REVIEW

Endothelial Nitric Oxide Synthase—Deficient Mice

A Model of Spontaneous Cerebral Small-Vessel Disease

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Age-related cerebral small-vessel disease (CSVD) is a major cause of stroke and dementia. Despite a widespread acceptance of small-vessel arteriopathy, lacunar infarction, diffuse white matter injury, and cognitive impairment as four cardinal features of CSVD, a unifying pathologic mechanism of CSVD remains elusive. Herein, we introduce partial endothelial nitric oxide synthase (eNOS)—deficient mice as a model of age-dependent, spontaneous CSVD. These mice developed cerebral hypoperfusion and blood-brain barrier leakage at a young age, which progressively worsened with advanced age. Their brains exhibited elevated oxidative stress, astrogliosis, cerebral amyloid angiopathy, microbleeds, microinfarction, and white matter pathology. Partial eNOS-deficient mice developed gait disturbances at middle age, and hippocampus-dependent memory deficits at older ages. These mice also showed enhanced expression of bone morphogenetic protein 4 (BMP4) in brain pericytes before myelin loss and white matter pathology. Because BMP4 signaling not only promotes astrogliogenesis but also blocks oligodendrocyte differentiation, we posit that paracrine actions of BMP4, localized within the neurovascular unit, promote white matter disorganization and neurodegeneration. These observations point to BMP4 signaling pathway in the aging brain vasculature as a potential therapeutic target. Finally, because studies in partial eNOS-deficient mice corroborated recent clinical evidence that blood-brain barrier disruption is a primary cause of white matter pathology, the mechanism of impaired nitric oxide signaling-mediated CSVD warrants further investigation. (*Am J Pathol* 2021, 191: 1932–1945; <https://doi.org/10.1016/j.ajpath.2021.02.022>)

Vascular cognitive impairment and dementia (VCID) is the second most common form of dementia after Alzheimer disease (AD).¹ The prevalence of dementia with clinical and pathological features of both VCID and AD has been

steadily increasing in the aging population, and overt cerebrovascular pathology is evident in at least half of all dementia cases.² This comorbidity of VCID in sporadic AD is thought to be a major driver of the overall clinical burden of

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This article is part of a review series on small blood vessel disease in the brain, addressing current knowledge, new mechanisms, biomarkers, and therapeutic approaches.

dementia.^{3,4} Cerebral small-vessel disease (CSVD) refers to diverse pathologic processes that affect small cerebral arteries, arterioles, capillaries, and small veins; CSVD is a major cause of stroke and dementia in elderly people.⁵ Evidently, at least 50% of cognitive impairment and dementia may be attributable to CSVD. Patients with relatively normal neuroimaging results may not be classified as having CSVD, but may have invisible pathologies, such as microinfarcts, only detectable at the microscopic level. Individuals with multiple cortical microinfarcts are at a higher risk for CSVD and stroke.^{6–9} CSVD is categorized into two main types: the amyloid form, which includes amyloid angiopathy; and nonamyloid form, commonly associated with old age, hypertension, diabetes, and metabolic syndrome.¹⁰ Pathologically, CSVD is characterized by white matter hyperintensities, lacunae, perivascular space, cerebral microbleed, cerebral microinfarcts, blood-brain barrier (BBB) disruption, and brain atrophy.^{11–14}

Despite recent advances in neuroimaging techniques and the discovery of potential biomarkers,¹⁵ the precise mechanistic underpinnings of the pathogenesis of CSVD have remained elusive. Damage to the BBB has been postulated to be a common feature of varied forms of CSVD.^{16–20} An association between BBB leakage and changes in white matter and cognition has also been demonstrated by neuroimaging in patients with a diagnosis of CSVD, as well as in apparently normal aging brains.^{21,22} White matter tracts are particularly susceptible to vascular damage,^{23,24} which is thought to be caused by chronic hypoperfusion, cerebrospinal fluid disturbance, BBB breakdown, and oxidative stress–induced inflammation.^{25–27} A pivotal role of BBB breakdown in cerebral white matter lesions has gained the most support from the examinations of the aging brains,²² in the brains of patients with acute and chronic CSVD,^{17–20} and in early AD patients.^{28–30} There is compelling evidence to indicate that pericytes play a crucial role in CSVD; pericytes are involved in maintaining normal cerebral blood flow, functional integrity of BBB, and white matter health.^{31–33} At present, the precise sequence of cellular and biochemical reactions that drive white matter pathology remain largely undefined.

NO Is a Key Regulator of Cardiovascular and Cerebrovascular Functions

Soon after its discovery as the endothelium-derived relaxing factor three decades ago,³⁴ endothelium-derived relaxing factor and nitric oxide (NO) were shown to be indistinguishable from each other.^{35,36} A few years later,³⁷ NO was characterized as a novel second messenger that activated guanyl cyclase–coupled receptors in the central nervous system, where it was shown to be involved in the autor-regulation of cerebral blood flow,³⁸ as well as a regulator of presynaptic plasticity of neurons.³⁹ In mammals, NO is synthesized from L-arginine by three distinct calcium-calmodulin-controlled NO synthases (NOSs), named

