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Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors

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Abstract

In the adult central nervous system, the vasculature of the neurogenic niche regulates neural stem cell behavior by providing circulating and secreted factors. Age-related decline of neurogenesis and cognitive function is associated with reduced blood flow and decreased numbers of neural stem cells. Therefore, restoring the functionality of the niche should counteract some of the negative effects of aging. We show that factors found in young blood induce vascular remodeling, culminating in increased neurogenesis and improved olfactory discrimination in aging mice. Further, we show that GDF11 alone can improve the cerebral vasculature and enhance neurogenesis. The identification of factors that slow the age-dependent deterioration of the neurogenic niche in mice may constitute the basis for new methods of treating age-related neurodegenerative and neurovascular diseases.

In the adult brain, neural stem cells reside in a three-dimensional heterogeneous niche where they are in direct contact with blood vessels and the cerebrospinal fluid. The vasculature can influence neural stem cell proliferation and differentiation by providing a local source of signaling molecules secreted from endothelial cells (1) as well as by delivering systemic regulatory factors (2). The hormone prolactin (3), dietary restriction (4) and an exercise/enriched environment (5), positively modulate neurogenesis, whereas increased levels of glucocorticoids associated with stress have the opposite effect (6). In the aging niche, the vasculature deteriorates with a consequent reduction in blood flow (7), and the neurogenic potential of neural stem cells declines, leading to reduced neuroplasticity and cognition (8-10). Systemic factors can also affect these aging-associated events, either positively

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Supplementary Material: Materials and Methods Figs. S1 to S12

where circulating monocytes enhance remyelination in aged mice (11, 12) or negatively where the accumulation of chemokines in old blood can reduce neurogenesis and cognition in young mice (10).

To test if the age-related decline of the neurogenic niche can be restored by extrinsic young signals, we used a mouse heterochronic parabiosis model. Our experiments reveal a remodeling of the aged cerebral vasculature in response to young systemic factors, producing noticeably greater blood flow, as well as activation of subventricular zone (SVZ) neural stem cell proliferation and enhanced olfactory neurogenesis leading to an improvement in olfactory function. Furthermore, we tested GDF11, a circulating TGF- β family member that reverses cardiac hypertrophy in aged mice (13), and found that it can also stimulate vascular remodeling and increase neurogenesis in aging mice. Thus, we have observed that age-dependent remodeling of this niche is reversible by systemic intervention.

To test our hypothesis we generated heterochronic parabiotic pairs between 15-month old (Het-O) and 2-month old (Het-Y) male mice, as well as control groups of age-matched pairs, namely isochronic young (Iso-Y) and isochronic old (Iso-O) pairs (Fig.S1). The average lifespan of this strain of mice in the National Institute of Aging's growth conditions is 27 months. All parabiotic pairs remained surgically joined for 5 weeks (14). Because aging leads to a reduced number of progenitor cells (15-17), we assessed how heterochronic parabiosis can affect the SVZ neural stem cell populations by analyzing coronal SVZ sections (Fig.S2A) of heterochronic and isochronic brains for different SVZ stem/progenitor cell types, such as proliferative Ki67⁺ cells, Sox2⁺ stem cells and Olig2⁺ transit amplifying progenitors (Fig.1A, B, C respectively). Quantification of these sections revealed an increase of 26.9% for Ki67⁺ cells (Fig.S2B), 112% for Sox2⁺ cells (Fig.1D) and 57% for Olig2⁺ cells (Fig.1E) in the Het-O compared to the Iso-O SVZ. However, these cell populations were unaffected in the Het-Y mice (Fig.S2C and Fig.1 D, E). Systemic factors in old blood can have detrimental effects on hippocampal neurogenesis in young animals (10); however, we saw no decrease in neural stem/progenitor cell numbers in the SVZ of young mice joined to 15-month old partners. We wondered if this discrepancy was related to differences between the SVZ and the hippocampus or to the fact that our old animals were younger than the old animals used in the previous study. We therefore joined 2-month old mice with 21-month old mice (Fig.S3A) and found that the older blood negatively affected young SVZ neurogenesis, as Het-Y₂₁ mice showed decreased proliferative Ki67⁺ (Fig.S3B, D) and Sox2⁺ cell populations (Fig.S3C, E) in the SVZ compared to Iso-Y mice. These data are consistent with the previously reported negative effect of older blood on hippocampal neurogenesis (10) and indicate an age-dependent accumulation of factors in the blood of older mice that affect neurogenic zones in both the hippocampus and SVZ.

