SUPPLEMENTAL MATERIAL

Diabetes Mellitus Impairs White Matter Repair and Long-term Recovery after Cerebral Ischemia

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Supplementary Methods

Animal model

Distal middle cerebral artery occlusion (dMCAO) was performed as described previously.[1] Briefly, mice were anesthetized and a skin incision was made in the middle of the neck. After ligation of the left common carotid artery (CCA), the skin was sutured and another incision was made at the midpoint between the infraorbital edge and outer canthus of the left side. The distal part of the MCA was coagulated by bipolar electrocautery. Cerebral blood flow (CBF) was measured using laser Doppler flowmetry.

Regional CBF measurements with laser speckle contrast imager

Regional CBF (rCBF) was monitored using a laser speckle contrast imager (PeriCam PSI HR System, Perimed Inc., Jarfalla-Stockholm, Sweden), as previously described.[2] Repeated measurements were made in each group prior to surgery (baseline), during occlusion of the left CCA, after left dMCAO, and 3d, 7d and 14d after dMCAO. After induction of anesthesia, the mouse was place under a laser speckle contrast imager in the prone position. A parietal middle incision was made to expose the skull. The speckle imager was placed at a working distance of ~10 cm above the skull to detect CBF. CBF measurements were made in the same areas (MCA territory) over time. CBF changes were expressed as percentages of baseline perfusion.

Measurement of infarct volume

Neuronal tissue loss was determined after immunofluorescent staining with a rabbit anti-NeuN antibody (Millipore, Billerica, MA). Images of six consecutive sections per animal were captured through the lesion area using a 1.25X objective. Infarct volume in all six sections was quantified using NIH Image J software (Bethesda, MD) by an investigator blinded to experimental conditions. The confounding effects of brain edema on infarct volume measurements were avoided by subtracting the non-infarcted volume of the ipsilateral hemisphere from the total volume of the contralateral.

Immunohistochemistry and cell counting

Brain sections were prepared and subjected to immunofluorescent staining, as described before.[1] Primary antibodies included rabbit anti–myelin basic protein (MBP; Abcam, Cambridge, MA), rabbit anti-Iba1 (Wako, Richmond, VA), goat anti-CD206 (R&D Systems, Minneapolis, MN), rat anti-CD16 (BD Biosciences, San Jose, CA), mouse anti-5-bromo-2-deoxyuridine (BrdU; BD Bioscience, San Jose, CA), rabbit anti-NG2 (Millipore, Billerica, MA), rabbit anti-Adenomatous Polyposis Coli (APC, Abcam, Cambridge, MA) and mouse anti-neurofilament heavy chain 200 (NF-200; Abcam, Cambridge, MA). For BrdU staining, brain sections were pretreated with 1N HCl for 1 hour followed by 0.1 mol/L boric acid (pH 8.5) for 10 minutes at 37 °C. Confocal microscopy (Fluoview FV1000; Olympus, Tokyo, Japan) was used to capture images. Cell counts and MBP fluorescence intensity measurements were made using NIH image J software (Bethesda, MD) by an investigator blinded to experimental groups. Means of positively stained cells or fluorescence intensities were calculated from two microscopic fields in the cortex (CTX) and two fields in the external capsule (EC) of each section. Three brain sections were analyzed for each animal. Cell numbers were expressed as numbers per square millimeter. Mean fluorescence intensity was expressed as a function of the fluorescence intensity of the contralateral hemisphere of the same animal using the same camera and microscope settings.

Neurobehavioral tests

Behavioral tests were performed to access sensorimotor function and long-term cognitive function. Adhesive removal, open field, and Morris water maze tests were performed as previously described.[1] Each mouse was trained for three trials per day (with randomly assigned starting positions) to locate the platform for three consecutive days before MCAO. Animals were tested at 22-26 days after tMCAO. Three trials were performed on each day. The path efficiency, calculated as the Euclidean distance between the starting location and the final location divided by the total distance travelled, was recorded. On day 27, the platform was removed and a single 60s probe trial was conducted for the spatial memory test. The path efficiency in the goal quadrant where the platform was previously located was recorded as spatial memory by a blinded observer.

The open field test was used to assess general locomotor activity levels, anxiety, and willingness to explore the environment. Briefly, mice were gently removed from their cages and immediately placed in a clean, empty-walled arena equipped with a video camera. Mice were allowed to explore the arena for 10 minutes. Time in the center and total distance travelled were recorded and analyzed by a blinded observer.

Blood sugar measurements

The mouse's tail was sterilized with alcohol before each snip. One drop of tail blood was collected under the same conditions on the pre-surgical day and 1d, 3d, 5d after surgery. Blood glucose was measured with a OneTouch Ultra glucometer (LifeScan, Chesterbrook, PA).