endothelial NO synthase (eNOS), neuronal NO synthase (nNOS), and inducible NOS (iNOS), each encoded by a unique gene. Although iNOS and nNOS are soluble proteins predominantly located in the cytosol, eNOS is a membrane-bound enzyme, and a major source of NO in the blood vessels.⁴⁰ Because expression of eNOS, nNOS, and iNOS does not occur in a strictly tissue-specific manner, determining individual pathophysiological functions of the three NOS isozymes posed a major challenge further exacerbated by the fact that most tissues contain innervation and vasculature. Fortunately, over the years, these conceptual hurdles have been steadily mitigated with the generation of transgenic mice engineered with deletions of functional NOS1, NOS2, or NOS3 genes from their genomes, which encode nNOS, iNOS, and eNOS, respectively. Detailed analyses of several lines of such transgenic mice unraveled not only how the lack of a particular NOS isozyme affects the basal phenotype of the mice but also their responses to varied experimental manipulations.⁴¹

eNOS-deficient mice were generated by three independent laboratories.^{42–44} Initially, these mice were used to elucidate a role of eNOS in cardiovascular pathophysiology. Their propensity to develop spontaneous hypertension and exacerbated stroke outcomes was reported in two independent lines of eNOS knockout mice.^{42,43} eNOS knockout mice have defects in systemic circulation, as indicated by increased myocardial infarctions, and other hematologic abnormalities such as renal thrombotic microangiopathy, atherosclerosis, and thrombosis.⁴⁴ eNOS knockout male mice have increased mortality attributable to cardiovascular defects,⁴⁵ suggesting potential sex differences in certain phenotypes in these mice. Although, in the original study, no gross anatomic and histologic changes were reported in the brain vasculature,⁴² it was later discovered that these mice displayed vascular hypertrophy and increased vascular tone.^{46,47} A critical role of eNOS in the brain was indicated by the eNOS-deficient mice developing increased ischemic infarctions and impaired arteriogenesis after stroke.⁴⁸

In humans, endothelium-derived NO plays a key role in the regulation of basal cerebral blood flow via vasodilation of cerebral vessels, and aberrant biogenesis of NO is associated with CSVD, cerebral hypoperfusion, and the impairment of the BBB. Genetic studies in humans have lent strong support to observations made in laboratory animals by demonstrating a positive link between eNOS gene polymorphisms and increased risk of CSVD in patients, including silent brain infarction.^{49,50} Significantly, some eNOS polymorphisms were also shown to be associated with metabolic syndrome,⁵¹ an umbrella term encompassing conditions such as obesity, dyslipidemia, hyperglycemia, hypertension, and arterial stiffness. Almost all systemic aspects of metabolic syndrome are manifested by mice with eNOS deficiency. These include insulin resistance, hyperlipidemia,^{52,53} and impaired mitochondrial β -oxidation.⁵⁴ Similarly, in humans, metabolic syndrome is associated with eNOS polymorphisms.^{55,56} A converging role of

metabolic disorders in both major forms of dementia (ie, AD and VCID) has been well recognized.⁵⁷ Several eNOS polymorphisms appear to interact with a genetic polymorphism associated with enhanced homocysteine levels; these dual polymorphisms are thought to worsen cognitive decline in patients with AD or dementia.⁵⁸ Although eNOS genetic variants are not exclusively associated with AD, reduced eNOS mRNA and protein expression levels in capillaries have been reported in the brains of AD patients and were positively correlated with apolipoprotein E4 genotype.⁵⁹ A comparative study between AD with a large subset of cases with superimposed vascular lesions (AD + VCID) revealed decreased expression of eNOS in cerebral vessels in both AD and AD + VCID subjects. This study also indicated that reduced eNOS expression was associated with increased vascular lesions and reduced AD pathology in AD + VCID brains.⁶⁰ Given the compelling clinical and experimental evidence presented herein, we therefore speculated whether eNOS deficiency might represent a common mechanism of CSVD and cognitive impairment, both stemming from metabolic syndrome. In this study, the age-dependent structural and functional changes in the brains of eNOS-deficient mice were re-examined.

Brains of Partial eNOS-Deficient Mice Display AD-Like Pathology

Sporadic cerebral amyloid angiopathy (CAA) is a cerebrovascular disorder that is characterized by β -amyloid ($A\beta$) deposits in the walls of small- to medium-sized blood vessels of the brain and leptomeninges. CAA is a major cause of lobar intracerebral hemorrhage and cognitive impairment in the elderly and is associated with a high prevalence of white matter hyperintensities and cerebral microbleeds, the two hallmarks of CSVD.⁶¹ Because varying degrees of CAA are present in nearly all brains with sporadic AD, it is reasonable to posit that a common $A\beta$ -based pathogenesis and comorbidity underlie both VCID and AD.

eNOS-derived NO signaling plays an important role in regulating the processing of amyloid precursor protein into amyloid peptides.⁶² On the basis of these observations, we speculated that some form of amyloid pathology may be directly linked to vascular dyshomeostasis seen in mice with insufficient eNOS gene expression. Consistent with this notion, increased amyloid load in eNOS-deficient mouse brains has been observed.^{63,64} Since eNOS^{+/-} mice were assumed to better mimic patients with eNOS polymorphisms, age-dependent changes in the brains of eNOS^{+/-} mice were compared with the brains of wild-type and eNOS^{-/-} mice. Key central nervous system anomalies seen in eNOS knockout mice also developed in the brains of mice with eNOS haploinsufficiency. However, the pathologic changes in the brains of eNOS^{+/-} mice were delayed by several months and were significantly milder, especially

during the first 12 months of their life span; the phenotypes were often not significantly different between eNOS^{+/-} and eNOS^{-/-} after 18 months of age.

