Age-Related Decline in Oligodendrogenesis Retards White Matter Repair in Mice

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Background and Purpose—Aging is one of the major risk factors for white matter injury in cerebrovascular disease. However, the effects of age on the mechanisms of injury/repair in white matter remain to be fully elucidated. Here, we ask whether, compared with young brains, white matter regions in older brains may be more vulnerable in part because of decreased rates of compensatory oligodendrogenesis after injury.

Methods—A mouse model of prolonged cerebral hypoperfusion was prepared by bilateral common carotid artery stenosis in 2-month and 8-month-old mice. Matching in vitro studies were performed by subjecting oligodendrocyte precursor cells to sublethal 7-day $CoCl_2$ treatment to induce chemical hypoxic stress.

Results—Baseline myelin density in the corpus callosum was similar in 2-month and 8-month-old mice. But after induction of prolonged cerebral hypoperfusion, older mice showed more severe white matter injury together with worse deficits in working memory. The numbers of newborn oligodendrocytes and their precursors were increased by cerebral hypoperfusion in young mice, whereas these endogenous responses were significantly dampened in older mice. Defects in cyclic AMP response element-binding protein signaling may be involved because activating cyclic AMP response element-binding protein with the type-III phosphodiesterase inhibitor cilostazol in older mice restored the differentiation of oligodendrocyte precursor cells, alleviated myelin loss, and improved cognitive dysfunction during cerebral hypoperfusion. Cell culture systems confirmed that cilostazol promoted the differentiation of oligodendrocyte precursor cells.

Conclusions—An age-related decline in cyclic AMP response element-binding protein–mediated oligodendrogenesis may compromise endogenous white matter repair mechanisms, and therefore, drugs that activate cyclic AMP response element-binding protein signaling provide a potential therapeutic approach for treating white matter injury in aging brains. **(*Stroke*. 2013;44:2573-2578.)**

Key Words: aging ■ animal model ■ cyclic AMP response element-binding protein ■ oligodendrocyte ■ white matter diseases

Aging is one of the most important risk factors for developing white matter injury in stroke and cerebrovascular disease.[1] The risk of stroke doubles every decade after 55 years of age,[2] and aged patients show less functional recovery from stroke compared with younger patients.[3] However, the mechanisms that underlie the increased vulnerability of aging white matter remains poorly understood.

Increasingly, it has been proposed that central nervous system pathophysiology is significantly influenced by the balance between deleterious versus beneficial responses to the initial insult.[4] Stroke and brain injury trigger a wide spectrum of neurovascular perturbations, glial activation, neuroinflammation, and neuronal cell death cascades. But many endogenous neuroprotective responses may also be induced at the same time. These include compensatory neurogenesis, angiogenesis, neuroplasticity, and remodeling.[5] Herein may lie a clue to the effects of aging on central nervous system disease. Although the adult brain retains plastic capabilities for regeneration and recovery, aging may significantly dampen these endogenous protective mechanisms. In particular, the capacity for neurogenesis seems to diminish with age. This is primarily because of a general reduction of neuronal precursor cell proliferation because of age-related alterations in the cellular microenvironment: decline of neurotrophic factor expression,[6,7] increase in cell death rate of neuronal precursors and mature neurons,[8] and decrease in the activation of cyclic AMP response element-binding protein (CREB) signaling.[9]

Similar to neurogenesis, oligodendrogenesis and white matter homeostasis might also be affected by white matter senescence.

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In healthy young adult brains, myelin-forming mature oligodendrocytes in the white matter can be newly generated from their precursor cells (oligodendrocyte precursor cells [OPCs]). After white matter injury, OPCs rapidly proliferate and migrate to fill the demyelinated area, differentiate into mature oligodendrocytes, and restore myelin sheaths.[10–12] Notably, however, myelin density, along with cognitive function, spontaneously declines with increasing age in both humans and rodents,[13,14] indicating that the capacity for oligodendrogenesis may be associated with white matter senescence. In this study, we ask whether analogous declines in endogenous recovery mechanisms may also occur after central nervous system injury, thus mediating the age-related increase in white matter vulnerability in stroke and cerebrovascular disease.

