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Rejuvenation of cerebromicrovascular function in aged mice through heterochronic parabiosis: insights into neurovascular coupling and the impact of young blood factors

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Abstract Age-related impairment of neurovascular coupling (NVC; "functional hyperemia") is a critical factor in the development of vascular cognitive impairment (VCI). Recent geroscience research indicates that cell-autonomous mechanisms alone cannot explain all aspects of neurovascular aging. Circulating factors derived from other organs, including pro-geronic factors (increased with age and detrimental to vascular homeostasis) and anti-geronic factors (preventing cellular aging phenotypes and declining with age), are thought to orchestrate cellular aging processes. This study aimed to investigate the influence of age-related changes in circulating factors on neurovascular aging. Heterochronic parabiosis was utilized to assess how exposure to young or old systemic environments could modulate neurovascular aging. Results demonstrated a significant decline in NVC responses in aged mice subjected to isochronic parabiosis (20-month-old C57BL/6 mice [A-(A)]; 6 weeks of parabiosis) when compared to young isochronic parabionts (6-month-old, [Y-(Y)]). However, exposure to young blood from parabionts

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significantly improved NVC in aged heterochronic parabionts [A-(Y)]. Conversely, young mice exposed to old blood from aged parabionts exhibited impaired NVC responses [Y-(A)]. In conclusion, even a brief exposure to a youthful humoral environment can mitigate neurovascular aging phenotypes, rejuvenating NVC responses. Conversely, short-term exposure to an aged humoral milieu in young mice accelerates the acquisition of neurovascular aging traits. These findings highlight the plasticity of neurovascular aging and suggest the presence of circulating antigeronic factors capable of rejuvenating the aging cerebral microcirculation. Further research is needed to explore whether young blood factors can extend their rejuvenating effects to address other age-related cerebromicrovascular pathologies, such as blood–brain barrier integrity.

Introduction

Age-related vascular cognitive impairment (VCI), characterized by cognitive deficits resulting from a spectrum of cerebrovascular pathologies, is a growing concern in many developed countries [1–3]. This concern is heightened by the increasing proportion of elderly individuals within their populations [4–8]. The demographic shift toward an older population is particularly evident in countries like Japan and several European Union nations, including Germany, Hungary, and Italy, where more than 20% of the populace is aged 65 or older [9, 10]. Projections suggest that by 2050, 27% of the European Union's population and 22% of the US population will have reached the age of 65 or older [9, 11, 12]. This demographic shift has profound implications, as a significant portion of these older individuals will experience VCI, leading to a gradual reduction in their functional independence and social engagement, and imposing substantial financial burdens on society [6, 7, 13–15]. Consequently, there is a pressing need to identify potentially reversible age-related pathophysiological mechanisms contributing to VCI and vascular dementia, as this knowledge could pave the way for

the development of clinically relevant preventative treatments.

A complex landscape of age-related microvascular changes contributes to the genesis of VCI, encompassing disruptions in the blood–brain barrier, the accumulation of amyloid pathologies, microvascular rarefaction, microvascular inflammatory phenotypic changes, microhemorrhages, and other interconnected factors [3, 16–25]. Among these multifaceted influences, emerging evidence underscores the critical role of aging-induced dysregulation of cerebral blood flow (CBF) in the genesis of cognitive impairment among older adults [26, 27]. Despite constituting only 2% of the body's weight, the brain consumes a remarkable 20% of its oxygen supply, necessitating a constant, tightly regulated supply of oxygen and glucose to maintain its metabolic demands. This requirement for metabolic homeostasis is further underscored during periods of heightened neuronal activity, where rapid adjustments in oxygen and glucose delivery, as well as efficient removal of harmful metabolites, are essential for sustaining the brain's microenvironment. Achieving this delicate balance relies on a fundamental homeostatic mechanism known as neurovascular coupling (NVC) or functional hyperemia [3, 28–40]. Mounting evidence has implicated aging-induced impairment of NVC responses as a contributor to age-related cognitive decline [3, 35–37]. First, NVC responses are diminished in older adults, a finding consistently associated with impaired cognitive performance [27, 30, 32, 41–44]. Second, pharmacological disruption of NVC in young mice can replicate aspects of the cognitive aging phenotype, further underscoring the link between NVC and cognitive function [45, 46]. Third, interventions targeting cellular and molecular mechanisms of aging have demonstrated the potential to rescue NVC responses and improve cognitive performance in experimental rodent models [35–37, 47–49].

The cellular underpinnings of NVC responses involve the release of vasodilator nitric oxide (NO) by cerebromicrovascular endothelial cells in response to gliotransmitters and mediators released from activated neurons [50, 51]. Endothelium-derived NO operates by inducing relaxation in smooth muscle cells and is presumed to affect contractile pericytes within resistance arterioles and precapillary sphincters. Furthermore, endothelial cells play a pivotal role in promoting the upstream propagation of conducted

vasodilation that initiates within the microcirculation, thus amplifying functional hyperemia [52]. Translational studies have highlighted compromised endothelial function in older adults [53] and aged laboratory animals [35, 36, 54], contributing significantly to the age-related decline in NVC responses. Understanding these cellular mechanisms affecting cerebromicrovascular aging holds immense promise for developing clinically translatable interventions aimed at neurovascular rejuvenation and enhancing cerebral blood flow, with the ultimate goal of preventing and delaying the onset of vascular cognitive impairment.