A myloid pathology was detected with diffused β -amyloid deposits in and around the cerebral vasculature in eNOS^{+/-} mice (Figure 1, A-C). To our knowledge, this is the first report of spontaneous development of amyloid pathology in mice that do not overexpress a mutant amyloid precursor protein or presenilin gene. $A\beta$ aggregation, as detected by thioflavin S staining, revealed only a diffusive signal, predominantly in the parietal region of eNOS^{+/-} mouse brains. This pattern of $A\beta$ aggregation is in stark contrast to the development of focal plaques seen in the brains of 5XFAD mice.⁶⁵ This diffusive nature of the CAA in eNOS^{+/-} mice seems to reflect a distinctly mild form of CAA in the majority of AD; advanced CAA is only detected in 25% of AD cases in humans.⁶³ Moreover, brains of AD + VCID patients display diffusive amyloid pathology instead of dense and focal amyloid parenchymal plaques seen in the brains of typical AD cases.⁶⁰ In addition to CAA, microbleeds and microinfarctions are detected in multiple brain regions of eNOS^{+/-} mice.⁶⁵ Using fluorescein isothiocyanate (FITC) angiography, multiple hypoperfused or nonperfused areas were detected in young eNOS^{+/-} mice (Figure 1D), and with markedly increased frequency of similar lesions in the brains of aged eNOS^{+/-} mice (Figure 1F). These lesions/occlusions were typically small (ranging from 100 to 500 μ m in diameter) and were frequently found bilaterally in the parietal association cortex (Figure 1E). This pathologic pattern is highly reminiscent of the bilateral temporoparietal hypoperfusion characteristic of AD patients. Occlusions were not uniformly located throughout the eNOS^{+/-} brain, but rather in defined areas, listed in order of higher to lower frequency: parietal association, temporal association, and retrosplenial granular cortices, hippocampus, and thalamus. No such lesions were detected in the wild-type eNOS littermates up to 24 months of age. As reported earlier,⁶⁵ the CAA pathology in eNOS^{+/-} mice displayed striking colocalization with hypoperfused areas of the brain, primarily in the parietal cortex. Of note, the predominant distribution of colocalized clusters in temporal-parietal cortex seen in the brains of eNOS^{+/-} mice precisely match the most vulnerable areas of hypoperfusion (detected by neuroimaging in early AD patients).⁶⁶⁻⁶⁸

Despite colocalization of the occluded vessels and CAA, its contribution to the process of neurodegeneration in our eNOS^{+/-} model remains elusive. This mechanistic quandary is further highlighted by the fact that endogenous murine $A\beta$ species are less aggregable and thus less toxic than their human counterparts. It is also noteworthy that focal $A\beta$ plaques or neurofibrillary tangles were not observed, despite the occurrence of tau hyperphosphorylation, even at 24 to 32 months of age in partial eNOS-deficient or knockout mice. This is even more

Figure 1 Evidence of cerebral hypoperfusion, microinfarction, and amyloid pathology in endothelial nitric oxide synthase (eNOS) model. **A** and **B**: Representative section of 18-month-old eNOS^{+/-} mice doubly immunostained with either anti- β -amyloid (A β ; 4G8; **A**) and glucose transporter-1 (GLUT1; **B**) plus DAPI or 4G8 and CD31 antibodies. **C**: Quantification of A β -positive arterioles in hippocampal fissure. **D** and **E**: Representative images of fluorescein isothiocyanate-dextran (2000 kDa) angiography performed in eNOS^{+/-} mice at young (6 months old; **D**) and old (18 months old; bilateral section; **E**) ages, as described previously.⁶⁵ **White double arrows** indicate bilateral nonperfused lesions located in parietal association cortex (**asterisks**), retrosplenial granular cortex (**daggers**), and temporal association cortex (**arrowheads**). **F**: Quantification of the number of cerebral nonperfusion lesions per mouse. **G–I**: Representative images of hematoxylin and eosin staining (6 μ m thick coronal sections). **Blue arrow line** indicates magnified fresh infarct lesion, and **blue arrows** indicate old lesions reminiscent of infarct scars. Notes: Figure is modified from the published figures used in Tan et al,⁶⁵ used with permission from Springer Nature and BMC. Data represent means $\pm$ SEM (**F**). $n = 5$ to 6 mice for each genotype (**C**); $n = 4$ to 6 mice for each genotype (**F**). * $P < 0.05$, ** $P < 0.01$. Scale bars: 300 μ m (**A**); 50 μ m (**B**); 100 μ m (**D** and **H**, right panel); 500 μ m (**E**, **G**, **H**, left panel, and **I**).

significant in light of the finding that the brains of aged eNOS^{+/-} mice show obvious cortical thinning and atrophy, clear signs of neurodegeneration.⁶⁵ Because of the lack of these two major pathologic manifestations of terminal AD, other cerebrovascular features commonly shared by AD and VCID, especially a putative involvement of CSVD in the brains of eNOS^{+/-} mice, were explored.