Methods

Cerebral Prolonged Hypoperfusion Model

All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Cerebral prolonged hypoperfusion stress was induced by bilateral common carotid artery stenosis. Male C57Bl/6 mice (2 months and 8 months old; Charles River Institute) were anesthetized with 4.0% isoflurane and then maintained on 1.5% isoflurane in 70% N_2O and 30% O_2 using a small-animal anesthesia system. Through a midline cervical incision, both common carotid arteries were exposed. A microcoil with a diameter of 0.18 mm (Sawane Spring Co) was applied to bilateral common carotid arteries, maintaining the rectal temperature between 36.5°C and 37.5°C using a heating pad. All experimental groups were randomized, and investigators responsible for surgical procedures or drug treatments were blinded. End point assessments (please see online-only Data Supplement for detailed methods of end point assessments) were performed by investigators blinded to the groups to which each animal was assigned.

Cell Culture

OPC cultures were prepared from rat neonatal cortex. Cultured OPCs were plated and maintained in Neurobasal medium containing glutamine, 1% penicillin/streptomycin, 10 ng/mL platelet-derived growth factor, 10 ng/mL fibroblast growth factor, and 2% B27 supplement onto poly-DL-ornithine–coated plates. Four to 5 days after plating, the OPCs were used for the experiments. To differentiate OPCs from myelin basic protein–positive oligodendrocytes, the culture medium was switched to Dulbecco's Modified Eagle Medium containing 1% penicillin/streptomycin, 10 ng/mL ciliary neurotrophic factor, 50 ng/mL T3, and 2% B27 supplement. To mimic chronic mild-hypoxic condition, OPCs were incubated with nonlethal $CoCl_2$ (Sigma). Please see online-only Data Supplement for detailed methods of in vitro cell culture experiments.

Statistical Analysis

On the basis of published and pilot data, power estimates were calculated based on α=0.05 and β=0.8 to obtain group sizes appropriate for detecting effect sizes in the range of 30% to 50% for in vivo models and 40% to 50% for cell culture models. A 1-way ANOVA followed by post hoc Fisher protected least significant difference test was used to determine the significant differences in various indices among the groups. A P value of <0.05 was considered statistically significant.

Results

Eight-Month-Old Mice Suffer More White Matter Injury Than 2-Month-Old Mice After Prolonged Cerebral Hypoperfusion

There were no clear differences in myelination and white matter integrity of the corpus callosum in young 2-month-old mice compared with older 8-month-old mice (Figure S1 in the online-only Data Supplement). We then asked whether in spite of similar baseline conditions, older white matter would still be more vulnerable to injury. Mice were subjected to a standard model of prolonged cerebral hypoperfusion by using microcoils to bilaterally narrow the luminal diameters of their common carotid arteries. There were no initial differences in carotid diameters (2-month-old mice: 341.4±24.6 μm; 8-month-old mice: 342.4±24.7 μm), and the surgical procedures produced similar degrees of cerebral hypoperfusion (2-month-old: 75.9±8.7%; 8-month-old: 78.0±6.6%, cerebral blood flow levels at 14 days relative to sham-operative animals).

For 14 days after onset of cerebral hypoperfusion, white matter integrity was assessed with fluoromyelin staining, myelin basic protein Western blot, and spectral domain optical coherence microscopy imaging (Figure S2 in the online-only Data Supplement). As expected, cerebral hypoperfusion induced a progressive degradation of white matter integrity in the corpus callosum in all mice. But loss of myelin immunostaining (Figure 1A) and myelin basic protein Western blot (Figure 1B) was more severe in older 8-month-old brains compared with the younger 2-month-old brains. Spectral domain optical coherence microscopy imaging confirmed that the 8-month-old brains showed increased myelin fiber derangement and white matter vacuoles (Figure 1C). Consistent with these morphological end points, 8-month-old mice demonstrated more severe neurological deficits compared with younger 2-month-old mice, as measured with the standard Y-maze test. At prehypoperfusion baseline conditions, all

