Supplemental Materials and Methods

Immunostaining

For immunostaining analysis, brain sections were incubated with blocking solution containing 0.1% Triton X-100 for permeabilization and 3% BSA for 20 minutes at room temperature and then with primary antibody (in PBS with 5% BSA and 0.1% Tween-20) at 4°C for overnight. The following primary antibodies were used: CD31 (BD Bioscience, 553369;1:500), SMA-594 (Sigma, C6198; 1:1000), SMA-647 (Santa Cruz Biotechnology, sc-32251; 1;1000), SLC16A1 (OriGene Technologies, TA321556; 1;200), Desmin (Dako, M0760; 1;500), PDGFR-β(Cell signaling technology, 3169; 1:200), Ki67 (Dako, M724001-2, RRID:AB_2631211; 1:200), cleaved Caspase 3 (Cell signaling technology, 9661, 1:200), BrdU (abcam, ab6326, 1:500), Laminin-γ1 (DSHB, 2E8, 1:500), Laminin-α5 (abcam,ab17107, 1:200), Collagen Ⅳ (abcam, ab6586, 1:200), ZO1 (Thermofisher, 61-7300, 1:200), claudin5 (abcam, ab15016, 1:200), PLVAP (abcam, ab27853, 1:200), Caveolin-1 (LSBio, Ls-B9776, 1:200), Apelin1 (Novus, NLS65, 1:200), and CD146 (Sigma, HPA008848, 1:200), Egfl7 (R and D systems, AF3089, 1:200), AQP4 (Sigma-Aldrich, A5971, 1:200), and VGLUT1 (abcam, ab227805, 1:200). The brain sections were then washed with 3x PBST buffer, and incubated with appropriate secondary antibodies at 1:1000 (Thermo Fisher Scientific, Alexa Fluor conjugates) for 1-h at room temperature. Brain sections were washed and mounted using Prolong Diamond Antifade mounting media with or without DAPI (Thermo Fisher Scientific). The images were taken by a Zeiss 710 multiphoton confocal microscope with 10×, 20× or oil-immersion 63× objective, with sequential-acquisition setting. Images were also taken with BZX fluorescence microscope with 4× or 10× objective. Quantitative analyses were performed using ImageJ software and ImageJ-vessel analyse plug in for measurements of vessel density. To precisely analyze vessel length and branch points, Neuron J software was used to trace each single vessel and branch.

Evans Blue Extravasation

The 8 weeks old mice were anesthetized and injected with Evans blue (4 mg/kg) via their caudal vein. 30 minutes after Evans blue injection, mice were perfused with 0.9% NaCl containing heparin (100 U/mL) to remove the dye from the blood vessels. Their cortex was dissected and weighted, and half of the cortex was homogenized in 0.75 ml of PBS and 0.25 ml of 100% TCA solution, which could precipitate macromolecular compounds. The other half of the cortex was put into a drying oven at 60℃ for 24h

and weighted. Homogenized samples were cooled overnight at 4 °C, and then centrifuged for 30 min at 1,000 x g at 4 °C. The EB in the supernatants of each sample were subsequently measured at 620 nm using a 96-well plate reader, 100 µl of each sample was measured. All measurements were within the range of the established standard curve. The dye concentration was calculated as the ratio of absorbance relative to the amount of tissue.

Cell cultures

Primary astrocytes from neonatal *Neo*$^{GFAP\text{-}CreER}$ or *Neo*$^{GFAP\text{-}Cre}$ mice and the control mice were cultured as described previously(1). Briefly, cerebral neocortex from P1-P3 neonatal pup was isolated without meningeal, cut into small pieces, and then incubated with 0.125% trypsin at 37°C for 20 min. The cerebral neocortex was then dissociated into a single-cell suspension by mechanical disruption using 100 µm meshes. The cells were planted on poly-l-lysine (0.1 mg/ml, Sigma) coated culture dishes and incubated in DMEM containing 10% FBS (Invitrogen). After 6–8 d culturing, the cells become confluent. To eliminate the microglia and oligodendrocyte precursor cells (OPC), the dishes were shaked at 200 rpm overnight. Astrocytes were subsequently detached using 0.25% trypsin-EDTA (Invitrogen) and plated into poly-l-lysine-coated 35 mm dishes or onto poly-l-lysine-coated coverslips. The purity of glial fibrillary acidic protein (GFAP) positive astrocytes in our culture system is >95%. For astrocytes from *Neo*$^{GFAP\text{-}CreER}$*;Ai9* and it's control mice (*GFAP-CreER;Ai9),* 4-OH-tamoxifen (1µM) was added into the culture medium for 24 hours to turn on the Cre activity. For BMP2 treatments, astrocytes were starved in DMEM serum-starve media for overnight before treatment.

Human umbilical veins endothelial cells (HUVECs) were kindly provided by Dr. William Caldwell (Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta University, Augusta, GA). Mouse brain microvaslular endothelial cells (MBMEC) were purchased from CellBiologics (C57-6023) and cultured with M-200 culture medium plus 10% FBS. Both MBMEC and HUVECs were plated in a 6-well plate. When these EC cultures reached to 60-70% confluence, the culture medium was changed to DMEM with 1% FBS for 12 hrs. For a parallel experiments, neonatal astrocytes from P 3~5 control and neogenin mutant mice were cultured and passaged to the 2nd generation and seeded in an inserted well with the culture medium DMEM+~1% FBS for 24 hrs, then the inserts were put into the six-well plate for

additional 6 hrs for the co-culture assays. The culture medium DMEM+~1% FBS was added with BrdU (at final concentration of 3μg/ml). Alternatively, HUVECs that were plated in a 6-well plate were cultured in the presence of CMs from astrocytes (DMEM+1% FBS cultured with control and neogenin KO astrocytes for 24 hrs) for 6 hrs. BrdU (at final concentration of 3μg/ml) was added at the same time. For netrin1 treatment, both MBMEC and HUVEC were starved for 24 hrs (DMEM with ~1% FBS), the CM of astrocytes was changed with new astrocyte CM with different dosage of recombinant NTN1 proteins and BrdU. For EC migration assay, both MBMEC and HUVEC were planted in the 24-well insert with 0.4μm pore for 16 hrs and the medium inside the insert was DMEM with 1% FBS while the medium outside of the insert was CM of astrocytes (~1%FBS DMEM) with or without recombinant NTN1. The insert membrane was then stained with crystal violet to check the migrated ECs.

Real-time PCR

The second passages of the primary cultured astrocytes from neogenin mutant and their control mice were harvested with Trizol and the Real-time PCR was performed as described previously(1).

Fluorescent in situ hybridization (FISH)

PGEM-T easy vector systems were purchased from Promega, and FISH Tag RNA Green Kit was purchased from Thermofisher (F32952). The experiment was follow the protocol as described previously (2) with some modification. The probe for netrin1 is 329bp with the forward primer 5'-tcctgtcacctctgcaactc-3', and the reverse primer 5'-tgctcgttctgtttggtgat-3'. All buffers and reagent were RNAase free prepared. Briefly, *Neo*$^{GFAP\text{-}CreER}$;Ai9 mice and the littermates *GFAP-CreER*;Ai9 were exposed to TAM at P30, sacrificed at P60, and perfused with 4% PFA. Their brain samples were post-fixed with 4% PFA overnight and were sectioned into 40 μm thickness. For pre-hybridization, brain slices were dehydrated with RNase-free 100% ethanol and fixed with 50% methanol/5% formaldehyde in PBT butter for 5 minutes and permeabilized with PBT/Proteinase K. Then the denatured netrin-1 probes were incubated in hybridization buffer at 56℃ for 36 hours. After the incubation, the probe/hybridization buffer was removed and replaced with fresh hybridization buffer without probe at 55℃ for 5 minutes and 30 minutes for washing and this step was repeated thrice. Then 50% PBT/ 50% hybridization buffer was used for rinsing the

slices. Finally, brain slices were counterstained with DAPI and mounted with the antifade reagent to take images under a confocal microscope.

