Stem Cell Reports, Volume *9*

Supplemental Information

The aPKC-CBP Pathway Regulates Post-stroke Neurovascular Remodeling and Functional Recovery

Ayden Gouveia, Matthew Seegobin, Timal S. Kannangara, Ling He, Fredric Wondisford, Cesar H. Comin, Luciano da F. Costa, Jean-Claude Béïque, Diane C. Lagace, Baptiste Lacoste, and Jing Wang

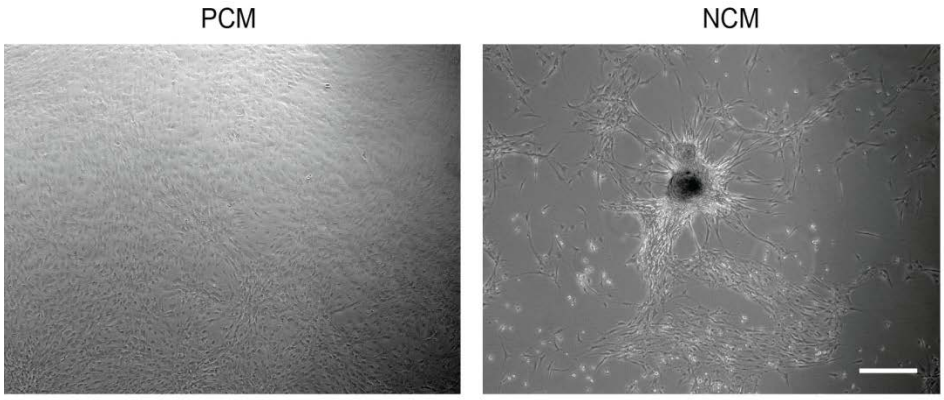

Figure S1. Reprogramming of i-pericytes to neural precursors. Ischemia-activated pericytes were cultured as a monolayer of cells under pericyte conditioned medium (PCM, left panel), while neurospheres were formed in the culture dish when PCM were switched to neural conditioned medium (NCM, right panel). Scale Bar=200μm