In the past, investigations into vascular aging predominantly concentrated on cell-autonomous mechanisms responsible for instigating functional decline [55]. These studies have contributed significantly to our grasp of essential factors, including mitochondrial dysfunction [36, 56], heightened production of reactive oxygen species (ROS) [24, 55, 57–66], compromised nitric oxide availability [10, 17–19], cellular NAD+depletion [35, 67–69], epigenetic alterations (including sirtuin dysregulation) [58, 70], and energetic dysfunctions [36] implicated in the etiology of vascular aging [55, 71]. Nonetheless, in recent years, there has been an increasing awareness of the significance of non-cell autonomous mechanisms in instigating aging processes and contributing to the pathogenesis of age-related diseases [55]. Geroscience research has accumulated compelling evidence suggesting that circulating pro-geronic factors (increased with age and detrimental to vascular homeostasis, e.g., inflammatory cytokines) and/or the presence of anti-geronic factors (preventing cellular aging phenotypes and declining with age), derived from various organs like adipose tissue, the brain, the endocrine system, and the immune system, orchestrate cellular aging processes within the vascular wall [55, 71]. Yet, further experimental investigation is warranted to elucidate the relative contributions of circulating factors versus cell-autonomous mechanisms to the development of neurovascular aging phenotypes.

To address this critical gap in our understanding, we employed heterochronic parabiosis [72–75], a surgical technique enabling the fusion of the circulatory systems of two animals, as a means to investigate the role of circulating factors in neurovascular aging. Building upon our previous research demonstrating the roles of circulating factors in driving vascular aging within the aorta [76, 77], our study sought to experimentally test the hypothesis that age-related changes in circulating pro-geronic and/or anti-geronic factors contribute to the regulation of neurovascular aging processes in a non-cell autonomous manner. Leveraging heterochronic parabiosis, combined with the assessment of NVC responses with laser speckle contrast imaging (LSCI), we aimed to determine the extent to which exposure to an old or young environment could transpose neurovascular aging phenotypes within the young and old brain, respectively.

Materials and methods

Animal procurement

Young (4- to 5-month-old, C57BL/6, $n=16$) and aged (18- to 19-month-old, C57BL/6, $n=16$) male mice (*Mus musculus*) were procured from the aging colony maintained by the National Institute on Aging at Charles River Laboratories (Wilmington, MA). The animals were housed under specific pathogen-free barrier conditions in the Rodent Barrier Facility at the University of Oklahoma Health Sciences Center (OUHSC). They were maintained under a controlled photoperiod (12 h of light and 12 h of darkness), provided unlimited water access, and fed a standard AIN-93G diet ad libitum.

Parabiosis surgery

Parabiosis, a surgical technique that involves the fusion of the circulatory systems of two animals, has gained wide prominence for investigating the roles of circulating factors in the pathogenesis of various diseases [78–81], as well as for studying the regulation of aging processes [74, 75, 82–102]. This experimental tool, particularly heterochronic parabiosis, enables the exploration of the intricate interplay between cell-autonomous and non-cell autonomous mechanisms of aging [74, 75, 82–100].

The parabiosis procedure comprised a series of steps, including a 5-day pair acclimation period, the surgical intervention itself, 2 weeks of postoperative care, and an additional 4 weeks of parabiosis to allow for the effects of blood exchange (Fig. 1B). Subsequently, after 6 weeks of parabiosis, neurovascular coupling responses were assessed in each mouse. Throughout the pairwise habituation and
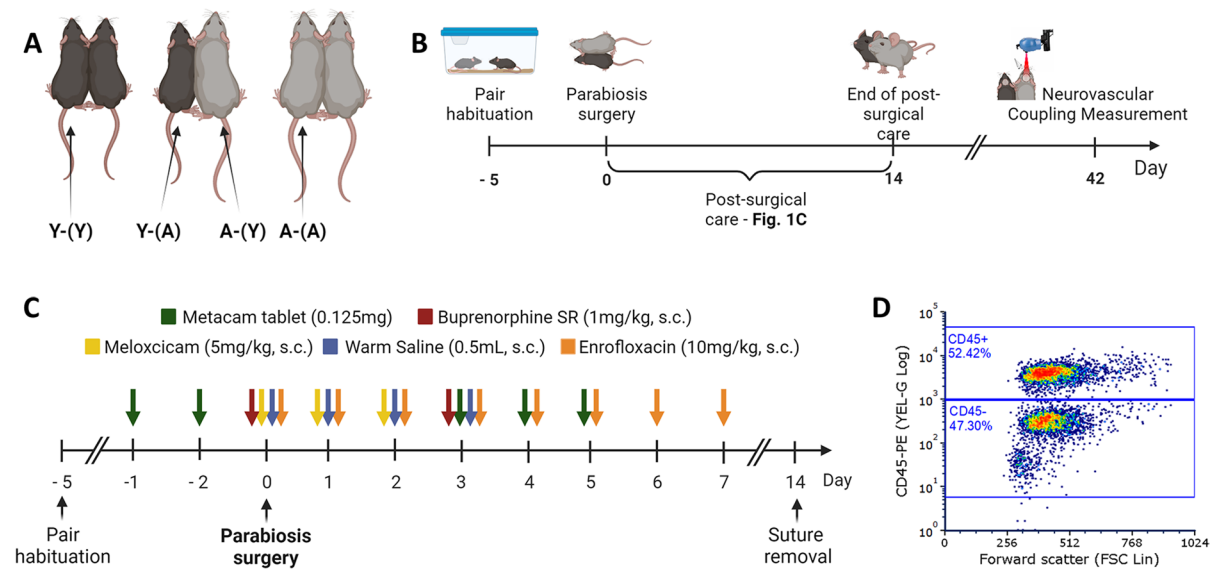
Fig. 1 Parabiosis types and timelines. **A** This figure illustrates the four distinct experimental groups in our study, each represented by a unique combination of young (Y) and aged (A) parabionts, designated by the co-parabiont in parentheses. These groups include isochronic young (Y-(Y)), heterochronic young (Y-(A)), heterochronic aged (A-(Y)), and isochronic aged (A-(A)) parabionts. **B** The timeline for the parabiosis surgery procedure is depicted. Five days prior to surgery, mice are acclimated and assessed for potential aggression. Following surgery, animals are subjected to a meticulous 14-day monitoring period. Neurovascular coupling responses are evaluated in parabionts 6 weeks post-surgery. **C** This graph provides an overview of the comprehensive pre- and postsurgical care regimen administered to parabionts. To effectively manage pain and inflammation, parabionts receive treatment with buprenorphine and meloxicam. To prevent infection, surgery is performed in aseptic conditions and parabionts receive daily enrofloxacin injections for seven consecutive days. To prevent dehydration, parabionts are injected with warm saline. **D** Representative flow cytometry graph displaying mononuclear cells isolated from the blood of the non-injected parabiont. The graph emphasizes the presence of phycoerythrin-labeled leukocytes within the top gate, which originate from the injected co-parabiont. This observation serves as conclusive evidence confirming the successful exchange of blood between the parabionts