Compound action potential measurements

Compound action potentials (CAPs) were measured by a blinded observer in the EC, as previously described.[3, 4] Mice were decapitated after CO_2 euthanasia and the brains were removed. Coronal slices (350 μm thick) were prepared -1.06 mm from Bregma and transferred to an incubation chamber containing pre-gassed (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF; 126 mmol/L NaCl, 2.5 mmol/L KCl, 1 mmol/L $Na_2H_2PO_4$, 2.5 mmol/L $CaCl_2$, 26 mmol/L $NaHCO_3$, 1.3 mmol/L $MgCl_2$, 10 mol/L glucose; pH 7.4) for 1 h at room temperature. Brain slices were then perfused with aCSF at a constant rate (3–4 mL/min) at 22 °C. For recording, a bipolar stimulating electrode (intertip distance, 100 μm) was placed across the corpus callosum at ~0.9 mm lateral to the midline. A glass extracellular recording pipette (5–8 MΩ tip resistance when filled with aCSF) was placed in the EC, 0.24–0.96 mm from the stimulating electrode in 0.24-mm increments.[3] Only the recording made at 0.75 mm from the stimulating electrode is reported here. The CAP signal was digitized (Digidata 1500B plus humsilencer; Molecular Devices, San Jose, CA), amplified (x 1K) and recorded by the Axoclamp 700B amplifier (Molecular Devices, San Jose, CA). The signal was then analyzed by pClamp 10 software (Molecular Devices, San Jose, CA). The input-output curves were generated by increasing the stimulating intensity in 250 mA intervals from baseline, up to 2000 mA. The amplitude of the N1 component of the CAP (representing myelinated fibers) was calculated as the variance from the first peak to the first trough. The amplitude of the N2 component (representing unmyelinated fibers) was measured as the variance from the last trough to baseline.

Flow cytometry

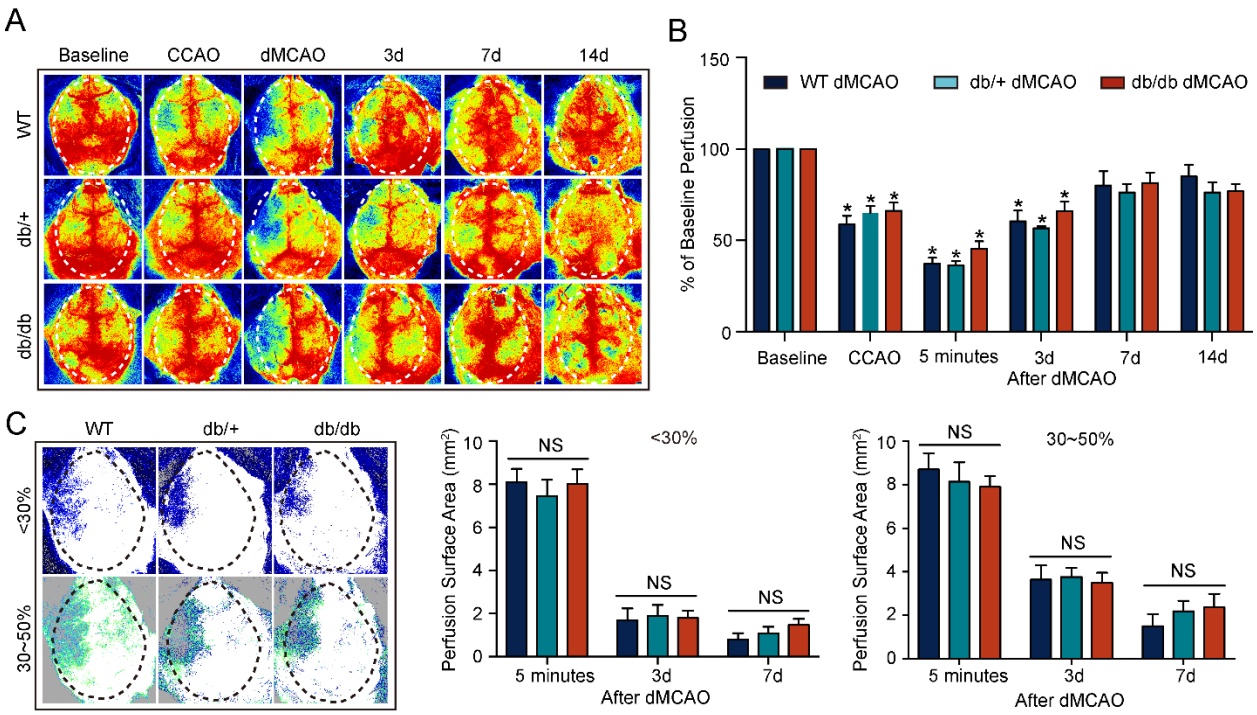
Brain single cell suspensions were prepared as we previously described.[5, 6] Single cells were stained with fluorescent-labeled mouse CD11b (eBioscience, San Diego, CA), CD45 (eBioscience, San Diego, CA), and CD16 (BD Biosciences, San Jose, CA). Cells were then permeablized/fixed using an intracellular staining kit (BD Biosciences, San Jose, CA) followed by Arginase 1 (Arg1, Biolegend, San Diego, CA) immunostaining. Flow cytometric analysis was performed using a FACS cytometer (BD Biosciences, San Jose, CA).

