Semaphorin 3A (Sema3A) increased significantly in mouse brain following cerebral ischemia. However, the role of Sema3A in stroke brain remains unknown. Our aim was to determine whether Sema3A functions as a vascular permeability factor and contributes to ischemic brain damage. Recombinant Sema3A injected intradermally to mouse skin, or stereotactically into the cerebral cortex, caused dose- and time-dependent increases in vascular permeability, with a degree comparable to that caused by injection of a known vascular permeability factor vascular endothelial growth factor receptors (VEGF). Application of Sema3A to cultured endothelial cells caused disorganization of F-actin stress fibre bundles and increased endothelial monolayer permeability, confirming Sema3A as a permeability factor. Sema3A-mediated F-actin changes in endothelial cells were through binding to the neuropilin2/VEGFR1 receptor complex, which in turn directly activates Mical2, a F-actin modulator. Down-regulation of Mical2, using specific siRNA, alleviated Sema3A-induced F-actin disorganization, cellular morphology changes and endothelial permeability. Importantly, ablation of Sema3A expression, cerebrovascular permeability and brain damage were significantly reduced in response to transient middle cerebral artery occlusion (tMCAO) and in a mouse model of cerebral ischemia/haemorrhagic transformation. Together, these studies demonstrated that Sema3A is a key mediator of cerebrovascular permeability and contributes to brain damage caused by cerebral ischemia.
Mical proteins are molecular conduit through which Sema3A affects actin reorganization in drosophila. Alteration of the actin cytoskeleton produces hallmarks of cell morphological changes, such as loss of polarity and the appearance of rounded shapes. Mics are an evolutionary conserved family of multidomain signal transduction proteins, and which can directly modify actin cytoskeleton depending on their monoxygenase enzyme activity and/or redox signaling.

In the present study, we show Sema3A increases vascular permeability mediated through NRP2/VEGFR1 receptors. In response to Sema3A, VEGFR1 directly interacts with Mic2 to cause F-actin disorganization, thereby changing cerebroendothelial cell morphology and permeability. Deletion of Sema3A expression (Sema3A-/-) reduced cerebrovascular permeability and protected mouse brain against two types of cerebral ischemia.

**Methods**

**Materials.** All chemicals and reagents, unless stated otherwise, were purchased from Sigma Chemical Co. (Burlington, ON, Canada) and were of analytical grade or higher. Recombinant Sema3A was purchased from R&D Systems (Minneapolis, MN). A battery of primary antibodies were purchased from commercial sources and used for blocking experiments and Western blots (WB) as shown in the Table 1. Chemical inhibitors Zm 306416 (Catalog No.S2897, specific for VEGFR1) and Kit8751 (specific for VEGFR2) were purchased from Selleckchem.com.

**Animals.** All procedures using animals were approved by the Institute for Biological Sciences Animal Care Committee (protocol 2007.13) following the guidelines established by the Canadian Council on Animal Care. Sprague Dawley male rats (200–250 g) were obtained from Charles River (St Fioque, PQ, Canada). Sema3A knockout (Sema3A-/-) backgrounded with C57BL/6 mice were kind gifts from Dr Masahiko Taniguchi (Sapporo Medical University, Japan) as described previously. Sema3A-/- mice were bred by mating between heterozygote littermates and the value obtained was reported as OD600/mg of tissue. For the Mies assay, after Evans blue extraction analysis. To do this, tissues were removed, weighted and fixed in PBS. Brains were removed, post fixed in 4% formaldehyde (pH 7.4) for 18 h and isofluorane and perfused transcardially first with saline followed by 4% formaldehyde in PBS. Brains were removed, post fixed in 4% formaldehyde (pH 7.4) for 18 h and cryoprotected in 30% sucrose for 36 h at 4°C. High resolution (1300 × 1300 d.p.i.) individual color layers were imported into NIH Image J software (http://rsb.info.nih.gov/ij/) and converted into gray scale mode. A common threshold was established for all images for image intensity analysis. At least five randomly selected areas were measured and averaged. All experiments were repeated at least three times.