Aging results in longer cell cycle times in precursor cells isolated from the SVZ (18). To assess the effect of heterochronic parabiosis on neural stem cell proliferation, we cultured neural stem cells from parabiotic brains as neurospheres (19,20). After the first passage, neurospheres derived from the Het-O SVZ were 43% larger in diameter than those derived from the Iso-O SVZ (Fig. S4) and, after removal of growth factors, they generated ~2.5-fold more TuJ1⁺ neurons than the Iso-O (Fig. S5). This suggests that neural stem cells exposed to young systemic factors increase their ability to proliferate and differentiate into neurons.

Collectively, these data demonstrate that youthful circulating factors can restore the self-renewal and differentiation potential of aged SVZ stem cells, and this effect can persist for some time after isolation from the mouse brain.

Adult SVZ neural stem and progenitor cells differentiate into neuroblasts and migrate through the rostral migratory stream to the olfactory bulb where they mature into interneurons (21). We asked if the increase in neural stem and progenitor cells could produce a subsequent change in olfactory neurogenesis in the Het-O mice. We pulsed parabiotic pairs with BrdU to label newborn neurons, and after 3 weeks the mice were analyzed for BrdU⁺/NeuN⁺ cells to quantify newborn neurons (Fig. 2A). As expected from our *in vitro* studies, we observed increased olfactory neurogenesis *in vivo*. Het-O newborn neuron populations were enriched by 92% compared to Iso-O populations (Fig. 2B). In accordance with our above results, the number of new neurons in Het-Y mice was only slightly negatively affected, although the decrease was not statistically significant (Fig. 2C).

To test the functional significance of these findings, we performed an olfaction assay where naïve single parabionts, separated from their parabiotic partners after 5 weeks, were exposed to different concentrations of an odorant (22). After a short habituation period, each parabiont was presented with different concentrations of an odorant, and the total time that each parabiont spent exploring the odorant for each concentration was measured. In this assay, Het-O (Fig. 2D) and young control (Fig. 2D) mice both spent more time exploring a low concentration of odorant (diluted 10⁵ times), whereas a high concentration (10-fold diluted) produced a negative response. By comparison, Iso-O mice spent roughly the same amount of time exploring the odorant regardless of its concentration (Fig. 2D). These results suggest that Het-O mice have a higher olfactory discrimination than the Iso-O mice. Therefore, exposure of the neurogenic niche to young systemic factors enhances functional neurogenesis, culminating in improved olfactory behavior.

Cerebrovascular architecture, capillary density and cerebral blood flow have been reported to decline with aging (23-25). Given the interconnection between the vasculature and neural stem cells, we asked if young blood factors can also rejuvenate blood vessel architecture and function. To test this we created “angiograms”, 3D reconstructions of the blood vessels (Fig. S6A). Volumetric analysis of these angiograms showed that aging causes a decrease in blood vessel volume, as expected (Fig. 3A, B). However, heterochronic parabiosis reversed this decline, increasing blood vessel volume by 87% in the Het-O compared to the Iso-O group (Fig. 3A, B). Furthermore, we observed that blood vessel branching increased by 21% in Het-O versus Iso-O mice (Fig. S6B). Some blood vessels in the Het-O mice were not associated with AQP4⁺ astrocytic endfeet, suggesting that these vessels are newly formed and potentially leaky, thus providing NSCs with enhanced nutritive support (Fig. S6E). This phenomenon of vascular remodeling in the Het-O mice extended to other neurogenic areas like the hippocampus (Fig. S7A, B) and also to non-neurogenic areas like the cortex (Fig. S7C). To test if the increased blood vessel volume led to functional improvement, we measured cerebral blood flow (CBF) by magnetic resonance imaging (MRI) in the parabiotic mice (Fig. 3E), since CBF is known to decrease with aging (27). We found that heterochronic parabiosis indeed restored CBF to the levels seen in young animals (Fig. 3C, D E), indicating that the vascular remodeling observed in Het-O mice changes the