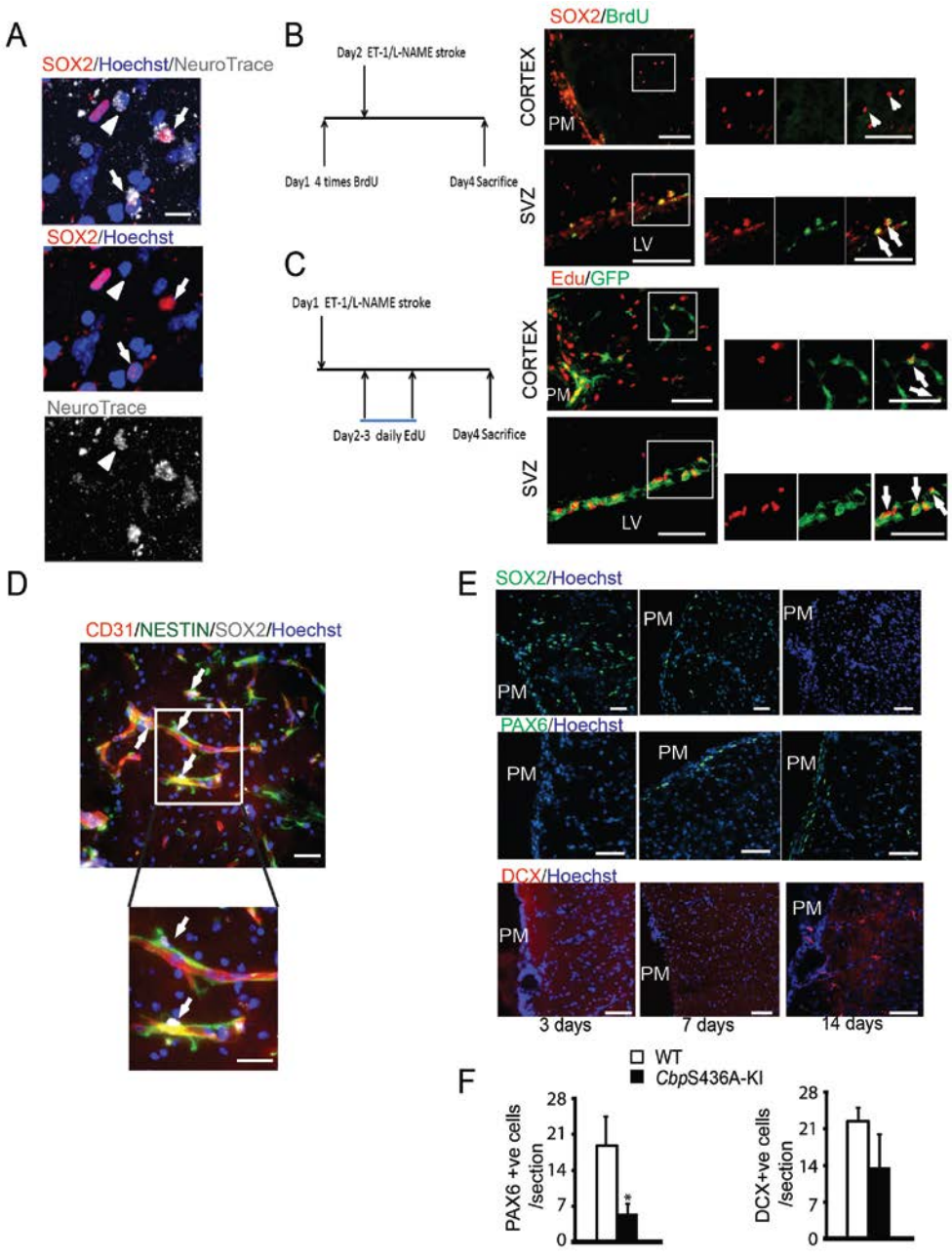

Figure S2. Locally-derived neural precursor cell lineage in cortex layer I following ischemic stroke injury. (A) Merged images from live two-photon imaging for NeuroTrace and confocal imaging for SOX2+NPCs. Scale bar=10μm. (B) Representative micrographs of the ipsilateral cortex and SVZ of mice who received stroke surgery 2 days prior and BrdU (i.p. 60mg/kg, 4

times every 3-hour) injections 24 h pre-surgery, stained for SOX2 (red) and BrdU (green). Arrows denote co-labelled cells, while arrow heads denote single-labeled cells with SOX2+. Scale bar= 40μm; PM: pia mater, LV: lateral ventricle. A flowchart of this experiment is on the left panel. (C) Representative micrographs of the ipsilateral cortex and SVZ of *Sox2-GFP* mice who received stroke surgery 3 days prior and EdU injections (i.p. 50mg/kg, daily) post stroke day 2-3, stained for GFP (green) and EdU (red). Arrows denote co-labelled cells. Scale bar= 40μm. A flowchart of this experiment is on the left panel. (D) Representative micrographs of the ipsilateral cortex 3 days post-stroke show that locally-derived SOX2 (white) + NPCs are adjacent to CD31 (red)+ endothelial cells, colabelled with NESTIN (green). Scale bar=20μm. (E) Representative images of the infarct cortex at days3, 7, 14 post-stroke, stained with SOX2 (green, top), PAX6 (green, middle), and DCX (red, bottom). Scale bar=20μm. (F) Quantitative analysis of PAX6 (left) and DCX (right) positive cells in WT and *Cbp*S436A ipsilateral cortex layer I below the pia surface, 14 days post-stroke, *$p<0.05$, n=3 per group.

