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ORIGINAL ARTICLE

Young blood-mediated cerebromicrovascular rejuvenation through heterochronic parabiosis: enhancing blood-brain barrier integrity and capillarization in the aged mouse brain

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Abstract Age-related cerebromicrovascular changes, including blood-brain barrier (BBB) disruption and microvascular rarefaction, play a significant role in the development of vascular cognitive impairment (VCI) and neurodegenerative diseases. Utilizing the unique model of heterochronic parabiosis, which involves surgically joining young and old animals, we investigated the influence of systemic factors on these vascular changes. Our study employed heterochronic parabiosis to explore the effects of young and aged systemic environments on cerebromicrovascular aging in mice. We evaluated microvascular density and BBB integrity in parabiotic pairs equipped with chronic cranial windows, using intravital two-photon imaging techniques. Our results indicate that short-term exposure to young systemic factors leads to both functional and structural rejuvenation of cerebral microcirculation. Notably, we observed a marked decrease in capillary density and an increase in BBB permeability to fluorescent tracers in the cortices of aged mice undergoing isochronic parabiosis (20-month-old C57BL/6 mice [A-(A)]; 6 weeks of parabiosis), compared to young isochronic parabionts (6-month-old, [Y-(Y)]). However, aged heterochronic parabionts (A-(Y)) exposed to young blood exhibited a significant increase in cortical capillary density and

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restoration of BBB integrity. In contrast, young mice exposed to old blood from aged parabionts (Y-(A)) rapidly developed cerebromicrovascular aging traits, evidenced by reduced capillary density and increased BBB permeability. These findings underscore the profound impact of systemic factors in regulating cerebromicrovascular aging. The rejuvenation observed in the endothelium, following exposure to young blood, suggests the existence of anti-geronic elements that counteract microvascular aging. Conversely, pro-geronic factors in aged blood appear to accelerate cerebromicrovascular aging. Further research is needed to assess whether the rejuvenating effects of young blood factors could extend to other age-related cerebromicrovascular pathologies, such as microvascular amyloid deposition and increased microvascular fragility.

Keywords Aging · Alzheimer's disease · Blood-brain barrier · Brain aging · Cerebromicrovascular aging · Cognitive health · Microvascular density · Systemic milieu · VCID

Introduction

The increasing prevalence of age-related vascular cognitive impairment (VCI), characterized by cognitive deficits attributable to a range of cerebrovascular pathologies, is becoming an urgent health concern, particularly in developed countries [1–5]. As demographic trends skew towards an older population [6–10], over 20% of the populace is 65 years or older in nations like Japan and many within the European Union [11, 12]. Projections indicate that by 2050, this demographic will constitute approximately 27% and 22% of the populations in the European Union and the United States, respectively [11, 13, 14]. This demographic shift is consequential, as a considerable segment of this older population is likely to suffer from VCI, leading to marked impairments in functional independence and imposing substantial socioeconomic burdens [8, 9, 15–17].

Central to the development of VCI is a complex array of aging-induced microvascular changes. These include the impaired dilatory capacity of cerebral resistance arterioles and consequential dysregulation of cerebral blood flow (CBF), impairment of neurovascular coupling (NVC) responses, microvascular amyloid pathologies, pro-inflammatory changes in endothelial phenotype, microhemorrhages, and other interconnected factors [3, 18–27]. Particularly important are microvascular rarefaction [3, 19, 28–31] and blood-brain barrier (BBB) disruption [3, 19, 21, 22, 32–43].

Microvascular rarefaction, characterized by the diminished density and complexity of cerebral microvasculature, leads to reduced capillarization and brain hypoperfusion [44–46]. There is strong experimental evidence that age-related microvascular rarefaction contributes to cognitive impairment [47–49]. Concurrently, aging is also associated with BBB disruption both in preclinical rodent models and humans [18, 35, 37, 40, 41, 48, 50–52]. The BBB is pivotal in maintaining a tightly controlled humoral microenvironment around the neurons, which is vital for brain health. A growing body of experimental and clinical evidence shows that age-related BBB disruption is strongly associated with cognitive decline [35, 53, 54]. BBB disruption is apparent early in the pathogenesis of both VCI and Alzheimer's disease (AD), where it is suspected to play a causative role [53, 55–57]. Significantly, increased BBB permeability has been observed to correlate with cognitive status [43, 53, 58, 59]. Animal studies have further elucidated that BBB disruption is a key factor in the development of neuroinflammation and cognitive impairment [60–65]. The entry of serum factors into the brain parenchyma through a damaged BBB activates microglia, potentially leading to synaptic pruning, amyloid pathology, demyelination, and white matter damage [66–71]. Importantly, experimental strategies aimed at preserving or restoring BBB integrity show promise in mitigating cognitive deficits in preclinical animal models of aging [18, 51].

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The underlying mechanisms of both microvascular rarefaction and BBB disruption are chiefly dependent on endothelial function. Age-related declines in endothelial angiogenic processes and an increase in endothelial cell apoptosis are believed to be significant contributors to microvascular rarefaction [72]. Cerebromicrovascular endothelial cells, forming the linchpin of the BBB, undergo several changes with aging. These include altered morphology and cytoskeletal organization, impaired energetics, altered cell-to-cell communications and transcellular transport mechanisms, and dysregulation of cell junctions, all of which contribute to the compromised integrity and functionality of the BBB in the aging brain. Understanding these endothelial phenotypic changes is crucial for developing interventions aimed at cerebromicrovascular rejuvenation, potentially preventing or delaying the onset of VCI.

Traditionally, vascular aging research has focused on cell-autonomous mechanisms [73, 74], such as mitochondrial dysfunction [75, 76], increased reactive oxygen species production [26, 74, 77–86] and related impairment in NO bioavailability [10, 17–19], cellular NAD^+ depletion [87–90], epigenetic changes [78, 91], and energetic dysfunctions [75]. However, recent geroscience research is increasingly highlighting the significance of non-cell autonomous mechanisms in aging [74]. Factors circulating in the bloodstream, derived from a variety of organs such as adipose tissue, the brain, the endocrine system, and the immune system, are now recognized as key drivers of cellular aging within the vascular wall [23, 25, 73, 74, 92–104]. These include pro-geronic factors, which increase with age and adversely affect vascular homeostasis, exemplified by inflammatory cytokines [105], and anti-geronic factors that act to counteract cellular aging phenotypes but tend to diminish with advancing age [23, 25, 95–104]. Despite this emerging understanding, the precise roles and interplay of these circulating factors, as opposed to cell-intrinsic mechanisms, in cerebromicrovascular aging remain to be fully elucidated.

Our study utilizes heterochronic parabiosis, a surgical technique that merges the circulatory systems of two animals, to explore the role of circulating factors in cerebromicrovascular aging [106–109]. Preliminary research indicates that exposure to young blood enhances brain endothelial function and neurovascular coupling responses in aged mice [110]. It also improves endothelial vasodilation in aged heterochronic parabionts and partially rejuvenates the endothelial transcriptome in the aorta [94]. Notably, cerebromicrovascular endothelial cells also exhibit significant rejuvenation potential upon exposure to young blood [111, 112].

Expanding upon these findings, our present study is designed to test the hypothesis that age-related shifts in circulating pro- and anti-geronic factors significantly contribute to the phenomena of cerebromicrovascular rarefaction and BBB disruption, operating through non-cell autonomous mechanisms. To rigorously test this hypothesis, we adopted the heterochronic parabiosis model in mice, enhanced with the use of cranial windows in both heterochronic and isochronic pairs. This setup allows for direct, longitudinal observation and analysis of cerebral microcirculation. Employing this innovative approach, coupled with state-of-the-art intravital two-photon microscopy, we conducted comprehensive assessments of BBB permeability and microvascular density and elucidated the impact of an aged or young systemic environment on transposing microvascular aging phenotypes within the young and aged brain, respectively.

Materials and methods

Animal procurement and housing conditions

For our study, we sourced young (4-month-old, n = 20) and aged (18-month-old, n = 28) male C57BL/6 mice (Mus musculus) from the National Institute on Aging's colony, maintained at Charles River Laboratories in Wilmington, MA. These mice were subjected to a consistent 12h:12h light-dark cycle, and provided unlimited access to a standard AIN-93G diet and water. Initially housed under specific pathogen-free conditions in the Rodent Barrier Facility at the University of Oklahoma Health Sciences Center (OUHSC), animals were later moved to the standard rodent colony at OUHSC in preparation for the surgical procedures. This transition ensured that the mice were acclimated to the environment where they would undergo and recover from surgery, maintaining the consistency and reliability of our experimental conditions.

Ethical considerations and animal welfare

Our study adhered strictly to the ethical guidelines outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (8th Edition, revised 2011). The experimental protocols were thoroughly reviewed and approved by the Institutional Animal Use and Care Committee of the University of Oklahoma Health Sciences Center. In line with these guidelines, we implemented stringent measures, both institutional and specific to our study, to minimize any potential distress or discomfort to the animals involved.

Surgical process and subsequent care were closely supervised by a qualified veterinarian from OUHSC's Department of Comparative Medicine. This ensured that all procedures were conducted with the utmost attention to the animals' well-being, maintaining the highest standards of care throughout the study. Our commitment to ethical research practices included continual assessments and adaptations to maximize animal welfare, reflecting our dedication to responsible and humane scientific inquiry.

Cranial window surgery protocol

Our laboratory has standardized the cranial window surgery procedure, as previously described [19]. This surgery was performed on individual mice 3 to 4 weeks before the parabiosis surgery and involves several critical steps.