post-intervention periods, animals were housed in the conventional rodent colony at OUHSC, with a controlled photoperiod (12 h of light and 12 h of darkness), unrestricted access to water, and ad libitum consumption of a standard AIN-93G diet.

Presurgical procedures

In preparation for the surgery, animals were paired for 5 days to facilitate their acclimation. During this period, close monitoring ensured that aggressive behaviors between pairs were minimal, and any animals displaying aggression towards each other were excluded from undergoing parabiosis surgery. Furthermore, two days prior to the surgery, animals received bacon-flavored Metacam tablets (one per animal, 0.125 mg, MD275-0125, Bio-Serv, NJ, USA) to acquaint them with oral meloxicam administration

and reduce the number of injections after the parabiosis surgery.

Parabiosis surgery

Parabiosis surgery adhered to established protocols by the Einstein Health Span Core, as previously reported [76–79, 101, 103]. Surgical unions were established between young animals (isochronic; young Y–(Y); $n=4$ pairs), aged animals (isochronic old; A–(A); $n=4$ pairs), and young and aged mice (heterochronic Y–(A) and A-(Y); $n=8$ 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 (3–4% induction, 2–3% maintenance, [Isoflurane USP, ref#: 029405, Covetrus, UK], 0.6–0.8 L/min O_2) 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, including 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 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, employing 4–0 silk suture (683G, Ethicon, OH, USA). The skin flaps on the dorsal and ventral surfaces were meticulously sutured together using a continuous interlocking suture technique. Additionally, two ends of 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 closely monitored until fully awake and then placed in a clean cage on a heating pad for recovery.

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 (1 mg/kg, s.c., Buprenorphine E.R., 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, s.c. Meloxicam injectable solution, Covetrus, UK; and Metacam, 0.125 mg/day, oral, 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 (72–06-5022, ClearH$_2$O, ME, USA) for the first 5 days. After the
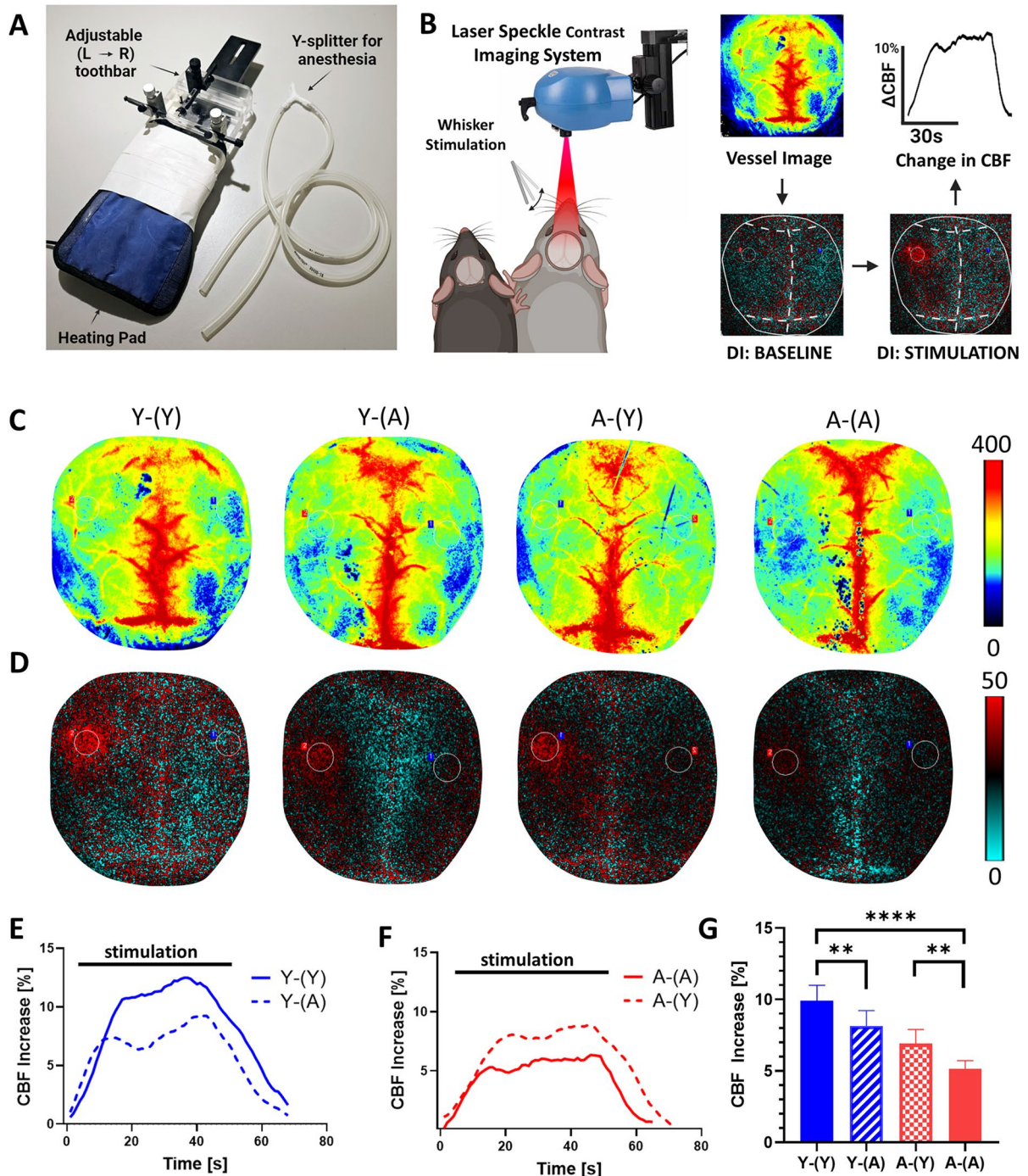
surgery, the parabionts' condition, weight, coordination, and sutures were monitored twice a day for the first 2 weeks. Skin sutures were removed 2 weeks after the surgery, and animals were monitored daily until the experimental end point. Any concerning symptoms were promptly assessed and treated in consultation with OUHSC veterinarians. Animals remained joined for 6 weeks before being subjected to terminal experimentation and euthanasia.