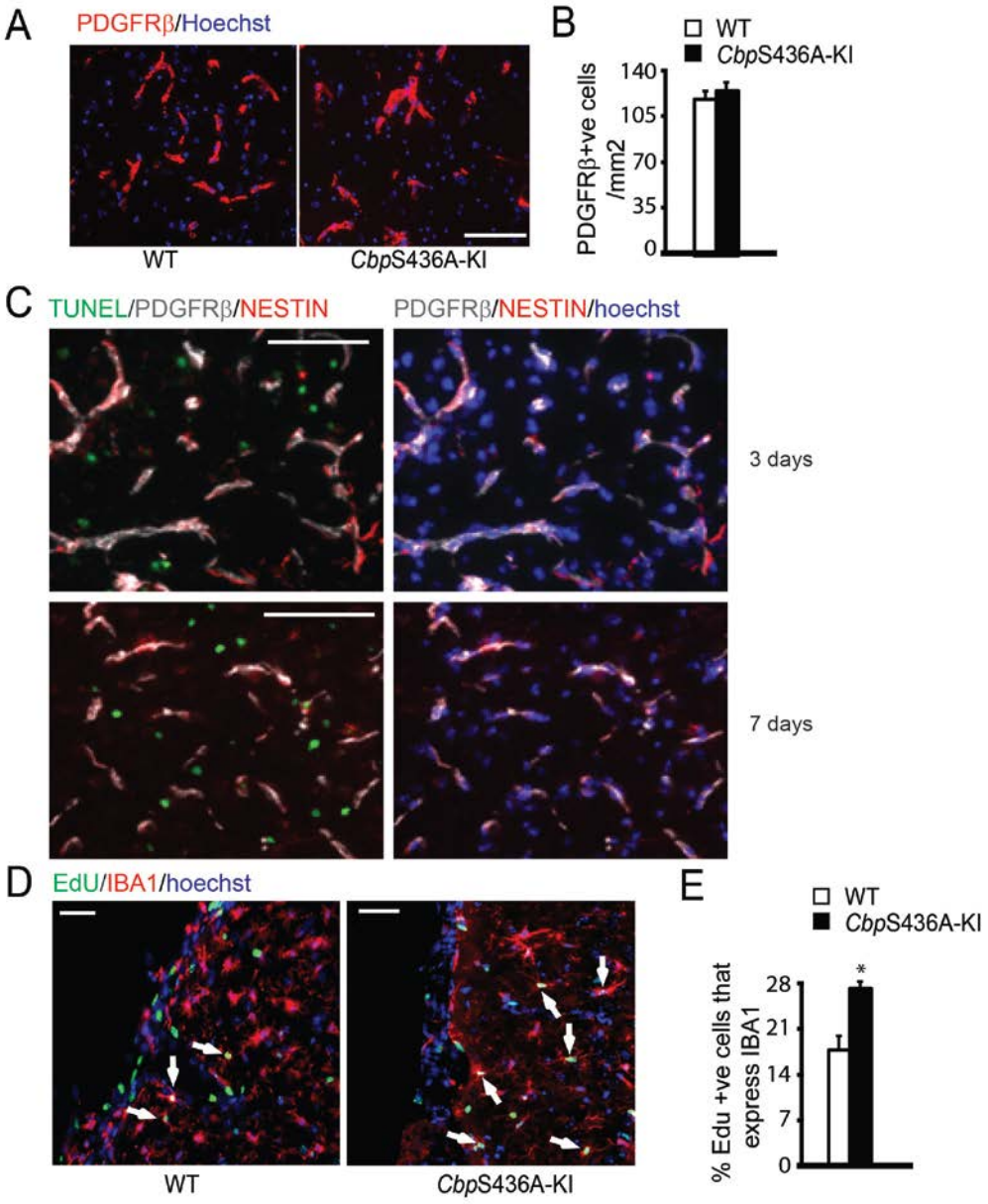

Figure S3. (A-C) Basal number of pericytes and apoptotic i-pericytes remain the same between WT and C*bp*S436A-KI. (A) Representative micrographs of the cortex from both WT and *Cbp*S436A-KI mice under physiological conditions, stained for PDGFRβ (red), counterstained with Hoechst (blue). Scale bar= 40μm (B) Quantitative analysis of PDGFRβ + pericytes in the WT and *Cbp*S436A cerebral cortex as shown in (A) under physiological condtions. n=3/group. (C) Representative micrographs of ipsilateral cortex from WT mice at 3