Primary microglia and OPC cultures

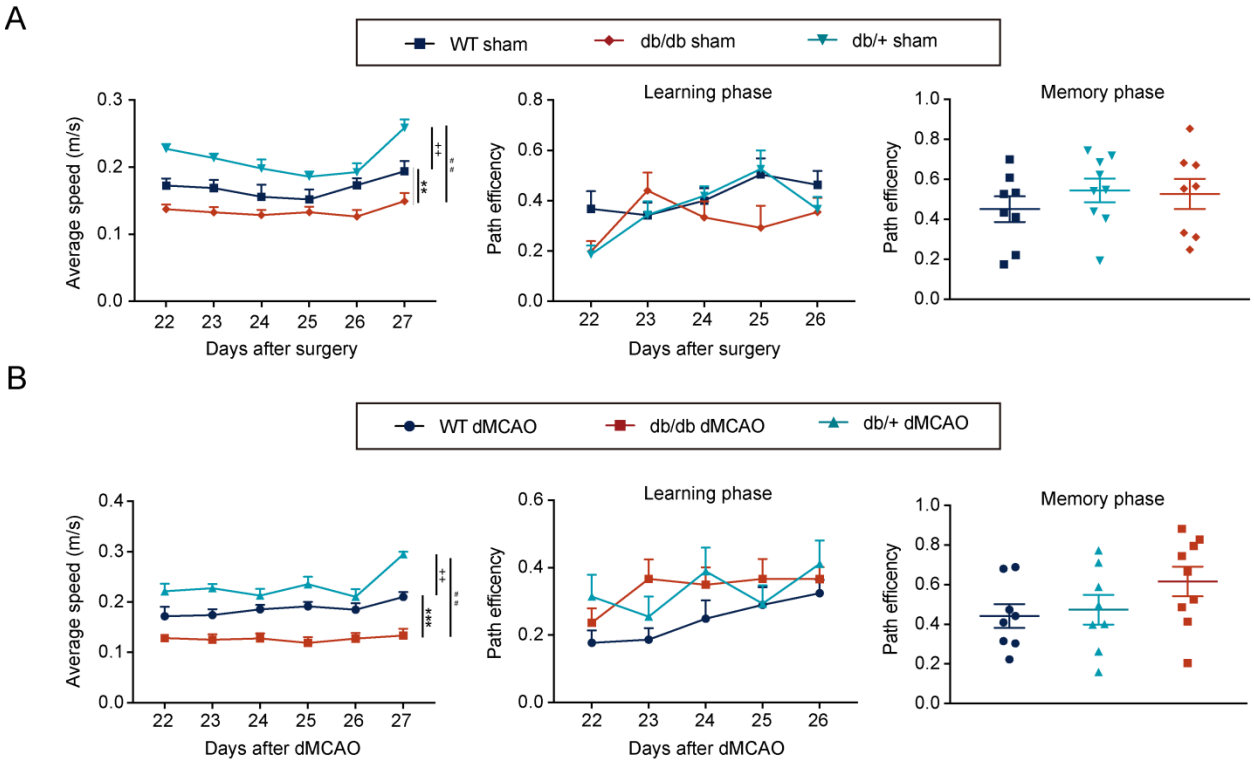
Primary microglia and OPCs were prepared from the whole brains of 1-day-old Sprague-Dawley rat pups, as previously described.[7] Microglia were cultured for 48 hours in media with a normal concentration of glucose (5.5 mM) or high glucose (15 mM), in the presence or absence of low concentrations of lipopolysaccharides (LPS, 2.5 ng/mL). The normal concentration of glucose (5.5 mM) approximates normal blood sugar levels *in vivo*. Brain extracellular glucose concentration is usually lower than plasma glucose with a steady-state value of approximately 3 mM,[8] but this may be higher in stroke patients due to loss of BBB integrity. We have compared the effects of culture media containing 5.5 mM or 3 mM glucose on microglia polarization and did not observe any significant differences (not shown). Therefore, we have used the regular culture media containing 5.5 mM glucose as the appropriate control. For co-culture experiments, pretreated microglia were co-cultured with OPCs for 3 days.

Supplementary Table I. Physiological parameters before and 10 min after dMCAO in WT, db/+, and db/db mice.

