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Testosterone attenuates sevoflurane-induced tau phosphorylation and cognitive impairment in neonatal male mice

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Abstract

Background: Sevoflurane anaesthesia induces phosphorylation of the microtubule-associated protein tau and cognitive impairment in neonatal, but not adult, mice. The underlying mechanisms remain largely to be determined. Sex hormones can be neuroprotective, but little is known about the influence of testosterone on age-dependent anaesthesia effects.

Methods: Six- and 60-day-old male mice received anaesthesia with sevoflurane 3% for 2 h daily for 3 days. Morris water maze, immunoassay, immunoblotting, co-immunoprecipitation, nanobeam technology, and electrophysiology were used to assess cognition; testosterone concentrations; tau phosphorylation; glycogen synthase kinase-3β (GSK3β) activation; binding or interaction between tau and GSK3β; and neuronal activation in mice, cells, and neurones.

Results: Compared with 60-day-old male mice, 6-day-old male mice had lower testosterone concentrations (3.03 [0.29] *vs* 0.44 [0.12] ng ml^{-1}; $P<0.01$), higher sevoflurane-induced tau phosphorylation in brain (133 [20]% *vs* 100 [6]% in 6-day-old mice, $P<0.01$; 103 [8]% *vs* 100 [13]% in 60-day-old mice, $P=0.77$), and sevoflurane-induced cognitive impairment. Testosterone treatment increased brain testosterone concentrations (1.76 [0.10] *vs* 0.39 [0.05] ng ml^{-1}; $P<0.01$) and attenuated the sevoflurane-induced tau phosphorylation and cognitive impairment in neonatal male mice. Testosterone inhibited the interaction between tau and GSK3β, and attenuated sevoflurane-induced inhibition of excitatory postsynaptic currents in hippocampal neurones.

Conclusions: Lower brain testosterone concentrations in neonatal compared with adult male mice contributed to age-dependent tau phosphorylation and cognitive impairment after sevoflurane anaesthesia. Testosterone might attenuate the sevoflurane-induced tau phosphorylation and cognitive impairment by inhibiting the interaction between tau and GSK3β.

Keywords: cognitive impairment; neuroprotection; sevoflurane; sex differences; tau phosphorylation; testosterone

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Editor's key points

- The mechanisms underlying volatile anaesthetic-induced phosphorylation of the microtubule-associated protein tau and cognitive impairment in neonatal, but not adult, rodents are unclear.
- Molecular, electrophysiological, and neurobehavioural experiments were used to assess cognition, testosterone concentrations, molecular interactions, and neuronal activation in mice and neuronal cells.
- Compared with 60-day-old male mice, 6-day-old male mice had lower testosterone concentrations, higher sevoflurane-induced tau phosphorylation in brain, and greater sevoflurane-induced cognitive impairment, all of which were attenuated by testosterone treatment.
- Lower brain testosterone contributes to age- and sex-dependent tau phosphorylation and cognitive impairment after sevoflurane anaesthesia.

The widespread and growing use of anaesthesia in children makes its safety a significant health issue. Several clinical studies show cognitive impairment in children after anaesthesia and surgery.[1–5] However, other studies show no association.[6,7] Thus, the effects of anaesthesia on cognitive function in children and the underlying mechanisms remain to be determined.

Anaesthetics induce neurotoxic effects and cognitive impairment in young rodents and monkeys.[8–12] For instance, anaesthesia with sevoflurane causes tau protein phosphorylation and impaired cognition in neonatal, but not in adult, mice.[13–17] Tau is a protein predominantly expressed in neurones and is associated with microtubule assembly and function.[18,19] Tau phosphorylation, aggregation, and spread contribute to Alzheimer's disease pathogenesis.[20] These studies suggest a molecular pathogenic mechanism for impaired cognition after anaesthesia; however, it remains unclear what contributes to the age-dependent effects of anaesthesia in these laboratory models.

As the sex hormones oestrogen and androgen are neuroprotective,[21] one potential link may be through sex hormone levels in neonatal or young compared with adult individuals. 17β-Oestradiol attenuates tau hyperphosphorylation induced during global cerebral ischaemia by inhibiting c-Jun N-terminal kinase/c-Jun/Dickkopf-1 signalling pathway activation.[22] 17β-Oestradiol also mitigates tau hyperphosphorylation induced by protein kinase A in human embryonic kidney cells stably expressing tau, potentially by inhibition and elevation of cyclic adenosine monophosphate and overactivation of protein kinase A.[23]

Sex- and age-dependent anaesthetic neurotoxicity in developing brain has been reported.[24–26] However, the role of sex hormones in these effects has not been specifically determined. Our previous studies[13–17,27] assessed male and female neonatal animals together, so did not differentiate effects by sex. Therefore, the objective of the study was to investigate whether testosterone contributes to the observed age-dependent effects of sevoflurane on tau phosphorylation and cognitive impairment in mice, and to determine the underlying mechanism. Sevoflurane may phosphorylate tau by activating glycogen synthase kinase-3β (GSK3β),[14] a kinase that contributes to tau phosphorylation.[28] Decreased phospho-GSK3β-Ser9[29,30] or increased phospho-GSK3β-Tyr216[31] indicates activation of GSK3β. We further hypothesised that increasing testosterone could attenuate sevoflurane-induced tau phosphorylation and cognitive impairment in neonatal male mice by inhibiting the interaction between tau and GSK3β.

Methods

Mice, anaesthesia, and treatment

The Standing Committee on Animals approved the animal protocol at Massachusetts General Hospital (protocol 2006N000219). Efforts were made to minimise the number of animals used; experiments and reporting follow the Animal Research: Reporting of In Vivo Experiments guidelines. Adult male mice (C57BL/6J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Neonatal male mice of the same strain at postnatal (P) Day 6 were generated from in-house breeding. Littermates were assigned randomly to all study groups. Neonatal and adult mice were randomly assigned to one of the following groups: (i) control plus vehicle (corn oil), (ii) control plus testosterone, (iii) sevoflurane plus vehicle, and (iv) sevoflurane plus testosterone. We also included female neonatal mice under control conditions and sevoflurane anaesthesia to compare tau phosphorylation between female and male neonatal mice.

Mice were anaesthetised in chambers with sevoflurane 3% and oxygen 40% for 2 h daily for 3 days at P6, P7, and P8 (neonatal group), or at P60, P61, and P62 (adult group), based on previous studies.[13,14] Control group mice received oxygen 40% at the identical flow rate in similar chambers and with the same separation time from their mothers. Previous studies showed that anaesthesia with sevoflurane 3% and oxygen 40% did not significantly affect blood gas values.[32]

Sevoflurane and oxygen concentrations were continuously monitored using a gas analyser (Dash 4000; GE Healthcare, Milwaukee, WI, USA). Anaesthesia chamber temperature was monitored and controlled by an automatic feedback-based system (World Precision Instruments Inc., Sarasota, FL, USA), keeping mouse rectal temperature at 37 [0.5]°C via a warming pad placed under the chamber. Testosterone (100 μg, dissolved in corn oil 25 μl to a concentration of 4 μg μl^{-1}; PHR2027; Sigma-Aldrich, St Louis, MO, USA) or vehicle only (corn oil 25 μl) was administered subcutaneously 1 h before sevoflurane anaesthesia based on methods described previously.[33] A pilot study showed that administration of testosterone 100 μg led to a peak concentration of testosterone in brain 1 h after administration (data not shown).

