White Matter Damage as a Consequence of Vascular Dysfunction in a Spontaneous Mouse Model of Chronic Hypoperfusion with eNOS Deficiency

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Abstract

Background: Vascular cognitive impairment and dementia (VCID) is the second most common form of dementia after Alzheimer's disease (AD). Currently, the mechanistic insights into the evolution and progression of VCID are not fully understood. White matter change represents an invariant feature of both VCID and AD. Compelling clinical neuroimaging and pathological evidence suggest a link between white matter changes and neurodegeneration. Our prior study detected non-perfusion lesions in mice with partial deficiency of endothelial nitric oxide (eNOS) expression at a very young age. These lesions developed in multiple brain regions in an age-dependent manner, precisely matching to those hypoperfused areas identified in preclinical AD patients (i.e., temporoparietal and retrosplenial granular cortexes, and hippocampus). We therefore reasoned that eNOS- deficient mice could serve as a spontaneous model of chronic hypoperfusion.

Methods/Results: White matter tracts are particularly susceptible to the vascular damage induced by chronic hypoperfusion. Using immunohistochemistry, we detected massive demyelination in the middle aged eNOS-deficient mice. The demyelinated areas were confined to cortical and subcortical areas including the corpus callosum and hippocampus, but did not involve the striatum. The intensity of demyelination correlated with behavioral gait deficits. By Evans blue angiography, we detected blood-brain barrier (BBB) leakage as another early pathological change affecting frontal and parietal cortex in eNOS-deficient mice, which occurs in as early as 3-4 months of age. Sodium nitrate fortified drinking water provided to young and middle aged eNOS-deficient mice completely prevented non-perfusion, BBB leakage, and white matter pathology, indicating that impaired endothelium-derived NO signaling may have caused these pathological events.

Conclusions: Using eNOS-deficient mice, we identified BBB breakdown and non-perfusion as the two earliest pathological events, resulting from insufficient vascular NO signaling. We speculate that the compromised BBB and chronic hypoperfusion trigger vascular damage, along with oxidative stress and astrogliosis, accounting for the white matter pathological changes in the eNOS-deficient mouse model. We conclude that eNOS-deficient mice represent an ideal spontaneous evolving model for studying the earliest events leading to spontaneous white matter changes, which will be instrumental to future therapeutic testing of drug candidates and for targeting novel/specific vascular mechanisms contributing to VCID and AD.

Background

Vascular cognitive impairment and dementia (VCID), is the second most common form of dementia in the elderly after Alzheimer's disease (AD) [1] and is caused by insufficient blood supply to the brain. The prevalence of mixed dementia with clinical and pathological features of both VCID and AD is increasing; cerebrovascular pathology is present in at least half of all dementia cases [2–4]. VCID encompasses a broad spectrum of cerebrovascular-driven cognitive impairment, from mild cognitive impairment to fully developed dementia. This disease state is often further complicated by genetic factors (ApoE4),

metabolic disorders (hypertension, type 2 diabetes, high cholesterol, hyperhomocysteinemia) and lifestyle factors (smoking, inactivity, excessive alcohol consumption) [5–7]. These risk factors often result in chronic cerebral hypoperfusion and microvascular pathology [8–11]. They are the most common pathologies that co-occurs in VCID and AD [12, 13]. Although there are several good models to stimulate individual aspects of VCID [e.g., *in vitro* models of the neurovascular units, surgical models of chronic cerebral hypoperfusion induced by bilateral common carotid artery stenosis (BCAS), animals with NOTCH3 mutations as a model of small vessel disease], the heterogeneity of the disease states limits any of them from being a perfect model of all aspects of the disease [14, 15]. Therefore, there is a pressing need to develop representative animal models to better understand the underlying mechanisms and identify preclinical biomarkers, prevention and treatment strategies.

Our prior work based on mice partially deficient in expressing endothelial nitric oxide (eNOS) gene has identified a potentially excellent model for studying the contribution of vascular dysfunction to dementia [16]. Endothelial eNOS-deficient mice spontaneously develop multiple hypoperfused/occluded areas, matching the most vulnerable areas of hypoperfusion in early AD patients [17–19]. Moreover, these mice also display microinfarctions, microbleeds, cerebral amyloid angiopathy, and hippocampus-dependent neurodegeneration in an age-dependent manner. However, another invariant feature of VCID patients, namely, white matter pathology, was not studied previously.

White matter health is of paramount importance to normal cognitive performance [20]; the recent comprehensive transcriptomic study on AD forebrain using single-cell RNA sequencing on different cell types revealed that genes associated with the myelination pathways are the most changed group correlated with early stages of disease progression [21]. White matter tracts are a region at heightened risk for vascular damage [22]. The exact molecular and cellular mechanisms of white matter changes are unclear. Chronic hypoperfusion, blood-brain barrier (BBB) breakdown, oxidative stress, and inflammation have all been postulated as potential mechanisms [23–26]. In this study, we present data on all these aspects of cellular events concerning white matter changes in the eNOS-deficient mouse model.

Methods

Materials And Methods

Animals

Mice deficient in eNOS gene expression (eNOS^{-/-}, NOS3^{tm1Unc}/J Jackson Laboratory, Bar Harbor ME) were crossbred with C57BL/6 mice to obtain eNOS^{+/-}. Animals eNOS^{+/-}, eNOS^{-/-} and littermate eNOS^{+/+} mice were derived from interbreeding between eNOS^{+/-} mice. Littermate mice were genotyped by genomic PCR using tail biopsy samples. For sodium nitrate (SN) feeding, NaNO₃ (S5506, Sigma, St. Louis, MO, USA) was added to the drinking water at a concentration of 85 mg·L⁻¹ (1 mM) [27]. All mice were housed and aged in well-ventilated cages under standard laboratory conditions on a 12:12 hour light-dark cycle with food and water ad libitum. All experimental animal procedures were conducted in

accordance with the animal care standards of the National Institute of Health and were approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center.