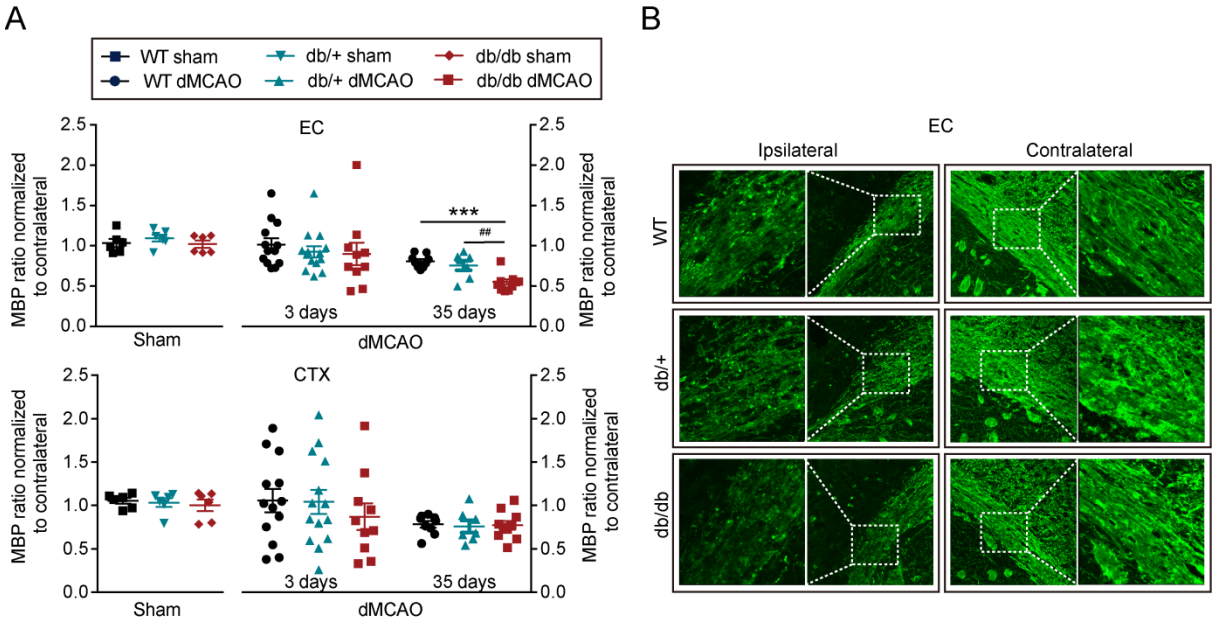
	WT mice			db/+ mice			db/db mice		
	Pre-operation	Post-dMCAO	P-value	Pre-operation	Post-dMCAO	P-value	Pre-operation	Post-dMCAO	P-value
PH	7.367±0.026	7.155±0.135	n.s.	7.483±0.024	7.388±0.038	n.s.	7.362±0.025	7.321±0.032	n.s.
PaO2(%)	99.38±0.69	99.13±0.56	n.s.	99.87±0.05	99.83±0.04	n.s.	99.6±0.29	99.77±0.05	n.s.
PaCO2 (mmol/L)	20.08±3.76	15.73±5.4	n.s.	19.6±5.02	16.88±4.33	n.s.	24.78±5.46	22.93±2.47	n.s.
Na+ (mmol/L)	146.2±1.47	149±4.42	n.s.	145.67±0.47	146.5±3.77	n.s.	147.5±4.27	151.33±4.19	n.s.
K+ (mmol/L)	5.24±1.42	3.98±0.79	n.s.	4.53±0.17	3.25±0.29	n.s.	5.68±2.67	3.5±0.22	n.s.
Ca++ (mmol/L)	1.15±0.05	1.19±0.08	n.s.	1.11±0.08	1.23±0.02	n.s.	1.11±0.11	1.22±0.07	n.s.
Cl- (mmol/L)	113.4±3.44	114.67±2.62	n.s.	113.67±4.92	113.25±1.3	n.s.	113.5±4.77	116.33±6.18	n.s.