Cell line, primary neurones, anaesthesia, and treatment

SH-SY5Y neuroblastoma cells were purchased from the American Type Culture Collection (ATCC) (ATCC Cat# CRL-2266; ATCC, Manassas, VA, USA). Primary hippocampal neurones were harvested and cultured as described.[34] SH-SY5Y cells and neurones were randomly assigned to one of the following groups: (i) control plus vehicle (corn oil), (ii) control plus testosterone, (iii) sevoflurane plus vehicle, and (iv) sevoflurane plus testosterone. Primary neurones were treated with sevoflurane 3% for 2 h daily on culture Days 6, 7, and 8. SH-SY5Y cells were treated on culture Day 7 with sevoflurane 4% for 6 h as described.[35] SH-SY5Y cells and neurones were

treated with testosterone (dissolved in medium to a final concentration of 100 nM; PHR2027; Sigma-Aldrich) 1 h before sevoflurane exposure.

Morris water maze

A Morris water maze test was performed on P30–P36 (neonatal group) or P84–P90 (adult group) mice with four trials per day for 7 days as the reference training. Details of the Morris water maze test have been described.[16] At the end of the reference training (P36 or P90), the platform was removed from the pool. Each mouse was placed in the opposite quadrant and allowed to swim for 90 s. The numbers the mouse swam to cross the platform area were recorded as platform crossing number.

Brain tissue harvest, lysis, and protein quantification

Mice were decapitated at the end of sevoflurane anaesthesia on P8 or P62. Cerebral cortex and hippocampus were harvested and homogenised on ice using a lysis buffer, including immunoprecipitation buffer (Mammalian Protein Extraction Reagent, Cat# 78501; ThermoFisher Scientific, Waltham, MA, USA) plus protease inhibitor cocktail (Cat# 11836170001; Sigma). Lysates were collected and centrifuged for 15 min at ~19 000 × *g*. Total protein amounts were quantified using the Pierce™ protein assay kit (Cat# 23225, ThermoFisher Scientific).

Western immunoblotting

Total tau and total GSK3β protein amounts were detected with anti-tau 46 antibody (Cat# T9450, 55 kDa, 1:1000; Sigma-Aldrich) and anti-GSK3β antibody (Cat# 22104-1-AP, 47 kDa, 1:1000; Proteintech, Chicago, IL, USA). Phospho-tau-Ser262 antibody (Cat# ab131354, 47 kDa, 1:1000; Abcam, Cambridge, MA, USA) was used to detect tau phosphorylated at Ser262. AT8 antibody (Tau-PS202/PT205, Cat# MN1020, 55 kDa, 1:500; ThermoFisher Scientific) was used to measure the amount of tau phosphorylated at Ser202 and Thr205. Phospho-GSK3β-Ser9 (Cat# MA5-14873, 47 kDa, 1:1000; ThermoFisher Scientific) and phospho-GSK3β-Tyr216 (Cat# ab75745, 47 kDa, 1:1000; Abcam) antibodies were used to measure GSK3β phosphorylated at Ser9 and Tyr216, respectively. Postsynaptic density protein-95 (PSD-95) amounts were detected with anti-PSD-95 antibody (Cat# 2507S, 85 kDa, 1:1000; Cell Signaling Technology, Danvers, MA, USA). Antibodies detecting β-actin (Cat# A5441, 42 kDa, 1:5000; Sigma-Aldrich) and glyceraldehyde-3-phosphate dehydrogenase (Cat# 5174S, 36 kDa, 1:5000; Cell Signaling Technology) served to control for differences in total protein amount loaded. Protein quantification was performed as described.[36]

Enzyme-linked immunosorbent assay

A mouse/rat testosterone enzyme-linked immunosorbent assay (ELISA) kit (Cat# TE187S-100; Calbiotech, El Cajon, CA, USA) was used to measure testosterone concentrations in brain homogenates, serum, or plasma according to the manufacturer. Another kit (Cat# KGE010, ThermoFisher Scientific) was used to measure testosterone concentration in aqueous buffers. However, we could not use this ThermoFisher kit to measure the testosterone concentrations in brain tissues potentially owing to the low sensitivity. We, therefore, used the Calbiotech kit (Cat# TE187S-100) to measure testosterone concentrations in brain tissues. In the validation studies, we used the diluted buffer provided in the Calbiotech kit (Test 2) and the aqueous buffer (Test 3) to dissolve the testosterone powder provided in the ThermoFisher kit, respectively, which generated two standard curves similar to the standard curve of the Calbiotech kit (Test 1) (Supplementary Fig. 1).

Immunostaining

AT8 antibody (Tau-PS202/PT205, Cat# MN1020, 55 kDa, 1:100; ThermoFisher Scientific) was used to measure the amount of tau phosphorylated at Ser202 and Thr205 in primary neurones and SH-SY5Y cells. Immunoglobulin G (IgG) Alexa Fluor Plus 594 (Cat# A32742, 1:500; ThermoFisher Scientific) served as the secondary antibody. Mounting medium with 4′,6-diamidino-2-phenylindole and aqueous fluoroshield (Cat# ab104139; Abcam) was used in total cell counts. Experiments and quantification were performed blind.

Cell viability

A LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells (Cat# L3224; Invitrogen, Waltham, MA, USA) was used to measure viability of primary neurones and SY5Y cells following manufacturer instructions.

Spontaneous excitatory postsynaptic current recordings

Electrophysiological recordings of spontaneous excitatory postsynaptic currents (sEPSCs) in cultured primary hippocampal neurones were performed as described.[37] Recordings were made at room temperature 4 h after sevoflurane anaesthesia or control conditions on culture Day 7. Our pilot study found that cultured neurones on Day 7 could generate stable sEPSCs. Recording electrodes were pulled from thin-walled borosilicate capillary glass (World Precision Instruments) using a PP-830 Puller (Narishige, Toyko, Japan). SEPSCs were recorded in the whole-cell configuration or using an Axopatch™ 200B amplifier (Molecular Devices, Foster City, CA, USA) with voltage clamped at −60 mV, low-pass filtered at 2 kHz, digitised at 10 kHz using Digidata® 1550B (Molecular Devices), and stored with Clampex software (Molecular Devices) for offline analysis. Neurones exhibiting a significant change in series resistance (>20%) were excluded from analysis. Patch electrode resistance was 3–5 MΩ, and series resistance (6–8 MΩ) was not compensated. Recording of each neurone lasted 30–60 min. Electrode internal solution contained (mM): CsCl, 140; ethylene glycol tetraacetic acid, 2.5; $MgCl_2$, 2; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 10; triethanolamine, 2; and K_2ATP, 4 (pH 7.3); osmolarity was 300–310 mOsmol L^{-1}. The extracellular (bath) solution contained (mM): NaCl, 140; $CaCl_2$, 1.3; KCl, 5.0; HEPES, 25; glucose, 33; strychnine, 0.001; and bicuculline, 0.02 (pH 7.4); osmolarity was 325–335 mOsmol L^{-1}. Amplitude and frequency of sEPSCs were analysed using Mini Analysis software (Synaptosoft, Inc., Decatur, GA, USA). The detection threshold for sEPSCs was 12 pA. Overlapping sEPSCs were excluded from analysis. We chose healthy neurones with a clear cell membrane, homogeneous cytoplasm, good refraction, and a transparent cell body for whole-cell recording. Only those sEPSC data from neurones with giga-seal and membrane resistance >100 MΩ were included in the data analysis.

