Effects of Chronic Secondhand Smoke (SHS) Exposure on Cognitive Performance and Metabolic Pathways in the Hippocampus of Wild-Type and Human Tau Mice

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BACKGROUND: Exposure to secondhand smoke (SHS) is a risk factor for developing sporadic forms of sporadic dementia. A human tau (htau) mouse model is available that exhibits age-dependent tau dysregulation, neurofibrillary tangles, neuronal loss, neuroinflammation, and oxidative stress starting at an early age (3–4 months) and in which tau dysregulation and neuronal loss correlate with synaptic dysfunction and cognitive decline.

OBJECTIVE: The goal of this study was to assess the effects of chronic SHS exposure (10 months’ exposure to ~30 mg/m³) on behavioral and cognitive function, metabolism, and neuroinflammation in mice.

METHODS: Wild-type (WT) and htau female and male mice were exposed to SHS (90% side stream, 10% main stream) using the SCIREQ® inExpose™ system or air control for 168 min per day, for 312 d, 7 d per week. The exposures continued during the days of behavioral and cognitive testing. In addition to behavioral and cognitive performance and neuropathology, the lungs of mice were examined for pathology and alterations in gene expression.

RESULTS: Mice exposed to chronic SHS exposure showed the following genotype-dependent responses: a) lower body weights in WT, but not htau, mice; b) less spontaneous alternation in WT, but not htau, mice in the Y maze; c) faster swim speeds of WT, but not htau, mice in the water maze; d) lower activity levels of WT and htau mice in the open field; e) lower expression of brain PHF1, TTMCl, IGF1β, and HSP90 protein levels in WT male, but not female, mice; and f) more profound effects on hippocampal metabolic pathways in WT male than female mice and more profound effects in WT than htau mice.

DISCUSSION: The brain of WT mice, in particular WT male mice, might be especially susceptible to the effects of chronic SHS exposure. In WT males, independent pathways involving ascorbate, flavin adenine dinucleotide, or palmitoleic acid might contribute to the hippocampal injury following chronic SHS exposure. https://doi.org/10.1289/EHP8428

Introduction

Several studies have shown detrimental effects of SHS on the adult brain (Akhtar et al. 2013; Heffernan and O’Neill 2013a, 2013b). Nonsmokers exposed to SHS have an increased risk of developing mild cognitive impairment (MCI) (Akhtar et al. 2013; Barnes et al. 2010; Llewellyn et al. 2009; Orsitt et al. 2012). Most MCI patients eventually develop dementia (Langa and Levine 2014). SHS also doubles the risk for dementia among individuals who never smoked (Cataldo et al. 2010; Chen 2012; Schick and Glantz 2005).

In Alzheimer’s disease (AD), a common form of dementia, the spread of neurofibrillary tangles (NFTs), which are made up of hyperphosphorylated tau aggregates, is associated with disease severity in individuals with AD. Tau seeding activity in cell- and animal-based studies was correlated with disease severity (Dujardin et al. 2020), and there is evidence that spread of tau pathology throughout the brain is correlated with progressive cognitive decline in AD and other forms of dementia (Mufson et al. 2014; Ossenkoppele et al. 2020; Vogel et al. 2020; Brier et al. 2016; Malpetti et al. 2020). The formation of tau oligomers is considered an early event that triggers the subsequent hyperphosphorylation and aggregation of tau in NFTs (Takeda 2019). Recent advances in tau oligomer research have increased our understanding about tau dysregulation in cognitive disorders (Mufson et al. 2014; Ossenkoppele et al. 2020; Vogel et al. 2020; Brier et al. 2016; Malpetti et al. 2020), but whether SHS induces dysregulation of wild-type (WT) human tau is unknown. In addition, although much is known about the influence of mutant human tau, less is known about the dysregulation of endogenous tau on brain integrity and function in animal models of dementia. A nonmutant human tau (htau) mouse model is available that exhibits age-dependent tau dysregulation, neurofibrillary tangles, neuronal loss, neuroinflammation, and oxidative stress starting at an early age (3–4 months) and in which tau dysregulation and neuronal loss correlate with synaptic dysfunction and cognitive decline (Polydoro et al. 2009).
MCI is characterized by a dysfunction in brain glucose metabolism (Chami et al. 2016; Willette et al. 2015a, 2015b) and other metabolic pathways are perturbed in neurodegenerative diseases (González-Domínguez et al. 2015; Liu et al. 2014; Trushina et al. 2013). SHS might increase the risk of MCI or dementia by perturbing brain metabolism (i.e., insulin signaling, oxidative stress) and the accumulation of pathological proteins (i.e., tau, amyloid).

Consistent with the human data, brain insulin signaling was impaired and the accumulation of pathological proteins induced following short-term exposure of 2-month old mice (6 h/d × 5 d/week) in a mixture of sidestream/mainstream cigarette smoke (Deochand et al. 2015). Lipid peroxides, DNA damage, and tau dysregulation (tau isomers, phosphotau) in the brain were induced following shorter durations of exposure of neonatal mice to mainstream/sidestream smoke (1 h/d × 1 month) (La Maestra et al. 2011), features frequently observed in MCI and dementia patients (Bradley-Whitman et al. 2013; Lovell and Markesbery 2007; Wirz et al. 2013). Longer exposures of 2-month-old WT rats or 3-month-old APP/PS1 transgenic mice to sidestream cigarette smoke (1 h/d × 5 d/week × 4 wk or 8 wk) resulted in tau and amyloid pathology like that reported in patients with MCI or dementia (Ho et al. 2012; Moreno-Gonzalez et al. 2013). The duration of SHS exposure in these studies (Avila-Tang et al. 2013) was much shorter in comparison with human studies that showed a strong correlation between the duration of SHS exposure (>25 y) and impaired cognitive function (Barnes et al. 2010; Liewellyn et al. 2009; Orsatto et al. 2012). To more closely replicate human SHS exposure, in the present study we assessed the effects of daily SHS exposure, for 10 months and starting at 5–9 wk of age, on behavioral and cognitive performance and neuropathology of WT and htau mice. In addition, the lungs of these mice were examined for pathology and alterations in gene expression. Because women are at increased risk of developing MCI and AD (Au et al. 2017; Barnes et al. 2003; Farrer et al. 1997) and one study showed that cognitive decline was greater in women than men (Sohn et al. 2018), we included female and male mice in these studies.

Animals, Exposures, Materials, and Methods

Animals and Exposures

Sixty four 5- to 9-wk-old htau mice on a C57BL/6J background and C57BL/6J WT mice (n = 16 mice/genotype/sex) were purchased from the Jackson Laboratory. Mice were housed four per cage. Food (PicoLab Laboratory Rodent Diet 5L00D) and water were available ad libitum except during the SHS or control exposures. Lights in the housing room were set to 12 h light:12 h dark cycle. All behavioral tests and procedures took place within the light cycle. For each group of 16 mice per genotype and sex, 8 were randomly assigned to SHS and 8 to air control (Sham) exposures. For the Sham exposures, the mice were put in the same pie-shaped holders and exposed to ambient air. The SHS and Sham exposure groups were offset by 1 month (Sham exposures started 1 month after SHS exposures) to allow for enough time for behavioral and cognitive testing. One male htau mouse and one male WT mouse died during the 10-month exposure window (312 d, 7 d per week). Mice were exposed to SHS (90% sidestream, 10% mainstream) using the SCIREQ® inExpose™ system or to air control (Sham) for 168 min per day. The exposures continued during the days of behavioral and cognitive testing. Each day, (24) 3R4F certified cigarettes (University of Kentucky, Lexington, KY, USA) were lit using a cigarette-smoking robot (CSR) and CSR lighter (SCIREQ®), with one puff taken per minute and a flow rate of 2 L/min. The amount of particulate matter was analyzed monthly by gravimetric analysis as described in Noël et al. (2017). Off-line determination of total mass concentration was measured gravimetrically by sampling and collecting the SHS particulate on glass fiber filters (MilliporeSigma) placed inside a cassette. The filters were weighed, before and after sampling, on a Mettler-Toledo balance (AG285). All mice were weighed every other week. After exposure for 4 and 8 months, blood was collected (submandibular) in tubes containing 5 μL of a 0.5 M EDTA solution and following centrifugation at 5,000 × g for 10 min, and the supernatant was stored at −80°C for analysis of steady-state plasma cotinine levels.

