

Original Article

Extracellular Space Barrier Dysfunction Disrupts Interstitial Fluid Drainage and Is Associated with Memory Heterogeneity in Cognitive Aging

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ABSTRACT: Abnormal cognitive aging is characterized by memory decline beyond normal age-related physiological changes. Nevertheless, the intrinsic mechanisms driving individual memory heterogeneity during aging remain poorly elucidated. Given the critical roles of brain interstitial fluid (ISF) dynamics and extracellular space (ECS) transport in maintaining neural homeostasis, the present study aimed to explore whether ECS compartmentalization and ISF drainage disturbance contribute to interindividual variations in memory performance during aging. Aged rats were stratified according to their memory performance. A multi-modal strategy combining behavioral assessment, microdialysis, metabolomics, and electrophysiological recording was applied to detect structural and functional deficits in ECS barrier integrity and ISF drainage, with the assistance of MRI-based tracer imaging, fluorescence imaging, and ultrastructural observation. The results demonstrated that memory-impaired aged rats exhibited region-specific neurotransmitter imbalance in the caudate nucleus and thalamus, which was closely associated with age-related ECS barrier dysfunction. Such barrier dysfunction induced ISF drainage disturbance, thereby reducing local neurotransmitter concentrations in the caudate nucleus and impairing thalamocortical oscillations during non-rapid eye movement sleep, and ultimately disturbing memory consolidation. Further tracer imaging and electron microscopy examinations confirmed that compromised myelin integrity in the internal capsule served as the structural basis for age-related ISF drainage disturbance. Collectively, these findings reveal that ISF drainage disturbance induced by age-related ECS barrier alterations acts as a non-degenerative mechanism underlying memory heterogeneity in cognitive aging. This work highlights ECS compartment integrity as a promising biomarker and therapeutic target for intervening age-related cognitive decline.

Keywords: Cognitive aging, Extracellular space, Interstitial fluid, ECS barrier, ISF drainage disturbance, Tracer-based MRI; Memory heterogeneity

INTRODUCTION

The global aging population is expanding at an unprecedented rate, with the number of individuals aged 65 years and older projected to reach 1.7 billion by 2030

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[1, 2]. A hallmark of cognitive aging is its pronounced heterogeneity, with memory decline ranging from subtle, age-related changes to severe impairments that precede dementia [3, 4]. Epidemiological studies estimate that approximately 40% of older adults experience some degree of memory decline, and 10–15% meet the criteria for abnormal cognitive aging—characterized by accelerated memory loss that is distinct from normal aging [4]. This heterogeneity poses substantial societal challenges, as unaddressed memory decline contributes to increased healthcare costs, reduced independence, and an elevated risk of dementia, which is projected to affect 152 million people globally by 2050 [5]. Therefore, elucidating the mechanisms underlying this variability may facilitate targeted interventions to preserve cognitive health and reduce the burden on aging societies.

Current explanations for the heterogeneity of cognitive aging primarily focus on neuronal degeneration, synaptic dysfunction, and vascular compromise [6–8]. Despite extensive research, investigations into molecular, cellular, and genetic mechanisms have yet to yield effective clinical treatments [9, 10], underscoring the inexorable progression of accelerated memory decline [11]. More importantly, these approaches fail to explain why a subset of older adults exhibit rapid memory decline without overt neuropathological lesions or structural brain abnormalities [12], suggesting that non-degenerative mechanisms may underlie the heterogeneity of cognitive outcomes [13, 14]. Emerging research has focused on extracellular fluid, highlighting its potential for rejuvenating the aging brain; in particular, brain interstitial fluid (ISF) has shown promise in mitigating the aging niche and restoring vital compounds [15–18]. Although the brain is primarily composed of neurons and glial cells, the growing recognition of ISF for its critical role in maintaining microenvironmental homeostasis warrants further investigation [19, 20]. However, the role of ISF in cognitive aging remains insufficiently explored. Hence, integrating insights from brain cells and extracellular ISF may offer a more comprehensive understanding of their combined contributions to age-related memory heterogeneity [21, 22].

Brain ISF contains various ions and neurometabolites and modulates neural excitability, regional substance transport, and the clearance of extracellular waste [23–25]. Notably, ISF, which is involved in diverse physiological processes, can be disrupted during aging and in pathological conditions [25, 26]. In Alzheimer's disease, for instance, the accumulation of A β has been shown to hinder ISF drainage, linking impaired clearance to both sleep deprivation and reduced ISF elimination during slow-wave sleep [27, 28]. Prior studies have also shown that ISF in the deep brain drains through a compartmentalized extracellular space (ECS), rather than

a highly interconnected one [29]. Specifically, the densely myelinated internal capsule (i.c.) compartmentalizes ISF drainage within the ECS of thalamus (Tha.) and caudate nucleus (C.n). Notably, the compartmentalized ECS corresponds to the regional distribution of neurotransmitters [30, 31], and its dysfunction may be associated with memory loss. Furthermore, the structural maturity of the compartmental barrier is essential for cognitive development [32]. According to the theory of retrogenesis [33], the myelinated barrier structure that mediates ECS compartmentalization may be vulnerable in older individuals, and even in the absence of overt pathology, age-related changes could modulate compartmentalized ISF drainage [28, 34]. Nevertheless, to the best of our knowledge, no studies have yet examined cognitive aging heterogeneity and ISF drainage stability in the deep brain.

In this study, we provide new insights into the brain's interstitial system, demonstrating how compartmentalized ISF drainage enhances our understanding of memory heterogeneity in the aging brain. Using a combination of behavioral tests, tracer-based MRI, electrophysiology, microdialysis, and histopathology in aged rats, we investigated how aging-associated ISF dysregulation affects neurotransmitter transport, thereby contributing to memory heterogeneity [35].

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley (SD) rats—including young rats (2 months old, 350–400 g) and aged rats (24 months old, 600–700 g)—were obtained from Peking University. The study was approved by the Peking University Biomedical Ethics Committee (permit number: LA2020356). Rats were housed individually in ventilated cages under controlled conditions: temperature ($22 \pm 1^\circ\text{C}$), humidity ($60 \pm 5\%$), and a 12-h day/night cycle. Food and water were provided ad libitum. All experiments were conducted during the light phase.

About 10–15 aged rats in each batch were subjected to the Morris water maze test. A total of 90 aged rats were stratified by independent investigators blinded to the experimental grouping to eliminate selection bias: the Normal Aging group (time spent in the target quadrant $\geq$ median, $N=43$) and the Abnormal Aging group (time spent in the target quadrant $\leq$ lower quartile, $N=17$). Rats with moderate behavioral performance were excluded from the study.

Morris Water Maze

The Morris water maze assessed spatial reference memory. The apparatus consisted of a 120-cm diameter, 50-cm high circular pool filled with water maintained at $23 \pm 1^\circ\text{C}$ and rendered opaque with nontoxic white paint. The hidden platform was 10 cm in diameter and submerged 1 cm below the water surface. The pool was located in a room with extra-maze visual cues and was conceptually divided into four quadrants. To acclimate the rats and reduce stress, initial training involved locating a visual platform 1 cm above the surface. Each rat underwent four trials per day for 5 days. In each trial, rats were released from a random quadrant facing the wall. A trial ended when the rat found and stayed on a submerged, hidden platform for ≥ 5 s. Escape latency was recorded. Rats failing to locate the platform within 120 s were guided to it and allowed to remain on it for 10 s. On Day 6, the platform was removed, and the rats swam freely for 60 s from a random starting point [36]. The time spent in the target quadrant, platform crossings, and swim paths were recorded using a video-tracking system (Tracking Master, version 4.0; Zhongshi Technology Development Co., Ltd., Beijing, China). All data analyses were performed by a double-blind researcher, and each scatter point in the figures represents an independent biological replicate.

Novel Object Recognition Test

In the training phase, rats explored two identical objects. In the test phase, one object was replaced with a novel one. Given the rodents' natural preference for novelty, exploration of the new object indicated memory of the familiar object. A 24-hour retention interval was used as previously described. The discrimination ratio was calculated using Tracking Master software. After each trial, the apparatus and objects were cleaned with 75% ethanol and dried to eliminate olfactory cues. A discrimination ratio of zero indicated no preference, while a positive value indicated a preference for the novel object [37]. All data analyses were performed by a double-blind researcher and each scatter point in the figures represents an independent biological replicate.

Object Place Recognition Test

Before testing, all rats underwent a 5-minute habituation in the test cage without objects. After this, the two objects were then placed in the cage, and the rats were allowed to explore freely for 10 min. After a 2-hour retention interval, one of the two objects was relocated to a novel position, and each rat was allowed 5 min of free exploration. Exploration behavior was recorded, and the discrimination ratio was calculated using Tracking Master software. No external stimuli were applied during the test

to minimize stress-related confounding factors [38]. All data analyses were performed by a double-blind researcher and each scatter point in the figures represents an independent biological replicate.

Elevated Plus Maze Test

Anxiety-like behavior was assessed using an elevated plus maze (EPM) with two open arms (50×5 cm) and two closed arms ($50 \times 5 \times 15$ cm) made of opaque gray plastic and elevated 60 cm above the floor. Rats were placed in the center, facing an open arm, and allowed to explore for 10 min. Movements were tracked using Tracking Master software (version 4.0), which recorded time spent and entries into the open and closed arms. Between trials, the apparatus was cleaned with 75% alcohol to ensure consistency [39]. All data analyses were performed by a double-blind researcher and each scatter point in the figures represents an independent biological replicate.

Microdialysis Procedure

Rats underwent stereotaxic surgery to implant guide cannulae (CMA, #830 9024), targeting the thalamus and C.n. The bregma was used as the coordinate origin (C.n.: A/P: 1.0 mm, M/L: -3.0 mm, D/V: -2.5 mm; thalamus: A/P: -2.0 mm, M/L: -2.0 mm, D/V: -2.5 mm). Following craniotomy at these coordinates, cannulae (Microbiotech Se/AB, Sweden) were implanted. Microdialysis began 7 days post-surgery. After recovery, rats were housed individually with free access to food and water. On the day of sampling, rats were anesthetized with isoflurane, and microdialysis probes (100-kDa cutoff; Microbiotech Se/AB, Sweden) were inserted. Subsequently, rats were allowed to fully recover from anesthesia and maintain a conscious, freely moving state throughout the subsequent sampling procedure. Artificial cerebrospinal fluid was perfused at $1 \mu\text{L}/\text{min}$ using a peristaltic pump (CMA, Sweden). After a 1-hour equilibration period, ISF samples were collected continuously for 6 h, and then aliquoted and stored at -80°C for targeted neuro-metabolite analysis and untargeted metabolomics [40].

Untargeted Metabolomics

Each sample (100 μL), including quality control (QC) samples, was aliquoted into Eppendorf tubes, and the remaining volume was stored at -80°C . Samples were treated with 700 μL of extraction solvent containing internal standard 1 (methanol:acetonitrile:water, 4:2:1, v/v/v). After 1-minute vortexing, mixtures were incubated at -20°C for 2 h, then centrifuged at $25,000 \times g$ for 15 min. Supernatants were vacuum-dried, reconstituted in 180 μL methanol:water (1:1, v/v), vortexed, and

centrifuged again at $25,000 \times g$ for 15 min. A 20 μL aliquot from each sample was pooled for QC and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for further analysis.

