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Supplementary Materials for

Mfsd2a and Spns2 are essential for sphingosine-1-phosphate transport in the formation and maintenance of the blood-brain barrier

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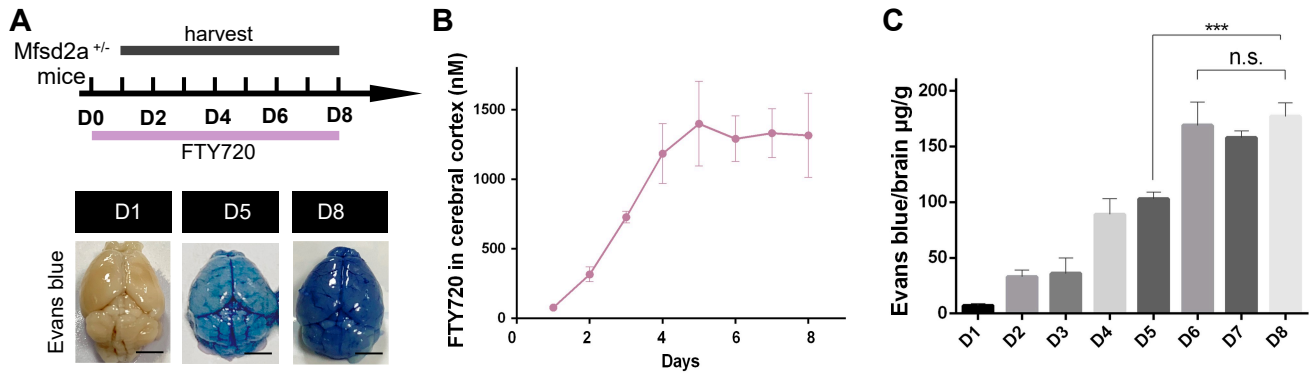

Figure S1. Interference with S1P in vivo leads to the gradual breakdown of the BBB. During a gradual increase in the FTY720 concentration in the brain parenchyma, the permeability of the BBB increases.

(A) Strategy used for FTY720 injection and Evans blue examination of the BBB; the permeability to Evans blue showed an increasing trend during continuous administration.

(B) The FTY720 concentration reached a stable value on the fifth day (D5).

(C) Evans blue examination showed that BBB leakage peaked at D6.

Scale bars: 4 mm in **a**. Error bars: SEM. Significance determined by Students t-test: *** $p<0.001$, n.s. $p>0.05$. n=5 mice per group.

Photo Credit: Zhifu Wang, State Key Laboratory of Medical Neurobiology, the Institutes of Brain Science and the Collaborative Innovation Center for Brain Science, Shanghai Medical College, Fudan University.

