

Stroke-induced gut microbiome dysbiosis accelerates Alzheimer's disease progression

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Abstract

Stroke survivors face an elevated risk of developing Alzheimer's disease (AD), yet the biological mechanisms linking these conditions remain poorly defined. Here, we show that a stroke-induced gut microbiome is a key driver of AD-related pathology. Fecal microbiota transplantation (FMT) from stroke patients into young triple-transgenic Alzheimer's disease (3xTg-AD) mice accelerated tau phosphorylation, increased neuroinflammation, and disrupted metabolic homeostasis in both the brain and gut, compared with FMT from healthy donors. Mice receiving stroke-derived microbiota exhibited persistent, donor-specific dysbiosis and broad metabolic reprogramming involving redox balance, nucleotide metabolism, and energy pathways in cecal contents and brain tissue. These metabolic disturbances were accompanied by widespread and region-specific transcriptional changes revealed by single-cell spatial transcriptomics, including glial activation, impaired neuron–glia communication, and dysregulation of mitochondrial, amyloid-processing and inflammatory pathways across cortical and hippocampal regions. Collectively, these findings identify post-stroke gut dysbiosis as a mechanistic contributor to heightened neurodegenerative vulnerability and AD risk, highlighting the gut–brain axis as a potentially modifiable target for preventing post-stroke dementia.

Keywords

Alzheimer's disease, fecal microbiota transplantation, gut–brain axis, neuroinflammation, stroke

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Introduction

Stroke is a major global health challenge, ranking as the second leading cause of death and the primary cause of long-term disability worldwide, with more than 12 million new cases and 6.5 million deaths reported annually.¹ Although advances in acute stroke care have improved survival, the growing population of stroke survivors faces serious long-term complications, particularly cognitive decline, affecting quality of life. Nearly one in three survivors develop dementia or another measurable cognitive impairment within 5 years, with many progressing to either Alzheimer's disease (AD) or vascular dementia.² Longitudinal studies and meta-analyses consistently show that stroke nearly doubles the risk of AD,³ particularly among individuals with recurrent cerebrovascular events, hippocampal atrophy, or extensive white matter lesions.⁴ Mechanistically, ischemic stroke can trigger sustained cerebral hypoperfusion, limiting nutrient and oxygen delivery to vulnerable brain regions and potentially accelerating neurodegenerative processes.

Beyond cerebrovascular injury, mounting evidence implicates the gut microbiome as a key modulator of post-stroke outcomes.⁵ Stroke is frequently accompanied by gut dysbiosis,^{5–8} an imbalance between beneficial and harmful bacteria, resulting in gastrointestinal dysfunction, increased gut permeability, and altered microbial composition. These microbial disturbances activate systemic inflammation, perturb immune–metabolic signaling, and impair neurological recovery. The gut–brain axis, a bidirectional communication network linking the central and enteric nervous systems through immune, endocrine, and microbial pathways, provides a plausible conduit by which peripheral dysbiosis could influence brain pathology.⁹ Both experimental and clinical evidence indicate that gut dysbiosis exacerbates neuroinflammation, synaptic dysfunction, and blood–brain barrier disruption,⁹ ultimately facilitating amyloid- β (A β) and tau accumulation and elevating the risk for AD.¹⁰

Despite these converging lines of evidence, the causal role of stroke-induced gut alterations in accelerating AD progression remains poorly understood. Addressing this gap is essential for clarifying how cerebrovascular injury interacts with neurodegenerative processes and for identifying modifiable targets to mitigate post-stroke dementia risk. To date, few studies have directly examined whether stroke-associated gut dysbiosis might influence the trajectory of AD-related pathology or contribute to neurodegeneration through post-stroke microbial shifts.^{6–8} Our work fills this critical gap by experimentally determining whether human stroke-derived microbiota can drive early neurodegenerative changes in a susceptible AD model.

In this study, we examined whether stroke-induced alterations in the gut microbiome create a permissive

environment for neurodegeneration and accelerate AD-related pathology. We used a young, presymptomatic triple-transgenic AD (3xTg-AD) mouse model that recapitulates key amyloid and tau features of human AD.^{11,12} Fecal microbiota transplantation (FMT) was performed using stool from post-stroke patients or age-matched healthy controls. To define the mechanisms by which stroke-altered gut microbiota exacerbates AD-related pathology, we employed an integrative multi-omics approach that combined microbiome sequencing, metabolomics, single-cell spatial transcriptomics, immunohistochemistry, and immunofluorescence. Together, this comprehensive framework enabled us to directly link post-stroke gut dysbiosis to accelerated AD-related neuropathology and neuroinflammatory remodeling.

Materials and methods

Human donor cohort characterization

Stool samples used in this study were obtained from a subset of participants enrolled in a previously published cohort.⁷ The current donor group included five post-stroke individuals (SD; $n=5$) and six age-matched healthy donors (HD; $n=6$) with mean ages of 69.8 ± 09.20 and 65.16 ± 6.49 years, respectively, and no significant differences in body mass index (BMI) between groups. Clinical characteristics of the fecal donors, including APOE genotype distributions, are summarized in Table 1. Compared with HD, SD showed lower educational attainment ($p=0.0059$) and a higher prevalence of vascular comorbidities, including diabetes, hyperlipidemia, hypertension, and depression.

Human fecal sample collection was conducted under Institutional Review Board (IRB) approval at the University of Kentucky, as previously described.⁷ The study was conducted in accordance with the ethical principles outlined in the Belmont Report, and written informed consent was obtained from all participants. Briefly, participants aged 55–85 years were recruited, with stroke patients enrolled during subacute rehabilitation following a first-time ischemic stroke. Exclusion criteria included clinically significant pulmonary, gastrointestinal, dermatologic, hepatic, or renal disorders requiring ongoing medical management, as well as a history of major gastrointestinal surgery within the past 5 years. Healthy donors were recruited through ResearchMatch.org and advertisements disseminated by the Center for Clinical and Translational Science at the University of Kentucky. All donors were non-Hispanic White and free of major inflammatory or neurological conditions. Stool samples were collected under standardized conditions, immediately frozen at -80°C , and validated for microbiome integrity prior to transplantation. Additional methodological details are provided in the prior publication.⁷

Table 1. Demographic and clinical characteristics of healthy and stroke donors.

Parameters	Healthy donors (N=6)	Stroke donors (N=5)	p value
Age (years)	65.16 ± 6.49	69.80 ± 9.20	0.353
BMI	27.65 ± 5.70	27.75 ± 6.15	0.978
Gender (M:F ratio)	0:6	3:2	
Ethnicity	Not Hispanic or Latino	Not Hispanic or Latino	
Race	White	White	
APOE genotype	E2/E3 (n=2), E3/E3 (n=3), E3/E4 (n=1)	E3/E4 (n=1), E3/E3 (n=1), E2/E3 (n=2), N/A (n=1)	
Education (years)	17.83 ± 1.60	11.50 ± 3.78	0.0059**
Diabetes (%)	16.66%	40.00%	
Hyperlipidemia (%)	0.0%	60.00%	
Hypertension (%)	0.0%	60.00%	
Depression (%)	16.66%	40.00%	

SD: standard deviation.

Values are presented as mean ± SD for continuous variables and as percentages for categorical variables. p values were calculated using unpaired two-tailed t-tests for continuous variables and Fisher's exact test for categorical variables.

**p < 0.01.

Fecal microbiota transplantation (FMT) procedure and experimental timeline

The experimental design and study timeline are illustrated in Figure 1. FMT was performed in 3-month-old 3xTg-AD mice, a presymptomatic stage at which overt AD-related pathology is not yet present. Mice were randomized into three groups: naïve control (no FMT), healthy-FMT (stool from HD), and stroke-FMT (stool from SD). To reduce endogenous gut microbiota and promote donor-dependent colonization, healthy-FMT and stroke-FMT mice received a 7-day course of oral broad-spectrum antibiotics (enrofloxacin 0.5 mg/mL and ampicillin 1 mg/mL in drinking water).¹³ FMT was then performed over 3 consecutive days by oral gavage using 200 µL of freshly prepared fecal suspensions in sterile PBS from either HD or SD donors. This 3-day regimen was selected based on established protocols to ensure maximal taxonomic diversity and stable engraftment of the donor microbiota.^{13,14}

Fecal samples from recipient mice were collected longitudinally at four time points: before antibiotic treatment (Pre-Abx), after antibiotic treatment (Post-Abx), and at 1 week (P1W-FMT) and 2 months (P2M-FMT) following FMT. Pre-Abx and Post-Abx fecal samples were included in the 16S rRNA amplicon sequencing dataset and specifically served as internal controls to confirm that the 7-day antibiotic regimen effectively depleted the endogenous gut microbiota. The P1W-FMT time point was utilized to validate donor-dependent microbial engraftment following antibiotic treatment, while the P2M-FMT interval was selected to assess the long-term persistence of dysbiosis and its cumulative impact on neurodegenerative progression in the 3xTg-AD model. Mice were monitored from ~3 to 6.5 months of age and euthanized at 6.5 months for tissue collection. Brain tissue and cecal contents were

harvested for downstream analyses, including 16S rRNA gene sequencing, GC-MS- and LC-MS-based metabolomics, immunohistochemistry (IHC) for phospho-tau, IBA1 (ionized calcium-binding adaptor molecule 1; a microglia marker) and glial fibrillary acidic protein (GFAP, an astrocyte marker), and single-cell spatial transcriptomic profiling using a Bruker CosMx™ Spatial Molecular Imager (SMI). Naïve control mice received neither antibiotics nor FMT; fecal samples were collected at time points corresponding to P1W-FMT and P2M-FMT in the FMT groups, and these animals were included in microbiome and brain IHC analyses.

Animal allocation and sample size

The 3xTg-AD transgenic mouse strain (B6;129-Tg(APPswe,tauP301L)1Lfa Psen1tm1Mpm/Mmjax; RRID: MMRRC_034830-JAX)^{11,12} was obtained from the Mutant Mouse Resource and Research Center (MMRRC) at The Jackson Laboratory. Mice were maintained under specific pathogen-free conditions with ad libitum access to food and water and were fed a standard irradiated rodent diet. Animals were routinely monitored during standard husbandry procedures to ensure normal health and welfare. Body weight was not collected as a quantitative study variable. Behavioral assessments, including locomotor and cognitive tests, were performed as described behavioral testing section below.