Metabolites were separated using a Waters 2777c UPLC system (Waters, USA) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, USA). Chromatographic separation was performed on a Waters ACQUITY UPLC BEH C18 column (1.7 μm , 2.1×100 mm) at 45°C . In positive mode, the mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B); in negative mode, 10 mM ammonium formate (A) and acetonitrile (B). The gradient elution was as follows: 0–1 min, 2% B; 1–9 min, 2–98% B; 9–12 min, 98% B; 12–12.1 min, 98–2% B; and 12.1–15 min, 2% B. The flow rate was 0.35 mL/min with an injection volume of 5 μL .

Mass spectra were acquired using full-scan range mode (70–1050 m/z) at 120,000 resolutions. The automatic gain control (AGC) target for MS was 3×10^6 with a 100 ms injection time. The top three precursors were selected for MS/MS fragmentation (AGC 1×10^5 , resolution 30,000, injection time 50 ms). Stepped collision energies were 20, 40, and 60 eV. Electrospray ionization (ESI) parameters included a sheath gas flow rate at 40, aux gas flow rate at 10, spray voltages of 3.80 kV (positive) and 3.20 kV (negative), capillary temperature at 320°C , and aux gas heater at 350°C .

Data were processed using Compound Discoverer 3.3 (Thermo Fisher Scientific) with the BGI metabolome database (bmdb), mzCloud, and ChemSpider. A metabolite matrix with peak areas and identification results were generated. MetaX software was used for preprocessing, including 1) probabilistic quotient normalization (PQN), 2) batch effect correction via QC-based locally weighted scatterplot smoothing, and 3) removal of metabolites with $>30\%$ coefficient variation in QC samples. Principal component analysis (PCA) was used to assess QC clustering and reproducibility. Partial least squares discriminant analysis (PLS-DA) identified group differences utilizing metabolite profiles [41, 42].

Targeted Metabolomics of Neurochemicals

Samples were derivatized by adding methanol (1:3, v/v), vortexed at 8000 rpm for 5 min, and centrifuged at $20,000 \times g$ for 10 min. The supernatant was collected for further analysis. Chromatographic separation was performed on a PFP C18 chromatographic column (1.8 μm , 2.1×50 mm; Waters Corporation, USA). The mobile phase consisted of solvent A (water with 10 mM ammonium formate and 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid), with gradient elution at 0.3 mL/min. The column and sample chamber temperatures were maintained at 35°C and 4°C , respectively. For mass

spectrometry, 2 μL of sample was injected using an electrospray ionization (ESI) source under the following conditions: curtain gas (N_2) at 35 psi, collision gas (N_2) at 9 psi, spray voltage at 5500 V, and atomization temperature at 550°C . Ion source gas (Ion Source Gas1, N_2) and auxiliary gas (Ion Source Gas2, N_2) were both set at 55 psi. Multiple reaction monitoring (MRM) in positive-ion scanning mode was used [43].

For adenosine detection, 10 μL of sample was mixed with methanol (1:3, v/v), vortexed, and centrifuged at 13,000 rpm for 10 min at 4°C . The supernatant was analyzed using a Waters AtlantisTM T3 column (2.1×100 mm, 3 μm). The mobile phase consisted of water (A, 0.1% formic acid) and methanol (B), with gradient elution at 0.3 mL/min. Column temperature was maintained at 30°C , and the injection volume was 1 μL . Mass spectrometry was performed in positive-ion mode using an ESI source with capillary voltage at 4000 V, source temperature at 300°C , and spray gas flow rate at 10 L/min. Adenosine was monitored using the ion pair m/z 268.2 $\rightarrow$ 136.2, with a fragmentation voltage of 110 V and collision energy of 15 V [44]. The regional distribution ratio of neuro-metabolites in ECS was calculated as: Percentage of metabolites in C.n. vs Total = Concentration in C.n./[Concentration in C.n. + Concentration in Tha.]. All metabolite quantifications and statistical analyses were performed double-blindly and each scatter point in figures represents an independent biological replicate.

Tracer-Based MRI Scanning

Gadolinium diethyltri-amine pentaacetic acid (Gd-DTPA)-enhanced MRI, developed in our laboratory, was used to assess contrast agent distribution in the brain's ECS and ISF. Gd-DTPA (Magnevist; Bayer Schering Pharma AG, Berlin, Germany) was diluted to 10 mmol/L in 154 mmol/L sodium chloride and injected into the C.n.. Rats were placed under compound anesthesia. An initial MRI scan confirmed injection site positioning, after which animals were fixed in a stereotaxic frame (Lab Standard Stereotaxic-Single; Stoelting Co., Wood Dale, IL, USA) [45].

The skull was exposed and drilled according to atlas-based coordinates, (AP: 1 mm, L: -3 mm, and V: -6 mm), to allow Gd-DTPA injection into the right C.n. using a microinjection pump (Harvard Apparatus, Holliston, MA, USA). A 10- μL microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) was used to deliver 2 μL at 0.2 $\mu\text{L}/\text{min}$. To prevent backflow, the needle remained in place for 10 min post-injection and was withdrawn over 5 minutes.

MRI was performed using a Magnetom Trio scanner (Siemens Medical Solutions, Erlangen, Germany) with a small animal coil. T1-weighted magnetization-prepared

rapid acquisition gradient-echo (MP-RAGE) sequences were used with the following parameters: echo time, 3.7 ms; repetition time, 1,500 ms; flip angle, 9°; inversion time, 900 ms; field of view, 267 mm; voxel size, 0.5 mm³; matrix, 512 × 512; average, 2; and phase-encoding steps, 96. Each scan lasted 290 s. Images were acquired at pre-injection and at 15, 30, 45, 60, 90, 120, 150, 180, and 240 minutes post-injection [16].

Images were processed using Nano-Detect Analysis System software (MRI Lab, Beijing, China), as described previously. Initial preprocessing included grayscale calibration and histogram equalization. Post-injection images were aligned with baseline scans using rigid transformation, mutual information metrics, high-order interpolation, and adaptive stochastic gradient descent optimization. Subtraction of pre- from post-injection scans highlighted tracer-enhanced regions. “Bright regions” were identified using a seed point and threshold within the region of interest. Post-processed images were generated in horizontal, sagittal, and coronal planes with 1-mm slice thickness [46].

Affine transformation was used to register MRI images to the Waxholm Space Atlas of the Sprague–Dawley Rat Brain using 3D-Slicer (www.slicer.org/) with the Elastix extension [47]. After registration, Mango software (<https://mangoviewer.com/>) was used to identify tracer distribution coordinates by clicking on points of interest in the registered images by another blinded radiologist. These coordinates were then entered into the online Waxholm Atlas preview tool to determine the corresponding brain regions [48].

The diffusion coefficient of each pixel within 1–2 mm of the injection site was calculated using a modified diffusion equation [20]. The volume fraction (α) represented the ECS volume fraction, while tortuosity (λ) reflected diffusion hindrance, calculated as the ratio of the free diffusion coefficient (D) to that in the ECS (D^*). ISF drainage routes were visualized by MRI following tracer injection. Parameters including α , λ , D^* , clearance coefficient (k'), and half-life ($T_{1/2}$) were computed using the D_{ECS} -mapping technique, version 3.1 (MRI Lab, Beijing, China). D_{ECS} mapping also quantified the scope and rate of C.n. diffusion. All image processing were conducted in a double-blind manner and each scatter point in figures represents an independent biological replicate.

Laser Scanning Confocal Microscopy (LSCM) for ISF Tracer Imaging

Lucifer yellow (Sigma-Aldrich, St. Louis, MO, USA) at 10 mM was used as the fluorescent tracer owing to its favorable visualization properties. A 2 μ L volume of fluorescent tracer was injected as described in the “Tracer-Based MRI Scanning” section. The C.n. was

localized using the bregma as a reference, at stereotaxic coordinates AP: 1 mm, L: -3 mm, and V: -6 mm. After a 2-hour diffusion period, brains were extracted and fixed with 4% paraformaldehyde (PFA). Fixed tissue was embedded in a slicing mold, and oblique sagittal sections (1 mm thick) were cut at a 15° angle relative to the axis connecting the thalamus and the C.n. Sections were mounted on confocal dishes and imaged using a Leica TCS SP8 MP FLIM system. Lucifer yellow was excited at 488 nm, with emission captured between 510–550 nm. The LSCM merging function with a water immersion objective (Leica; magnification, 25×; numerical aperture, 1.2) was used to visualize myelin. Myelin was excited at 633 nm and appeared purple in the images. Acquired images were reconstructed to assess tracer distribution and myelin architecture [32].

Electron Microscopy

Rats were deeply anesthetized and perfused with saline followed by 4% PFA (Sigma-Aldrich) in 0.1 M phosphate buffer (PB). Brains were removed, and 1.0 mm³ i.c. samples were dissected and fixed in 2.5% glutaraldehyde (Sigma-Aldrich) for 2 h. Samples were dehydrated in graded acetone and embedded in Epon 812 resin. Ultrathin sections were stained with 4% uranyl acetate and lead citrate, mounted on copper grids, and examined via transmission electron microscopy (TEM; HT7700, Hitachi). Myelin ultrastructure was assessed as described previously [28]. Myelin sheath thickness, area of myelinated axon and number of myelinated axon were measured using ImageJ software via a double-blind researcher.

Luxol Fast Blue Staining

Rats were transcardially perfused with 4% PFA at 4°C for 24 h. Brains were dehydrated overnight in graded sucrose (15%, 20%, and 30%). Oblique sagittal sections (20 μ m) were cut with the cerebellum as the central axis, rotated 15° from the sagittal plane. Sections were incubated at 37°C for 12 h and stored at -20°C. Before staining, the tissue was rinsed in distilled water, hydrated in 95% ethanol, and stained with 0.1% Luxol Fast Blue (LFB) at room temperature. Excess stains were removed with 95% ethanol, and differentiation was performed with 0.05% lithium carbonate in 70% ethanol until the gray matter became distinct. Sections were rinsed with distilled water, mounted, and cover-slipped. Images were acquired by optical microscopy and analyzed with CAM-MS software (CNC Software Inc.). Myelin sheaths appeared blue, while other structures remained mostly unstained. The i.c. area was analyzed by a blinded observer and each scatter

point in figures represents an independent biological replicate [49].

Immunofluorescence Histochemistry

Dissected rat brains were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc.), frozen in liquid nitrogen, and stored at -80°C . Oblique sagittal sections ($20\text{ }\mu\text{m}$, 15° angle relative to the axis connecting the thalamus and caudate nucleus) were cut using a cryostat. Sections were fixed with ice-cold 4% PFA for 15 min, washed thrice with 10 mM PB saline (PBS), and permeabilized with 0.3% Triton-X 100 for 30 min. Non-specific binding was blocked with 5% normal horse serum (Gibco) for 30 min at room temperature. Sections were incubated overnight at 4°C with a rabbit polyclonal antibody against myelin basic protein (MBP; 1:200, Abcam). After three PBS washes, sections were incubated with Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500; Invitrogen) for 2 h at room temperature in the dark. Nuclei were counterstained with Hoechst 33342 (1:1000; Sigma-Aldrich) for 15 min. Sections were mounted with anti-fading mounting medium and cover-slipped. Negative controls were processed using PBS in place of the primary antibody. Fluorescence images of oligodendrocytes were acquired with using a 3DHISTECH (MIDI, Budapest, Hungary) with the Panoramic Scanner software. Red fluorescence indicated MBP, while blue fluorescence indicated nuclei. MBP intensity was quantified with ImageJ software by a blinded observer each scatter point in figures representing an independent biological replicate [49].