Anesthesia and preparation

Initially, mice were anesthetized using isoflurane (3–4% for induction, 2–3% for maintenance, at a flow rate of 0.6–0.8 L/min). They were then positioned on a stereotactic stage under a Zeiss Stemi 2000 stereomicroscope. To protect their eyes during the procedure, ophthalmic eye ointment was applied. For sterility, the head hair was removed, and the skin was thoroughly cleaned with povidone-iodine surgical scrub and a 70% ethanol wipe. Once the mouse was fully anesthetized, as indicated by the absence of paw or tail reflexes, an oval section of skin covering the skull (10 × 15 mm) was carefully excised using pointed-end scissors, and the surface of the skull was gently scraped with a disposable scalpel.

Craniotomy

A drop of lidocaine (2 % saline solution, ref: L7757, Millipore Sigma, MA, USA) was applied to the skull and allowed to sit for 2 min before drilling. The craniotomy was performed over the somatosensory cortex, 2–3 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture, with a diameter of approximately 4 mm. First, a shallow circular outline was drilled, which was gradually and evenly thinned with the drill. Drilling was paused every 2–3 min to prevent tissue damage caused by heat. When the bone of the skull within the groove was thin enough, the circular skull piece was detached using fine forceps under a drop of sterile saline. Sterile gelatin sponges soaked in sterile saline were used in case of bleeding (Dengofoam, ref: 600034, Dengen Dental, WY, USA).

Window placement and attachment

A glass round coverslip (⌀5 mm, ref: 64-0700, Warner Instruments, MA, USA) was disinfected with 70% ethanol, wiped clean, and rinsed with sterile saline. The glass window, with a drop of sterile saline underneath, was then carefully placed over the exposed area. Subsequently, a small amount of cyanoacrylate glue was applied around the window to attach it to the bone. After 3 min, a layer of acrylic cement (Dental Cement, ref: 51459, Stoelting, IL, USA) was added around the window and on the exposed skull to secure the edges of the glass window.

Post-surgical care

Once the acrylic layer had hardened, the mouse received buprenorphine (1 mg/kg body weight, sc injection, Buprenorphine ER, 1 mg/mL, ZooPharm, WY, USA) for pain management and enrofloxacin (10 mg/kg body weight, sc injection, 2.27%, Baytril, Elanco, IN, USA) to prevent infection. Subsequently, the mouse was moved to a clean cage and closely observed until fully awake. Hydrogel (70-01-5022, ClearH2O, ME, USA) and food pellets were provided on the cage floor to aid in the recovery process. The mouse's well-being and recovery were closely monitored for up to a week following the surgery,

and any necessary actions were taken in case of rare complications, with consultation from OUHSC veterinarians.

Parabiosis surgery protocol

Parabiosis, a surgical procedure uniting the circulatory systems of two animals, has become a pivotal tool in understanding the role of circulating factors in disease pathogenesis [113–116] and aging processes [108, 109, 117–137]. Heterochronic parabiosis provides a unique window into the complex interplay between cell-autonomous and non-cell-autonomous aging mechanisms [108, 109, 117–135].

The parabiosis experiment is a multi-step procedure. It begins with a 5-day presurgical pair- and treatment-wise habituation, followed by the intricate surgical procedure itself. After surgery, a critical 2-week post-operative care period ensues. Six weeks post-surgery, vital intravital measurements, including assessments of BBB integrity and microvascular density, were conducted (as depicted in Fig. 1B). Throughout the experiment, the animals were housed in the standard rodent facility at OUHSC, receiving consistent care and monitoring from our laboratory staff and the OUHSC Division of Comparative Medicine.

Presurgical procedures

In preparation for the surgery, prospective partner mice were co-housed to facilitate mutual acclimation and allow for close monitoring for incompatibility. Pairs exhibiting aggressive behavior were not considered for parabiosis surgery. Furthermore, 1 and 2 days prior to the surgery, bacon-flavored Metacam tablets (one per animal, 0.125 mg meloxicam, MD275-0125, Bio-Serv, NJ, US) were placed on the cage floor to familiarize the animals with the oral administration of meloxicam, thereby reducing the number of injectable treatments after the parabiosis surgery.

Parabiosis surgery

Parabiosis surgery adhered to established protocols by the Einstein Chronobiosis and Energetics/Metabolism of Aging Core, as previously reported [93, 94, 110, 113, 114, 136, 138]. Surgical unions were established between young animals (isochronic; young Y-(Y); in this designation, the first "Y" indicates the animal whose measurements were taken first in the experiment, and the second "(Y)" signifies its young parabiont partner; $n = 5$ pairs), aged animals (isochronic old; A-(A); $n = 9$ pairs), and young and aged mice (heterochronic Y-(A) and A-(Y); $n = 10$ pairs) at the Oklahoma Center for Geroscience, resulting in four experimental groups: young mice exposed to young blood (Y-(Y)), young mice exposed to aged blood (Y-(A)), aged mice exposed to young blood (A-(Y)), and aged mice exposed to aged blood (A-(A)) (Fig. 1A).

During the parabiosis procedures, strict adherence to aseptic surgical protocols was maintained according to OUHSC guidelines. Mice were sedated using isoflurane anesthesia (3% induction, 1.5–2.5% maintenance, with 0.6–0.8 L/min flow rate) and subsequently transferred to the surgical preparation site. In isochronic young (Y-(Y)) and aged (A-(A)) pairs, anesthesia was maintained using a single vaporizer with a Y bifurcation splitter. In contrast, heterochronic pairs required two separate vaporizers to adjust isoflurane levels independently in the young and aged co-parabionts. The level of animal sedation was routinely assessed by monitoring paw and tail reflexes. The surgical procedure involved several steps: preparation, incision, bone exposure, and connecting the two mice.

Preparation

The corresponding body sides of both animals were carefully shaved using clippers (Mini Arco®, Wahl, IL, USA), and any remaining hair was meticulously removed using a specialized hair removal lotion. The shaved areas were thoroughly cleaned with 70% ethanol, followed by the application of povidone-iodine surgical scrub (Betadine, Avrio Health, CT, USA), with this disinfection process repeated up to three times to ensure optimal sterility. Subsequently, an incision line was marked using a surgical marker, and the animals were then transferred to a surgically sterile environment.

Incision and bone exposure

A longitudinal incision was made on the side of the first animal, followed by a gentle blunt dissection