Both male and female mice were included in this study. In total, 83 animals (50 males, 33 females) contributed to the datasets, with different subsets used for specific analyses. Animals were randomly assigned to experimental groups (naïve control, healthy-FMT, and stroke-FMT) using a stratified approach to ensure balanced representation of sexes. Based on our data and prior studies,^{15,16} we

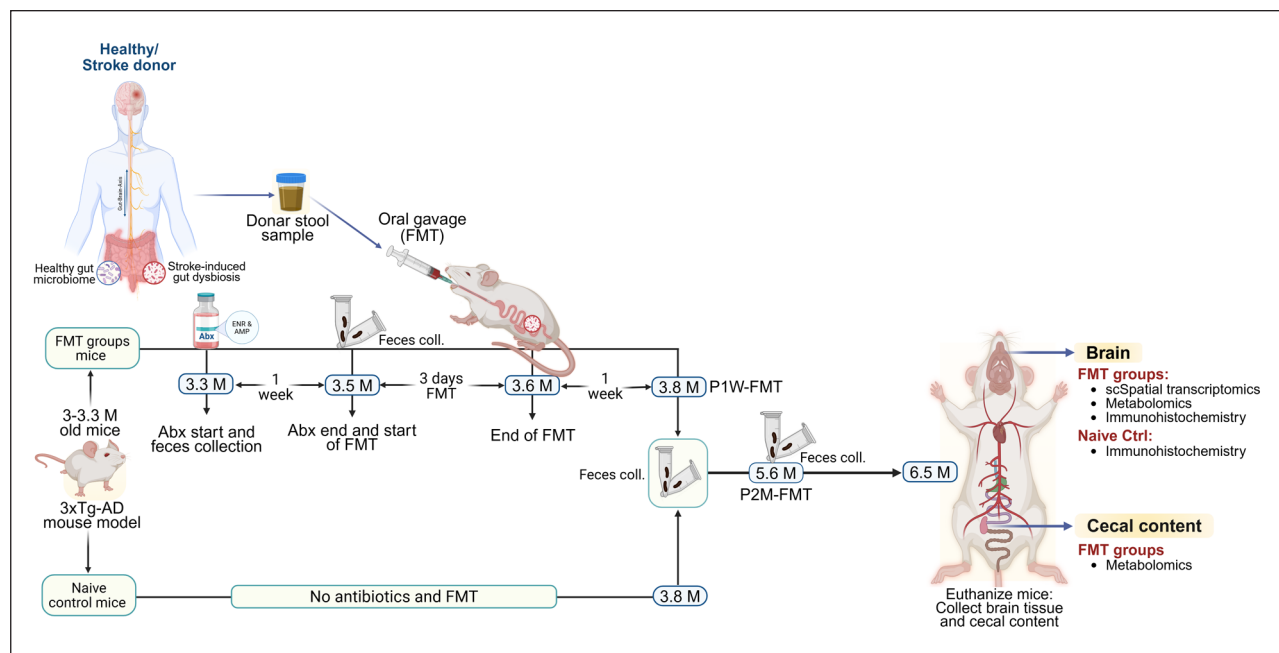


Figure 1. Experimental design and study timeline. Young 3xTg-AD mice received a 7-day course of oral antibiotics, followed by a 3-day FMT using fecal material from either HD or post-SD. Fecal samples were collected at four time points: Pre-Abx, Post-Abx, and at P1W-FMT and P2M-FMT. Mice were monitored from ~3 to 6.5 months of age and euthanized at 6.5 months for downstream analyses, including microbiome profiling, metabolomics, IHC, and single-cell spatial transcriptomics. Naïve control mice did not receive antibiotics or FMT; fecal samples were collected at time points corresponding to P1W-FMT and P2M-FMT in the FMT groups. Naïve controls were included for microbiome and brain IHC analyses. Figure created with BioRender.com. FMT: fecal microbiota transplantation; HD: healthy donors; IHC: immunohistochemistry; Post-Abx: after antibiotic treatment; Pre-Abx: prior to antibiotic treatment; P1W-FMT: 1 week following FMT; P2M-FMT: 2 months following FMT; SD: stroke donors.

estimated that 10 animals/group would provide 80% power to detect microbiome differences (effect size ≥ 1.5) and AD-related pathology (effect size = 0.75), with $\alpha = 0.05$.

We used a multimodal design that included microbiome profiling, metabolomics, immunohistochemistry (IHC), and single-cell spatial transcriptomics. To minimize procedural interference and preserve tissue integrity, animals were prospectively assigned to specific assays. As a result, sample sizes varied across experiments due to platform requirements and technical quality control, rather than exclusion based on biological outcomes. No animals were excluded based on results, and no unexpected mortality occurred.

For microbiome analyses, longitudinal fecal samples were collected at P1W-FMT (naïve control ($n = 5$), healthy-FMT ($n = 23$), stroke-FMT ($n = 19$)) and P2M-FMT (naïve control ($n = 5$), healthy-FMT ($n = 19$), stroke-FMT ($n = 22$)). Metabolomics included cecal content analyzed by GC-MS and LC-MS (healthy-FMT ($n = 14$), stroke-FMT ($n = 14$)) and brain tissue analyzed by GC-MS (healthy-FMT ($n = 14$), stroke-FMT ($n = 13$)). IHC was performed on hippocampal and cortical sections stained for p-tau, GFAP, and IBA1, with sample sizes varying by marker and region after section-level quality control (p-tau: hippocampus $n = 12/21/26$, cortex $n = 14/23/26$; GFAP: hippocampus

$n = 12/21/21$, cortex $n = 13/23/21$; IBA1: hippocampus $n = 15/19/19$, cortex $n = 15/22/21$; control/healthy-FMT/stroke-FMT).

Single-cell spatial transcriptomics (CosMxTM SMI) was performed on three mice per group, yielding tens of thousands of spatially resolved cells per section and enabling robust cell-type and region-specific analyses despite the smaller group size.

Although both sexes were included in each group, the study was not designed or powered to test sex-specific effects, so sex was not included as a variable in the primary analyses. All animal experiments were conducted at the University of Missouri (MU), in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC; protocol #60881). Animal experiments are reported in accordance with ARRIVE 2.0 guidelines.¹⁷

Microbiome profiling by 16S rRNA gene sequencing

DNA extraction. Microbial DNA was extracted from fecal samples using the QIAamp PowerFecal Pro DNA Kit (Qiagen) following the manufacturer's instructions, with a

minor modification. Samples were homogenized in bead tubes using a TissueLyser II (Qiagen, Venlo, Netherlands) for 10 min at 30 Hz instead of the standard vortex adapter. Subsequent steps followed the standard protocol, and DNA was eluted in 100 μ L of elution buffer. DNA concentration was measured using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) with the Quant-iT Broad Range dsDNA Assay Kit, and samples were normalized for downstream applications.

16S rRNA library preparation and sequencing. Microbiome profiling was performed by amplification and sequencing of the bacterial 16S rRNA genes at the University of Missouri Genomics Technology Core, as previously described.^{18,19} Briefly, the V4 hypervariable region of the 16S rRNA gene was amplified from fecal DNA using universal primers (U515F/806R) containing standard Illumina adapter sequences with dual indexing. Following amplification, libraries were pooled and purified according to established core facility protocols. Sequencing was performed on an Illumina MiSeq platform using a 2 $\times$ 250 bp paired-end format, generating high-quality reads suitable for downstream taxonomic and diversity analyses.

Single-cell spatial transcriptomic profiling (CosMx™ SMI)

We performed single-cell spatial transcriptomic profiling with CosMx™ SMI, following standardized protocols from NanoString University (Bruker Spatial Biology, Inc., Seattle, WA, USA; <https://university.nanostring.com/page/document-library>). Formalin-fixed, paraffin-embedded sagittal brain sections were mounted onto Superfrost Plus slides and baked overnight at 60 °C. Sections were deparaffinized using sequential xylene and ethanol washes, followed by antigen retrieval and protease digestion at 40 °C for 30 min. Transcript detection was performed using a 1000-plex RNA in situ hybridization panel with ribosomal RNA probes for cell segmentation. Probes were hybridized overnight at 37 °C, followed by stringent washes and application of nuclear and cell segmentation markers, including Histone and GFAP, in addition to rRNA-based segmentation. Flow cells were assembled and loaded onto the CosMx SMI according to the manufacturer's instructions. Imaging and barcode readout were performed using standard acquisition settings.

Metabolomic profiling of brain and cecal content

GC–MS analysis of brain and cecal metabolites. Fresh-frozen brain and cecal content samples were homogenized by bead milling in ice-cold methanol:acetonitrile: water (2:2:1, v/v/v; 18 μ L/mg tissue) containing isotopically

labeled internal standards (D₄-citric acid, D₄-succinic acid, D₈-valine, and U¹³C-labeled amino acids; Cambridge Isotope Laboratories). Extracts were incubated at –20 °C for 1 h, centrifuged (21,000g, 10 min, 4 °C), and supernatants were dried under vacuum (SpeedVac). Dried samples were derivatized with methoxyamine hydrochloride (11.4 mg/mL in pyridine, 60 °C, 1 h) followed by BSTFA (60 °C, 30 min). One microliter was injected (20:1 split) into a Trace 1300 GC equipped with a TG-5SilMS column and coupled to an ISQ 7000 mass spectrometer. Helium carrier gas was maintained at 1.2 mL/min, with oven temperature held at 80 °C for 3 min, ramped to 280 °C at 20 °C/min, and held for 8 min. Data were acquired in electron ionization mode (–70 eV) using selected ion monitoring (3.9–21.0 min).

LC–MS (high energy/redox panels) analysis of cecal metabolites. Fresh-frozen cecal content samples were homogenized in ceramic bead tubes using 2:2:1 acetonitrile: methanol:water (v/v/v; 18 $\times$ w/v) containing nine heavy internal standards used for internal normalization. Extracts were reconstituted in acetonitrile:water (1:1, v/v), incubated at –20 °C overnight, centrifuged, and transferred to autosampler vials. Metabolite analysis was performed using a Q Exactive Hybrid Quadrupole–Orbitrap mass spectrometer coupled to a Vanquish Flex or Horizon UHPLC system. Separation was achieved on a SeQuant ZIC-pHILIC column (2.1 $\times$ 150 mm, 5 μ m) with a matching guard column using mobile phases of 20 mM ammonium carbonate with 0.1% ammonium hydroxide (A) and acetonitrile (B). The gradient started at 80% B, decreased to 20% B over 20 min, returned to 80% B in 0.5 min, and was held for 7 min. Data were acquired in full-scan polarity-switching mode (m/z 70–1000) at 70,000 resolution with an AGC target of 1 $\times$ 10⁶ and a maximum injection time of 200 ms. HESI source settings included a spray voltage of 3.0 kV, capillary temperature of 275 °C, probe temperature of 350 °C, and sheath, auxiliary, and sweep gas flows of 40, 15, and 1 unit.