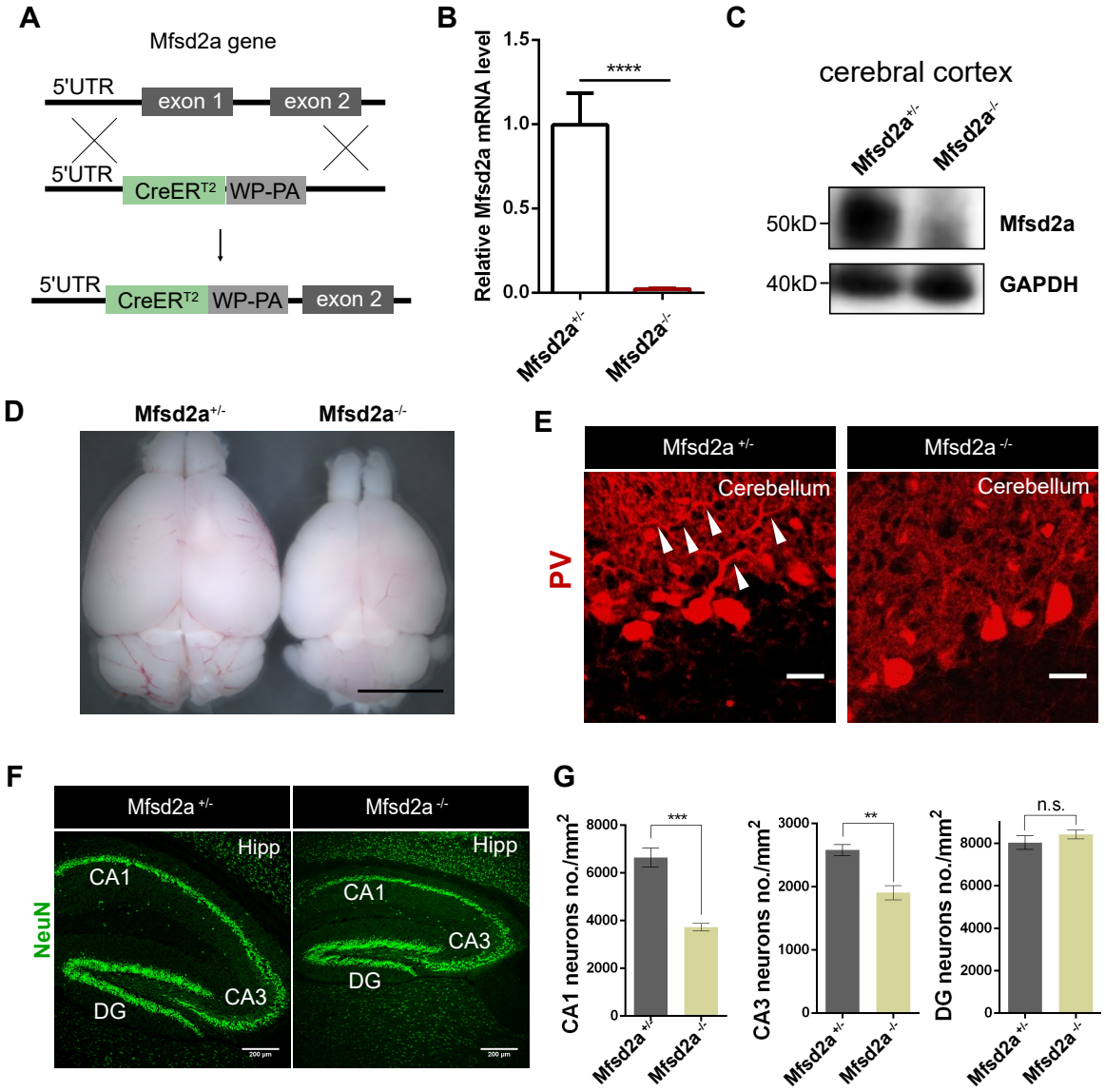

Figure S2. Homozygous Mfsd2a-CreERT2 mice are Mfsd2a-deficient mice.
(A) Strategy used for the generation of the Mfsd2a-CreER knock-in line by homologous recombination.
(B) Quantification of Mfsd2a mRNA by qPCR (n=5 mice per group), indicating that the Mfsd2a gene was not transcribed in homozygous Mfsd2a-CreER^{T2} mice.
(C) Western blot showing that homozygous mice did not express Mfsd2a protein (n=3 mice per group).
(D) The brain sizes of homozygous mice were smaller than those of heterozygous mice (n=10 male mice per group).
(E) Immunostaining for parvalbumin (PV) in cerebellum sections from homozygous mice showed abnormal Purkinje neurons (n=3 male mice per group).
(F-G) Immunostaining for NeuN in hippocampus sections; homozygous mice had fewer neurons in CA1 and CA3 (n=5 male mice per group).
Scale bars: 4 mm in D; 20 μm in E; 200 μm in F. Error bars: SEM. Significance determined by Students t-test: *** $p<0.001$, n.s. $p>0.05$.
Photo Credit: Zhifu Wang, State Key Laboratory of Medical Neurobiology, the Institutes of Brain Science and the Collaborative Innovation Center for Brain Science, Shanghai Medical College, Fudan University.