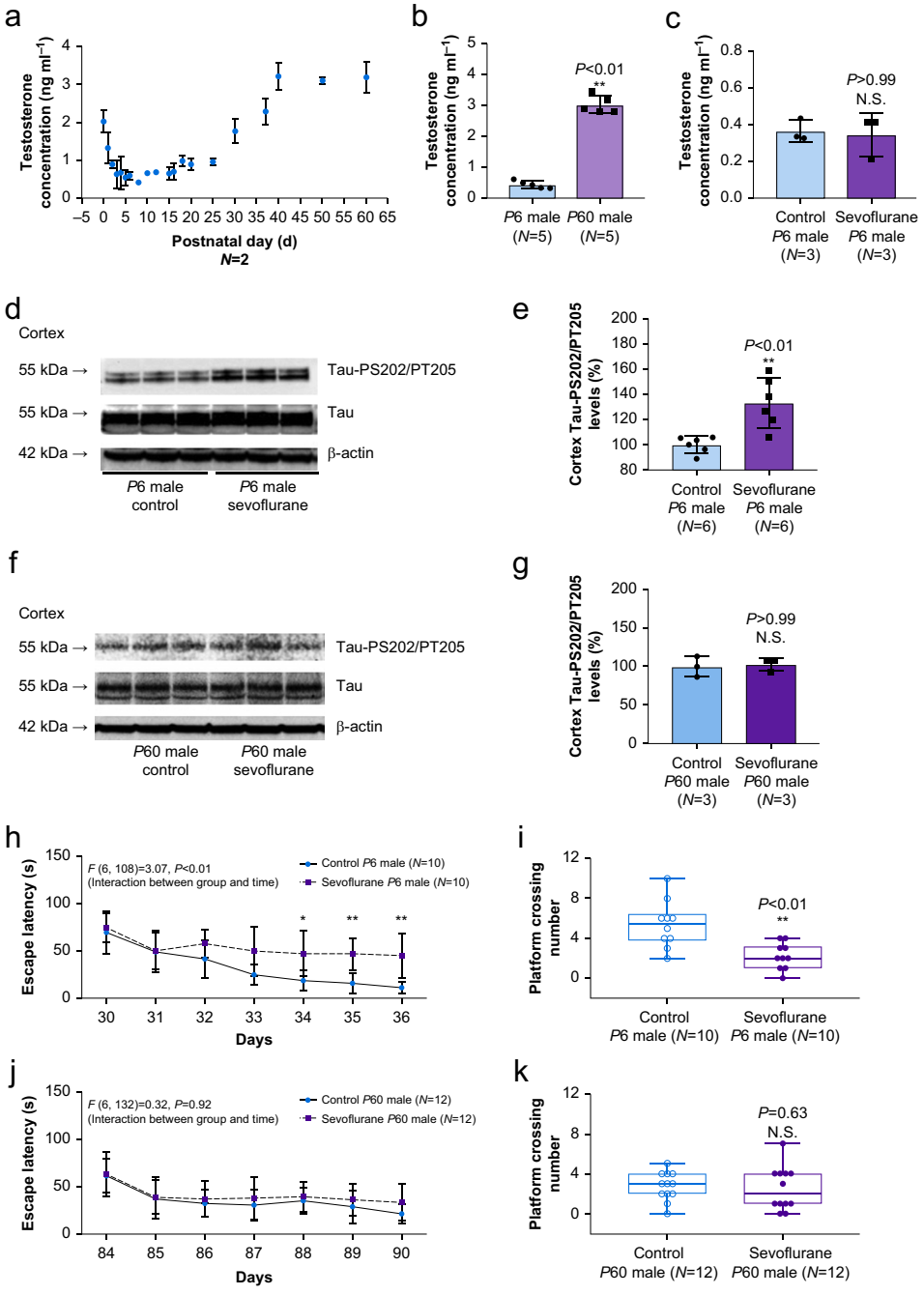

Fig 1. Differences between neonatal and adult male mice in brain testosterone concentrations, tau phosphorylation, and cognitive function after sevoflurane anaesthesia. (a) Concentrations of testosterone in brain of neonatal and adult mice (n=2 mice per group per day). (b) Brain concentrations of testosterone in P6 and P60 male mice (n=5 mice per group). (c) Effect of sevoflurane on brain testosterone concentrations in P6 male mice (n=3 mice per group). (d) Western immunoblot showing the effects of sevoflurane on the amounts of tau and tau-phospho-Ser202/Thr205 in the cerebral cortex of P6 male mice. (e) Quantification of western immunoblots in P6 male mice (n=6 mice per group). (f) Western immunoblot showing effects of sevoflurane on the amounts of tau and phosph-tau-Ser202/Thr205 in cerebral cortex of P60 male mice. (g) Quantification of western immunoblots in P60 male mice (n=3 mice per group). Effects of sevoflurane on escape latency and platform crossing number in the Morris water maze test in (h and i) neonatal male mice (n=10 mice per group) and (j and k) adult male mice (n=12 mice per group). Student's t-test was used to analyse difference in (b) and (e). Mann–Whitney U-test was used to analyse difference in (c), (g), (i), and (k). The overall P-values in (h and j) refer to interaction of group (control *vs* sevoflurane) and day (Days 30–36 or Days 84–90) in two-way repeated-measures analysis of variance. Day-specific *post hoc* P-values are Bonferroni corrected for multiple comparisons. Boxes in (i) and (k) indicate median and inter-quartile range; upper and lower bars indicate minimum and maximum. *P<0.05; **P<0.01. Error bars indicate standard deviation. MWM, Morris water maze; P, postnatal; PS, phosphorylated serine; PT, phosphorylated threonine; N.S., not significant.

Co-immunoprecipitation

We used SY5Y cells for co-immunoprecipitation (co-IP) experiments because these cells are easier to culture and can provide large amounts of protein for co-IP. Cultured cell co-IP experiments required more sensitive nanobeam studies to confirm potential interactions between tau and GSK3β, and to identify the influence of testosterone on such interactions. Specifically, protein extracted from SY5Y cells was immunoprecipitated overnight with a total tau antibody (anti-tau, Cat# 556319, 1:1000; BD Pharmingen, San Diego, CA, USA), and then incubated in a Protein A Sepharose® IgA (25 μl, Cat# 17-5280-01; GE Healthcare, Chicago, IL, USA) and Protein G Sepharose IgG (25 μl, 17-0618-01, GE Healthcare) agarose mixture for 3 h. The antibody–antigen–agarose mixture was then centrifuged and analysed via a western immunoblot with GSK3β antibody (Cat# 22104-1-AP, 47 kDa, 1:1000; Proteintech). We did not quantify the co-IP data, but independent experiments were performed three times separately.

Nanobeam sensor assessment of tau–GSK3β interactions

We used nanobeam sensor technology to study the interaction of tau and GSK3β at a single molecule level as described.[38,39] Tau (1 μM) was placed in the nanobeam sensor, and recombinant active GSK3β (50 nM) (Cat# ab60863; Abcam) was added to the microfluidic channel. When tau interacted with GSK3β, the nanobeam spectrum shifted to a longer wavelength. A subsequent wash removed proteins from the nanobeam sensor, shifting the spectrum back to the shorter wavelength. After one detection cycle, the nanobeam sensor was regenerated. Testosterone (100 nM) (58-22-0; MilliporeSigma, St Louis, MO, USA) and GSK3β were injected into the microfluidic channel to measure testosterone inhibition of the interaction of tau with GSK3β. A home-made microfluidic channel device (100 × 50 μm) with two inlets and one outlet was used for sample delivery. The peak of each spectrum was obtained using a Lorentzian fitting algorithm.[38]

Statistical analysis

Based on our previous studies,[13–17,27] sufficient power to detect a significant effect should be achieved using 10–12 mice per group for behavioural experiments, three to six mice or cell (neurone) samples per group for the western immunoblot and ELISA analyses, five neurone samples per group for the sEPSC recordings, and three to four cell (neurone) samples per group for the immunostaining studies.