Electrophysiological Analysis

Electroencephalogram (EEG), electromyogram (EMG), and local field potential (LFP) signals were acquired with a digital headstage (CerePlex μ) and processed using the CerePlex Direct system at 2 kHz. Signals were amplified and filtered as follows: EEG and LFP (low-pass at 250 Hz); EMG (band-pass at 10–250 Hz). Time-frequency analysis was performed with NeuroExplorer (version 5.0) to generate spectrograms. Video tracking (Tracking Master, version 4.0;) was used as an auxiliary method to assess sleep states [50].

Sleep recordings were scored in 4-s epochs over 6 h during the light phase based on EEG. Scoring was semi-automated using threshold and clustering methods. Non-rapid eye movement (NREM) was defined by low-frequency (0.5–4.0 Hz), high-amplitude (± 150 –250 μV) delta waves with low-amplitude EMG. Rapid eye movement (REM) was identified by a mixed frequency (mainly theta: 6–9 Hz), low-amplitude EEG (± 50 –100 μV), and flat EMG. Wakefulness was indicated by a high-

frequency (8–50 Hz), low-amplitude (± 50 –100 μV) EEG, and high-amplitude EMG. Automated scoring was manually verified by the blinded senior researcher, and epochs containing with motion artifacts were excluded. Sleep stages were further validated using artificial intelligence-based software (Lunion Stage; Lunion Data Co., Ltd., Shanghai, China) [51].

Slow oscillations during NREM were identified by band-pass filtering EEG at 0.5–4.0 Hz. Events were marked based if the duration was 0.4–2.0 s, amplitude exceeded 66.6% of the median, and minimum amplitude fell below the mean -1.5 standard deviation (SD) of the filtered signal. Thalamic LFPs were filtered at 7–15 Hz for spindle detection; and the envelope was computed using the Hilbert transform. A spindle event was identified when the envelope exceeded mean +3.5 SD of the NREM LFP envelope. Onset and end points were marked at the 2 SD threshold. Events lasting <0.4 s or >3 s were excluded. Spindles were marked at envelope peak time. Event density was calculated as the number of events divided by NREM duration. Spindle-slow oscillation (SO) coupling was assessed within ± 2 s of SO events. Time shift was defined as the time difference between the spindle oscillation and SO. The Hilbert transform of the 0.5–4.0 Hz EEG signal was used to determine phase shift, defined as the phase of spindle occurrence [52]. All quantitative analyses of sleep architecture, slow oscillations, spindle events, and thalamocortical coupling were by the blinded senior researcher with expertise in electroencephalography. For all analyses of sleep architecture, slow oscillations, thalamic spindles, and thalamocortical coupling, data from multiple recording channels within the same animal were first averaged to generate a single representative value per animal.

Statistical Analysis

All statistical analyses were performed using SPSS (version 20.0; SPSS, Chicago, IL, USA). Data are presented as mean $\pm$ standard error of the mean (SEM). Comparisons among three groups were analyzed using one-way analysis of variance (ANOVA) with Tukey's post-hoc test, while two-group comparisons used independent or paired t-tests, where the choice was determined based on the characteristics of the two-group comparison. Interaction effects were assessed using two-way ANOVAs with Bonferroni correction. Data normality was assessed using the Shapiro-Wilk test. For non-normally distributed data, the Kruskal-Wallis test was applied. When variance equality was not met, the Games-Howell test was used. Pearson or Spearman correlation analyses were performed based on the data distribution. All correlation analyses in this study were

pre-specified, hypothesis-driven tests focused on targeted metabolite-behavior relationships, rather than large-scale exploratory screening. Each correlation represents a

single independent hypothesis; therefore, no correction for multiple comparisons was applied. Statistical significance was set at $P < 0.05$.

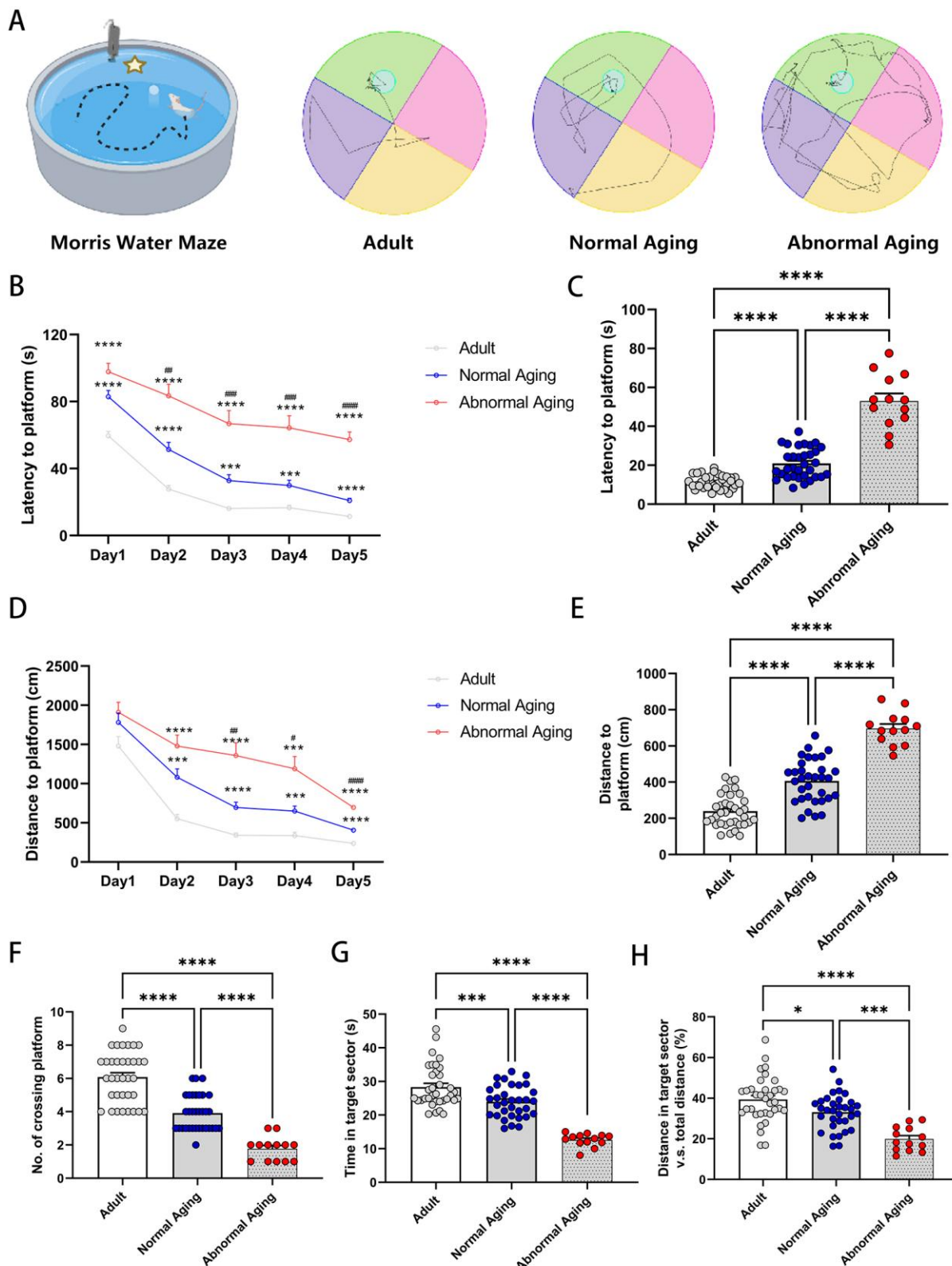


Figure 1. Long-term memory heterogeneity in aged rats. (A) Representative Morris water maze tracks revealed extended and disorganized trajectories in Abnormal Aging rats. (B) Quantification of latency to platform showed a progressive decrease during training in all groups, and (C) latency on the fifth day of the acquisition phase was significantly longer in Normal Aging and further prolonged in Abnormal Aging rats. (D) Distance to platform similarly decreased during training, (E) but was significantly greater in both aging groups on the fifth day. (F-H) During the probe phase, Abnormal Aging rats exhibited markedly reduced platform crossings (F), shorter time spent in the target sector (G), and a smaller proportion of swimming distance in the target sector (H). Adult group, N = 35; Normal Aging group, N = 33; Abnormal Aging group, N = 13. Each scatter point in the graphs represents data from an individual rat (independent biological replicate) and data are presented as Mean $\pm$ SEM. Panels (B) and (D) were analyzed using two-way repeated-measures ANOVA followed by Bonferroni's post hoc test; **** P < 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05 vs. Adult group; #### P < 0.0001, ### P < 0.001, ## P < 0.01, # P < 0.05 vs. Normal Aging group. The other panels were analyzed using one-way ANOVA followed by Tukey's post hoc test. **** P < 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05; ns, no significant difference (P > 0.05).

RESULTS

Aged Rats Exhibit Long-Term Memory Heterogeneity with Overnight Effects

Using the Morris water maze to evaluate spatial learning, aged rats were stratified into Normal Aging and Abnormal Aging groups based on their residence time in the target quadrant during the probe test [53]. Rats with a residence time more than median were classified as Normal Aging, and those less than the first quartile as Abnormal Aging. This classification revealed age-related memory heterogeneity with a prominent overnight effect.

In the water maze, Abnormal Aging rats showed more erratic search trajectories (Fig. 1A, Adult: N=35; Normal Aging: N=33; Abnormal Aging: N=13). While all groups demonstrated reduced escape latency over training days, the decline was slower in the Abnormal Aging group, suggesting impaired spatial memory acquisition (Fig. 1B). On Day 5, escape latency was longer in both aging groups vs. Adult (Abnormal Aging: P < 0.0001; Normal Aging: P < 0.0001; Fig. 1C), and Abnormal Aging was worse than Normal Aging (P < 0.0001; Fig. 1C). The repeated-measures two-way ANOVA revealed a significant interaction between the aging subgroup and long-term memory learning (escape latency: training time $\times$ group interaction, F (8, 356) = 2.562, P = 0.0100, Bonferroni's post hoc test), implicating memory consolidation deficits. Similar group differences were observed in the path distance to the platform (Fig. 1D), with the Abnormal Aging groups covering significantly longer distances than the Adult and Normal Aging groups on Day 5 (P < 0.0001; Fig. 1E). In the probe trial, the Abnormal Aging group rats had fewer platform crossings (P < 0.0001 vs. Adult/Normal Aging; Fig. 1F), shorter residence time (P < 0.0001 vs. Adult/Normal Aging; Fig. 1G), and reduced distance in the target quadrant (P < 0.0001 vs. Adult; P = 0.0002 vs. Normal Aging; Fig. 1H), supporting lower long-term memory. Swimming speeds during the training and probe phase did not differ among the groups (Supplementary Fig. 1A-B).