Immunohistochemistry

Mice were perfused transcardially with ice-cold phosphate buffered saline (PBS) followed by ice-cold 4 % paraformaldehyde (PFA) solution in PBS. Mouse brains were then removed from the cranium. After post-fixation in 4 % PFA in PBS at 4°C overnight, brains were either processed for OCT-embedding after passing through 20 % to a final of 30 % sucrose solution prepared in PBS for 36–48 h at 4°C until fully sank or transferred to PBS for vibratome sectioning of “fresh” tissue. Brains were then cut into serial coronal sections by Cryostat (CM1900; Leica, Heidelberg, Germany) (16 µm) or 30 µm thickness on a vibratome for immunostaining after permeabilization with 0.1% Triton X-100 (v/v) in 0.01 M PBS (pH 7.4) for 15 minutes, followed by blocking in 10% normal goat serum (NS02L; Sigma-Aldrich, Inc.) for 1 hour at room temperature. Table 1 summarizes the primary antibodies used in immunohistochemistry. After incubation with primary antibodies diluted in blocking solution overnight at 4°C, tissue sections were washed in PBS and incubated with the following secondary antibodies diluted in blocking solution for 60 min at room temperature: Alexa Fluor® 594 or 488 conjugated goat anti-rabbit or mouse IgG (H + L) (Cat. A11037, A11032, A11034, A11029; 1:1,000; Thermo Fisher Scientific, Inc.). Sections were washed in PBS and counterstained with DAPI (Roche Diagnostics, Lot# 70237122, 1:1000). After washing with PBS three times, sections were mounted on microscopy slides and covered with Fluoromount-G™ (Cat. 00-4959-52; Thermo Fisher Scientific, Inc.). Fluorescence images were captured by inverted fluorescence microscopy (Olympus IX50, Olympus Optical, Inc.) or Keyence's All-in-One fluorescence microscope (BZ-X800E). Fluorescent signals were quantified by NIH's Image J software.

Histochemistry

Luxol Fast Blue (LFB) staining was performed following the manufacture's protocol (NovaUltra Luxol Fast Blue Stain Kit, Cat IW-3005, IHC World). To measure brain reactive oxygen species (ROS) production, the brain sections were exposed to dihydroethidium (DHE) (D1168; Thermo Fisher Scientific, Inc.), according to a previously published validated method. Briefly, brain sections were incubated in PBS for 30 minutes at 37°C and then encircled with the hydrophobic pen. DHE (5 mmol/L) was topically applied, followed by incubation at 37°C in the dark for 30 minutes. Sections were rinsed in PBS, and oxidized DHE fluorescence was detected with an Olympus inverted fluorescence microscopy, and the fluorescence staining was quantified by ImageJ.

Cerebral fluorescent angiography

Cerebral fluorescein isothiocyanate (FITC)-dextran fluorescent angiography was performed as described in detail previously [16] using FITC-dextran (FD2000S-1G; 2000 kDa; 0.1 mL 50 mg/mL in sterile saline; Sigma, St. Louis, MO, USA). Mice were euthanized without perfusion 5 min after injecting FITC-dextran via tail vein and whole brains removed and post-fixed in 4 % PFA in PBS at 4°C overnight. Sequential coronal sections (100 µm thickness) were processed using vibratome for fluorescent imaging.

Evans blue angiography

Evans Blue (E2129, Sigma, St. Louis, MO, USA) was injected in 150 ml of 2% (m/v) in sterile saline through mouse tail vein. Mice were euthanized 5 min later without perfusion and whole brains removed and post-fixed in 4 % PFA in PBS at 4°C overnight. Sequential coronal sections (100 μ m thickness) were processed using vibratome for fluorescent imaging using rhodamine filter of inverted fluorescence microscopy (Olympus IX50, Olympus Optical, Inc.). Serial images were taken at Bregma: 2.0 mm, 1.42 mm, 0.26 mm, -0.22 mm, and -1.82 mm. For cerebral double fluorescent angiography, mice received a mixture of FITC-dextran and Evans blue dye in 200 μ l volume and euthanized 5 min later without perfusion. Brain tissue was processed for individual angiography.

White matter micro punch

Mice were euthanized by isoflurane overdose and brains were excised and placed in ice-cold PBS. Brains were subsequently sectioned with a vibratome to obtain two 1 mm coronal sections containing the genu and body of the corpus callosum. White matter microsamples were collected via micro punch using a 0.5 mm blunt-end needle attached to an air-filled syringe under a dissecting microscope. Micro punches of the corpus callosum were collected and aspirated into microcentrifuge tubes in 0.5 ml TRIzol (Invitrogen) and stored at -80°C until RNA extraction. The corresponding size of gray matter tissue was collected via micro punch from the adjacent subcortical region as control.

Western blot analysis and quantitative real-time RT-PCR (qRT-PCR)

Western blots and qPCR were performed as described previously [16]. Briefly, mice were transcardially perfused with ice-cold PBS and brains were removed. Half brains were post-fixed and used in immunohistochemistry and the other half brains were further dissected to forebrain tissue and then homogenized in cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1% NP-40, 1% sodium deoxycholate 0.1% SDS) with complete protease inhibitor cocktail (Roche, Inc.). Lysate preparation and Western blot analysis were performed as previously described [16]. For qPCR, RNA was extracted from the dissected brain tissue using TRIzol according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 1 mg of total RNA using High Capacity cDNA Reverse Transcription kits (Applied Biosystems). qPCR was performed with RealMasterMix SYBR Green (Applied Biosystems). Primary antibodies and qPCR primers used are summarized in Tables 2 and 3, respectively.

Gait balance test (CatWalk)

Mice were subjected to gait assessment using the CatWalk (Noldus Information Technology, Wageningen, The Netherlands) which consists of an enclosed walkway on a glass plate that is traversed by mice from one side of the walkway to the other. The room light is off. A camera is set below the glass floor to record paw prints. The red light installed inside the deck generates the background in the video. The glass floor sheds green fluorescence-like light so that paw prints are distinguished when the paw touches the floor. The digitalized paw prints are then automatically classified into four cohorts, namely left forepaw, left hind paw, right forepaw, and right hind paw. Mice from the same housing cage were trained to traverse a glass walkway toward their home cages from one end to another without interruptions for 30 minutes for

five consecutive days. On subsequent test day, three non-stop complete runs across the walkway were recorded. The test starts once the mouse enters the visual field of the camera and stops once the mouse disappears. If an animal failed to complete a run, walked backwards, or reared during the run, it was given an additional run. Quantitative analysis of gait performance was done by CatWalk XT version 10.0.408 to include the (1) spatial parameters related to individual paws (intensity, maximum area, print area, etc); (2) relative spatial relationship between different paws (base of support, relative paw placement, and stride length); (3) interlimb coordination (step pattern, regularity index, and phase lag); and (4) temporal parameters (swing, stance, cadence, and walk speed). Data are presented as mean $\pm$ s.e.m.