The difference in learning between treatment groups, based on escape latency in the Morris water maze test, was analysed using a repeated-measures two-way analysis of variance (ANOVA). *Post hoc* comparisons with Bonferroni correction were used to compare escape latencies across groups on each day of the test only when there was an effect of days within a given group. Platform crossing number was compared across groups using Mann–Whitney *U*-tests. Student's *t*-test was used to analyse differences in brain testosterone concentrations between P6 and P60 male mice (Fig. 1b; $N=5$), phosphorylated tau (phospho-tau) amounts between control and sevoflurane in P6 male mice (Fig. 1e; $N=6$), and testosterone concentrations between the control condition and testosterone treatment in P6–P8 male mice (Fig. 2b; $N=6$). The Mann–Whitney *U*-test was used to analyse differences in brain testosterone concentrations between control and sevoflurane in P6 male mice (Fig. 1c; $N=3$), and brain phospho-tau concentrations between control and sevoflurane in P60 male mice (Fig. 1g; $N=3$). The effects of sevoflurane and testosterone on brain, cultured cells, and primary neurones were analysed using two-way ANOVA and *post hoc* tests with Bonferroni correction. Given the unequal variance amongst groups, sEPSC amplitude and frequency were compared using unequal variance Student's *t*-test. Data are expressed as percentages, arbitrary units, or real numbers, and presented as mean (standard deviation) for protein analysis and Morris water maze escape latency, or as median with interquartile range (IQR) for Morris water maze platform crossing numbers. $P<0.05$ was considered statistically significant, and significance testing was two-tailed in a two-group comparison. For Bonferroni correction, adjusted *P*-values, calculated by dividing real *P*-values by sample size, are reported. Statistical analysis was conducted using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) and SPSS Statistics version 21.0 (IBM; Armonk, NY, USA).

Results

Sevoflurane increased tau phosphorylation and cognitive impairment in neonatal male mice, but not in adult male mice

Brain testosterone levels increased from P0 to P60 (Fig. 1a), with adult male mice having higher levels than neonatal male mice (Fig. 1b; 3.03 [0.29] *vs* 0.44 [0.12]; $P<0.01$). Sevoflurane did not significantly alter testosterone concentration in neonatal male mouse brain (Fig. 1c). Sevoflurane increased tau phosphorylation in neonatal male mouse cortex, as evidenced by increased phospho-tau at S202 and T205 without a significant increase in total tau (Fig. 1d). The ratio of phospho-tau to tau was 133 (20)% in sevoflurane-treated *vs* 100 (6)% in control neonates (Fig. 1e; $P<0.01$). Sevoflurane did not increase tau phosphorylation in adult mouse cortex (Fig. 1f and g; 103 [8]% *vs* 100 [13]%; $P=0.77$).

The standard curve generated by the testosterone ELISA kit (Test 1) (Cat# TE187S-100; Calbiotech) and the two standard curves generated employing the diluted buffer of this Calbiotech kit (Test 2) and aqueous buffer (Test 3) used to dissolve the testosterone provided by the ThermoFisher testosterone ELISA kit (Cat# KGE010) were very similar (Supplementary Fig. 1). There were no significant differences in either tau or phospho-tau amounts in brain between neonatal male or neonatal female mice under either control condition or sevoflurane anaesthesia (Supplementary Fig. 2).

For the Morris water maze test, there was a significant interaction between treatment (sevoflurane *vs* control) and time (P30–P36) on escape latency in neonatal male mice (Fig. 1h; $F=3.07$; $P<0.01$), with sevoflurane increasing the escape latency on the final 3 days of the 7 day test (P34, P35, and P36). Sevoflurane-treated neonates performed less platform crossings than control mice (Fig. 1i; 2.2 [IQR: 1.0–3.3] *vs* 5.4 [IQR: 3.8–6.5]; $P<0.01$). Sevoflurane did not cause cognitive impairment in adult mice (Fig. 1j and k).

Testosterone attenuated the sevoflurane-induced tau phosphorylation and cognitive impairment in neonatal male mice

Given the higher testosterone levels in adult *vs* neonatal male mouse brain, and that only neonatal male mice had increased tau phosphorylation and cognitive impairment after sevoflurane anaesthesia, we asked whether testosterone could