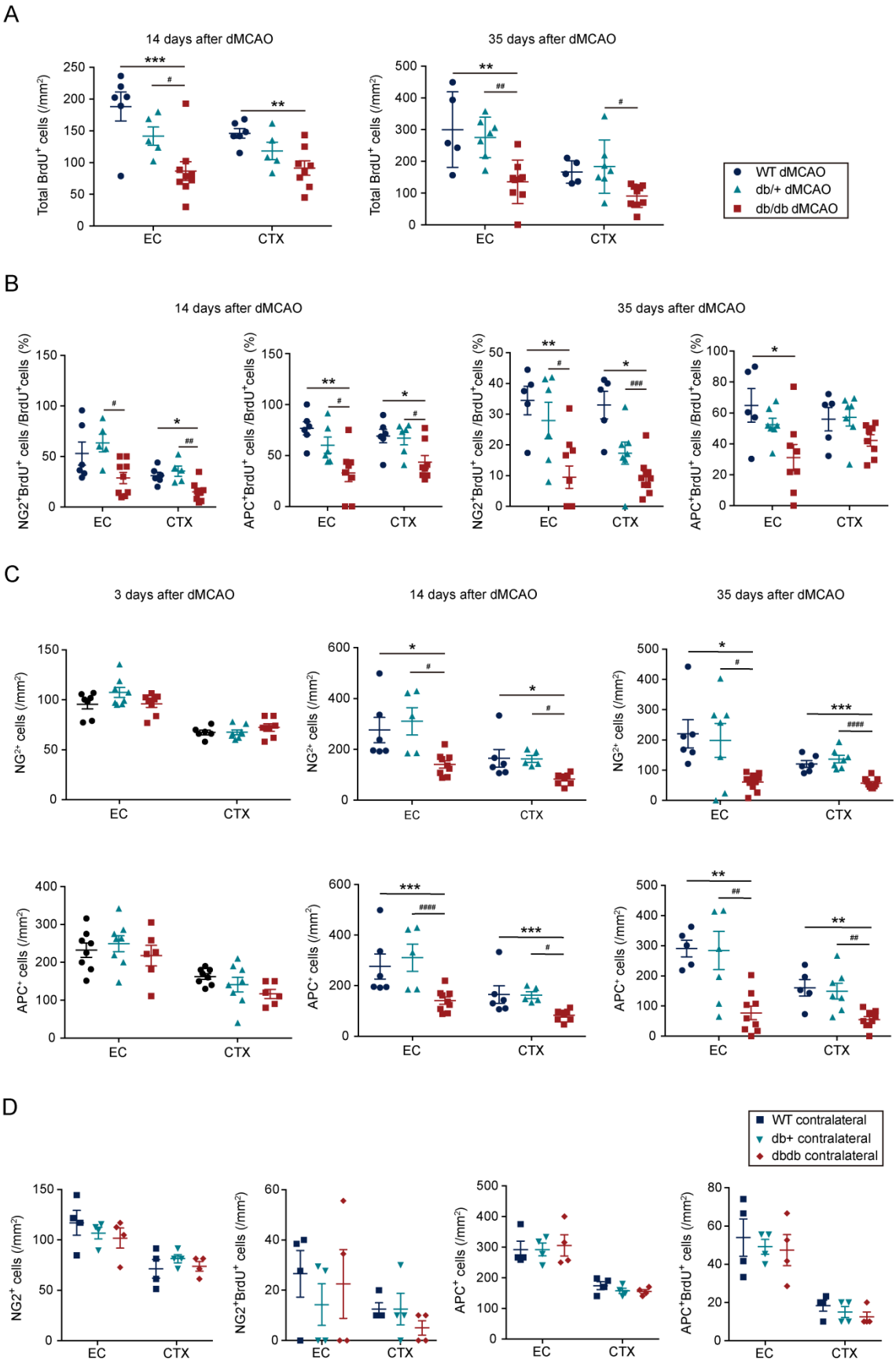
Supplementary Figure I. WT, db/+, and db/db mice exhibit similar regional cerebral blood flow (rCBF) over time after dMCAO. rCBF was monitored in WT, db/+, and db/db mice using the 2-dimensional laser speckle technique before surgery, after CCAO, immediately after dMCAO, and 3d, 7d, 14d after dMCAO. **A,** Representative 2-D laser speckle images of the axial surface view from the top. The white dotted line outlines the boundaries of the skull. **B,** rCBF in the infarct regions was quantified and expressed as the percentage change from baseline. **C,** Representative images and quantification of surface areas where the rCBF decreased to less than 30% (blue), or where the rCBF was between 30-50% (green) of baseline at 0d, 3d, and 7d after dMCAO. Data are expressed as means ± SEM. n = 6-8 per group. NS, not significant. *p < 0.001 vs corresponding baseline; repeated measures ANOVA followed by Bonferroni *post hoc* test.



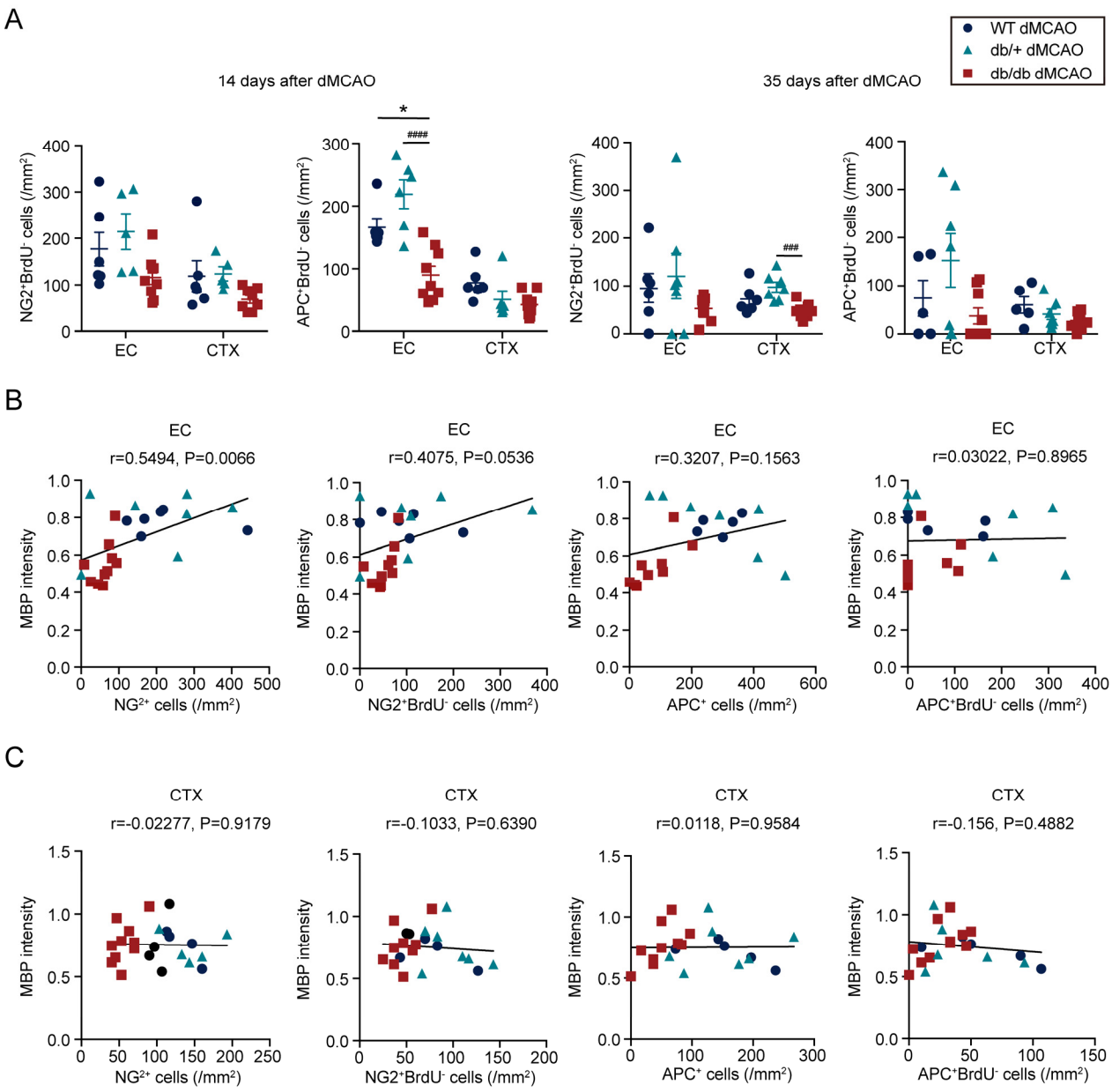
Supplementary Figure II. WT, db/+ mice, and db/db mice exhibit similar cognitive function after dMCAO. The Morris water maze was used to evaluate cognitive function. The following parameters were measured: escape latency, swimming speed, path efficiency in learning phase, and path efficiency when the platform was removed (memory phase). n = 8 - 9/sham group, n = 12-14/dMCAO group. Data are expressed as means ± SEM. **p < 0.01, ***p < 0.001, WT vs. db/db; ++p < 0.01, WT vs. db/+. ##p < 0.01, db/+ vs. db/db; two-way repeated measures ANOVA followed by Bonferroni *post hoc* test.