Immunohistochemistry for tau and glial markers

Immunohistochemistry (IHC) for phospho-tau (Thr231), GFAP, and IBA1 was performed on formalin-fixed, paraffin-embedded sagittal brain sections (5 μ m) using the Dako Link 48 Autostainer. Antigen retrieval was carried out in citrate buffer (pH 6.0) for phospho-tau or EDTA buffer (pH 9.0) for GFAP and IBA1 at 95 °C for 20 min with pre-heat and cooldown steps. Endogenous peroxidase activity was blocked using enzyme block (Dako SM801) for phospho-tau and GFAP or 3% hydrogen peroxide for IBA1. After protein blocking (Dako X0909 for IBA1), sections were incubated with primary antibodies against phospho-tau (Thr231; Invitrogen 44-746G, 1:100, 20 min), GFAP (Dako IR524, RTU, 20 min), or IBA1 (Abcam EPR16588,

1:2000, 60 min). Detection was performed using appropriate secondary antibodies and HRP polymer systems, and immunoreactivity was visualized with DAB+ chromogen followed by hematoxylin counterstaining. Slides were digitized, and quantitative analysis was performed using QuPath (v0.4.3) to measure the percentage area of positive immunostaining within predefined regions of interest.²⁰ Positive and negative controls were included in each staining run. Image acquisition and quantitative analysis were performed with the investigator blinded to the experimental group.

Behavioral testing in mice

Behavioral testing was performed after collection of P2M-FMT fecal samples during the light phase of a 12 h light/dark cycle (9:00 a.m.–6:00 p.m.). Tests were conducted in the following order: novel object recognition (NOR), Y-maze, and open field test (OFT). All behaviors were recorded and analyzed using ANY-maze software (v7.4; Stoelting Co., USA), and raw data were exported for statistical analysis.

Open field test (OFT). The OFT was used to assess locomotor activity and anxiety-like behavior. Mice were placed in the center of a 40 × 40 cm open-field arena and allowed to explore freely for 5 min. Movements were tracked using ANY-maze, and parameters including total distance traveled, time spent in center, peripheral, and corner zones, and number of zone entries were quantified.²¹ Total distance traveled was used as the primary measure of locomotor activity. The arena was cleaned with 70% ethanol between animals.

Y-maze test. Spontaneous alternation behavior was assessed using a Y-maze consisting of three arms (35 × 5 × 10 cm) positioned at 120° angles. Each mouse was placed at the end of one arm and allowed to explore for 5 min. Arm entries and their sequence were recorded using ANY-maze.²² Working memory was quantified as the percentage of spontaneous alternations, calculated as:

Percentage spontaneous alternation = (total alternations / (total arm entries – 2)) × 100.

Novel object recognition (NOR) test. Recognition memory was assessed using the NOR test. During habituation, mice explored an empty arena (40 × 40 cm) for 5 min. In the training phase, two identical objects were placed in opposite corners and explored for 5 min. After a 24 h inter-trial interval, one familiar object was replaced with a novel object, and exploration was recorded for 5 min. Object interaction was defined as sniffing or contact within 2 cm of the object and was tracked using ANY-maze.²³ The discrimination index was calculated as:

Discrimination index (%) = (time with novel – time with familiar) / total exploration time × 100.

Data processing and bioinformatic analysis

Microbiome sequencing data were processed by the Bioinformatics and Analytics Core at the University of Missouri. Primer sequences were trimmed using Cutadapt²⁴ (v2.6), requiring primer matches with a maximum error rate of 0.1 and a minimum overlap of 3 bp. Reads lacking valid primer matches were excluded. Denoising, dereplication, and amplicon sequence variant (ASV) inference were performed using the DADA2²⁵ plugin (v1.10.0) in QIIME2.²⁶ Reads were truncated at 150 bp, those with expected errors >2 were removed, and chimeric sequences were filtered using the consensus method. Taxonomic classification was performed using a naïve Bayes classifier trained on the SILVA v132 database²⁷ via the classify-sklearn method. Downstream analyses and visualizations were conducted in QIIME 2, R (v3.5.1), and Biom (v2.1.7).

Single-cell spatial transcriptomic data generated with the CosMxTM Spatial Molecular Imager were initially processed using the AtoMxTM Spatial Informatics Platform for cell segmentation, transcript quantification, and quality control. Expression matrices were exported to Python (v3.10) for downstream analysis. Anatomical regions were annotated based on field-of-view metadata, and data were loaded into AnnData objects using Squidpy's read_nanostring function.^{28,29}

Transcripts corresponding to negative control probes were excluded, and cells with >10% negative probe counts were removed. Additional filtering excluded cells with fewer than 100 total transcripts or fewer than 20 detected genes, and genes expressed in fewer than 100 cells. Data were normalized and log-transformed using Scanpy.³⁰ For anatomical region-specific classification and trajectory-based (flight-path) analyses, data subsets were processed in AtoMx using the InsituType algorithm.³¹

Raw GC–MS data were processed using TraceFinder 5.1 (Thermo Fisher Scientific). Metabolites were identified based on the detection of at least two ions with retention times matching in-house reference standards. Pooled quality control samples were analyzed throughout the run, and signal drift was corrected using NOREVA.³² Corrected peak intensities were normalized to the total signal per sample. LC–MS data were processed using TraceFinder 4.1, with metabolite identities assigned using a standard-confirmed in-house library curated by the University of Iowa Metabolomics Core. Drift correction and normalization were applied using NOREVA, and normalized data were used for downstream analyses. Natural log-transformed metabolite concentrations were visualized in R using the ComplexHeatmap package (v2.14)³³ with metabolites grouped by biological pathway.

For immunohistochemistry, regions of interest were manually defined and quantified using QuPath (v0.4.3) to measure the percentage area occupied by positive immunostaining.²⁰

Behavioral data were processed using ANY-maze software (v7.4) to extract locomotor and cognitive parameters, including distance traveled, time spent in predefined zones, and task-specific metrics.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (v10.2.0) and Stata 19 unless otherwise specified. Data normality was assessed using the Shapiro–Wilk test, and non-normally distributed data were log-transformed where appropriate prior to parametric analyses. Group comparisons were conducted using unpaired *t*-tests, Wilcoxon rank-sum tests, one-way ANOVA, repeated-measures ANOVA (rANOVA), or Kruskal–Wallis tests, as appropriate. Baseline characteristics between human donor groups were compared using two-sided unpaired *t*-tests and chi-square tests. Microbial alpha diversity was assessed using unpaired *t*-tests and confirmed using Wilcoxon rank-sum tests, while Simpson diversity was analyzed using rANOVA with Bonferroni correction and confirmed by sensitivity analysis using the Friedman test. Effect sizes for immunohistochemistry and metabolomics outcomes were estimated using linear regression models and are reported as mean differences with 95% confidence intervals. Cecal metabolomics data were analyzed on log-transformed values where appropriate, while brain metabolomics data were analyzed on untransformed values, with multiple-comparison adjustment applied. Spatial transcriptomic clustering and differential gene expression analyses were performed in Python using Scanpy, with differentially expressed genes identified using the `rank_genes_groups` function with the Wilcoxon rank-sum test. Region- and cell-type-specific analyses incorporated FMT group, anatomical region, and cell-type annotations. Statistical significance was defined as $p < 0.05$, with corrections applied for multiple comparisons.

Results

Stroke donor microbiota exhibits reduced diversity and dysbiosis signatures

Simpson alpha diversity, reflecting microbial richness and evenness, was modestly lower in stroke donors (SD; 0.92 ± 0.04) compared with healthy donors (HD; 0.95 ± 0.02), although this difference did not reach statistical significance (mean difference = 0.025; 95% CI: -0.015 to 0.064 ; $p = 0.19$; Figure 2(a)). In contrast, beta diversity analysis revealed a clear separation between HD and SD microbial communities (Bray–Curtis PCoA; PERMANOVA $p = 0.0093$, $F = 1.6$; Figure 2(b)).

Hierarchical clustering confirmed distinct compositional profiles (Figure 2(c)). At the genus and species levels, HD samples exhibited higher abundance of *Terrisporobacter*, *Actinomyces graevenitzi*, *Lachnospiraceae* UCG-009, *Dorea*, *Oscillospiraceae*, *Lachnospiraceae* GCA 90006 6575, *Fusicatenibacter*, and *Romboutsia*. SD samples were enriched in *Eubacterium brachy* group, *Lachnospiraceae*, *Ruminococcus torques*, *Leuconostoc*, *Escherichia–Shigella*, *Papillibacter*, *Blautia*, and *Oscillospiraceae* UCG-002.

Donor-specific gut microbiota colonization following FMT in 3xTg-AD mice

Naïve control mice maintained high and stable microbial diversity at both P1W-FMT and P2M-FMT, providing a consistent reference for FMT-induced changes. In contrast, microbial diversity in recipient mice shifted in a donor-dependent manner. Repeated-measures ANOVA revealed a significant main effect of donor group on the Simpson index ($p = 0.019$). At P1W-FMT, diversity was highest in controls, intermediate in healthy-FMT mice, and lowest in stroke-FMT mice, with a significant difference between the two FMT groups (mean difference = -0.046 ; 95% CI: -0.089 to -0.004 ; $p = 0.033$; Figure 2(d)). By P2M-FMT, both FMT groups continued to show lower diversity than controls but no longer differed from each other ($p = 0.941$). Nonparametric sensitivity analyses supported these patterns at both time points (P1W $p = 0.001$; P2M $p = 0.016$), indicating early donor-dependent differences in microbial diversity that converged over time (Figure 2(d)). Principal coordinates analysis (PCoA) further demonstrated clear clustering by donor group and time point (PERMANOVA $p = 0.0001$; Figure 2(e)).