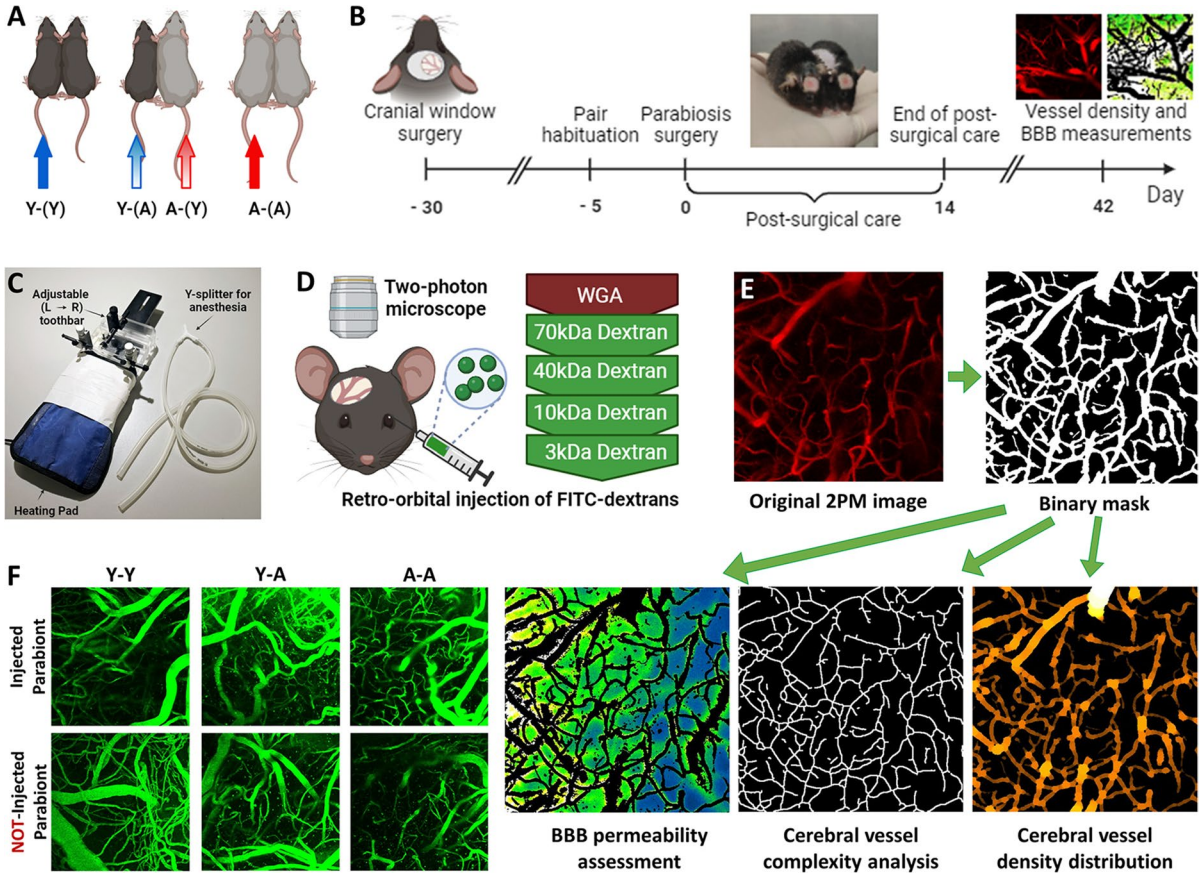


Fig. 1 Overview of parabiosis surgery, experimental design, and two-photon microscopy analysis. **A** Experimental groups: This diagram illustrates the four experimental groups used in our parabiosis study, represented by combinations of young (Y) and aged (A) mice. The groups include isochronic young (Y-(Y)), heterochronic young (Y-(A)), heterochronic aged (A-(Y)), and isochronic aged (A-(A)) parabionts, each denoted by the age of the co-parabiont in parentheses. **B** Experimental timeline: 1 month prior to parabiosis surgery, mice are fitted with chronic glass cranial windows. Five days before surgery, they undergo habituation and aggression monitoring. Parabiosis surgery occurs on day 0, followed by a 14-day period of post-surgical treatments and monitoring. After 6 weeks of parabiosis, intravital two-photon microscopy assesses BBB permeability, microvascular density and complexity, and vessel size distribution. **C** Parabiont-adjusted stage: This setup facilitates simultaneous anesthesia of parabionts and enables consecutive in vivo measurements under a two-photon microscope. **D** Experimental design for BBB permeability and microvascular complexity measurements: Parabionts receive retro-orbital injections of WGA-AF594 (AlexaFluor594-conjugated wheat germ agglutinin, labelling the glycocalyx layer of endothelial cells) followed by sequential retro-orbital injections and imaging using FITC-conjugated dextrans of decreasing molecular weights. **E** Image analysis processing: A maximal projection of Z-stack images of the microvasculature from the somatosensory cortex (50–150 µm depth, 509 µm × 509 µm) is used to generate a binary map of the vasculature. For BBB permeability assessment, baseline-normalized cumulative fluorescence intensities within the extravascular space are calculated. The binary masks are skeletonized to evaluate cerebrovascular complexity and density. The Local Thickness tool in ImageJ is utilized to determine vessel size distribution. **F** Confirmation of blood exchange: The success of blood exchange in each experimental group is verified by detecting FITC-conjugated tracers in the non-injected parabionts

of the superficial fascia and surrounding muscles to expose the femur bone. The periosteum of the femur was carefully scraped using a disposable scalpel. The shoulder blade was localized, and the supraspinatus and infraspinatus muscles were gently repositioned to partially expose the shoulder blade. To prevent tissue drying, a sterile saline solution was generously applied to the exposed bones and surrounding fascia. These steps were subsequently replicated for the co-parabiont.

Connecting the two mice

Two strands of 2-0 silk suture (SP118, Surgical Specialties, MA, USA) were passed underneath the femur of the first parabiont. The longer ends of the sutures were threaded beneath the femur of the co-parabiont, and the ends were securely fastened with square knots. The shoulder blades of the two mice were connected using a combination of one primary horizontal mattress suture and two additional surgical square knots (4-0 silk suture, ref: 683G, Ethicon, OH, USA). The skin flaps on the dorsal and ventral surfaces were meticulously sutured together using a continuous interlocking suture technique (4-0 silk suture, ref: 683G, Ethicon, OH, USA). Then, the loose ends of both continuous sutures were tied together using a single surgical knot between the necks of two parabionts. Three to four additional interrupted sutures (square surgical knots) were added to ensure the stability of the sutured connection. Subsequently, the animals were placed in a clean cage on a heating pad for recovery and closely monitored until fully awake.

Postsurgical care

Following surgery, animals were placed on a partial heating pad overnight, and a multifaceted pharmacological treatment plan was initiated to ensure their well-being (Fig. 1C). To alleviate pain during and post-surgery, animals received two subcutaneous injections of buprenorphine extended release (1 mg/kg body weight, sc injection, Buprenorphine ER, 1 mg/ml, ZooPharm, WY, USA). The first dose was administered briefly before the surgery, followed by a second injection on day three post-surgery. Additionally, animals received meloxicam during and after surgery (5 mg/kg, sc injection, Meloxicam injectable solution, Covetrus, UK; and Metacam, 0.125 mg/day, oral, ref: MD275-0125, Bio-Serv, NJ, USA; on days 0–2 and 3–5, respectively). To prevent infections, parabionts were subcutaneously injected with enrofloxacin (10 mg/kg, Baytril, 2.27%, IN, USA) once daily for 7 days, starting on the day of the surgery. To prevent dehydration caused by reduced water intake post-surgery, animals were subcutaneously injected with 0.5 mL warmed saline during the surgery and on postsurgical days 1, 2, and 3. Additionally, animals received fresh DietGel Recovery (ref: 72-06-5022, ClearH_2O, ME, USA) for the first 5 days. After the surgery, the parabionts' condition, weight, coordination, and sutures were monitored twice a day for the first 2 weeks. Skin sutures were removed 2 weeks after the surgery, and animals were monitored daily until the experimental end point. Any concerning symptoms were promptly assessed and treated in consultation with OUHSC veterinarians. Animals remained joined for 6 weeks before being subjected to terminal experimentation and sacrifice.

Measurement of blood-brain barrier integrity and vessel density using intravital two-photon microscopy

In each group, parabiont pairs were anesthetized with isoflurane (3% induction, 2% maintenance, with 0.6–0.8 L/min flow rate) via the Y bifurcation splitter. The mice were transferred to a parabiont-adjusted stage, which featured a 3D-printed base equipped with a lateral movable adaptor holding a mouse anesthesia mask (ref: 51625 and 514609M, Stoelting, USA) and a heating pad (Fig. 1C). The head of measured parabiont was tightly secured in a leveled position using ear bars. Stage with parabionts was carefully moved under the XLPLN25XWMP, 25× water immersion objective (1.05 numeric aperture; Olympus, Tokyo, Japan) installed on a FluoView 1000 MPE two-photon microscope (Olympus, Tokyo, Japan) coupled with a MaiTai HP DeepSee-OL 690 to 1040 nm (Spectra-Physics, San Jose, CA). An 800-nm laser line was used for excitation. The emitted light was collected by PMT detectors. Three channels with the following filter sets were used: 420–460, 495–540, and 575–630 nm [19].

The surface of the glass cranial window was cleaned with 70% ethanol, and a droplet of water was added between the cranial window and the objective to ensure optical coupling. The eyepiece (green fluorescence filter) was used to adjust the focus to the level of meningeal vessels. Subsequently, Alexa Fluor594-conjugated Wheat Germ Agglutin (WGA-AF594, 1 mg/mL, 4 µL/g body weight, ref: W11262, ThermoFisher Scientific, MA, USA) was retro-orbitally injected to visualize blood vessels (Fig. 1D). Acquisition was changed into a two-photon mode (the same detection settings and laser power (5–10%) were used regardless of used tracer or imaging depth). The optimal location within the cranial window was selected to obtain a well-represented view of various

blood vessels throughout a depth of at least 150 µm. Subsequently, a Z-stack consisting of 31 images with 5-µm intervals was captured, providing a baseline image. Next, fluorescein isothiocyanate (FITC)-conjugated dextrans of decreasing molecular weights were retro-orbitally injected (70-, 40-, 10-, and 3-kDa FITC-dextrans, 4 µL per gram of body weight, 2 mg/mL; ref: D1823, D1845, D1821, D3305, respectively, ThermoFisher Scientific, MA, US; Fig. 1D). After each tracer injection, a 15-min time-stack (one Z-stack per minute) was captured, resulting in a total of sixty-one Z-stack images per measurement, saved as a hyperstack.

After completing the imaging process for the first animal, its head was carefully released from the ear bars. Subsequently, we positioned the head of the co-parabiont under the microscope to assess the presence of green fluorescent tracers in its cerebral blood vessels. This step was critical for confirming successful blood exchange between the parabionts, as illustrated in Fig. 1F. Due to the high efficiency of blood exchange across all parabiosis types, conducting immediate measurements of BBB integrity in the co-parabiont was not feasible.

To establish an appropriate timeline for measuring BBB integrity in the co-parabiont, we investigated the clearance rate of FITC-conjugated dextrans from the brain parenchyma, as detailed in Supplementary Fig. 1A-G. Our observations indicated that the levels of green fluorescence in the co-parabiont's brain parenchyma reverted to baseline 2 days post-injection. Based on this finding, we scheduled the BBB integrity measurements for the co-parabiont 3 days after completing the assessment in the initial parabiont. This approach ensured accurate and reliable evaluation of BBB integrity, accounting for the dynamics of tracer movement and clearance within the shared circulatory system of the parabionts.

Analysis of cerebrovascular measurements

For image analysis, we utilized ImageJ software (version 1.53t, NIH, USA) [139] based on the improved version of our previously established image processing and analysis protocol [19]. The process started with concatenating the baseline image and four time-stacks, each comprising 15 post-tracer images (one z-stack image per minute for each of the four tracers, resulting in 60 images), to create a complete time-stack of 61 z-stack images. These images underwent three-dimensional alignment using the "*Correct 3D drift*" plugin in ImageJ. The uppermost layer of meningeal blood vessels (first 50 µm from the surface) was excluded from the z-stacks, focusing on the depth range of 50–150 µm. These images were then converted into two-dimensional representations using the "*Max Intensity*" function. The brain microvasculature images, prepared as outlined above, were instrumental in evaluating several key parameters: BBB integrity, microvascular density and complexity, the distribution of vessel sizes, and capillary density.

BBB integrity assessment

To quantify BBB permeability, we summarized the changes in green fluorescence intensity over the baseline in the brain parenchyma (extravascular space) for each FITC-labelled tracer. This involved subtracting the vasculature binary mask from the 61-frame time stack. The masks of the vasculature, created using the "*Trainable Weka Segmentation*" tool [140], included the autofluorescent signal, which was subsequently subtracted from the brain parenchyma to refine the analysis. The cumulative changes in green fluorescence for each tracer were averaged for each experimental group and presented as means ± SD.