RNA-Scope based In Situ hybridization

RNA-Scope reagent (RNAscope Multiplex Fluorescent Reagent Kit v2 Assay), the NTN1 probe, as well as the hybrid oven were purchased from ACDbio company, and all the procedures were performed according to the manufactural protocol. Briefly, *Neo*$^{GFAP\text{-}CreER}$;Ai9 mice and the littermates *GFAP-CreER*;Ai9 were sacrificed at P60 and perfused with 4% PFA; their brain samples were post-fixed with 4% PFA for 3 hrs, dehydrated with 1.5% and 3% sucrose PBS, and then cryo-sectioned (12 µm thickness). The brain sections were dehydrated in an ethanol series; then incubated in retrieval buffer maintained at a boiling temperature (100°C to 103°C) using a hot plate for 15 minutes, rinsed in deionized water, and immediately treated with protease at 40°C for 30 minutes in a HybEZ hybridization oven. Hybridization with the NTN1 probe as well as the negative control probe (a gene anti soil bacteria) and the positive control probe (Polr2-RNA polymerase 2) in 40℃ hybrid oven for 2 hours. After washing, the slides were treated with pre-amplifier for 30 min and amplifier for 15 min at 40℃ oven. Chromogenic detection was performed using Opal^{TM} 520 to label the NTN1 RNA with fluorescence and imaged under confocal microscope, DAPI was used as the counterstain.

Adeno-Associated Virus (AAV) generation and injection

AAV5-GFAP-Netrin1-myc-his-mCherry was synthesized by Vigene Biosciences, Inc. AAV5-GFP was the negative control virus, Neo CKO and the control mice (p30 age) were anesthetized with 1% isoflurane and placed in a stereotaxic frame, and then two tiny holes were made on left side of the skull overlying sensorimotor cortex according to the coordinates from the mice brain atlas of Paxinos and Watson. AAV were injected into the sensorimotor cortex. The coordinates relative to bregma for the injection sites were -1.06 and -2.06 mm, 1.5 mm lateral, and 0.8 mm depth. One group of mice (n = 4) were injected with 0.2 µl volume of AAV-EGFP, and another group mice (n = 4) were injected with 0.2 µl volume of AAV-Netrin1 using a microliter syringes (Hamilton) at a speed of 200 nl/min controlled by UltraMicroPump. The needle was left in place for 2 min and gradually withdrawn to allow diffusion of viral vector from the injection site and to prevent leakage from the needle track. 3 days after injection, mice were

injected with tamoxifen (100 mg/kg/d for 4 days) to induce the Cre protein expression. The mice were sacrificed at P60 and the brains were harvested and sectioned as described above. mCherry, c-Myc and GFP in control group were stained to check the AAV expression.

1. Huang Z, Sun D, Hu JX, Tang FL, Lee DH, Wang Y, et al. Neogenin Promotes BMP2 Activation of YAP and Smad1 and Enhances Astrocytic Differentiation in Developing Mouse Neocortex. *J Neurosci.* 2016;36(21):5833-49.
2. Lee D, Xiong S, and Xiong WC. General introduction to in situ hybridization protocol using nonradioactively labeled probes to detect mRNAs on tissue sections. *Methods Mol Biol.* 2013;1018:165-74.

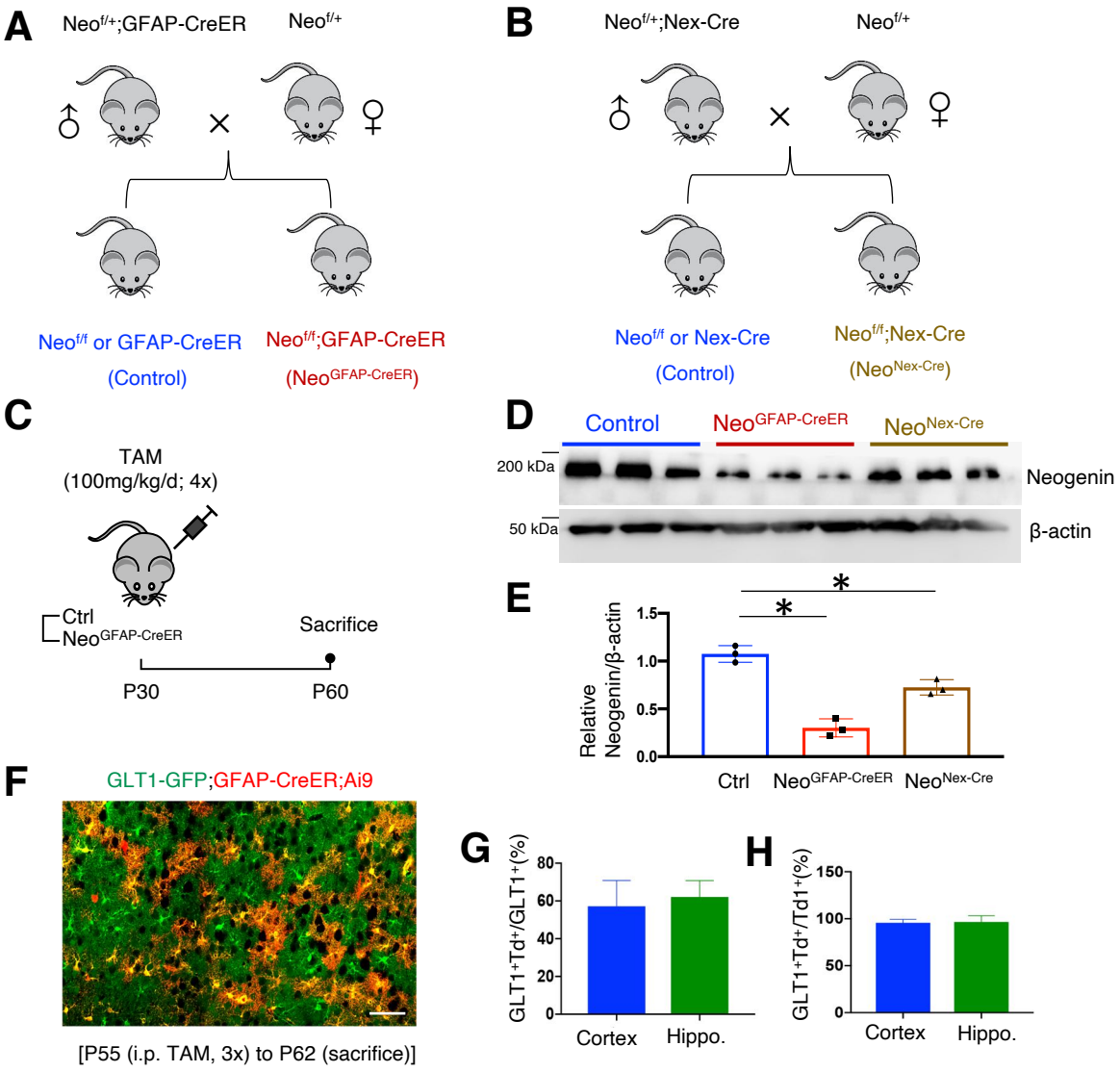

Figure S1. Generation of *Neo*$^{GFAP\text{-}CreER}$ and *Neo*$^{Nex\text{-}Cre}$ mice. (A-B) Illustrations of the breeding strategies for generation of *Neo*$^{GFAP\text{-}CreER}$ and *Neo*$^{Nex\text{-}Cre}$ mice, respectively**. (C)** Illustration of tamoxifen injection protocol in control and *Neo*$^{GFAP\text{-}CreER}$ mice. **(D)** Western blot analysis of cortex homogenates from indicated genotyped mice and using indicated antibodies. **(E)** Quantification of data in (D). **(F)** Representative images showing the GFAP promoter driven Cre (*GFAP-CreER*) induced td-Tomato positive astrocytes that were positive for GLT1-GFP in young adult mice (*GFAP-CreER;Ai9;GLT1-GFP*) (1 week after tamoxifen administration at P55). **(G-H)** Quantification analyses of data in (F). Data shown are mean±SEM (n=3 mice/group in E, and n=6 slices from 1 mouse in G-H). *, $P < 0.05$, Mann-Whitney test for (G)and (H); one-way ANOVA plus post-hoc analysis for (E).