Current knowledge of the pathophysiological roles of eNOS-derived NO in vascular functions is primarily based on studies in young eNOS knockout mice. Therefore, older mice (eNOS^{+/-} and eNOS^{-/-}) were investigated in the current study. As previously reported,⁶⁵ 18-month-old eNOS^{+/-} mice displayed severe learning and memory deficits, as assessed by the water maze test, while no significant difference was observed between older eNOS^{+/-} and eNOS^{-/-} mice (F.F.L., unpublished data). These behavioral data are consistent with the report⁶³

showing that late middle-aged eNOS knockout animals (14 to 15 months old) commit more errors than their wild-type littermates in an eight-arm radial maze test. In addition, there were no differences in locomotor activity between all three genotypes of eNOS mice, regardless of their ages. Interestingly, younger eNOS heterozygous and knockout mice (<12 months of age) displayed slightly increased anxiety. On the basis of the well-established crucial roles of NO/eNOS in mediating various forms of neocortical and hippocampal long-term potentiation,³⁹ it is generally believed that impaired long-term potentiation would be paralleled by memory impairments measured by the water maze learning test. An independent group reported seemingly contradictory results where young adult eNOS knockout mice showed superior place learning and performance in water maze but unaltered performance in radial arm maze.^{69,70} Moreover, these eNOS knockout

mice showed increased anxiety in the elevated plus maze and the open field test of anxiety. The learning tasks in these testing paradigms were speculated to depend on different neurotransmitters. eNOS knockout mice used in the aforementioned studies were generated by disrupting the exons 24 and 25,⁴⁴ whereas the eNOS knockout mice were used in prior studies^{63,65} were generated by disrupting the exon 12 (42, B6.129P2-*Nos3^{tm1Unc}/J* on a pure C57bl/6 background; JAX stock 002684). It is unclear why NOS3 gene ablation, achieved by targeting different exons, would lead to different cognitive behavioral phenotypes. This is somewhat puzzling because all three lines of eNOS knockout mice display similar phenotypes in terms of synaptic or cardiovascular dysfunctions.^{42–44} These seemingly inconsistent behavioral data underline the importance of age, sex, and strain differences with respect to evaluating a role of eNOS in learning and memory.

Partial eNOS-Deficient Mice Display Key Features of CSVD

Alteration of the arteriosclerosis-dependent brain perfusion is considered to be a major determinant of CSVD. Because cerebral hypoperfusion and cerebrovascular pathology occur before the emergence of functional deficits in the brain, as deduced by neuroimaging and other experimental techniques,⁷¹ a more detailed characterization of younger eNOS-deficient mice was performed. Regions of hypoperfused brain and microvascular occlusion appear in eNOS^{+/-} mice by 3 to 6 months of age.⁶⁵ Hematoxylin and eosin staining of brain sections revealed lesions with markedly reduced cellularity and necrosis in the core in eNOS^{+/-} mice (Figure 1H), as compared to littermate control mice (Figure 1G). These lesions met all criteria required by the accepted definition of microinfarcts: sharply delineated microscopic regions of cellular death or tissue necrosis, often with cavitation.⁷² Moreover, different stages of microinfarcts based on hematoxylin and eosin histology were detected in eNOS^{+/-} brain at various ages; the multiple pale lesions detected in older mice most likely corresponded to chronic or late-stage infarcts (Figure 1I), presumably with damaged tissue phagocytized. No similar lesions at any stage were detected in eNOS^{+/+} mice up to 24 months of age.

To determine the cause(s) of vascular obstruction, of fibrinogen/fibrin immunohistochemistry was used to detect the presence of intravascular microthrombi in multiple cortical and subcortical regions, blocking the vessels in aged eNOS^{+/-} mice (Figure 2, A and B) and, to a lesser degree, in young eNOS^{+/-} mice. Most of the intravascular thrombi were much smaller than the lumen of the arterioles and were not washed out by transcardial perfusion used for the preparation of tissues before histochemistry. Thus, the *in situ* mural thrombi most likely represent thrombosis rather than thromboembolism.

BBB Leakage Is One of the Earliest Pathologies Detected in Partial eNOS-Deficient Mice

BBB leakage was observed in eNOS^{+/-} mice in age-dependent manner based on FITC–dextran angiography as well as quantitative mouse IgG extravasation (Figure 2, C–E).⁶⁵ Due to the sensitivity of FITC–dextran (150 kDa), the only quantifiable leakage detected in eNOS^{+/-} brain was at <6 months of age. Use of FITC–dextran of a lower molecular weight range (3 to 30 kDa) as a tracer failed to indicate fluorescence in the brain, presumably due to rapid systemic clearance. Evans blue dye was subsequently used to determine the youngest age at which BBB leakage occurs in the brains of eNOS-deficient mice. Evans blue (980 Da) is an azo dye that has a high affinity for serum albumin (67 kDa). Because serum albumin cannot cross the BBB and virtually all Evans blue is bound to albumin, in case of compromised BBB, albumin-bound Evans blue enters the parenchymal tissue in central nervous system. Because Evans blue fluoresces with excitation peaks at 470 and 540 nm and an emission peak at 680 nm, it can be detected in brain sections under fluorescent microscope using the rhodamine filter.

BBB leakage was detected as early as 4 months of age in eNOS^{+/-} mice (Figure 2F), and in even younger eNOS knockout mice (Figure 2G, H), while no leakage was detected in WT control mice at 26 months of age (Figure 2I). BBB leakage primarily occurred in the frontal and parietal cortex (ie, 2.0 to –2.0 mm as to bregma) of the eNOS-deficient mice; as mice aged, BBB leakage expanded to deeper brain areas (eg, temporal-parietal cortex and hippocampus). Of note, the areas of BBB leakage correlated spatially with hypoperfused tissues and CAA clusters in parietal cortex of young eNOS^{+/-} mice. Because of the well-recognized roles of eNOS-mediated signaling in angiogenesis and vasculogenesis during development as well as in vascular permeability (eg, modulating vascular endothelial growth factor signaling), it is not surprising to observe hypoperfusion and compromised BBB integrity at this young age. However, whether the BBB damage is a result of the intrinsic hypoperfusion in eNOS model remains to be determined.