Ethical considerations.

All experiments were conducted in strict adherence to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and were approved by the Institutional Animal Use and Care Committee of OUHSC. Rigorous measures were taken to minimize animal suffering and distress, and a highly skilled veterinarian affiliated with the Department of Comparative Medicine provided expert oversight at every stage of the surgical procedure, ensuring the highest standards of animal care and welfare throughout the study.

Measurement of neurovascular coupling responses

Parabiont pairs of mice in each group were anesthetized with isoflurane (induction at 3% and maintenance at 1%), facilitated by a Y bifurcation splitter. Endotracheal intubation was achieved using a 20Gx1

catheter (Ref: 266,741, Exel, CA, USA), followed by mechanical ventilation using a MousVent G500 system (Kent Scientific Co, Torrington, CT). To maintain a consistent body temperature of 37 °C, a thermostatic heating pad (Kent Scientific Co, Torrington, CT) was utilized [51]. End-tidal CO_2 levels were controlled within the range of 3.2% and 3.7% to keep blood gas values within the physiological range, as described [39, 104]. The parabiont mice were securely immobilized and affixed to a specially adapted stereotaxic frame designed for this study (Fig. 2A). This customized frame comprised a 3D-printed base featuring a lateral movable adaptor equipped with a mouse anesthesia mask (51,625 and 514609 M, Stoelting, USA). This configuration enabled sequential measurements on the left and right parabionts. Subsequently, the scalp and periosteum were retracted and secured aside, and a thin layer of nail polish was applied to the cranial surface to provide a smooth optical interface. Dual vaporizers were employed for anesthesia administration, affording independent control of sedation levels in each parabiont (1% and 2%, isoflurane, in measured and co-measured parabiont, respectively) A laser speckle contrast imager (Perimed, Järfälla, Sweden) was positioned 10 cm above the cranial surface, and to achieve the highest CBF responses, the left and right whiskers were stimulated for approximately 45 s at 5 Hz from side to side [36]. Differential perfusion maps of the brain surface were acquired, and changes in CBF were evaluated above the barrel cortex. This procedure was repeated in eight trials with 5-–10-min intervals between each trial. Changes in CBF were subsequently averaged and expressed as a percentage (%) increase relative to the baseline value [105].

Blood exchange confirmation using flow cytometry

The primary objective of parabiosis surgery is to establish a shared circulatory system between two animals, thereby enabling the evaluation of the impact of circulating factors between the paired animals. To validate a blood exchange between parabionts, we employed an antibody-based circulating leukocyte labelling approach [106]. Briefly, 3 h prior to planned euthanasia, 50 μL of blood was collected from facial vein of both parabionts into EDTA-coated tubes (MiniCollect Tube, ref: 450,476, Greiner Bio-One, Austria). Subsequently, one of the parabionts in

each pair received a retro-orbital injection of phyco-erythrin-labeled anti-CD45 antibody (10 μg, 0.2 mg/mL, cat#: 103,106, BioLegend, CA, USA). After a 3-h interval, blood samples were once again collected from both parabionts following the same procedure. All blood samples were promptly processed, and peripheral blood mononuclear cells were isolated using the density gradient method with Ficoll-Paque (ref: 17,144,002, Cytiva, Sweden) as per the manufacturer's instructions. The middle layer containing mononuclear cells was transferred into a sterile 1.5-mL centrifuge tube and washed once with PBS (500 g, 10 min, 20 °C). Subsequently, cells were resuspended in 150 μL of PBS, and the ratio of labeled to non-labeled leukocytes was determined for each blood sample (both pre- and post-injection in both parabionts) using flow cytometry. Ratios of CD45-positive and negative cells were acquired using the easyCyte flow cytometer (Guava easyCyte BGR HT System, Cytek, CA, USA), and data analysis and graph preparation were conducted using FCS ExpressPro (version 7.18, De Novo Software, CA, USA).

Statistical analysis

Graphs and statistical analyses were conducted using GraphPad Prism software (version 8.0.1, GraphPad Software, MA, USA). To assess group differences, one-way ANOVA followed by Sidak's post hoc test was employed, as deemed appropriate for the analysis. Statistical significance was defined at $p < 0.05$ (**$p < 0.01$, ****$p < 0.0001$). Data are presented as the mean $\pm$ standard deviation. In the neurovascular coupling experiments, we conducted at least eight independent measurements for each experimental group.

