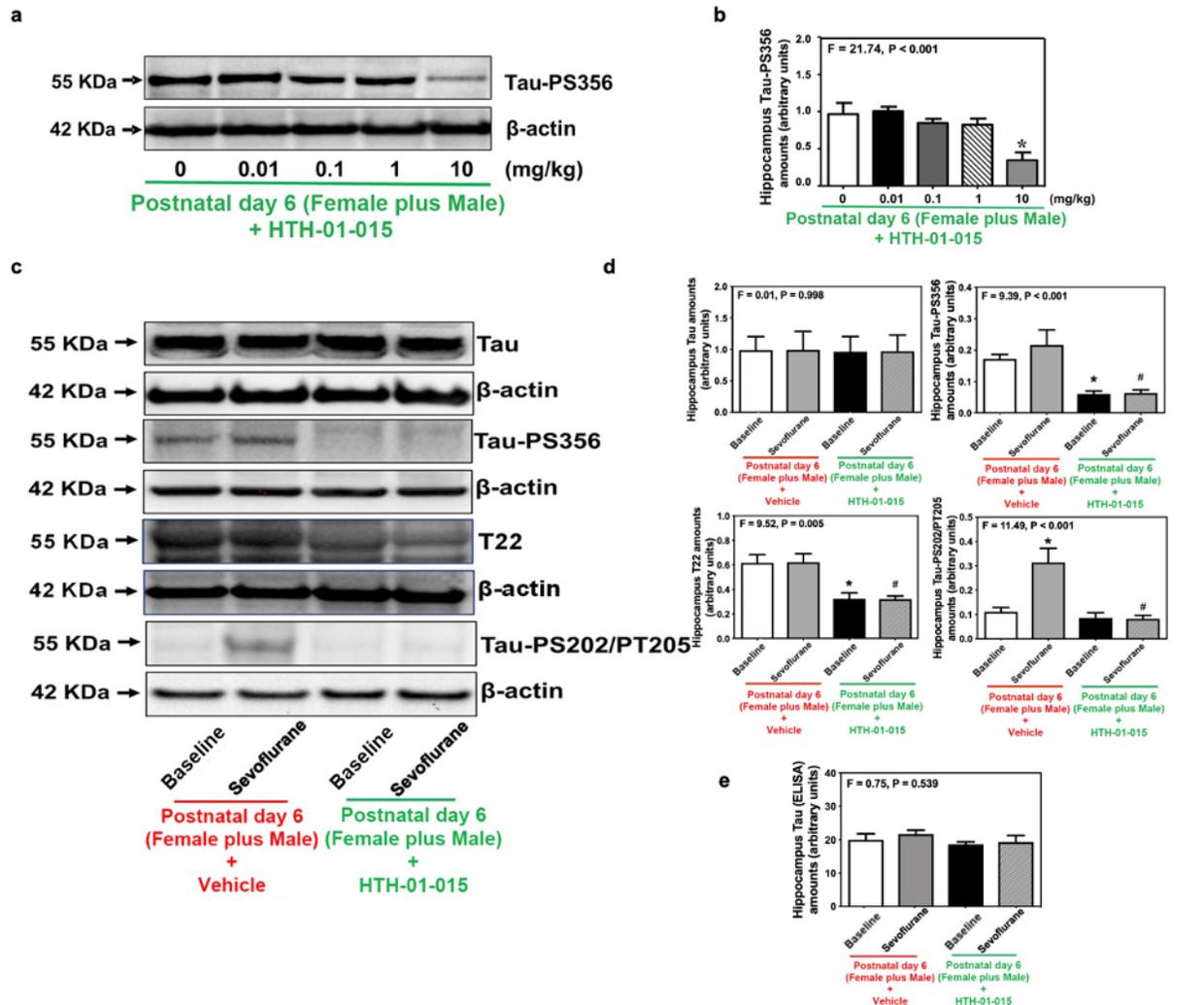
Supplemental Figure 6.



**Supplemental Figure 6. Brain amounts of Nuak1, ATP concentration, and cognition in female and male 60-day-old (postnatal day 60) mice.** (a) Western blot of Nuak1 protein amounts in the cortex of postnatal day 60 female and male mice in baseline and sevoflurane anesthesia groups. β-actin is a loading control. (b) Summary of western blots of Nuak1 amounts in the cortex of female and male postnatal day 60 mice in baseline and sevoflurane anesthesia groups. (c) ATP concentration in the cortex of female and male postnatal day 60 mice (n=6