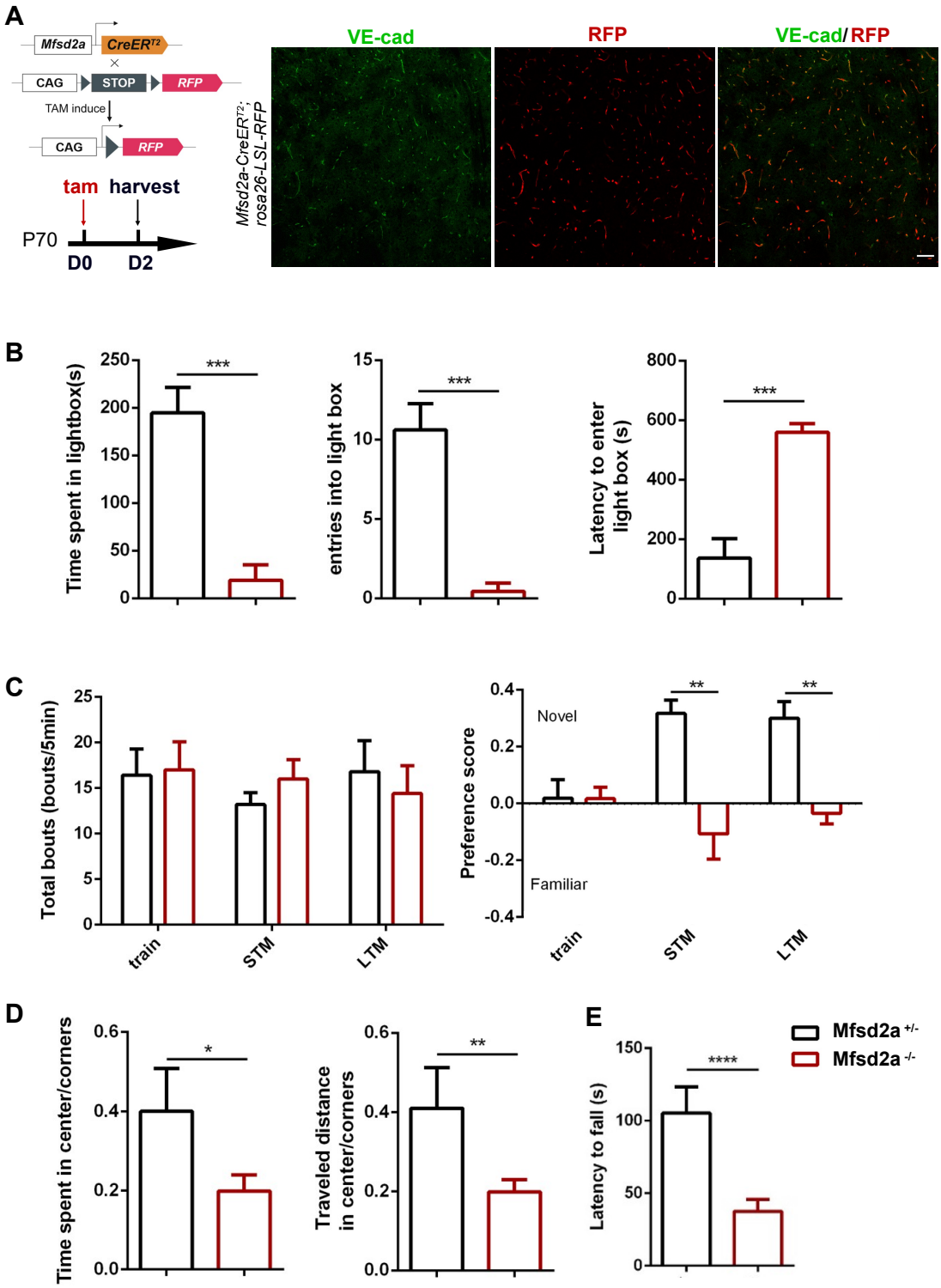

Figure S3. Homozygous Mfsd2a-CreER^{T2} mice are Mfsd2a-deficient mice. Homozygous mice exhibit learning and memory deficits as well as severe anxiety.
(A) Lineage tracing showed that Mfsd2a was specifically expressed in the ECs in the brain.
(B) Homozygous mice spent a longer time in the dark box during the lightdark box test; decreased entry and increased latency indicated anxiety (heterozygous: n=10, homozygous: n= 9 male mice).
(C) The novel object recognition test showed that homozygous mice had decreased preference for novel objects, indicating short-term (STM) and long-term memory (LTM) defects (heterozygous: n=11, homozygous: n= 9 male mice).
(D) The open field test indicated that homozygous mice had anxiety.
(E) The rotarod test for assessing activity indicated that homozygous mice had motor dysfunction (heterozygous: n=9, homozygous: n= 12 male mice).
Scale bars: 100μm in A. Error bars: SEM. Significance determined by Students t-test: **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, n.s. p>0.05.

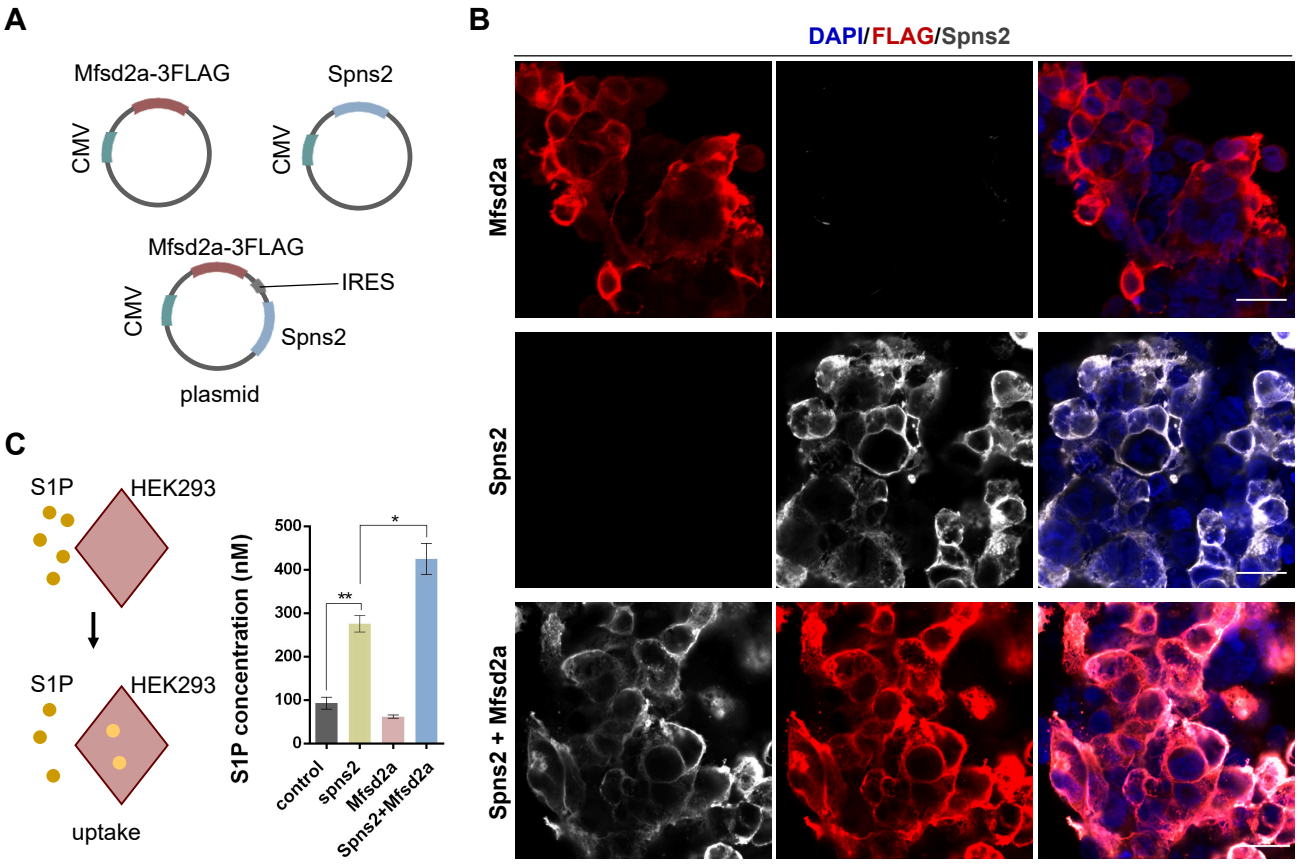

Figure S4. S1P is exported by Spns2, and Mfsd2a supports higher efficiency S1P transport.
(A) Strategy used for overexpressing Spns2, Mfsd2a or both in the HEK293 cell line.
(B) Immunostaining for FLAG (Mfsd2a) and Spns2, showing HEK293 cells expressing Spns2, Mfsd2a or both.
(C) S1P examination by MS showed the increased uptake of S1P in Spns^{-} Mfsd2a^{+} cells compared with Spns^{+} cells, while Spns^{+} cells showed higher uptake than Mfsd2a^{+} cells.
Scale bars: 20 μm in B. Error bars: SEM. Significance determined by Students t-test: **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, n.s. $p>0.05$. All results are representative of three individual samples.