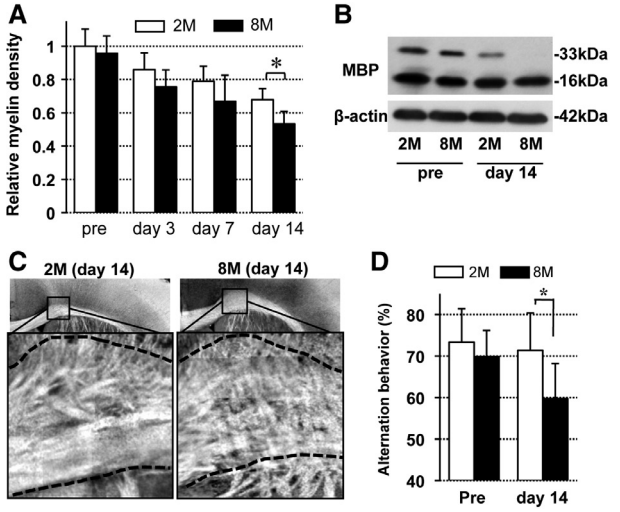

Figure 1. White matter lesion after prolonged cerebral hypoperfusion. **A**, Relative density of fluoromyelin intensity in mouse corpus callosum. Values were calculated based on the density of pretreatment in 2-month-old mice (n=5). **B**, Western blot images for myelin basic protein expression in mouse corpus callosum before and 14 days after the stress onset. β-Actin is an internal control. **C**, Spectral domain optical coherence microscopy images of corpus callosum in 2-month-old and 8-month-old mice at day 14 after white matter injury. **D**, Alternation behavior (index of working/spatial memory) of 2-month and 8-month-old mice at pretreatment and day 14 after injury (n=10). Values are mean±SD (*P<0.05). 2M indicates 2-month-old mice; and 8M, 8-month-old mice.

mice showed normal Y-maze function. But after 2 weeks of cerebral hypoperfusion, Y-maze function was more severely affected in the older 8-month-old mice (Figure 1D).

Eight-Month-Old Mice Show Decreased Oligodendrogenesis After Prolonged Cerebral Hypoperfusion

To assess the hypothesis that older brains possess dampened endogenous repair capacities, we used 5-bromodeoxyuridine (BrdU) incorporation experiments to ask whether oligodendrogenesis is suppressed in aging white matter (Figure S3 in the online-only Data Supplement). In young 2-month-old white matter, the number of BrdU-incorporated cells was increased after cerebral hypoperfusion (Figure 2A). In older 8-month-old white matter, the increase in BrdU-incorporated cells was detectable but significantly reduced compared with 2-month-old white matter (Figure 2A). Double-immunofluorescence labeling with cell-specific markers revealed that the major cell type of BrdU-incorporated cells comprised NG2-positive OPCs in both groups (Table S1 in the online-only Data Supplement).

To confirm these findings, immunostaining was used to quantify the number of NG2 positive OPCs up to 14 days after cerebral hypoperfusion onset. In young 2-month-old brains, the number of NG2-positive OPCs in the corpus callosum was gradually increased over the course of prolonged hypoperfusion (Figure 2B and 2C). In contrast, older 8-month-old brains did not show a significant increase in OPC numbers over time (Figure 2B and 2C). The ratio of single stranded DNA/NG2–double-positive cells (damaged OPCs) at day 14 was significantly larger in the 8-month-old brains (Figure 2D), consistent with the observation that OPC numbers did not increase in this older group of mice.