Middle-Aged eNOS-Deficient Mice Elicit Severe White Matter Pathology, Neurodegeneration in Cortical Layers II/III and V, and Gait Disturbances

In contrast to the extensively studied gray matter, which contains the cell bodies of neurons (somata), the relatively understudied white matter consists mostly of glial cells and myelinated axons encompassing the largest and deepest parts of the brain. Long-range myelinated axons are crucial for transmitting electrical nerve signals among distant brain nuclei and are thus required for the formation of proper

Figure 2 Evidence of age-dependent blood-brain barrier leakage and thrombosis in endothelial nitric oxide synthase (eNOS) model. **A:** Evidence of thrombosis. Representative images of fibrinogen/fibrin (red) and/or CD31 (green) immunohistochemistry in the brain sections of 18-month-old $eNOS^{+/-}$ mice. **Boxed areas:** **White arrows** indicate individual fibrinogen clots inside the arterioles. **B:** Western blot of eNOS forebrain lysate probed with anti-fibrinogen antibody. Quantification was done on the basis of three mice per genotype, run three times. P value obtained by unpaired t -test. **C:** Representative images of anti-mouse IgG immunohistochemistry with DAPI counterstaining show parietotemporal cortical vascular extravasations of mouse serum IgG (green) in old $eNOS^{+/-}$ mice. Mice were perfused transcardially, vibratome sectioned (60 μ m thick), followed by immunostaining with Alexa 488–goat anti-mouse IgG antibody. **White arrows** in the **right panel** indicate leaky vascular areas. **D:** Quantification of extravasated mouse IgG based on anti-mouse IgG immunoblot analysis from mice after perfusion. Note less expression of β -actin in hippocampus (Hipp) than in cortex (Cort). Values are normalized to β -actin of 20 μ g total proteins. **E:** Representative image of FITC–dextran angiography taken from 18-month-old $eNOS^{+/-}$ mice. Angiography taken 60 minutes after injection of FITC–dextran (150 kDa), showing diffusive fluorescent signal as an indication of leaky vessels surrounding the occluded area located in parietal association cortex. **F–I:** Representative images of Evans blue angiography taken in young $eNOS^{+/-}$ mice taken at 1.85 to 2.0 mm bregma. Evans blue was injected in 200- μ L volume through mouse tail vein. Mice were euthanized 5 minutes later without perfusion, and whole brains were removed and vibratome processed to 100- μ m serial sections for fluorescent imaging using rhodamine filter. Notes: Figures in **A** and **C** through **E** are modified from the published figures used in Tan et al.,⁶⁵ used with permission from Springer Nature and BMC. Data are expressed as means $\pm$ SEM (**D**). $n = 4$ animals for each genotype (**D**). $*P < 0.05$. Scale bars: 500 μ m (**A**, left panel); 50 μ m (**A**, right panels); 100 μ m (**C**, left panel); 20 μ m (**C**, right panel); 200 μ m (**E**); 300 μ m (**F–I**).

neuronal circuits and their function.⁷³ White matter has received increasing attention as a locus of heightened injury in dementia over the last decade. White matter hyperintensities are an invariant pathologic feature of CSVD,^{23–27} and manifest as loci of demyelination and vascular degeneration at the histopathologic level. As the specialized myelin-producing cells in the central nervous system, oligodendrocytes have also received much attention in recent years as a new player in neuroglial vascular unit.⁷⁴ Cells at different stages of oligodendrocyte lineage have been differentiated by distinct molecular markers detectable by flow cytometry analysis.⁷⁵

White matter pathology was examined in eNOS-deficient mice at various ages. While Luxol fast blue staining

indicated myelin loss in $eNOS^{+/-}$ brains at 12 months of age (**Figure 3**, A and D), more severe loss was detected in $eNOS^{-/-}$ brain at the same age. Quantification of immunostaining with an antibody against myelin-binding protein indicated significant myelin loss in the cortex (**Figure 3**, G–I) and corpus callosum (CC) (**Figure 3**, J–L), but not in the striatum in $eNOS^{+/-}$ mice (data not shown). Thinning of the CC, the largest white matter tract in the mouse brain, was found at the older age of 24 months. Reduced numbers of mature oligodendrocytes ($GalC^{+}/O4^{+}/MOG^{+}$)⁷⁵ were detected by both immunohistochemistry and flow cytometry. Concomitantly, significantly enhanced expression of oligodendrocyte progenitors ($A2B5^{+}/PDGFR\alpha^{+}$)⁷⁵ was seen at the onset of disease (12 months of age), suggesting

LFB-Nissl stain: eNOS^{+/+}

MBP + NF-200 stain

MBP stain

an attempt by the degenerating oligodendrocytes to repair myelin. Moreover, reduced immunostaining of both oligodendrocyte transcription factor 1 (oligo1) and the mature somatic marker CC1, which label immature and functionally mature oligodendrocytes (the neuroglial cell type responsible for myelin production), respectively, was also indicated. No significant changes were observed in the number of endothelial cells, astrocytes, or microglia (F.-F.L., unpublished data).