Statistical analysis

Statistical analyses were performed using unpaired two-tailed or one-tailed Student's t-test. For Western blot quantification, statistics were performed using one-way ANOVA test. Data are reported as mean $\pm$ s.e.m., except as mean $\pm$ s.d.m. for qPCR. Statistical significance was set at $P < 0.05$.

Results

Middle aged eNOS-deficient mice display severe myelin loss and axonal pathology

In our previous work, we characterized AD-like pathologies in eNOS-deficient mice at old age [16]. We have recently focused on characterizing middle-aged mice, since metabolic vascular factors pose the highest risk in this age group [5–7]. As reported, eNOS-deficient mice manifested most of the phenotypes of metabolic syndrome such as hypertension, insulin resistance, hyperlipidemia, hypertension, and hyperhomocysteinemia [28–30]. We now determined white matter changes at three ages (7 MO, 12 MO, and 24 MO) in eNOS-deficient (eNOS^{-/-}) and littermate control mice (eNOS^{+/+}). Coronal sections encompassing the brain regions corresponding from frontal to temporal cortical areas [+ 2.0 mm to -2.5 mm] were systematically examined by histological means for the expressional changes in axonal and myelin protein markers. Firstly, myelin integrity was assessed in eNOS mouse brains by both LFB (luxol fast blue) staining (Fig. 1A) and immunohistochemistry on myelin basic protein (MBP) (Fig. 1B). Significant myelin loss was first detected in the cortex and corpus callosum (CC), but not in the striatum in eNOS^{-/-} mice at 12 months of age (12 MO) (Fig. 1 and SFigure 1). Myelin loss was also detected in eNOS heterozygous mice at mid-age (SFigure 1B) and thinning of the CC region, the largest white matter zone, was detected at old age (24 MO).

Surprisingly, Western blot data based on forebrain tissue lysates showed a nearly 2-fold increase in MBP protein in eNOS^{-/-} at mid-age (12 MO) on all four isoforms from alternative splicing, with 17 kDa and 21.5 kDa being the predominant species (SFigure 1C) which presumably play differential roles of functional significance in oligodendrocytes. Notably, there was markedly increased colocalization of MBP immunosignals in the nuclei of eNOS^{-/-} brain sections (SFigure 1D). It is unclear if these nucleus-translocated MBP species represent mis-aggregated dysfunctional protein which warrants further investigation via biochemical analysis. Nuclear MBP has been reported to display different roles in myelination compared to cytoplasmic protein species, most often seen with the 17 kDa and 21.5 kDa

isoforms [31], appearing to be associated with the process during oligodendrocyte maturation before compact myelin formation [32]. Indeed, we observed similar results on two additional myelin protein markers Olig1 (oligodendrocyte transcription factor 1) and CC-1 (mature oligodendrocyte somata marker) (SFigure 2A and 2B), indicating the impaired function of myelin-producing oligodendrocytes which was supported by additional data to be presented later.

Severe gait imbalance and cortical pyramidal layer neurodegeneration were detected in middle aged eNOS-deficient mice

Cortical white matter change is often associated with gait dysfunction. We used the CatWalk system to assess gait performance of eNOS littermate mice in all three genotypes at 12 months of age. The test started once the mouse entered the visual field of the camera and stopped once the mouse disappeared. The averaged running speed is therefore reciprocal to the duration of the trial. Since the traces of each limb are marked, the moving speed can be alternatively calculated based on each limb. The immediate speed can be constant or vary heavily, so the variability is evaluated as well. We present data in Fig. 2A that the averaged running speed was consistently reduced in eNOS^{+/-} mice and was even slower in eNOS^{-/-} mice. Of note, eNOS^{-/-} mice not only moved slowly, but also failed to run at a constant speed compared to eNOS^{+/+} and eNOS^{+/-} mice. Moreover, eNOS-deficient mice (both eNOS^{+/-} and eNOS^{-/-}) displayed significantly altered patterns of gait sequence inter-limb coordination compared to eNOS^{+/+} mice. The same cohort of eNOS-deficient mice displayed significant deficits in two additional tests of rotarod and grip strength for motor functions at mid-age compared to eNOS^{+/+} mice (data not shown).

Cortical white matter demyelination predictably leads to cortical circuit dysfunction. We then examined the overall health of the neurons in both cortical and hippocampal regions of eNOS^{-/-} and control mice by LFB-Nissl double staining. Strikingly we detected disorganized cortical structure with signs of severe neurodegeneration in cortical pyramidal neuronal layers II/III and V/VI at 12 months of age, revealing the presence of an empty vacuole surrounding the seemingly degenerated neurons due to loss of myelin sheath (Fig. 2B). Of note, we did not detect apparent neurodegeneration of hippocampal neurons at this age. Consistently, we detected a significant increase in the expression of activated caspase-3 in the forebrain samples of eNOS^{-/-} mice compared to control littermate eNOS^{+/+} forebrain (SFigure 2C). Notably, myelin loss appeared to occur before the cortical pyramidal neurodegeneration since no neurodegeneration was detected at 7 months of age while there was a sign of demyelination (SFigure 3).

Elevated ROS and astrogliosis and neuroinflammation detected selectively in the white matter before the middle age

Since myelin-producing oligodendrocytes are the most energy-demanding cell type in CNS at the highest metabolic rate, they are known to be most vulnerable to stress such as ROS and hypoxia/ischemia. Indeed, we observed markedly elevated ROS levels by DHE staining, selectively in the parietal cortical region (Fig. 3A); no significant increase in DHE staining signal in the CC or striatal brain regions at middle age (SFigure 4A).

Furthermore, we found markedly increased fibrous astrocytes based on the expression of the glial fibrillary acidic protein (GFAP) in the CC region in middle-aged eNOS-deficient mice; they were aligned in rows between the axon bundles in parallel which displayed smooth and long processes of a fingerlike shape (Fig. 3B). Heterozygous eNOS^{+/-} brains also displayed an increase in GFAP positive cells at 12 months of age, but at lesser degrees than eNOS^{-/-} brains (SFigure 4B). Surprisingly, there was no significant increase in Iba-1 positive microglia in the brains of 12 months-old eNOS-deficient mice; a significant global increase was found at 24 months of age (Fig. 4C). It remains to be determined the precise order of these events at molecular and cellular levels.