If there is a difference in OPC response in older brains, does this mean that the numbers of mature oligodendrocytes would also be different? To answer this question, 2 BrdU treatment schedules were used: (1) BrdU was injected on day 7 after the hypoperfusion onset, and brains were removed and examined on the same day (‘7→7’), and (2) BrdU was injected on day 7, and brains were examined 1 week later (‘7→14’; Figure 3A). In young 2-month-old mice, the number of BrdU-positive cells on day 14 was similar to that on day 7 (Figure 3B). In contrast, for 8-month-old mice, the number of BrdU-positive cells on day 14 was less than that on day 7 (Figure 3B), indicating that newly generated OPCs after hypoperfusion in the older brains would die in 1 week without maturing into oligodendrocytes. Double-immunofluorescence labeling with cell-specific markers showed that some of newly generated OPCs on day 7 were successfully differentiated into mature oligodendrocytes on day 14 in young mice. But the older 8-month-old mice did not show equivalent levels of oligodendrogenesis (Figure 3C), indicating that OPCs in the older mice would die before differentiating into mature oligodendrocytes.

CREB Signaling Is Involved in Oligodendrogenesis After White Matter Injury

Older white matter regions may have a dampened recovery response to prolonged cerebral hypoperfusion compared with young brains. To this point, our findings suggest that this may be partly because of a decline in the endogenous capacity for compensatory oligodendrogenesis. But what mechanisms are involved? Because CREB signaling is known to be generally important for neurogenesis and oligodendrogenesis, we asked whether differences in CREB responses may also be involved in our models. Immunostaining showed that, in both young

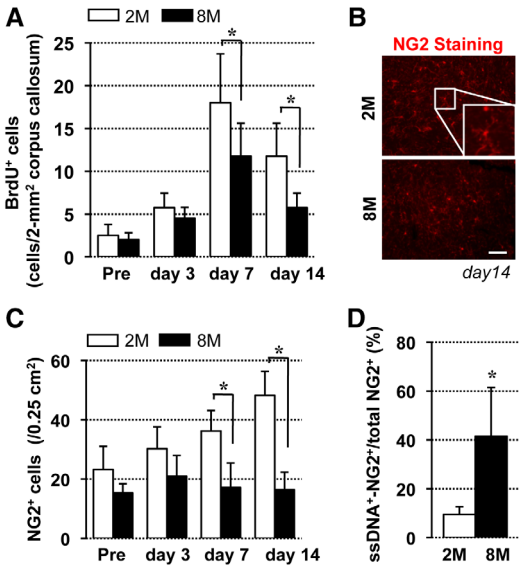

Figure 2. Oligodendrocyte precursor cell proliferation in vivo. **A**, Number of 5-bromodeoxyuridine (BrdU)-positive (BrdU⁺) cells in the corpus callosum (n=5). **B**, Representative images of NG2 staining in the lateral side of corpus callosum at day 14 after white matter injury (bar=50 μm). **C**, Number of NG2-positive (NG2⁺) cells in mouse corpus callosum during white matter damage (n=5). **D**, Ratio of single-stranded DNA (ssDNA)/NG2–double-positive cells in total NG2 cells in the mouse corpus callosum at day 14 (n=5). 2M indicates 2-month-old mice; and 8M, 8-month-old mice.