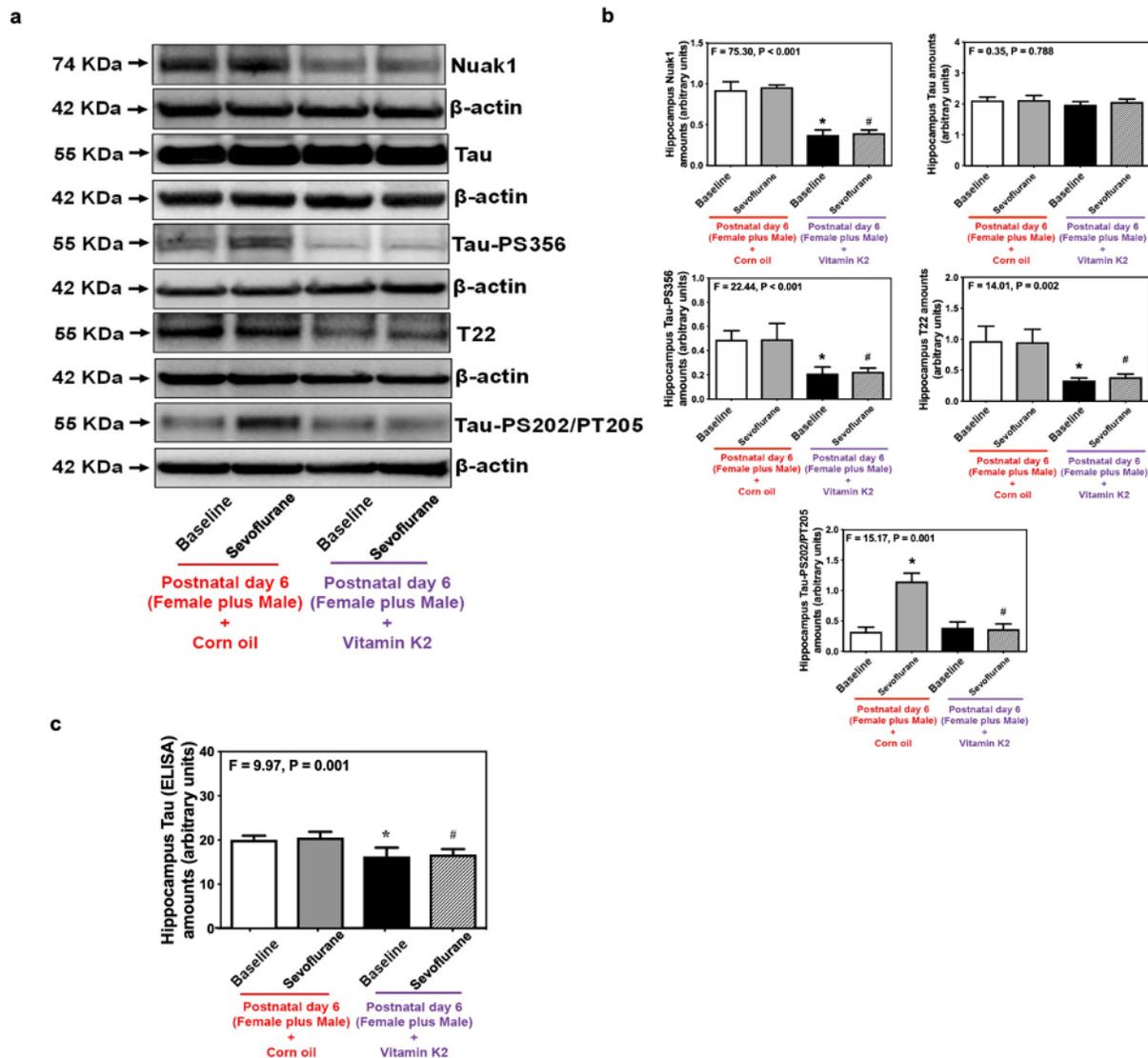
mice/group). (d) Escape latency (left) and platform crossing time (right) of female postnatal day 60 mice in baseline and sevoflurane anesthesia groups (n=10). (e) Escape latency (left) and platform crossing time (right) of male postnatal day 60 mice in baseline and sevoflurane anesthesia groups (n=10). All data are quantified and expressed as arbitrary units or real numbers compared to the reference group (male adult mice: c) or baseline group [female (b and d) or male (e) adult mice in non-anesthetized group]. Two-way ANOVA: (b), (d left), and (e left). Student's t-test: (c). Mann-Whitney test: (d right) and (e right). \*\*\* $P < 0.001$ .

Supplemental Figure 7.