days and 7 days following stroke surgery, stained for TUNEL (green), PDGFRβ (grey), NESTIN (red), counterstained for Hoechst (blue). Scale bar= 40μm. (D-E) **C*bp*S436A-KI mice show an increase in the population of Iba1+microglia in injured cortex layer I following stroke.** Representative images (D) and quantitative analysis (E) of co-labelled IBA1+/EdU+ cells in WT and *Cbp*S436A ipsilateral cortex 14 days post-stroke, injected with EdU (i.p. 50mg/kg) at post-stroke days 2-5. Scale bar= 25μm. *$p<0.05$, n=3-4/group.

Supplemental Experimental Procedures:

Animals

Only wild type (WT) and homozygous (*Cbp*S436A-KI) mice (Zhou, et al., 2004) were used as experimental mice and heterozygous of *Cbp*S436A were used for breeding. The *Sox2*-GFP reporter transgenic line was provided by Dr. Ruth Slack. The *Nestin*-GFP reporter transgenic line (Yamaguchi, et al., 2000) and *Nestin*Cre-ER^{T2} (Lagace, et al., 2007)/tdTomato$^{flx/Stop/flx}$ transgenic line were provided by Dr. Diane Lagace.

ET-1/L-NAME Surgery

Injections were performed using a Hamilton 10uL gastight syringe with a 0.49mm diameter needle (Hamilton Robotics, Reno NV, 7653-01). Injections of saline, or ET-1 (Abcam, Cambridge, UK, AB120471) (2 µg/ µl) + L-N^{G}-Nitroarginine methyl ester (L-NAME) (Sigma-Aldrich, St. Louis MS, N5751) (2.7 µg/ µl) were performed at +0.0mm anterior-posterior (AP), -2.0mm Medial-Lateral (ML), -1.6mm Dorsal-Ventral (DV); +0.2AP, -2.0ML, -1.4DV; and +0.4AP, -2.0ML, -1.3DV. ET-1 and L-NAME were dissolved in PBS and sonicated in a 4^{o}C water bath for 15 minutes before use. The injection was performed at 0.2 µL/minute for 5 minutes per site for a total of 1µL/injection. Upon needle insertion, 1 minute waiting time was used to allow for settling of tissue. Following injection, a 3-minute waiting time before needle removal was used to reduce back-flow. Body temperatures were continually monitored and maintained at 37^{o}C during surgery using a heating pad and anal thermometer. Local Bupivacaine and subcutaneous buprenorphine were administered post-surgery and 4 hours later. All animals that received strokes were included in the study since Cresyl Violet histological staining (method below) confirmed strokes were produced in all mice and there was no lethality post stroke.

Pericyte Culture and Sphere Formation

The infarct/periinfarct cortical tissue was removed and digested in HBSS for 30 minutes using papain (Worthington Biochemicals, LS003126) and 100 units DNAse (Sigma-Aldrich, D5025-15KU) at 37^{o}C. The samples were then triturated through 18- and 23- gauge needles. Single dissociated cells were plated at 600,000 cells/mL in an uncoated plastic dish with high glucose DMEM/F-12 containing 5µg/mL EGF (VWR, CACB354052), 5µg/mL FGF (Peprotech, 100-18B), 1% N2 supplement (Thermo Fisher, 17502048), and 2% FBS (Life Technologies, 12484010). Full media changes were performed on days 1 and 2 post-plating and half media changes every other day following that.