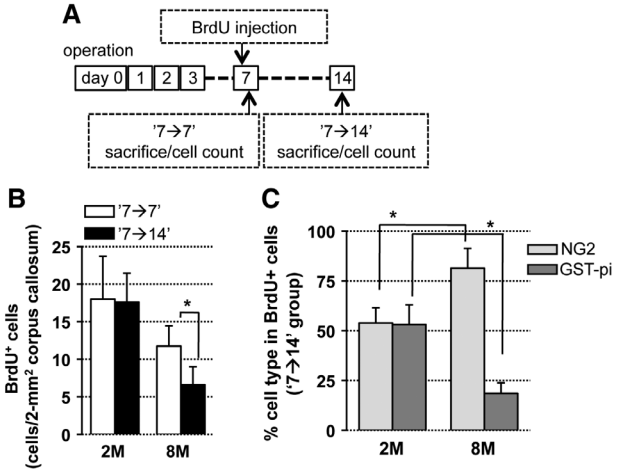

Figure 3. Oligodendrocyte precursor cell differentiation in vivo. **A**, Experimental schedule to assess cell differentiation in the corpus callosum using 5-bromodeoxyuridine (BrdU). **B**, Number of BrdU-positive (BrdU⁺) cells in corpus callosum at days 7 and 14 (n=5). **C**, Ratio of BrdU/NG2- and BrdU/GST-π–double-labeled cells in total BrdU cells in corpus callosum at day 14 (BrdU injection: day 7; n=5). Values are mean±SD (*P<0.05). GST-π indicates glutathione S-transferase-π; 2M, 2-month-old mice; and 8M, 8-month-old mice.

and older white matter, phosphorylated CREB signaling was observed in mature oligodendrocytes (CNPase-positive) and OPCs (platelet-derived growth factor receptor-α–positive) under normal conditions, but phosphorylated CREB was primarily expressed within OPCs (Figure 4A; Figure S4 in the online-only Data Supplement). After prolonged cerebral hypoperfusion, phospho-CREB signals decreased in both groups, with a larger reduction present in the 8-month-old brains (Figure 4B and 4C). Western blots confirmed that CREB phosphorylation was significantly lower in 8-month-old brains compared with 2-month-old brains (Figure 4D).

PDE III Inhibitor Cilostazol Promoted OPC Differentiation In Vitro and Rescued OPC Recovery In Vivo

Differences in CREB-mediated OPC proliferation and differentiation may, in part, explain why older white matter was more vulnerable to prolonged cerebral hypoperfusion. So we next asked whether promoting CREB signaling with the phosphodiesterase (PDE) III inhibitor cilostazol could restore these compensatory OPC responses after prolonged stress and injury. To answer this question, we performed both cell culture and in vivo experiments.

First, primary OPCs were cultured from rat neonatal brain cortex. Under normal growth conditions, these OPCs matured over 7 days and decreased the expression of NG2, a marker for OPCs. In contrast, glutathione S-transferase-π and myelin basic protein expression were increased (Figure 5A), indicating that our cultured OPCs successfully differentiated into mature oligodendrocytes in this model system. To mimic the hypoxic state of prolonged cerebral hypoperfusion, OPCs were treated with 0.01 to 1 μM of $CoCl_2$ for 7 days. As expected, 7 days of $CoCl_2$ treatment increased hypoxia-inducible factor 1-α expression in our cultured OPCs in a dose-dependent manner (Figure 5A). CREB signaling may also be involved as the phosphorylation level of CREB was also decreased by $CoCl_2$-induced chemical hypoxia (Figure 5A). Correspondingly, OPC maturation was inhibited by the chronic $CoCl_2$ treatment (Figure 5A). Lactate dehydrogenase assays showed that 0.01 to 1 μM of $CoCl_2$ did not induce cell death in our OPCs (Figure 5B), indicating that the reduction of OPC maturation by $CoCl_2$ was not merely because of cell death. Next, we asked whether activating CREB with cilostazol could rescue OPC maturation after prolonged chemical hypoxia. Cilostazol alone did not change OPC state (Figure S5 in the online-only Data Supplement) but significantly increased CREB phosphorylation and promoted OPC differentiation under $CoCl_2$-induced hypoxic conditions (Figure 5C and 5D; Figure S5 in the online-only Data Supplement).