Although GFAP is the main astrocytic intermediate filament, immunohistochemistry using multiple GFAP antibodies only labeled astrocytes in the corpus callosum, cerebral peduncle, and the hippocampus in mouse brain under physiological condition. Owing to the increasingly recognized heterogeneity of astrocytes from different brain regions [33], we used a second marker S100b which is more suitable for capturing the overall distribution of astrocytes in all brain regions. Indeed, we observed more S100b-positive astrocytes in the cortical area in eNOS^{-/-} brain at mid-age; not only the number but also the fluorescent intensity of each astrocyte was markedly increased (SFigure 4C). Most strikingly, contrary to the globally increases S100b-positive astrocytes in both the CC and cortical areas in eNOS^{-/-} brains, the GFAP-positive astrocytes were selectively found around the cortical layer II/III and V/VI regions, surrounding the degenerating/degenerated pyramidal neurons (SFigure 4D). Astrocytes are best known for multi-faces, in tissue repair, and in mediating an inflammatory response. Indeed, at higher magnification, both the GFAP- and S100b-positive astrocytes displayed thicker cellular process, indicating reactive astrocytes upon injury (SFigure 4C and 4D).

To verify that these newly increased astrocytes are indeed reactive astrocytes upon injury, we undertook a different approach of investigating cytokine gene expressional changes from the largest CC region (white matter, WM) and the adjacent subcortical region (gray matter, GM) (Fig. 4A). Most strikingly, markedly upregulated levels of three pro-inflammatory cytokine genes were detected only in the white matter of the eNOS^{-/-} brain at 10 months of age (Fig. 4B). Because we did not observe increased microgliosis at this age, we reasoned that these cytokines were most likely released from the activated reactive GFAP-positive astrocytes. Given the sparse nature of the newly emerged GFAP-astrocytes in the specific regions of cortical areas in eNOS^{-/-} mouse brains by mid-age, the upregulated inflammatory gene expression in gray matter may be oversight from the micro samples taken via micro punch which consisted of a majority of dense neuronal bodies (somas). Nevertheless, the prominently elevated cytokines in the CC region are the unambiguous evidence for neuroinflammation, specifically white matter neuroinflammation, as an early contributing mechanism to the cortical pathology such as demyelination and selective cortical neurodegeneration. Consistently, all four major myelin pathway genes (i.e., *Mbp*, *Plp1*, *Mog* and *Mag*) were found to be markedly downregulated in the CC region in eNOS^{-/-} mice at 10 months of age as compared to littermate control mice based on quantitative qPCR data (Fig. 4C). The implication of impaired integrity of the myelinated axons and white matter axon-glial coupling upon eNOS deficiency will be discussed later.

Hypoperfusion and BBB leakage are the two earliest pathologies detected in eNOS-deficient mice

Endothelium-derived NO (EDNO) signaling has long been known to be crucial for angiogenesis and vasculogenesis during the early developmental stage. Using FITC-angiography, we detected reduced vessels from a dorsal view of eNOS-deficient mice at a young age (3 MO) (Fig. 5A, left two panels), which was similarly reported previously not attributable to impaired collateral formation in the embryo-neonate stage [34]. We therefore reasoned that this phenomena indicated arterial occlusion resulting from impaired NO signaling. Indeed, as we reported previously [16], regions of the hypoperfused brain and microvascular occlusion appear in eNOS-deficient mice at a young age, with markedly increased frequency of similar lesions in an age-dependent manner. As shown in a representative cortical section from young eNOS-deficient mice (3 MO) (the right panel of Fig. 5A), these lesions/occlusions are typically small (ranging from 100 to 200 μm in diameter); by middle age, they are frequently found bilaterally in the parietal association cortex (Table 1). This pathological 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 WT eNOS littermates up to 24 months of age.

In our prior work we also reported compromised BBB in middle to old-aged eNOS-deficient mice based on FITC-dextran (150 kDa) angiography as well as quantitative mouse IgG extravasation [16]. We speculated that BBB integrity and function may be compromised at a much younger age in eNOS-deficient mice owing to the indispensable role of EDNO signaling in endothelial vascular permeability via modulating VEGF signaling. To detect early BBB leakage, we systematically tested various molecular tracers reportedly used for this purpose including FITC-dextran species at different molecular weights and Evans blue dye. Surprisingly, when we used FITC-dextran of a lower molecular weight range (3–30 kDa) as a tracer, fluorescence was not detected in the brain, presumably due to rapid systemic clearance. In the end, we concluded that Evans blue dye is an optimal fluorescent tracer for the degrees of leakage in the eNOS model.

Evans blue (980 Da) is an azo dye with a very high affinity for serum albumin (67 kDa). Because serum albumin cannot cross the BBB and virtually all Evans blue is bound to albumin, albumin-bound Evans blue enters the parenchymal tissue in CNS when the BBB has been compromised. Since 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 a fluorescent microscope using a Rhodamine filter. After injecting Evans blue dye to mouse tail vein, the brains were removed shortly and post-fixed and processed to serial 100 μm coronal sections for angiographic examination. By this method, we detected massive BBB leakage in eNOS^{-/-} mice beginning at a very young age (6–8 week-old). As shown in Fig. 5B, eNOS-deficient brain at 3 months of age displayed numerous diffusive spots indicating BBB leakages spanning from frontal to parietal cortical regions compared to no leakage found in littermate control mice. The gradually decreased leakage spots in the five representative angiographs spanning Bregma + 2.0 mm to -1.82 mm indicates that leakages start predominantly from an anterior to posterior direction. Indeed, we detected

more severe leakage expanded to deeper and posterior areas in older eNOS-deficient mice. Strikingly, like FITC-non-perfusion lesions, there was no leakage detected in wild-type eNOS mice even at 24 months of age using this methodology (Table 1). Interestingly, we examined mouse brain sections after co-injection of FITC-dextran (2000 kDa) and Evans blue dye and found largely non-overlapping fluorescent signals in eNOS^{-/-} brain at a young age (data not shown), suggesting that these two early pathological events of non-perfusion and BBB leakage are independent of each other, both resulting from the impaired EDNO signaling.