hemodynamics of the vascular system in the CNS. Young vasculature, on the contrary, retained the same volumetric (Fig. 3A, B), blood flow (Fig. S6D), and branching (Fig. S6C) characteristics in both isochronic and heterochronic parabiosis.

New vessels can form either by sprouting from existing capillaries or *de novo* from circulating endothelial progenitors. To test which of these mechanisms is taking place we parabiotically joined young GFP mice with old non-GFP mice for 5 weeks. Analysis of these brains excluded any detectable contribution of young circulating endothelial progenitors to the vascular remodeling in Het-O animals (Fig. S8). As pericytes play a role in vasoconstriction in capillaries (26), we sought to investigate if their numbers were altered by heterochronic parabiosis. The number of pericytes associated with blood vessels was unaffected by parabiosis (Fig. S9). The likelihood that systemic factors can act directly on endothelial cells was further supported when we cultured primary mouse brain capillary endothelial cells and treated them with serum isolated from either young or old mice. Young serum stimulated endothelial cell proliferation by 88% compared to old serum (Fig. S10).

Several factors, including Sonic Hedgehog, erythropoietin, nitric oxide, Notch ligands, Fibroblast Growth Factor and Vascular Endothelial Growth Factor (28-31), that affect neurogenesis are also involved in blood vessel maintenance and proliferation. Of most relevance to our study are those factors that decrease with aging. Recently, one such factor, GDF11/BMP11, a circulating member of the BMP/TGF- β family, was found to be present in higher concentrations in young and heterochronic old than in old mouse serum. GDF11 administration to older mice reproduces many of the beneficial effects of parabiosis on aging hypertrophic cardiac muscle (13). This prompted us to test if GDF11 could also restore the age-related decline in neurogenesis and participate in vascular remodeling. For that purpose, 21-23 month old mice were treated with daily injections of either recombinant GDF11 (rGDF11, 0.1mg/kg mouse body weight), a dosing regimen that increases GDF11 levels in old mice toward youthful levels (30), or PBS (vehicle) for 4 weeks and their blood vessels were subsequently analyzed using the volumetric assay described above. The volume of blood vessels in GDF11-treated old mice increased by 50% compared to the PBS-treated mice (Fig. 4A, C). Moreover, the population of Sox2⁺ cells in GDF11-treated old mice increased by 29% compared to the control (Fig. 4B, D).

In vitro experiments confirmed that GDF11 acts, at least in part, on brain capillary endothelial cells. First, treating endothelial cells with rGDF11 (40ng/ml) activates the well-known TGF- β signaling pathway in these cells, revealed by an increase in SMAD phosphorylation cascade (Fig. 4E, F). Second, a 6-day treatment of primary brain capillary endothelial cells with rGDF11 (40ng/ml) increased their proliferation by 22.9% compared to controls (Fig.S11), but not in the presence of a TGF- β inhibitor (Fig.S12), confirming that GDF11 has a direct biological effect on these cells through the p-SMAD pathway.

The physiology of the brain is intimately dependent on its vasculature during aging. In the normal brain there is a close association between stimulation of neural stem cells and blood vessels in the SVZ (4-6) and in the dentate gyrus. Here we show that heterochronic parabiosis increases neurogenesis and improves vascularity and blood flow of the neurogenic niche. GDF11, a factor that also rejuvenates heart and skeletal muscle in aged

mice (35), also was able to increase blood flow and neurogenesis in aged mice. Its effects were not as large as those of parabiosis itself, although that may relate to using sub-optimal doses of this factor. In addition, some of its actions may be direct, and others may be indirect. Additional experiments will be needed to address this issue.