Luxol fast blue–Nissl double staining also revealed disorganized cortical structure with signs of severe neurodegeneration in cortical pyramidal neuronal layers II/III and V, as evident from the presence of an empty vacuole (Figure 3E and F, as compared to Figure 3B, C), suggestive of loss of myelin sheath. Of note, similar neurodegenerative pathology is detected in superficial cortical layers II/III and V in clinical specimens of both early demented AD⁷⁶ and post-stroke brains.⁷⁷ Markedly elevated levels of not only oxidative stress (reactive oxygen species) by dihydroethidium staining, most prominently in the parietal cortical region, but also astrogliosis (glial fibrillary acidic protein) were observed, specifically around the degenerating neurons in the cortical layers II/III and V. However, there was no significant increase in Iba-1–positive microglia in the brains of 12-month-old eNOS^{+/-} mice. In addition, gait deficiencies were detected in eNOS-deficient mice (both eNOS^{+/-} and eNOS^{-/-}) at this age, determined by significantly impaired performance during the catwalk testing. We posit that these motor deficits may be caused by loss of sensory neuronal function in cortical layer V. No hippocampus-dependent spatial memory deficits were detected by 12 months of age, as determined by cross maze and water maze tests (F.-F.L., unpublished data). Because the corticohippocampal circuits have long been established to be crucial for memory,⁷⁸ the demyelinated axons in white matter at early age was expected to negatively impact the cortical, and ultimately, hippocampal neurodegeneration. This hypothesis is being tested by *in vitro* and *in vivo* electrophysiology in eNOS model. Furthermore, disrupted axonal integrity has been reported by cerebral hypoperfusion⁷⁹; it remains to be determined if impaired cortical impulse axonal conduction is present in eNOS^{+/-} mice at a young age. Impaired axonal impulse conduction is likely to occur in these animals based on the presence of obvious demyelination in their brains. In light of the recent seminal studies that have demonstrated crucial roles of pericyte/BBB in white matter health³³ and in neuronal circuit regulation,⁸⁰ pericyte-

mediated events seen in the brains of eNOS^{+/-} mice were explored.

Potential Molecular Mechanisms Linking Hypoperfusion and BBB Leakage with White Matter Pathology

Members of transforming growth factor- β superfamily, which include bone morphogenetic proteins (BMPs), are implicated in aging and cardiovascular diseases,⁸¹ as well as in the AD,⁸² and various forms of CSVD.⁸³ A mutation in *HtrA1* gene, encoding a serine protease that represses signaling by transforming growth factor- β family members, has been associated with a major cause of an inherited form of CSVD—cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy.⁸⁴ The BMPs, the largest subfamily of the transforming growth factor- β superfamily, have recently been postulated to be involved in CSVD pathogenesis.⁸⁵ BMP4 signaling has been widely reported as a key negative regulator of neurogenesis and brain development, adult hippocampal neurogenesis, astrogliogenesis,⁸⁶ and oligodendrocyte differentiation.⁸⁷ Expression of BMP4, BMP6, and BMP7 is up-regulated after acute injury in areas of white matter damage in experimental autoimmune encephalomyelitis models.⁸⁸ Blocking BMP signaling by the pan-inhibitor Noggin or other compounds promotes remyelination in various experimental models. This strategy is currently considered a promising treatment in multiple sclerosis.⁸⁹ It remains unclear whether this strategy will also benefit BBB integrity and restore white matter functions in the eNOS^{+/-} mouse model.

BMP4 is up-regulated in the brains of patients with vascular dementia as well as in the hypoperfused brain of mice undergoing bilateral common carotid artery stenosis.⁸³ Pericytes are proposed to be the source of up-regulated BMP4. Interestingly, up-regulated BMP4 gene expression was detected on the isolated microvessels from eNOS^{+/-} mouse brain at young age (7 months of age) (F.-F.L., unpublished data). More importantly, no significant white matter changes were detected at this young age; loss of CC1-positive mature myelin-producing oligodendrocytes occurred only around 12 months of age. Unfortunately, the immunohistochemistry signals of BMP4 could not be optimized in spite of using multiple commercial antibodies. As an alternative approach, BMP4 reporter mice with fusion of the cyan fluorescent protein gene (CFP) under mouse BMP4

Figure 3 Myelin loss in endothelial nitric oxide synthase (eNOS) model at 12 months of age. **A–F:** Representative images of Luxol fast blue (LFB) and Nissl double-stained sections (coronal; 16 μ m thick) of brains of eNOS^{+/-} and control littermates in whole brain (**A** and **D**) and frontal cortical regions (**B**, **C**, **E**, and **F**). **B:** Red double arrows and the corresponding Roman numerals indicate cortical layers I through V. **G** and **H:** Double-staining immunohistochemistry on myelin-binding protein (MBP; red) and NFP-200 (green). **I:** Quantification of MBP immunofluorescence signals in the cortical region based on three pairs of eNOS^{+/-} and control mice. **J** and **K:** Representative images of MBP immunohistochemistry. The dashed white lines (upper left) delineate the cortical surface. **L:** Quantification of MBP immunofluorescence signals in the corpus callosum region based on three pairs of eNOS^{+/-} and control mice. **P* < 0.05, ***P* < 0.01. Scale bars: 1 mm (**A** and **D**); 150 μ m (**B** and **E**); 40 μ m (**C** and **F**); 50 μ m (**G** and **H**); 500 μ m (**J** and **K**).