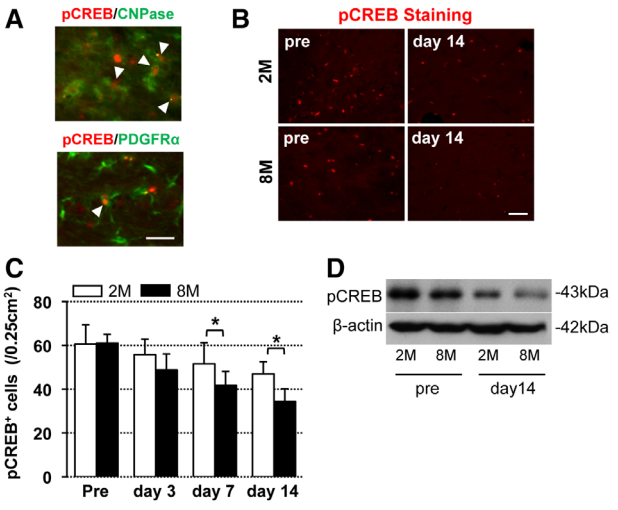

Figure 4. Phospho-CREB (pCREB) in white matter in vivo. **A**, Double immunofluorescent staining of pCREB with CNPase (a marker for mature oligodendrocytes) or PDGFRα (a marker for oligodendrocyte precursor cells) in 2-month-old mice under normal conditions. Arrowheads are double-positive cells (bar=25 μm). **B**, Representative images of pCREB staining in the lateral side of corpus callosum (bar=50 μm). **C**, Number of pCREB-positive (pCREB⁺) cells in the lateral side of corpus callosum during the hypoperfusion stress (n=5). **D**, Western blot image of pCREB expression in the corpus callosum. β-Actin is an internal control. Values are mean±SD (*P<0.05). CREB indicates cyclic AMP response element-binding protein; PDGFRα, platelet-derived growth factor receptor-α; 2M, 2-month-old mice; and 8M, 8-month-old mice.

Finally, we turned back to our in vivo model of prolonged cerebral hypoperfusion. Cilostazol treatment (10 mg/kg per day for 14 days) was performed by intraperitoneal injection in 8-month-old mice subjected to cerebral hypoperfusion. This treatment schedule was previously reported to effectively inhibit PDE III in brain in vivo.[15] As expected, mice treated with cilostazol showed larger numbers of phospho-CREB–positive cells than the vehicle-treated group did at day 14 (Figure S6 in the online-only Data Supplement). Correspondingly, the cilostazol-treated mice exhibited less white matter injury (Figure 6A; Figure S6 in the online-only Data Supplement), larger number of OPCs (Figure 6B; Figure S6 in the online-only Data Supplement), and better cognitive function (Figure 6C). The BrdU-incorporation/differentiation assay (Figure S7 in the online-only Data Supplement) revealed that cilostazol promoted the proliferation of white matter cells (Figure S8 in the online-only Data Supplement), as well as differentiation of OPCs into mature oligodendrocytes (Figure 6D).

Discussion

Demyelination and cognitive decline are major pathological hallmarks of ischemic white matter diseases, which are mostly associated with increasing age. Although adult brains retain neuroplasticity and regenerative capacities to compensate for lost brain cells, aged brains tend to slowly lose these endogenous repair systems. Our current study demonstrated that white matter regions in aging 8-month-old mice are more vulnerable to prolonged cerebral hypoperfusion, and this is caused in part by a loss of endogenous CREB-mediated oligodendrogenesis. Therefore, drugs that can activate protein kinase A-CREB signaling cascade may provide novel therapeutic approaches for white matter injury in vascular dementia and stroke.

Aging lowers the endogenous capacities for brain regeneration and remodeling. Past studies have demonstrated that aging reduces the differentiation of neuronal precursor cells into neuronal phenotypes,[16] increases the number of dying/dead brain cells,[17] and decreases CREB activation in neurons.[18] Our current data may expand these findings—the capability