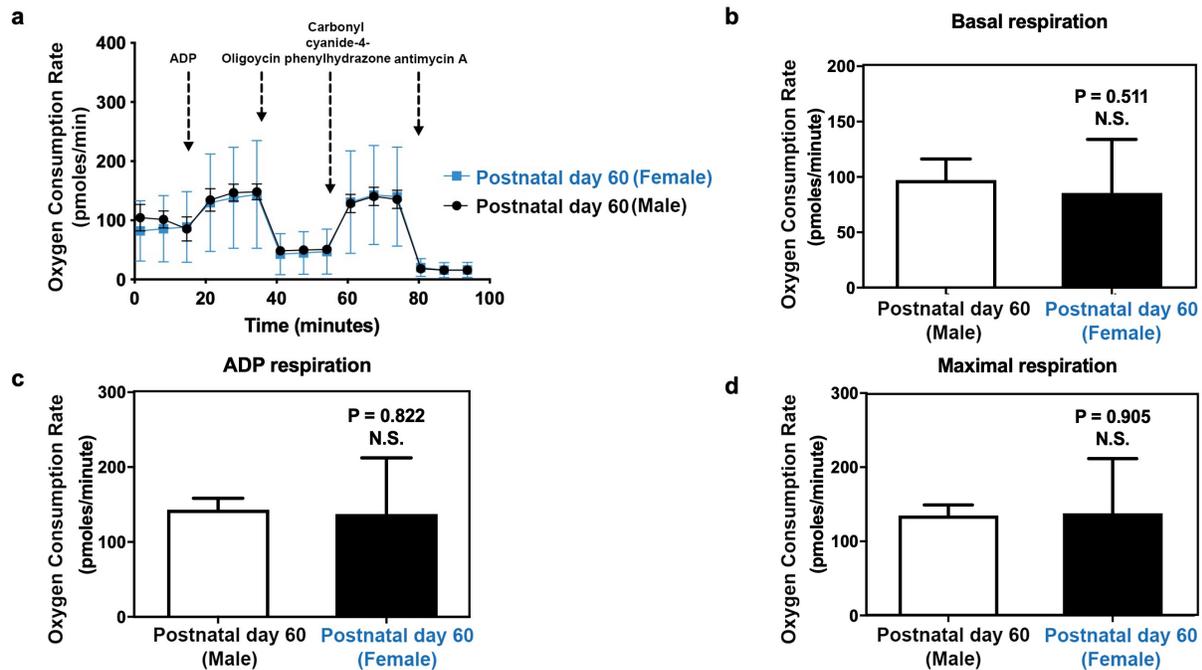
**Supplemental Figure 7. Regulation of Tauopathy by Nuak1 in neonatal mice.** (a) Western blot of amounts of Tau-PS356 in the hippocampus of 6-day-old (postnatal day 6) mice from both sexes (female plus male) following treatment with different doses of HTH-01-015. (b) Summary of western blots of hippocampus Tau-PS356 amounts in mice treated with different doses of HTH-01-015 (n=6 mice/group). (c) Western blots of the amounts of Tau, Tau-PS356, oligomer Tau (T22), and Tau-PS202/PT205 in the hippocampus of 6-day-old (postnatal day 6) mice following vehicle or HTH-01-015 treatment in sevoflurane anesthesia and baseline groups. (d) Summary of western blots of amounts of Tau (top left), T22 (bottom left), Tau-PS356 (top right), and Tau-PS202/PT205 (bottom right) (n=6 mice/group). (e) ELISA measurement of Tau in the hippocampus of neonatal mice from both sexes treated with vehicle or HTH-01-015 in sevoflurane anesthesia and baseline groups (n=4 mice/group). All data are quantified and expressed as arbitrary units compared to the baseline (neonatal mice from both sexes in non-anesthetized and vehicle group). PS, phosphorylated serine; PT, phosphorylated threonine. One-way ANOVA: (b), (d), (e). \*P<0.05; #P<0.05.