At the genus and species levels, hierarchical clustering analysis confirmed distinct microbial compositional profiles between healthy-FMT and stroke-FMT mice (Figure 2(f)). *Lachnospiraceae* members showed sample-specific enrichment ($z > 4$). *Bacteroides* spp., including *B. dorei* and *B. uniformis*, were more abundant in healthy-FMT mice. The butyrate-producer *Intestinimonas butyriciproducens* was enriched in healthy-FMT relative to stroke-FMT mice. *Akkermansia* showed differential representation across groups. In contrast, *Clostridium sensu stricto*, UC *Lachnospiraceae*, and GCA-900066575 were strongly enriched in stroke-FMT mice ($z > 4$), indicating distinct post-transplant microbiota structures.

Stroke-FMT accelerates phospho-tau pathology and glial reactivity

Sagittal brain sections from FMT-treated and naïve control mice were analyzed by IHC for phospho-tau (p-tau), IBA1, and GFAP. Naïve control mice exhibited low baseline levels of tau pathology and gliosis and did not differ

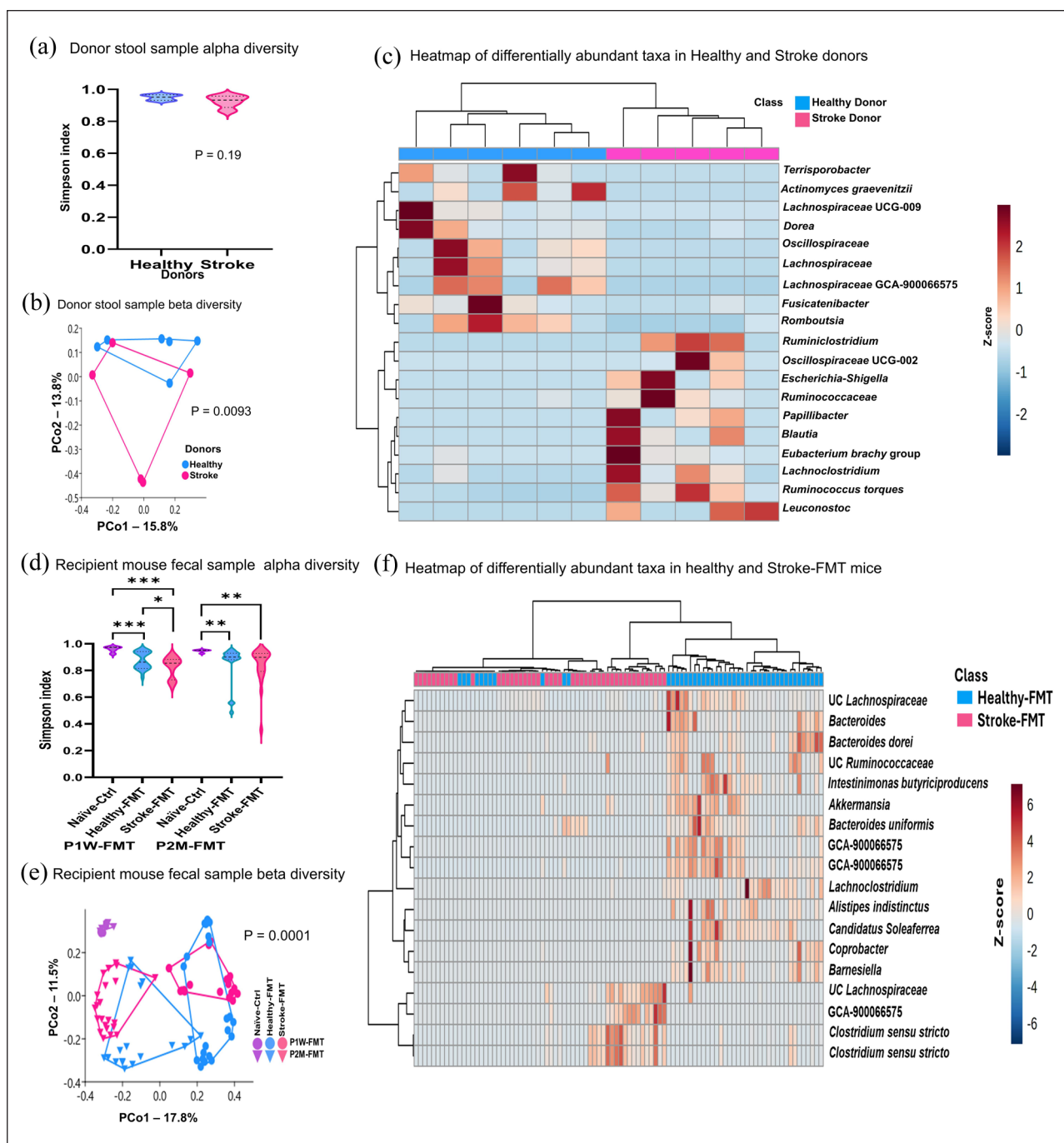


Figure 2. Gut microbiome profiling of human donors and recipient 3xTg-AD mice following FMT: (a) alpha diversity (Simpson index) of stool samples from HD and SD (two-sided Student's *t*-test), (b) beta diversity of donor stool samples based on Bray–Curtis dissimilarity, visualized by PCoA, showing separation between HD and SD groups (PERMANOVA: $p = 0.0093$, $F = 1.6$). Axes represent PCo1 and PCo2, (c) heatmap of differentially abundant microbial taxa in donor stool samples (row-scaled z-scores), (d) alpha diversity (Simpson index) of fecal samples from recipient 3xTg-AD mice at P1W-FMT and P2M-FMT microbiota (repeated-measures ANOVA: donor $p = 0.019$; time $p = 0.93$; interaction $p = 0.74$), (e) beta diversity of recipient fecal microbiota based on Bray–Curtis dissimilarity, visualized by PCoA, demonstrating donor- and time-dependent community shifts (two-way PERMANOVA: donor $p = 0.0001$; time $p = 0.0001$; interaction $p = 0.41$), and (f) heatmap of fecal microbial taxa in recipient mice following healthy-FMT or stroke-FMT (row-scaled z-scores).

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

FMT: fecal microbiota transplantation; HD: healthy donors; PCoA: principal coordinate analysis; PCo1: principal coordinate 1; PCo2: principal coordinate 2; P1W-FMT: 1 week following transplantation with healthy-FMT or stroke-FMT; P2M-FMT: 2 months following transplantation with healthy-FMT or stroke-FMT; SD: stroke donors.

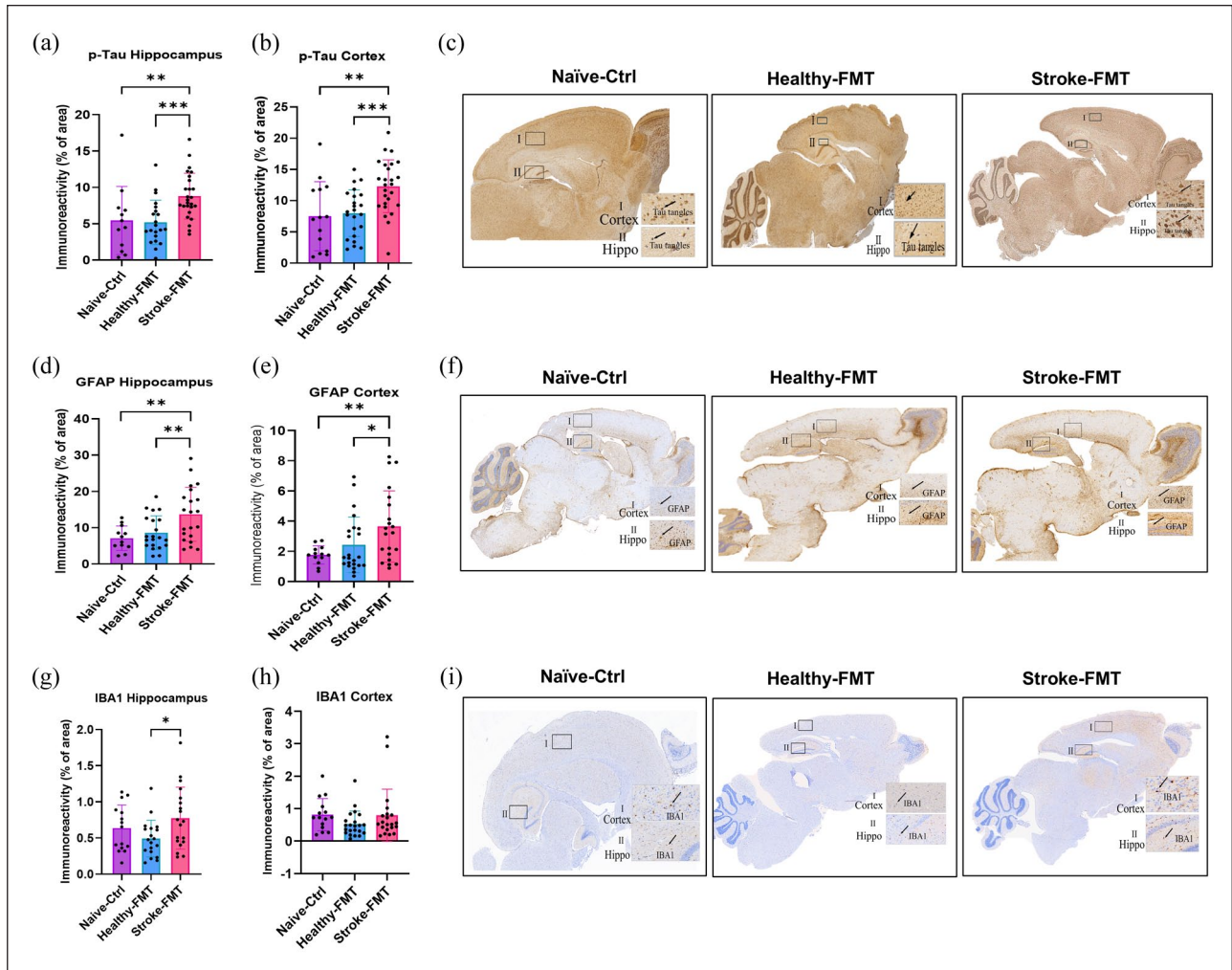


Figure 3. Immunohistochemical analysis of p-tau, GFAP, and IBA1: (a, b) quantification of p-tau (% area) in hippocampus (a) and cortex (b), (c) representative p-tau IHC images. (d, e) GFAP quantification in hippocampus (d) and cortex (e), (f) representative GFAP images, (g, h) IBA1 quantification in hippocampus (g) and cortex (h), and (i) representative IBA1 images. Quantification (a, b, d, e, g, h) was analyzed by linear regression with 95% CIs.