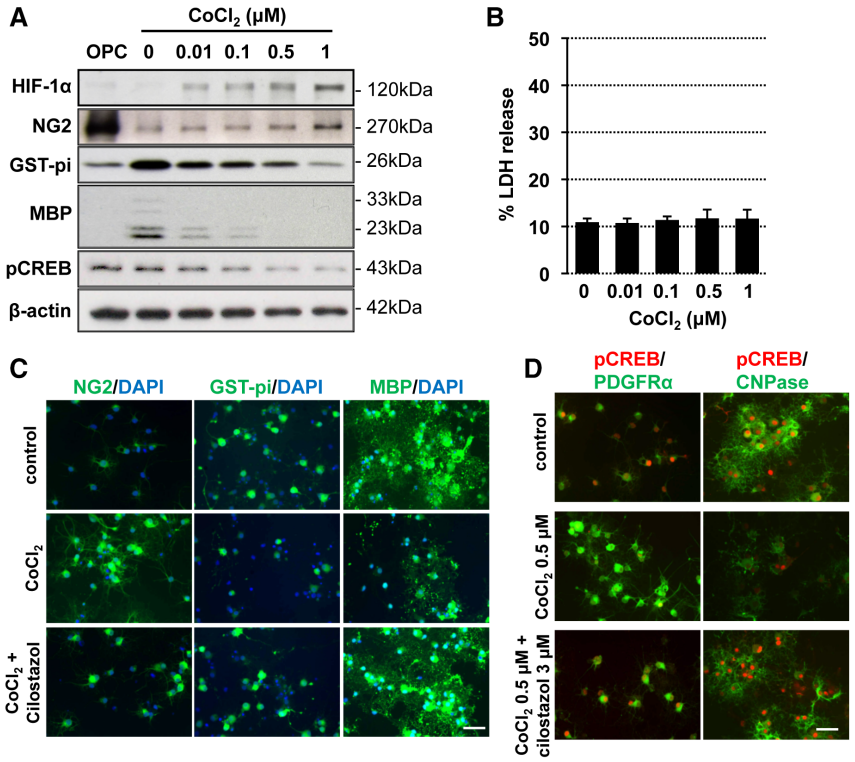

Figure 5. Cilostazol-induced OPC proliferation/differentiation in vitro. **A**, Western blot images using samples from cultured rat OPCs with 7-day $CoCl_2$ treatment. HIF-1α is a marker for hypoxic conditions, NG2 is a marker for OPCs, GST-π and MBP are markers for mature oligodendrocytes, and β-actin is an internal control. **B**, LDH assays showed that 0.01 to 1 μM of $CoCl_2$ (7-day treatment) did not induce cell death in our OPCs (n=6). Values are mean±SD. **C**, Representative images of NG2, GST-π, and MBP staining in cultured rat OPCs at day 7 after the $CoCl_2$ treatment with or without cilostazol (bar=25 μm). **D**, Representative images of pCREB, PDGFR-α, and CNPase staining in cultured rat OPCs at day 7 after $CoCl_2$ treatment with or without cilostazol (bar=25 μm). DAPI indicates 4',6-diamidino-2-phenylindole; GST-π, glutathione S-transferase-π; HIF-1α, hypoxia-inducible factor 1-α; LDH, lactate dehydrogenase; MBP, myelin basic protein; OPC, oligodendrocyte precursor cells; and pCREB, phosphorylated cyclic AMP response element-binding protein.

of oligodendrogenesis in the white matter also decreases with aging through deactivation of CREB signaling. In this study, middle-aged mice showed lower phospho-CREB level in the white matter than young mice under pathological conditions. Moreover, activating CREB by a PDE III inhibitor cilostazol alleviated the white matter dysfunction in middle-aged mice by protecting newly generated OPCs and enhancing OPC proliferation/maturation. Cilostazol has been approved for the treatment of intermittent claudication and has been shown to improve pain-free walking distance worldwide in patients with peripheral arterial disease[19] and help in the secondary prevention of ischemic stroke in Japan.[20] Our findings provide initial proof-of-concept that cilostazol (or other PDE III inhibitors) might be a promising drug for age-related white matter diseases. But of course, more extensive preclinical studies are required before this approach can be translated into clinical applications.