Accordingly, we detected massive upregulation of metalloproteinases 9 (MMP9) at middle age by both immunohistochemistry and Western blot analysis, with MMP9-positive signals largely colocalizing with the Evans blue leakage signals (SFigure 5A-C), indicating breakdown of the endothelial basement membrane. Surprisingly, we detected no significant changes in MMP2 expression, and laminin expression was only elevated at 24 months of age (SFigure 5D). Since astrocytes contact blood capillaries with their endfeet as an integral part of the BBB interface, they are crucial in governing and maintaining water homeostasis in the CNS. We therefore also investigated aquaporin-4/Aqua4, a marker of astrocytic endfeet surrounding the capillaries by immunohistochemistry. Aqua4 proteins displayed more intense expression in microvessels in gray matter than in white matter, consistently because the microvessel density is much lower in white matter. Although Western blot data showed no significant change in Aqua4 protein expression levels from forebrain tissues of eNOS^{-/-} mice at 12 months of age, Aqua4 proteins appeared to undergo redistribution upon eNOS deficiency by middle age; there were slightly more intense Aqua4 immunosignals overlapping with GFAP-positive astrocyte endfeet along the microvessels (SFigure 6B), suggesting altered BBB functions. Western blot analysis revealed nearly complete loss of Aqua4 protein expression in old eNOS^{-/-} mouse brain at 24 months of age (SFigure 6C). Similarly, we detected a significant reduction of glucose transporter 1/GLUT1 expression only at an older age (SFigure 6D).

Restoration of NO signaling via sodium nitrate (SN) feeding rescues white matter pathologies in eNOS-deficient mice

Restoration of NO signaling with 1 mM SN in drinking water for up to 10 weeks has been reported to reverse hypertension, systemic hyperlipidemia, and glucose intolerance in 14- to 22-month-old eNOS^{-/-} mice [27]. This prompted us to use this agent to test our hypothesis that feeding eNOS-deficient mice with SN in drinking water at a very young age, starting from 6-week-old for 6 weeks, would prevent the development of the two early pathological events namely hypoperfusion and BBB leakage. Strikingly, 6-week SN feeding completely prevented BBB leakage and non-perfusion lesions in eNOS^{-/-} mice (Fig. 5C and Fig. 6B and 6C Cohort 1 mice; n = 3/group). These promising data encouraged us to further testing the possibility of chronic feeding sodium nitrate in drinking water to eNOS-deficient mice at older post-symptomatic age.

In addition to the first cohort of mice that received 6-week-SN feeding which resulted in nearly complete prevention of BBB leakage (Fig. 5C and Fig. 6B), we also fed mice for a longer period in eNOS^{-/-} mice for

over 6 months (Cohort 2) and post-symptomatically (Cohort 3, 11–16 months) (Fig. 6A). We focused on ROS, white matter inflammation, and myelin preservation in the Cohort 2 mice. Specifically, SN feeding completed prevented pro-inflammatory cytokine gene upregulation in eNOS^{-/-} white matter tissue at 10 months of age (Fig. 4B) and the decline of myelin pathway gene expression (Fig. 4C). It also prevented ROS elevation and astrogliosis in selective cortical layers (data not shown). Furthermore, loss of myelin neurofilaments (SMI 132) in eNOS^{-/-} brains was significantly rescued by SN feeding (Fig. 6C). Lastly, gait performance was significantly improved in Cohort 3 mice when tested at 16 months of old age, after SN feeding was initiated from 11 months of age in eNOS^{-/-} mice (Fig. 6D). Although the SN eNOS^{-/-} mice could not perform as well as the eNOS^{+/+} mice at this old age (16 MO), this result is most encouraging with translational value for a potential reversal effect of cortical neuronal connectivity and functions. Taken together, chronic SN feeding resulted in significant prevention or improvement in white matter pathologies and function in eNOS^{-/-} mice.

Discussion

White matter comprises half of the brain and has expanded more than gray matter during evolution, continuing from childhood through adulthood. Over the past decade, white matter is increasingly recognized as equally critical as the cerebral cortex and hippocampus for cognition. This is not surprising given the fact that white matter tracts form an indispensable component of the complex neural connectivity that supports neurobehavioral operations. Nowadays, there is compelling evidence supporting that white matter lesions disturb and probably directly contribute to cognitive dysfunctions [35].

Myelination, a process that continues for decades in the human brain is crucial for information processing by regulating the velocity and synchrony of axonal impulse conduction between distant cortical regions. It is now well recognized that the process of myelination can be modifiable by experience [36, 37]. Compelling evidence collected from aging and AD brains suggests that demyelination within the cerebral white matter is an upstream event that initiates amyloid deposition and then tau as later end products comprising cortical pathology in AD [38]. Although this “myelin model” was initially instated in the context of AD, the same theory may be generalized to other neurodegenerative and psychiatric conditions including vascular dementia. Thus far, this model has been largely supported by compelling evidence including recent genetic data [21], which is in line with the term “white matter dementia” brought up two decades ago to highlight the importance of white matter dysfunction to cognitive decline. The tight association between the degrees of dementia with the involvement of white matter lesions [39] provides additional strong support to this theory. Since dementia has been most extensively studied in the form of Alzheimer’s type, our current therapeutic development towards dementia is still dominated by anti-Aβ/tau strategies with all trials without success in clinics. If the myelin model proves to be correct as a unifying theory for all types of dementia including cortical, white matter and subcortical dementia, it would be instrumental in redirecting our focus from amyloid and tau to myelin-oriented preventive and treatment intervention. Therefore, a deeper understanding of the mechanisms of

demyelination/remyelination at both cellular and molecular levels would be fundamentally important in designing novel targeting strategies long before the onset of cognitive dysfunction. This relies not only on the knowledge gained from clinical neurology (e. g., neuroimaging and neuropathology) to identify early white matter pathology but also from various representative experimental models of dementia conditions to validate the earliest white matter mechanisms that drive cognitive decline.

This report presents evidence that genetic eNOS-ablation spontaneously induces chronic hypoperfusion, BBB leakage, elevated ROS/astrogliosis, myelin loss, and axonal pathology as well as cortical neurodegeneration/gait imbalance in mice. All these phenotypes are considered the essential components as potential contributing mechanisms to dementia, based on both clinical [20, 22–26] and experimental [40, 41] evidence. Clinically, dementia has been most widely studied in AD type so-called cortical dementia, the other two types of dementia (i.e., subcortical dementia, often seen with stroke patients), and white matter dementia are featured by distinct core cognitive dysfunctions, especially at the early stages of dementia, likely due to different white matter lesions [35]; of note, these features become indifferent as neuropathologies progress to late stages, all three types of dementias become indistinguishable in the end. Again, these clinical notions underscore the paramount importance of studying white matter myelin mechanisms using representative experimental models.