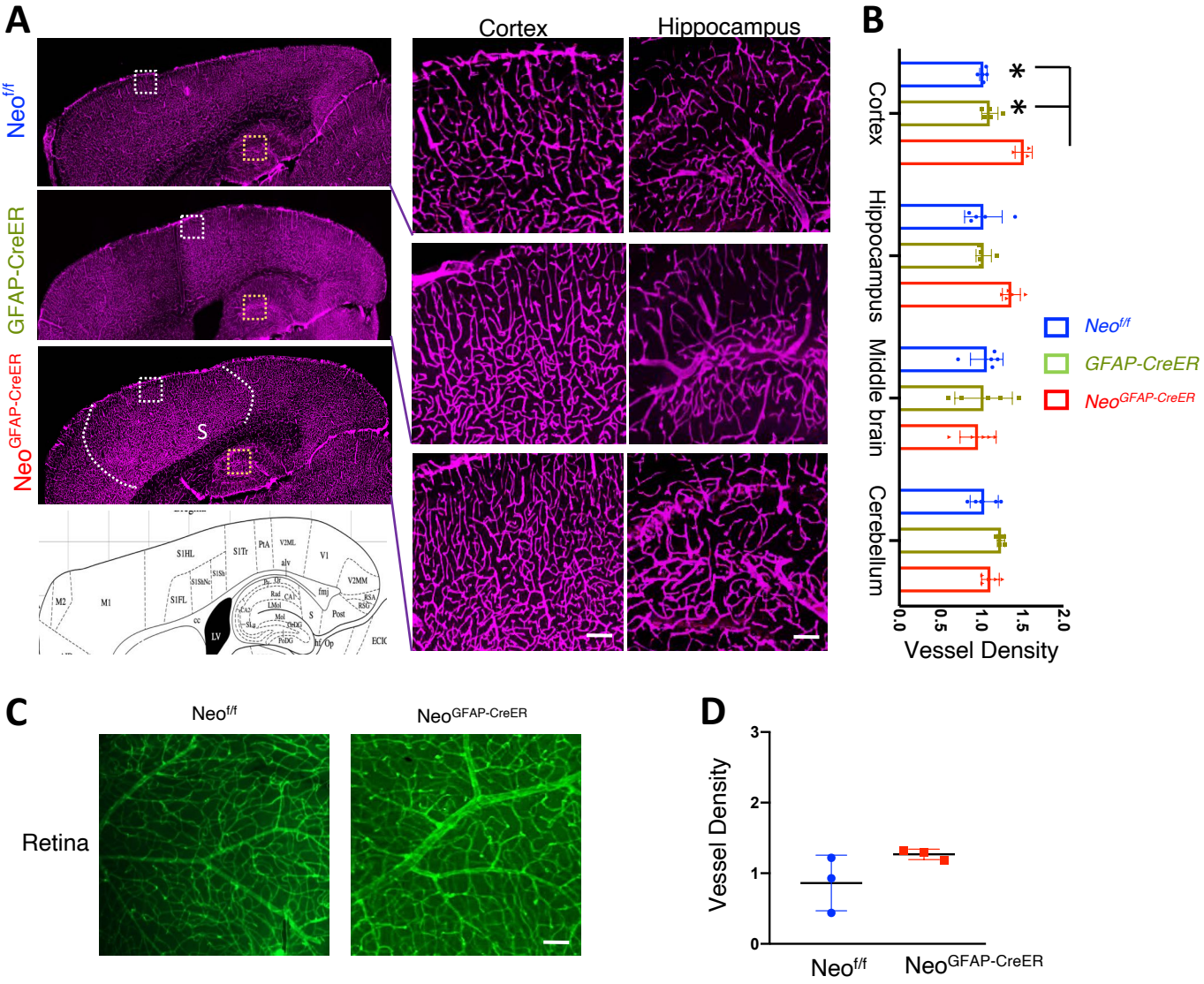

Figure S2. Increased vessel density in somatosensory cortex in *neo*$^{GFAP\text{-}CreER}$ mice. (A) Representative images of PECAM1^{+} BVs in *neo*$^{GFAP\text{-}CreER}$ mice and their littermate controls, *GFAP-CreER* and *Neo*$^{f/f}$ mice. **(B)** Quantification analyses of vessel density in indicated brain regions. **(C)** Representative images of PECAM1^{+} BVs in retina of *neo*$^{GFAP\text{-}CreER}$ mice and littermate *Neo*$^{f/f}$ mice. **(D)** Quantification analyses of data in (C). Data shown are mean±SEM (n=3-5 mice/group). *, P<0.05, Two-way ANOVA analysis.

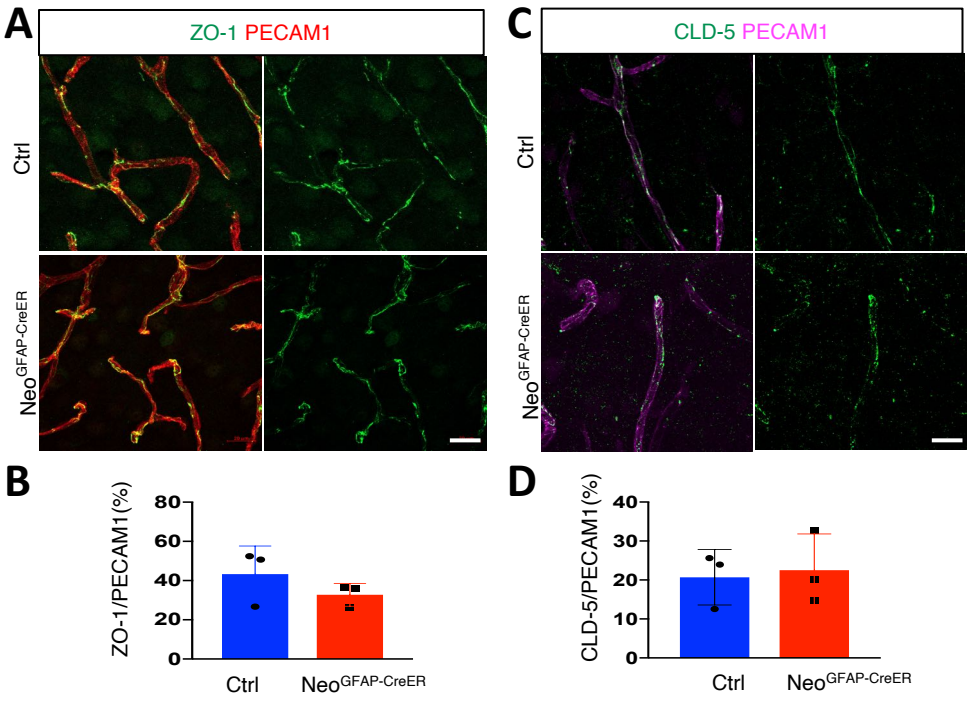

Figure S3. No obvious change in tight junctions in *Neo*$^{GFAP\text{-}CreER}$ mice. (A, C) Representative images of ZO-1 (zonula occludens-1) (A) and Claudin-5 (C) immunostaining analyses in control and *neo*$^{GFAP\text{-}CreER}$ mice. **(B, D)** Quantification analyses of data in (A) and (C), respectively. Data shown are mean±SEM (n=3 mice/group). Mann-Whitney test. Scale bars: 20µm.