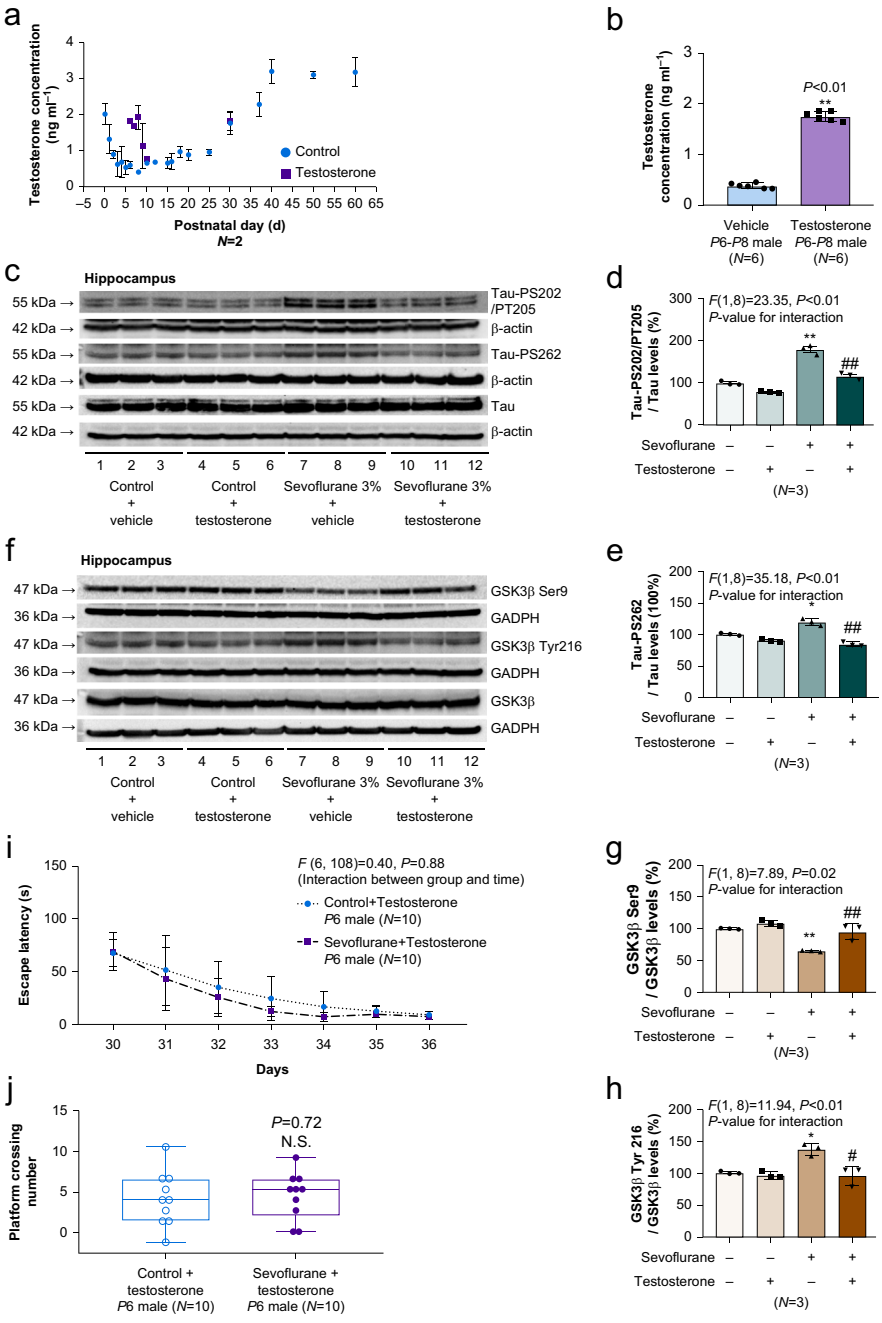

Fig 2. Testosterone attenuates sevoflurane-induced tau phosphorylation and cognitive impairment in neonatal male mice. (a) Concentrations of testosterone in the brain of neonatal male mice (P6–P10 and P30; red dots) after administering testosterone from P6 to P8 (n=2 mice per group per day). (b) Concentration of testosterone in the brain tissues of P8 male mice after injection of testosterone from P6 to P8 (n=6 mice per group). (c) Effects of sevoflurane, testosterone, and sevoflurane plus testosterone on the amounts of tau, phospho-tau-Ser202/Thr205, and phospho-tau-Ser262 in the hippocampus of neonatal (P8) male mice. Quantification of the ratio of (d) phospho-tau-Ser202/Thr205 to total tau and (e) phospho-tau-Ser262 to total tau (n=3 mice per group). (f) Effects of sevoflurane, testosterone, and sevoflurane plus testosterone on the amounts of glycogen synthase kinase-3β (GSK3β), phospho-GSK3β Ser9, and phospho-GSK3β Tyr216 in the hippocampus of neonatal (P8) male mice. Quantification of (g) the ratio of phospho-GSK3β Ser9 to total GSK3β and (h) the ratio of phospho-GSK3β Tyr216 to total GSK3β (n=3 mice in each group). (i) Effect of sevoflurane on escape latency of neonatal mice pretreated with testosterone in the Morris water maze test. (j) Effect of sevoflurane on platform crossing number of neonates pretreated with testosterone in the Morris water maze test (n=10 mice per group). Student's t-test was used to analyse difference in (b). Two-way analysis of variance (anova) was used to analyse difference in (d), (e), and (g–i). Mann–Whitney U-test was used to analyse differences in (j). The overall P-value in (i) refers to the interaction of group (control *vs* sevoflurane) and day (Days 30–36) in the two-way repeated-measures anova. Day-specific *post hoc* P-values are Bonferroni corrected for multiple comparisons. Boxes in (j) indicate median and inter-quartile range; upper and lower bars indicate minimum and maximum. *P<0.05; ** or ##P<0.01. Error bars indicate standard deviation. MWM, Morris water maze; P, postnatal; PS, phosphorylated serine; PT, phosphorylated threonine; Ser9, serine-9; Tyr216, tyrosine-216.

attenuate sevoflurane-induced cognitive impairment in neonatal male mice.

Treatment with testosterone three times (P6, P7, and P8), but not once (P6), attenuated sevoflurane-induced tau phosphorylation (Supplemental Fig. 3a and b). Testosterone treatment on P6, P7, and P8 increased testosterone levels in neonatal brain at P8 (Fig. 2a and b; 1.76 [0.1] *vs* 0.39 [0.05] ng ml^{-1}; $P<0.01$) and attenuated sevoflurane-induced tau phosphorylation in neonatal hippocampus (Fig. 2c), both at S202 and T205 (Fig. 2d; $F=23.35$; $P<0.01$) and S262 (Fig. 2e; $F=35.18$; $P<0.01$).

A similar interaction of sevoflurane and testosterone on tau phosphorylation occurred in neonatal cortex (Supplementary Fig. 3c–e). Compared with vehicle treatment, testosterone-treated neonates showed a smaller decrease in GSK3β phospho-Ser9 (Fig. 2f and g; $F=7.89$; $P=0.02$) and a smaller increase in GSK3β phospho-Tyr216 (Fig. 2f and h; $F=11.94$; $P<0.01$) in the hippocampus after sevoflurane anaesthesia. A similar interaction between sevoflurane and testosterone on GSK3β phospho-Ser9 and phospho-Tyr216 also occurred in neonatal male mouse cortex and primary neurones (Supplementary Fig. 3f–h). These results showed that testosterone attenuated sevoflurane-induced tau phosphorylation in neonatal brain by inhibiting sevoflurane-induced activation of GSK3β.

Sevoflurane induced cognitive impairment in neonates not treated with testosterone before anaesthesia, as evidenced by increased escape latency and decreased platform crossing in the Morris water maze test. Neonates treated with testosterone before sevoflurane anaesthesia showed no significant difference in either measure (Fig. 1h and i *vs* Fig. 2i and j).

Testosterone inhibited sevoflurane-induced interaction between tau and GSK3β

We used SY5Y cells for co-IP studies because they are easier to culture and yielded sufficient protein for co-IP experiments. After determining the testosterone concentration that attenuated sevoflurane-induced tau phosphorylation and activation of GSK3β in SY5Y cells (100 nM; Supplementary Fig. 4a–c), we showed that sevoflurane, testosterone, and their interaction do not decrease cell viability (Supplementary Fig. 4d–g), validating the use of these cells to study binding or interaction of sevoflurane with testosterone. Sevoflurane anaesthesia increased the interaction between GSK3β and tau (Fig. 3a), demonstrating that sevoflurane promoted binding or interaction between the two proteins. Testosterone mitigated this binding or interaction (Fig. 3a).

We used nanobeam technology (Fig. 3b), including a microfluidic channel for tau and GSK3β interaction with or without testosterone (Fig. 3c), to study the real-time dynamic interaction between tau and active GSK3β at the single-molecule level (Fig. 3d). The shift from green or purple to red represented the specific and nonspecific binding or interaction of tau and GSK3β; the shift from red to blue or light blue represented specific interaction or binding between tau and GSK3β after the washout of the weak interaction between tau and GSK3β. The interaction between tau and active GSK3β was reduced in the presence of testosterone, as evidenced by a larger signal reduction between GSK3β plus testosterone and the second wash (Fig. 3e, Phase II, uncoloured background, $\Delta\lambda=0.20$ nm) *vs* between GSK3β plus vehicle and the first wash (Fig. 3e, Phase I, coloured background, $\Delta\lambda=0.36$ nm). Thus, testosterone may reduce sevoflurane-induced tau phosphorylation and GSK3β activation by inhibiting the interaction between tau and GSK3β.

Testosterone attenuated sevoflurane-induced tau phosphorylation and sEPSC reduction in neurones

Testosterone attenuated sevoflurane-induced tau phosphorylation in primary neurones, rescuing the increases in phospho-tau-S202/T205 and tau-262 (Fig. 4a–c) and in the number of phospho-tau-S202/T205-positive cells (Fig. 4d and E; $F=13.66$; $P<0.01$). Testosterone also attenuated sevoflurane-induced decreases in GSK3β phospho-Ser9 (Fig. 4f and g; $F=18.85$; $P<0.01$) and increases in GSK3β phospho-Tyr216 (Fig. 4f and h; $F=10.86$; $P=0.01$).

Testosterone attenuated sevoflurane-induced reduction in the amount of PSD-95 in primary neurones (Fig. 5a and b) and in the amplitude (Fig. 5c and d; $F=4.31$; $P=0.05$) and frequency (Fig. 5c and e; $F=1.42$; $P=0.25$) of their sEPSCs. These data suggest that sevoflurane may decrease neuronal activation, which can be attenuated by testosterone treatment.