Procedures were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, with approval from the Oregon Health & Science University (OHSU) Institutional Animal Care and Use Committee (IACUC) and consistent with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Plasma Cotinine Levels

For the analysis of plasma cotinine and its metabolites in the mice, a hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC-MS) method adapted from Li et al. (2012) was used; (+)-Cotinine (COT) was purchased from Sigma-Aldrich. (R,S)-norcotinine (NCOT), trans-3’-hydroxycotinine (OHcot) and (S)-cotinine-N-oxide were obtained from Toronto Research Chemicals. Stable isotope labeled internal standard (IS) (±)-cotinine-D3 (COT-d3) solution (1 mg/mL in methanol) was obtained from Sigma-Aldrich. (R,S)-norcotinine-d4 (NCOT-d4), trans-3’-hydroxycotinine-d3 (OHcot-d3) and (R,S)-cotinine-N-oxide-d3 (COTNO-d3) were also obtained from Toronto Research Chemical. Stock solutions of all analytes and internal standards were prepared by dissolving 1.0 mg of each compound in 1.0 mL of methanol to obtain drug concentrations of 1.0 mg/mL, with the exception of COT-d3, which was prepared as a 1.0-mg/mL methanol solution. Serial dilution of each compound with 90% acetonic (ACN)/water (v/v, 9/1) was used to obtain combined working solutions at concentrations of 10.0, 20.0, 50.0, 100.0, 200.0, 500.0, and 1,000.0 ng/mL. Quality control (QC) working solutions were 10.0, 30.0, 300.0, and 750.0 ng/mL. IS working solutions containing COT-d3, NCOT-d4, OHcot-d3, and COTNO-d3 were prepared at a single concentration of 500.0 ng/mL in 90% ACN/water (v/v, 9/1).

Liquid Chromatography–Mass Spectrometry (LC-MS/MS) Conditions for Plasma Cotinine Levels

An Agilent 1100 binary pump high-performance liquid chromatography (HPLC) system was interfaced to a Waters Micromass Quattro micro™ triple quadrupole mass spectrometer. The analytes were separated on a Phenomenex Kinetex® HILIC ultra-high-performance liquid chromatography (UHPLC) column (50 × 2.1 mm ID, 2.6 μm) coupled with a SecurityGuard™ ULTRA HILIC guard column. The mobile phase A was a 10 mM ammonium formate aqueous buffer with 0.1% formic acid. The mobile phase B was acetonitrile; 10 μL of samples were injected onto the column. The analytes were separated with the following gradient (time in minutes, % mobile phase B): (0, 95), (8, 50), (8.1, 95), (15, 95) at a flow rate of 0.3 mL/min and a column temperature of 25°C. The LC system was interfaced by a six-port divert valve to the mass spectrometer, introducing eluents from 1.0 to 6.0 min to the ion source. After each injection, the autosampler needle was washed with methanol.

The mass spectrometer was run in positive ion electrospray mode, with nitrogen as the desolvation gas at a flow rate of 500 L/h and a temperature of 500°C. The cone gas flow was set to 0 L/h. Argon was used as the collision gas. The collision cell pressure was 3.5 × 10⁻³ mbar. The source temperature was 120°C, and the capillary voltage was set at 3.0 kV. For the quantification of analytes, multiple reaction monitoring (MRM)
functions were used: cotinine, norcotinine, cotinine-N-oxide, and \textit{trans}-3'-hydroxy-cotinine. The cone voltage was 20 V, and collision energy was 26 eV. Ion transitions monitored for analytes were 177 → 80 for COT, 163 → 80 for NCO(T, 193 → 80 for OHCO(T, and 193 → 96 for COTNO. Ion transitions for IS were 180 → 80 for COT-d3, 167 → 84 for NCO(T-d4, 196 → 80 for d3-OHCO(T and 196 → 96 for d3-COTNO.

\textbf{Plasma Cotinine Standards}

Blank plasma with sodium EDTA was purchased from Bioreclamation. In addition, 10 µL of standard or QC working solution was spiked into 100 µL of blank plasma to generate standard/QC samples. For the calibration standards, the final concentrations were 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 ng/mL in plasma.

\textbf{Sample Preparation for Assessment of Plasma Cotinine Levels}

The mouse plasma samples were subjected to protein precipitation and solid phase extraction (SPE). To remove plasma proteins, 10 µL of the IS working solution (500 ng/mL) was added to 100 µL of plasma, 800 µL of water, and 100 µL of 25% (wt/vol) trichloroacetic acid (TCA), and the mixture vortexed for 10 min. For SPE, the supernatant from protein precipitation was loaded onto an OASIS MCX SPE cartridge (Waters Corporation), which was preconditioned with 1 mL of methanol and equilibrated with 1 mL of water and allowed to flow by gravity. The cartridge was washed twice with 1 mL of 5% methanol, 5% formic acid in water (v/v), followed by vacuum drying for 5 min. Analytes were eluted with 1 mL of fresh 20% methanol, 5% ammonia in water (v/v). The eluent was evaporated to dryness in a centrifuge evaporator at 50°C. The lyophilized sample was reconstituted with 100 µL of 95% ACN/water (v/v, 9/1) with 2% formic acid prior to injection.

\textbf{Calibration Curve Plasma Cotinine Levels}

To generate calibration curves for cotinine and its metabolites, a \(1/x\)-weighted linear regression was used. The response was reported from peak area ratios between the analyte and the internal standard. For all analytes, the calibration curves exhibited good linearity \((R^2 > 0.99)\) within the range of 1 to 100 ng/mL.

\textbf{Behavioral and Cognitive Testing}

\textbf{Y maze.} Mice were tested for hippocampus-dependent spontaneous alternation in the Y maze, exploratory behavioral and measures of anxiety, and object recognition in week 1. Performance in the Y maze was described using the mazes from O’Haras as described in Saito et al. (2014). The Y-shaped maze was purchased from O’Haras. It has raised sides (3.8 cm bottom width, 12.55 cm top width, 12.55 cm height) with plastic, opaque gray arms (37.98 cm length) at a 120° angle from each other. At the beginning of a 6-min trial, mice were placed in the center of the maze. To isolate mice from the surrounding room as well as from the experimenter, the mazes were surrounded with a white curtain. The mazes were cleared with 0.5% acetic acid between trials. Performance of the mice was tracked during testing with Ethovision XT v14 software (Wageningen). To measure the number of arm entries and to calculate the percent spontaneous alternations, the videos were analyzed. The criterion for an arm entry was when all four limbs were within the arm. The spontaneous alternation percentage was calculated by dividing the number of 3-arm alternations by the number of possible 3-arm alternations and multiplying the value by 100.

\textbf{Open field and object recognition.} Exploratory activity and measures of anxiety were assessed in the open field test, as described (Benice et al. 2006). The open field consisted of a well-lit square (L 40.6 x W 40.6 x H 40.6 cm) with a central light intensity of 100 lux. On each of 2 subsequent days, mice were allowed to explore the open field for 5 min. Object recognition was assessed as described (McGinnis et al. 2017), with the following modifications. On day 3, the open field contained two identical objects, and the mice were exposed to the open field with the objects for a 10-min trial. The objects were placed 10 cm apart and 15 cm from the adjacent walls of the arena. On day 4, one object was replaced with a novel object, and mice were allowed to explore for 10 min. During object recognition trials, the objects were affixed with masking tape to the floor of the arena. Physical interaction with the object in the form of sniffing within a 2-cm proximity was coded as object exploration. The enclosures were cleaned with 0.5% acetic acid between trials. Performance of the mice during all trials was analyzed using Ethovision XT software (version 7.0). Time spent in the center of the open field was analyzed to assess measures of anxiety. Videos were later hand scored to measure object exploration. The percent time exploring the novel object out of the total time spent exploring both objects on day 4 was calculated.

\textbf{Water maze.} The mice were tested for spatial learning and memory in the water maze in week 2, with a 72-h probe trial on the following Monday. The maze was 140 cm in diameter and was filled with opaque water containing nontoxic, white chalk. Large visual cues surrounded the maze to make this a hippocampus-dependent task. An escape platform was submerged 1 cm below the water surface. First, the mice were trained to locate a visible platform over 2 d, each consisting of three trials. During each training trial, mice were dropped off in counterbalanced locations and allowed to explore the water maze. When mice located the escape platform and remained on it for 3 s, the trial ended. Mice that did not find the platform within the 60-s trial time were gently led to the platform by the experimenter. Following visible platform training, the mice were trained to locate a hidden platform over 3 d, each consisting of three trials. To assess spatial memory retention, there was a probe (no platform) before starting the third day of hidden platform training. Following the third day of hidden-platform training, there was a second probe trial 72 h later. For the visible and hidden platform learning curves, time to reach the platform (latency), distance moved, and swim speeds were analyzed using Ethovision XT v14 software. Percent time spent in the quadrant that contained the target location during the hidden platform training trial (target quadrant) and the three non-target quadrants and cumulative distance to the target (since there is no platform in the probe trial) location were used as performance measures for analyzing performance in the probe trials.

\textbf{Contextual fear conditioning.} Mice were tested for hippocampus-dependent contextual fear learning and memory in week 3. Contextual fear conditioning was assessed over the 2 consecutive days using a Med Associates mouse fear conditioning system (PMED-VFC-NIR-M; Med Associates, Inc.) and Med Associates VideoFreeze automated scoring system. Mice were placed inside the fear conditioning chamber. The chamber lights were turned on at the beginning of the trial. Following a 120-s baseline habituation period, four 30-s tones (80 dB) were presented which coterminated for the last 2 s of each tone with a 0.50 mA foot shock. Twenty-four hours later the mice were placed in the same chamber as during training (acquisition) for a 5-min trial. The chamber lights were on, but no tones or shocks were presented. Between trials, the enclosures were cleaned with 0.5% acetic acid. Motion during the baseline (prior to the first
tine on the training day) and percent freezing during the tones and between the tone-shock pairings on the training day and during the contextual fear memory test were analyzed using VideoFreeze® software.