In addition to the transgenic/knock-in rodent models based on mutant familial AD genes (e.g., APP, PS1, and tau) in which white matter dysfunction was occasionally reported, there are currently more than a dozen of rodent models that develop major features of human dementia with the vascular origin [42]; none of a single model recapitulates all features of human dementia. The optimal choice of model depends on the aspect of pathophysiology being studied. Chronic cerebral hypoperfusion has been suggested to be a key mechanism leading to dementia [15]. Over the last decade, bilateral common carotid occlusion/stenosis (BCAS) has been the most widely used chronic hypoperfusion model. This BCAS surgery in both rats and mice induces several major white matter changes including reduced parenchymal cerebral blood flow, oligodendrocyte loss, glial activation, and disrupted clearance of intracerebral fluid and BBB, and cognitive impairment [43]. However, sizable intrinsic variance in cerebral hypoperfusion with blood flow dropping too suddenly and too severely after occlusion surgery is one major shortcoming of this type of surgical model. Moreover, all pathological events occur within 2–3 months, making the model difficult to consider the aging component. In our opinion, the spontaneously developed white matter and cognitive phenotypes in an age-dependent manner in the mouse model based on eNOS-deficiency (both heterozygous eNOS and eNOS knock-out mice) makes these mice ideal for studying white matter mechanisms, linking chronic hypoperfusion and demyelination directly to neurodegeneration. Below, we will discuss in detail our findings based on the eNOS-deficient mice.

White matter changes, including myelin loss and axonal pathology

The largest white matter tract, the corpus callosum (CC), is particularly vulnerable to stress. This region has been extensively examined and reported for various pathological changes in the brains of healthy

elderly individuals, AD, multiple sclerosis and vascular dementia. Although we observe CC atrophy in eNOS^{-/-} brain at very old age (24 MO), we detect demyelination in cortical and CC regions, but not striatum, at a much younger age (starting from 7 months of age). The mechanism for CC atrophy is poorly understood, resulting from axonal disruption due to white matter damage.

Maintenance of the integrity of the myelinated axons and the functional coupling between axon and the responsible glial cells (i.e., oligodendrocytes) are well-known to be critical for action potential propagation and thus for regulating efficient neuronal connectivity. Axonal dysfunction in white matter disorders is well recognized to confer a worse prognosis than that implied by myelin damage alone [40]. White matter axon-glial integrity has been reported to be disrupted in mice by mild cerebral hypoperfusion in an experimental model (BCSA) [44]. We used the pan-antibody SMI-32 to visualize the distribution of mixed neurofilaments in eNOS^{-/-} brain at middle age and observed significantly reduced immunosignals in multiple regions, including CC and subcortical areas (Fig. 6C); the thinner and more “chaotic” staining pattern displayed in eNOS^{-/-} brain also indicated reduced axonal diameters and aberrantly sprouting axons derived from cortico-cortical fibers, predicting the loss of synaptic circuitry underlying the basis of memory. Taken together, our data combined between immunohistochemistry and quantitative gene expression by qPCR analysis, we conclude that both the integrity of the myelin sheath (assessed by MBP) and integrity of axons (assessed by SMI-32) are compromised upon eNOS deficiency. Moreover, the reduced expression of the gene encoding for myelin-associated protein MAG (Fig. 4C) also indicates disruption of the axon-glial integrity. We speculate that this axonal pathology occurs early, before the neuronal cell body pathology. Indeed, we observe demyelination at a young age in eNOS^{-/-} mice (7 months of age) while no cortical layer pyramidal neurodegeneration is detected at this age. Surprisingly, the demyelination occurs from the outer layers of cortical region to the inner layers (e.g., layer I to VI), consistently with the notion that the outer cortical layers II/III are most vulnerable to hypoperfusion in the clinical specimen of both early demented AD and post-stroke brains [45, 46].

Chronic hypoperfusion versus BBB dysfunction

We have collected evidence to identify hypoperfusion and BBB leakage as the two earliest pathological events in eNOS^{-/-} brains from newly weaned mice (6 weeks of age) via FITC and Evans blue angiographies, respectively. We discovered a general pattern through systemic examination of the coronal brain sections from these angiographies for these two events, both expanding from the anterior to the posterior brains in an age-dependent manner. While FITC-dextran non-perfused lesions occur predominantly at the frontal parietal cortical area, Evans blue leakages are present globally in both dorsal and ventral cortical areas. The overall “anterior-to-posterior” hypoperfusion gradient is a reminiscent of the clinical hypoperfused human brains correlating with various stages of dementia [47, 48]. For example, the dis-executive individuals often display significant hypoperfusion of the left superior, medial frontal and cingulate cortex. In contrast, the amnesic mild cognitive impaired individuals show significant hypoperfusion in the left hippocampus, para-hippocampal gyrus, and fronto-parieto-temporal areas [49].

Perhaps limited by the nature of the two angiographic methods we used, we could not determine which of these two events occurs first. Given the different angiographic profiles from FITC-dextran and Evans blue, we posit that they are independent events, both resulting from impaired EDNO signaling. Indeed, we observe non-overlapping fluorescent signals from the brains receiving a co-injection of both dyes. However, the main question remains of how these initial pathological events lead to the subsequent white matter pathologies. White matter is long speculated to be most vulnerable to chronic hypoperfusion due to intrinsic lower microvessel density [11, 26]. Despite a recent report excluding BBB leakage as an invariant feature of the white matter lesions based on case studies from the genetic paradigm of small vessel disease so called cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [50], more experimental evidence supports a prevailing view that disrupted BBB can contribute directly to white matter changes. For example, using a model of pericyte-deficiency, white matter pathology and impaired executive functions were detected. In this model study, oligodendrocyte degeneration was suggested to be caused by an accumulation of toxic blood-derived fibrin(ogen) deposits and blood-flow reductions; the latter further leads to a loss of oligodendrocytes, and thus myelin, and axonal structure and function [39]. Our data are consistent with the observations from this study: both the accumulation of fibrin(ogen) deposits [16, 51], as well as a reduced expression of immature and mature oligodendrocyte markers (Oligo1 and CC1) (SFigure 2), are detected in the eNOS model. In addition, oligodendrocyte health, as the most vulnerable cell type in CNS, may also be affected by elevated oxidative stress and inflammatory environment in white matter, in particular, as discussed in the next section.