Microvascular complexity and size distribution analysis

Binary masks were created using the same method as in the BBB integrity assessment. The *Trainable Weka Segmentation* plugin classified vasculature and extravascular spaces. Unlike the binary masks for BBB integrity assessment, the binary masks for microvascular complexity analysis excluded autofluorescent particles ("*Analyze particles*", size ≤ 25 px^2), to prevent misidentification as vasculature. The edges of the vasculature in these masks were smoothed using "*Dilate*" and "*Erode*" functions in ImageJ. In some cases, manual adjustments were made to the masks to accurately represent the vasculature. These masks were then skeletonized for the analysis of total vessel length, number of branches, and number of junctions; normalized to the image area; and presented as means ± SD. The "*LocalThickness*" tool in ImageJ was then applied to these vasculature masks to evaluate vessel size distribution, generating

histograms of vessel diameters. Additionally, the mean area under the curve for small vessels (diameter ≤ 12 μm) was calculated to provide a simplified analysis of capillary density. Vessels up to 12 μm in diameter were considered capillaries, accounting for a slight increase in apparent vessel diameter in binary masks due to segmentation and smoothing processes.

Statistical analysis

Statistical analyses and graphs were prepared using GraphPad Prism software (version 8.0.1, GraphPad Software, MA, USA). One-way ANOVA was used, followed by Sidak's post-hoc test to compare multiple groups. Student's *t*-test was used to compare capillary density between Y-(Y) and Y-(A) and A-(Y) and A-(A) groups. Statistical significance was defined at $p < 0.05$ ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). The results are presented as the mean ± standard deviation. Two-photon assessments of changes in brain microvasculature were assessed in 7–9 biological replicates per experimental group. The two-photon images impacted by the low quality of its cranial window were excluded from the study.

Results

Outcomes, complications, and validation of parabiosis surgery

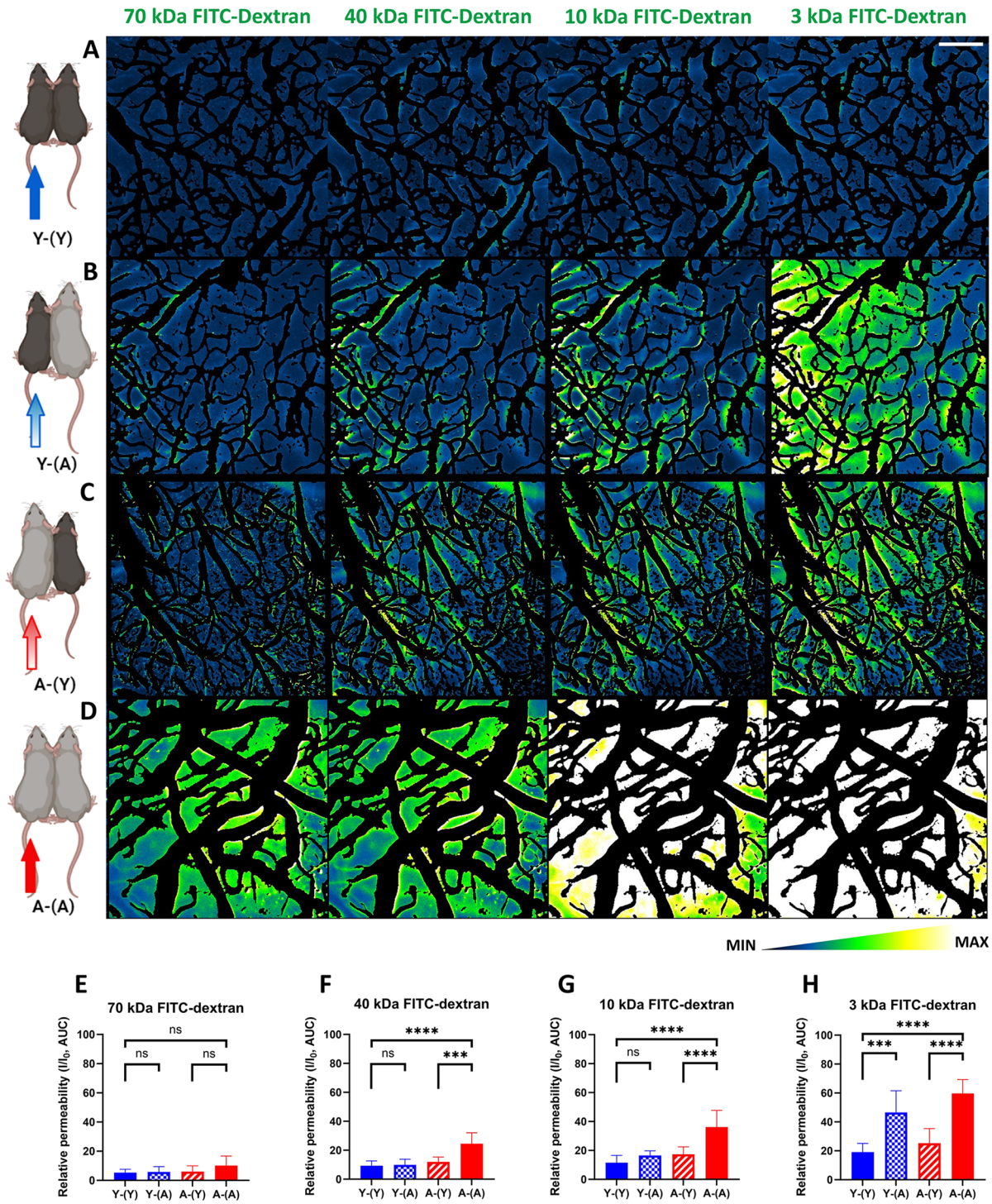
Parabiosis creates a shared circulatory system for investigating the impact of young and aged blood. To control for confounding factors like distress and reduced mobility, we included isochronic young (Y-(Y)) and aged (A-(A)) pairings as control groups (Fig. 1A). The primary objective of this study was to intravitally examine the influence of young and aged blood on BBB integrity and cerebral microvascular density. A month before the parabiosis surgery, individual mice were fitted with glass cranial windows (Fig. 1B), with a success rate above 95%. The overall post-parabiosis survival rate was 92% (Table 1), consistent with our previous studies on parabionts without cranial windows [110]. Post-surgery recovery averaged at 75%, with all isochronic young parabionts recovering, while recovery rates for heterochronic and isochronic aged parabionts were 80% and 56%, respectively (Table 1). The lower recovery rate in aged parabionts likely reflects increased sensitivity to anesthesia and diminished resilience.

Parabiosis surgery can lead to a range of post-operative complications, including dehydration, discoordination, wound opening, and reduced food intake, which can result in significant weight loss [136]. Our observations mirrored previous findings, with all parabiosis types experiencing initial weight loss. Notably, heterochronic and isochronic aged parabionts faced challenges in regaining weight compared to the young pairs. We implemented various post-surgery interventions, such as dietary gels, easily accessible food (placing food pellets on the cage floor), and familiarizing the animals with these dietary adjustments before the parabiosis surgery to promote weight gain and recovery.

The use of cranial windows on both parabionts and a specialized stereotactic frame allowed

Table 1 Surgical survival and post-surgical recovery rates, extent of weight loss, and blood exchange confirmation for each type of parabiosis. Parabiosis is a major surgical procedure, exhibiting an elevated incidence of surgical complications and reduced recovery rates, particularly in parabiont settings involving aged mice. Surgical survival and recovery rates were expressed as proportions of surgeries devoid of intra-surgical and post-surgical mortalities, respectively. One of the most prevalent complications following parabiosis surgery is weight loss. Notably, our observations indicated that both heterochronic and isochronic aged pairs displayed a diminished likelihood of restoring their pre-surgery weights. Data is shown as mean ± SD. Furthermore, using two-photon microscopy, we evaluated the blood exchange between the parabionts. Our findings demonstrate a robust blood exchange in each validated pair

Type of parabiosis	Post-surgery survival (animals/total)	Post-surgery recovery (animals/total)	Weight change at day 3 (%)	Weight change at day 14 (%)	Weight change at day 42 (%)	Confirmation of blood exchange (animals/total)
Y-Y	100% (5/5)	100% (5/5)	−12 ± 2	−14 ± 4	−7 ± 5	Yes (2/2)
Y-A	90% (9/10)	80% (8/10)	−14 ± 3	−17 ± 4	−11 ± 3	Yes (2/2)
A-A	89% (8/9)	56% (5/9)	−11 ± 1	−12 ± 7	−13 ± 8	Yes (2/2)



for the confirmation of effective blood exchange. We verified this exchange in all types of parabiosis settings (Table 1), with injected FITC-conjugated dextran tracers appearing rapidly in the non-injected partners, confirming fast and robust blood exchange in our laboratory's parabiosis procedures.