**Histopathological Analysis of the Lungs**

Mice were killed by cervical dislocation the day after the last SHS or sham exposure, and the lungs were examined for histopathology. The lungs that were excised were not previously lavaged, and therefore the histopathology is representative of intact lungs. No mice in this study were used to collect broncho-alveolar lavage (BAL). After euthanasia, the lungs of mice were excised and either fixed or processed for RNA extraction. The left lung of each mouse (n = 8 mice per group) was fixed with buffered formalin (10%) for 24–48 h. Subsequently, standard histological processing, sectioning and hematoxylin-eosin (H&E) staining were conducted as previously described (Noël et al. 2020). Slides containing three sections (5 µm) of lung lobes were coded randomly. The stained sections were then evaluated by a board-certified veterinary pathologist with expertise in pulmonary pathology and blinded to treatments. Lung tissues were evaluated for: a) hyperplasia of bronchus-associated lymphoid tissue (BALT); b) bronchial, peribronchial, and perivascular infiltration of lymphocytes and plasma cells; c) bronchial, peribronchial, and peri-vascular infiltration of neutrophils; d) bronchial, peribronchial, and perivascular infiltration of eosinophils; e) bronchial goblet cell hyperplasia; and f) alveolar inflammation. Ten to 20 random sections were evaluated to determine the spectrum of lesions in the study from the least to most affected for each parameter. On that basis, the least affected ones were considered to be normal, and the spectrum of changes was used to determine the range of changes from normal to severe (if applicable). The lungs were scored for each of the six aforementioned parameters on a 0–4 scale, with 0 = normal, 1 = minimal increase, 2 = mild increase, 3 = moderate increase, and 4 = severe increase.

**Lung mRNA Extraction**

Half of the right lung of each mouse (n = 8 mice per group) was harvested and placed in RNA later and stored at −80°C. Prior to mRNA extraction from these lung samples, RNA was purified from the aqueous phase of the lung homogenate using a RNeasy Mini Kit (Qiagen) that included a RNase-free DNase treatment, according to the manufacturer’s instructions. A NanoDrop™ ND-1000 Spectrophotometer (Nano-Drop Technologies) was used to assess the quantity and purity of the RNA samples, as previously described (Noël et al. 2017, 2020).

**Gene Expression Analysis via RT2 Profiler Polymerase Chain Reaction (PCR) Array**

The expression of 84 genes included in the Molecular Toxicology Pathway Finder RT² PCR array (PAMM-401Z, Qiagen) was analyzed in the lungs of mice (n = 4 mice per group) following the manufacturer’s instructions. As previously described in Noël et al. (2020), after DNase treatment of lung tissues, total RNA (0.5 µg) was reverse transcribed using the RT² First Strand Kit (Cat. No. 330401; Qiagen). The cDNA was diluted with RNase-free water and mixed with RT² SYBR Green qPCR Master mix (Cat No. 330503; Qiagen). Equal aliquots (25 µL) were added to the corresponding wells of a PCR Array plate. The PCR was performed according to the cycling conditions of an Applied Biosystems model 7300 real-time cycler. Gene expression and fold-change compared with the respective air control group were calculated using the 2^−ΔΔCt method. ΔCt data for male mice were calculated using the average arithmetic means of Actb, B2m, Gapdh, Gusb, and Hspa90ab1 to normalize the raw data, whereas ΔCt data for female mice were calculated using the geometric means of Actb and Gapdh as the normalization factor. The fold changes were calculated with the Qiagen web-based PCR Array data analysis software. Gene expression results were considered significant with fold-changes > ± 1.5 compared with the respective air control group.

**Ingenuity Pathway Analysis**

Gene expression data obtained from the RT² PCR array were analyzed with Ingenuity Pathway Analysis (IPA; version 60467501; Qiagen Ingenuity Systems), as previously described (Noël et al. 2020; Rouse et al. 2008). The Ingenuity Analysis Knowledge Database was used to identify gene networks and canonical pathways from our gene expression datasets.

**Dot Blot Analysis**

The hippocampus, cortex, and cerebellum were obtained from the left hemispheres of 4–5 mice per exposure/genotype/sex, immediately snap frozen in liquid N₂ prior to storage at −80°C. The left hemisphere was used for dot blotting and metabolomics analysis, whereas the right hemispheres were immersion fixed in 4% buffered paraformaldehyde and used for immunohistochemistry. Protein extracts were prepared from freshly frozen brain regions of mice and immunopробed for 21 molecular markers (see Table 1 for details of primary antibodies) by dot blot with near-infrared imaging as previously described (Chlebowski and Kisby 2020). The hippocampus, cerebral cortex and cerebellum of mice were sonicated in extraction buffer [Tris HCl, 10 mM, pH 7.8; dithiothreitol, 0.5 mM; MgCl₂, 5 mM; adenosine triphosphate, 30.8 mg/10 mL; cOmplete Mini Protease inhibitor, EDTA-free (1 tablet/10 mL); double-distilled water, 7.8 mL/10 mL solution]. All buffer components were from Millipore-Sigma. The tissue homogenates were centrifuged at 15,000 × g for 90 min at 4°C, and the supernatants were examined for protein concentration by the Bradford assay (BioRad Laboratories, Inc.). The supernatants were diluted with TBS for dot blot analysis and stored at −80°C until use. Diluted samples (1 µg protein/well) were first applied on a 0.45 µm nitrocellulose membrane in a dot blot apparatus (BioRad). The membranes were then treated with the Revert® total protein stain kit (LiCor) before imaging on an Odyssey® CLx imager (LiCor Biosciences) to determine protein loading. Membranes were then blocked with LiCor Intercept™ (TBS) blocking buffer, and the primary and secondary antibodies (see Table 1 for the list of primary antibodies) were diluted in 50:50 Intercept® Blocking Buffer:TBS with 0.01% Tween-20. The membranes were incubated with the primary antibodies overnight at 4°C with gentle rocking. Subsequently, the membranes were washed 3 × 10 min in TBS + 0.1% TWEEN-20 (TBST) before incubation with secondary antibodies for 1 h at 20–22°C in the dark (the wash container was wrapped in foil). All secondary antibodies were from LiCor and used at a 1:10,000 dilution. The secondary antibodies used were 926-32210, 926-32211, 926-68070, 926-68071, and 926-32219, depending on the species and detection wavelength. After secondary antibody incubation, the membranes were washed 2 × 10 min in TBST, and 1 × 15 min in TBS. Following incubation with antibodies, the membranes were imaged and quantified in the 800 channel using a LiCor Odyssey® CLx imager. Membranes were scanned on the imager at 0.0 µm offset, 84 µm resolution, and medium scan quality. For each dot, data are expressed as (antibody signal - residual Revert® signal)/(Revert® signal).

Dots were removed from analysis if the surrounding background was too high for the dot to be distinctly defined.
Table 1. Antibodies used for dot blot analyses.

| Antibody/target | Source | Dilution factor | Species | Catalog number |
|-----------------|--------|----------------|---------|----------------|
| Tau5            | Invitrogen | 1:1,000 | Mouse   | MA5-12808      |
| Anti-Tau phos Thr231 | MilliporeSigma | 1:1,000 | Rabbit | AB9668SSP      |
| Anti-Tau (3repea isoform R3), clone 8E6/C11 | MilliporeSigma | 1:1,000 | Mouse | 05-803 |
| Tau phos Ser202 (CP13) | Dr. P. Davies | 1:1,000 | Mouse | — |
| Tau phos ser 396Ser404 (PHF1) | Dr. P. Davies | 1:50 | Mouse | — |
| Tau (Alz50)     | Dr. P. Davies | 1:50 | Mouse | — |
| Tau pSer202/pThr205 (AH36) | StressMarq | 1:1,000 | Rabbit | SMC-601 |
| T22             | Dr. R. Kayed | 1:500 | Rabbit | — |
| TOMA2           | Dr. R. Kayed | 1:1,000 | Mouse | — |
| TTCM1           | Dr. R. Kayed | 1:500 | Mouse | — |
| IGF1B-specific | Proteintech | 1:1,000 | Rabbit | 20215-1-AP |
| INSR            | Proteintech | 1:1,000 | Rabbit | 20433-1-AP |
| IRS1            | Proteintech | 1:1,000 | Rabbit | 17509-1-AP |
| 4G8             | Dr. R. Woltjer | 1:5,000 | Mouse | — |
| Amyloid oligomers (A11) | StressMarq | 1:1,000 | Rabbit | SPC-506D |
| NeuN            | Proteintech | 1:1,000 | Rabbit | 26975-1-AP |
| Iba1            | Dr. R. Woltjer | 1:3,000 | Rabbit | 019-19741 |
| GFAP            | Proteintech | 1:5,000 | Mouse | 60190-1-lg |
| HSP90x          | Abcam | 1:1,000 | Rabbit | Ab2928 |
| HSC70/HSP73     | Enzo Life Sciences | 1:1,000 | Rat | ADI-SPA-815-D |

Note: —, non-commercially acquired antibodies.