To further assess memory heterogeneity, we conducted a novel object recognition test (Fig. 2A, Adult:

N=15; Normal Aging: N=11; Abnormal Aging: N=8). After a 24-hour interval, abnormally aging rats explored the novel object less (Fig. 2B). Quantitative analysis confirmed a lower discrimination ratio (P < 0.0001 vs. Adult/Normal Aging; Fig. 2C) and fewer novel object interactions (P < 0.0001 vs. Adult; P = 0.0003 vs. Normal Aging; Fig. 2D) of Abnormal Aging group rats. Locomotor activity did not differ between aging groups (Fig. 2E). In contrast, in a separate object location recognition test with a 2-h interval (Fig. 2F-G), aged rats explored the novel place of object less than adults (P < 0.0001; Fig. 2H-I). However, no significant differences were observed between the aging subgroups in discrimination ratio (P = 0.1626; Fig. 2H) or exploration frequency (P = 0.8880; Fig. 2I). The movement speeds of the two aging subgroups were no difference (Fig. 2J). These findings suggest that memory differences become more pronounced with longer intervals, consistent with an overnight effect. This pattern was also evident during the water maze training phase (Fig. 1B, D), with the Normal Aging rats outperforming the Abnormal Aging rats from day 2.

We also examined other aspects of brain function. In the elevated plus maze, the Abnormal Aging group showed a trend toward reduced open arm exploration (Supplementary Fig. 1C). Although entry frequency did not differ between aging subgroups (Supplementary Fig. 1D), the Abnormal Aging group spent significantly less time in the open arms (P = 0.0301; Supplementary Fig. 1E). Total arm entries, travel distance, and speed were similar across groups (Supplementary Fig. 1F-H), suggesting increased anxiety in the Abnormal Aging group without confounding motor differences.

Region-Specific Neurochemical Disturbances in the ECS of Abnormal Aging Rats

Small-molecule neurometabolites enriched in the ISF are closely linked to cellular metabolism, secretion, and extracellular signaling (Supplementary Fig. 2A). Hence, ISF drainage which supports extracellular homeostasis and metabolic connectivity is essential to cognitive function [32, 54]. To examine region-specific

neurometabolic interactions, we analyzed the ISF from the thalamus (Tha.) and caudate nucleus (C.n.) under ECS compartmentalization [31]. To explore regional differences, PLS-DA was performed to predict relationships between regions and metabolite expression groups (Supplementary Fig. 2B). Principal components reflecting regional variation between Tha. and C.n. were

more distinct in Adult rats (PC1=16.49% and PC2=13.63%) than that in Normal Aging rats (PC1=8.77% and PC2=11.91%) and Abnormal Aging rats (PC1=7.36% and PC2=10.10%). Moreover, volcano plot analysis ($|\log_2FC| \geq 1$, $P < 0.05$, $VIP \geq 1$) highlighted regional metabolic differences (Supplementary Fig. 2C, Supplementary Table1).

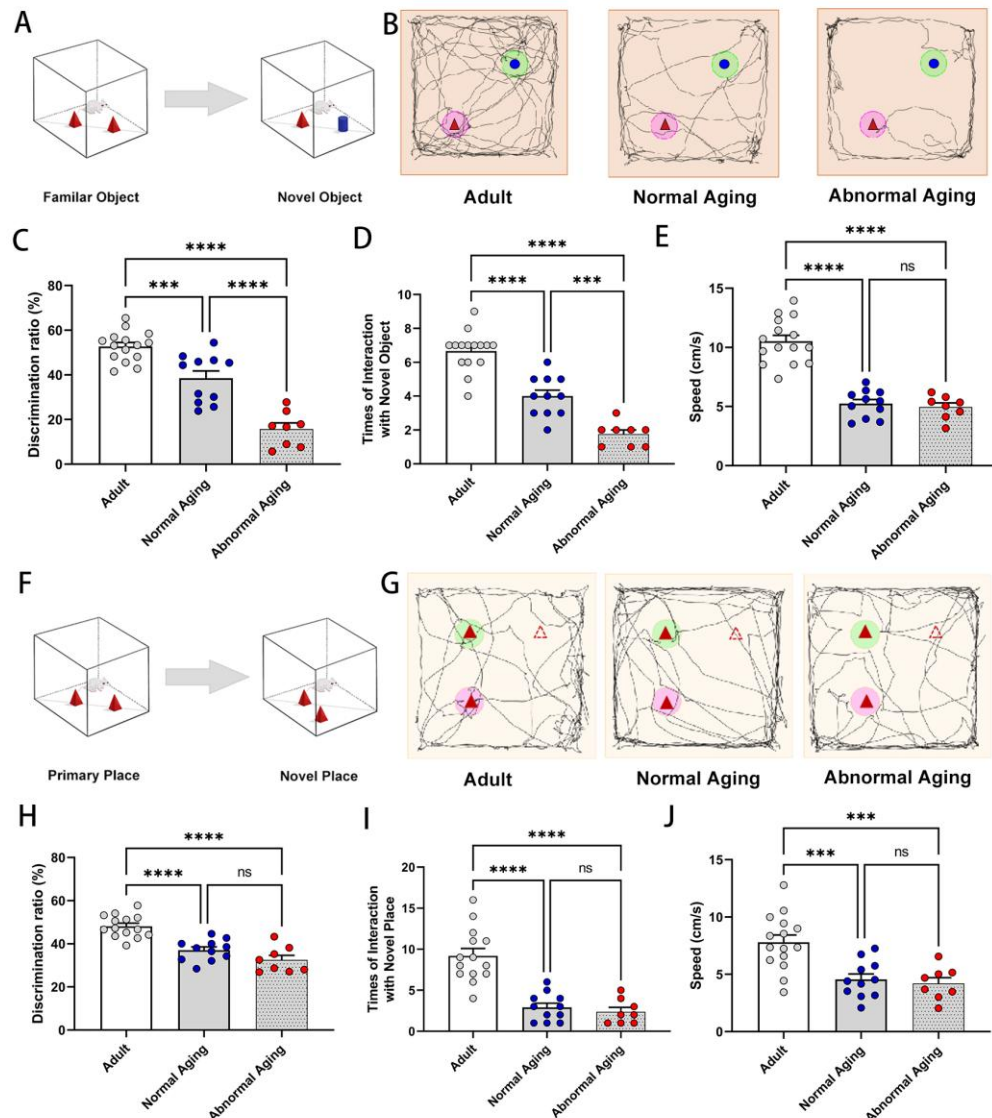


Figure 2. Abnormal Aging rats exhibit no early spatial memory dysfunction but an overnight recognition deficit. (A-B) Schematic of the novel object recognition (NOR) test (A) and representative tracks (B) illustrate differences among groups. (C-D) During testing, Abnormal Aging rats showed significantly lower discrimination ratios (C) and fewer interactions (D) with the novel object. (E) Locomotor speed did not differ between the Normal and Abnormal Aging groups. (F) Schematic of the object place recognition (OPR) test. (G) Representative exploration trajectories during the OPR test across groups. Green circles mark the novel location, and pink circles mark the familiar location. (H-I) During the test phase, quantitative analysis revealed no significant differences in discrimination ratio (H) or number of interactions with the object in the novel location (I) between the Normal Aging and Abnormal Aging groups. (J) Quantification of exploration speed during the OPR test showed no significant differences among the three groups. N = 8–14 rats per group for the object place recognition test and elevated plus maze test. Each scatter point in the graphs represents data from an individual rat (independent biological replicate), and data are presented as Mean $\pm$ SEM. One-way ANOVA with Tukey's post hoc test was applied within three groups comparison. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; ns, no significant difference ($P > 0.05$).

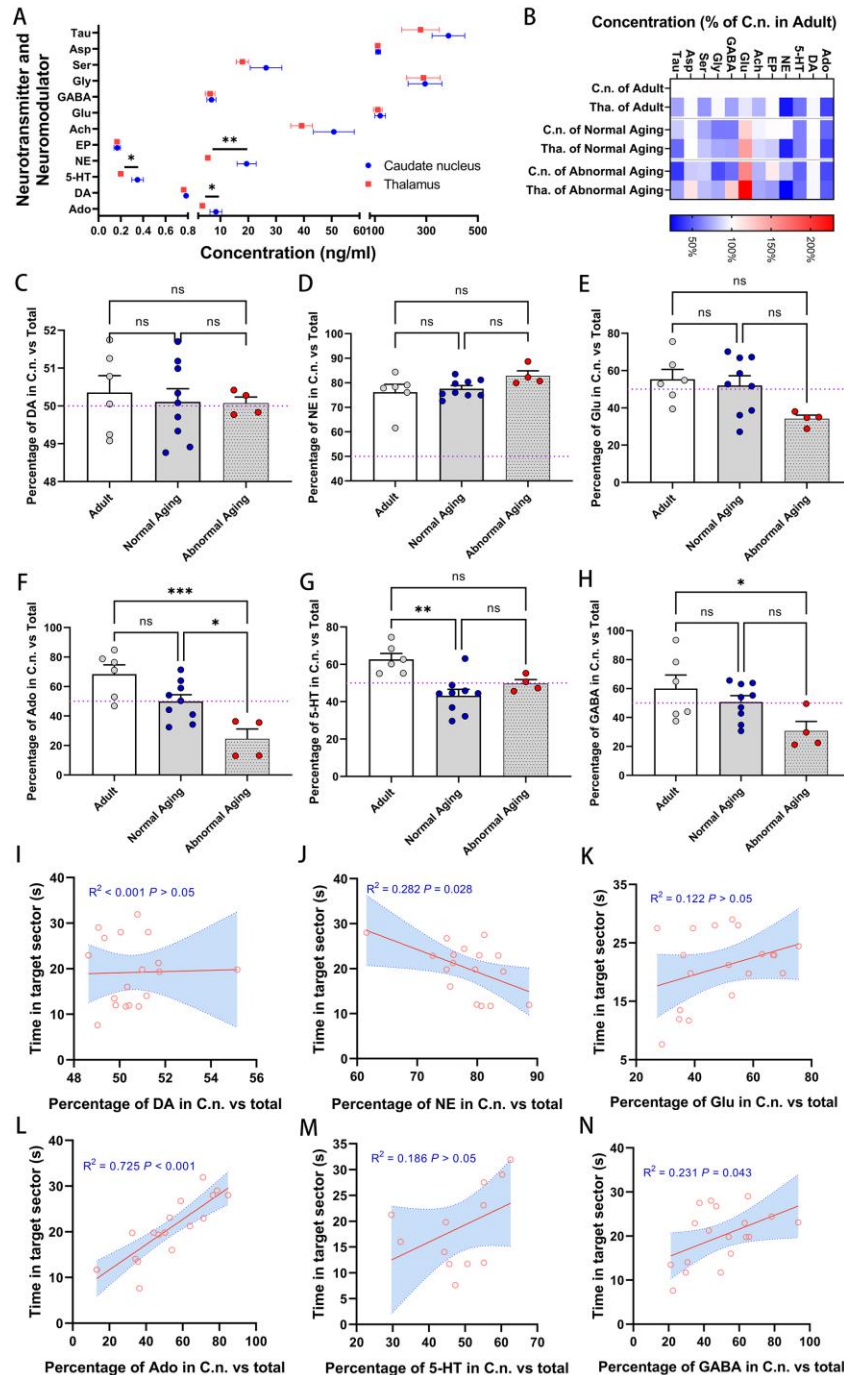


Figure 3. Neurotransmitter imbalance and cognitive performance correlation during aging. (A) Quantification of neurotransmitter and neuromodulator concentrations in ISF revealed regional differences in adenosine (Ado), norepinephrine (NE), and serotonin (5-HT) between the caudate nucleus (C.n.) and thalamus (Tha.) of adult rats via paired *t* test. (B) A heatmap showed relative neurotransmitter levels (% of adult C.n. baseline) across groups. (C–H) The regional distribution ratio of neurotransmitters in ECS was compared among Adult, Normal Aging, and Abnormal Aging rats. (I–N) Correlation analyses between neurotransmitter distribution and spatial memory performance (time in the target sector). *N* = 4–9 for each group; Each data point represents an independent biological replicate (single rat). One-way ANOVA with Tukey's post hoc test was applied within three groups comparison. Data are presented as Mean ± SEM. *****P* < 0.0001, ****P* < 0.001, ***P* < 0.01, **P* < 0.05; ns, no significant difference (*P* > 0.05). Abbreviations: ISF, interstitial fluid; C.n., caudate nucleus; Tha., thalamus; Ado, adenosine; NE, norepinephrine; 5-HT, serotonin; Glu, glutamate. Tau, taurine; Asp, aspartate; Gly, glycine; GABA, γ-aminobutyric acid; ACh, acetylcholine; EP, epinephrine; DA, dopamine; Ser, serine.