Figure 4 Mechanistic hypothesis and supporting evidence. **A–C:** Up-regulated bone morphogenetic protein 4 (BMP4) distribution on blood-brain barrier (BBB) predominantly on descending arterioles in frontal-parietal cortex of endothelial nitric oxide synthase (eNOS)—deficient mice at young age. **A:** Microscopic image of BMP4-CFP reporter mice on eNOS^{+/+} and eNOS^{+/-} background (5 to 6 months of age) using wide DAPI filter. **B** and **C:** Quantification graphs are based on the BMP4-CFP—positive cells (**B**) and on the fluorescent intensity of the positive cells (**C**). **D:** Approximate locations of the occluded lesions and cerebral amyloid angiopathy (CAA; white clouds) and BBB leakage (yellow staggers) in young eNOS^{+/-} brain; fluorescent image of hemispheric cerebrovasculature shown was taken from young mouse brain with Pdgfrb-positive cells labeled in tdTomato; PDGFRβ—tdTomato mice were derived from crossing between Pdgfrb—Cre with Ai14 (JAX stock number 007914). Pdgfrb: Platelet-derived growth factor receptor beta. **E:** Schematic diagram depicts the multiple pathologic events occurring along the life span of eNOS^{+/-} mice. P0: postnatal day 0. We hypothesize that the up-regulated BMP4 is an initial critical pathologic factor derived from BBB, leading to BBB leakage/pericyte degeneration, elevated generation of reactive oxygen species (ROS), astrogliosis, and oligodendrocyte lineage changes that underpin the white matter pathology; these pathologic sequelae are followed by subcortical and ultimately hippocampal neurodegeneration in aging eNOS^{+/-} mice. *n* = 3 each for genotype (**A**). ***P* < 0.01, ****P* < 0.001. Scale bar = 100 μm (**A**). Amyg, amygdala; Hippo, hippocampus.

promoter⁹⁰ were used to image BMP4 changes under conditions of acute and chronic hypoperfusion. In BMP4-CFP reporter mice crossed with eNOS knockout mice, up-regulated CFP signal was detected in young mice with partial eNOS deficiency: **Figure 4A** shows fluorescent pattern of a string of beads, possibly along the descending arterioles in frontal-parietal cortex. Because the CFP expression was under the BMP4 promoter, which was restricted to cell nuclei, it made it convenient to count the number of BMP4-CFP—positive cells (**Figure 4B**). BMP4-CFP—positive cell number increased by severalfold in young eNOS^{+/-} mice (**Figure 4C**). Similarly, microinjection of L-NIO (a selective eNOS inhibitor) into the subcortical region (CC) also induced BMP4 signals locally 24 hours later (F.-F.L., unpublished data), suggesting a link between impaired NO signaling and BMP expression. Microinjection

of L-NIO into the subcortical region has been reported to induce white matter demyelination.⁹¹ The identity of the cell type(s) with up-regulated BMP4 expression on hypoperfusion is currently under investigation. Nevertheless, in most experimental cases studied, BMP4 signaling is known to act in a non—cell-autonomous and paracrine manner.

Because both the BBB leakage and the up-regulated BMP4 in microvessels occur at young age in eNOS^{+/-} mice, it is unclear whether the up-regulated BMP4 is directly involved in BBB breakdown (**Figure 4D**). As summarized in **Figure 4E**, we hypothesize that up-regulation of BMP4 is an early molecular event that triggers BBB dysfunction in the brains of eNOS^{+/-} mice, before demyelination. Advanced mouse imaging tools (ie, BMP4 and pericyte reporter mice) are anticipated to help unambiguously determine the spatial and temporal events of BMP4

up-regulation, including the responsible cell type(s), in relation to BBB leakage and pericytic changes in the eNOS model. We also speculate that the impaired NO signaling is the key factor underlying early pathologic events in the eNOS^{+/-} mouse model, and have compelling data supporting this theory. Feeding eNOS-deficient mice sodium nitrate in drinking water for 6 to 8 weeks to restore NO signaling and vascular homeostasis,^{92,93} not only rescued BBB leakage and prevented BMP4 up-regulation, it also restored myelin levels in white matter (F.-F.L., unpublished data). On the basis of these observations, we posit that regardless of the cellular source for BMP4, its known paracrine actions could directly trigger white matter degeneration via promoting astrogliosis and interfering with oligodendrocyte differentiation.^{84,85} Although pericyte loss/BBB disruption has emerged as an early biomarker of cognitive deficits in patients, strategies to repair the dysfunctional BBB are limited.⁹⁴ Further characterization of BMP4 signaling cross talk between microvessels and neurovascular units and the elucidation of gene networks involved in these interactions in our eNOS model are likely to unravel novel therapeutic interventions that will preserve integrity of BBB and optimal functionality of the white matter.

Comparison with Other Models of Chronic Hypoperfusion

Currently, there are more than a dozen rodent models that develop major features of human CSVD.⁹⁵ Systematic reviews of these models reveal five major categories: i) embolic injuries; ii) hypoperfusion-based injuries (bilateral common carotid occlusion/stenosis, subcortical injection of L-NIO, or striatal injection of endothelin-1); iii) hypertension-based injuries; iv) blood vessel damage-based injuries; and v) diet-induced hemorrhage/microbleed (eg, hyperhomocysteinemia). None of these experimental models elicits all features of the human disease. The optimal choice of a model depends on the aspect of pathophysiology being studied.