Immunohistochemistry

The right hemispheres of air (sham) or SHS exposed wild type (C57BL/6J) male mice were immersed in 4% buffered paraformaldehyde, cryoprotected in sucrose (10%–30%), and the tissue was rapidly frozen in Tissue-Tek®, as previously described (Kisby et al. 2009). Sagittal brain tissue sections (20 μm) were generated with a cryostat (Leica) using the hemibrain of both sham and SHS exposed WT male mice. The corresponding sections of each treatment were placed on SuperFrost® Plus slides (VWR) (2 sections/slide). The sections were air-dried overnight. The next day, the sections were stained with cresyl violet or subjected to immunohistochemistry.

For cresyl violet (Nissl) staining, sections were washed 2 × 5 min in 10 mM PBS, and in ddH2O for 1 min. The slides were put in cresyl violet (Sigma) solution for 10 min at room temperature, then washed 2 × 5 min in ddH2O. Slides were then subjected to sequential washes in 90% EtOH, 95% EtOH, 2 × washes in 100% EtOH, and 3 × washes in Shandon™ Xylene Substitute (Thermo Scientific) each for 3 min, followed by slide mounting with CytoSeal™-60 (Electron Microscopy Services). Imaging was performed using a Leica DM100 microscope fitted with an Infinity1 camera.

For immunohistochemistry, brain tissue sections were subjected to antigen retrieval by incubating the slides in a citrate-based solution (Vector Labs) at 70°C for 15 min. The sections were then washed in PBS, incubated with 0.3% hydrogen peroxide for 5 min in blocking buffer and then washed again with PBS before incubating the sections overnight at 4°C with primary antibodies (IGF1β or HSP90). Tissue sections were then incubated for 30 min with Vectastain Elite™ ABC reagent and washed again in PBS before incubation with ImmPACT NovaRED™ (Vector Labs) to detect marker proteins. Slides were then washed again in PBS before they were mounted with VectaMount AQ™ (Vector Labs). Immunoprobed sections were examined by light microscopy using a Leica DM100 microscope fitted with an Infinity1 camera.

PHF1 Western Blot Analysis

Male cortical samples (30 μg/lane) were also analyzed for PHF1 immunoreactivity by western blot. The samples were run on a NuPAGE™ 4–12% Bis-Tris precast gel (1.5 mm thick, 15-well gels) (NP0336BOX; ThermoFisher) on an Invitrogen™ Novex™ mini-cell system for protein gel and western blots (X Cell SureLock™ system and blot module; ThermoFisher), using NuPage™ MOPS SDS running buffer (NP0001; ThermoFisher) NuPage™ Transfer buffer (NP0006; ThermoFisher), and a MagicMark™ XP Western Protein standard (LC5603; ThermoFisher). The gel was run at 110 V for 2 h. Subsequently, the proteins were transferred from the gel to a nitrocellulose membrane at 45 V for 1 h. The membrane was processed for PHF1 immunoreactivity using revert stain and anti PHF1 antibody hybridization and quantification as described above for the dot blot analysis.

Metabolomics

Hippocampi were dissected and homogenized in 300 μL (per 30 mg of tissues) of cold methanol:water (8:2, v/v). From 100 μL of hippocampal homogenates and plasma, metabolites were extracted, and untargeted metabolomics was completed as described (Kirkwood et al. 2013). LC was performed using a Shimadzu Nexera system with an Insertil Phenyl-3 column (4.6 × 150 μm, 100 Å, 5 μm; GL Sciences) coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF) (AB SCIEX, TripleTOF® 5600) operated in information-dependent MS/MS acquisition mode. Samples, including multiple QC samples, were ordered randomly, and automatic mass calibrations were performed every hour. QC samples, which were generated by pooling 10 μL aliquots from hippocampi tissues or plasma samples extracts, were analyzed along with the samples. Samples were run in the positive and negative ion mode. The column temperature was held at 50°C, and the samples were kept at 10°C. The metabolomics data were processed using MarkerView™ (SCIEX) and PeakView™ (SCIEX) software. Metabolites were individually identified based on mass error (<5 ppm), MS/MS fragment ions, mass to charge ratio, retention time and matching with spectra on Metlin, HMDB and Lipidmaps databases, and in-house library consisting of 619 IROA standards (IROA Technologies). MetaboAnalyst pathway analysis was performed as described by Kirkwood et al. (2013) and Xia and Wishart (2010).

Statistical Analyses

All data are presented as means ± standard error of the mean (SEM). Behavioral and cognitive data were analyzing with SPSS software (version 25; IBM). For most analyses, analyses of variance (ANOVA), with genotype and SHS condition as between
group factors were carried out with repeated measures when appropriate. For some analyses, as indicated and appropriate, two-sided t-tests were used to assess effects of SHS in each genotype and/or sex. We set statistical significance to \( p < 0.05 \). Greenhouse-Geisser corrections were used if sphericity was shown to be violated (Mauchly’s test). Paired Student’s t-tests were used to assess object preference in the object recognition test. Graphs show the data with sexes collapsed if sex was not a significant factor.

Statistical analyses of lung data were performed using the GraphPad Prism 8 software (version 8.2.0). Histopathological lung scoring was analyzed using the nonparametric Kruskal-Wallis statistical method. Results were considered statistically significant at \( p < 0.05 \). Graphs were generated using GraphPad software (version 8.2.0).

Metabolomics in plasma and the hippocampus were analyzed separately by genotype and sex. To identify potential plasma biomarkers of SHS exposure on cognitive performance, we used regression analyses and examined the 1 to 5 parameter metabolomics plasma panel that explained most of the variation (top 5 model for each 1 to 5 parameter metabolomics plasma panel). We selected those metabolites that were most consistently included in the models (at least 6 times out of potentially 21 times). MetaboAnalyst software was used to generate impact plots.

**Results**

**Gravimetric Analysis, Cotinine and Cotinine Metabolite Levels in Plasma**

Female and male mice were exposed to SHS in distinct chambers. There was no sex difference in the gravimetric analysis at the 4-month time point (\( t = 0.52, p = 0.61 \); Figure 1A). There were no genotype differences in plasma cotinine (WT: 6.3 ± 1.4 ng/mL; htau: 4.0 ± 0.4 ng/mL), norcotinine (WT: 2.5 ± 0.6 ng/mL; htau: 2.2 ± 0.4 ng/mL), cotinine-N-oxide (WT: 3.1 ± 0.5 ng/mL; htau: 3.0 ± 0.5 ng/mL), or trans-3′-hydroxy-cotinine (WT: 10.5 ± 2.4 ng/mL; htau: 10.2 ± 1.9 ng/mL). Therefore, the genotypes were combined for analysis of plasma cotinine levels and cotinine metabolites. There were no sex differences in plasma cotinine levels at the 4-month time point (Figure 1B). At the 8-month time point, the plasma cotinine levels were lower than those at the 4-month time point, and there were no sex differences in plasma cotinine levels (WT: 6.0 ± 1.3 ng/mL; htau: 4.3 ± 0.8 ng/mL, \( t = 1.082, p = 0.29 \); Figure 1C) or norcotinine levels (WT: 2.5 ± 0.6 ng/mL; htau: 2.2 ± 0.4 ng/mL, \( t = 1.0, p = 0.33 \), not shown). However, plasma levels of cotinine-N-oxide (\( t = 2.631, p = 0.016, 2\text{-}t\text{ailed} t\text{-test} \); Figure 1D) and trans-3′-hydroxy-cotinine (\( t = 3.216, p = 0.0034, 2\text{-}t\text{ailed} t\text{-test} \); Figure 1E) were higher in females than males.

**Figure 1.** Gravimetric analysis of the SHS and plasma cotinine at 4- and 8-month, body weight in WT and htau mice, and cumulative food intake in mice exposed or sham exposed to SHS for 312 d. All data are shown as mean ± SEM. (A) Mass of SHS to which male and female mice were exposed as determined by gravimetric analysis. The genotypes were combined for analysis of plasma cotinine levels and cotinine metabolites. (B) Plasma cotinine levels at the 4-month time point. (C) Plasma cotinine levels at the 8-month time point. (D) Plasma cotinine-N-oxide levels (at least 6 times out of potentially 21 times). MetaboAnalyst software was used to generate impact plots.