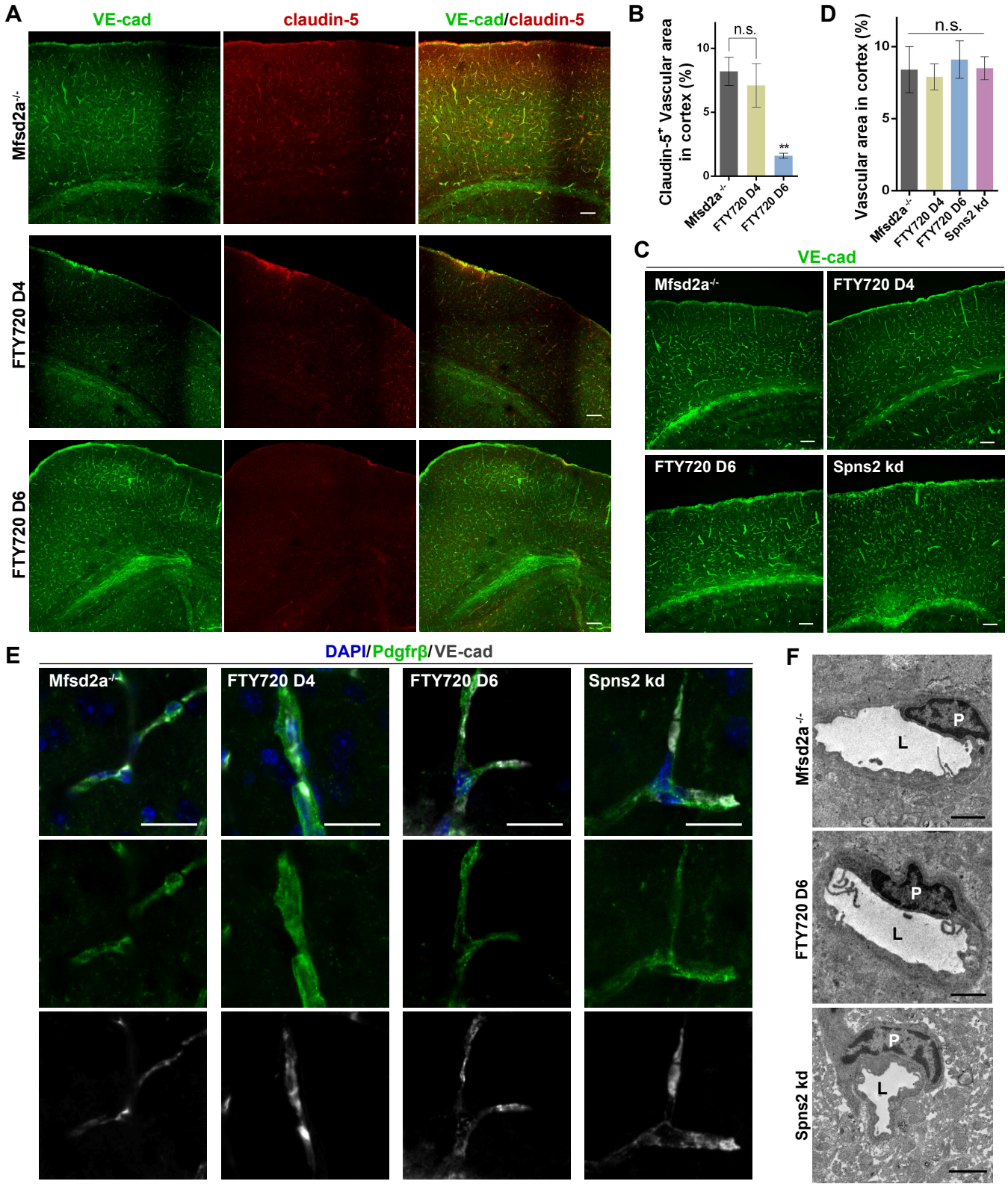

Figure S5. Spns2 deficiency leads to more severe BBB disruption than Mfsd2a deficiency. Inhibiting Spns2 or increasing the FTY720 concentration can cause the loss of TJs.
(A) Immunostaining for VE-cad and claudin-5 in brain sections showing TJ failure in D6 mice (n=3 male mice per group).
(B) Quantification of the claudin-5^{+} area in ECs implied a significantly decreased proportion in D6 mice (n=5 male mice per group).
(C and D) Quantification of the EC area in the brain showed no significant differences between the groups (n=3 male mice per group).
(E) Immunostaining of brain sections with Pdgfrβ and VE-cad; there is no lack of pericytes.
(F) Electron micrographs of longitudinal capillary sections showed that the pericytes had a normal appearance and were well located on the vessel wall of Mfsd2a$^{-/-}$, FTY720 D6 and Spns2 kd adult mice; the pericytes were adjacent to endothelial cells and shared a common basement membrane.
L, lumen; P, pericyte. Scale bars: 100μm in A and C; 20μm in E. Error bars: SEM. Significance determined by Students t-test: ** $p<0.01$, n.s. $p>0.05$.