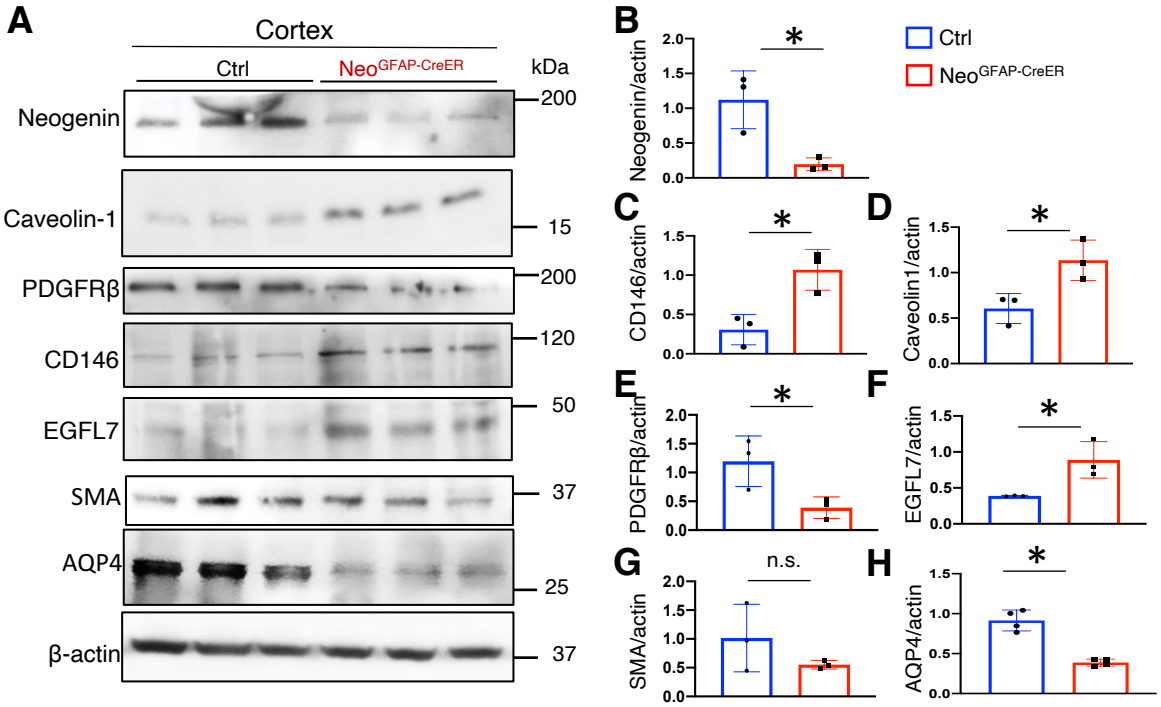

Fig.S4. **Western blot analyses of homogenates of cortex from indicated genotyped mice and using indicated antibodies. (A)** Representative blots. **(B-H)** Quantification analyses of data in (A). Data shown are mean±SEM (n= 3-4 mice/group). *, P < 0.05; n.s., no significance, Mann-Whitney test.

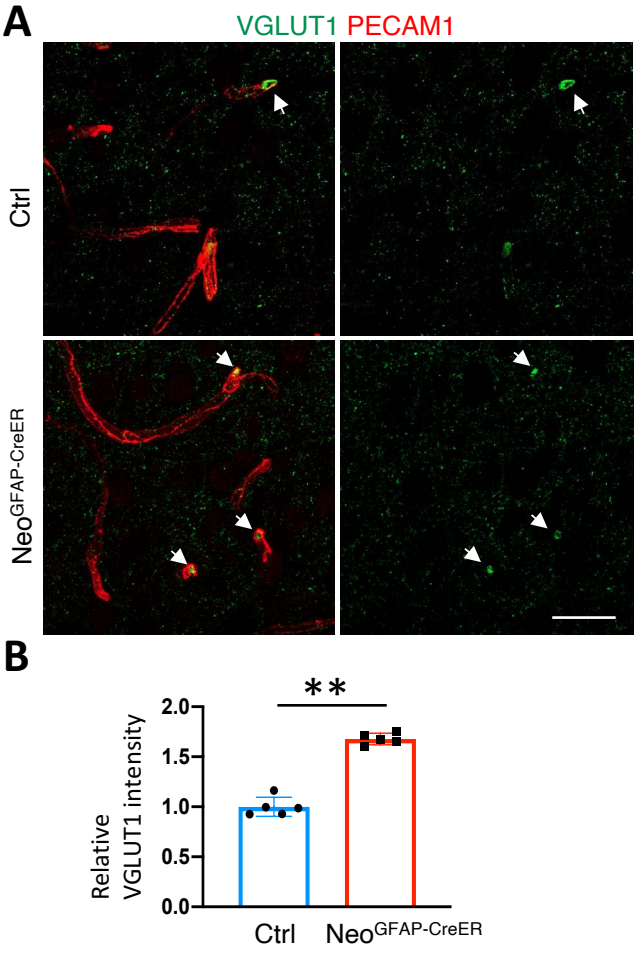

Figure S5. More VGlut1 positive tip cells in *Neo*$^{GFAP\text{-}CreER}$ cortex. (A) Representative images of VGlut1 and PECAM1 co-immunostaining analysis. Scale bar: 20µm. **(B)** Quantification of data in (A). Data shown are mean±SEM (n=5 mice/group). *P<0.05. Mann-Whitney test.

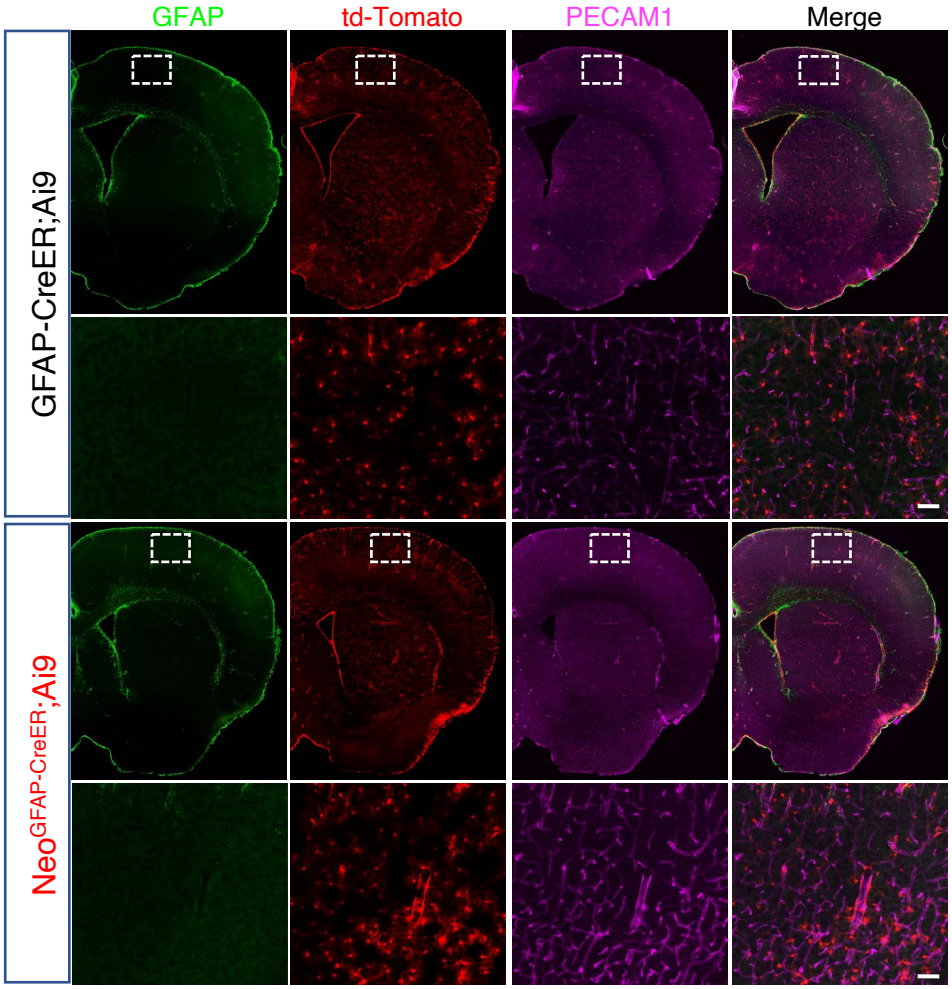

Figure S6. Little to no GFAP⁺ astrocytes in *neo*$^{GFAP\text{-}CreER}$ cortex. Representative images of co-immunostaining analyses using indicated antibodies in control *(GFAP-Cre;Ai9)* and *Neo*$^{GFAP\text{-}CreER}$*;Ai9* brain sections [P30 (TAM) to P60]. The area marked in the dot box are amplified and showed in the bottom. Scale bars: 20μm.