To further evaluate region-specific metabolomic disruptions, PLS-DA was performed separately for the C.n. and Tha., revealing group differences (Supplementary Fig. 2D). Both regions showed marked separation between the three rat groups, indicating altered ISF metabolite profiles with aging. Overlap analyses identified differentially expressed metabolites (DEMs) across group comparisons (Supplementary Fig. 2E) and the mean heatmaps (Supplementary Fig. 2F) showed inverse expression patterns between the Tha. and C.n. in Adult and Normal Aging rats, which were reversed in Abnormal Aging rats. Further PLS-DA confirmed a distinct separation between Normal and Abnormal Aging groups in both regions (Supplementary Fig. 2G). Volcano

plots (Supplementary Fig. 2H, Supplementary Table2) and cluster heatmaps (Supplementary Fig. 2I) revealed opposite expression patterns of DEMs between the C.n. and Tha., suggesting that aging disrupts the regional specificity of ECS metabolite homeostasis. These results are intended as exploratory evidence of metabolic differences, not definitive predictive or biomarker findings, due to its small sample size ($N=8-9$ rats per group). Notably, the high R^2 but negative Q^2 values indicate limited predictive validity and potential overfitting, consistent with the small sample size. These results are not interpreted as strong evidence of group differences, but only as preliminary trends to guide future hypothesis-driven studies.

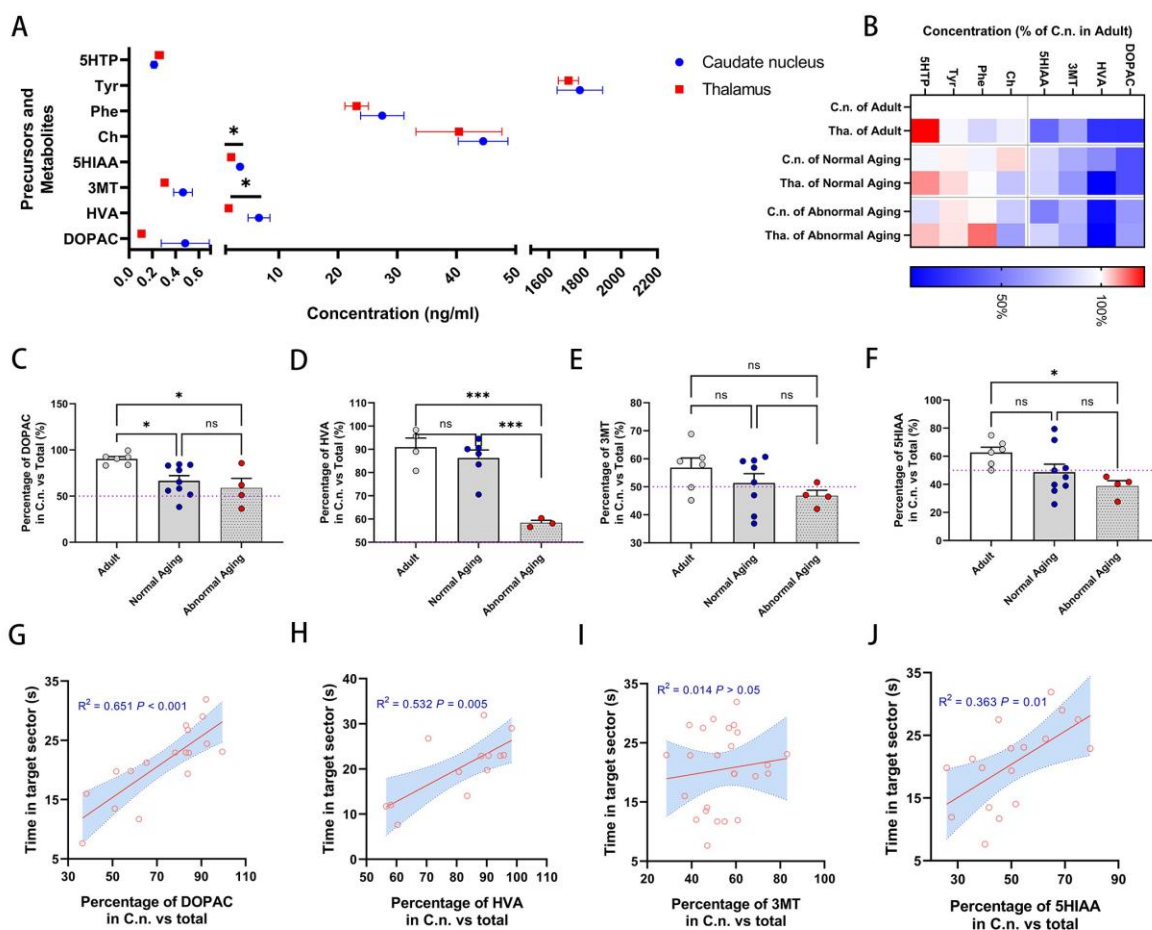


Figure 4. Neurotransmitter precursor and metabolite imbalance in aging. (A) Neurochemical profiling of neurotransmitter precursors (5-HTP, Tyr, Phe, Ch) and metabolites (5-HIAA, 3-MT, HVA, DOPAC) in ISF of the adult C.n. and Tha. revealed regional differences in choline (Ch) and homovanillic acid (HVA), as determined by paired t test. (B) A heatmap displayed relative metabolite concentrations across groups. (C–F) The regional distribution ratio of precursors and metabolites was compared among the three groups. (G–J) Correlation analyses linked these distributions to memory performance in the water maze. $N = 4-9$ for each group; Each data point represents an independent biological replicate (single rat). One-way ANOVA with Tukey's post hoc test was applied within three groups comparison. Data are presented as Mean $\pm$ SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; ns, no significant difference ($P > 0.05$). Abbreviations: 5-HTP, 5-hydroxytryptophan; Tyr, tyrosine; Phe, phenylalanine; Ch, choline; 5-HIAA, 5-hydroxyindoleacetic acid; 3-MT, 3-methoxytyramine; HVA, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid.

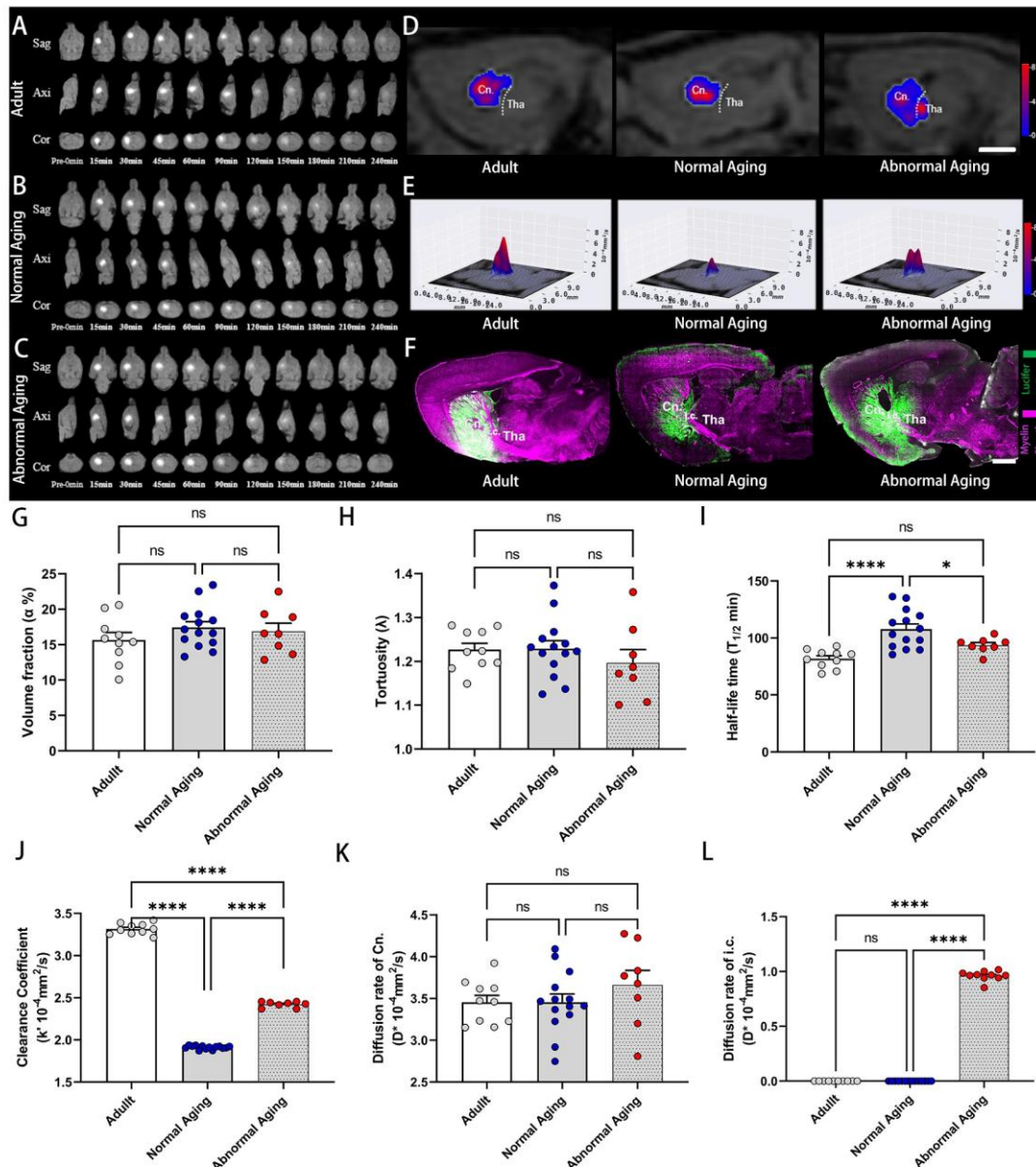


Figure 5. Spontaneous ISF reflux in Abnormal Aging rats. (A–C) Tracer-based MRI revealed complete tracer clearance over time in (A) Adult, (B) Normal Aging, and (C) Abnormal Aging rats. (D) Diffusion mapping demonstrated regional ISF reflux from the C.n. to Tha. in Abnormal Aging rats. (E) Three-dimensional mapping identified diffusion in the internal capsule (i.c.) and Tha. (F) Laser-scanning confocal microscopy (LSCM) confirmed fluorescent tracer reflux from C.n. to Tha. in Abnormal Aging rats. (G–K) Although no significant changes were observed in ECS volume fraction (G), tortuosity (H), or local diffusion rate (K), Abnormal Aging rats showed reduced ISF half-life (I) and clearance coefficient (J). (L) Only Abnormal Aging rats demonstrated detectable diffusion rate in the i.c. N = 8–14 for each group; Each data point represents an independent biological replicate (single rat). One-way ANOVA with Tukey's post hoc test was applied within three groups comparison. Data are presented as Mean ± SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; ns, no significant difference ($P > 0.05$).