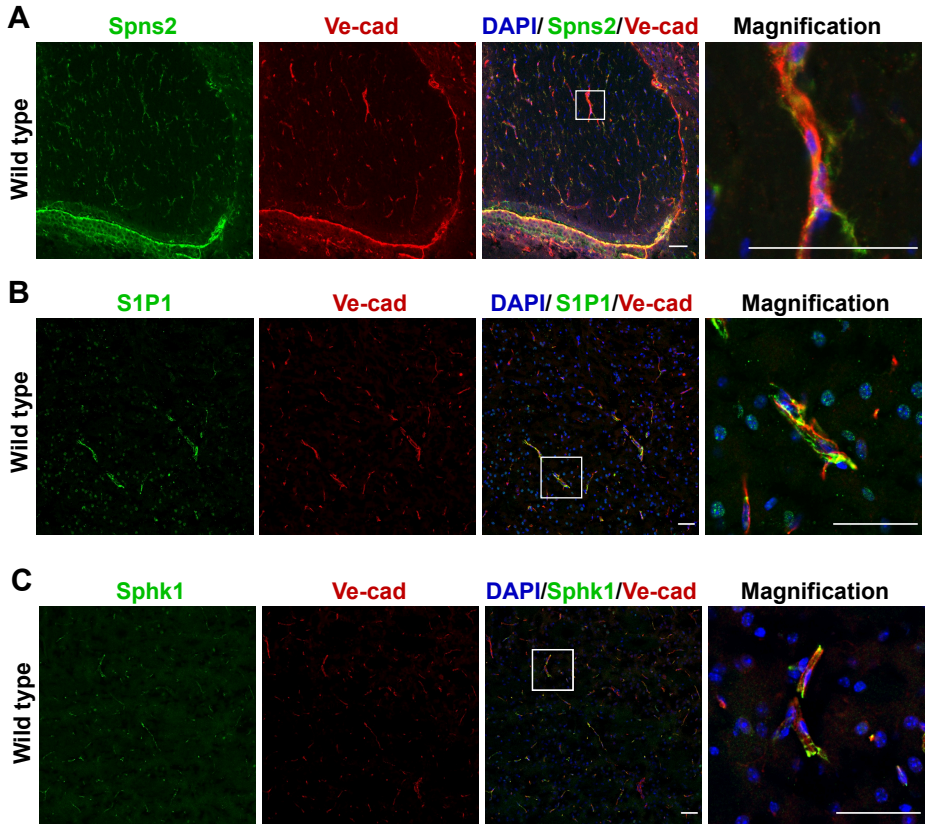

Figure S6. The ECs in the brain express S1P1, Spns2 and Sphk1.
(A) Immunostaining for Spns2 and VE-cad showing the colocalization of Spns2 with ECs, indicating the expression of Spns2 in brain ECs.
(B) Immunostaining for S1P1 and VE-cad in brain sections showing brain ECs expressing S1P1.
(C) Immunostaining for Spns2 and VE-cad showed brain ECs expressing Sphk1. The magnified views of the boxed fields are shown in the right panels.
Scale bars: 100 μm in A-C.