After 1 week, ischemia-activated pericytes (i-pericyte) were selectively grown, showing 100% population of cells expressing pericyte markers, PDGFRβ and NG2. These i-pericytes were exposed to neural conditioned medium (NCM): DMEM/F-12 media containing 5µg/ml FGF, 5µg/ml EGF, 10µg/mL leukemia inhibitory factor (LIF) (Peprotech, 250-02), and 1% N2 for 2-4 weeks. 100µL of media was added once a week to account for evaporation. After 3 weeks, the total spheres in each well were counted before single spheres were collected onto glass slides using a Cytospin (Thermo Fisher).

Single spheres were also picked and placed into a dish coated with matrigel in neural basal media containing retinoic acid (RA, 200nM) for differentiation. After 1 week cells were stained for βIII TUBULIN, GFAP and O4.

Drug treatment

To label dividing cells, mice received an intraperitoneal injection (i.p.) with 50mg/kg 5 ethynyl 2' deoxyuridine (EdU) (Cedarlane, Burlington, ON, AB16186) prepared in sterile PBS. Injections were given daily for day 2-5 post-stroke for *Cbp*S436A strain, and day 2-3 post-stroke for *Sox2*-GFP mice. A separate cohort of mice received i.p. injections with 60mg/kg 5 bromo2' deoxyuridine (BrdU) (Sigma-Aldrich, B9285) prepared in sterile PBS. Injections were performed beginning 24 hours before surgery and were repeated 4 times, once every 3 hours. To trace SVZ NPCs using *Nestin-CreER^{T2}/tdtomato floxed* transgenic line, tamoxifen (180mg/kg, i.p. daily for 5 days) were injected for 5 days beginning at 12 days before ET-1/L-NAME stroke surgery.

Immunohistochemistry, microscopy, and quantification

At 3 or 14 days post-surgery, mice were anesthetized and transcardially perfused with 4% Paraformaldehyde (PFA) (Sigma-Aldrich, 1518127) in PBS. Then brains were removed and sectioned in a CRYOSTAT (Leica Biosystems, Buffalo Grove IL, CM1850) at 20 μm. Serial sections were mounted on 10 glass slides. Brain sections that were stained for CD31 or PDGFR-β were perfused with PBS, followed by immediate dissection and snap freezing in 100% ethanol cooled with dry ice. The sections were fixed with acetone at room temperature immediately after sectioning. The slides were dried and then were stored at -80^{o}C until needed.

For PFA perfused sections, Two 5-minute washes with PBS were performed before 15-minute fixation with 4^{o}C 4% PFA. For acetone fixed sections; the sections were directly fixed with -20^{o}C acetone for 15 min and dehydrated with -20^{o}C 100% ethanol. Three 5-minute washes

with PBS were performed on all slides sections before proceeding to the permeabilization /blocking step.

Sections were blocked and permeabilized with 10% goat serum (Jackson Immuno-research, West Grove PA, 008-000-121) (horse serum if primary antibody was produced in a goat) and 0.3% Triton-X, and then were incubated with primary antibodies at 4ºC overnight, with secondary antibodies at room temperature for 1 hour, counterstained with Hoechst 33343 (1:2000, Sigma-Aldrich) and mounted using GelTol (Fisher). For BrdU colabelling with SOX2, sections were incubated in 1 N HCl at 60°C for 30 min, rinsed in PBS, incubated in rat anti-BrdU antibody at 4°C overnight, in Alexa 488 donkey anti-rat antibody for 1 hour and then sequentially immunostained for anti-Sox2 followed by Alexa Fluor-conjugated secondary antibodies.