(F) Body weight in male WT mice over the course of SHS or sham exposure. "\( p = 0.0112, 2\text{-}t\text{ailed} t\text{-test} \). Note: ANOVA, analysis of variance; SHS, secondhand smoke; WT, wild type.
**Effects of SHS on Body Weight (BW)**

To evaluate the role of SHS on general metabolism, we measured food intake and BW. When BWs were analyzed, there were effects of SHS \((p = 0.037)\), sex \((p < 0.001)\), and a genotype × SHS interaction \((p = 0.033)\). In WT males, BWs were lower following SHS exposure \(F(1,13) = 15.69, p = 0.0016; \text{Figure 1F}\). This was not seen in htau males \(\text{Figure 1G}\). There was a trend toward an effect of SHS on BWs in WT female mice, but that did not reach significance \(F(1,14) = 3.249, p = 0.0931; \text{Figure 1H}\). No effect of SHS was seen in htau females \(\text{Figure 1I}\). We next assessed whether differences in food intake might have contributed to the effects on BWs in WT males. There were no effects of SHS on BWs of WT males or htau males or females \(\text{Figure 1F–H}\). In WT females, the cumulative food intake was lower in mice exposed to SHS than in those exposed to air controls \(t = 2.921, p = 0.0112; \text{Figure 1J}\).

**Effects of SHS on Spontaneous Alternation in the Y Maze**

Hippocampus-dependent spontaneous alternation was assessed in the Y maze \(\text{Figure 2A}\). WT mice exposed to SHS had less spontaneous alternation in comparison with genotype-matched controls \(t = 2.259, p = 0.0316; \text{Figure 2B}\). No significant effect of SHS on spontaneous alternation was seen in htau mice \(\text{Figure 2B}\). There was a trend toward lower spontaneous alternation in air-exposed htau than in WT mice, but that did not reach significance \(t = 1.860, p = 0.0728; \text{Figure 2B}\). There were no group differences in 

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**Figure 2.** Behavioral performance of SHS- and air-exposed WT and htau mice. (A) Schematic illustration of the Y maze. (B) Spontaneous alternation of SHS- and air-exposed WT and htau mice. \(^{**}p = 0.0316, 2\text{-tailed }t\text{-test}\); \(^{***}p = 0.0728, 2\text{-tailed }t\text{-test}\). (C) Total arm entries, a measure of activity, of SHS- and air-exposed WT and htau mice. (D) Mice were tested for two sequential days for behavioral performance in the open field. On the third day, two identical objects were placed in the open field. On the fourth day, one familiar object was replaced by a novel one. (E) Activity levels of SHS- and air-exposed WT and htau mice on days 1 and 2 in the open field. \(^{*}p < 0.05\) vs. sham, ANOVA. (F) Entries of SHS- and air-exposed WT and htau mice into the center of the open field. \(^{*}p < 0.05\) vs. air exposed htau, ANOVA. \(^{*}p < 0.05\) vs. sham htau on day 1, 2-tailed \(t\)-test. (G) Time SHS- and air-exposed WT and htau mice spent in the center of the open field. There was a genotype × exposure interaction \(F(1,58) = 4.704, p = 0.034, \text{ANOVA}\). There was an effect of SHS in htau mice \(F(1,29) = 6.077, p = 0.0199, \text{ANOVA}\). \(^{*}p < 0.05\) vs. sham htau, ANOVA. \(^{*}p < 0.01\) vs. sham htau on day 1, 2-tailed \(t\)-test. (H) Object recognition of SHS- and air-exposed WT and htau mice. \(^{*}p < 0.05\) vs. the familiar object, 2-tailed \(t\)-test. (I) Spatial learning and memory of SHS- and air-exposed WT and htau mice in the water maze. (J) Swim speeds of SHS- and air-exposed WT and htau mice in the water maze. \(^{**}p = 0.0009\), ANOVA. (K) Learning curves of WT mice to locate the visible and hidden platform. (L) Learning curves of htau mice to locate the visible and hidden platform. (M) Spatial memory retention of WT and htau mice in the first probe trial. \(^{*}p < 0.05\), Dunnett’s. (N) Spatial memory retention of WT and htau mice in the second probe trial. \(^{*}p < 0.05\), Dunnett’s. (O) Cumulative distance to the platform location of WT and htau mice in the water maze probe trials. \(^{***}p < 0.0001\), ANOVA. (P) Baseline motion of SHS- and air-exposed WT and htau mice in the fear conditioning test. \(^{*}p < 0.01\), 2-tailed \(t\)-test. (Q) Freezing of SHS- and air-exposed WT and htau mice between the aversive stimuli during fear learning. (R) Contextual fear memory of SHS- and air-exposed WT and htau mice. All data are shown as mean ±SEM. Note: ANOVA, analysis of variance; SEM, standard error of the mean; SHS, second hand smoke; WT, wild type.
differences in total arm entries (Figure 2C), indicating that potential group differences in activity likely did not contribute to the group differences in spontaneous alternation.

**Effects of SHS on Activity in the Open Field and Object Recognition**

Mice were tested for 2 sequential days for behavioral performance in an open field (Figure 2D). SHS-exposed mice (both WT and htau) had less activity on day 1 [effect of SHS on Day 1: F(1,58) = 5.601, p = 0.021] and day 2 [F(1,58) = 8.910, p = 0.004] in the open field (Figure 2E). To assess whether SHS affects measures of anxiety, entries into and time spent in the more anxiety-provoking center of the open field were analyzed as well. For center entries, there was an overall effect of SHS [F(1,29) = 5.826, p = 0.0223] and a SHS × day interaction for both WT [F(1,29) = 9.115, p = 0.0052] and htau [F(1,29) = 7.22, p = 0.0118] mice (Figure 2F). We also analyzed the effect of SHS in each genotype separately. In WT mice, entries into the center were higher on day 2 following SHS than following air exposure. In htau mice, entries into the center were higher on day 1 following SHS than following air exposure. In addition, there was an effect of genotype on day 1 [F(1,58) = 13.097, p = 0.001] and day 2 [F(1,58) = 6.365, p = 0.014] with more center entries in WT than htau mice. Center duration showed a pattern similar to that of center entries (Figure 2G). There was a genotype × exposure interaction [F(1,58) = 4.704, p = 0.034] and effects of genotype on day 1 [F(1,58) = 6.634, p = 0.013] and day 2 [F(1,58) = 5.370, p = 0.024]. In addition, there was an effect of SHS in htau mice [F(1,29) = 6.077, p = 0.0199]. The htau mice exposed to SHS spent more time in the center on day 1 than genotype-matched mice spent following air exposure. There were no effects of SHS or genotype on distance traveled in the open field in the presence of objects (WT air: 19.1 ± 2.5; WT SHS: 18.6 ± 2.0; htau air: 18.2 ± 1.8; htau SHS: 16.8 ± 1.8).

When object recognition was assessed, sham- and SHS-exposed WT mice and sham-exposed htau mice preferentially explored the novel object (Figure 2H). However, SHS-exposed htau mice showed cognitive impairments and did not spend significantly more time exploring the novel than familiar object (Figure 2H).

**Effects of SHS on Spatial Memory in the Water Maze**

Next, spatial learning and memory was assessed in the water maze (Figure 2I). WT mice exposed to SHS had greater swim speeds to locate the platform than WT mice exposed to air [F(1,29) = 13.70, p = 0.0009; Figure 2I]. Therefore, distance moved was used to analyze performance. There was no effect of SHS on ability of WT (Figure 2K) or htau (Figure 2L) mice to locate the visible or hidden platform. However, effects of SHS on spatial memory retention were seen in the probe trials (no platform). When time spent in the target quadrant vs. the three non-target quadrants was analyzed in the first (Figure 2M) and second (Figure 2N) probe trials, there was an effect of quadrant in sham WT and htau mice; the sham mice spent more time in the target quadrant than any nontarget quadrant (p < 0.05, Dunnett’s). Consistent with these results, when cumulative distance to the platform location was analyzed, there was an effect of SHS, with WT and htau mice exposed to SHS swimming further away from the platform than WT and htau mice exposed to air [F(3,58) = 9.415, p < 0.0001; Figure 2O].

**Effects of SHS on Fear Learning and Memory**

When mice were placed in the fear conditioning chamber prior to receiving the first aversive stimulus, htau mice exposed to SHS moved less than htau mice exposed to air (p < 0.01; Figure 2P).

This effect was genotype-dependent and not seen in WT mice. There was a trend toward reduced freezing between the aversive stimuli during fear learning, but it did not reach statistical significance [F(1,58) = 3.664, p = 0.061; Figure 2Q]. When the period between the first and second aversive stimuli was excluded from the analysis to exclude contributions of baseline differences between the groups, fear learning was delayed following SHS in comparison with following air exposure [F(1,57) = 5.60, p = 0.0214; Figure 2Q]. Hippocampus-dependent contextual fear memory the day after fear learning was not significantly less in mice exposed to SHS than in mice exposed to air [F(1,57) = 2.93, p = 0.0924; Figure 2R].