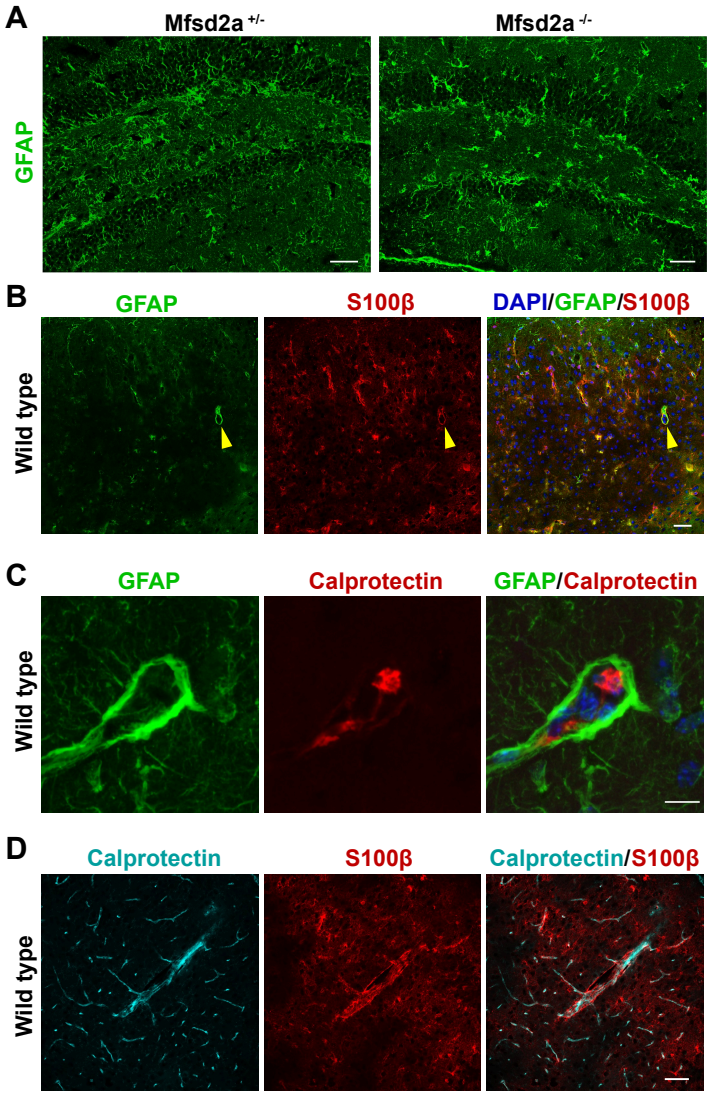

Figure S7. S100A8/A9 complex is not coexpressed with S100β. S100A8/A9 complex (Calprotectin) is specifically expressed in ECs but not in astrocytes.
(A) Immunostaining for GFAP (astrocytes marker) on the brain sections showed normal astrocytes in Mfsd2a $^{-/-}$ mice.
(B) Immunostaining for GFAP and S100β showed that S100β is mainly expressed in astrocytes in cortex.
(C and D) Immunostaining images showed S100A8/A9 complex is not expressed in astrocytes.
Scale bars: 100μm in A and D; 50μm in B; 10μm in C. All images are representative of three individual mice.

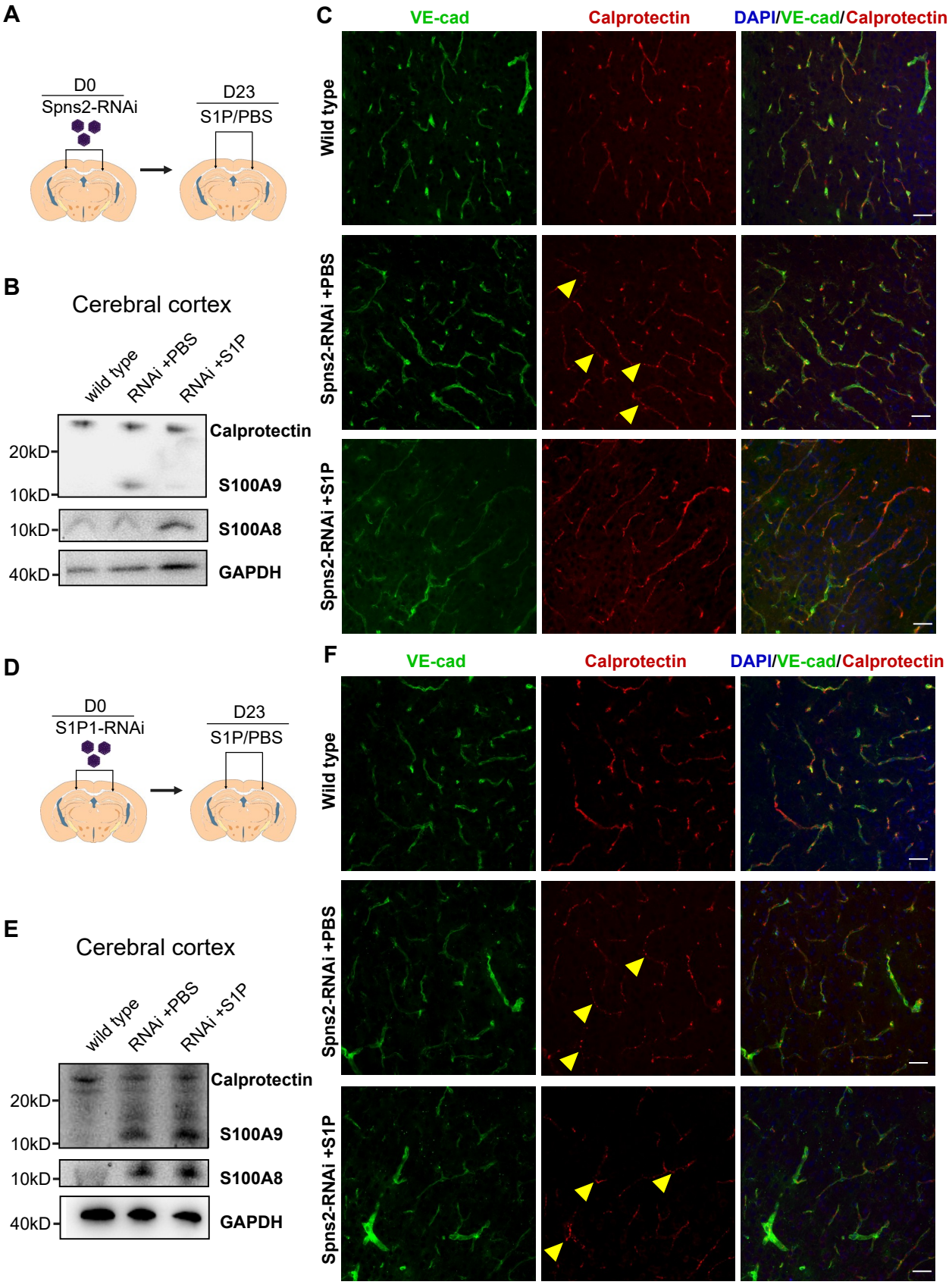
A
D0
Spns2-RNAi
D23
S1P/PBS
B
Cerebral cortex
wild type
RNAi +PBS
RNAi +S1P
Calprotectin
S100A9
S100A8
GAPDH
20kD
10kD
10kD
40kD
C
VE-cad
Calprotectin
DAPI/VE-cad/Calprotectin
Wild type
Spns2-RNAi +PBS
Spns2-RNAi +S1P
D
D0
S1P1-RNAi
D23
S1P/PBS
E
Cerebral cortex
wild type
RNAi +PBS
RNAi +S1P
Calprotectin
S100A9
S100A8
GAPDH
20kD
10kD
10kD
40kD
F
VE-cad
Calprotectin
DAPI/VE-cad/Calprotectin
Wild type
Spns2-RNAi +PBS
Spns2-RNAi +S1P