A question that arises from our work relates to aging-associated changes in the balance of positively- and negatively-acting circulating factors. We show here that blood from 15-month old mice does not have a detrimental effect on young mice whereas older blood (21-month old) dramatically decreases neural stem cell populations in the young brain, an effect also observed in the hippocampus (10). This observation suggests that there is an age at which deleterious systemic factors accumulate and/or young factors are reduced. However, regardless of the age of the old brain, we and others (10, 11) have shown that young blood is still able to rejuvenate the aged brain.

In conclusion, circulating factors, specifically including GDF11, have diverse positive effects in aging mice, including enhancing neurogenesis. Interestingly, aging also affects the microvascular network in non-neurogenic regions of the brain (7). Circulating factors improved the vasculature in the cortex, as well as in other parts of the aging mouse brain. It is possible that increased blood flow might result in increased neural activity and function, opening new therapeutic strategies for treating age-related neurodegenerative conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Rejuvenation of progenitor cells by heterochronic parabiosis

(A-C) Confocal images showing the effects of parabiosis on proliferative (A), neural stem (B) and progenitor cells (C) in the SVZ of isochronic and heterochronic mice. Scale bar: 50 μ m. (D-E) Quantification of neural stem (D) and progenitor cell (E) populations of the above images (n=9 animals for each experimental group, *p<0.05, **p<0.01, ***p<0.001). Data shown as mean $\pm$ S.E.M; statistical analysis by ANOVA.

Fig. 2. Heterochronic parabiosis enhances neurogenesis and cognitive functions in the aging mouse

(A) Representative images of olfactory bulbs showing newborn neurons in isochronic and heterochronic parabionts. Scale bar: 100 μ m. Circles in higher magnification inserts indicate BrdU⁺/NeuN⁺ double-positive cells. (B, C) Quantification of neurogenesis in the olfactory bulbs of old (B) and young (C) parabionts. (n=4, *p<0.05). (D) Measurement of the exploratory time during the olfactory sensitivity assay (n=3). Data shown as mean $\pm$ S.E.M; statistical analysis by t-test. “n=” indicates the number of animals for each experimental group.

Fig. 3. Young blood induces vascular remodeling and increases blood flow in old mice
(A) Confocal images of the SVZ area showing the changes in vasculature after heterochronic parabiosis. Scale bar: 50μm. **(B)** Measurement of blood vessel volume in isochronic and heterochronic parabionts ($n_{\text{old}}=9$, $n_{\text{young}}=6$). **(C, D)** Measurements of cerebral blood flow in the SVZ region of the parabionts: Iso-O versus Iso-Y (C) or Het-O (D) mice ($n=4$). **(E)** Perfusion MRI images of the brain. "V" indicates the ventricles. Data shown as mean±S.E.M; statistical analysis by ANOVA in (B) and t-test in (C,D); *p<0.05, **p<0.01, ***p<0.001. "n=" indicates the number of animals for each experimental group.

Fig. 4. GDF11 enhances vascular remodeling and neurogenesis

(A, B) Confocal images of coronal SVZ sections showing that 22-month old mice injected with rGDF11 for 4 weeks have enhanced vascularization (A) as well as increased Sox2⁺ neural stem cell populations (B) compared to control. (C) Measurement of blood vessel volume in rGDF11-treated and control mice (n=9). (D) Quantification of Sox2⁺ cells in the SVZ area (n=6); “n=” indicates the number of animals for each experimental group. (E, F) Quantification (E) and representative images (F) of the percentage of phospho-SMAD2/3⁺ cells in primary brain capillary endothelial cell cultures treated with either GDF11 (40ng/ml) or TGF- β (10ng/ml) in the presence of sodium orthovanadate used to inhibit phosphatase activity for 30 minutes (n=7). Scale bar: 100 μm . Data shown as mean $\pm$ S.E.M; statistical analysis by t-test, between each experimental condition and the untreated control, *p<0.05, **p<0.01, ***p<0.001.