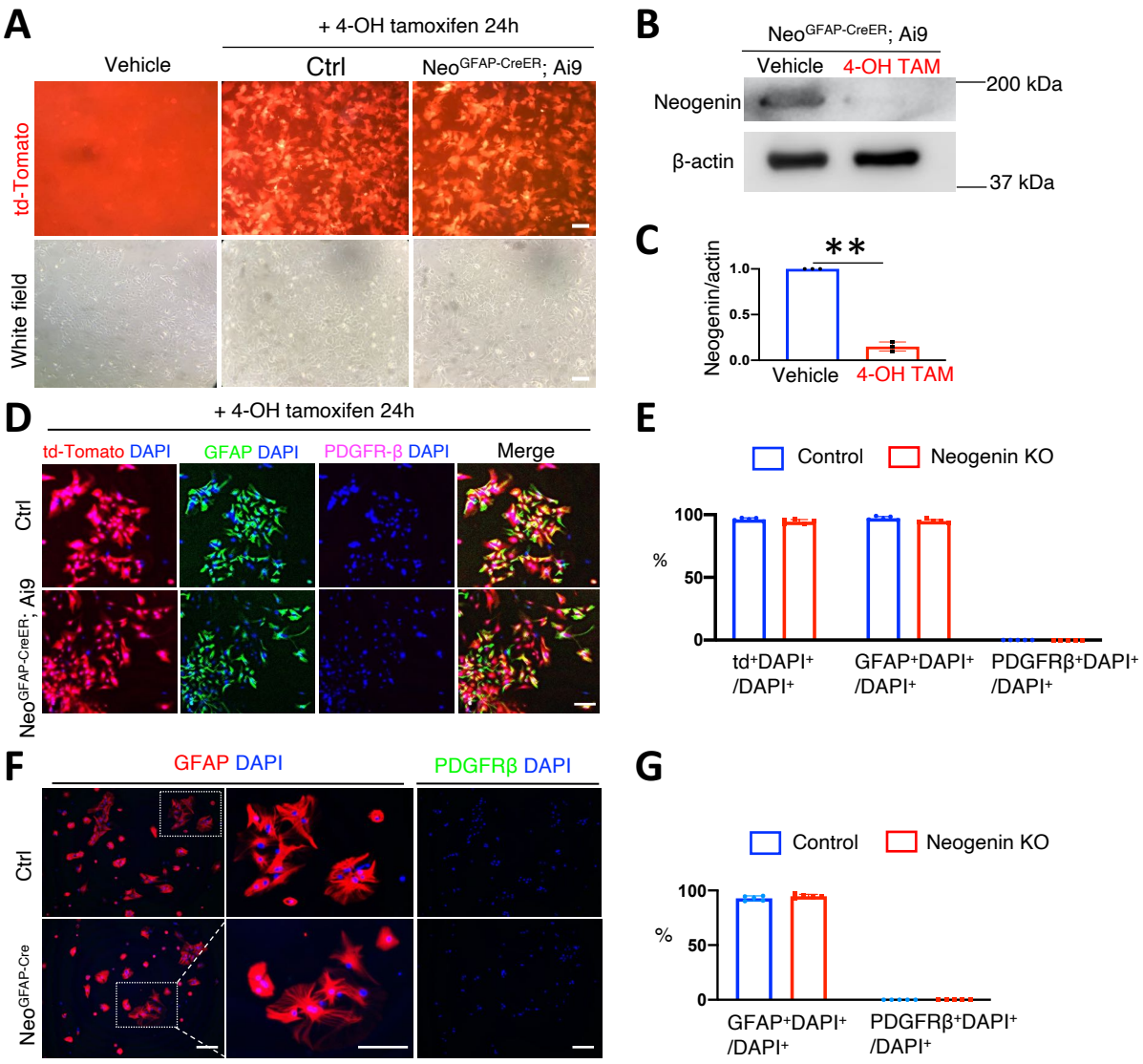

Figure S7. Characterizations of primary cultured astrocytes from indicated genotyped mice. (A) Representative images showing 4-OH-tamoxifen induced td-tomato expression in astrocytes from control (*GFAP-CreER;Ai9*) and *Neo*$^{GFAP\text{-}CreER}$*; Ai9* neonatal pups. **(B)** Western Blot analysis showing neogenin depletion after 4-OH-tamoxifen treatment. **(C)** Quantification of data in (B). **(D-E)** Co-immunostaining analysis showing that >95% of cells were GFAP$^+$/tdTomato$^+$ astrocytes, and no detectable PDGFRβ$^+$ pericytes in astrocyte cultures (+/- 4-OH TAM) from *Neo*$^{GFAP\text{-}CreER}$ neonatal mice. D, representative images; E**,** quantification of data in (D). **(F-G)** Co-immunostaining analysis showing that >95% of cells were GFAP$^+$ astrocytes, without PDGFRβ$^+$ pericytes in astrocyte cultures from control and *Neo*$^{GFAP\text{-}Cre}$ neonatal mice. F, representative images; and G, quantification of data in (F). Scale bars: 20μm. Data presented are mean±SEM (n=5 coverslips in each experiment). **, P < 0.01. Mann-Whitney test analysis for (C); One-way ANOVA plus post-hoc analysis for (E) and (G).

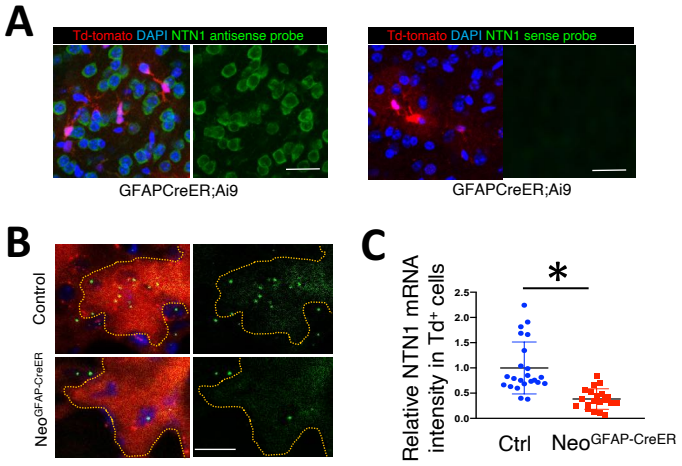

Figure S8. Netrin1 mRNA expression detected by FISH and RNA scope. (A) FISH In Situ hybridization of netrin-1 (NTN1) expression (by antisense, but not sense, probes) in GFAP-CreER;Ai9 mouse cortex sections [P30 (+TAM) to P60]. Netrin-1 signal was detected in both neurons and td-Tomato^{+} astrocytes. Bar=20µm. (**B-C**) RNA scope analysis of netrin1 mRNAs in td-Tomato^{+} astrocytes in control *(GFAP-CreER;Ai9)* and *Neo$^{GFAP\text{-}CreER}$;Ai9* cortex. B, Representative images, Bar=5µm; and C, Quantification analysis of data in (B). Data shown are mean±SEM (n=3 mice/group).*, $P < 0.05$, Mann-Whitney test.