ECS Compartmental Neurotransmitter Disturbance Links Age-Related Memory Heterogeneity

Given that several neurotransmitters display region-specific expression in tissue homogenates, we examined whether aging ISF disrupts regional neurotransmitter

balance in the Tha. and C.n. [30, 31]. In adult rats, serotonin (5HT, $P=0.0190$), norepinephrine (NE, $P=0.0027$), and adenosine (Ado, $P=0.0315$) concentrations were significantly higher in the C.n. than in the Tha. (Fig. 3A). This region-specific distribution shifted in both Normal and Abnormal Aging rats (Fig. 3B, Adult: N=6;

Normal Aging: N=9; Abnormal Aging: N=4). Comparison between aging groups revealed that region-specific neurotransmitter distribution significantly interacted with memory heterogeneity (neurotransmitter distribution $\times$ group interaction, $F(11, 298) = 3.141$, $P=0.0005$, mixed-effects two-way ANOVA with Bonferroni's post hoc), suggesting that cognitive aging alters neurotransmitter balance in a region-dependent pattern.

To further assess these effects, we analyzed group differences in the regional distribution ratio of the ECS neurotransmitter. Among excitatory neurotransmitters, dopamine (DA) and glutamate (Glu) did not differ significantly across groups (Fig. 3C, E) and showed no correlation with memory performance (Fig. 3I, K). Similarly, no group differences or correlations were observed for epinephrine (EP, Supplementary Fig. 3A, G), Acetylcholine (ACh, Supplementary Fig. 3B, H) or aspartate (Asp, Supplementary Fig. 3E, K). NE levels showed a trend toward increase in both aging groups (Fig. 3D), with a weak correlation to cognitive performance ($R^2=0.282$, $P=0.028$; Fig. 3J).

In contrast, among inhibitory neurotransmitters, the distribution ratio of Ado was significantly reduced in Abnormal Aging rats ($P=0.0004$ vs. Adult; $P=0.0197$ vs. Normal Aging; Fig. 3F) and positively correlated with memory ($R^2=0.725$, $P<0.001$; Fig. 3L). Gamma-aminobutyric acid (GABA) distribution were also lower in the C.n. of Abnormal Aging rats ($P=0.0356$ vs. Adult; Fig. 3H) and significantly associated with memory function ($R^2=0.231$, $P=0.043$; Fig. 3N). The distribution ratio of glycine (Gly) showed a downward trend (Supplementary Fig. 3C) and was similarly associated with memory; however, the differences were not significant ($R^2=0.268$, $P=0.028$; Supplementary Fig. 3I). Interestingly, the distribution ratio of 5HT was only significantly reduced in Normal Aging rats ($P=0.0013$ vs. Adult; Fig. 3G) but was not linked to cognitive outcomes (Fig. 3M), potentially contributing to group differences in anxiety-like behavior.

Among neuromodulators, the distribution ratio of Ser decreased significantly in Normal Aging rats compared with adult rats ($P=0.0036$ vs. Adult; Supplementary Fig. 3D), but not in Abnormal Aging rats. The extracellular distribution of taurine (Tau) in the C.n. differed significantly in the Abnormal Aging group ($P=0.0483$ vs. Normal Aging; Supplementary Fig. 3F). None of these neuromodulators correlated with memory changes (Supplementary Fig. 3J, L). Overall, among neurotransmitters and neuromodulators, we found that GABA and Ado imbalances appear to be closely linked to accelerated cognitive decline in aging.

We also measured the distribution levels of neurotransmitter precursors and metabolites within the

ECS compartment (Fig. 4A, B). While DA itself did not differ among groups, its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC, $P=0.0143$ vs. Adult) and homovanillic acid (HVA, $P=0.0005$ vs. Adult) were reduced in Abnormal Aging rats, whereas 3-methoxytyramine (3MT) did not differ (Fig. 4C-E). These findings may explain the absence of region-specific DA distributions, as rapid metabolic inactivation may mask regional differences. The 5HT metabolite 5-hydroxyindoleacetic acid (5HIAA) showed region-specificity in Adult rats ($P=0.025$), consistent with 5HT distribution patterns, and its distribution was altered in Abnormal Aging rats ($P=0.0377$ vs. Adult; Fig. 4F). Regarding the correlation with long-term memory, only DOPAC, HVA and 5HIAA showed a positive relationship (Fig. 4G-J). In contrast, precursors such as choline (Ch), 5-hydroxytryptophan (5HTP), phenylalanine (Phe), and tyrosine (Tyr), showed no regional specificity or group differences; moreover, their distribution was not correlated with memory (Supplementary Fig. 4A-H). These findings suggest that ISF neurotransmitter specificity reflects both functional release and extracellular inactivation, with aging-related changes appearing independent of precursor supply in the ECS.

ECS Compartmental Barrier Breakdown Induced ISF Reflux from Caudate Nucleus to Thalamus in Abnormal Aging Rats

Given that the compartmental ECS governs the regional distribution of neurometabolites, ISF drainage plays a key role in maintaining extracellular homeostasis [31]. We used tracer-based MRI and fluorescence imaging to assess ISF dynamics in the deep brain. The maximal diffusion area of ISF at 1.5 h differed significantly in Abnormal Aging rats compared with Adult and Normal Aging rats, whose ISF drainage did not extend into the thalamus (Supplementary Fig. 5A). Moreover, Abnormal Aging rats exhibited spontaneous ISF reflux not only toward the ipsilateral cortex but also in reverse toward the thalamus—crossing a ECS compartmental barrier typically formed by compact myelin fascicles [29]. Using the Waxholm Space atlas for spatial registration, we confirmed altered ISF drainage areas (Supplementary Fig. 5B), showing that reflux could cross the i.c. and reach the thalamic reticular nucleus (Supplementary Fig. 5C).

Of note, tracer clearance appeared faster in Abnormal Aging rats than in Normal Aging rats (Fig. 5A-C). Importantly, this accelerated global clearance does not reflect improved ISF drainage efficiency; instead, it may result from age-related redirection of ISF drainage. Further application of D_{ECS} -mapping technology revealed that the ISF in Adult rats diffused toward the cortex, whereas in Abnormal Aging rats, diffusion slowed and

extended into the i.c. and thalamus (Fig. 5D). In 3D reconstructions, Abnormal Aging rats displayed diffusion peaks in both the caudate nucleus and thalamus (Fig. 5E), indicating possible neurometabolite transport to the

thalamus. Fluorescence tracer imaging of the oblique sagittal sections verified that ISF reflux in Abnormal Aging rats crossed the i.c. to the thalamus (Fig. 5F).

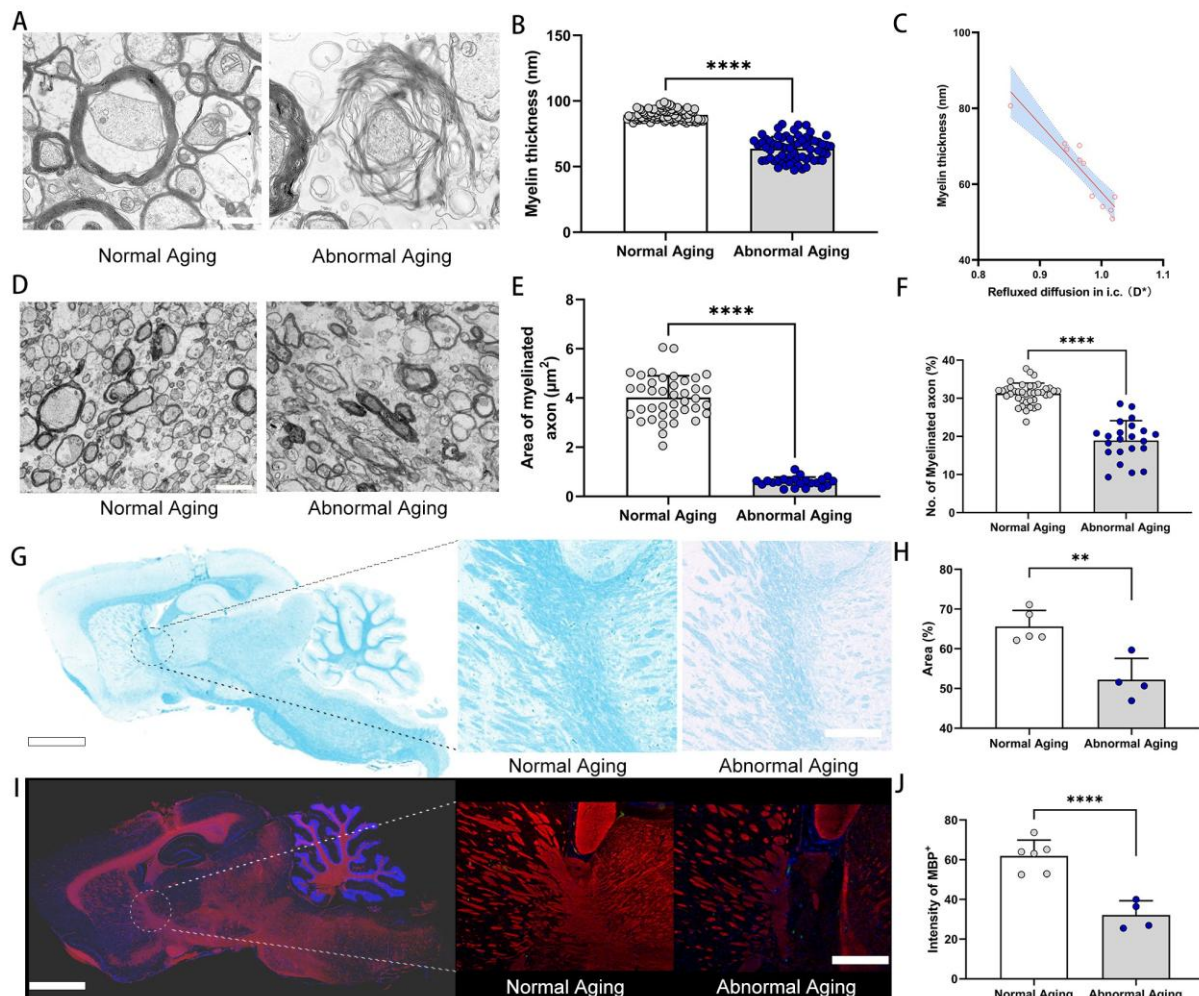


Figure 6. Impaired ECS compartmental barrier is associated with ISF reflux in abnormal aging rats. (A) Representative transmission electron microscopic (TEM) images of myelin sheaths in the internal capsule (i.c.). Scale bar = 500 nm. (B) Quantification of myelin sheath thickness in the internal capsule. (C) Correlation between the extent of ISF reflux and myelin sheath thickness in Abnormal Aging rats. (D) Representative low-magnification TEM images of myelin sheaths in the internal capsule. Scale bar = 5 μ m. (E–F) Quantification of the total area (E) and number (F) of myelinated axons in the internal capsule. (G) Representative Luxol Fast Blue (LFB) histochemical staining images of the internal capsule barrier. Scale bar = 3 mm (left), 1 mm (right). (H) Quantification of the LFB⁺ area of the ECS compartmental barrier. (I) Representative immunofluorescent images of myelin basic protein (MBP) staining in the internal capsule barrier. Scale bar = 3 mm (left), 1 mm (right). (J) Quantification of the relative fluorescence intensity of MBP in the ECS compartmental barrier. N = 11–15 for TEM and except panels (C) where each data point represents a single rat, the other scatter points in TEM quantification represent data from all technical replicates. N = 4–6 rats per group for LFB and MBP staining and each scatter point represents an independent biological replicate. Data are presented as Mean $\pm$ SEM and independent *t* test was conducted in two groups comparison. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; ns, no significant difference ($P > 0.05$).