ROS, astrogliosis and white matter neuroinflammation-what causes demyelination?

Astrocytes contact blood capillaries with their end feet and are therefore an important part of the blood–brain interface, presenting differences between gray (GM) and white matter (WM). Interestingly, astrocytes exert different properties of protoplasmic and fibrous subtypes in GM and WM, respectively. Therefore, it is likely that these different astrocyte subtypes contribute to the observed differences in BBB properties in GM and WM. We detect marked BBB leakage in eNOS^{-/-} brain at a very young age, followed by increased expression of GFAP-positive reactive astrocytes in the CC region and selected cortical layers II/III and V/VI by mid-age. Given the exclusive upregulated pro-inflammatory cytokine gene expression in the CC white matter region, we reason that the increased astrogliosis in the CC region likely contribute directly to oligodendrocyte degeneration and demyelination while the sparse presence of the GFAP-astrocytes in the cortical areas surrounding the degenerated layer II/III and V/VI pyramidal neurons likely reflect the well-recognized role of astrocyte in tissue repair upon injury. The precise astroglial mechanisms in various pathogenic stages of the white matter changes in the eNOS model will be further investigated at cellular and molecular levels. Our finding of the upregulated genes of the transforming growth factor-beta (TGFB) super-family (i.e., TGFB-1 and BMP4; Fig. 4D) selectively in white matter tissue opens up a new window of investigating impaired signaling of the TGFB family as a potentially crucial glial-vascular mechanism of the white matter changes in the eNOS model. Indeed, we observe

upregulation of BMP4 expression in the descending arterioles in eNOS^{-/-} brain at a young age [51], likely from pericytes as reported from mice undergoing BCAS [52]. Accordingly, the well-known paracrine actions of BMP4 signaling could directly trigger white matter degeneration via promoting astrogliosis and simultaneously interfering with oligodendrocyte differentiation, and maturation; a theory is being tested experimentally. How an impaired EDNO signaling induces upregulated TGFb-1 and BMP4 signaling is also an interesting and challenging question to be addressed.

NO-based therapy – inorganic nitrate/nitrite

In humans, EDNO plays a key role in regulating basal cerebral blood flow via vasodilation of cerebral vessels, and aberrant biogenesis of NO is associated with arterial stiffness, hypertension, atherosclerosis in patients with cardiovascular diseases, AD and vascular dementia patients [51]. Our data demonstrate that impaired EDNO signaling can first lead to cerebral hypoperfusion and the impairment of the BBB, subsequently to white matter changes and neurodegeneration from cortical to hippocampal regions. Developing NO-based therapeutics thus represents a viable strategy for treating white matter dementia.

Since the seminal discoveries of NO as the most crucial endothelial-derived relaxing factor, and subsequently, the biology of nitrite anion (i.e., the “nitrate-nitrite-NO pathway”) [53], developing NO-based therapeutics have been actively pursued. Several mechanisms probably contributed to the decrease in NO bioavailability, including defects in the cascade of NO generation (e.g., eNOS activity) and reduced NO donors. Thus far there are only three agents are approved by FDA for uses in treating acute angina by organic nitroglycerine, NO inhalation for pulmonary hypertension in neonates and phosphodiesterase inhibitors to prevent the breakdown of the downstream second messenger of NO, cyclic guanosine monophosphate/cGMP [54]. Although the first line cholesterol-lowering drugs statins also demonstrate a potential to enhance the bioavailability of EDNO, principally by up-regulating and activating eNOS [55, 56]; convincing clinical data is still lacking [57, 58]. Importantly, like L-arginine supplementation based on the discovery of it being the physiological substrate of eNOS, statins belong to the same category of NO-enhancing strategies requiring functional NOS system as a prerequisite. In theory, using strategies independent of a functional NOS system to increase bioavailability of NO production is more ideal.

Historical evidence suggests that using dietary supplements with natural products represents a safest strategy [59]. Numerous experimental evidence indicates that nitrite anion represents an important reservoir of NO bioequivalents to increase tissue NO levels during pathophysiological states in which NO synthesis from the eNOS-mediated enzymatic pathways are unavailable; nitrite anion production and distribution throughout the body can act in an endocrine manner to augment NO bioavailability [60]. Administration of nitrate or nitrite to humans and rodents clearly led to increased NO-like bioactivity from numerous studies. Feeding eNOS-deficient mice with oral sodium nitrite or sodium nitrate for as short as one to several weeks has been demonstrated by independent groups to restore NO signaling and vascular homeostasis [27, 61]. Our recent data not only reproduces these observations but also expands to the BBB and white matter beneficial effects (Figs. 5 and 6). These promising findings provide additional mechanistic insights to the NO-based therapy, highlighting the paramount importance of the EDNO signaling.

Although more than a dozen clinical reports revealing significant beneficial effects from administration of nitrate or nitrite to humans in multiple symptoms, including blood pressure, abnormal platelet aggregation, reduced triglycerides/hypercholesterolemia cardiac ischemia, proving absolute safety [54]. It should be noted that so far the majority of the clinical testing in patients used inorganic nitrate. In humans, a normal NO production scheme requires nitrate to first be reduced to nitrite via two electron reduction, followed by another one electron reduction to NO. Inorganic nitrate is nearly inert in humans due to an intrinsic lack of the functional gene to reduce nitrate to nitrite; the conversion relies on symbiotic oral bacteria and host enzymes which is inefficient (5 %) [54]. Therefore, the required conditions for nitrate to have therapeutic efficacy in humans are difficult to meet. On the other hand, nitrite is more efficacious but also slightly more toxic than nitrate. Therefore caution should be taken in terms of using optimal dosing. Both inorganic nitrite and nitrate are naturally abundant in green leafy vegetables and beets which are safe for humans, though it remains to be determined how much daily intake is sufficient to reach therapeutic benefits. Nevertheless, healthy lifestyle to combine dietary supplements and exercise is the key for a safest and most beneficial NO-based therapy.