◂**Fig. 2** Differential effects of young and old blood on BBB integrity in heterochronic parabionts. These representative two-photon microscopy images display the extravasation of FITC-conjugated dextrans within the brain's microvasculature (**A–D**). In young parabionts, BBB permeability is minimal, as indicated by the dark blue color in the extravascular space (**A**). In contrast, isochronic aged parabionts show significant permeability, with green, yellow, and white colors in the brain parenchyma representing tracer leakage for molecules of 3 to 70 kDa (D). Interestingly, tracer extravasation is substantially reduced in heterochronic aged parabionts (**C**), suggesting rejuvenating effects of young blood. However, in heterochronic young parabionts, increased extravasation for the 3 kDa dextran is observed (**B**), indicating augmented BBB permeability due to aged blood exposure. Scale bar: 100 µm. Quantitative analysis of extravasation for FITC-conjugated tracers of 70 kDa, 40 kDa, 10 kDa, and 3 kDa (**E–F**). The isochronic aged parabionts (A-(A)) show a significantly higher BBB permeability compared to their young counterparts (Y-(Y)) for tracers ranging from 3 to 40 kDa molecular weight (**F–H**). Notably, exposure to young blood in aged parabionts leads to a reduction in tracer extravasation for molecules ranging from 3 to 40 kDa (**F–H**), illustrating a mitigation of age-related BBB impairment. Conversely, exposure to aged blood in young parabionts increases BBB permeability specifically for the 3 kDa tracer (**H**), underscoring the detrimental impact of aged blood. Data are presented as mean ± SD (n = 7–9 per group, analyzed using one-way ANOVA with post-hoc Sidak's tests; $^{***}p < 0.001$, $^{****}p < 0.0001$)

Young blood rejuvenates BBB integrity in aged heterochronic parabionts

In our study, we utilized heterochronic parabiosis to explore the rejuvenating effects of systemic young blood factors on BBB integrity. This model enabled us to examine the influence of young blood on aged heterochronic parabionts (A-(Y)) and the impact of aged blood on BBB permeability in young heterochronic parabionts (Y-(A)). To account for changes in BBB integrity that might arise from the parabiosis procedure itself, we compared these findings with isochronic young (Y-(Y)) and aged (A-(A)) parabionts.

We conducted BBB permeability assessments on cranial window-equipped parabionts using a specially designed stage for intravital measurements. This stage facilitated simultaneous anesthesia and imaging of both parabionts, featuring an adjustable tooth-bar holder that has an adjustable left/right positioning (Fig. 1C). BBB integrity was evaluated in vivo using two-photon microscopy, measuring the extravasation of fluorescent tracers (ranging from 70 to 3 kDa FITC-conjugated dextrans, Fig. 1D). The extent of tracer extravasation into the brain parenchyma was quantified by comparing cumulative fluorescence intensities (relative to baseline) after subtracting the vascular mask from the green channel time-stack (1 baseline image and 15 images taken every minute after each tracer injection; Fig. 1D, E).

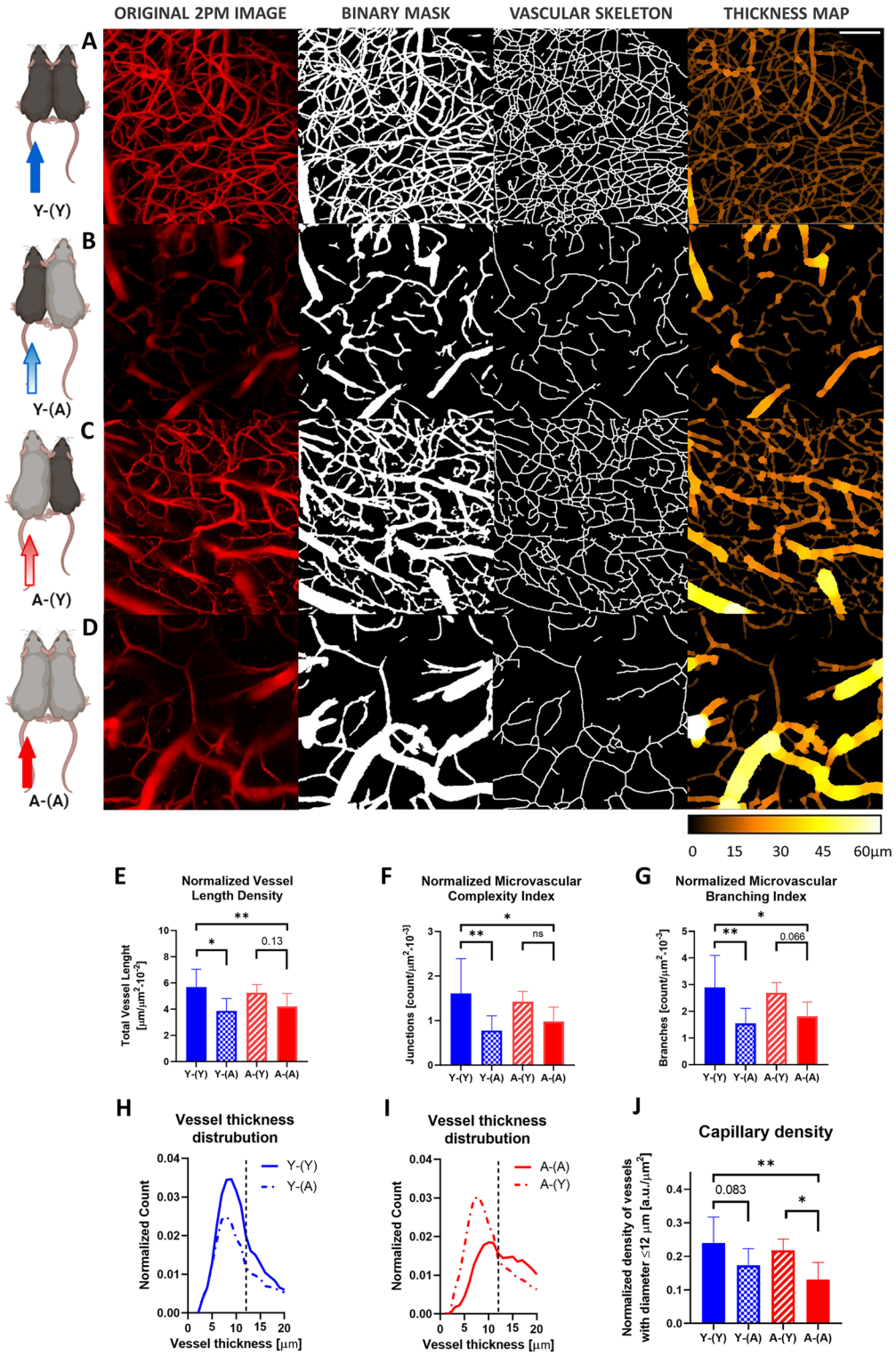
Representative pseudocolor images shown in Fig. 2 illustrate increased extravasation of FITC-dextran tracers in aged isochronic parabionts compared to young isochronic counterparts, signaling an age-related compromise in BBB integrity. In aged isochronic parabionts, we observed heightened permeability for tracers of molecular weights 40, 10, and 3 kDa (Fig. 2F–H, $p < 0.0001$). Remarkably, our data revealed that exposure to young blood significantly enhanced BBB integrity in aged heterochronic parabionts across tracers from 3 to 40 kDa (Fig. 2C, F–H). Conversely, exposure to aged systemic factors resulted in significantly increased BBB permeability for the 3 kDa tracer in young heterochronic parabionts (Fig. 2B, H; $p < 0.001$). These findings underscore the potential of circulating factors to influence age-related BBB dysfunction.

Effects of circulating factors on microvascular density in aging

Cerebral microvascular rarefaction is a hallmark of aging both in rodent models and humans [44, 45, 48]. To evaluate the effects of young and aged blood on cerebral microvascular networks [101, 127], we employed two-photon in vivo microscopy to analyze blood vessel coverage, total vessel length density, vascular complexity, branching, and capillary density in the somatosensory cortex of both isochronic and heterochronic parabionts.

Figure 3 shows representative maximal projection images of the WGA-AF594-labelled cerebral microcirculation (depth, 50–150 µm; Fig. 3A–D, first column) from each experimental group. The original two-photon images were segmented, and binary masks were used to create vascular skeletons and thickness maps (Fig. 3A–D, third and fourth column, respectively). Vascular skeletons were used to assess normalized vessel length density, microvascular complexity, and branching indices.

Our analysis confirmed significant decreases in microvascular length density and network complexity in the aged brain. These age-related changes were


ORIGINAL 2PM IMAGE
BINARY MASK
VASCULAR SKELETON
THICKNESS MAP
A
Y-(Y)
B
Y-(A)
C
A-(Y)
D
A-(A)
0 15 30 45 60µm
E
Normalized Vessel Length Density
Total Vessel Lenght [µm/µm²·10⁻²]
**
*
0.13
Y-(Y) Y-(A) A-(Y) A-(A)
F
Normalized Microvascular Complexity Index
Junctions [count/µm²·10⁻³]
*
**
ns
G
Normalized Microvascular Branching Index
Branches [count/µm²·10⁻³]
*
**
0.066
H
Vessel thickness distrubution
Normalized Count
Y-(Y)
Y-(A)
Vessel thickness [µm]
I
Vessel thickness distrubution
Normalized Count
A-(A)
A-(Y)
Vessel thickness [µm]
J
Capillary density
Normalized density of vessels with diameter ≤12 µm [a.u./µm²]
**
0.083
*