ECS microstructural analysis revealed no significant differences in volume fraction or tortuosity among the three groups (Fig. 5G–H, Adult: N=10; Normal Aging: N=14; Abnormal Aging: N=8); however, Normal Aging rats showed increased half-life ($P < 0.0001$ vs. Adult;

$P = 0.0423$ vs. Abnormal Aging; Fig. 5I) and decreased clearance coefficients compared with Abnormal Aging rats ($P < 0.0001$ vs. Abnormal Aging; Fig. 5J), suggesting an alternation of extracellular substance transportation in Abnormal Aging rats. However, this faster clearance

differed from the cortical drainage seen in adult rats and likely reflected redirection toward the thalamus (Supplementary Fig. 6). Although diffusion rates

remained unchanged in caudate nucleus (Fig. 5K), a notable ISF diffusion rate saw in the i.c. of Abnormal Aging rats (Fig. 5L).

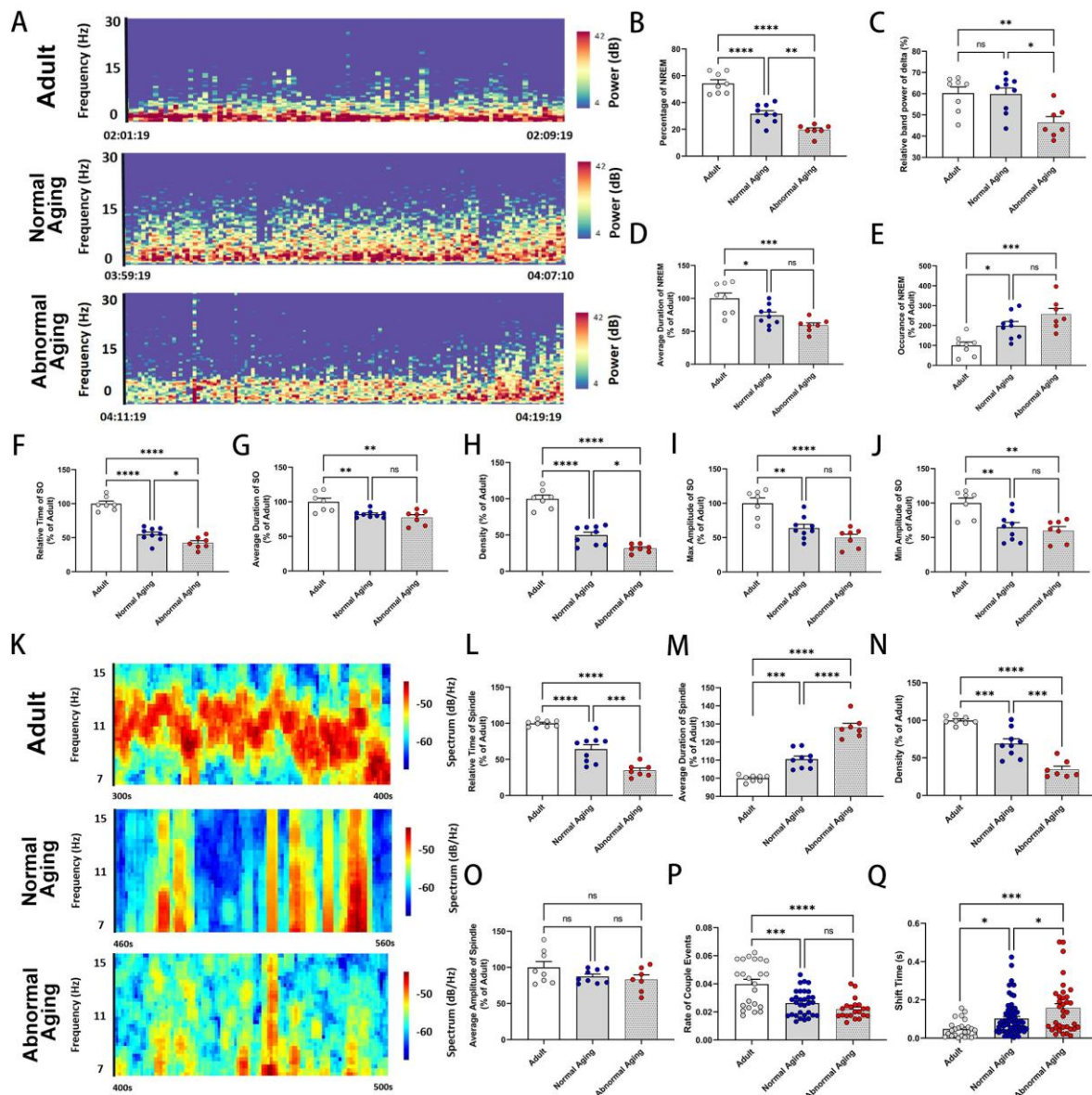


Figure 7. Disrupted NREM sleep and thalamocortical coupling in abnormally aging rats. (A) Electroencephalogram (EEG) recordings revealed alterations in spectral profiles across groups. (B-E) Quantification of NREM sleep (B) time, (C) delta power, (D) mean episode duration, and (E) episode count showed impaired sleep architecture in Abnormal Aging rats. (F-J) Analysis of slow oscillation (SO) showed reduced (F) relative time, (G) duration, (H) density, and (I-J) maximum/minimum amplitude in Abnormal Aging rats. (K) Local field potentials (LFPs) in the thalamus showed altered spindle oscillations (7–15 Hz) with aging. (L-O) Quantification of thalamic spindles revealed a (L) reduced time, (M) increased duration, (N) decreased density, but no difference in (O) amplitude. (P-O) Thalamocortical coupling, as indicated by coupled (P) event frequency and (Q) spindle-SO shift timing, was impaired in Abnormal Aging rats. $N = 7-9$ for each group; Expect panels (P-Q) where scatter points represent data from all recording channels, each data point in the other panels represents an independent biological replicate (single rat). Data from multiple recording channels per animal were averaged and all statistical analyses for sleep and thalamocortical coupling were performed at the individual animal level; One-way ANOVA with Tukey's post hoc test was applied within three groups comparison. Data are presented as Mean $\pm$ SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; ns, no significant difference ($P > 0.05$). Abbreviations: NREM, non-rapid eye movement; EEG, electroencephalogram; SO, slow oscillation; LFP, local field potential.

Given that dense myelin supports ECS compartmental integrity, we examined myelin ultrastructure in the i.c. using transmission electron microscope [29]. Under high magnification (Fig. 6A), myelin thickness was significantly reduced in the Abnormal Aging group ($P<0.0001$ vs. Normal Aging; Fig. 6B), and this reduction correlated with the degree of ISF reflux ($R^2=0.869$, $P<0.0001$; Fig. 6C), suggesting that myelin degradation may permit spontaneous reflux. Similar reductions in myelin integrity were also observed at low magnification (Fig. 6D), with significantly smaller area ($P<0.0001$ vs. Normal Aging; Fig. 6E) and number of intact myelin sheaths in Abnormal Aging rats ($P<0.0001$ vs. Normal Aging; Fig. 6F). Further LFB staining showed decreased i.c. area ($P=0.0036$ vs. Normal Aging; Fig. 6G-H), and the immunofluorescence intensity for MBP markedly reduced in Abnormal Aging rats as well ($P<0.0001$ vs. Normal Aging; Fig. 6I-J).

Impaired Sleep Architecture and Memory Consolidation in Abnormal Aging Rats

Given the disrupted distribution of inhibitory neurotransmitters and ISF reflux to the thalamus observed in Abnormal Aging rats, we examined sleep architecture and thalamic oscillations to determine whether ISF-mediated neurotransmitter imbalance impairs sleep-dependent memory consolidation [52]. For sleep architecture (Fig. 7A, Adult: $N=8$; Normal Aging: $N=9$; Abnormal Aging: $N=7$), Abnormal Aging rats exhibited a significantly lower proportion of NREM sleep ($P<0.0001$ vs. Adult; $P=0.0042$ vs. Normal Aging; Fig. 7B) and reduced delta wave energy ($P=0.0097$ vs. Adult; $P=0.0102$ vs. Normal Aging; Fig. 7C). Moreover, significantly decreased average durations of NREM ($P=0.0005$ vs. Adult; Fig. 7D) and significantly increased frequencies of NREM episodes ($P=0.0123$ vs. Adult; Fig. 7E) were observed in the Abnormal Aging group, indicating greater sleep fragmentation. Furthermore, because Slow Oscillations (SOs) during NREM sleep are linked to learning and memory, we also evaluated SO activity. The Abnormal Aging group showed significantly reduced SO time proportion ($P<0.0001$ vs. Adult; $P=0.0448$ vs. Normal Aging; Fig. 7F), duration ($P=0.0012$ vs. Adult; Fig. 7G), density ($P<0.0001$ vs. Adult; $P=0.0122$ vs. Normal Aging; Fig. 7H), and peak amplitude ($P<0.0001$ vs. Adult for max amplitude; $P=0.0020$ vs. Adult for min amplitude; Fig. 7I-J).