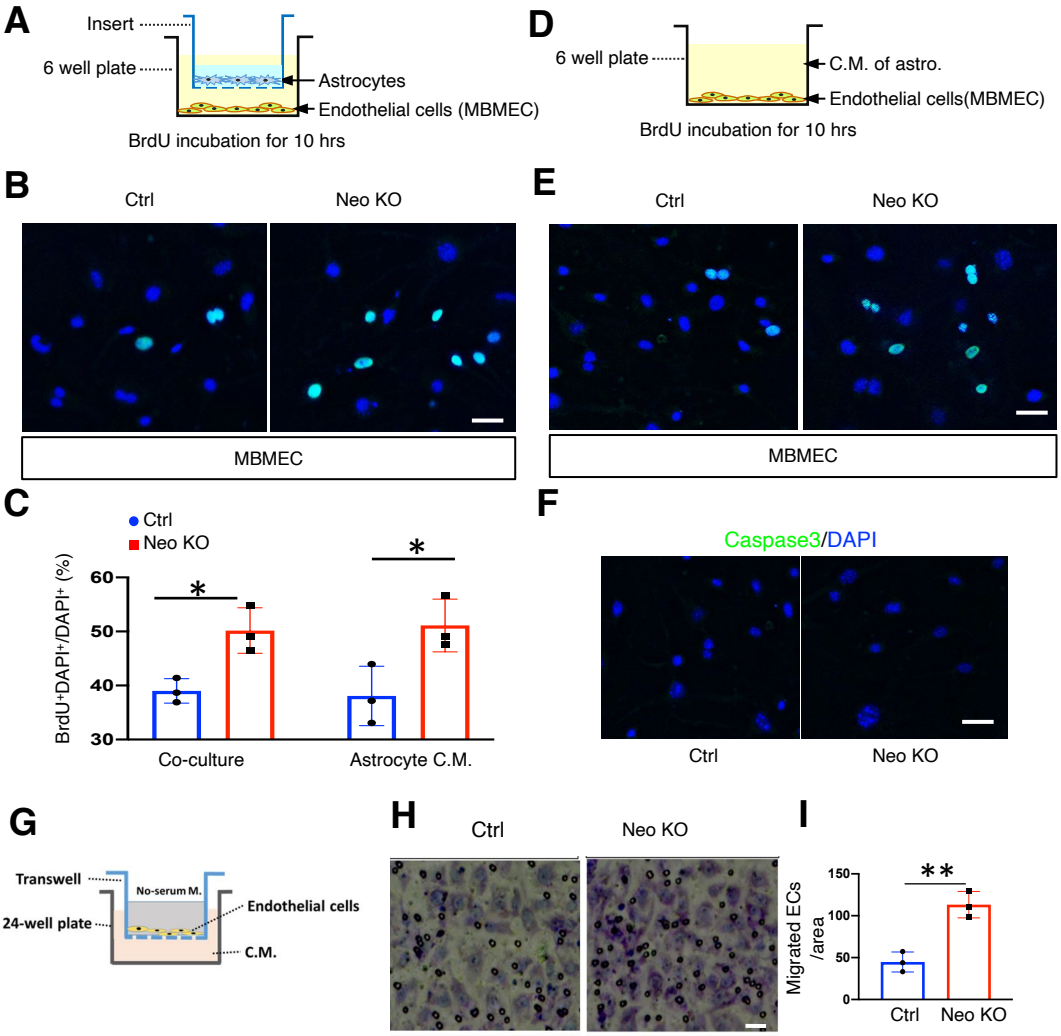

Figure S9. Increased EC proliferation and migration by co-culture of MBMECs with neogenin KO astrocytes or their CM. (A) Schematic illustration of the co-culture of Mouse brain vessel endothelial cells (MBMECs) with astrocytes from control and *Neo*$^{GFAP\text{-}Cre}$ cortex. BrdU was incubated for 10 hrs. **(B)** Representative images of BrdU^{+} MBMECs. **(C)** Quantitative analysis of data in (B) and (E). **(D)** Schematic illustration of MBMECs cultured with C.M. of astrocytes. BrdU was incubated for 10 hrs. **(E)** Representative images of BrdU^{+} MBMECs. **(F)** Representative images of MBMECs stained with anti-caspase 3. **(G)** Illustration of HUVEC trans-well experiments cultured with C.M. of astrocytes. **(H)** Crystal violet staining of the trans-membrane of HUVECs. **(I)** Quantification analysis of trans-migrated HUVECs. Data shown are mean±SEM (n=5 to 6 coverslips from 3 separated experiments). **P*<0.05; ***P*<0.01. Two-way ANOVA analysis for (C); Student's *t*-test for (I).

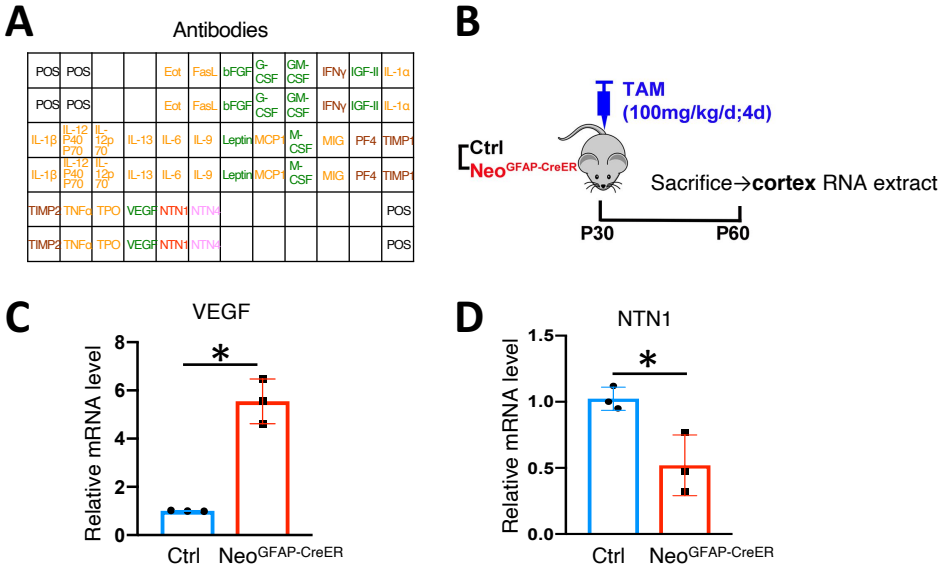

Figure S10. Increased expression of VEGF, but decreased NTN1 mRNAs in *Neo*$^{GFAP\text{-}Cre}$ cortex. (A) 26 antibodies on the nitrocellulose membrane for antibody array analysis. **(B)** Schematic illustration of the RNA extraction at P30 from the *Neo*$^{GFAP\text{-}Cre}$ mice cortex and its littermate controls. **(C-D)** Quantification analyses of VEGF and NTN1 mRNA expression. Data shown are mean±SEM (n=3 mice). *, $P < 0.05$, Student's *t*-test.

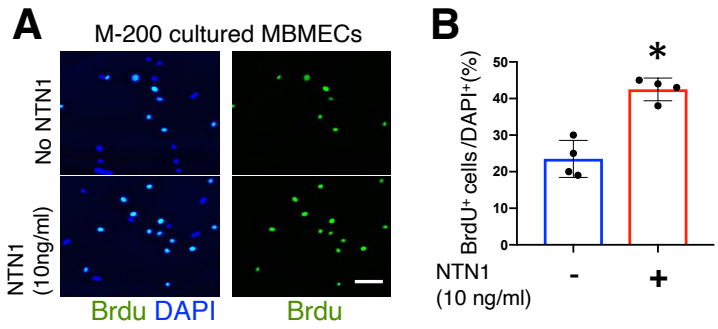

Figure S11. Increase of MBMEC proliferation by lower dose of netrin-1. MBMECs were cultured in M-200 culture medium (without FBS) in the presence or absence of netrin-1 (10 ng/ml) and BrdU (3µg/ml) for 10 hrs. **(A)** Representative images and **(B)** quantification of data in (A). Data shown are mean±SEM (n=4 cover slips /group). *, $P < 0.05$, Mann-Whitney test. Scale bar: 20 µm.