Results

Successful parabiosis surgery rate, post-surgical complications, and blood exchange confirmation

Heterochronic parabiosis is a complex surgical procedure characterized by its extended duration, intricate surgical maneuvers, comprehensive pre- and post-operative care and protracted recovery phase. Our surgical team underwent rigorous training under the guidance of the small animal surgery experts at the Einstein Chronobiosis and Energetics/Metabolism of Aging Core (Bronx, NY, USA), who bring years of expertise in performing this intricate surgical procedure. Within our laboratory, we have consistently achieved a parabiosis surgical success rate of 95%. The overall post-surgical recovery rate stands at 80%, with the lowest recovery rate observed in the case of isochronic aged parabionts (67%, Table 1). This reduced recovery rate among aged parabionts can be attributed to age-related declines in resilience, heightened vulnerability to anesthesia, and a heightened incidence of post-surgical complications. These complications encompass dehydration, compromised motor coordination, limb irritation, wound dehiscence, and weight loss [101]. A noteworthy observation, both in our investigations and in related studies [101], is the substantial weight loss experienced by parabionts following the surgical procedure, with aged parabionts being particularly susceptible (Table 1). To address this challenge, we implemented a range of interventions aimed at ameliorating weight loss. These strategies encompassed dietary supplementation through gels, placement of food pellets on the cage floor, and acquainting the animals with these dietary adjustments prior to the parabiosis surgery. The ultimate objective of parabiosis is to establish a

Table 1 Post-surgery outcomes and recovery in heterochronic parabiosis models. This table presents key post-surgery outcomes and recovery statistics for different types of parabiosis models, including information on post-surgery survival rates, recovery rates, weight changes at various time points after surgery, and confirmation of successful blood exchange (flow cytometry) between parabionts. Y-A: Heterochronic parabiont pairs; Y-Y and A-A: Isochronic parabiont pairs

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% (4/4)	100% (4/4)	-15 ± 2	-16 ± 4	-7 ± 3	Yes (2/2)
Y-A	100% (10/10)	80% (8/10)	-14 ± 2	-15 ± 3	-11 ± 1	Yes (2/2)
A-A	80% (5/6)	67% (4/6)	-17 ± 1	-16 ± 2	-14 ± 3	Yes (2/2)

shared circulatory system between parabionts, primarily through wound healing during the initial days post-surgery [101]. To derive meaningful conclusions from parabiosis experiments, it is imperative to validate blood exchange between parabionts. To achieve this, we administered a retro-orbital injection of a fluorescently labelled anti-CD45 antibody to label circulating leukocytes in one of the parabionts. Subsequently, we employed flow cytometry to assess the presence of CD45$^+$ cells in the bloodstream of both the injected and non-injected parabionts. This approach afforded us definitive confirmation of blood exchange in each category of parabiosis pairing (Table 1, Fig. 1D).

Young blood rejuvenates NVC responses in aged heterochronic parabionts

Substantial experimental evidence underscores the pivotal role of endothelial dysfunction in age-related neurovascular impairments in the murine brain. Additionally, exposure to young blood has been shown to effectively restore endothelial function in the aorta of aged heterochronic parabionts [77]. To investigate the impact of systemic young blood factors on NVC responses within the aging mouse brain, we harnessed the heterochronic parabiosis model (Fig. 1A). This experimental framework enabled us to explore the effects of systemic young blood factors on aged heterochronic parabionts (A-(Y)), as well as the consequences of systemic factors enriched in aged blood on young heterochronic parabionts (Y-(A)). To mitigate potential confounding influences stemming from surgical stress associated with parabiosis, we incorporated isochronic young (Y-(Y)) and isochronic aged (A-(A)) parabionts as control groups.

To assess NVC responses within parabionts, we employed a stereotactic frame equipped with a heating pad, specifically designed to accommodate two animals simultaneously (Fig. 2A). This apparatus featured a tooth-bar holder with adjustable left/right positioning during measurements, facilitating the sequential assessment of NVC responses in both parabionts. Utilizing the LSCI system, we quantified the average change in cerebral blood flow within the somatosensory cortex in response to whisker stimulation (Fig. 2B).

Pseudocolor baseline CBF maps, generated via LSCI, were instrumental in offering enhanced anatomical and orientation details (Fig. 2C). Our results revealed a significant reduction in NVC responses within the somatosensory whisker barrel cortex of aged mice compared to their youthful counterparts, indicative of compromised functional hyperemia in advanced age ($p < 0.0001$, Fig. 2G) [31, 35–37, 40, 107–109]. Notably, our study demonstrated a substantial increase in NVC responses among aged heterochronic parabionts following a 6-week exposure to a youthful systemic milieu when compared to their aged isochronic parabiont counterparts ($p < 0.01$, Fig. 2D–G). Although this exposure to young blood partially rejuvenated neurovascular function, NVC responses in aged heterochronic parabionts remained significantly lower than those observed in young isochronic parabionts.

Old blood impairs NVC responses in young heterochronic parabionts, recapitulating the aging phenotype

Remarkably, our investigations unveiled that young heterochronic parabionts exposed to the aged systemic milieu for 6 weeks exhibited a significant attenuation in NVC responses when compared to their young isochronic parabiont counterparts ($p < 0.01$, Fig. 2D–G), effectively mirroring the aging phenotype. Notably, the accelerated neurovascular aging triggered by exposure to aged blood was only partial, as NVC responses in young heterochronic parabionts remained significantly more robust than those observed in aged isochronic parabionts.

Discussion

The pivotal finding of this study lies in the demonstration that even a relatively brief exposure to a youthful humoral environment can mitigate neurovascular aging phenotypes, leading to the rejuvenation of NVC responses. Conversely, a short-term encounter with an aged humoral milieu in young mice accelerates the acquisition of neurovascular aging traits characterized by impaired NVC responses (Fig. 3).