**Effects of SHS on Lung Pathology and Expression of Common Lung Genes**

The SHS exposure used in this study (10 months of exposure to ~ 30 mg/m³ of SHS) did not induce significant differences in lung tissue histology compared to sham-exposed mice (Figure S1). In WT male mice, the expression of 11 genes as analyzed using a PCR array was significantly different in mice exposed to SHS in comparison with mice exposed to air (Figure 3). Two of those 11 genes (Cyp1a1 and Cyp3a11) were more highly expressed in SHS-exposed animals, whereas 9 genes (Cpt1b, Hspa1b, Abcd4, Adrn2, Inhbe, Pdyn, Srebf1, Trib3, and Trim10) were expressed at a lower level. SHS exposure also resulted in differences in the expression of 11 genes in htau male mice (Figure 3). Six of those genes (Cyp1a1, Cyp2el, Cpt1b, Hspa1a, Hspa1b, and Nqo1) were more highly expressed in SHS-exposed animals, whereas 5 genes (Abcc2, Fasl, Inhbe, Pdyn, and Trim10) were expressed at a lower level. There were four genes in common (Cyp1a1, Inhbe, Pdyn, and Trim10) in SHS-exposed WT and htau male mice that were dysregulated in the same direction (Figure 3). WT female mice exposed to SHS exhibited higher expression of seven lung genes (Cyp1a1, Cyp3a11, Hspa1a, Hspa1b, Hsp90ab1, Fasl, and Trim10) in comparison with WT female mice exposed to air (Figure 3).

To identify biological pathways altered by SHS, we used IPA of the gene PCR array data. This analysis revealed that in all WT males and female and htau male mice SHS exposure groups the dysregulated genes were related to canonical pathways associated with nicotine degradation, supporting a chronic effect of SHS exposure on pulmonary function (Figure S2). In addition, male mice

![Figure 3](image.png)
exposed to SHS had lower expression of the ABC transporters Abcb4 and Abcc2 than male mice exposed to air (Figure 3 and Figure S2.). Moreover, the expression of three heat shock protein coding genes (Hspa1a, Hspa1b, and Hsp90ab1; Figure 3 and Figure S2) was altered following SHS exposure. Overall, 13% of the 84 genes on the PCR array were significantly different in male mice exposed to SHS in comparison with those exposed to air, supporting molecular lung toxicity following chronic SHS exposure in this paradigm.

**Effects of SHS on Brain Protein Levels of PHF1, TTCM1, IGF1β, and HSP90**

Dot blot analyses were used for quantification of 21 proteins in three brain areas, as described (Chlebowski and Kisby 2020). Using the cortical samples of male mice, we assessed the relationship between the PHF1 signal intensity as analyzed by western blot with that analyzed by dot blot using identical antibodies and methods of visualization and quantification. There was a significant relationship between the western blot and dot blot immunoreactivity for PHF1 when the three protein bands of PHF1 on the western blot were combined for analysis [$r = 0.7429, p = 0.0022$ (2-tailed), Spearman; $n = 15$ samples; Figure S3A]. There was also a significant relationship between the dot blot and western blot immunoreactivity when only one protein band on the western blot was analyzed [$r = 0.6786, p = 0.0068$ (2-tailed), Spearman; $n = 15$ samples]. When the PHF1 immunoreactivity levels were analyzed, the levels of the three combined protein bands [$F(1,10) = 21.66, p = 0.0009$; Figure S3B] and of the single protein band [$F(1,10) = 22.69, p = 0.0008$; Figure S3C] were significantly higher in htau than WT mice (see Figure S4 for the western blot, Figure S5 for the total protein levels, and Figure S6 for dot blot images). These data support the use of dot blot analysis for quantification of levels of proteins in selected brain regions following SHS. Although brain levels of PHF1, as assayed by dot blot analysis, were higher in htau than in WT male mice, the genotype difference in htau levels was several-fold higher when assessed by western blot analysis than when assessed by dot blot. When the three PHF1 protein bands were combined in the analysis, the levels were $1.9 \pm 0.7$-fold higher by western blot analysis than by dot blot analysis. When the single PHF1 protein band was analyzed, the levels were $11.1 \pm 2.7$-fold higher by western blot analysis than dot blot analysis.

Of the 21 proteins analyzed by dot blot (see Table 1 for list of primary antibodies) in hippocampus, cortex, and cerebellum using repeated-measures ANOVA, with brain region as the repeated measure, the levels of only four proteins (PHF1, TTCM1, IGF1β, and HSP90) were significantly affected by SHS. For PHF1, there were effects of genotype [$F(1,30) = 32.68, p < 0.001$] and sex [$F(1,30) = 24.118, p < 0.001$] and a sex by exposure interaction [$F(1,30) = 4.600, p = 0.040$]. In female mice, there was only an effect of genotype [$F(1,16) = 20.388, p < 0.001$], with higher levels in htau than in WT mice (Figure 4A–D). In contrast, in males there were effects of genotype [$F(1,14) = 12.990, p = 0.003$], with higher levels in htau than in WT mice, and of exposure [$F(1,14) = 12.990, p = 0.003$; Figure 4E–H]. In males, PHF1 levels were lower following SHS exposure than following air exposure. This SHS effect was most pronounced in the hippocampus of WT males (Figure 4F).

TTCM1 is a monoclonal antibody that recognizes all tau toxic conformations including some misfolded monomers. It was generated using the same antigen as used for polyclonal antibody TTC-35 (Sengupta et al. 2017). For TTCM1, there was an effect of sex [$F(1,30) = 34.578, p < 0.001$] and a sex by exposure interaction [$F(1,30) = 5.033, p = 0.032$] and a trend toward an effect of genotype [$F(1,30) = 3.550, p = 0.069$; Figure 4I–P]. In females, there were no effects of genotype or SHS (Figures 4I–L). However, in males there were effects of genotype [$F(1,14) = 7.051, p = 0.019$] and exposure [$F(1,14) = 16.992, p = 0.001$; Figure 4M–P]. TTCM1 levels were lower in males exposed to SHS than in males exposed to air and higher in htau than in WT males.

For IGF1β levels, there were effects of sex [$F(1,30) = 4.859, p = 0.035$] and exposure [$F(1,30) = 4.627, p = 0.040$]. When sexes were analyzed separately, in females SHS did not affect IGF1β levels (Figure 5A–D). However, in males there was an effect of exposure [$F(1,14) = 1.030, p = 0.035$]; IGF1β levels were lower following SHS exposure than those following air exposure (Figure 5E–H). This was most pronounced in the cerebellum (Figure 5G). This finding was consistent with the staining pattern observed in the cerebellum of WT male mice that had been exposed to SHS when compared with air-exposed mice (Figure S7).

Because HSP90 gene expression was different in the lungs of SHS- and sham-exposed mice (Figure 3) and HSP90 entangles proteins that are normally involved in synaptic connectivity and cognition (Inda et al. 2020), HSP90 levels in brain were analyzed as well. For HSP90, there were effects of genotype [$F(1,23) = 31.972, p < 0.001$], sex [$F(1,23) = 4.849, p = 0.038$], exposure [$F(1,23) = 16.291, p = 0.001$], and a genotype × sex interaction [$F(1,23) = 5.332, p = 0.030$], and a trend toward an exposure × sex interaction [$F(1,23) = 3.094, p = 0.092$]. In females, there was only an effect of genotype [$F(1,12) = 5.195, p = 0.042$], with lower levels in htau than WT mice (Figure 5I–L). However, in males there were effects of genotype [$F(1,11) = 35.549, p < 0.001$] and exposure [$F(1,11) = 18.826, p = 0.001$]. HSP90 levels were lower in mice exposed to SHS than in mice exposed to air and lower in htau than in WT mice (Figure 5M–P). This finding was also consistent with the staining pattern of the prefrontal cortex of WT mice exposed to SHS when compared with those exposed to air (Figure S9).

Eight markers (Tau5, 3Rtau, Thr231, CP13, Alz50, INSR, IRS1, and GFAP) showed only effects of genotype and/or sex but not of SHS (Table S1). Eight other markers were not affected by genotype, sex, or SHS (pSer202, T22, TOMA2, amyloid, 4GB, NeuN, Iba1, and S100β).

**Effects of SHS on Metabolic Pathways in the Hippocampus**

To examine the effect of SHS on metabolism in the hippocampus, we performed a metabolomics pathway analysis of hippocampal brain tissue. Based on the sex and genotype differences observed in the initial inspection of the metabolic profiles, the genotypes and sexes were analyzed separately. Effects of SHS on hippocampal pathways in WT and htau mice are shown in Figure 6. Consistently, the effects of SHS were more pronounced in WT males than in females, more pronounced in htau males than in females, and more pronounced in WT mice than in htau mice. In WT male mice, taurine and hypotaurine metabolism, alanine, aspartate and glutamate metabolism, glycine, serine and threonine metabolism, and arginine and proline metabolism showed the largest pathway impact of SHS (Figure 6A). In WT females, taurine and hypotaurine metabolism, glycine, serine and threonine metabolism, arginine and proline metabolism, and arginine biosynthesis showed the largest pathway impact of SHS (Figure 6B). Thus, SHS affected taurine and hypotaurine metabolism, glycine, serine and threonine metabolism, and arginine and proline metabolism in both WT males and females.

In htau males, arginine and proline metabolism, glutathione metabolism, pentose phosphate pathway, and beta-alanine metabolism showed the largest pathway impact of SHS (Figure 6C). In htau females, arginine and proline metabolism, phenylalanine metabolism, ascorbate and aldarate metabolism, and beta-alanine metabolism showed the largest pathway impact of SHS (Figure 6D). Thus, SHS affected arginine and proline metabolism and other metabolic pathways in htau male mice.
beta-alanine metabolism in both htau males and females and arginine and proline metabolism in WT and htau males and females.