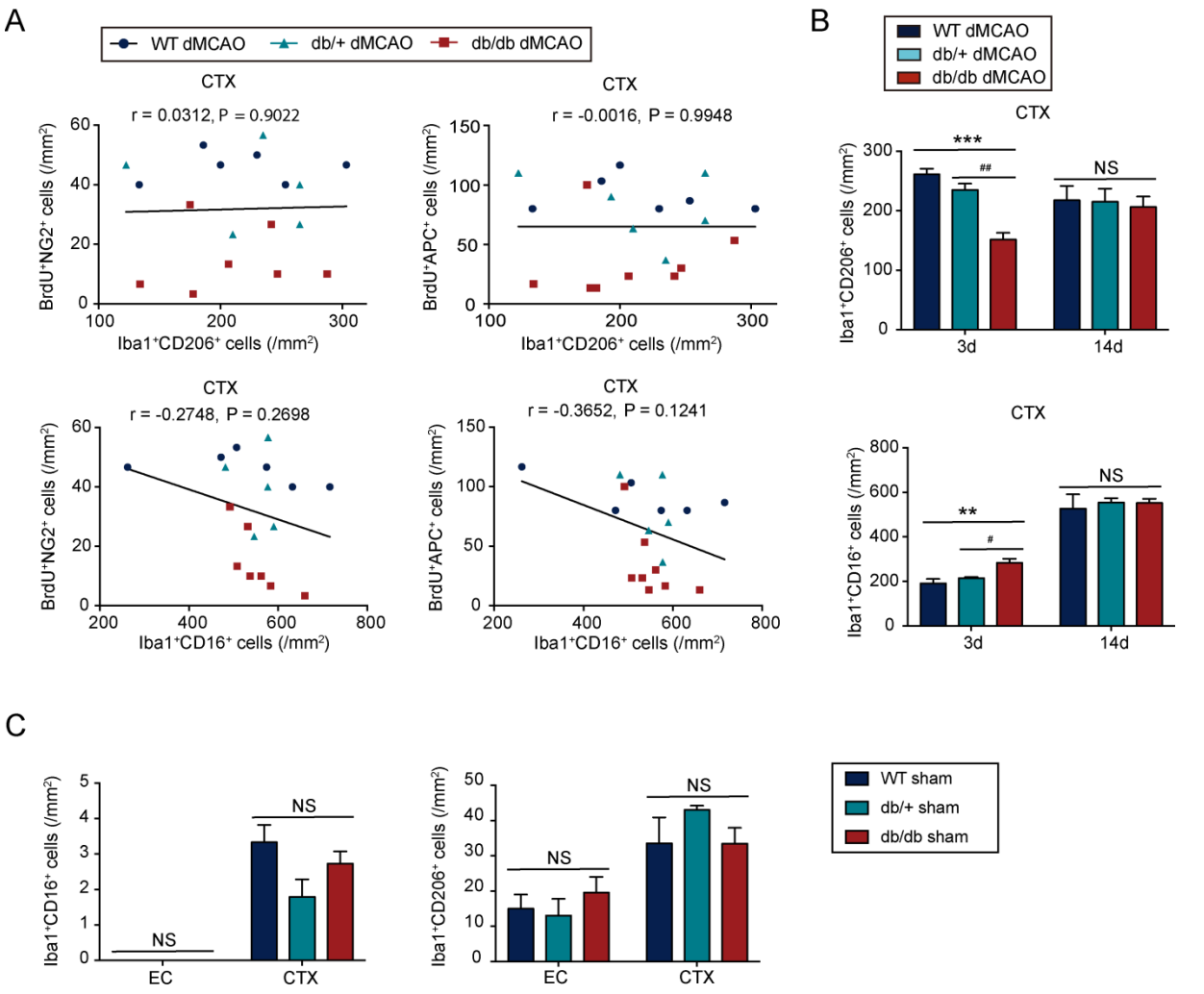
Supplementary Figure III. **White matter deterioration is significantly higher in db/db mice than WT mice or db/+ mice 35 days after stroke.** Myelin basic protein (MBP) immunostaining was used to measure the myelination of white matter in the EC and CTX of WT mice, db/+ mice, and db/db mice 3 days and 35 days after stroke. **A,** Quantification of MBP intensity of EC and CTX. Sham: n = 6/group; dMCAO 3d: n = 10-14/group; dMCAO 35d: n = 7-10/group. **B,** Representative images of MBP-immunostained sections in the ipsilateral and contralateral EC 35 days after dMCAO. Scale bar: 50 μm. Data are expressed as means ± SEM. ***p < 0.001, WT vs. db/db; ##p < 0.01, db/+ vs. db/db; one-way ANOVA followed by Bonferroni *post hoc* test.