◂**Fig. 3** Impact of young and aged blood on cerebral microvasculature in heterochronic parabionts. **A–D** This series of representative images showcases brain microvasculature in each experimental group. The original two-photon images (first column) depict cortical blood vessels (WGA-AF594, red), from which binary vascular masks (second column) were derived. These masks were then used to generate both vascular skeleton images (third column) and vessel thickness maps (fourth column). **E–F** The skeletonized images facilitated the quantification of vessel length, microvascular complexity, and branching indices, while **H–J** the thickness maps aided in assessing vessel size distribution and capillary density. Scale bar: 100 µm. **E** Vessel length density, normalized to the image area, demonstrates that aging correlates with a decrease in total vessel length ($p < 0.01$). In young heterochronic parabionts, total vessel length density is notably reduced, whereas exposure to young blood does not significantly alter vascular density. **F–G** The analysis of microvascular complexity and branching indices, normalized to the area, reveals that isochronic aged parabionts exhibit significantly impaired microvascular complexity ($p < 0.05$). Young heterochronic parabionts exposed to aged blood show decreased microvascular complexity and branching indices. **H–I** Histograms representing the distribution of vessel thickness for each experimental group highlight variations in vessel size across different conditions. **J** The density of small vessels, defined as those with a diameter of 12 µm or less, was calculated as the mean area under the curve. It was found that isochronic aged parabionts had the lowest capillary density, significantly reduced compared to isochronic young parabionts ($p < 0.01$). Exposure to young blood preserved capillary density in aged heterochronic parabionts, whereas aged blood exposure adversely affected capillary density in young animals ($p < 0.05$ and $p = 0.083$, respectively). Data are mean ± SD (n = 7–9 per group, one-way ANOVA with post-hoc Sidak's tests, $^{*}p < 0.05$, $^{**}p < 0.01$)

partially reversed in aged heterochronic parabionts exposed to young blood and exacerbated in young heterochronic parabionts exposed to aged blood. In isochronic aged parabionts, the cortical vessel length density was significantly lower when compared to isochronic young parabionts (Fig. 3E, $p < 0.01$). Exposure to young blood only partially improved cortical vascular length density when compared to isochronic aged parabionts (Fig. 3E, $p = 0.13$). Conversely, in heterochronic young parabionts, exposure to aged blood significantly decreased cortical vascular length density to the levels observed in isochronic aged parabionts (Fig. 3E, $p < 0.05$). Analyses of the junction and branching numbers in skeletonized images (normalized to the image area, Fig. 3F, G) mirrored these effects. In skeletonized images obtained from isochronic aged parabionts, the normalized numbers of junctions and branches were significantly lower when compared to isochronic young parabionts (Fig. 3F, G; $p < 0.05$ for both). Changes in microvascular complexity and branching indices were also assessed in heterochronic parabionts. In aged parabionts exposed to young blood, the numbers of junctions and branches increased but did not reach statistical significance (Fig. 3F, G; $p = 0.18$ and 0.07, respectively). Conversely, the normalized numbers of junctions and branches were significantly decreased in heterochronic young parabionts (Fig. 3F, G; $p < 0.01$ for both).

Subsequently, we generated vessel size distribution histograms from the vessel thickness maps (Fig. 3H, I). Given that brain aging is associated with a reduction in capillary density, we assessed the differences in normalized densities of vessels with diameter 12 µm or smaller among experimental groups (Fig. 3J). We found that this index of capillary density in isochronic aged parabionts was significantly lower when compared to that in isochronic young parabionts (Fig. 3J, $p < 0.01$). In heterochronic aged parabionts, there was a significant improvement in this index of capillary density (Fig. 3J, $p < 0.05$), whereas in young heterochronic parabionts, exposure to aged blood tended to decrease it (Fig. 3J, $p = 0.08$). These findings collectively underscore that age-related changes in cerebral microvascular network architecture are mediated, at least in part, by cell non-autonomous mechanisms. Furthermore, they suggest that age-related microvascular rarefaction could be alleviated through the replenishment of circulating factors enriched in young blood or by inhibiting progeronic factors found in aged circulation.

Discussion

This study's key discovery is that even short-term exposure to a youthful systemic milieu can mitigate cerebromicrovascular aging phenotypes, resulting in an enhancement in microvascular density and restoration of BBB integrity. In contrast, brief exposure of young mice to an aged systemic environment rapidly induces the acquisition of cerebromicrovascular aging traits, evidenced by reduced microvascular density and partial BBB disruption (summarized in Fig. 4). Our research contributes a crucial piece to the intricate puzzle of cerebromicrovascular and brain aging by demonstrating the rejuvenating influence of young systemic factors on microvascular density and the BBB. The literature is replete with studies underscoring the

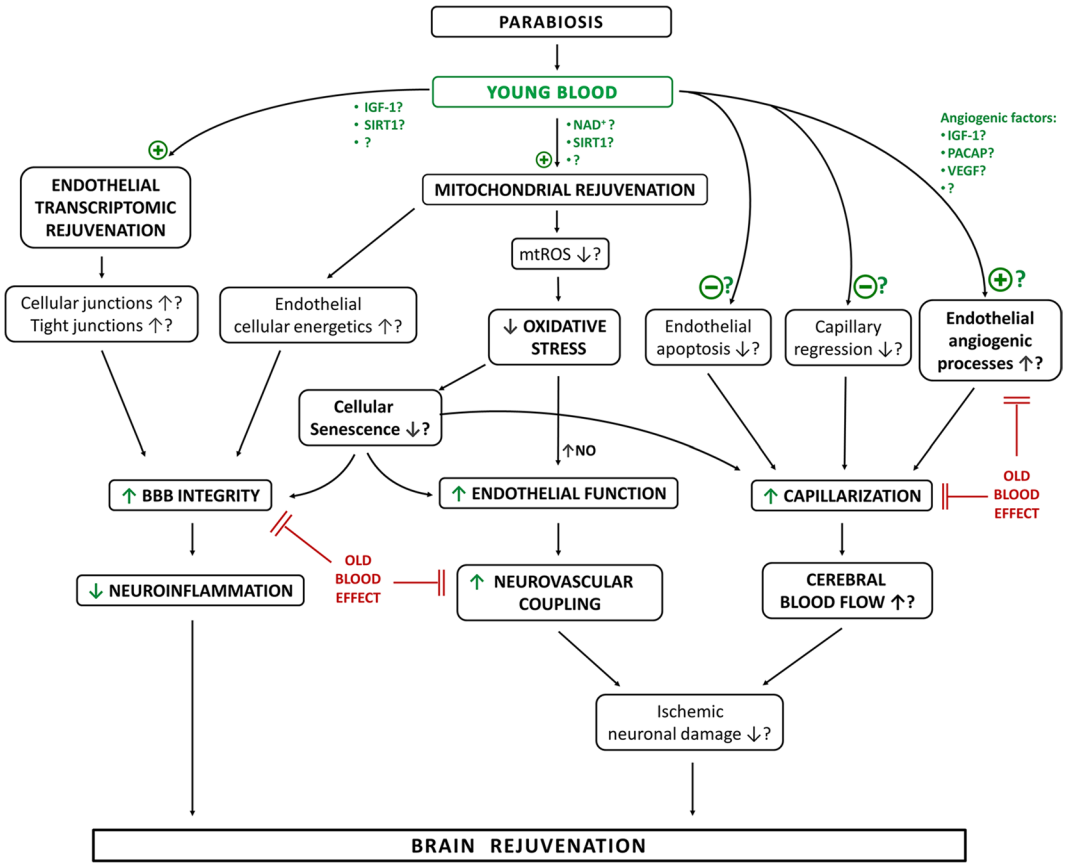


Fig. 4 Schematic overview of the impact of young blood on microvascular mechanisms involved in brain rejuvenation. This schematic flow chart encapsulates the current understanding and theoretical molecular, cellular, and functional impacts of systemic anti-geronic factors abundant in young blood on brain microvasculature. It contrasts these effects with the inhibitory processes associated with pro-geronic factors prevalent in aged blood. Young blood factors are postulated to rejuvenate cerebromicrovascular endothelial cells at the transcriptomic and mitochondrial levels. This rejuvenation could involve pathways such as IGF-1 signaling and NAD^+-dependent SIRT1 activation. The rejuvenation is hypothesized to improve cellular junctions, including tight junctions, thereby restoring blood-brain barrier (BBB) integrity. This restoration leads to reduced microvascular permeability to plasma proteins and other potentially harmful substances, consequently lowering microglia activation and neuroinflammation. Improved mitochondrial homeostasis in endothelial cells is believed to reduce the production of mitochondrial reactive oxygen species (mtROS) and oxidative stress, thereby enhancing NO bioavailability. This improvement supports neurovascular coupling (NVC) responses and increases cerebral blood flow (CBF), contributing to overall brain health. The increased microvascular density observed in our study is likely linked to reduced endothelial apoptosis and capillary regression, coupled with enhanced angiogenic processes driven by pro-angiogenic factors like IGF-1, VEGF, and PACAP, present in young blood. The overall impact of these processes includes prevention of brain ischemia and neuronal damage, alongside the potential preservation of cognitive function. Circulating factors in aged blood are associated with adverse effects on brain microvascular endothelium, characterized by decreased BBB integrity, impaired NVC responses, and reduced microvascular density. These negative outcomes are likely driven by elevated levels of pro-inflammatory mediators and a decline in protective factors. In summary, this schematic illustrates the profound influence of cell non-autonomous mechanisms in cerebromicrovascular aging and highlights potential targets for promoting cerebrovascular health and cognitive function

role of microvascular rarefaction and BBB disruption in age-related cognitive decline [48, 49, 54, 56, 141]. Our study adds to this body of work, providing further evidence that cell non-autonomous mechanisms play a substantial role in orchestrating cerebrovascular and endothelial aging processes.

Previous studies by our laboratories and others found that anti-geronic factors in young blood could

rejuvenate the endothelium and improve neurovascular coupling responses, which are critical for the aged brain's functionality [103, 110–112]. Our earlier work aligns with these findings, showing that young blood can similarly rejuvenate endothelial vasorelaxation in the aorta [93, 94].

The present study extends these insights by linking the functional and transcriptomic rejuvenation of cerebromicrovascular endothelial cells to an increase in capillarization within the aged brain. Semi-quantitative analyses using immunofluorescent labelling for the endothelial marker CD31 in brain sections from heterochronic parabiont pairs support the idea that young blood exposure may increase endothelial cell content in the aged mouse brain [127]. These results likely indicate increased microvascular angiogenesis in response to young blood factors.