Discussion

We performed *in vivo* (mice), *in vitro* (primary neurones and SY5Y cells), and nanobeam (single molecule) studies to explore the underlying mechanisms of age-dependent sevoflurane-induced tau phosphorylation and cognitive impairment. Neonatal male mice, which were more vulnerable to the neurotoxic effects of sevoflurane, had lower levels of brain testosterone than adult male mice did. Treatment with testosterone before sevoflurane anaesthesia attenuated sevoflurane-induced tau phosphorylation and cognitive impairment in neonatal male mice. Testosterone also attenuated sevoflurane-induced tau phosphorylation in SY5Y neuroblastoma cells and primary neurones, and reduced the interaction between tau and GSK3β. In addition, testosterone rescued sevoflurane-induced inhibition of the amplitude and frequency of neuronal sEPSCs. These results suggest possible protective effects of testosterone on anaesthetic neurotoxicity, potentially via inhibiting the tau–GSK3β interaction, pending further confirmative studies.

The objective of the present study was not to investigate whether supplementation of testosterone could be used to treat anaesthetic neurotoxicity, but rather to determine the potential underlying mechanism by which sevoflurane induces age-dependent cognitive impairment in mice.

Previous studies have shown that anaesthesia with one minimum alveolar concentration isoflurane for 4 h in P7 rats can cause neuronal death at 12 h after anaesthesia in both male and female rats, but only impairs recognition of objects and produces social memory deficits in male rats at P38.[24] Isoflurane anaesthesia at P4 induces greater neurotoxicity and neurobehavioural deficits than at P7 in female mice.[25] Male progeny, but not female progeny, of sevoflurane-exposed parents showed abnormalities in behavioural testing and expression of the KCC2 co-transporter. Male F1 rats of both exposed parents showed impaired spatial memory and KCC2 expression.[26] These results suggest that there could be sex-dependent differences in anaesthetic neurotoxicity. However, Loepke and colleagues[40] showed no sex difference in long-term neuronal density, spontaneous locomotion, or cognitive function after isoflurane anaesthesia in P7 mice. Boscolo and colleagues[41] reported that female P7 rats were more vulnerable to cognitive impairment after anaesthesia with midazolam, nitrous oxide, and isoflurane.

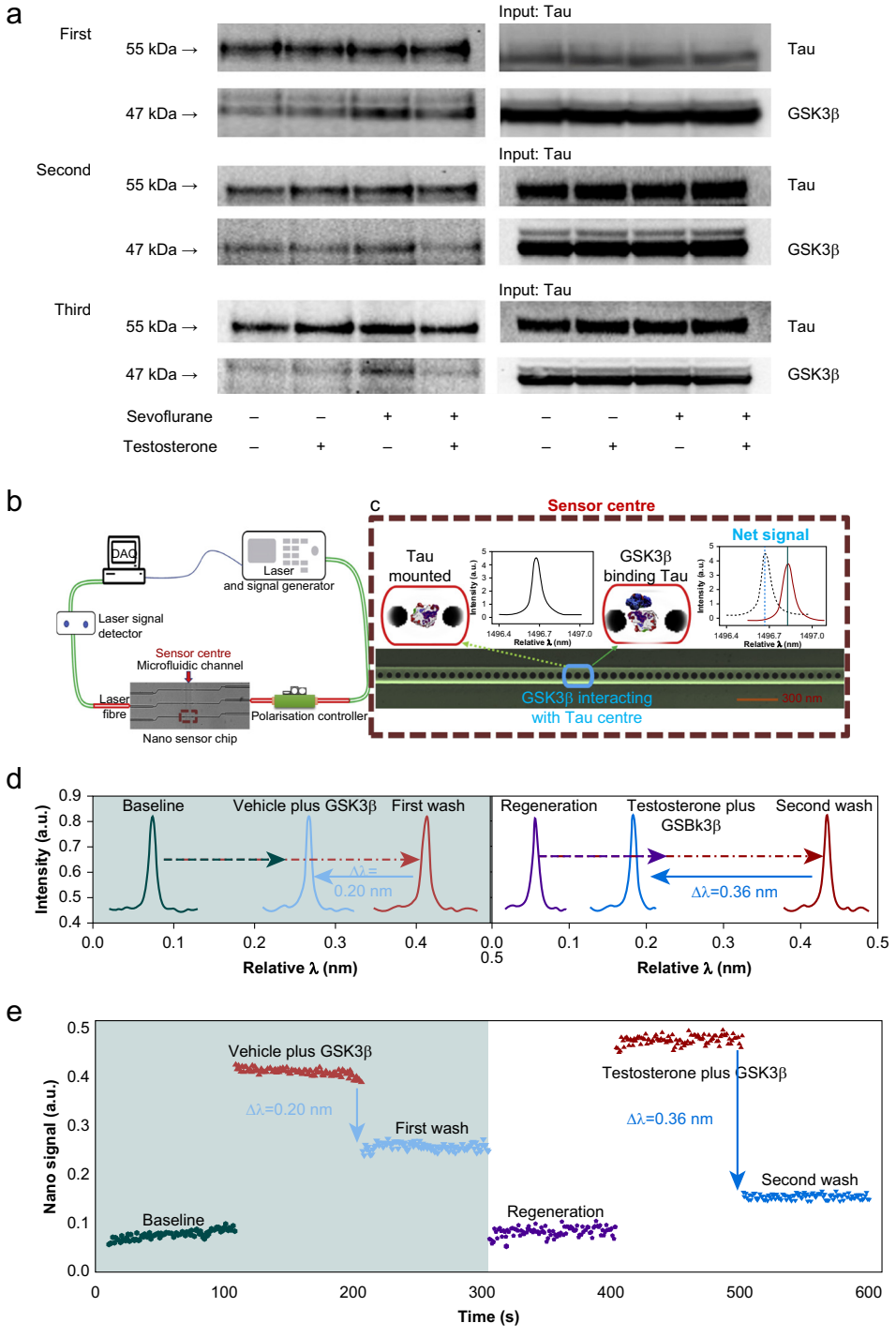

Fig 3. Binding and interaction between tau and glycogen synthase kinase-3β (GSK3β) in co-immunoprecipitation and nanobeam experiments. (a) Co-immunoprecipitation of tau and GSK3β in SY5Y cells with or without sevoflurane or testosterone treatment. (b) Nanobeam sensor centre and resonance measurement set-up. (c) Schematic of the microfluidic channel showing tau interacting with GSK3β. (d) Real-time recording schematic of the interaction between tau and GSK3β. The shifting of spectrum wavelength indicates changes in binding signals in different conditions. Specifically, shifting from black or purple to red or dark blue represented the interaction of tau and GSK3β; shifting from red or purple to blue or dark blue reflects binding between tau and GSK3β after wash-off of weak interactions between tau and GSK3β. (e) Nanobeam signal recording of all time points showing the interaction between tau and GSK3β without testosterone treatment (Phase I; coloured area) and with testosterone treatment (Phase II; uncoloured area). There was a larger signal reduction between GSK3β plus testosterone and the second wash (e) (Phase II; uncoloured background) *vs* between GSK3β plus vehicle and the first wash (e) (Phase I; coloured background), suggesting that the interaction between tau and GSK3β was reduced by testosterone. a.u., arbitrary unit; Δ, delta, representing changes; λ, nanobeam wavelength.