A
14 days after dMCAO
35 days after dMCAO
Total BrdU+ cells (/mm2)
EC
CTX
WT dMCAO
db/+ dMCAO
db/db dMCAO
B
14 days after dMCAO
35 days after dMCAO
NG2+BrdU+ cells /BrdU+cells (%)
APC+BrdU+ cells /BrdU+cells (%)
C
3 days after dMCAO
14 days after dMCAO
35 days after dMCAO
NG2+ cells (/mm2)
APC+ cells (/mm2)
D
WT contralateral
db+ contralateral
dbdb contralateral
NG2+ cells (/mm2)
NG2+BrdU+ cells (/mm2)
APC+ cells (/mm2)
APC+BrdU+ cells (/mm2)

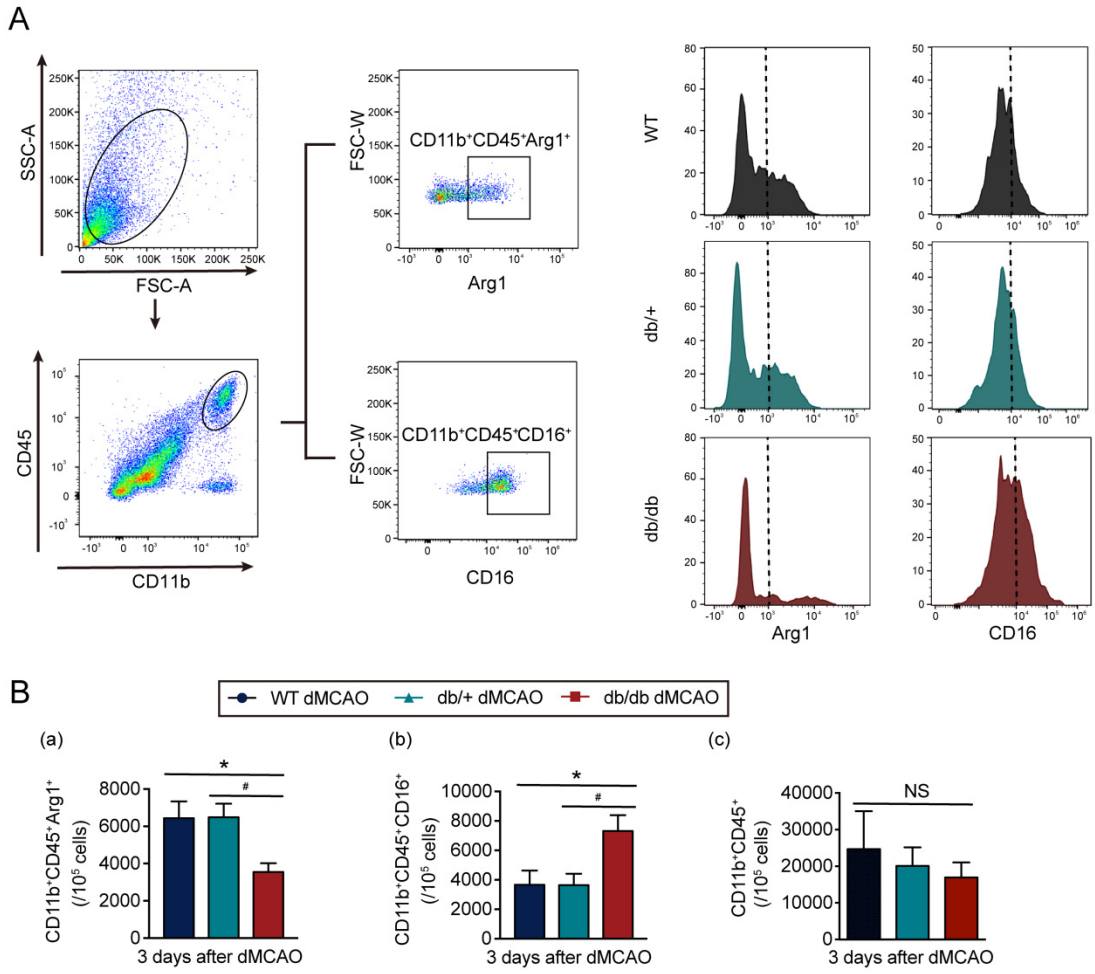
Supplementary Figure IV. The number of total oligodendrocytes is reduced in db/db mice compared to WT mice and db/+ mice. A, Quantification of the total number of $BrdU^+$ cells in the EC and cortex of WT mice, db/+ mice, and db/db mice 14 days and 35 days after stroke. **B,** Percentages of newly proliferated $BrdU^+$ cells expressing NG2 or APC in the EC and cortex of WT mice, db/+ mice, and db/db mice 14 days and 35 days after stroke. **C,** Quantification of the number of NG2-stained and APC-stained cells in the EC and CTX of WT mice, db/+ mice, and db/db mice 3 days, 14 days and 35 days after dMCAO. n = 5-10/group. Data are expressed as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, WT vs. db/db; #p <0.05, ##p < 0.01, ###p < 0.001, db/+ vs. db/db; one-way ANOVA followed by Bonferroni *post hoc* test. **D,** Quantification of the numbers of $NG2^+$ cells, APC^+ cells, $NG2^+BrdU^+$ cells and APC^+BrdU^+ cells in the contralateral EC and contralateral CTX of WT mice, db/+ mice, and db/db mice 35 days after dMCAO. Data are expressed as means ± SEM. n = 3-4/group.