To identify potential biomarkers for the detrimental effect of SHS on cognitive performance and neurophysiology, we performed a regression analysis and linked cognitive performance [lower cumulative distance to the target location values in the second probe trial (72 h after the last hidden platform training)] to both SHS and plasma metabolites. We examined 1 to 5 parameter metabolomics plasma panel that explained most of the variation (top 5 model for each 1 to 5 parameter metabolomics plasma panel). We selected those metabolites that were most consistently included in the models (at least 6 times out of potentially 21 times). The metabolite panel that best explained cognitive performance differed among genotype and sexes with the most consistent metabolite panel observed for WT male mice and included ascorbate \( (p = 0.00015) \), flavin adenine dinucleotide (FAD; \( p = 0.0002 \)), and palmitoleic acid (PA) (C16:1, \( p = 0.0002 \)), all of which decreased with cognitive impairment (Table S2). This metabolite panel could explain 93% of the variation in the data set, including all that was associated with SHS, which by itself explained 32% of the variation in cognitive performance \( (p = 0.03) \). These results suggest that the detrimental effects of SHS on spatial memory retention of WT males are mediated by oxidative stress. The Nissl staining pattern of the prefrontal cortex of WT male mice exposed to SHS is consistent with such a mechanism (Figure S8).

**Effects of SHS Exposure on Pathways in Plasma**

Plasma metabolites were not significantly different between WT or htau male or female mice exposed to SHS or air (Figures S9, S10).

Finally, we performed a regression analysis to assess whether specific metabolites were associated with cognitive performance. Consistent with the other data, the most conclusive results were
revealed in WT males. In Table S2, the metabolites in WT males that were correlated with better cognitive performance are indicated (lower cumulative distance to the target location values). Ascorbate, FAD, and PA were indicated at least six times in the top five models and were each associated with cognitive performance ($p = 0.00015$, $p = 0.0002$, and $p = 0.0002$, respectively; Table S3). SHS alone was significantly associated with cognitive performance ($r^2 = 0.3165$, $p = 0.029$). The $p$ value dropped to $p = 0.92$ when ascorbate, FAD, and PA were included in the model (Table S3). These data suggest that SHS alters ascorbate, FAD, and PA, which in turn alters spatial memory retention.

**Discussion**

Exposure to SHS is a major risk factor for the sporadic forms of dementia (Chami et al. 2016; Chen 2012; Yu et al. 2015). SHS exposure might start early in life. Although SHS exposure declined in 3- to 11-y-old children between 1999 and 2014, disparities persist with a measured increased exposure of children among low-income families (Merianos et al. 2020). Exposure to SHS is a risk factor for developing neurodevelopmental disorders (e.g., ADHD, depression, anxiety) (Pagani 2014) as well. Therefore, early-life SHS exposure might have short- and long-term effects. By examining SHS-exposed WT and htau mice, we determined that cognitive injury and neuropathological changes were associated with specific alterations of metabolic pathways, including those involving oxidative stress, that are perturbed in human neurodegenerative disease (González-Domínguez et al. 2015; Liu et al. 2014; Trushina et al. 2013) and a mouse model of AD (González-Domínguez et al. 2015). Data from the current study show that chronic SHS exposure for 10 months to $\sim 30$ mg/m$^3$ affected main brain and body functions, including $a$) reduced body weight in WT, but not htau, mice, without affecting cumulative food intake; $b$) reduced spontaneous alternation in...
WT, but not htau, mice without affecting activity levels in Y maze; e) increased swim speeds of WT, but not htau, mice in the water maze; f) reduced activity levels of WT and htau mice in the open field; g) reduced brain PHF1, TTCTM1, IGF1β, and HSP90 protein levels in WT male, but not in female, mice; and h) had more profound effects on metabolic pathways in WT male than in female mice and more profound effects in WT than in htau mice. These data suggest that WT mice, and particularly WT male mice, might in general be more susceptible to the detrimental effects of chronic SHS exposure than htau mice. With the exception of spontaneous alternation in the Y maze, which was lower in htau than in WT mice, these genotype-dependent responses did not seem to be related to genotype differences in the controls. However, this pattern was not generally seen for all outcome measures. Chronic SHS exposure impaired object recognition in htau, but not in WT, mice. In addition to performance in the object recognition test, chronic SHS exposure affected htau but not WT mice at baseline in the fear conditioning test. In contrast, chronic SHS exposure impaired spatial memory retention of both WT and htau mice in the water maze and did not affect contextual fear memory in either genotype. In the object recognition and contextual fear conditioning tests, memory was assessed 24 h after training. As in the first water maze probe trial memory retention was assessed 24 h after the last hidden platform training, these data indicate that among these three cognitive tests involving hippocampal function (Clark et al. 2007; Kim and Cho 2020; Maren et al. 1997; Maren and Quirk 2004), the water maze probe trial might be particularly sensitive to detect detrimental effects of SHS in WT mice. These data are consistent with visuospatial-related cognitive injury seen in children and adolescents exposed to SHS (Ling and Hefferman 2016; Yolton et al. 2005). Because impaired spatial memory in adults with MCI can predict conversion to AD (Wood et al. 2015), the water maze probe trial data highlight the translational relevance of the detrimental long-term effects of SHS on cognitive function revealed in this study.

In contrast to the hippocampus, there were no clear lung tissue histological changes following 10 months of SHS exposure. Although unexpected, both DBA/2 and C57BL/6J mouse strains exhibited bronchial epithelial cell injury, whereas there was no overt lung morphological changes associated with obstructive retention of air in the lungs following chronic cigarette smoke exposures (3 cigarettes/d, 5 d/wk, for up to 10 months) (Bartalesi et al. 2005). These studies suggest that the lungs of different strains of mice can display distinct lung responses to cigarette smoke exposures (Bartalesi et al. 2005). Similarly, C57BL/6J mice exposed to 4 wk of subchronic exposures to 70–80 mg/m³ of SHS had no significant effect on pulmonary tissue inflammation or pathology but induced lower/lesser lung function that was characterized by decreased elastance (Hartney et al. 2012). In another study, C57BL/6J mice exposed to 150 mg/m³ of cigarette smoke for 3 or 6 months, showed changes in lung function at those two time points, which preceded changes in lung morphology that were only seen at 6 months (Rinaldi et al. 2012). These data strongly suggest that impaired lung function may be a more sensitive indicator than lung pathology and that earlier and transient effects on lung function may be more pronounced in C57BL/6J mice exposed to SHS or mainstream cigarette smoke. These studies also suggest that lung pathology, including measures of inflammation, might be also concentration- and duration-dependent. Some of the detrimental effects of SHS and mainstream cigarette smoke on the lungs of mice following long-term exposures might be transient due to the acclimation of pulmonary responses, including those involving inflammatory mediators (Lerner et al. 2016; Liu et al. 2018). Although no lung pathology was observed and murine lung function was not measured in the present study, we cannot exclude that lung physiology might have been affected by this chronic 30 mg/m³ SHS exposure (levels that are much lower than those previously reported). Regardless, 13% of the 84 genes on the PCR array were significantly altered in male mice exposed to SHS. SHS altered the expression of three heat shock protein genes (Hspa1a, Hspa1b, and Hsp90ab1; Figure 3 and Figure S2), which are associated with protein homeostasis via their roles in chaperone-mediated autophagy and the degradation of misfolded proteins, is related to neuroinflammation and neurodegenerative diseases, including Huntingtons disease signaling (Leak 2014). Two genes that were altered by SHS, Srebfl and Trib3, are related to insulin resistance pathways. Thus, molecular toxicity might be present, and future efforts are warranted to assess chronic molecular effects using broader spectrum of genes for analysis.

In male mice, SHS exposure down-regulated the expression of the ABC transporters Abcb4 and Abcc2. ABC transporters play key roles in lung lipid homeostasis (Sonett et al. 2018). Cigarette smoke down-regulates the expression of pulmonary ABC transporters in vitro and in vivo, and this effect on lung lipid homeostasis might be an early effect of chronic cigarette smoke-induced inflammation (Sonett et al. 2018). At the 8-month time point, the plasma cotinine levels were lower than those at the 4-month time point, in the same range as

**Figure 6.** Hippocampal pathways in WT male (A), WT female (B), htau male (C), and htau female (D) mice exposed to SHS. Red letters within each panel highlight the pathways most affected within each group and are listed here. For WT males (A): A. Taurine and hypouracil metabolism; B. Alanine, aspartate and glutamate metabolism; C. Glycine, serine and threonine metabolism; and D. Arginine and proline metabolism. In WT females (B): A. Taurine and hypouracil metabolism; B. Glycine, serine and threonine metabolism; C. Arginine and proline metabolism; and D. Arginine biosynthesis. In htau males (C): A. Arginine and proline metabolism; B. Glutathione metabolism; C. Pentose phosphate pathway; and D: beta-Alanine metabolism. In htau females (D): A. Arginine and proline metabolism; B. Phenylalanine metabolism; C. Ascorbate and aldarate metabolism; and D. beta-Alanine metabolism.
those seen in C57BL/6j male mice exposed to SHS for 1 h per day, 5 d per week, for 12 or 24 wk (Xu et al. 2014). The pattern of the sex differences in plasma cotinine levels in the current study is consistent with higher plasma nicotine levels in female than in male rats following intravenous nicotine injections (Harrod et al. 2007) and higher plasma nicotine and cotinine in plasma of female than in male mice following nicotine aerosol exposure in mice (Lefever et al. 2017).