Primary antibodies used were: rat anti-CD31 (1:200) (BD Pharminogen, Franklin Lake, NJ, 550274), rat anti-BrdU (1:200) (AbD Serotec, Oxford UK, OBTOO30G), rabbit anti-SOX2 (1:200) (Millipore, Billiereca MA, 49005), goat anti-DCX (1:200) (Santa Cruz Biotechnologies, Dallas TX, sc-8066), rabbit anti-PDGFR-β (1:100) (Santa Cruz Biotechnologies, sc-1627), Rabbit anti-PAX 6 (1:200)(Biolegend, 901301), chicken anti-NESTIN (1:1000) (Aves labs, NES), Chicken anti-GFP(1:1000)(Abcam, ab13970), rabbit anti-NG2(1:1000)(Milipore, AB5320), rabbit anti-IBA1(1:1000)(Wako Chemicals, 019-19741), rabbit anti-DSRED (1:1000)(Living Colors, 632496), mouse β III TUBULIN (1:1000) (Covance, MMS-435P), rabbit GFAP (1:1000) (Abcam, ab7260), mouse O4 IgM (1:400) (Millipore, MAB345).

Digital image acquisition was performed using either a Zeiss Axioplan 2 fluorescent microscope with Zeiss Axiovision software that contains z-axis capability, or a Zeiss LSM 510 confocal microscope using Zeiss Zen Pro software V2.0 (Oberkochen, Germany). 10-15 images

were captured in the Z-axis per section at a maximum of 1µm apart and processed as an optical stack of 10-15 scanned slices for quantification.

For quantification, positive cells in the desired region were quantified using imageJ software (National Institute of Health, Bethesda MD). Images were obtained at 20x magnification. Three images per section for 6-10 sections were quantified, dependent on the extent of the stroke.

Particle analysis for vascular coverage was performed using ImageJ. The auto threshold function was used in conjunction with particle analysis for area coverage. Particles below 5 pixels were excluded from analysis for area coverage.

Three Dimensional (3-D) Blood Vessel Staining, Imaging and Quantification

The cortices were removed from between the glass and washed with PBS, then embedded in 2% agarose in PBS. The cortices were cut tangentially into 120µm sections using a vibratome (Leica WT1000S). Sections were blocked with 10% horse serum, permeabilized with 0.2% tritonX100 and 0.5% fish gelatin followed by overnight incubation with rat anti-CD31 (BD Pharminogen 550274, 1:200). The sections were then rinsed in PBS and incubated for two hours in anti-rat Alexa fluor 488 conjugated antibodies (Jackson Immunoresearch, 1:300). Slides were mounted with fluoromount G and visualized using fluorescent confocal microscopy (LSM510/Axioimager.M1, Zeiss). Z-Stacks between 60-80µm were obtained.

Two images per hemisphere per section for three sections were analyzed for vessel length and branch points as previously described (Lacoste et al., 2014). Briefly, first, the images were smoothed, followed by application of an adaptive thresholding procedure (Gonzalez & Woods, 2007). The obtained arteries had their medial axes extracted by using a thinning algorithm

(Palàgyi & Kuba, 1998), giving rise to a network (Viana et al., 2009; Rafelski et al., 2012). Bifurcations in such structures were detected as corresponding to pixels with three or more neighbors, while pixels with a single neighbor were understood as terminal nodes. The arc-length of the segments between the bifurcations were then estimated (Cesar Jr. & Costa, 1999). The number of branch points was also obtained.