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

GFAP: glial fibrillary acidic protein; IBA1: ionized calcium-binding adapter molecule 1; IHC: immunohistochemistry; p-tau: phosphorylated tau.

significantly from healthy-FMT mice in p-tau, GFAP, or IBA1 across hippocampal and cortical regions ($p > 0.15$). In contrast, stroke-FMT mice showed significant elevations in p-tau and GFAP relative to naïve controls ($p = 0.002$ – 0.008), highlighting the pathological divergence between the healthy-FMT and stroke-FMT groups. In the hippocampus, p-tau levels were higher in stroke-FMT compared with healthy-FMT (8.7% vs 5.2%), with a significant difference in effect sizes (3.60; 95% CI: 1.56–5.63; $p = 0.001$; Figure 3(a)). A similar pattern was observed in the cortex (12.2% vs 7.9%; effect size 4.30; 95% CI: 1.79–6.80; $p = 0.001$; Figure 3(b)). Figure 3(c) shows more widespread and intense tau staining in stroke-FMT brains. GFAP immunoreactivity was also increased in stroke-FMT mice. Hippocampal GFAP was significantly higher than in healthy-FMT (13.6% vs 8.6%; effect

size 5.07; 95% CI: 1.53–8.61; $p = 0.006$; Figure 3(d)). Cortical GFAP showed a smaller but significant increase (3.6% vs 2.4%; effect size 1.22; 95% CI: 0.08–2.35; $p = 0.036$; Figure 3(e)). Representative images are shown in Figure 3(f). Microglial activation reflected similar trends. Hippocampal IBA1 was higher in stroke-FMT than healthy-FMT (0.77% vs 0.49%; effect size 0.28; 95% CI: 0.05–0.50; $p = 0.015$; Figure 3(g)), whereas cortical IBA1 did not differ significantly between groups ($p = 0.147$; Figure 3(h)). Representative IBA1 images are shown in Figure 3(i). These differences remained significant even after accounting for multiple comparisons. In summary, p-tau and GFAP immunoreactivity were markedly increased in the hippocampus of stroke-FMT mice, with more pronounced changes than those observed for cortical GFAP or IBA1 when compared with healthy-FMT mice.

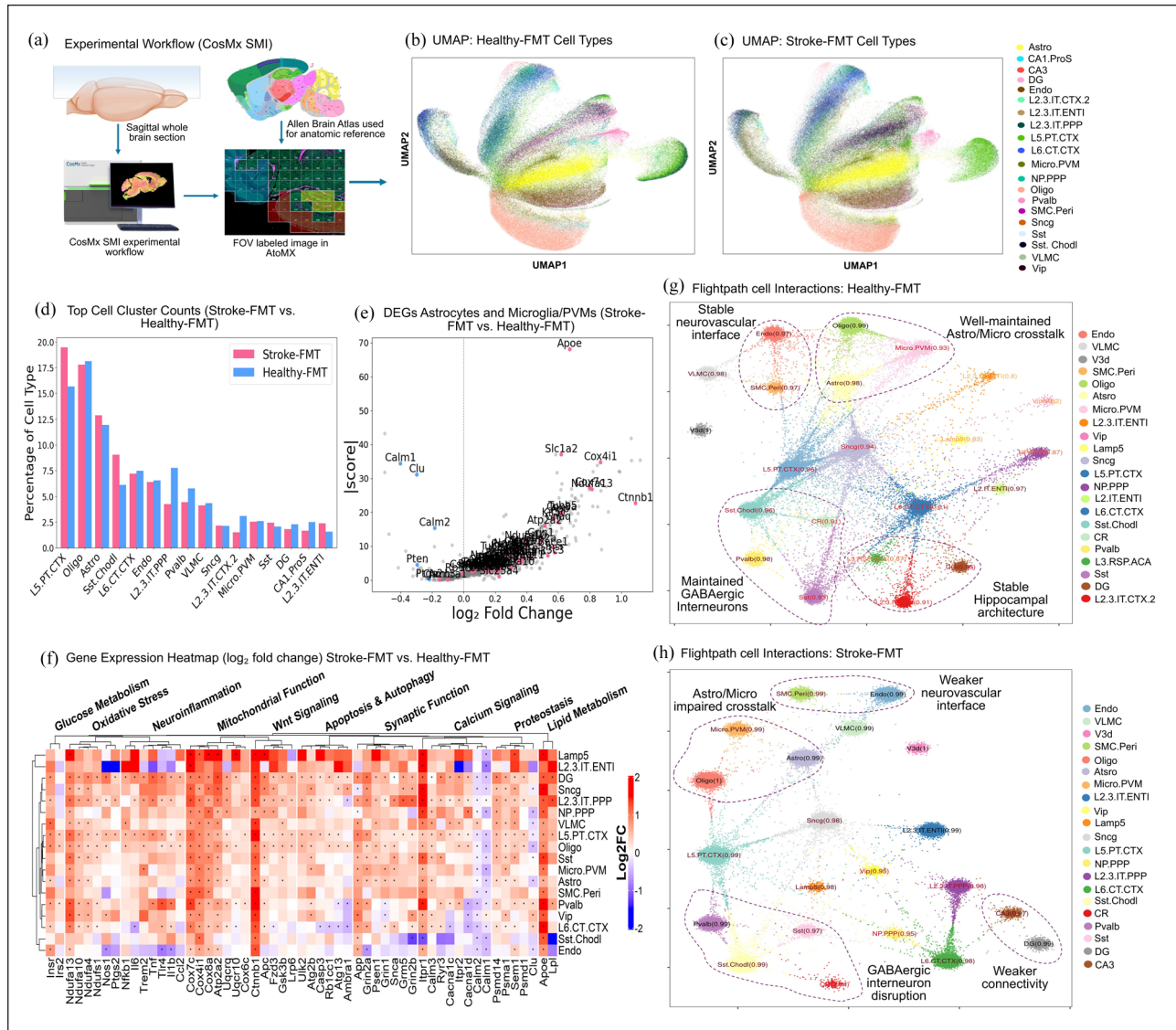


Figure 4. Whole-brain spatial transcriptomics after healthy- or stroke-FMT: (a) CosMxTM SMI workflow for whole-brain profiling, (b, c) UMAP projections showing donor-driven shifts in cell-type composition, (d) percent abundance of major cell types, with increases in Sst.Chodl neurons, astrocytes, and L5.PT.CTX neurons in stroke-FMT, (e) volcano plots of differentially expressed genes in astrocytes, microglia, and perivascular macrophages (e.g. upregulated Apoe, Cox4i1; downregulated Calm1–2, Clu, Pten), (f) heatmap of log₂ fold changes across functional gene modules (lipid metabolism, neuroinflammation, mitochondrial pathways, oxidative stress, synaptic signaling, calcium homeostasis), and (g, h) cell–cell interaction networks showing preserved neuronal–glial connectivity in healthy-FMT (g) and disrupted astrocyte–microglia and reduced neuronal connectivity in stroke-FMT (h). FMT: fecal microbiota transplantation; SMI: spatial molecular imager; UMAP: uniform manifold approximation and projection.

Stroke-altered microbiota induces global transcriptional remodeling across brain cell types

To determine how stroke-associated microbiota reprogram brain-wide gene expression, we compared whole-brain single-cell spatial transcriptomic profiles between the healthy-FMT and stroke-FMT groups (Figure 4(a)). Uniform Manifold Approximation and Projection (UMAP), a nonlinear dimensionality-reduction method for visualizing high-dimensional single-cell transcriptomic

data, identified all major neuronal and glial populations in both groups; however, stroke-FMT brains showed broader, less compact clustering for some cell types, most prominently in parvalbumin-positive interneurons (Pvalb), vasoactive intestinal peptide-expressing interneurons (VIP), dentate gyrus (DG) and layer 2/3 intratelencephalic (L2.3.IT) excitatory neurons, suggesting altered transcriptional states and cellular organization (Figure 4(b) and (c)). Stroke-FMT brains also exhibited more prominent clustering of Layer 5 Pyramidal Tract Cortical (L5.PT.CTX) neurons.

Cell-type composition analysis revealed that healthy-FMT brains retained higher proportions of excitatory neurons, whereas stroke-FMT mice exhibited increased abundance of L5.PT.CTX and Somatostatin-expressing chondrolectin-positive (Sst.Chodl) interneuron populations (Figure 4(d)). Differential expression analysis uncovered widespread transcriptional reprogramming, most notably in astrocytes and microglia/perivascular macrophages (Micro.PVM; Figure 4(e)). Stroke-FMT brains showed robust upregulation of genes associated with mitochondrial activity (e.g. *Cox4i1*, *Cox7c*, *Ndufa13*), amyloid processing (*Apoe*, *Bace1*), synaptic and excitatory signaling (*Grin1*, *Gnaq*), immune activation (*Tnf*, *Il1b*, *Trem2*), and oxidative stress pathways. In contrast, downregulated transcripts included calcium signaling genes (*Calm1/2*), proteostasis regulators (*Clu*), and neuroprotective/autophagy-related genes (*Pten*, *Ambra1*).

A hierarchical clustered heatmap further illustrated extensive cell-type-specific remodeling across 10 functional gene modules, including lipid metabolism, proteostasis, synaptic signaling, neuroinflammation, mitochondrial activity, autophagy, oxidative stress, and Wnt signaling (Figure 4(f)). Overall, stroke-FMT brains displayed consistently pro-inflammatory and metabolically activated transcriptional states across multiple cell classes.

Furthermore, Spatial Flightpath analysis revealed significant disruption of cell–cell communication networks in stroke-FMT mice. Specifically, we observed impaired astrocyte–microglia signaling and weakened neurovascular coupling, as well as reduced connectivity among interneuron populations (e.g. *Pvalb*, *Sst*, *CR*, *Sst.Chodl*), and diminished CA3–DG coordination. In contrast, healthy-FMT brains maintained a coherent and functionally integrated network architecture, characterized by stable neurovascular interfaces and maintained hippocampal coordination. These findings provide a structural and molecular basis for the impaired neuron–glia communication observed following the transplantation of stroke-derived microbiota (Figure 4(g) and (h)).