Supplementary Figure V. Pre-existing BrdU$^-$ OPCs or oligodendrocytes are not correlated with MBP intensity. A, Quantification of the numbers of NG2$^+$BrdU$^-$ and APC$^+$BrdU$^-$ cells in the EC and CTX of WT mice, db/+ mice, and db/db mice 14 days and 35 days after dMCAO. n = 5-10/group. Data are expressed as means ± SEM. *p < 0.05, WT vs. db/db; ###p < 0.001, ####p < 0.0001, db/+ vs. db/db; one-way ANOVA followed by Bonferroni *post hoc* test. **B-C,** Pearson correlation between the number of NG2$^+$ total OPCs or NG2$^+$BrdU$^-$ OPCs or APC$^+$ total oligodendrocytes or APC$^+$BrdU$^-$ oligodendrocytes and MBP intensity in the EC (B) or CTX (C) 35 days after dMCAO. n = 5-10/group.



Supplementary Figure VI. Microglia/macrophage responses in the cortex (CTX) are not associated with impaired oligodendrogenesis. A, Pearson correlation between the number of $NG2^+$ OPC or APC^+ oligodendrocytes and the number of $CD16^+Iba1^+$ M1-like microglia/macrophages or $CD206^+Iba1^+$ M2-like microglia/macrophages in the cortex 14 days after dMCAO. **B**, Upper: Quantification of the number of Iba1 and CD206 double-stained cells in the CTX of WT mice, db/+ mice, or db/db mice 3 days and 14 days after dMCAO. Lower: Quantification of the number of Iba1 and CD16 double-stained cells in the CTX of WT mice, db/+ mice, or db/db mice 3 days and 14 days after dMCAO. Three days: n = 3-4/group, 14 days: n = 5-8/group. Data are expressed as means ± SEM. **p < 0.01, ***p < 0.001, WT vs. db/db; #p <0.05, ##p < 0.01, db/+ vs. db/db; NS, not significant; one-way ANOVA followed by Bonferroni *post hoc* test. C, Quantification of the number of $Iba1^+CD206^+$ cells and $Iba1^+CD16^+$ cells in the EC and CTX of WT mice, db/+ mice, or db/db mice after sham operation. n = 3-4/group.



Supplementary Figure VII. Microglia/macrophage phenotypic changes 3 days after stroke in WT, db/+, and db/db mice. Flow cytometry was used to determine microglia/macrophage phenotypes in the brain 3 days after dMCAO. **A,** Gating strategy and representative histograms for $CD11b^+CD45^+Arg1^+$ populations and $CD11b^+CD45^+CD16^+$ populations in the ischemic brains. **B**, Quantification of $CD11b^+CD45^+Arg1^+$ cells (a), $CD11b^+CD45^+CD16^+$ cells (b) and total number of $CD11b^+CD45^+$ cells (c) in the ischemic brains. Data are mean ± SEM, n = 3-4 per group, *$p < 0.05$, WT vs. db/db; #$p < 0.05$, db/+ vs. db/db; NS, not significant; one-way ANOVA followed by Bonferroni *post hoc* test.

References:

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*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	Yes

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	Yes
Type and methods of randomization have been described:	Yes
Methods used for allocation concealment have been reported:	Yes

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	Yes

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	Yes

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	Yes
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	Yes
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	Yes

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	Yes

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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