## Supplemental Figure 8.



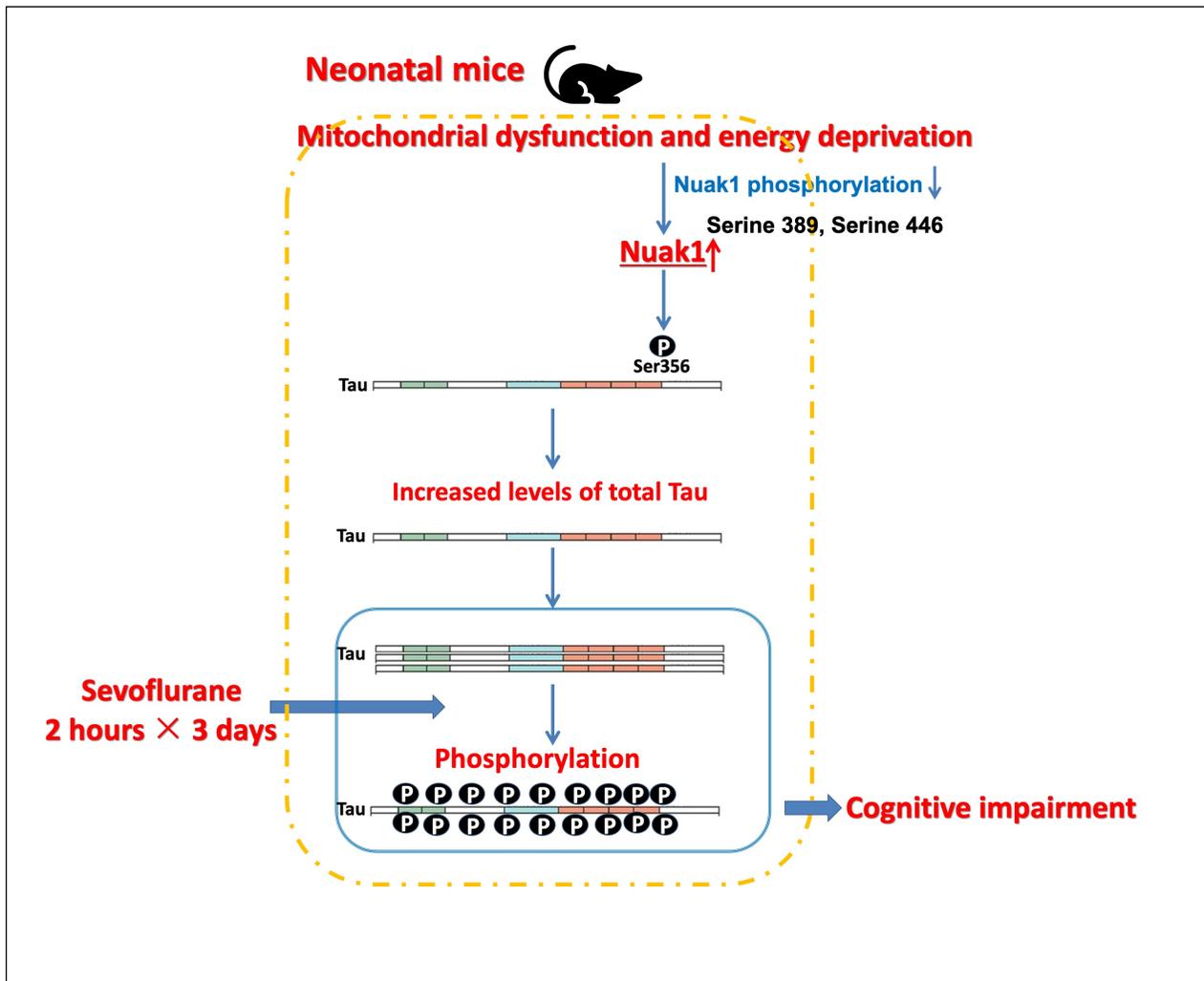
**Supplemental Figure 8. Vitamin K2 rescues Tauopathy in neonatal mice.** (a) Western blots of the amounts of Nuak1, Tau, Tau-PS356, oligomer Tau (T22), and Tau-PS202/PT205 in the hippocampus of 6-day-old (postnatal day 6) mice from both sexes (female plus male mice) treated with corn oil (vehicle) or vitamin K2 in sevoflurane anesthesia and baseline groups. (b) Summary of western blots of amounts of Nuak1 (top left), Tau (top right), Tau-PS356 (middle left), T22 (middle right), and Tau-PS202/PT205 (bottom) ( $n=6$  mice/group). (c) ELISA measurement of Tau amounts in the hippocampus of postnatal day 6 mice following treatment with corn oil or vitamin K2 in sevoflurane anesthesia and baseline groups ( $n=4$  mice/group). All data are quantified and expressed as arbitrary units compared to the baseline (neonatal mice from both sexes in non-anesthetized group). PS, phosphorylated serine; PT, phosphorylated threonine. One-way ANOVA: (b) and (c). \* $P<0.05$ ; # $P<0.05$ .

## Supplemental Figure 9.

**Supplemental Figure 9. Brain mitochondrial metabolism of adult female and male mice.**

Overall oxygen consumption rate (a), basal respiration (b), ADP respiration (c), and maximal respiration (d) in the hippocampus of 60-day-old (postnatal day 60) female and male mice (n=6; unpaired t test). All data are quantified and expressed as real number compared to female reference group mice. Student's t-test.

Supplemental Figure 10.



**Supplemental Figure 10. Hypothesized pathway.** In neonatal mice, Nuak1, which is increased during energy deprivation, can phosphorylate Tau protein at serine 356 and increase total Tau amounts. The anesthetic sevoflurane then acts on increased amounts of Tau to induce Tau phosphorylation, leading to cognitive impairment in neonatal mice.

**Reference**

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