Two-photon *ex vivo* imaging. Briefly, 3 days following NeuroTrace 500/525 and ET-1/L-NAME injections, mice were deeply anesthetized with isofluorane (Baxter Corporation, Canada), and transcardially perfused with ice-cold, oxygenated choline-based artificial cerebrospinal fluid (choline-aCSF), containing the following (in mM): 119 Choline-Cl, 2.5 KCl, 4.3 $MgSO_4$, 1.0 NaH_2PO_4, 1.0 $CaCl_2$, 11 Glucose, and 26.2 $NaHCO_3$ (pH 7.2-4, 295-310 mOsm/L). Mice were then decapitated and the brain was quickly removed. Coronal slices (300 µm) containing the full extent of the ET-1-induced infarct were generated using a Leica VT1000 S vibratome blade microtome (Leica Microsystems). Brain sections were then transferred to an incubation chamber, and allowed to recover for at least one hour in oxygenated artificial cerebrospinal fluid (aCSF), containing the following (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 11 Glucose, and 26.2 $NaHCO_3$ (pH = 7.2-4, 295-310 mOsm/L) initially maintained at 30^{o}C, then recovered at room temperature. Slices were transferred to a perfusion chamber and immersed in oxygenated aCSF (2 mL/minute) at room temperature. Bright-field imaging of cells within Layer 1-2 of the peri-infarct cortex was conducted using an AxioCam ICm1 CCD camera (Zeiss), coupled to a Zeiss LSM710 multiphoton microscope with a 20x (1.0 NA) objective. Bright-field acquisition allowed the aligning of 2-photon imaging, performed using a Ti:Sapphire pulsed laser tuned to 1000 nm (MaiTai-DeepSee, Spectra Physics), similar to

previously reports *in vivo* (Damisah et al., 2017). Once imaging was complete, slices were post-fixed in 4% paraformaldehyde for 1-3 hours, and then placed in 30% sucrose (in 1x PBS) at 4^{o}C.

Slices were permeabilized in PBS with 0.3% triton for 24 hours at 4^{o}C. The primary antibody against SOX2 was then added for 24 hours at 4^{o}C in 10% normal goat serum. Three PBS washes were performed for 10 minutes and secondary antibody was added overnight at 4^{o}C. The section were mounted and imaged by fluorescent confocal microscopy (LSM510/Axioimager.M1, Zeiss) for the first 30μm of depth. The two-photon and confocal images were compared and landmarked in order to align physical features and cells. Once aligned, the images were merged. All land marking and merging was performed using ImageJ.

TUNEL staining

Mice were sacrificed either 3 or 7 days post-stroke and stained for nicked DNA, a hallmark of apoptosis, using R&D systems Tacs in situ fluorescence TUNEL kit (#4812-60-K). Following TUNEL staining the tissue was stained for NESTIN and PDGFRβ as described above. The ipsilateral cortex was analyzed for the presence of TUNEL^{+}/NESTIN^{+}/PDGFRβ^{+} cells.

Horizontal Ladder Test

Mice were placed at the beginning of a horizontal ladder consisting of two plexiglass walls and irregularly placed metal bars across from their home cage. The home cage was placed at the end of the ladder. The mice were videotaped crossing the bar from slightly below in order to have full view of all four limbs. Five trials per day were videotaped and only trials in which mice made clear, uninterrupted movement across the ladder were later analyzed. The mice were given one day of pre-training and one day of baseline measurements prior to surgery. Mice were re-

tested at 7 and 14 days post-stroke. The videos were later analyzed in a blinded fashion using VLC media player at a reduced speed. Steps in which the mice slipped on or missed a bar, or the use of the wall to support their weight, were counted as an error. The percentage of steps containing an error was reported.

Cylinder Test

Mice were placed in a clear plastic cylinder under red light and were videotaped from above for the time it took for the mice to perform 20 rears. One pre-surgery baseline value was obtained and the mice were re-tested at 7 and 14 days post-stroke. The videos were later analyzed frame-by-frame in VLC media player (Version 2.2.3). The total number of ipsilateral, contralateral, and double paw touches against the cylinder wall was counted. The percentage ipsilateral preference was calculated as:

(total ipsilateral forepaw touches – contralateral forepaw touches) *100) / total forepaw touches

Cresyl Violet Staining and **Infarct Volume Measurement**

Slides containing serially collected sections were dried at 37^{o}C for 15 minutes. The slides were then submerged in cresyl violet solution (Sigma-Aldrich, C5402) (0.2% cresyl violet dissolved in 0.5% acetic acid solution, pH = 3.5) for 20 minutes. Sequential washes in 70%, 95% and 100% ethanol were performed before clearance with citrosolv (Fisher, 22-143-975) clearing agent. The slides were mounted with permount solution and placed on a 37^{o}C slide warmer to dry overnight. Cresyl violet images were captured using an Aperio digital pathology slide scanner (Leica Biosystems) and analyzed using Image J. Infarct volume was calculated based on the previously published paper (Huang, et al., 2013).