In general, chronic SHS exposure had more profound effects on various outcome measures in male mice than in female mice. Although there were no sex differences in the gravimetric analysis, plasma levels of cotinine-N-oxide and trans-3'-hydroxy-cotinine were higher in females than in males at the 8-month time point. Based on the sex differences observed in plasma cotinine levels, which were also seen following nicotine aerosol exposure in mice (Lefever et al. 2017), we cannot exclude that these sex differences in cotinine pharmacokinetics might have contributed to the more profound effects observed following chronic SHS exposure in male than in female mice.

Chronic SHS exposure also had a more pronounced effect on BW of WT males but only a trend toward significance in WT females. The duration and regimen (exposure for 7 d per week) and the source of the WT mice might explain these differences. Exposure of C57BL6 mice from Charles River to three cigarettes per day for 5 d per week for 6 months similarly affected the BWs of females and males (Tam et al. 2020). In contrast to BWs, SHS exposure reduced the food intake of WT female, but not male, mice. Consistent with these mouse data, nicotine-related changes in BW were shown to be accompanied by changes in food consumption in female, but not male, rats (Grunberg et al. 1986). The relative higher levels of plasma cotinine-N-oxide and trans-3'-hydroxy-cotinine in female than in male mice and reduced food intake following SHS in females but not in males seem counterintuitive with the reduced BWs seen in WT males but not in females. Sex differences in the gut microbiome and the gut-brain axis following SHS might have contributed to these divergent findings. Chronic cigarette smoke exposure was shown to induce sex-dependent changes in the mouse cecal microbiome (Tam et al. 2020). The gut microbiome of genetically identical mice is modulated by the local environment and can differ in distinct institutions. Therefore, the source of the mice as well as differences in the gut microbiome due to the local environment might have contributed to the divergent findings related to sex differences in body weight changes in the current study compared to a recent study performed at St. Paul’s hospital in Vancouver, British Columbia, Canada (Tam et al. 2020).

Although women are at increased risk of developing MCI and AD (Au et al. 2017; Barnes et al. 2003; Farrer et al. 1997) and were associated with a higher body mass index (p = 0.03) and with higher fasting glucose in men (p = 0.01), but not in women. There was also a sex x SHS interaction for fasting glucose levels (p = 0.007). (Reed et al. 2017). Similarly, a stronger correlation was observed between SHS and elevated glucose levels in men than in women in a meta-analysis (Pan et al. 2015). The relative protection of females against SHS might be mediated by estrogens. For example, baroreflex-protective effects of estrogens following nicotine exposure have been reported (El-Mas et al. 2012).

Hyperphosphorylation of tau is a hallmark of tau pathological inclusions in human brains, and pathological findings indicate that phosphorylation of tau at specific residues, including PHF1 epitopes, occurs early in tau inclusion formation (Strang et al. 2017). As expected, brain levels of PHF1 and TTCM1, both markers of tau pathology, were higher in htau than in WT mice. A striking finding was that chronic SHS exposure reduced the levels of both tau markers in male, but not female, mice. Thus, although as described above, chronic SHS exposure had more profound effects on many outcome measures and metabolic pathways in the hippocampus of males than in that of females, these effects were associated with reduced tau pathology in males only. Immunization with monoclonal anti-PHF1 antibodies reduced tau pathology and hippocampal atrophy in tau transgenic mice (Liu et al. 2016).

Brain IGF1 levels can be neuroprotective. AD patients and AD mouse models show decreased levels of circulating IGF-I that enter the brain as evidenced by a lower ratio of cerebrospinal fluid/plasma IGF-I (Trueba-Sáiz et al. 2013). Nontoxic monomeric form of β-amyloid can activate IGF1 receptor signaling, and circulating IGF1 can enter the brain and promote the clearance of amyloid peptides (Giuffrida et al. 2012). AD patients and human amyloid precursor protein (APP) and presenilin 1 (PS1) mouse models of AD showed decreased levels of circulating IGF1 entering the brain as evidenced by a lower ratio of cerebrospinal fluid/plasma IGF1 (Trueba-Sáiz et al. 2013). In the current study, SHS exposure reduced IGF1 levels in male, but not in female, mice. HSP90 can trap proteins involved in cognitive performance, and a HSP90 inhibitor is in clinical AD trials (Inda et al. 2020). Male mice, but not female mice, exposed to SHS had lower IGF1 levels. No sex differences in detrimental effects of SHS exposure on cognitive injury were seen and the neuropathological measures described above were lower in SHS-exposed males but not females. Thus, this finding illustrates the complex relationship between cognitive measures and AD-related neuropathology. This complexity between the sexes in neuropathology is also observed in cognitively healthy elders. In the entorhinal cortex of cognitively healthy amyloid-positive elders, more tau pathology was seen in women than in men (Buckley et al. 2019). These data illustrate that although tau pathology is a valuable diagnostic biomarker of AD, but tau pathology does not necessarily correlate with cognitive injury or treatment response in terms of disease progression and clinical status, highlighting the need for the development of nontau pathology biomarkers in AD (Park et al. 2020).

The more profound genotype difference found by western blotting are consistent with that reported in other studies with htau and WT mice (Neddens et al. 2020). Thus, although we noted a significant correlation between PHF1 immunoreactivity by dot blot and western blot analyses, the systematic underestimation of PHF1 levels by dot blot analysis illustrates the limitations of using dot blot analysis for comparing genotype differences in markers of tau-related pathology.

Eight markers (Tau5, 3Rtau, Thr231, CP13, Alz50, INSR, IRS1, and GFAP) showed only effects of genotype and/or sex but not of SHS, whereas eight other markers were not affected by genotype, sex, or SHS (pSer202, T22, TOMA2, amyloid, 4GB, NeuN, Iba1, and S100b).

The higher levels of PHF1 and CP13 in htau than in WT mice is consistent with those previously reported in htau mice (Polydoro et al. 2009). These data highlight the specific effects of SHS on levels of particular markers of neurodegeneration and the challenge of identifying specific molecular neuropathological markers that might relate to the effects of SHS on behavioral and cognitive performance. An interesting finding is that when brain sections were stained with Nissl, similar patterns of Nissl-stained cerebral neurons were observed like those found in ischemia of TBI (Ooigawa et al. 2006).
The data in our study suggest that htau mice might be more resilient than WT mice to the detrimental effects of SHS. This result may relate to the fact that WT human tau is expressed in the htau mouse model. WT, but not mutant, human tau was able to restore Aβ42-mediated inhibition of long-term potentiation, a molecular measure of memory (Vargas-Caballero et al. 2017). Therefore in our experimental design, WT human tau might similarly provide relative protection against SHS. Future efforts are warranted to determine whether mice expressing mutant human tau instead of murine tau are indeed more susceptible than WT mice to SHS.

Regression analysis in WT males revealed that hippocampal ascorbate, FAD, and PA might mediate spatial memory retention in the second probe trial (72 h after the last hidden-platform training). This result is consistent with the antioxidant properties of ascorbate, the proposed vitamin C depletion caused by environmental smoking (Preston et al. 2003), and the relationship of ascorbate with spatial memory in the water maze (de Oliveira et al. 2019).

FAD plays an important role in mitochondrial function (Balasubramaniam and Yaplito-Lee 2020). Because mitochondrial dysfunction is one of the hallmarks of cognitive decline, and FAD is a coenzyme involved in the conversion of the inactive form of glutathione, GSSG, to the active form, GSH (Deponte 2013), this establishes a direct link between depletion of FAD and oxidative stress. Not surprisingly, depletion of C16:1 is also linked to oxidative stress because C16:1 can prevent C16:0-induced oxidative injuries (Wu et al. 2017). The three metabolites might prevent oxidative injury via distinct mechanisms, which explains their additive effect on spatial memory retention. Moreover, chronic SHS exposure may cause oxidative injuries during detoxification of toxic tobacco components, which in turn may result in neurological injuries and subsequently learning and memory deficits, with males being more susceptible, which warrants follow-up studies.

In summary, independent pathways involving ascorbate, FAD, or PA contributed to hippocampal injury following chronic SHS exposure in a mouse model. These data support a critical role of alterations in metabolic pathways, including those involving oxidative stress, in the detrimental effects of SHS on the brain. Increased efforts are warranted to test the ascorbate, FAD, and PA 3-hit hypothesis of detrimental effects of chronic SHS exposure on the hippocampus and on AD risk.

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