Stroke-derived microbiota reprograms cortical and hippocampal transcriptional networks underlying AD vulnerability

To define region-specific consequences of stroke-altered microbiota, we profiled the cortical and hippocampal transcriptome data from healthy-FMT and stroke-FMT 3xTg-AD mice (Figure 5(a)). UMAP projections revealed compact, well-organized neuronal and glial clusters in healthy-FMT brains (Figure 5(b)), whereas stroke-FMT samples displayed broader dispersion of DG and intratelencephalic L2/3 IT excitatory neurons, along with increased scatter across glial and vascular populations

(Figure 5(c)), suggesting altered regional cellular organization.

Cell-abundance analysis (Figure 5(d)) showed that stroke-FMT brains were enriched in layer 6 corticothalamic (L6.CT.CTX) neurons, gamma-synuclein expressing (*Sncg*) interneuron, as well as Astrocytes and Oligodendrocytes. Healthy-FMT brains retained higher proportions of L2/3 IT neurons in the pre/para/post-subiculum, (L2.3.IT.PPP) and L2/3.IT.CTX excitatory populations as well as near projecting pre/para/post-subiculum (NP.PPP) and CA1 prosubiculum (CA1.ProS) neurons.

Differential gene expression revealed extensive cortical and hippocampal transcriptional remodeling (Figure 5(e)). Stroke-FMT brains showed broad upregulation of genes involved in excitatory signaling, neuroinflammation, amyloid processing, and mitochondrial metabolism—including *Apoe*, *Slc1a2*, *Cox4i1*, *Cox7c*, *Ndufa13*, *App*, *Gnaq*, *Grin1*, and *Ctnnb1*. Downregulated transcripts included *Clu*, *Calm1/2*, and *Pten*, key regulators of calcium homeostasis, proteostasis, and neuroprotection.

Hierarchical clustering (Figure 5(f)) highlighted convergent upregulation across functional pathways, lipid metabolism (*Apoe*, *Lpl*), proteostasis (*Psmd14*, *Sem1*), synaptic signaling (*App*, *Grin2b*), neuroinflammation (*Tnf*, *Il1b*, *Trem2*), mitochondrial activity (*Cox7c*, *Uqcrc*), oxidative stress (*Ndufa13*, *Nos1*), and Wnt signaling (*Ctnnb1*, *Apc*). These changes were most pronounced in glial and immune-responsive populations. Downregulated pathways included calcium signaling (*Calm1/2*, *Cacna1d*), proteostasis (*Clu*), and mitochondrial function (*Uqcrc10*, *Cox6c*).

The top region-specific up- and downregulated genes in stroke-FMT compared with healthy-FMT further underscored enhanced expression of AD-relevant drivers (*Apoe*, *Slc1a2*, *Cox4i1*) and reduced expression of neuroprotective factors (*Clu*, *Calm1/2*; Figure 5(g)). Collectively, these results indicate that stroke-derived microbiota drive pronounced, region-specific reprogramming of cortical and hippocampal transcriptional networks associated with increased AD susceptibility.

Stroke-altered microbiota disrupts gut–brain redox and energy metabolism

To determine how stroke-derived microbiota alter host metabolism, we performed untargeted GC–MS of cecal contents and brain tissue and targeted LC–MS of redox- and energy-associated metabolites in FMT-recipient mice.

Untargeted GC–MS of cecal contents revealed pronounced metabolic divergence between groups (Figure 6(a)). Stroke-FMT mice showed significant increases ($p < 0.001$) in metabolites linked to glycolytic flux, lipid metabolism, and redox balance, including pyruvate (0.48; 95% CI: 0.29–0.66), docosanoate (0.84; 95% CI:

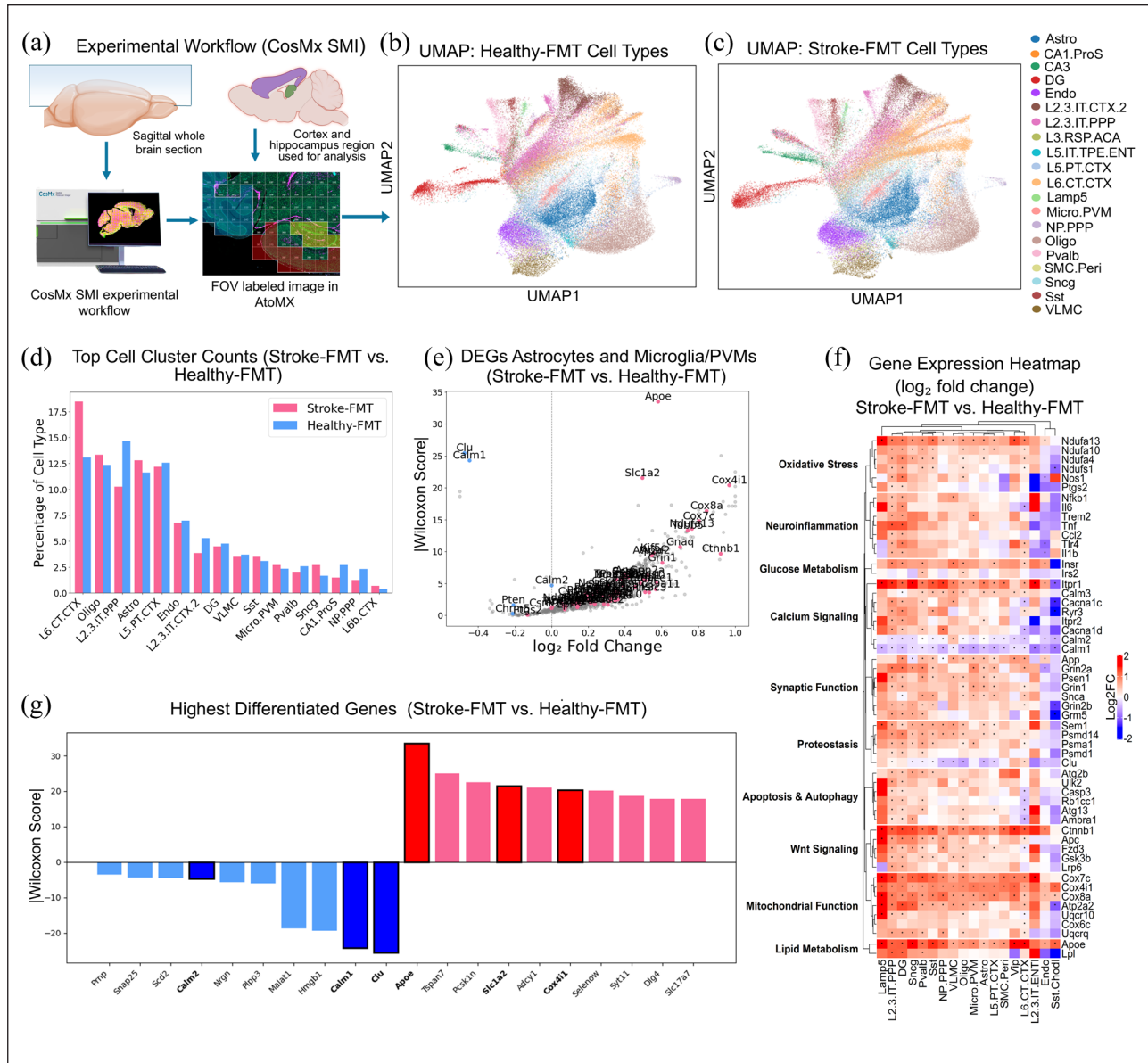


Figure 5. Spatial transcriptomics of cortex and hippocampus following healthy- or stroke-FMT: (a) CosMxTM SMI workflow for region-specific profiling, (b, c) UMAP projections showing donor-dependent shifts in cortical and hippocampal populations, (d) percent abundance of major cell populations, with increased L6.CT.CTX neurons, astrocytes, oligodendrocytes, and Sncg⁺ cells in stroke-FMT, (e) volcano plots of differentially expressed genes in astrocytes and microglia/perivascular macrophages (upregulated Apoe, Slc1a2, Cox4i1; downregulated Calm1, Clu), (f) heatmap of log₂ fold changes across functional modules, and (g) top upregulated and downregulated genes by Wilcoxon scores in stroke-FMT compared with healthy-FMT; AD-related genes in bold. AD: Alzheimer's disease; FMT: fecal microbiota transplantation; SMI: spatial molecular imager; UMAP: uniform manifold approximation and projection.

0.51–1.17), cholesterol (0.41; 95% CI: 0.22–0.60), cysteine (0.39; 95% CI: 0.19–0.60), and erythrose (0.38; 95% CI: 0.20–0.56). Reductions were observed in adonitol (−0.57; 95% CI: −0.86 to −0.27) and orotate (−0.61; 95% CI: −0.88 to −0.33). Additional elevations included spermidine (0.46; 95% CI: 0.21–0.70; $p=0.001$), heptanoate (0.28; 95% CI: 0.11–0.44; $p=0.002$), and phosphoenolpyruvate (0.28; 95% CI: 0.10–0.46; $p=0.003$). Broader perturbations encompassed amino acid, nucleotide, and

TCA-associated metabolites, with increases in GABA, guanosine, taurine, fumarate, and cytosine and reductions in glucose-6-phosphate, fructose-6-phosphate, succinate, methionine, and lysine (all $p < 0.05$ after multiple-comparison adjustment).