**Brain tissue processing and sectioning.** Brain tissue was processed as previously described. Briefly, at various repercussion time points, mice were anesthetized with isofluorane and perfused transcardially first with saline followed by 4% formaldehyde in PBS. Brains were removed, post fixed in 4% formaldehyde (pH 7.4) for 18 h and cryoprotected in 30% sucrose for 36 h at 4°C. Coronal sections (10 μm thickness) were cut using a cryostat (Leica Microsystems, Wetzlar, Germany) and mounted on superfrust slides (Fisher Scientific, Toronto, ON, Canada), stored at −80°C.

**Immunohistochemistry and image quantification.** Immunohistochemistry was performed exactly as we previously described. Immunostained sections were visualized on a Zeiss inverted microscope and digital images were obtained using the Axiosvion v.4.7.2.0 software as we previously described. High resolution (1300 × 1300 d.p.i.) individual color layers were imported into NIH Image J software (http://rsb.info.nih.gov/ij/) and converted into gray scale mode. A common threshold was established for all images for image intensity analysis. At least five randomly selected areas were measured and averaged. All experiments were repeated at least three times.

**Generation and purification of replication defective adenovirus for injection.** The methods used to generate replication defective adenovirus were exactly as previously described. Replication defecitve recombinant adenoviruses were generated from the constructed pShuttle trans vectors using the AdEasy vector system (QBiogene) according to the manufacturer’s instructions. Human embryonic kidney 293A cells (HEK-293A) (Q BIOgene, Carlsbad, CA) were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 40 μg/ml gentamycin sulfate. The identity of all recombinant adenoviruses was confirmed by amplification and EGFP fluorescence. Reombinant constructs were verified by sequencing and recombinant adenoviral particles were CsCl purified and titrated as described previously.

To prepare for injection of the viral particles, Ad viruses were subjected to two rounds of CsCl ultracentrifugation purification and extensive dialysis to remove the CsCl.

In vitro culture of rat brain capillary endothelial cells (RBEC). Primary cultures of RBEC were prepared from 3-week-old rats, as previously described. Meninges were carefully removed from forebrains and gray matter was minced into small pieces of approximately 1 mm3 in ice-cold Dulbecco’s modified Eagle medium (DMEM), then dissociated by 25-times of up- and down-strokes with a 5-ml pipette in DMEM containing collagenase type 2 (1 mg/ml, Worthington Biochemical Corp., NJ, USA), 300 μl DNase (15 μg/ml), gentamycin (50 μg/ml) and then digested in a shaker for 1.5 h at 37°C. The cell pellet was separated by centrifugation in 20% bovine serum albumin (BSA)-DMEM (1000 × g, 20 min). The microvessels obtained in the pellet were further digested with collagenase-dispase (1 mg/ml, Roche Applied Sciences, Basel, Switzerland) and DNase (6.7 μg/ml) in DMEM for 1 h at 37°C. Microvessels were collected into a 50-ml centrifuge tube, and the pellet was resuspended in 5 ml of DMEM supplemented with 10% fetal calf serum and 40 μg/ml gentamycin sulfate. After centrifugation at 350 × g, the microvessels were further digested with collagenase-dispase (1 mg/ml, Roche Applied Sciences, Basel, Switzerland) and DNase (6.7 μg/ml) in DMEM for 1 h at 37°C. Microvessel