Statistical Analysis

Data analysis was performed using GraphPad Prism 6 (Graphpad Software, La Jolla CA). Behavioural analysis was performed using two-way ANOVA with Tukey's post-hoc test. Single comparisons were performed using two-tailed Student's T-test.

References

Cesar, R.M., Jr., and Costa, L.D. (1999). Computer-vision-based extraction of neural dendrograms. J. Neurosci. Methods *93*, 121–131.

Gonzalez, R.C., and Woods, R.E. (2007). Digital Image Processing, Third Edition. (New Jersey: Prentice Hall).

Damisah, E.C., Hill, R.A., Tong, L., Murray, K.N., and Grutzendler, j. (2017) A fluoro-Nissl dye identifies pericytes as distinct vascular mural cells during *in vivo* brain imaging Nature Neurosci *20*, 1023-1032.

Huang, J., Li, Y., Tang, Y., Tang, G., Yang, G.Y., Wang, Y. (2013) CXCR4 antagonist AMD3100 protects blood–brain barrier integrity and reduces inflammatory response after focal ischemia in mice. Stroke *44*, 190-197.

Lacoste, B., Comin, C.H., Ben-Zvi, A., Kaeser, P.S., Xu, X., Costa, L.F., and Gu, C. (2014). Sensory-related neural activity regulates the structure of vascular networks in the cerebral cortex. Neuron *83*, 1117–1130.

Lagace, D.C., Whitman, M.C., Noonan, M.A., Ables1, J.L., DeCarolis, N.A., Arguello, A.A., Donovan, M.H., Fischer, S.J., Farnbauch, L.A., Beech, R.D., DiLeone, R.J., Greer, C.A., Mandyam, C.D. and Eisch, A.J. (2007). Dynamic Contribution of Nestin-Expressing Stem Cells to Adult Neurogenesis. J Neurosci. *27*, 12623–12629.

Lee K.F., Soares C., Thivierge J.P., Beique J.C. (2016). Correlated Synaptic Inputs Drive Dendritic Calcium Amplification and Cooperative Plasticity during Clustered Synapse Development. Neuron *89*,784-799.

Palàgyi, K., and Kuba, A. (1998). A 3D 6-subiteration thinning algorithm forextracting medial lines. Pattern Recognit. Lett. *19*, 613–627.

Rafelski, S.M., Viana, M.P., Zhang, Y., Chan, Y.H., Thorn, K.S., Yam, P., Fung, J.C., Li, H., Costa, Lda.F., and Marshall, W.F. (2012). Mitochondrial network size scaling in budding yeast. Science *338*, 822–824.

Soares C., Lee K.F., Nassrallah W., Beique J.C. (2013). Differential subcellular targeting of glutamate receptor subtypes during homeostatic synaptic plasticity. J Neurosci 33:13547-13559.

Viana, M.P., Tanck, E., Beletti, M.E., and Costa, Lda.F. (2009). Modularity and robustness of bone networks. Mol. Biosyst. *5*, 255–261.

Yamaguchi, M., Saito, H., Suzuki, M., and Mori, K. (2000). Visualization of neurogenesis in the central nervous system using nestin promoter-GFP transgenic mice. Neuroreport. *11*, 1991-6.

Zhou, X.Y., Shibusawa, N., Naik, K., Porras, D., Temple, K., Ou, H., Kaihara, K., Roe, M.W., Brady, M.J., Wondisford, F.E. (2004). Insulin regulation of hepatic gluconeogenesis through phosphorylation of CREB-binding protein. Nat. Med. *10*, 633-637.