NVC stands as a critical homeostatic mechanism that ensures adequate blood supply to active brain regions. Neurons, devoid of energy or oxygen storage, heavily rely on the closely regulated delivery of nutrients and oxygen by the surrounding microvasculature

Fig. 3 The impact of circulating factors on neurovascular aging. This schematic representation illustrates the potential presence of anti-geronic factors in young blood (depicted in green) and pro-geronic factors in old blood (depicted in red). These circulating factors act upon endothelial cells, influencing pathways responsible for endothelium-dependent vasodilation. Neurovascular coupling (NVC) responses are partially reliant on endothelial function; therefore, exposure to young blood can partially rejuvenate NVC responses in old parabionts. Conversely, exposure to old blood induces aging-like changes in endothelial cells, resulting in impaired NVC responses. These alterations in NVC are anticipated to have a direct impact on cognitive function
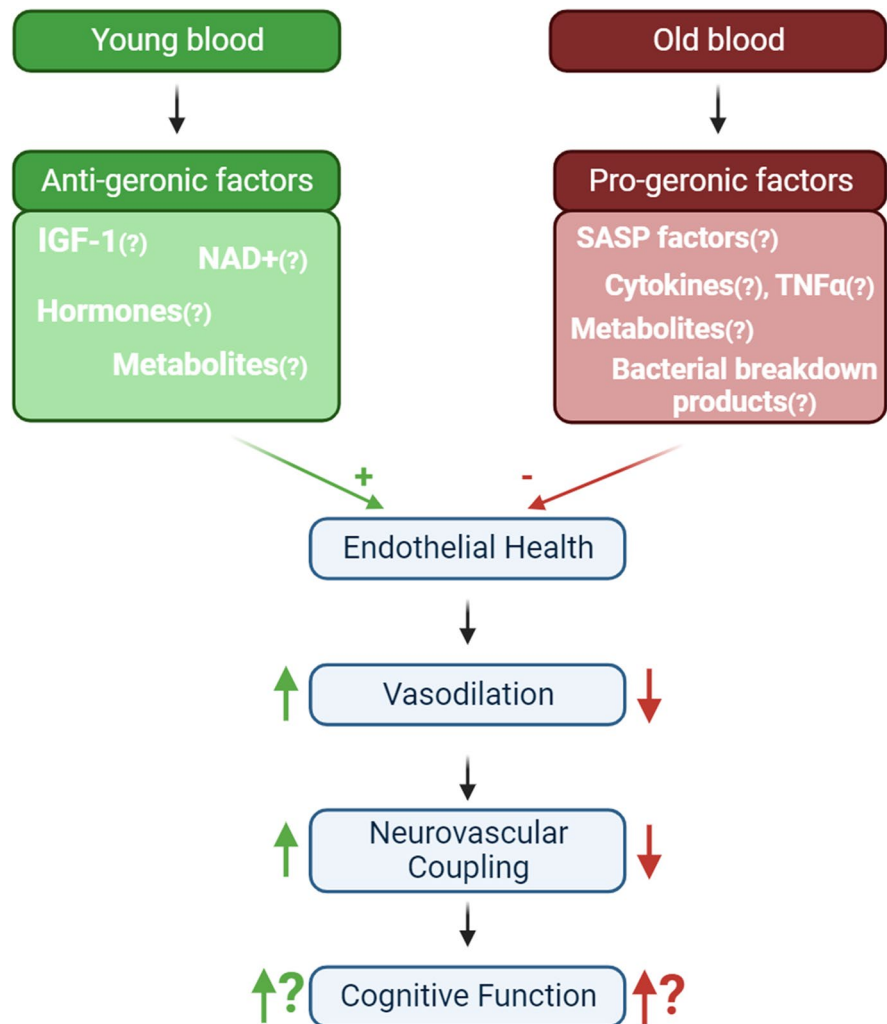
to sustain their functionality. During neuronal activation, NVC orchestrates the adjustment of regional cerebral blood flow to increased demand. This intricate coupling between neuronal activity and the vascular response guarantees an uninterrupted supply of oxygen and nutrients, preventing any spatiotemporal functional impairment within the brain. Upon neuronal activation neurotransmitters are released and astrocytes undergo activation, releasing gliotransmitters (e.g., ATP). These signaling molecules act on endothelial cells, subsequently triggering endothelial NO synthase (eNOS) activation, NO release, and the relaxation of smooth muscle cells in precapillary arterioles and pericytes around capillaries, increasing regional CBF [110, 111]. Furthermore, the microvascular endothelium plays a vital role in the retrograde transduction of vasorelaxation from capillary beds to pial arterioles, ensuring precisely localized vasodilation and safeguarding against "flow steal" from neighboring vascular networks [110].

With age, there is a progressive decline in endothelial function within the cerebral circulation, leading to diminished vasodilatory response and disrupted regulation of cerebral blood flow. The mechanisms of vascular aging [55, 71] contributing to age-related endothelial dysfunction are multifaceted and include mitochondrial dysfunction [36, 56, 109], heightened production of reactive oxygen species (ROS) [24, 55, 57–66], compromised nitric oxide availability [10, 17–19], cellular NAD + depletion [35, 67–69], epigenetic alterations (including sirtuin dysregulation) [58, 70], and

energetic dysfunctions [36]. Impaired endothelium-mediated vasodilation compromises the aged brain's ability to meet neuronal metabolic demands through NVC, ultimately contributing to cognitive deficits [112–114]. Our findings are of substantial importance, providing conclusive evidence that circulating factors play a central role in neurovascular aging and the age-associated decline in NVC responses.