**Table 1 | Source of primary antibodies and the amount used**

| Antibody         | Source          | Catalogue Number | Amount Used |
|------------------|-----------------|------------------|-------------|
| Anti-Sema3A      | Santa Cruz      | sc-1148          | 2 μg (blocking) |
| Anti-Neuropilin1 | Calbiochem      | PC343            | 2 μg (blocking) |
| Anti-Neuropilin2 | Zymed           | 361500           | 2 μg (blocking) |
| Anti-Flexin A1   | Santa Cruz      | sc-25639         | 2 μg (blocking) |
| Anti-VEGFR1      | R&D Systems     | AF471            | 2 μg (blocking) |
| Anti-VEGFR2      | R&D Systems     | AF442            | 2 μg (blocking) |
| Anti-hemoglobin subunit alpha | abcam | Ab92499         | 2 μg (blocking)  |
| Anti-CRMP3       | gift            |                  |             |
endothelial cell clusters were separated on a 33% percolate Percoll (Pharmacia, Uppsala, Sweden) gradient, collected and washed twice in DMEM before plating on 35 mm plastic dishes coated with collagen type IV and fibronectin (both 0.1 mg/ml). RBEc cultures were maintained in DMEM/F12 supplemented with 10% plasma derived serum (PDS, Animal Technologies Inc., MD, USA), basic fibroblast growth factor (bFGF, Roche, Applied Sciences, Basel, Switzerland, 1.5 ng/ml), heparin (100 U/ml), sodium selenite (5 ng/ml), insulin-transferrin-sodium selenite supplement, gentamyces (50 μg/ml) and pyruvnic (4 μg/ml) (RBEc medium I) at 37°C with a humidified atmosphere of 5% CO2/95% air, for 2 days. On the third day, the cells received a new medium which contained all the components of RBEc medium I except pyruvnic (RBEc medium II). When the cultures reached 80% confluency (4th day in vitro), the purified endothelial cells were passaged by a brief treatment with trypsin (0.05%, w/v)-EDTA (0.02%, w/v) solution, and used in vitro BBB models.

Phallolidin staining. RBEc were cultured in a 24 well plate on glass coverslips. Cells grew vigorously and did not appear to enter quiesence and de-activation even at relatively low density. Cells were subjected to the following treatment: 1) Sema3A treatment: 0.2, 1, 2, 5 or 10 μg recombinant Sema3A was added to 200 μl medium. Cells were treated for 1 h; 2) Denatured Sema3A treatment: Sema3A (2 μg) was denatured in the boiling water for 10 min. After it was cooled to room temperature, denatured Sema3A was added into cells for 1 h; 3) Antibody pre-treatment: specific antibodies to Neuropilin 1, Neuropilin 2, PlexinA1, VEGFR1 or VEGFR2 (2 μg) were added to 200 μl cell culture medium to treat cells for 1 h, followed by Sema3A (2 μg) treatment for another 1 h. After these treatments, the culture medium was removed and the cells were fixed in freshly prepared 4% paraformaldehyde (in 1 X PBS) for 20 min. Cells were washed in PBS twice, 5 min each, incubated with blocking solution (1.5% BSA in PBS, containing 0.2% Triton X-100 and 0.02% NaN3) for 30 min, and stained with rhodamine labelled phallolidin (1: 100, in blocking solution) for 1 h. After washing with PBS (3 min each for 3 times), deionized water (once), cells were mounted in Dako mounting medium (spiked with Hoechst 33258) and dried for microscopic analysis.

Western blotting. Western blotting was performed with the indicated amount of protein as previously described[44,45,46]. Proteins were electrophoresed in an 8% sodium dodecyl sulfate mini gel and then electroblotted onto a nitrocellulose membrane in transfer buffer. Primary antibody to a specific dilution was used at 4°C overnight or at room temperature for 1 h. After washing with TBS (10 mM Tris.HCL, pH 7.8, 150 mM NaCl and 0.1% Tween 20), a horseradish peroxidase-conjugated secondary antibody to a dilution of 1: 5000 dilution was applied to the membrane at room temperature for 1 h. After washing with TBS (10 mM Tris-HCl, pH 7.8, and 150 mM NaCl), a horseradish peroxidase-conjugated secondary antibody at a dilution of 1: 50000 was applied to the membrane for 1 h at room temperature. Enhanced chemiluminescence detection of the target protein was performed using Western blotting detection reagents and X-ray film.