Targeted LC–MS analysis further identified disruptions in nucleotide and NAD-related metabolism in stroke-FMT mice (Figure 6(b)). Cytidine (0.58; 95% CI: 0.29–0.86; $p < 0.001$), adenosine (0.59; 95% CI: 0.20–0.98; $p=0.004$),

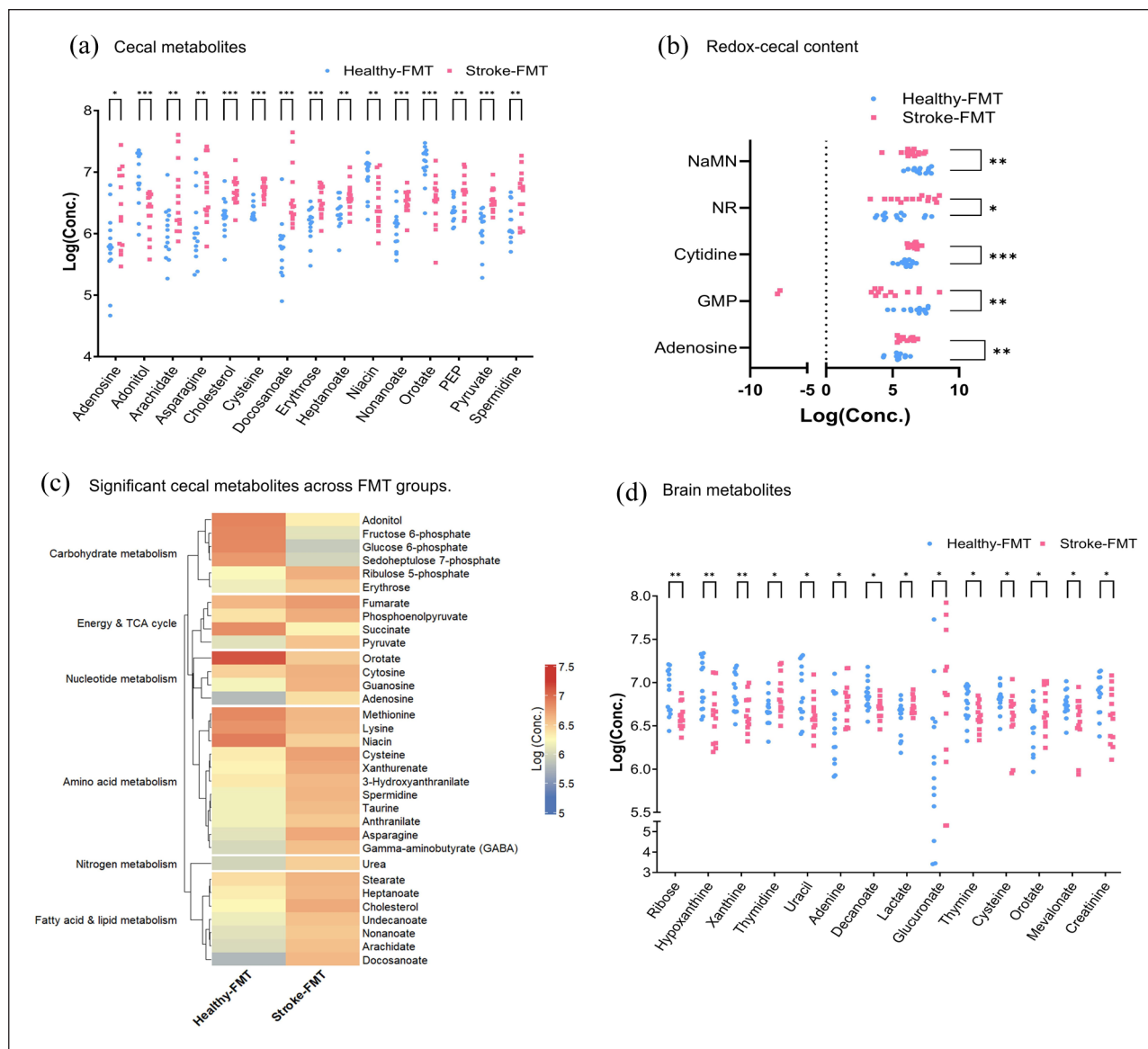


Figure 6. Cecal and brain metabolomic profiling: (a) untargeted GC–MS analysis of log-normalized cecal metabolites in stroke-FMT versus healthy-FMT mice, (b) targeted LC–MS quantification of energy- and redox-related cecal metabolites, (c) heatmap of significantly altered cecal metabolites, grouped by major metabolic pathways and shown as row-scaled relative abundances, and (d) untargeted GC–MS profiling of log-normalized brain metabolites in stroke-FMT and healthy-FMT groups.

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

FMT: fecal microbiota transplantation; GC–MS: gas chromatography–mass spectrometry; LC–MS: liquid chromatography–mass spectrometry.

and nicotinamide riboside (1.08; 95% CI: 0.05–2.10; $p = 0.040$) were increased, whereas GMP (−2.26; 95% CI: −3.55 to −0.97; $p = 0.001$) and nicotinic acid mononucleotide (−0.73; 95% CI: −1.23 to −0.22; $p = 0.006$) were significantly reduced after multiple-comparison adjustment. Hierarchical clustering (Figure 6(c)) revealed coordinated elevation of metabolites associated with glycolysis, TCA flux, redox balance, and polyamine metabolism in stroke-FMT mice, alongside relative depletion of glycolytic intermediates in healthy-FMT mice.

Brain metabolomic profiling revealed significant depletion of energy- and nucleotide-related metabolites in stroke-FMT mice (Figure 6(d)). Reductions were observed in ribose (−0.34; 95% CI: −0.55 to −0.14; $p = 0.002$), hypoxanthine (−0.47; 95% CI: −0.77 to −0.17; $p = 0.003$), xanthine (−0.29; 95% CI: −0.51 to −0.08; $p = 0.009$), uracil (−0.32; 95% CI: −0.58 to −0.07; $p = 0.015$), and additional metabolites linked to redox buffering and mitochondrial function, including cysteine, mevalonate, and creatinine ($p < 0.05$). In contrast, stroke-FMT brains showed

increased thymidine (0.19; 95% CI: 0.04–0.34; $p=0.014$), adenine (0.25; 95% CI: 0.04–0.46; $p=0.022$), lactate (0.13; 95% CI: 0.02–0.24; $p=0.024$), glucuronate (0.73; 95% CI: 0.05–1.41; $p=0.037$), and orotate (0.18; 95% CI: 0.003–0.37; $p=0.046$). Together, these changes indicate impaired oxidative metabolism and disrupted energy homeostasis, accompanied by compensatory shifts toward glycolytic and nucleotide salvage pathways in the stroke-FMT brain.

Stroke-altered microbiota shows trends toward impaired behavior and memory in 3xTg-AD mice

Naïve control mice generally exhibited numerically higher locomotor activity and cognitive performance than FMT-recipient groups across behavioral assays, although no global group-wise differences reached statistical significance. Behavioral performance was evaluated around 6 months of age using a validated battery targeting locomotion, spatial working memory, and recognition memory: open field test (OFT), Y-maze test (YMT), and novel object recognition (NOR).

In the OFT, representative trajectories (Figure S1(A)) and total distance traveled (Figure S1(B)) showed reduced locomotor activity in stroke-FMT mice compared to healthy-FMT and naïve control groups. However, the healthy-FMT versus stroke-FMT contrast was not significant (-536.5 ; 95% CI: -1237.7 to 164.6 ; $p=0.129$), which was confirmed in nonparametric sensitivity analyses.

A similar pattern emerged in the YMT. Stroke-FMT mice exhibited lower spontaneous alternation than both healthy-FMT and control groups (Figure S1(C) and (D)), but the healthy-FMT versus stroke-FMT difference again did not reach significance (-2.34 ; 95% CI: -9.34 to 4.65 ; $p=0.498$), which was confirmed in nonparametric sensitivity analyses. All groups performed within the expected range for 3xTg-AD mice at this age.

In the NOR task (Figure S1(E) and (F)), stroke-FMT mice displayed a lower discrimination index (-0.39%) relative to healthy-FMT (0.55%) and naïve control (0.88%). Although the healthy-FMT versus stroke-FMT contrast was not significant (-0.94 ; 95% CI: -2.48 to 0.60 ; $p=0.222$), which was confirmed in nonparametric sensitivity analyses, the NOR showed the largest numerical separation, suggesting a trend toward reduced recognition memory in stroke-FMT mice.

Discussion

In this study, we provide multi-omics and spatial transcriptomic evidence that stroke-derived human microbiota is sufficient to accelerate AD-related pathology in young, pre-symptomatic 3xTg-AD mice. Although prior

epidemiological and preclinical work suggested links between stroke, gut dysbiosis, and neurodegeneration,^{1,4–8,34} our findings uniquely demonstrate that the gut microbiome acts as a mechanistic bridge connecting cerebrovascular injury to early AD-related molecular changes. Specifically, within 2 months, post stroke-FMT induced persistent dysbiosis, widespread metabolic disruption, targeted brain transcriptional reprogramming, glial activation, and exacerbated amyloid–tau pathology in otherwise healthy young mice. Together, these results establish vascular injury-associated microbiota as a causal modifier of neurodegeneration through gut–brain inflammatory crosstalk.

Consistent with prior reports in patients and animal models,^{5–7} stroke donors in our study exhibited classical post-stroke dysbiosis, characterized by reduced diversity and restructured microbial communities. While species richness (alpha diversity) remained comparable to healthy controls ($p=0.19$), the stroke-associated microbiota was characterized by a profoundly restructured microbial community (beta diversity, $p=0.0093$). This aligns with the principles established by Lozupone et al.,³⁵ who argued that the health and resilience of the gut microbiota are better characterized by compositional structure and the relative abundance of taxa than by total species counts alone. Healthy donors were enriched in taxa such as *Terrisporobacter*, *Actinomyces graevenitzi*, *Romboutsia*, *Fusicatenibacter*, *Dorea*, and *Lachnospiraceae* UCG-009. Several of these genera have been associated in prior studies with short-chain fatty acid production and maintenance of epithelial barrier function.^{36,37} In comparison, stroke donors showed increased abundance of *Eubacterium brachy*, *Ruminococcus torques*, *Leuconostoc*, *Lachnospiraceae*, and *Escherichia/Shigella*. Although these taxa are common commensals, some members have been associated with mucin degradation, altered bile acid pathways, or inflammation in specific contexts.^{38,39} Such compositional differences raise the possibility that donor-derived microbial functions may contribute, at least in part, to the distinct pathological and transcriptional phenotypes observed in 3xTg-AD mice following FMT.