To assess subsequent effects on thalamic spindle activity during NREM, we recorded LFPs from the thalamic reticular nucleus, focusing on the 7–15 Hz frequency band (Fig. 7K, Adult: $N=8$; Normal Aging: $N=9$; Abnormal Aging: $N=7$). Quantitative analysis revealed that both aging groups, particularly the

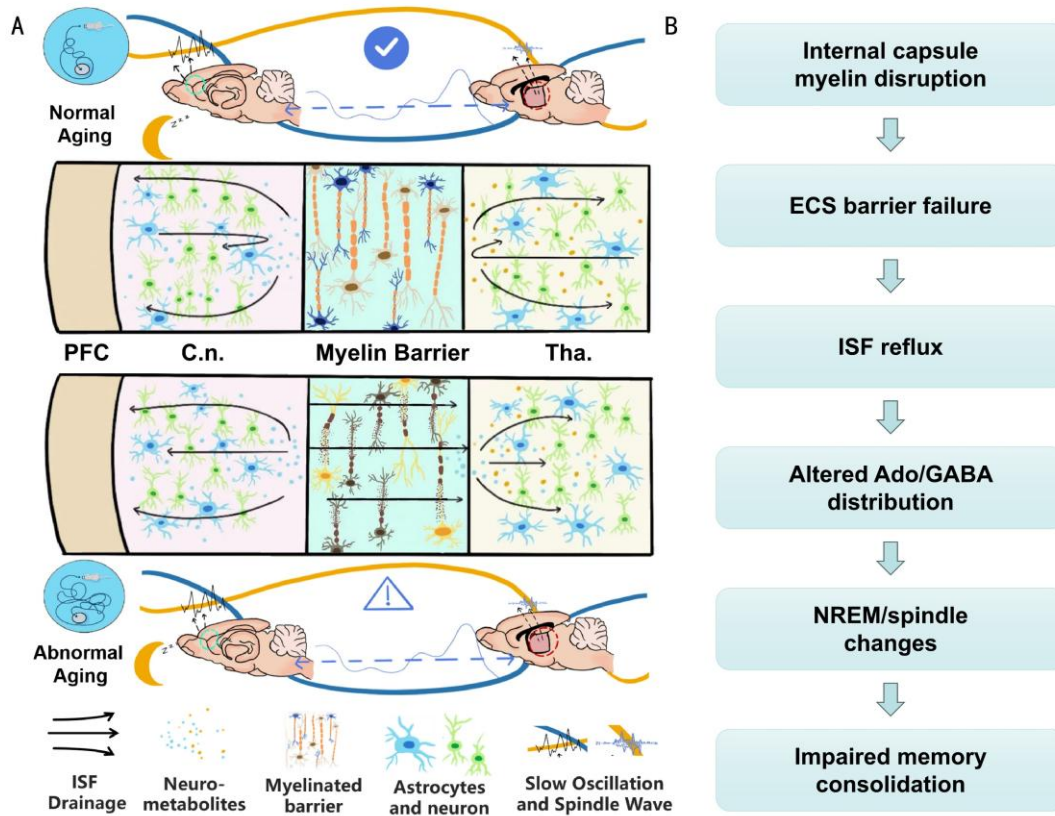
Abnormal Aging group, had reduced spindle time proportion ($P<0.0001$ vs. Adult; $P=0.0004$ vs. Normal Aging; Fig. 7L) and density ($P<0.0001$ vs. Adult; $P=0.0001$ vs. Normal Aging; Fig. 7N). Additionally, the average spindle duration was increased in the Abnormal Aging group ($P<0.0001$ vs. Adult; $P<0.0001$ vs. Normal Aging; Fig. 7M), although no significant group differences were observed in spindle amplitude (Fig. 7O). Next, we examined thalamocortical coupling, which contributes to memory consolidation. Phase-phase coupling analysis showed that although the rate of couple events did not differ between aging subgroups, they were significantly lower in Abnormal Aging rats than in Adult rats ($P<0.0001$ vs. Adult; Fig. 7P). Furthermore, the shift time between spindle and SO events was longer in Abnormal Aging rats ($P=0.0001$ vs. Adult; $P=0.0211$ vs. Normal Aging; Fig. 7Q). These findings suggest that ISF reflux in Abnormal Aging rats disrupts memory consolidation by impairing thalamocortical coordination.

DISCUSSION

Memory performance varies among older adults, with some at higher risk of dementia [55]. However, the basis of this heterogeneity remains unclear. Our findings suggest that ISF reflux and its downstream effects are associated with accelerated memory decline in aging rats (Fig. 8).

In the water maze and novel object recognition tests, a subset of aged rats exhibited long-term impairment with an overnight effect—unlike the shorter 2-hour interval in the object place recognition task [28, 56]. Continued training phase during water maze showed an interaction between age and learning, suggesting impaired memory consolidation in Abnormal Aging rats [57]. Therefore, ISF reflux may contribute to heightened vulnerability to cognitive decline and dementia [58, 59]. Notably, memory consolidation is closely tied to sleep, and sleep disruption is a recognized risk factor for dementia in older adults [60–62]. Furthermore, our study highlights that Abnormal Aging rats with marked memory deficits also showed greater sleep fragmentation and impaired memory consolidation [63]. Importantly, to explore mechanisms underlying memory heterogeneity, we stratified aged rats using an extreme phenotype strategy, focusing on animals with the most pronounced memory deficits and preserved cognitive function to maximize group contrast. While this design improves our power to detect biological differences between the two groups, it inherently enriches for extreme behavioral phenotypes, which may limit the generalizability of our findings to the full spectrum of cognitive aging. Future studies incorporating intermediate phenotypes and larger, more representative cohorts will

help validate these results across a broader range of cognitive trajectories.



ISF drainage is coupled to sleep, supporting the clearance of proteins and cellular debris, and promoting cognitive function [64]. While no structural abnormalities of the ECS were detected, we observed altered ISF drainage pathways in Abnormal Aging rats. Given age-related neuronal loss and gliosis, changes in ISF may result from the disruption of anatomical barriers within ECS compartments [29, 32]. Extracellular homeostasis depends on the local brain microenvironment, and ISF reflux may disturb regional neurometabolite distributions [31]. Therefore, in addition to glymphatic clearance, maintaining extracellular neurobiochemical stability appears essential for cognition, particularly for inter-individual differences in memory [65, 66].

Disrupted compartmentalized ISF drainage may alter regional brain microenvironments and the composition of

regional extracellular metabolites, leading to functional impairment [67, 68]. Neurotransmitter distributions differed across brain regions and are susceptible to age-related disruption [30]. These imbalances may result from altered ISF directionality [30]. Prior studies suggest that ISF determines the local diffusion range of neurotransmitters [69, 70]; our data extend this by showing that aging-related ISF changes also disrupt the distributional balance of neurotransmitters across adjacent brain regions [31, 71]. Specifically, ISF reflux in Abnormal Aging rats was associated with an imbalanced distribution of the inhibitory neurotransmitters such as Ado and GABA.

Notably, Ado promotes NREM sleep in the caudate nucleus [72]. ISF reflux may reduce Ado levels in the caudate nucleus, thereby impairing NREM sleep. As

NREM sleep is essential for memory consolidation, such disruption likely contributes to memory decline [73]. Both Glu and GABA are involved in spindle generation [74, 75]. In our study, Abnormal Aging rats showed a disturbance in spindle waves and thalamocortical coupling during NREM sleep. This was accompanied by altered GABA concentrations in thalamic ISF, differing significantly from those in Normal Aging rats [74]. These findings suggest that regional ISF reflux from the caudate nucleus to the thalamus may disrupt mesoscale neurochemical networks and contribute to memory heterogeneity via impaired sleep-dependent memory consolidation [76].

Previous studies show that myelinated structures maintain ECS compartmentalization [29]. Aging-related demyelination—common in Alzheimer disease and aging—may compromise this barrier [77, 78]. In the present study, we found reduced i.c. integrity and subsequent ISF reflux in rats with abnormal cognitive aging. This is consistent with cohort studies showing that decreased i.c. integrity serves as a biomarker for abnormal cognitive aging [79, 80]. Beyond the internal capsule, other white matter tracts including the corpus callosum, cingulum also revealed age-related demyelination, which may contribute to interstitial fluid dynamics and cognition [81, 82]. Considering the retrogenesis hypothesis and the role of myelination in memory consolidation, we propose that i.c. demyelination disrupts ISF compartmentalization, contributing to memory heterogeneity [50, 83]. Given the sleep fragmentation observed in Abnormal Aging rats with ISF reflux, and the association between sleep disorder and dementia risk in older adults, the integrity of the ECS compartmental barrier may serve as an early biomarker for cognitive decline, particularly in individuals reporting sleep disturbances [84, 85].

Our tracer-based MRI and D_{ECS} -mapping techniques provide a preclinical platform for quantifying ECS barrier integrity and ISF dynamics in aged rats. Notably, multicompartment decoupling imaging (MDI), a non-invasive, tracer-free quantitative MRI technique, is being developed for in vivo, whole-brain voxel-level mapping of the extracellular space (ECS), cellular compartments, and vascular compartments in the human brain, which sensitively reflects ECS structural integrity and fluid transport efficiency [86]. This translational tool represents a preliminary exploratory approach toward non-invasive biomarkers for evaluating ECS barrier function in human aging. From a biomedical engineering translation perspective, optimizing MDI sequences for clinical scanners, validating its performance in multi-center aging cohorts, and establishing normative ECS parameter ranges will enable longitudinal monitoring of age-related ECS barrier deterioration and ISF reflux [87]. These preclinical findings offer preliminary exploratory value

for early detection of aberrant ECS-ISF changes associated with memory heterogeneity and may provide a potential translational direction for preclinical-to-clinical extension in age-related cognitive decline.

Despite these insights, several limitations warrant consideration. First, the molecular mechanisms regulating ECS compartment integrity remain unclear. Multiple key upstream regulators may govern myelin integrity in the ECS compartmental barrier, including oligodendrocyte maturation, myelin structural synthesis and neuro-inflammation [78, 88]. Identifying regulators of i.c. myelin sheath stability may reveal upstream drivers of ISF reflux and provide novel therapeutic targets for dementia. Future research should explore intervention strategies aimed at preserving the ECS compartment integrity [89]. Second, this study exclusively adopted male rats to rule out the interference of age-related hormonal changes in female rats on cognitive performance. Given the evident gender disparities in aging progression, sleep architecture, white matter and myelin integrity, future investigations are warranted to clarify sex-specific alterations in the ECS-ISF system and their contributions to gender-dependent cognitive heterogeneity [90, 91]. Besides, although consistent anesthesia was applied across groups to minimize confounding effects, the potential effects of anesthesia on ISF drainage and neurotransmission should be further investigated [92]. In addition, anxiety-like alterations in the Abnormal Aging subgroup are consistent with emotional disturbances frequently observed in early dementia, and their potential contribution to cognitive dysfunction merits further in-depth exploration [93].

In summary, our study identified disrupted ISF drainage within compartmentalized ECS as a contributor to accelerated memory decline in aging. We demonstrate that impairment of the ECS barrier permits ISF reflux from the C.n. to the thalamus, revealing a nondegenerative mechanism underlying memory heterogeneity. This ISF reflux may alter regional neurotransmitter homeostasis, impair thalamocortical synchrony during sleep-dependent memory consolidation, and correlate with cognitive dysfunction in aging populations.

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Contributions

H.H. conceived and designed the overall study, was responsible for funding acquisition, supervised the whole

project, and guided manuscript drafting and revision. X.M. performed the core experiments; X.M. and R.W. wrote the initial manuscript draft and participated in revision. T.G. developed the methodological framework and completed data analysis. H.T., S.Y., and C.W. processed MRI, morphological and electrophysiological data. M.X. offered suggestions, analysed data, wrote and revised the manuscript. W.F. offered academic guidance and key research suggestions. D.D., S.S., Y.F. and Y.S. participated in result discussion and critically revised the manuscript. All authors have read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

Data and materials availability

The data that support the findings of this study are presented within the article and Supplementary Materials. Raw data generated and analyzed during this study are available and further inquiries can be directed to the corresponding authors.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2026.0422.

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