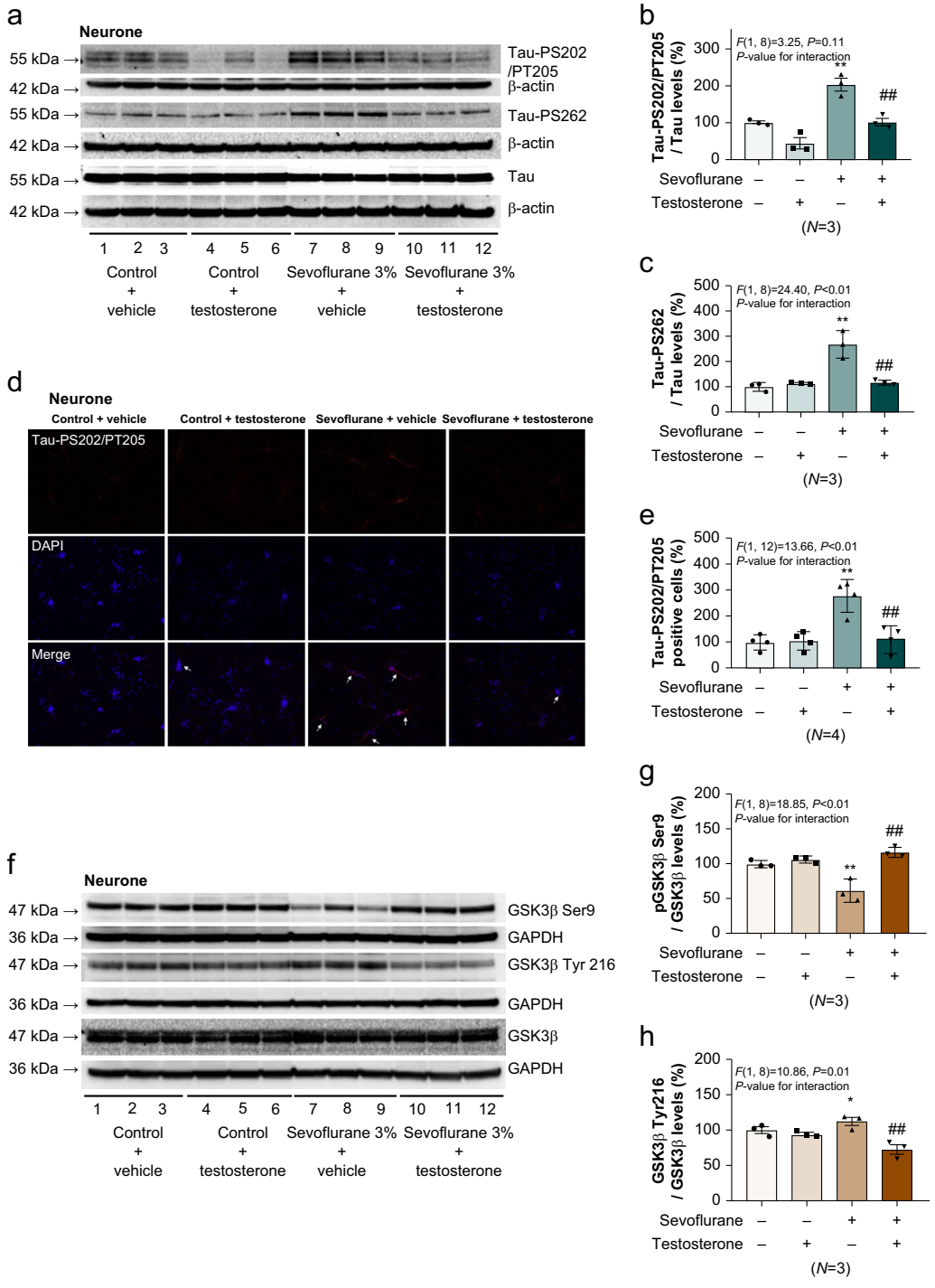

Fig 4. Testosterone attenuates sevoflurane-induced tau phosphorylation and activation of glycogen synthase kinase-3β (GSK3β) in primary neurones. (a) Effects of sevoflurane, testosterone, and sevoflurane plus testosterone on the amounts of tau, phospho-tau-Ser202/Thr205, and phospho-tau-Ser262 in primary neurones. Quantification of the ratio of (b) phospho-tau-Ser202/Thr205 to total tau and (c) phospho-tau-Ser262 to total tau (n=3 biological samples per group). (d) Immunohistochemistry imaging showing phospho-tau-Ser202/Thr205-positive primary neurones after treatment with sevoflurane, testosterone, and sevoflurane plus testosterone. (e) Quantification of the immunohistochemistry imaging (n=4 biological samples per group). (f) Effects of sevoflurane, testosterone, and sevoflurane plus testosterone on the amounts of GSK3β, phospho-GSK3β Ser9, and phospho-GSK3β Tyr216 in primary neurones. Quantification of (g) the ratio of phospho-GSK3β Ser9 to total GSK3β and (h) the ratio of phospho-GSK3β Tyr216 to total GSK3β (n=3 biological samples per group). Two-way analysis of variance was used to analyse differences in (b), (c), (e), (g), and (h). *P<0.05; ** and ##P<0.01. Error bars indicate standard deviation. DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PS, phosphorylated serine; PT, phosphorylated threonine; Ser9, serine 9; Tyr216, tyrosine 216.

Brain testosterone concentrations were shown to be higher in P6 male rats than in P60 male rats.[42] In contrast, we showed that P6 male mice had lower concentrations of brain testosterone than P60 male mice. The reason for this difference is not known. To our knowledge, there have been no studies that compared brain concentrations of testosterone between P6 and P60 male mice. Moreover, the developmental stages between rats and mice are different, as puberty occurs between P42 and P55 in male rats,[43] but at P30 in male mice,[44,45] which could contribute to the different findings in brain testosterone concentrations between rats and mice. Finally, blood testosterone concentrations in P6 male rats or P6 male mice are lower than those in P60 male rats or P60 male mice,[45] consistent with our study.

Previous studies showed that an androgen receptor antagonist[46] or gonadectomy[47] attenuated isoflurane-induced behavioural deficiencies in neonatal male rats. Treatment with testosterone plus isoflurane caused cognitive impairment in neonatal female rats.[48] However, the data from the present study suggest that treatment with testosterone can mitigate sevoflurane-induced cognitive impairment in neonatal male mice. The reason for such difference remains unknown. In the aforementioned studies, neonatal rats received isoflurane for 6 h at P7; androgen receptor antagonist flutamide at P2, P4, and P6[46]; or testosterone at P1–P6. In the present study, neonatal mice received sevoflurane 3% for 2 h with and without testosterone on P6, P7, and P8. In both studies, the rodents received anaesthesia with oxygen 40%.