Figure S8. Spns2 or S1P1 deficiency cause Calprotectin dissociation. The dissociation of Calprotectin caused by Spns2 deficiency is reversible through adding S1P, but not in S1P1-deficient cortex.
(A and D) Schematic showing the experimental strategy used for the injection of AAV and addition S1P/PBS.
(B) Western blotting showed the recovery of calprotectin after the in situ addition of S1P in the Spns2-deficient mouse cortex.
(C) Immunostaining for VE-cad and calprotectin showed that the distribution of calprotectin was continuous after adding S1P.
(E) Western blotting showed that S1P failed to reverse calprotectin dissociation.
(F) Immunostaining for VE-cad and calprotectin showed the discontinuous distribution of calprotectin (yellow arrowheads) in brain ECs.
Scale bars: 20μm in C and F. Error bars: SEM. Significance determined by Students t-test: ** $p<0.01$, n.s. $p>0.05$. All results are representative of three individual mice.

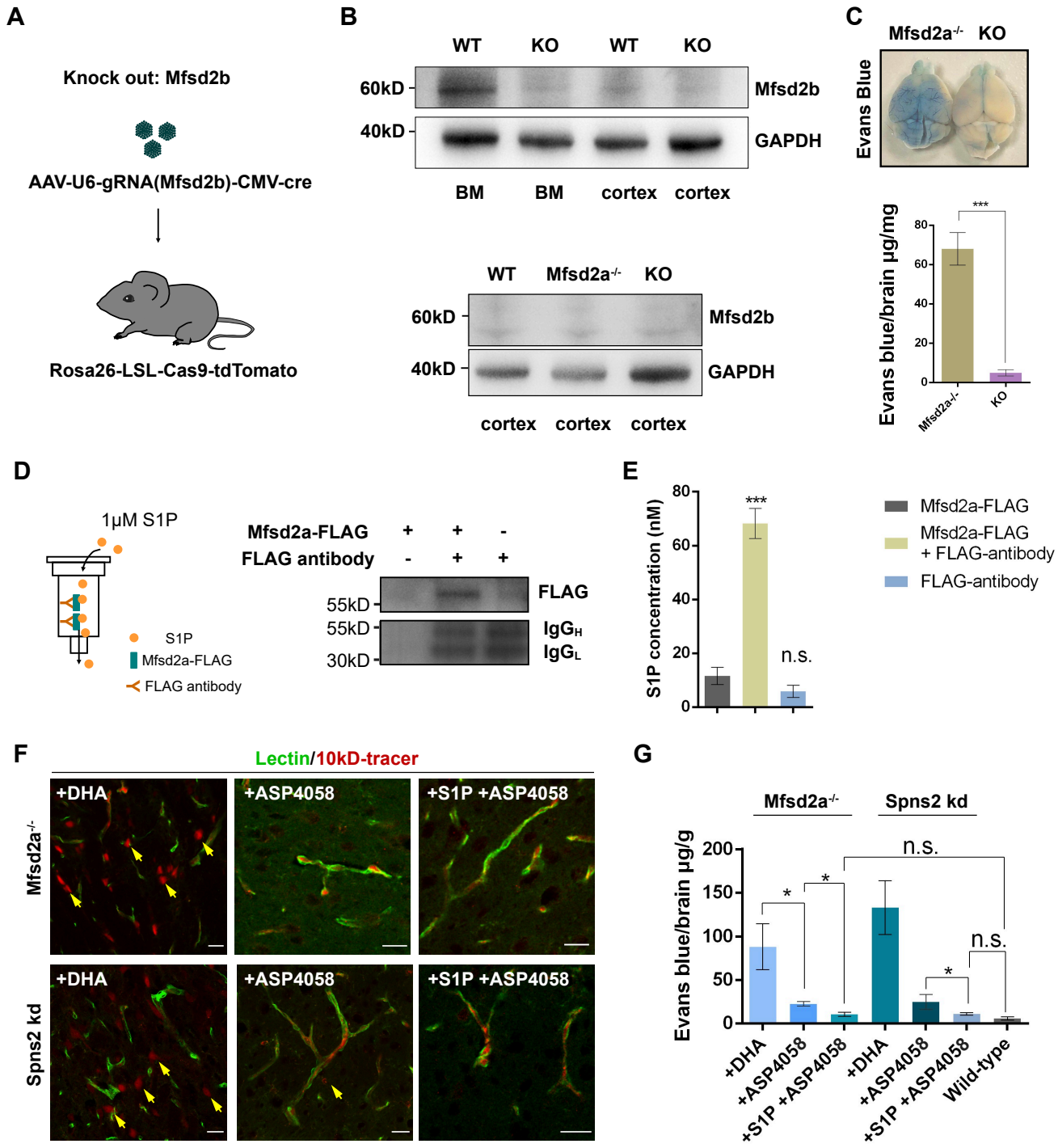

Figure S9. Mfsd2a can bind to S1P to promote the export of S1P. Mfsd2a and Mfsd2b share high similarity(~60%) in sequence, and Mfsd2b is minimally expressed in brain.

(A) Strategy of knocking out Mfsd2b using the Rosa26-LSL-Cas9 line and AAV. AAV was injected in femur and cerebral cortex at 28 days before harvesting bone marrow(BM) and cortex.