Conclusions

White matter lesions can directly contribute to cognitive dysfunctions via dis-coupled glia-vascular functions. Since we observed hypoperfusion and BBB breakdown before myelin loss in white matter, we speculate that the compromised BBB with vascular injury along with oxidative stress and astrogliosis all contribute to the subsequent white matter change. Moreover, we also detect selective neurodegeneration in the superficial cortical layers (II/III and V/VI), likely due to cortical demyelination. The chronically evolving nature of the spontaneous model of hypoperfusion in eNOS-deficient mice will allow us to tease out the sequential pathological events in a future longitudinal study, at both molecular and cellular levels. These mice offer a unique opportunity to generalize and further validate the “myelin model” in a mouse system without being compounded by the amyloid and tau as dominating factors. We conclude that eNOS-deficient mice may represent an ideal spontaneous model for studying the earliest events leading to white matter changes. Moreover, it would be useful for future testing drug candidates for therapeutic use via targeting novel/specific vascular mechanisms contributing to VCID and AD.

Abbreviations

Vascular cognitive impairment and dementia (VCID), Alzheimer’s disease (AD), Endothelial nitric oxide synthase (eNOS), Endothelium-derived nitric oxide (EDNO), Blood-brain-barrier (BBB), Corpus callosum (CC), White matter (WM), Gray matter (GM), Bilateral common carotid artery stenosis (BCAS).

Declarations

Ethical Approval and Consent to participate: All animal work had been reviewed and approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center, and was conducted in accordance with the animal care standards of the National Institute of Health.

Consent for publication: All authors read and approved the final manuscript.

Availability of supporting data: We will make all materials and data including supporting data available to scientific community upon acceptance of this manuscript.

Competing interests: The authors declare that they have no competing interests.

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Tables

Due to technical limitations, table 1 to 3 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Severe myelin loss in middle-aged eNOS-deficient mice. A) Representative images of LFB (coronal, 16 μ m) staining taken from mice at 12 months of age. Scaled bars are indicated in each picture. B) Representative images of MBP immunohistochemistry at 12 months of age. Scaled bars: 500 μ m. C) Representative images of MBP immunohistochemistry from the frontal cortical regions at 12 months of age. Scaled bars: 100 μ m. Quantification was performed based on the mean fluorescent intensity from the frontal cortical regions and expressed as mean $\pm$ s.e.m; N=5-6 mice/group.

Figure 2

Gait disbalance and cortical pyramidal neurodegeneration. A) Gait performance by CatWalk testing. The test started once the mouse entered the visual field of the camera, and stopped once the mouse disappeared. The averaged running speed and additional parameters (e.g., altered sequence patterns and inter-limb coordination) were recorded, analyzed and expressed as mean $\pm$ s.e.m; N=13-15 mice/group per genotype. * $P < 0.05$ and ** $P < 0.005$. B) Representative images of LFB and Nissl double-stained sections of brains of eNOS^{-/-} and control littermates showing frontal cortical regions. Red double arrows and the corresponding Roman numerals indicate cortical layers I through V. Red arrowheads indicate degenerating neuron with swollen soma. Scaled bars are indicated in each picture.

Figure 3

Elevated reactive oxygen species (ROS) and astrogliosis. A) Representative images of dihydroethidium (DHE)-stained frontal forebrain cortical regions from mice at 12 months of age. Roman numerals in yellow indicate cortical layers II/III and V where we detected most elevated ROS signals. Lower right panel images with zoned area showing intracellular DHE signals presumably deriving from the clustered mitochondria in cytoplasm. Quantification was performed based on the mean fluorescent intensity from the frontal cortical regions and expressed as mean $\pm$ s.e.m; N=5-6 mice/group. B) Representative images of GFAP immunohistochemistry on coronal brain sections of mice at 12 and 24 months of age. C) Quantification of GFAP and Iba-1 immunofluorescence intensity based on 5-6 mice/group and expressed as mean $\pm$ s.e.m.

Figure 4

Gene expressional changes in white matter tissue as determined by quantitative qPCR. A) Diagram of the specific brain region tissue for micro punch. B) three representative genes encoding for pro-inflammatory cytokines. *Il-1 β* : interleukine-1 β ; *Il-6*: interleukine-6; *Tnf- α* : tumor necrotic factor- α . C) myelin-pathway genes: *Mbp*: myelin binding protein; *Plp*: myelin proteolipid protein or lipophilin; *Mog*: myelin oligodendrocyte glycoprotein; *Mag*: myelin associated glycoprotein. D) Two genes of the transforming growth factor TGF α family. *Tgf-1 β* : transforming growth factor-1 β ; *Bmp4*: bone morphogenetic protein 4. Data are presented as mean $\pm$ s.d.m., normalized by *gapdh* gene expression. N=3-4 mice/group.

Figure 5

Cerebral FITC-dextran and Evans blue angiography. A) Representative FITC-dextran angiograms of whole brain (left two panels) from dorsal view from eNOS mice at 3 months of age. Red boxed indicates parietal cortical zone from dorsal view. Right two panels show the representative FITC angiograms taken from 100 μ m coronal sections of frontal brains from the same mice as in the left two panels. B) Representative images of Evans blue angiograms of young eNOS^{+/+} and eNOS^{-/-} mice (3 months of age) taken at 2.0 to -1.82 mm Bregman, showing BBB leakage detected at young age which can be prevented by sodium nitrate (SN) feeding in drinking water for 6 weeks starting from 8-week-old (SN eNOS^{-/-}, N=3 mice/groups). Evans Blue (2 % in sterile water) was injected in 150 μ l volume through mouse tail vein. Mice were euthanized 5 min later without perfusion and whole brains removed and vibratome-processed to 100 μ m serial sections for fluorescent imaging using an rhodamine filter. Scaled bars: 500 μ m.

Figure 6

Restoring NO signaling by sodium nitrate feeding rescues white matter pathology and functional phenotypes. A) Schematic diagram illustrating sodium nitrate (SN) feeding schedules in three cohorts of mice. B) Quantification of Evans blue fluorescent signals based on the angiograms presented in Figure 5B. Data are presented as mean $\pm$ s.e.m based on N=3 mice each group. C) Representative images of SMI-32 immunohistochemistry (Cohort 2 mice). D) Gait performance in post-symptomatic $eNOS^{-/-}$ mice with and without SN feeding for 4 months (Cohort 3 mice); data are presented as mean $\pm$ s.e.m., N=17-19 mice/group. * indicates $P < 0.05$.

Supplementary Files

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