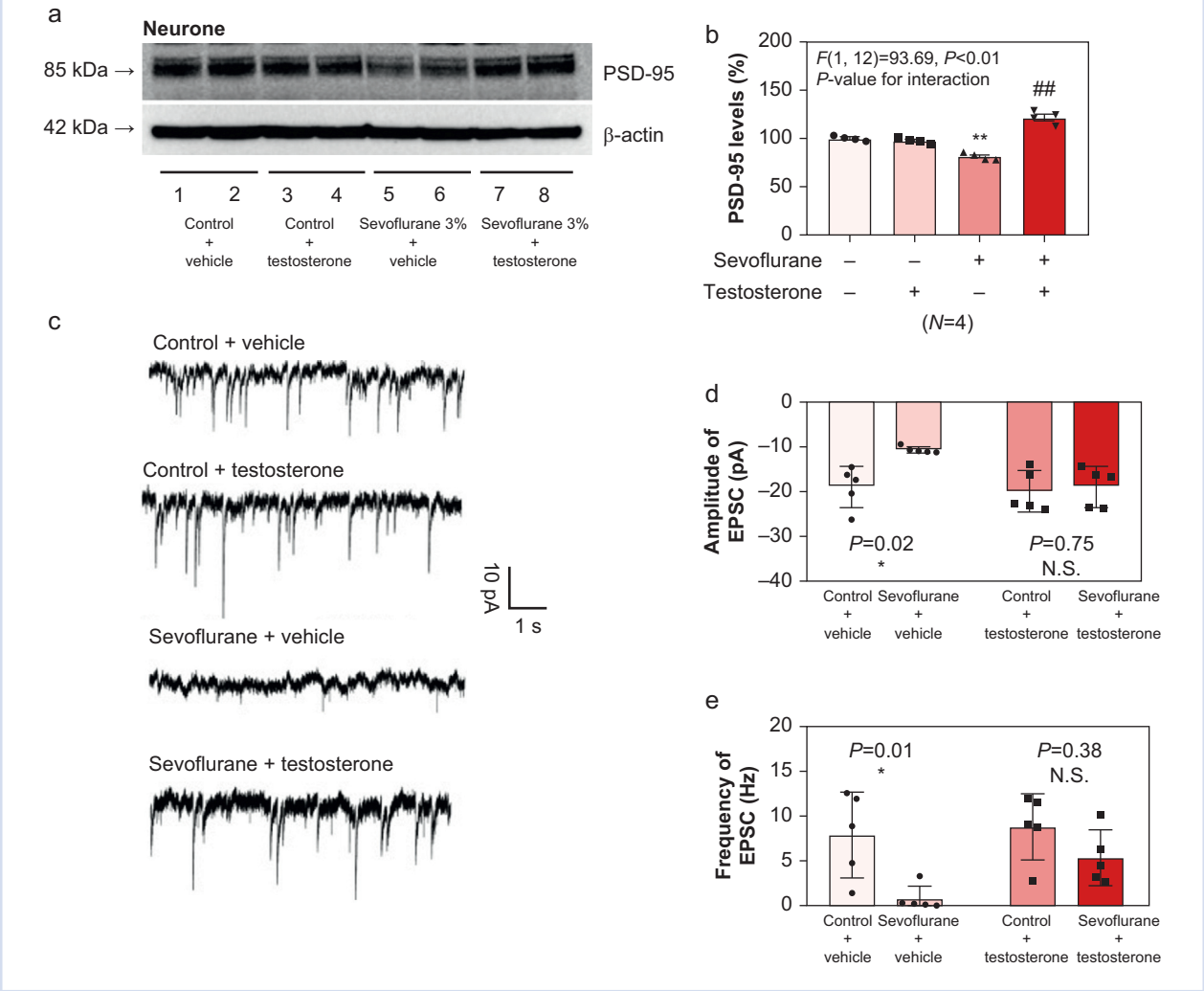

Fig 5. Testosterone attenuates sevoflurane-induced reduction in postsynaptic density protein-95 (PSD-95) and spontaneous excitatory postsynaptic currents (sEPSCs) in primary neurones. (a) Effects of sevoflurane and testosterone on the amounts of PSD-95 in primary neurones. (b) Quantification of the western immunoblots of the effects of sevoflurane and testosterone on the amounts of PSD-95 in primary neurones (n=4 biological samples per group). (c) Representative whole-cell current traces showing the effects of sevoflurane and sevoflurane plus testosterone on sEPSCs in primary neurones. Quantification of the effects of sevoflurane and sevoflurane plus testosterone on the (d) amplitude and (e) frequency of sEPSCs in primary neurones (n=5 biological samples per group). Two-way analysis of variance was used to analyse the differences amongst different groups in (b). Unequal variance Student's t-test was used to analyse the difference in different groups in (d) and (e). * and #P<0.05; *** and ##P<0.01. Error bars indicate standard deviation; N.S., not significant.

The role of testosterone in cognitive function is still not conclusive. Both human[49] and animal[50] experiments suggest dose-dependent effects of testosterone on cognitive function. Although testosterone may have neuroprotective effects via inhibiting tau phosphorylation (present study), androgen receptor antagonism and gonadectomy may have neuroprotective effects via changes in chloride transporters[46] and being more developmentally similar to females,[47] respectively. Testosterone plus isoflurane could cause cognitive impairment in female neonatal rats via changes in chloride transporters.[48] Other studies have shown that testosterone can improve cognitive function in animals and humans,[49–53] but conflicting reports exist.[54–56] Future studies should compare the effects of testosterone, androgen receptor antagonist, or gonadectomy under the same anaesthetic techniques in rats or mice with measurement of testosterone concentrations in both blood and brain.

We did not find significant differences in sevoflurane-induced brain tau phosphorylation between neonatal male and female mice (Supplementary Fig. 2), consistent with another study.[40] These findings suggest potential ceiling effects of sevoflurane-induced tau phosphorylation in neonatal mice by different mechanisms (e.g. mitochondrial dysfunction[17]).

We recently showed that tau levels in hippocampus and cortex are higher in neonatal than in adult mice, which could account for the increased tau phosphorylation in neonatal mice after sevoflurane anaesthesia.[17] The increased tau level in neonatal mice was attributable to increased amounts of Nuak1, an adenosine monophosphate-activated protein kinase-related kinase[57] that phosphorylates tau at Ser356, preventing tau degradation, ultimately leading to accumulation of total tau.[58] In turn, the increased level of Nuak1 in neonatal brain was caused by decreased impaired mitochondrial function and reduced ATP levels.[17] Testosterone may protect mitochondrial function by improving cell survival and mitochondrial membrane potential, and reducing nuclear fragmentation and reactive oxygen species generation, as shown in human astrocytes.[59] Testosterone also enhanced mitochondrial function in rat brain by increasing mitochondrial reduced nicotinamide adenine dinucleotide–ubiquinone oxidoreductase chain 1 protein level and mitigating oxidative damage.[60] Future work should determine whether neonatal mice accumulate more tau in brain attributable to impaired mitochondrial function resulting from lower testosterone levels.

Despite its strengths, our study has several limitations. First, we used only *in vitro* electrophysiology to show that testosterone could attenuate sevoflurane-induced reduction in neuronal activation, which was not necessarily associated with the cognitive impairment observed in the Morris water maze. Second, we only performed a single blood gas analysis during the three 2-h anaesthetic exposures. This measurement probably does not rule out disturbances of blood gas values at other time points. Third, Deng and colleagues[61] reported that isoflurane anaesthesia predominantly caused caspase-3 activation in the cortex of neonatal mice and in the hippocampus of adult mice, suggesting brain-region- and age-dependent anaesthetic neurotoxicity. We did not determine brain-region-dependent tau phosphorylation between neonatal and adult male mice because the objective of the present study was to reveal testosterone-associated mechanisms by which sevoflurane causes age-dependent changes in tau phosphorylation and cognitive function.

In conclusion, our results suggest that the lower brain testosterone level in neonatal male mice is one of the mechanisms underlying age-dependent tau phosphorylation and cognitive impairment after sevoflurane anaesthesia. Testosterone attenuated sevoflurane-induced tau phosphorylation and cognitive impairment by decreasing binding and interaction between tau and GSK3β, a kinase that phosphorylates tau. Testosterone might also mitigate sevoflurane-induced inhibition of action potential firing in neurones. These findings pave the way for further research on the effects of anaesthesia on the developing brain.

Authors' contributions

Project conception/design: ZX, FL, YY
Performance of experiments: YY, FL, JG, YD
Performance of electrophysiology experiments: JG
Data analysis: YY, FL, JG, YD
Preparation of figures: YY, FL, JG, YD
Writing of paper: ZX, FL, YY, H-JF
Provision of critical comments on the study design and paper: GY, SGS, H-JF, YZ
Review of final paper: all authors.

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These studies were performed in the Department of Anesthesia, Critical Care and Pain Medicine at the Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA.

Declarations of interest

The authors declare no competing financial interests. ZX is a consultant for Novartis, Baxter (invited speaker), and the Shanghai 9th and 10th hospitals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2021.08.028.

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