Moreover, our results also underscore the detrimental influence of pro-geronic factors present in aged blood, which induce aging-like changes in cerebral microvessels. This leads to impaired neurovascular coupling and a decrease in capillary density [110, 127]. These findings propose that a youthful systemic environment could enhance cerebral perfusion, potentially preventing brain ischemia. However, additional research is necessary to empirically validate these possibilities and fully understand their implications.

Our measurements of BBB function using fluorescent tracers of varying sizes revealed a critical aspect of cerebromicrovascular aging. The mechanisms underlying the permeability of the BBB vary depending on the size of the molecules involved. We observed that BBB permeability to small (3 kDa), medium-sized (10 kDa), and large (40 kDa) FITC-Dextran tracers increases with age, indicating that aging compromises multiple mechanisms of barrier function. For smaller molecules, such as those around 3 kDa, permeability is often governed by paracellular pathways, where molecules pass through the spaces between endothelial cells [38]. This process is typically regulated by tight junction proteins, which likely become compromised with aging, leading to increased permeability. In contrast, the passage of larger molecules, in the range of 10 kDa to 40 kDa, is more reliant on transcellular mechanisms [142]. This involves the transport of molecules across the endothelial cell membranes, potentially through vesicular processes like receptor-mediated transcytosis or caveolae-mediated transport. Age-related changes in these transcellular transport mechanisms likely also contribute to the differential permeability observed in aging brains for medium-sized and larger molecules [38].

Our findings reveal that anti-geronic factors in young blood play a crucial role in maintaining and rejuvenating the BBB in the aged brain. Notably, exposure to young blood restored BBB permeability across all these size ranges, indicating that anti-geronic factors effectively rejuvenate both the paracellular pathways and the transcellular transport mechanisms. Conversely, the introduction of an aged blood milieu led to increased BBB permeability to the 3 kDa tracer. This suggests that pro-geronic factors in aged blood contribute significantly to the disruption of tight junctions [38].

The biological implications of these findings are profound, as they suggest that a myriad of molecules present in the circulation, varying in size and function, could potentially infiltrate the aged brain. While permeability to larger molecules like fibrinogen and IgG (which are significantly larger than 40 kDa) would be expected to increase at older ages [21, 32], several smaller proteins and factors within the blood, such as various cytokines and metabolic byproducts, fall within the 3–40 kDa range. These can include important signaling molecules like interleukins and chemokines, smaller peptide hormones, and potentially neurotoxic metabolic byproducts. The increased penetration of these molecules in aging into the brain could contribute to neuroinflammation, altered neuronal signaling, and other age-related neurological changes. Conversely, the restoration of BBB integrity by young blood suggests a therapeutic potential in controlling the cerebral milieu, thereby preserving neural function and preventing age-related neuropathology. This rejuvenation of BBB function likely contributes to reduced neuroinflammation, a process intimately connected with synaptic pruning and cognitive decline [127, 131, 143]. Crucially, the alleviation of neuroinflammation, coupled with enhanced regulation of cerebral blood flow, may be key mechanisms behind the observed improvements in synaptic plasticity and cognitive function following exposure to a youthful systemic environment [127, 131, 144, 145]. This interplay highlights the therapeutic potential of young blood factors in preserving and restoring neurological health.

Age-related changes in endothelial phenotype and function are governed by a complex network of cellular mechanisms, which appear to be significantly influenced by a variety of factors present in young blood [73, 74]. The processes of endothelial rejuvenation may be orchestrated by either a single set of factors or a synergistic interaction of multiple pathways. Given the complex nature of aging, it is plausible that a diverse array of pathways is involved, each contributing to the enhancement of endothelial health and function. Our research, demonstrating the distinct and contrasting impacts of young and old blood, lends further support to this hypothesis. The rejuvenating influence of young blood and the aging acceleration induced by old blood suggests a dynamic interplay of various pathways. This complex interaction underscores the need for further research to dissect the specific contributions of different circulating factors and pathways in the processes of endothelial aging and rejuvenation.

Studies have associated the phenomenon of capillary rarefaction during aging, at least partially, with a decrease in circulating angiogenic trophic factors like insulin-like growth factor 1 (IGF-1) [28, 29, 101], vascular endothelial growth factor (VEGF) [146], and pituitary adenylate cyclase-activating peptide (PACAP) [147]. These factors are pivotal not only in promoting endothelial angiogenesis and capillary formation but also in maintaining overall vascular health [72]. We propose that rejuvenation of endothelial cells following exposure to young blood observed in our study may, in part, be attributed to the replenishment of anti-geronic, pro-angiogenic trophic factors via blood exchange.

This hypothesis is supported by observations that sera from calorically restricted non-human primates and rodents can induce angiogenic processes in cultured endothelial cells [148], indicating the presence of beneficial, pro-angiogenic factors in a youthful systemic environment. Additionally, it is plausible that aged blood contains factors that either reduce the free concentration of these angiogenic mediators, such as IGF-1 binding proteins [149, 150]). Furthermore, it is important to consider that some aspects of the age-related decline in endothelial angiogenic capacity are cell-autonomous [72, 77, 151–153]. This suggests that intrinsic changes within the endothelial cells themselves contribute to the reduced capacity for angiogenesis and capillary rarefaction in aging. Hence, the rejuvenating effects of young blood may be mediated through a combination of replenishing systemic angiogenic factors and counteracting the intrinsic, cell-autonomous aging processes within the endothelial cells.

The mechanisms underlying age-related endothelial vasodilator dysfunction are complex and multifaceted [72–74]. Alongside mitochondrial dysfunction [75, 87], heightened production of reactive oxygen species (ROS) by mitochondria [75] and NADPH oxidases [154], cellular NAD^+ depletion [87, 88, 155, 156], and epigenetic changes such as sirtuin dysregulation [87, 156, 157], circulating factors also play significant roles. Notably, previous studies have pointed out that circulating anti-geronic vasoprotective hormones, like IGF-1, are instrumental in orchestrating vascular aging processes [23, 25, 96, 99, 102–104, 158–160]. Additionally, there is growing evidence suggesting the involvement of increased levels of pro-inflammatory cytokines, such as TNFα [105, 161] and IL-6 [162, 163], in the onset of age-related endothelial dysfunction. These cytokines can exacerbate oxidative stress in the vascular endothelium, diminishing the bioavailability of NO and leading to impaired vasodilation [105, 163]. Given these insights, it becomes evident that the vascular aging process is influenced by a delicate balance between protective and detrimental circulating factors. The role of heterochronic parabiosis in modulating these factors is particularly intriguing. However, pinpointing the exact circulating factors responsible for the observed effects of heterochronic parabiosis remains a challenge. Future studies using transgenic animals and specific inhibitors are required to elucidate the specific contributions of these diverse humoral factors.

The age-related disruption of the BBB may be attributed to several factors, including the lack of trophic factors regulating the expression and assembly of tight junctions, the increased paracrine release of inflammatory mediators by senescent cells, and the increased presence of inflammatory cytokines in the circulation. Potential circulating factors present in young or aged blood that impact the BBB include trophic factors, such as IGF-1 [98, 104]. These factors can enhance the expression and function of tight junction proteins, crucial for maintaining the barrier properties of the BBB.

Serum Response Factor (SRF) plays a significant role in regulating the BBB. SRF is an evolutionarily

conserved transcription factor known for its involvement in cellular processes such as growth, cytoskeletal organization, and energy metabolism. It mediates the effects of various growth factors, including PDGF and VEGF [164–173]. In the context of the BBB and microvascular physiology, SRF is expressed in microvascular endothelial cells and is essential for the maintenance of endothelial cell integrity, cytoskeletal organization, and angiogenesis [164]. It regulates the expression of various genes involved in the formation and maintenance of tight junctions [164]. Intriguingly, in the context of heterochronic parabiosis models, the inhibition of SRF signaling has emerged as a potential mediator of the effects of old blood on the vasculature [93]. This hypothesis is grounded in gene expression profiles obtained from RNA sequencing experiments, which indicate downregulation of SRF-related pathways in the vasculature in response to old blood [93]. This suggests that the diminished activity of SRF in the presence of old blood could contribute to the observed changes in BBB function and microvascular integrity, highlighting the significance of SRF as a regulatory factor in vascular aging and its potential as a therapeutic target for ameliorating age-related vascular and BBB dysfunction. Sex hormones and other endocrine factors in young blood, which tend to decline with age (e.g., estrogen [174, 175]), may also play a role in maintaining BBB integrity. Their reduction in older individuals could contribute to the weakening of the BBB. Old blood often contains higher levels of pro-inflammatory cytokines like TNFα and IL-6, which can disrupt the BBB by weakening tight junctions [34, 176, 177]. Emerging research also suggests that microRNAs and extracellular vesicles [178, 179], which can differ significantly in young versus old blood, might influence BBB integrity as well. These components can regulate gene expression and cellular responses, potentially affecting the functionality of BBB endothelial cells.

Other heterochronic parabiosis experiments revealed that circulating factors may also have effects in other organ systems outside the brain. Factors found in young plasma, such as growth differentiation factor 11 (GDF11) [127, 130, 180] and exerkines [135, 181, 182], have been linked to beneficial effects not only in the brain but also in other tissues, suggesting their potential role in promoting overall organismal rejuvenation. Conversely, aged plasma contains factors like CCL11 [130], B2M [135] TGF-β [183], and glucocorticosteroids [184], which have been associated with pro-aging effects in multiple organs. While these factors have shown promising results in certain organs, it remains to be methodically tested whether they exert similar effects on vascular health and function in the context of heterochronic parabiosis. The interplay of these factors in endothelial aging, in particular, warrants further investigation.