(B and C) Western blot showed that the expression of Mfsd2b is quite low in cortex. Evans blue test showed that knockout of Mfsd2b did not lead to the BBB breakdown, indicating that Mfsd2b has minimal effect on S1P transport of brain.

(D) Immunoprecipitation for Mfsd2a-FLAG expressed by HEK293 cells, then test the binding of Mfsd2a and S1P.

(E) MS analysis for (D) showed high S1P concentration resided in the column containing Mfsd2a-FLAG, indicating that Mfsd2a can bind to S1P.

(F and G) Immunostaining images and Evans blue tsets showed that combination of S1P and ASP4058 can restore BBB breakdown cause by Mfsd2a and Spns2 deficiency.

KO: Mfsd2b-knockout. Scale bars: 20 μm in (F). Error bars: SEM. Significance determined by Students t-test: *** $p<0.001$, * $p<0.05$, n.s. $p>0.05$. Each image is representative of 3-6 individual samples.

Photo Credit: Zhifu Wang, State Key Laboratory of Medical Neurobiology, the Institutes of Brain Science and the Collaborative Innovation Center for Brain Science, Shanghai Medical College, Fudan University.

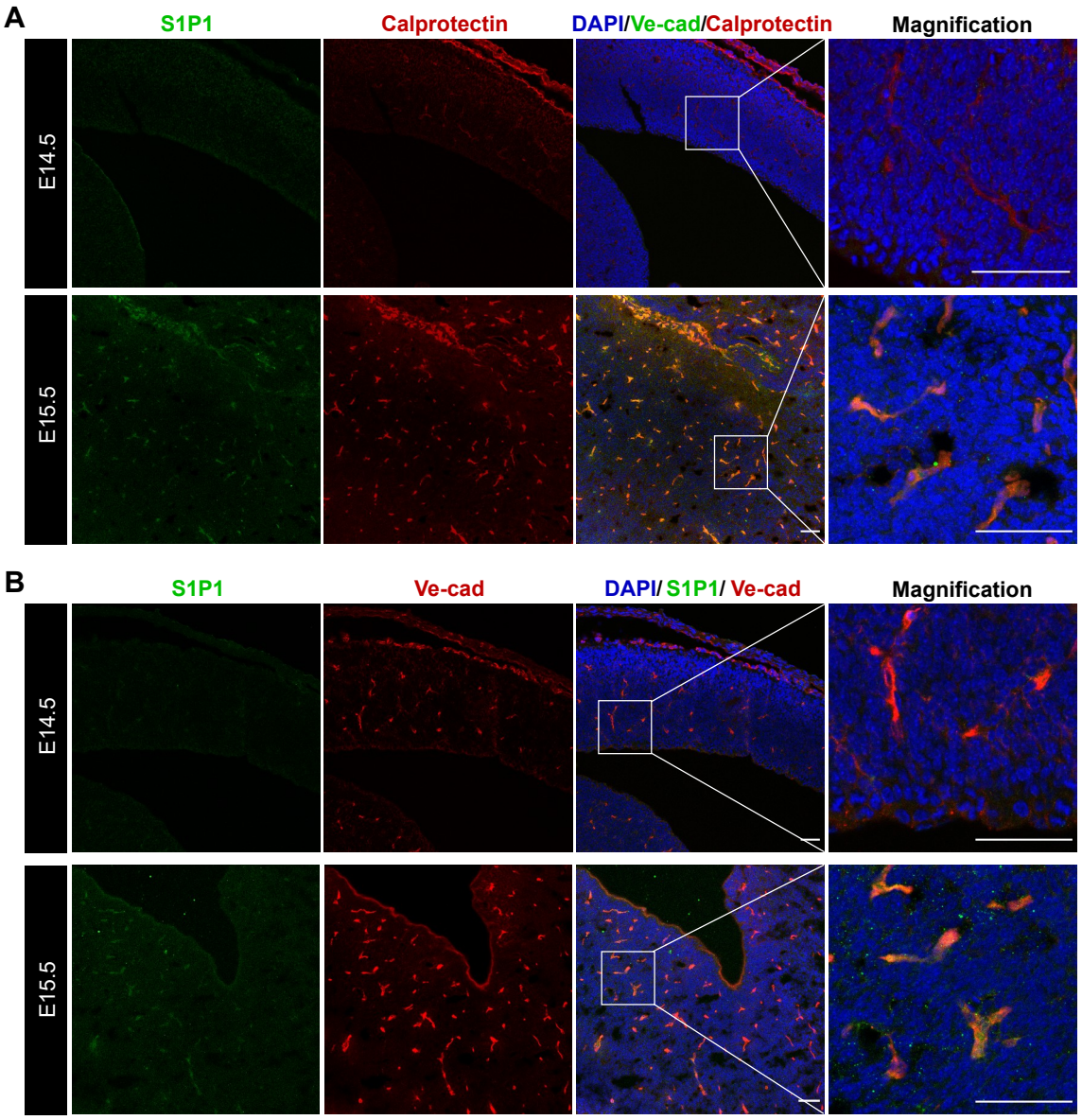

Figure S10. Calprotectin and S1P1 are highly expressed in brain ECs at E15.5.
(A) Immunostaining for S1P1 and calprotectin in brain sections from embryos showing the robust expression of S1P1 and calprotectin at E15.5 but not at E14.5.
(B) Immunostaining for S1P1 and VE-cad showed that S1P1 was expressed in ECs in the brain at E15.5.
Scale bars: 100 μm. Each image is representative of three individual embryos.