Immunoprecipitation (IP) and Western blotting. IP was performed using the Protein A beads method (Dynal Inc., Lake Success, NY) as exactly described by the manufacturer[47]. Briefly, 200 μg of total protein was incubated for 2 h at 4°C with an antibody to target protein followed by incubation with protein A beads. After washing away unbound proteins, the immunoprecipitated protein complex was subjected to Western blotting for the proteins of interests. The procedures for Western blotting were exactly described previously[48]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control. The intensities of the bands were quantified using NIH Image J software.

Matrix metalloproteinase (MMP) activity loaded to the apical chamber (CTop) to the bottom chamber (Cbottom) and permeability was calculated as a percentage ratio (R) of the amount of radioactivity loaded to the apical chamber (Ctop) to the bottom chamber (Cbottom) and expressed as R = (Cbottom/Ctop) × 100.

MMP2/9 gelatin Zymography. Brain tissue was freshly collected, homogenized in a buffer solution containing 50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid (pH 7.4), and protease inhibitors. Protein concentrations were analyzed with a Bio–Rad system (Heracles, CA). Equal amounts of protein (20 μg/ lane) for each sample were mixed with 2X sample buffer and loaded on a 10% polyacrylamide gel incorporated with 0.1% gelatin for electrophoresis. MMP2 and MMP9 zymographic standards were used as positive controls (Chemicon). After electrophoresis, gels were washed in 2.5% Triton X-100 for 1 h, incubated for 18 h at 37°C in collagenase buffer, and stained for 1 h with 0.1% Coomassie brilliant blue dye. Gelatinolytic activity was visualized as a transparent band against a blue background and the band intensity was measured using Image J to quantify MMP2 and MMP9 activities.

Data analysis. Statistical analysis was performed using an unpaired Student’s t-test with a 2-tailed p value, or using an ANOVA with Tukey’s post hoc test for multiple comparisons using GraphPad Prism 5 from the GraphPad Software, Inc. (La Jolla, CA). In cases as specified, a non-parametric Mann–Whitney U test was performed. Differences were considered significant when the p value was less than 0.05, with * indicating p < 0.05 and ** indicating p < 0.01. All experiments were independently repeated at least three time. All values were expressed as mean ± S.E.M.

Results

Sema3A induces peripheral vasculature permeability. To determine if Sema3A increases blood vessel permeability, a Mile’s assay was performed on the skin of anesthetized rats. Sema3A was injected intradermally into the shaved rat abdominal skin, or the rat ear skin which is semi-translucent and excellent for visualization of microcirculation. PBS or VEGF were used as controls. As shown in Fig. 1A (A, B, C), both VEGF and Sema3A caused increased extravasation of the Evans blue dye into the tissue compared to PBS-injected tissue, indicating an increased vascular permeability. Importantly, Sema3A induced vascular permeability were dose- and time-dependent (Fig. 1D–G). Quantitative analysis of Evans blue dye retained in the bleeding area demonstrated significantly increased vascular leakage of the dye after injection with Sema3A (Fig. 1C, E, G; *p < 0.01 compared to the PBS-treated group). Together, these data showed that Sema3A elevated permeability of peripheral vasculature.
Sema3A elevates cerebrovascular permeability. We examined whether Sema3A plays a role in affecting the permeability of the cerebrovasculature, as its anatomical structure is more complex than that of the peripheral vasculature system. To this end, Sema3A was sterotactically injected into mouse cerebral cortex with a Bragma coordination of 0.9 mm lateral, 0.1 mm posterior, and 2.0 mm ventral using a Hamilton microsyringe at 1 μl/min at a dosage of 200 ng/2 μl. After thorough perfusion with saline to remove the Evans blue dye, the tissue containing residual Evans blue dye was removed surgically. Evans blue was extracted as described in the Methods section. The OD reading against tissue weight was calculated and shown in graphs C, E and G. At least three independent repeats were performed and ** indicates statistical significance with p < 0.01 by one-way ANOVA followed by Tukey’s post hoc analysis to identify significant groups.