The plasma dilution hypothesis, which suggests that diluting aged plasma can mitigate the effects of aging in various organ systems [185, 186], also presents intriguing implications for vascular health. In the aged brain, for instance, plasma dilution has been shown to enhance neurogenesis and cognitive functions [185, 186], potentially hinting at improved cerebral blood flow or microvascular health. Similarly, rejuvenating effects in liver and muscle tissues may also indirectly suggest improvements in local vascular structures. Therefore, future studies are essential to explicitly explore the impact of plasma dilution on the vascular system. These studies could include assessments of endothelial function, BBB integrity, and capillary density following plasma dilution in aged models. Understanding whether and how plasma dilution can influence vascular aging processes will be crucial for developing comprehensive therapeutic strategies targeting systemic aging and its associated vascular effects.

As we look towards the future of plasma interventions and the identification of crucial aging-related factors, several key areas emerge as vital for exploration. First, the effort to pinpoint the specific components in young and aged plasma that drive aging processes is critical. This effort could be advanced through sophisticated proteomic and genomic techniques, potentially uncovering new factors responsible for observed rejuvenating or aging-promoting effects. Exploring the mechanisms by which these plasma factors exert their influence is also critical. It involves understanding their interaction with mitochondrial pathways and other cellular mechanisms and their specific roles in vascular rejuvenation. Further, the potential of therapeutic plasma exchange or the transfusion of young plasma into aged animals for cerebromicrovascular rejuvenation warrants thorough evaluation. Extending the investigations of heterochronic parabiosis or the transfusion of young plasma into aged animals to models of age-related diseases can provide valuable insights into the therapeutic potential of plasma interventions for conditions like

Alzheimer's, cardiovascular, and cerebrovascular diseases. Another crucial aspect is examining the long-term effects and reversibility of changes induced by plasma interventions to ascertain if they lead to lasting improvements in organ function and overall health. After the discontinuation of heterochronic parabiosis, the adverse alterations observed in young mice due to aged blood exposure are hypothesized to undergo a normalization process. This recovery could unfold over weeks to months, showcasing the young cerebromicrovascular system's inherent resilience and capacity for regeneration. Similarly, the beneficial effects of young blood on aged mice prompt questions about the reversibility of these rejuvenating outcomes. Future investigations will be essential to determine the duration of these effects post-separation and to explore whether a reversal to pre-parabiosis conditions occurs. Such studies would not only illuminate the dynamics of cerebromicrovascular aging and rejuvenation but also guide the development of interventions mimicking the beneficial effects of young systemic environments. Transitioning from animal models to human clinical trials represents a crucial next step in advancing our understanding of these interventions. Future studies in this area need to comprehensively evaluate not only the efficacy and safety of these therapeutic approaches but also to meticulously develop and refine optimal treatment protocols. Additionally, these studies should incorporate a detailed assessment of changes in microvascular physiological outcomes. This will provide a clearer picture of the impact of these interventions on human vascular health, helping to bridge the gap between preclinical findings and clinical applicability.

In evaluating the results of the heterochronic parabiosis experiments, it is crucial to recognize the model's limitations and confounding factors. The primary strength of this model is its capacity to continuously expose both young and aged animals to the circulating factors of their co-parabiont. This continuous exposure makes the model highly effective for detecting subtle pro- and anti-geronic physiological effects. However, parabiosis, involving the systemic integration of blood from young and old mice, creates a biologically complex environment, making it challenging to isolate the effects of specific factors or to establish causal relationships. Furthermore, the model involves more than just exposure to the circulating factors of the co-parabiont; it also includes indirect exposure to their organs, such as the liver and kidneys, as well as their microbiome. This aspect introduces an added layer of complexity, potentially enhancing metabolic clearance and the production of co-factors, which could improve overall health, particularly in aged heterochronic parabionts. Our study exclusively utilized male mice to maintain consistency with previous parabiosis research and control for variables introduced by hormonal fluctuations. Considering the estrous cycle in female mice, which could influence cerebrovascular physiology and, potentially, the BBB through hormonal changes, future studies incorporating female mice would be valuable in assessing whether the rejuvenating effects of young blood and the aging effects of old blood manifest differently or similarly compared to male counterparts, acknowledging the potential for greater or varied impacts due to added hormonal activity. Additionally, the generalizability of results across different mouse strains is limited, as responses to aging and systemic factors can vary. The surgery itself and the ensuing shared circulation can induce stress in the mice, potentially affecting physiological responses. Another factor adding to the complexity is the altered physical activity levels in young and aged heterochronic parabionts, which could influence their cardiovascular and metabolic health outcomes. Nutritional status plays a pivotal role in influencing the processes of aging and cardiovascular health. In the context of parabiosis experiments, the dietary intake and metabolic condition of one parabiont can have indirect consequences on the other. This interplay may lead to skewed results, particularly in studies focusing on circulating factors, as the nutritional and metabolic profiles of the parabionts are intertwined. Additionally, it is noteworthy that parabionts commonly experience considerable weight loss following surgery, a phenomenon reminiscent of the effects observed in caloric restriction studies. Such a change in body weight could influence a range of measured outcomes in these experiments and should be carefully considered when interpreting results. Finally, the use of mouse models poses inherent limitations. While mice provide a valuable model for studying aging and systemic factors, there are significant physiological and genetic differences between mice and humans. This limits the direct translatability of our findings to human physiology and aging processes. Understanding and addressing these limitations is vital for accurately interpreting the results and for the continued advancement of research in this field. Equally important is the intersection of findings from mouse parabiosis studies

with those from studies investigating circulating factors in humans. This cross-species comparison can help validate the relevance of the findings and provide a more comprehensive understanding of the mechanisms at play in the aging process. By integrating insights from both animal models and human studies, a more robust and translational framework can be established, enhancing the potential for clinical applications and interventions based on our understanding of systemic factors in aging.

In conclusion, our findings reinforce the concept that aging is a systemic process, influenced by a multitude of factors circulating in the blood. Although our present and previous [110] studies primarily concentrated on rejuvenating the BBB, capillarization, and neurovascular coupling responses, the potential for young systemic factors to alleviate other age-related cerebromicrovascular pathologies, such as microvascular amyloid pathologies, glymphatic dysfunction, and microvascular fragility, remains an area ripe for exploration. Studying these aspects is likely to yield valuable insights into the extensive impact of young blood-mediated brain rejuvenation.

To further investigate the rejuvenating effects of young blood, a series of comprehensive and targeted future experiments are warranted. Young blood is rich in a diverse array of factors that may impart anti-geronic effects, encompassing hormones, cytokines, proteins, peptides, lipid mediators, micropeptides, metabolites, and circulating exosomes. Advanced proteomic, lipidomic, metabolomic, and genomic techniques should be employed to profile the components of young blood, identifying specific molecules contributing to anti-geronic effects. Concurrently, research should focus on determining the cellular sources of these key circulating factors. Functional assays, both in vitro and in vivo, are crucial to test the effects of these identified factors on endothelial cells, pericytes, astrocytes, and vascular smooth muscle cells, thereby ascertaining their specific rejuvenating impacts on the neurovascular unit. Mechanistic studies investigating the pathways through which these factors exert their effects, including signal transduction, gene expression changes, and cellular responses, will provide deeper insights into their mechanisms of action. Utilizing transgenic animal models to examine the effects of overexpressing or knocking down specific young blood factors will further illuminate their roles in aging and rejuvenation processes. Additionally, observational studies in humans to correlate the levels of these factors with aging biomarkers and vascular and brain health outcomes would help in validating the findings from animal models.

The idea of treating age-related conditions in humans with young plasma, while intriguing, is currently not recommended due to several critical concerns, particularly in the fields of immunology and virology. Firstly, the repeated introduction of plasma from young donors into an older recipient poses significant immunological risks. Secondly, plasma transfusion carries the risk of transmitting infectious agents. Despite screening, there is always a possibility of transferring viruses or other pathogens that might be present in the donor's blood, including emergent pathogens that current screening methods may not detect. This risk is particularly concerning for immunocompromised or elderly individuals, who are more susceptible to infections and may have a less robust immune response to fight off new pathogens. Given these significant immunological and virological risks, along with the current lack of comprehensive understanding of the long-term effects of such treatments, the administration of young plasma to humans as an anti-aging therapy is not advisable. Instead, controlled interventional trials using a combination of selected young blood factors in aged animal models are necessary to assess their efficacy in reversing age-related changes across various organ systems. Prior to considering human trials, extensive safety and efficacy studies in animal models are imperative to understand potential side effects and optimal dosages of these factors.

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Author contribution The study's conception, design, and data interpretation involved contributions from all authors. Parabiosis surgeries were executed by RG and BC, while the post-surgical monitoring of animals was carried out by RG, BC, BP, JF, and SS. The assessment of blood-brain barrier permeability and microvascular density and subsequent data analysis were undertaken by RG, ANT, SN, and RP The initial draft of the manuscript was jointly composed by RG, ANT, ST, and ZU. Subsequent revisions to the manuscript were conducted by all authors, who also collectively reviewed and provided their approval for the final version of the manuscript.

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Declarations

Competing interests Dr. Anna Csiszar serves as Associate Editor for The Journal of Gerontology, Series A: Biological Sciences and Medical Sciences and GeroScience. Dr. Zoltan Ungvari serves as Editor-in-Chief for GeroScience. Dr. Stefano Tarantini, Dr. Adam Nyul-Toth, Dr. Shannon Conley, Dr. Peter Mukli, Dr. Derek M. Huffman, and Dr. Andriy Yabluchanskiy serve as Associate Editors for GeroScience.

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