Over the last decade, bilateral common carotid occlusion/stenosis has been the most widely used model of chronic hypoperfusion. This bilateral common carotid occlusion/stenosis surgery in both rats and mice recapitulates several major white matter changes, including reduced parenchymal cerebral blood flow, oligodendrocyte loss and glial activation, disrupted clearance of intracerebral fluid and BBB, and cognitive impairment.^{96,97} However, there are two shortcomings of bilateral common carotid occlusion/stenosis models: large intrinsic variance in cerebral hypoperfusion for each animal and a sudden and severe blood flow reduction; all pathologic events occur relatively quickly (within 2 to 3 months after the induction of hypoperfusion). Although none of the bilateral common carotid occlusion/stenosis studies went beyond age of 12 months, likely

because of higher mortality in older animals, it is difficult to identify the earliest causative events using these models.

Chronic hypertensive rodent models display several key features of CSVD seen in hypertensive patients who develop SVD with age. The stroke-prone spontaneously hypertensive rat is the most widely used model. Evidently, these rats are genetically susceptible to stroke independent of severe hypertension. Studies on these rats may therefore provide useful information about the genetic underpinnings of particular types of cerebrovascular disease, such as lacunar infarction and intracerebral hemorrhage.⁹⁸ In our opinion, heterozygous eNOS-deficient mice brains more closely mimic the sequelae of silent infarcts. To our knowledge, this is the first age-dependent spontaneous model linking chronic hypoperfusion and demyelination directly to neurodegeneration and thus represents an ideal model for studying mechanisms underlying degeneration of BBB and white matter. However, because of the sporadic nature of microinfarctions in this model, it is difficult to determine the degrees of hypoperfusion; magnetic resonance imaging did not detect significant cortical microinfarctions, possibly because microinfarcts in mice become more difficult to discern by structural/diffusion imaging in the subacute to chronic stage of injury.⁹⁹ It remains to be determined if chronic and potentially mild hypoperfusion can be detected with more advanced imaging module, such as the arterial spin labeling magnetic resonance perfusion technique.

Concluding Remarks and Perspective

A unique age-dependent spontaneous mouse model with aspects of VCID pathology. Mice deficient in eNOS expression, both partially (eNOS^{+/-}) or completely (eNOS^{-/-}), represent a unique spontaneous model for studying pathologic mechanisms induced by chronic hypoperfusion. The heterozygous eNOS mice develop all phenotypes identified in knockout mice, but at an older age (3 to 4 months older). Moreover, studies examining eNOS mice of various ages led to the identification of early molecular and cellular mechanisms involved in cerebrovascular pathology of these animals. Of note, several early pathologic events we identified (eg, nonperfusion, BBB leakage, and BMP4 up-regulation on descending arterioles) were all first detected in frontal cortex, which subsequently progressed to parietal and temporal cortices as mice age. This pattern appears to mirror to a large degree the patterns detected in AD patients,⁶⁶⁻⁶⁸ presumably because of intrinsic vessel architecture (eg, watershed areas).

An ideal model for studying CSVD in both gray and white matter. Partially eNOS-deficient mice spontaneously developed chronic cerebral hypoperfusion in multiple areas that precisely matched the most vulnerable areas of hypoperfusion seen in early dementia patients. These areas also demonstrated elevated reactive oxygen species and

neuroinflammation, microbleeds, CAA in middle-aged mice, followed by hippocampal neurodegeneration in older mice. Moreover, BBB leakage and striking demyelination and oligodendrocyte loss in parietal cortical areas of these mice occur at young-middle age; these pathologic manifestations are also accompanied by marked astrogliosis and selective loss of pyramidal neurons in cortical layers II/III and V. Interestingly, in this mouse model, neurodegeneration was first observed in the cortical pyramidal neurons, before the deeper hippocampal neuronal change. This is consistent with the sequence of the pathologic changes in dementia patients with AD and vascular dementia (eg, after stroke).

Limitations of studying mechanisms of white matter pathology in rodents. Despite the fact that rodent models are widely used in dementia research, there are two main shortcomings of using brains of rodents as proxies of human brains (ie, the white matter content of rodent brains is considerably smaller, and there are significant differences in the cortical microvasculature between rodents and humans). While there are more penetrating arterioles than penetrating venules in the human cortex, it is the opposite in rodents.¹⁰⁰ The microvascular architecture that supports white matter likely also differs between humans and rodents but remains to be investigated in detail. Finally, there are major differences in the white matter tracts that are commonly studied in rodents and humans. Although most studies in mice focus on the corpus callosum or external capsule, the equivalent juxtacortical tracts in the human brain correspond to superficial white matter fibers.

Mechanistic hypothesis: Given an increasingly appreciated role of pericytes in maintaining the functional integrity of the BBB and the neurovascular unit, and in the regulation of capillary blood flow, up-regulated BMP4 is proposed to be the initial critical pathologic factor acting on other cell types within the neurovascular unit. We theorize that this aberrant BMP4 signaling leads to BBB leakage, elevated generation of reactive oxygen species, astrogliosis, and oligodendrocyte lineage changes that underpin white matter pathology; these pathologic sequelae are followed by subcortical and ultimately hippocampal neurodegeneration in aging eNOS^{+/-} mice. Therefore, normalization of BMP4 signaling may be a potential therapeutic strategy for preventing age-dependent white matter pathology and neurodegeneration.

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Author Contributions

R.R., F.-M.Z., and A.Y.S. contributed to the framework of this review. F.-F.L. wrote and R.R. and A.Y.S. edited the review. X.-L.T., G.L., X.C., L.C., and W.Z. performed most of the experiments. All authors read and approved the final manuscript.

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