The mechanisms underlying the promotion of neurovascular rejuvenation by young blood factors likely involve the rejuvenation of cerebromicrovascular endothelial cells. Several lines of evidence support this hypothesis. Firstly, our previous studies have established that aging-induced endothelial dysfunction is a pivotal contributor to neurovascular dysfunction in aged mice [31, 35–37, 40]. Crucially, we have demonstrated that heightened mitochondrial oxidative stress in microvascular endothelial cells is a critical mechanism through which aging impairs cerebromicrovascular endothelial dysfunction [31, 35, 36]. Notably, the administration of agents that mitigate mitochondrial oxidative stress, such as mitochondria-targeted antioxidative peptide SS-31 and resveratrol, has been shown to ameliorate NVC in mice, as well as improve cognitive function [36, 109]. Secondly, our previous investigations have established that exposure to young blood rejuvenates endothelial vasodilation in the aorta using the same mouse parabiosis model employed in this study [76, 77]. Importantly, this functional endothelial rejuvenation was accompanied by the rejuvenation of the vascular mitochondrial transcriptome and the mitigation of vascular oxidative stress [76, 77]. Thirdly, recent research by Ximerakis et al. has demonstrated that, within the brain, endothelial cells are the most susceptible to age-related mitochondrial transcriptomic changes, while exhibiting the highest degree of responsiveness to rejuvenation induced by young systemic factors in a mouse model of heterochronic parabiosis [115]. Taken together, we propose that young blood-mediated neurovascular rejuvenation primarily arises from the rejuvenation of mitochondria in cerebromicrovascular endothelial cells. Further research is necessary to evaluate the roles of other related mechanisms, such as the reduction of DNA damage-induced cellular senescence, which may be attributed to the alleviation of oxidative stress,

in the overall rejuvenation of cerebromicrovascular endothelial function.

Our observations regarding the rejuvenation of NVC responses by young blood factors align with prior findings from heterochronic parabiosis, plasma transfer, and plasma dilution experiments, all highlighting the beneficial impact of a youthful systemic milieu on brain aging. Exposure to factors enriched in young plasma has also been shown to reduce neuroinflammation, enhance hippocampal neurogenesis, improve cognitive function, and rejuvenate the transcriptome of various brain cell types [88, 92, 115]. Indeed, considering the direct exposure of endothelial cells to blood factors, it is tempting to envision them as the central hub for young blood-mediated rejuvenation. This scenario suggests that other cells might undergo a form of rejuvenation, albeit indirectly, facilitated by factors released from the rejuvenated endothelium, potentially encompassing cytokines, growth factors, and diverse signaling molecules. Furthermore, the enhancement of endothelial transport and barrier functions could potentially exert an indirect rejuvenating influence on parenchymal cells within the organ.

The precise nature of the anti-geronic factors present in the circulation of young animals remains a subject of active investigation (Fig. 3). Our recent bioinformatics analysis has identified a spectrum of potential pathways likely contributing to the rejuvenating effects of young blood. These pathways and factors include the inhibition of rapamycin-insensitive companion of mTOR (RICTOR) and the activation of insulin-like growth factor 1 receptor (IGF1R), VEGF receptors, and sirtuins. Notably, insulin-like growth factor 1 (IGF-1), a hormone responsible for mediating the anabolic effects of growth hormone and essential for normal growth and development, has been shown to significantly decrease in serum levels during aging [116] both in humans and laboratory animals. Strong evidence links circulating IGF-1 deficiency to cardiovascular aging and, in particular, neurovascular and brain aging [21, 23, 33, 34, 39, 116–132]. Age-related IGF-1 deficiency has been causally associated with endothelial dysfunction, impaired neurovascular coupling responses, and cognitive deficits in both rodent models and humans [33, 34, 38, 39]. In a significant demonstration of the importance of endothelial IGF-1 signaling, cell-specific

IGFR1 knockdown in cerebrovascular endothelial cells resulted in an accelerated neurovascular aging phenotype and impaired NVC responses. Circulating IGF-1 levels have also been observed to correlate with NVC responses in older adults [38]. Consequently, further investigation is warranted to delineate the role of IGF-1 in young blood-mediated neurovascular rejuvenation. Another putative mechanism underlying the young blood-mediated-improvement of NVC responses is the restoration of cellular NAD^+ levels [35, 133]. NAD^+ serves as a co-factor for over four hundred enzymes, including vasoprotective and lifespan-extending sirtuins. With age, circulating and cellular NAD^+ levels decline, including a decrease within cerebromicrovascular endothelial cells [35]. The replenishment of NAD^+ through the administration of NAD precursors has been shown to rescue endothelial function, reverse age-related endothelial transcriptomic changes, and rejuvenate NVC responses [35, 133]. Although initial evidence suggests that sirtuin activation may contribute to endothelial rejuvenation in the aortas of aged heterochronic parabionts, comprehensive mechanistic studies are needed to assess the role of sirtuin activation in the rejuvenation of cerebrovascular endothelial function and NVC responses. Other systemic factors (e.g., tissue plasminogen activator [tPA] [134–136], growth differentiation factor 11 [GDF-11] [92, 137, 138], tetrahydrobiopterin [BH_4] [139, 140]) may also contribute to the beneficial effects of young blood on NVC. However, the exact contributions of the aforementioned circulating factors to NVC rejuvenation are currently theoretical and warrant further exploration. Future studies, particularly those employing transgenic mice lacking specific circulating factors combined with heterochronic parabiosis models, are essential to pinpoint the precise factors and pathways involved in the rejuvenation effects of young blood on NVC. In addition, the observed enhancement of NVC responses in aged heterochronic parabionts could be linked to the dilution of pro-geronic factors present in aged plasma. This hypothesis aligns with the increasing evidence from both preclinical and clinical research, indicating beneficial outcomes from diluting aged plasma, such as improvements in neuroinflammation, cognitive function, and biological age [141–143]. Therefore, it is essential to explore further whether the dilution of pro-geronic

factors by young blood significantly contributes to the improved NVC seen in aged heterochronic parabionts.