Transferring these donors' microbial communities into 3xTg-AD mice demonstrated that such dysbiotic profiles are sufficient to initiate neurodegenerative processes. The 3xTg-AD mice receiving FMT resulted in stable, donor-specific engraftment, consistent with previous studies demonstrating long-term microbial persistence.¹⁴ Hierarchical clustering further confirmed that each group developed distinct community-level signatures that recapitulated key features of their respective donor microbiota.³⁵ Healthy-FMT mice displayed greater representation of *Bacteroides* species, including *B. dorei* and *B. uniformis*, as well as enrichment of the butyrate-producing organism *Intestinimonas butyriciproducens*, taxa associated with SCFA metabolism and epithelial barrier integrity

in human and mouse studies.⁴⁰ In contrast, stroke-FMT mice showed selective enrichment of *Clostridium sensu stricto* and several *Lachnospiraceae* subgroups—patterns aligned with emerging evidence that specific *Lachnospiraceae* and *Eubacterium*-related taxa exert causal effects on stroke outcomes and promote pro-inflammatory gut states through impaired barrier and immune regulation.⁴¹ *Akkermansia* species also differed between groups, suggesting altered mucin turnover and shifts in barrier-related nutrient dynamics, consistent with data showing that *Akkermansia* preferentially utilizes host-derived mucins and metabolites such as lactate.⁴² Collectively, these compositional signatures indicate that stroke-FMT mice developed gut communities characterized by reduced butyrate-producing taxa and increased pro-inflammatory organisms a profile previously linked to impaired metabolic and immune homeostasis in neurological and inflammatory disease models.⁴³ This donor-specific microbial configuration provides a mechanistic foundation for the downstream metabolic and immune alterations observed in stroke-FMT recipients and supports the concept that donor microbiota shape host physiology through strain-level functional effects.⁴⁴ Our results demonstrate that the stroke-associated microbial signature not only successfully engrafted but remained stable throughout the experimental period. The observation of donor-specific signatures at both early (P1W-FMT) and late (P2M-FMT) time points confirms the robustness of the FMT procedure and supports the role of the gut microbiome as a persistent driver of host physiological changes.

Metabolomic profiling of cecal contents and brain tissue further revealed pronounced disruptions in redox and energy metabolism in stroke-FMT mice. In the cecum, stroke-FMT recipients displayed enhanced glycolytic flux, mitochondrial stress, and lipid metabolic reprogramming, consistent with ischemic and dysbiotic states.⁴⁵ Key glycolytic and TCA intermediates—including glucose-6-phosphate, succinate, and malate were reduced, consistent with impaired oxidative metabolism and mitochondrial dysfunction.^{46,47} Targeted redox analyses showed altered NAD⁺ metabolism, disrupted nucleotide turnover, and redox imbalance,⁴⁸ while elevated spermidine reflected activation of the polyamine pathway, a known inflammatory and stroke-associated metabolic signature.⁴⁹

Brain metabolomics similarly revealed distinct metabolic perturbations. Purine and pyrimidine metabolites were markedly decreased, indicating impaired nucleotide turnover and reduced energetic support typical of ischemic and neuroinflammatory states.⁵⁰ Reductions in cysteine, mevalonate, decanoate, and creatinine further suggested compromised redox buffering, lipid precursor availability, and mitochondrial energy capacity.^{51,52} In contrast, increases in adenine, lactate, glucuronate, and orotate were consistent with heightened glycolytic reliance, activation

of nucleotide salvage pathways, and pyrimidine metabolic stress associated with mitochondrial dysfunction.^{53,54} Collectively, these data indicate that stroke-derived microbiota impose a substantial metabolic burden on the host, characterized by reduced oxidative capacity, disrupted nucleotide homeostasis, and impaired redox.

At the cellular level, spatial single-cell transcriptomics revealed regionally distinct transcriptional reprogramming across glial and neuronal populations. Stroke-FMT UMAP clusters for excitatory neurons and Pvalb interneurons displayed diffuse boundaries and expansion, suggesting altered transcriptional states rather than discrete cell-type loss. Excitatory intratelencephalic (IT) a major class of glutamatergic neurons that project within the telencephalon to connect cortical layers and hemispheres and Pvalb neurons were depleted in stroke-FMT brains, mirroring cell-type vulnerabilities described in human AD brain atlases.^{55–57}

Transcriptomic profiling further revealed upregulation of genes involved in amyloid processing, mitochondrial metabolism, and inflammatory signaling, alongside downregulation of autophagy and calcium-buffering pathways. These changes align with evidence that APOE–TREM2 signaling promotes microglial transition to a neurodegenerative, lipid-dysregulated, and phagocytosis-impaired state.^{58,59} Reduced expression of *Clu*, *Pten*, and *Ambra* parallels studies showing that impaired calcium handling and defective proteostasis contribute to neuronal loss in AD.^{60–62} The fragmentation of astrocyte–microglia and neurovascular networks observed here is also consistent with mechanisms of vascular–glial dysfunction known to underlie cognitive decline.^{63,64} Disruption of these vascular-associated transcriptional networks in stroke-FMT brains suggests impaired neurovascular coupling and inefficient metabolic substrate delivery following cerebrovascular injury. Furthermore, fragmentation of these neurovascular networks was accompanied by robust downregulation of genes essential for endothelial maintenance and neuroprotection, specifically *Pten* and *Ambra1*, within vascular-associated cell populations.^{65,66} This molecular signature coincided with a significant depletion of the butyrate-producing species *Intestinimonas butyriciproducens* in the gut of stroke-FMT mice, a taxon linked to epithelial and vascular barrier support in prior work.⁴⁰ Although classical exogenous tracer assays of barrier permeability were not performed in this cohort, these spatially resolved transcriptomic and microbial changes together suggest impaired BBB integrity and heightened neurovascular vulnerability in response to stroke-associated microbiota. When considered alongside the observed redox and nucleotide metabolic deficits, these changes support a model in which stroke-altered microbiota destabilize the neurovascular unit and increases vulnerability to neurodegeneration. Consistent with this framework, stroke-induced dysbiosis

amplified AD-associated transcriptional signatures across hippocampal and cortical regions, positioning gut microbial perturbations as an upstream driver of glial activation and neuronal susceptibility.

Tissue-level analyses supported these molecular findings. Stroke-FMT brains exhibited elevated p-tau, GFAP, and IBA1 immunoreactivity in hippocampal and cortical regions, consistent with hallmark AD-related gliosis and tau pathology.^{63,67,68} These features align with prior reports that reactive glia closely track plaque and tangle accumulation in AD models.^{63,67,68} Together, these observations confirm that gut-derived metabolic and immune disturbances can manifest as canonical AD proteinopathies.

Further, behavioral testing demonstrated consistent, though non-significant, trends toward reduced exploratory activity and impaired cognition in stroke-FMT mice compared with healthy-FMT and naïve controls.¹² Recognition memory exhibited the most pronounced deficits, consistent with early functional decline that typically precedes overt neuronal loss. These behavioral findings align with prior evidence linking gut microbiota composition to cognitive and affective performance^{9,69} and suggest that post-stroke dysbiosis primes neural circuits for early functional impairment, potentially accelerating AD-like progression. Notably, these subtle changes were observed within 2 months of FMT in young, asymptomatic mice. We speculate that more robust and statistically significant cognitive deficits may emerge at later time points as the mice age.

The present findings highlight the therapeutic potential of maintaining a healthy gut microbiome to protect the brain function after neurovascular injury. Notably, microbiome composition changes with age^{70,71} and advanced age has been a major risk factor for stroke. Stroke, traumatic brain injury (TBI), and other neurovascular insults consistently induce dysbiosis, which amplifies neuroinflammation, metabolic stress, and vulnerability to cognitive decline. Restoring or preserving microbial balance, through dietary fiber, prebiotics, probiotics, or targeted microbial metabolites, has emerged as a promising strategy to interrupt these pathogenic cascades. For example, recent clinical and preclinical studies show that microbiome-directed interventions can reduce neuroinflammatory burden and improve cognitive outcomes in individuals with mild cognitive impairment and in animal models of stroke and TBI.^{5,72} Our group also demonstrated that prebiotic inulin, administered before or after TBI, reduces neuroinflammation and supports cognitive recovery,^{73,74} demonstrating the capacity of diet-derived microbial substrates to modulate brain health. Together with the current results, these findings suggest that early correction of dysbiosis, particularly that triggered by neurovascular dysfunction, may help preserve metabolic homeostasis, enhance immune resilience, and slow progression toward

post-stroke cognitive impairment and AD. Microbiome-targeted therapies, therefore, represent a modifiable and feasible approach to reducing long-term dementia risk in populations vulnerable to cerebrovascular injury.

We acknowledge the sex imbalance in our human donor cohort, specifically the exclusive inclusion of female healthy donors as a study limitation. While the stroke group included both sexes, the lack of male healthy controls may influence baseline microbial comparisons. Although our recipient mice included both males and females, the overall sample size and modest sex imbalance limited our ability to resolve sex-specific effects. Future studies with fully sex-matched donor-to-recipient cohorts are needed to further parse sex-specific microbial contributions to AD. In addition, we did not quantify circulating inflammatory mediators in this cohort, preventing a direct assessment of systemic cytokine changes. Future studies incorporating serum profiling alongside gut and brain measures will be needed to define this systemic inflammatory axis in post-stroke dysbiosis and AD.

In summary, this multi-omics investigation provides the first causal, mechanistic evidence that stroke-induced dysbiosis is sufficient to trigger rapid molecular and pathological neurodegenerative changes in an Alzheimer's-prone model. Stroke-associated microbiota disrupts metabolic and redox pathways, activates glial responses, and intensifies amyloid-tau interactions, culminating in early cognitive impairment. By integrating microbial, metabolic, and transcriptomic analyses, these results demonstrate that the gut-brain axis represents a modifiable conduit linking cerebrovascular injury to neurodegeneration, opening new opportunities for microbiome-based strategies to reduce post-stroke dementia risk.

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Author contributions

CA, ACE, PB, and A-LL conceived and designed the study. CA coordinated the experiments, performed animal studies, and drafted the manuscript. CW assisted with spatial transcriptomic data processing and analysis. MG and KI assisted with animal experiments and data collection. PK and EG contributed to experimental optimization and data interpretation. AZ, WY, JC, and ZG performed immunohistochemistry experiments. ACE contributed to microbiome analysis and interpretation. AKD provided statistical analysis and biostatistical guidance. PB and RZ provided scientific consultation. CA, ACE, AKD, CW, MG, KI, JC, ZG, and A-LL edited the manuscript and provided scientific interpretation. A-LL and PB supervised the study and

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical considerations

Human stool sample collection was approved by the Institutional Review Board (IRB) of the University of Kentucky. Following approval, the principal investigator (Dr. Ai-Ling Lin) transitioned to the University of Missouri (MU). All animal experiments were subsequently conducted at MU and approved by the MU Institutional Animal Care and Use Committee (IACUC) under Protocol #60881, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Data availability statement

Data supporting the findings of this study are presented in the main text and figures. Processed datasets and analysis code are available from the corresponding author upon reasonable request. Raw sequencing and spatial transcriptomics files will be shared in accordance with institutional and participant-consent requirements.

Supplemental material

Supplemental material for this article is available online.

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