Although our current findings demonstrate that aged white matter possesses less capacity for oligodendrogenesis, many important caveats remain. First, we focused on only oligodendrocyte lineage cells in this study. But there are other cell types in white matter, including endothelial cells and astrocytes. The concept of the neurovascular unit suggests that interactions between different cell types maintain brain function under normal conditions and facilitates brain remodeling after injury in the gray matter.[5] In white matter, cell–cell trophic coupling might similarly participate in white matter homeostasis. Indeed, both cerebral endothelial cells and astrocytes show supportive effects for oligodendrocytes/OPCs.[21] Hence, future studies should examine whether the trophic coupling between oligodendrocytes and neighboring cells diminishes with aging in the white matter. Second, our current study demonstrates that loss of CREB activation in the aging

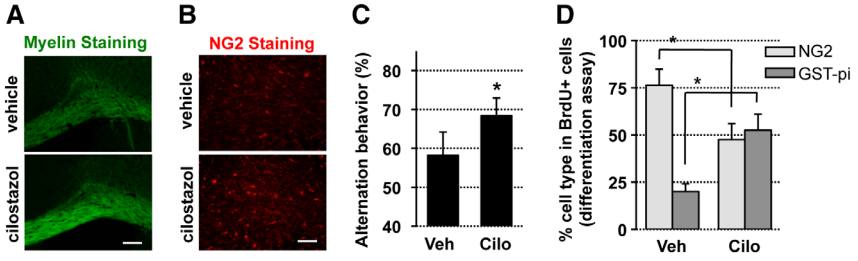

Figure 6. Cilostazol-induced white matter repair in middle-aged mice. **A**, Representative images of fluoromyelin staining in 8-month-old mice with or without cilostazol treatment at day 14 (bar=100 μm). Quantitative data are shown in Figure S6D in the online-only Data Supplement. **B**, Representative images of NG2 staining in the lateral side of corpus callosum in 8-month-old mice with or without cilostazol (10 mg/kg per day for 14 days, IP) at day 14 after white matter injury (bar=50 μm). Quantitative data are shown in Figure S6E in the online-only Data Supplement. **C**, Alternation behavior (index of working/spatial memory) of 8-month-old mice with or without cilostazol treatment at day 14 (n=8). **D**, Ratio of BrdU/NG2- and BrdU/GST-π–double-labeled cells in total BrdU cells in 8-month-old mice corpus callosum at day 14 (n=5). Values are mean±SD. *P<0.05. Experimental schedule for the oligodendrocyte precursor cells differentiation assay is shown in Figure S7 in the online-only Data Supplement. BrdU indicates 5-bromodeoxyuridine; Cilo, cilostazol-treatment group; GST-π, glutathione S-transferase-π; and Veh, vehicle group.

8-month-old white matter leads to deficits in oligodendrogenesis. But what factors/mechanisms lower the CREB signaling in the middle-aged white matter? CREB activation is regulated by several growth factors, such as brain-derived neurotrophic factor, and growth factor expressions are decreasing in aging.[22] Hence, reduction of growth factor expression may fail the CREB activation during the stress in aged white matter. These questions should be explored in future studies. Finally, it is important to acknowledge that trying to correlate aging mouse models to the aging human brain is not straightforward. To date, most aging studies with rodents have focused on differences between young (2–3 months old) and very old (>12–15 months old) brains.[23] But it might be possible that the age-related decline in oligodendrogenesis would occur even before obvious declines in myelin density or cognitive function. In this study, we compared young 2-month-old mice with older 8-month mice. It is possible that our models may mimic middle-aged humans. But further studies are warranted to carefully track the temporal profile of these CREB-mediated mechanisms in a wider age range of mice.

In conclusion, our current study demonstrates novel mechanisms by which aging white matter in mouse brain is more vulnerable to prolonged cerebral hypoperfusion and hypoxic stress. Importantly, these phenomena occurred in mice that had normal myelin density and cognitive function at baseline; no deficits were present yet. But these aging regions had lost their ability to recruit CREB-mediated oligodendrogenesis for responding to injury and stress. Hence, drugs that can activate CREB signaling may be a promising approach for aging patients with white matter–related diseases such as vascular dementia or stroke.

Please see online-only Data Supplement for methods and additional data.

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Disclosures

None.

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