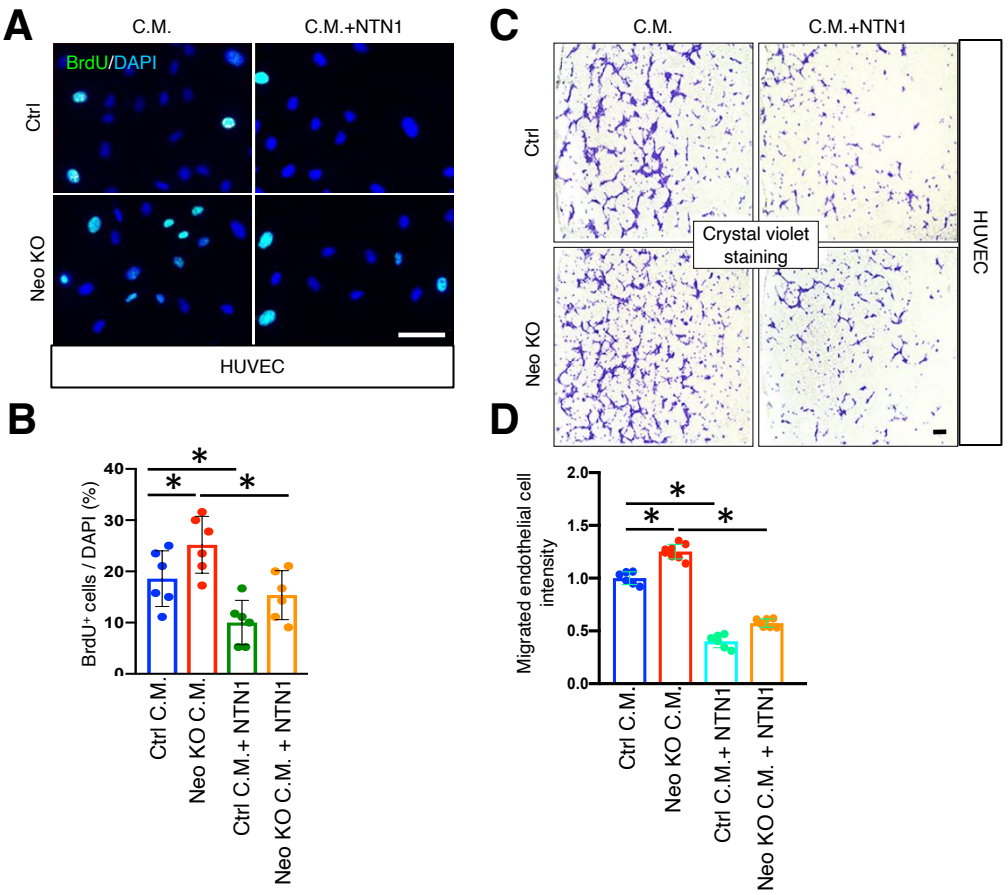

Figure S12. Netrin-1 inhibition of HUVEC proliferation and migration in culture. (**A-B**) Netrin-1 (1 μg/ml) inhibition of HUVEC proliferation. HUVECs were cultured with CMs of control and neogenin KO astrocytes in the absence or presence netrin-1 (1μg/ml) and BrdU (3μg/ml) for 6 hrs. A, Representative images of BrdU⁺ HUVECs. B, Quantification analysis of data in (A). **(C-D)** Netrin-1 inhibition of HUVECs migration. HUVECs were cultured in transwell plates, which exposed to the CMs of control and neogenin KO astrocytes with or without netrin-1 (1μg/ml) for 16 hrs. C, Representative images of migrated HUVECs (stained with Crystal violet). D, Quantification analysis of data in C. Data shown are mean±SEM (n=6 cover slips /group). **P*<0.05. Two-way ANOVA analysis. Scale bars: 20 μm.

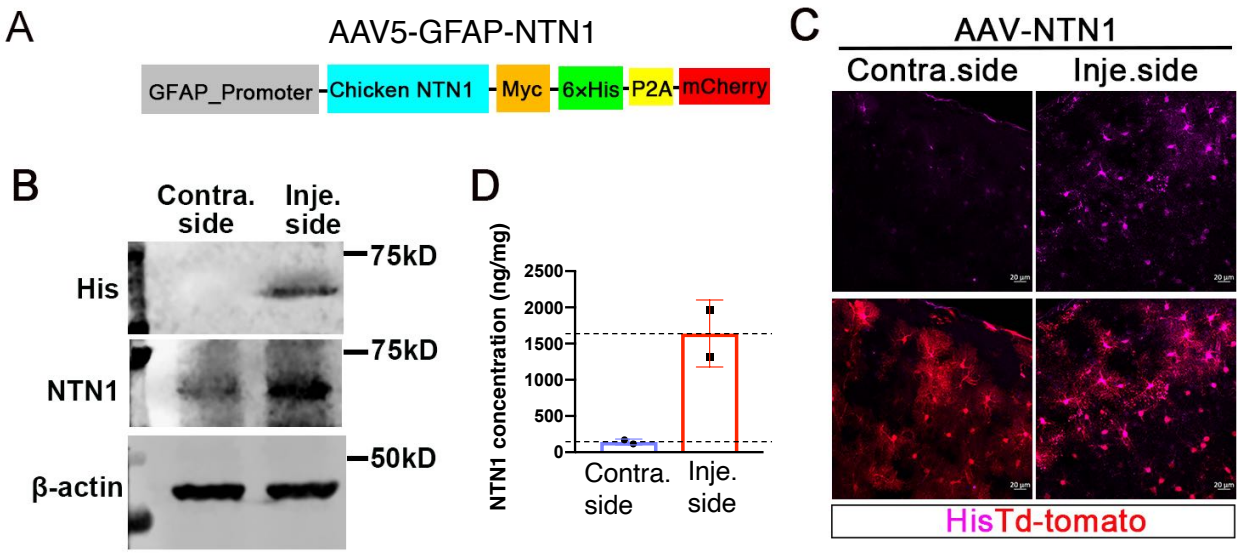

Figure S13. AAV5-GFAP-NTN1 generation and expression. (A) Illustration of AAV5-GFAP-NTN1, which expresses chicken-netrin1 fused with myc and 6×His tags, P2A, and mCherry, under the control of GFAP promoter. **(B)** Western blot analysis showing NTN1 expression upon AAV-GFAP-NTN1 injection into the cortex (ipsilateral side compared with contralateral side). **(C)** Representative images of His and tdTomato staining in the injection side and contralateral side. **(D)** Quantification analysis of netrin-1 concentration in AAV-GFAP-NTN1 injected cortex based on Western blot analysis using various concentrations of purified recombinant netrin-1 as controls. Data shown are are mean±SEM (n=2 separated experiments).

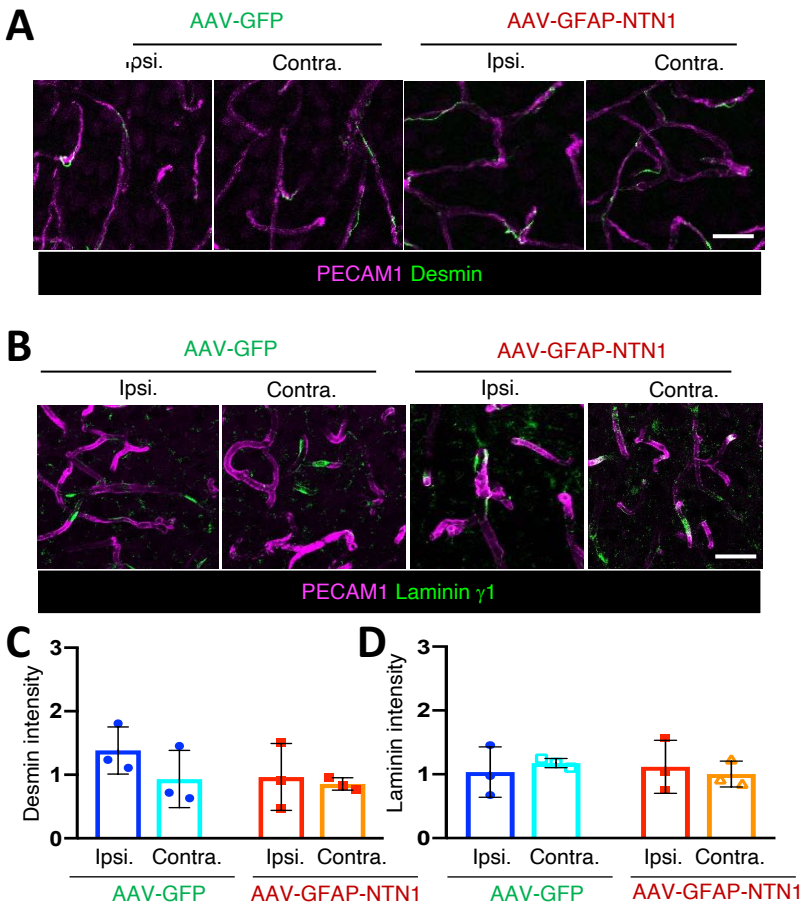

Figure S14. Little effect on laminin-γ1 and pericyte coverage in *Neo*$^{GFAP\text{-}CreER}$ cortex injected by AAV-GFAP-NTN1. (A) Representative images of desmin immunostaining in the *Neo*$^{GFAP\text{-}CreER}$ cortex injected with AAV-GFP or AAV-GFAP-NTN1.**(B)** Representative images of laminin-γ1 staining in the *Neo*$^{GFAP\text{-}CreER}$ cortex injected with AAV-GFP or AAV-GFAP-NTN1. **(C-D)** Quantification analyses of desmin^{+} **(C)** and laminin-γ1^{+} **(D)** over PECAM1+ BVs in ipsilateral and contralateral sides. Data shown are mean±SEM (n= 3 mice /group). Two-way ANOVA analysis. Scale bars: 20 µm.