To further demonstrate that Sema3A elevates vascular permeability, Sema3A was over-expressed in Sema3A+/+ and Sema3A−/− littermate mice brain using replication defective adenovirus expressing an eGFP-tagged Sema3A (Ad-Sema3A-eGFP). Ad-Sema3A-eGFP was injected into the lateral tail vein. After 3 d of infection, mouse brain was subjected to Western blot. As shown in Fig. 2(C and D), significant increase in Sema3A protein level occurred in Ad-Sema3A-eGFP infected Sema3A+/+ and Sema3A−/− mice brain.
Figure 2 | Sema3A elevates cerebrovascular permeability. Sema3A+/+ mice were injected with Evans blue through the lateral tail vein, followed by a stereotactical injection with PBS, VEGF, or Sema3A with the amount and time as indicated in panel A. Sema3A+- mice were also treated similarly to show increased vascular permeability when endogeneous Sema3A was absent. After thorough transcardial perfusion with saline to remove Evans blue dye, the tissue containing residual Evans blue dye in the brain was surgically cut out and extracted as described in the Methods section. Quantification of permeability (OD of Evans blue/tissue weight) was shown in panel B. Statistics were performed as indicated in the Methods section with ** indicating p < 0.01 against the PBS-injected brains. Panels C-E: Replication defective adenovirus expressing Sema3A (Ad-Sema3A-eGFP) was injected in into the brain through the lateral tail vein. After 3 days of infection, mice were injected with Evans blue and the brain residual Evans blue was quantified after a thorough transcardial perfusion with saline. Ad-eGFP-injected brain and no virus injected mouse brain were used as controls. Western blotting was preformed to show increased Sema3A expression in the brain (C). The cropped blots are shown in the figure and the full-length blots are presented in supplementary figure S1. Quantification of Sema3A expression was performed using Image J (D). Brain section was stained with DAPI to visualize brain cells (blue color in E) and EGFP positive blood vessels (green color in E). Anti-CD31 staining (Rhodamine, red color) was performed on Ad-Sema3A-eGFP injected brain to indicate endothelial expression of Sema3A (yellow color in E lower right hand panel). Cerebrovascular permeability was determined by injecting Evans blue through the lateral tail vein. After transcardial perfusion with saline, brain residual Evans blue was quantified as shown in F. ** indicates p < 0.01 compared to the no virus treatment group. Scale bars = 80 μm.
Figure 3 | Sema3A induces permeability of endothelial cells in vitro. Cultured RBECs were grown on a transwell to a confluent monolayer. After treating the transwell with Sema3A (as indicated in A), or pre-treating cells with antibodies or inhibitors against known Sema3A receptors as indicated in B and C, freshly prepared mouse blood labeled with fluorescent Gr1 antibody (to label PMNs) was applied onto the transwell to perform permeability assay. 

** indicating $p < 0.01$ by one-way ANOVA with Tukey’s post hoc analysis compared with Sema3A treatment group. Panels D – F show morphological changes of RBEC cells due to Sema3A treatment. RBECs were fixed after the indicated treatment and stained with phalloidin conjugated with rhodamine (red color) and counter stained with DAPI (blue color). F-actin stress fibres are very clear in untreated cells (D). Sema3A treatment caused disruption of F-actin inside the cells. Densely packed bundles of cortical actin filaments started to appear along cell membranes (arrows) over the course of treatment with Sema3A. Antibodies and inhibitors against receptors of Sema3A were pre-incubated with the cells for 15 min before the addition of Sema3A. Antibodies to VEGFR1 and NRP2 (panel E) and the inhibitor Zm 306416 (selective to VEGFR1, panel F) were effective in ameliorating the effect of Sema3A. Scale bar = 20 μm.
Figure 4 | VEGFR1 directly interacts with Mical2 in response to Sema3A.