This study also highlights a crucial discovery that a relatively brief exposure of young mice to an aged humoral environment can accelerate the onset of neurovascular aging phenotypes. Notably, we demonstrate, for the first time, that mediators circulating in the blood of aged mice can expedite neurovascular aging. These findings build upon our prior research, which elucidated pro-geronic transcriptional alterations induced by aged blood in the aorta [76]. Moreover, our results further substantiate the notion that non-cell-autonomous mechanisms are pivotal in driving neurovascular aging processes, likely contributing to the pathogenesis of VCI. Earlier investigations have revealed that the presence of aged blood in the circulation of young heterochronic parabionts also prompts age-like phenotypic transformations in the liver, heart, and brain [31, 47, 49, 52, 55, 98]. Interestingly, it has been demonstrated that old blood factors exert pro-geronic effects on the central nervous system [143, 144]. This suggests that specific circulating anti-geronic factor(s) may traverse the blood–brain barrier or induce their detrimental impact on the brain by fostering accelerated neurovascular aging. The remarkable plasticity of neurovascular aging in response to both pro-geronic and anti-geronic circulating factors underscores the potential for therapeutic interventions aimed at counteracting the age-related dysfunction of cerebral microcirculation, either by directly or indirectly targeting the systemic milieu. Despite these findings, the precise nature and cellular origins of circulating pro-geronic factors, responsible for inducing accelerated neurovascular aging remain enigmatic. In a recent transcriptomic investigation, we identified potential cellular pathways induced by pro-geronic factors present in aged blood, including the possible involvement of TGFβ signaling, inhibition of the p53 pathway, serum response factor (SRF)-driven pathways, and inhibition of VEGF-A and IGF-1 signaling [76]. Furthermore, other circulating factors whose levels are modulated in young mice through heterochronic parabiosis or systemic administration of aged plasma or blood and which may bestow aging-like effects in the vasculature, encompass fibroblast growth factor 23 (FGF23) [145, 146], β2-microglobulin, TGFβ family cytokines [93, 100], TNFα [147], and other pro-inflammatory

mediators secreted by senescent cells, collectively known as the senescence-associated secretory phenotype (SASP) [71, 148, 149]. Future studies should aim to identify pro-geronic factors responsible for accelerated endothelial aging. Uncovering these factors could be achieved through heterochronic parabiosis experiments using genetically engineered animals lacking certain pro-geronic factors[134,150]. Furthermore, the adverse effects on NVC noted in our study may partly stem from the dilution of protective young blood factors.

Recognizing the limitations and confounding factors of the heterochronic parabiosis model is crucial. This model's primary strength lies in its ability to continuously expose both young and aged animals to their co-parabiont's circulating factors, making it highly effective for detecting subtle pro- and anti-aging effects. However, this same strength poses a challenge in isolating and identifying specific factors and pathways responsible for the observed effects from heterochronic blood exchange. Additionally, the model involves not only exposure to the systemic factors of the co-parabiont but also indirect exposure to their organs, such as the liver and kidneys. This aspect could potentially enhance metabolic clearance and overall health, especially in the case of aged heterochronic parabionts. The surgery and subsequent shared circulation can induce stress in the animals, potentially influencing physiological responses. The altered physical activity levels in young and aged heterochronic parabionts add another layer of complexity, potentially influencing their health outcomes. Nutritional status can significantly impact aging processes and vascular health. In parabiosis experiments, the nutritional intake and metabolic status of one parabiont can indirectly influence the other, potentially skewing results related to circulating factors. It is pertinent to note that post-parabiosis surgery, parabionts often exhibit a significant weight reduction, which could impact various measured outcomes, similar to observations in caloric restriction studies. Despite controls like isochronic pairings, reduced mobility in heterochronic parabiosis poses challenges in specific physiological assessments, such as cognitive function evaluation or procedures requiring animal imaging and anesthesia. These challenges necessitate additional methodological adjustments, including the use of equipment and adaptation of protocols specifically designed for parabionts.

In summary, our study highlights the promising potential for reversing neurovascular aging by leveraging the systemic influences of both anti-geronic and pro-geronic circulating factors. While this study focused primarily on the rejuvenation of NVC responses, it remains to be explored whether young systemic factors can extend their rejuvenating effects to address other age-related cerebromicrovascular pathologies, such as blood–brain barrier disruption or microvascular rarefaction. Investigating these facets promises valuable insights into the broader implications of young blood-mediated brain rejuvenation. The circulation exposes each plasma constituent in blood, encompassing circulating hormones, cytokines, proteins, peptides, lipid mediators, micropeptides, metabolites, and circulating exosomes, to the vasculature. Each of these constituents may exert either anti-geronic or pro-geronic effects, warranting further investigation into the precise nature and cellular origins of circulating factors that mediate neurovascular rejuvenation and the induction of accelerated vascular aging, as observed in our study.

Utilizing transgenic animals in parabiosis experiments is a promising approach to uncover the factors that drive the rejuvenating effects of young blood and the detrimental impacts of aged blood, thereby enhancing the potential for translational applications in future research. Additionally, methods like plasma transfer, heterochronic blood exchange, and plasma dilution experiments provide valuable alternative models. These approaches can augment the findings from heterochronic parabiosis and contribute to a more comprehensive understanding of the mechanisms involved. The versatility of these models offers an avenue to extend our observations beyond the confines of parabiosis studies, shedding light on the broader implications of young blood factors in the context of age-related VCI and rejuvenation strategies.

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Declarations

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