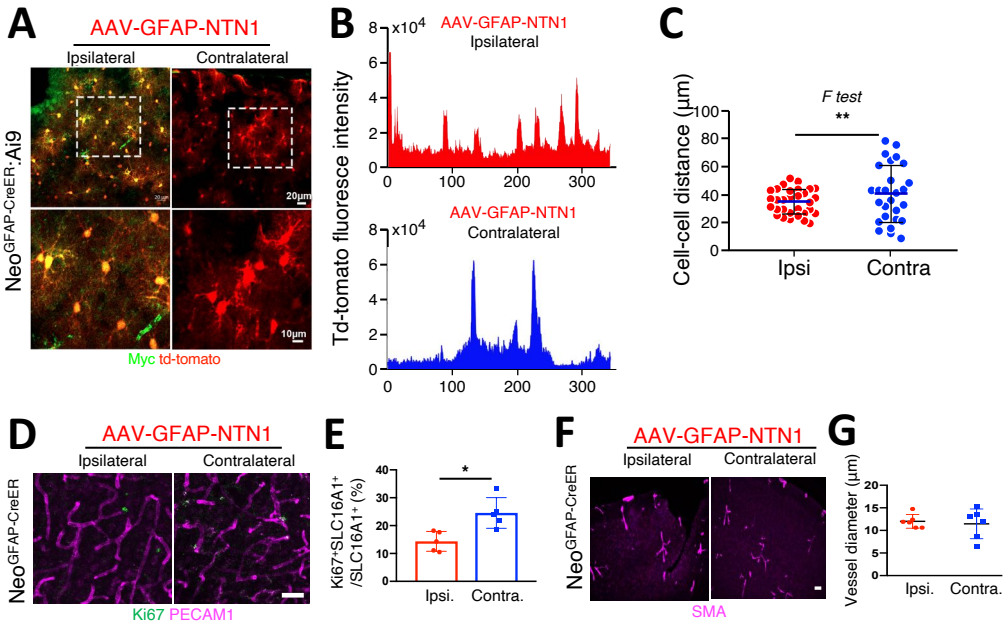

Figure S15. Netrin-1 amelioration of the deficits of astrocyte distribution and EC proliferation in *Neo*$^{GFAP\text{-}CreER}$ cortex. (**A**) Representative images showing Myc$^+$ (injected netrin-1) and td-Tomato$^+$ signals in the injection (ipsilateral) and contralateral sides of neogenin mutant mice. Note that netrin-1 (Myc$^+$) was largely expressed in td-Tomato$^+$ astrocytes. (**B**) Profile of the td-Tomato signal intensity of the diagonal line in the panel A. **(C)** Quantification analysis of cell-cell nuclear distance in AAV-GFAP-NTN1 injection and contralateral sides. Data are mean±SEM (n=30 cells /group). Unpaired t test of each side of the two groups was analyzed and the *p* value showed from F test for data variance. ***P*<0.01. Mann-Whitney test for data in C. **(D)** Representative images of Ki67$^+$;PECAM1$^+$ signals in the injection (ipsilateral) and contralateral sides of *Neo*$^{GFAP\text{-}CreER}$ cortex. (**E**) Quantification analyses of data in (D). Data shown are mean±SEM (n=5 mice /group), **P*<0.05. Mann-Whitney test. **(F)** Representative images of SMA$^+$ signals in the injection (ipsilateral) and contralateral sides of *Neo*$^{GFAP\text{-}CreER}$ cortex. (**G**) Quantification analyses of data in (E). Data are mean±SEM (n=6 mice /group). F test analysis.

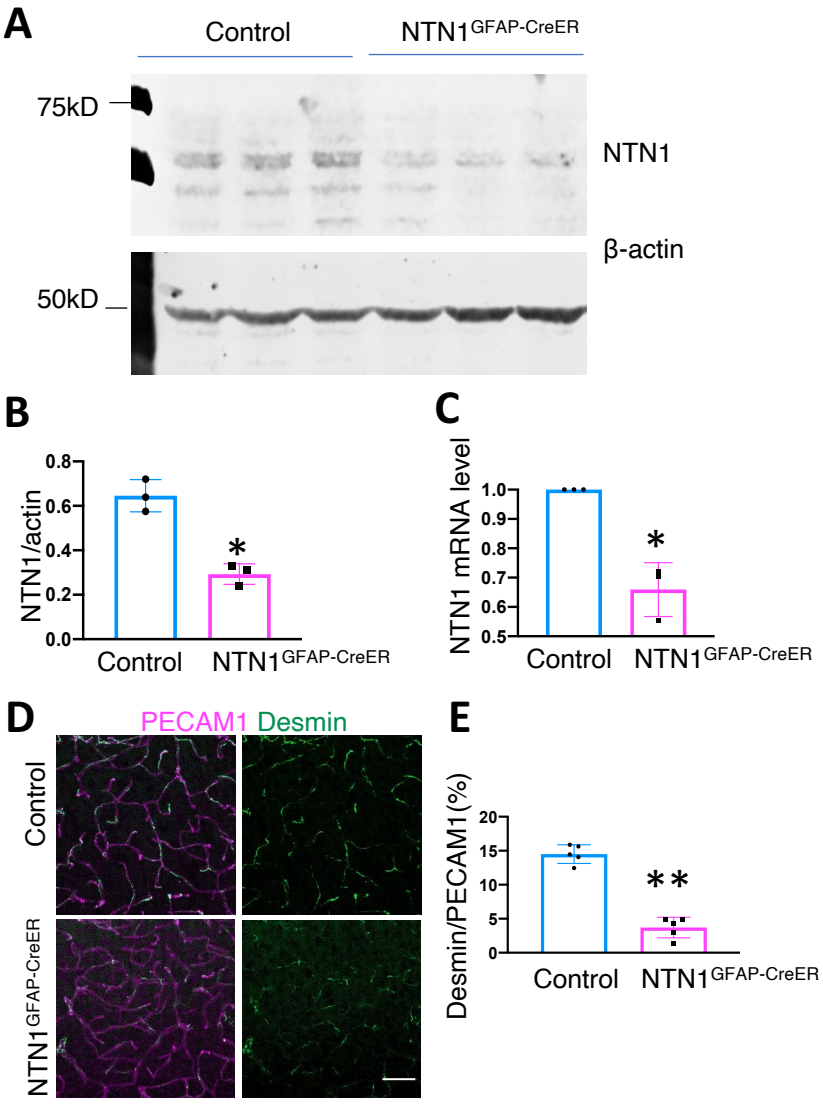

Figure S16. Decreased Netrin-1 expression and desmin⁺ pericytes in *NTN1*$^{GFAP\text{-}CreER}$ cortex. The control *(NTN1*$^{f/f}$*)* and *NTN1*$^{GFAP\text{-}CreER}$ mice were treated with TAM at P30, and examined at P60. (**A**) Western blots of cortex homogenates using indicated antibodies. **(B)** Quantification of data in (A). **(C)** RT-PCR analysis of netrin1 mRNA levels in control *(NTN1*$^{f/f}$*)* and *NTN1*$^{GFAP\text{-}CreER}$ cortex. (**D-E**) Representative images (D) and quantification (E) of desmin⁺ BVs in cortexes of control and *NTN1*$^{GFAP\text{-}CreER}$ mice. Scale bars: 20 µm. Data shown are mean±SEM (n=3-5 mice /group), **P*<0.05, **, P < 0.01, Mann-Whitney test.