(A) Cultured RBEC cells were grown on glass cover slips and treated with Sema3A at 10, 50, 100 and 150 nM and the intracellular calcium concentrations were measured. KCl was added to cells which caused a drastic increase in intracellular calcium due to depolarization of cellular membrane. (B) Cultured RBECs were treated with Sema3A and followed by protein extraction as described in the Methods section. Protein isolations were immunoprecipitated with antibodies to VEGFR1 or VEGFR2 (B, C) and followed by Western blotting against Mical1 (B; the right side lane is recombinant Mical1 protein from Abnova, serving as a positive control) or Mical2 (C). Total Mical2 proteins were probed as loading controls. Western blot against IgG of the same gel was also performed to show equal protein loading as an internal control. The cropped blots are shown in the figure and the full-length blots are presented in supplementary figure S4. Quantification of the VEGFR1 band against IgG is shown in F. Increased VEGFR1, but not VEGFR2, associated with Mical2 occurred in response to Sema3A treatment (average of three experiments as shown in F). ** indicates statistical significant with p < 0.01 by Student’s t-test.

These experiments demonstrated that Sema3A indeed plays a role in elevating the permeability of cerebrovasculature.

Sema3A elevates permeability in an in vitro model of blood brain barrier (BBB). To determine the mechanisms underlying Sema3A-induced vascular permeability, we used RBEC cultured on transwell to determine the permeability of fluorescent-labeled Gr1 positive polymorphonuclear neutrophils (PMNs). This assay is a powerful tool to investigate mechanisms of BBB permeability51. Indeed, as shown in Fig. 3A, Sema3A caused a dose- and time-dependent increase in transmigration of the PMNs across the RBEC monolayer of cells, demonstrating elevated permeability.

Radiolabeled [14C]sucrose was also performed on RBEC serving as an additional control of barrier tightness besides the measurement of PMN transmigration. Since [14C]sucrose is not subjected to transporters or taken up by endothelial cells, it can only permeate to the corresponding chamber in the transwell system via the paracellular pathway. Indeed, as shown in Fig. 3A’, exposing RBEC cells to various concentrations of Sema3A increased the permeability of radiolabeled [14C]sucrose in a similar pattern and to a similar degree compared to those of PMN transmigration assay (Fig 3A). This experiment thereby confirmed the validity of PMN transmigration assay to monitor RPEC permeability, at least in the current experimental condition. Subsequently, all permeability experiments were performed using the PMN assay.

Sema3A interacts with VEGFRs to mediate vasculogenesis and vascular patterning. To understand whether Sema3A-evoked permeability also involves in its cognate receptors, several VEGFR-specific inhibitors were used before Sema3A treatment in the PMN paracellular transmigration assay. Sema3A-induced paracellular permeability of RBEC was blocked by ZM 306416, an inhibitor for VEGFR1 with a known IC50 of 0.33 μM (Fig. 3B; p < 0.01 compared to Sema3A treatment group). In contrast, Ki8751, a potent and selective inhibitor of VEGFR2 with a known IC50 of 0.9 nM, was not effective at 10 nM and 50 nM; suggesting that Sema3A elevates paracellular permeability through VEGFR1. In addition, cantharidin, a potent and selective PP2A inhibitor known to affect Sema3A-mediated vasculogenesis, and other known Sema3A intracellular downstream kinase inhibitors such as okadaic acid, LY294002 and Y-27632, were also not effective (not shown), serving as negative controls to demonstrate that Sema3A selectively interacts with VEGFR1 to mediate RBEC paracellular permeability. Mannitol caused shrinkage of the RBEC and maximum paracellular permeability serving as a positive control for maximum barrier leakiness.

To further confirm the involvement of specific receptors for Sema3A-induced permeability, a battery of receptor specific antibodies were used. Preliminary experiments were performed using Western blot to demonstrate the specificities of these antibodies recognizing their respective receptors (not shown). Moreover, these antibodies alone neither altered the cellular morphology seen under phallloidin staining, nor changed RBEC permeability to PMNs (not shown). As shown in Fig. 3C, antibodies blocking VEGFR1 and NRP2 were effective in ameliorating Sema3A-induced permeability to PMNs; while Sema3A pre-mixed with the antibody against Sema3A or heat-denatured Sema3A did not cause increased per-
meability to PMNs, serving as negative controls (Fig. 3C; p < 0.01 compared to Sema3A treatment group). Together, these data confirmed VEGFR1/NRP2 as receptors for Sema3A during Sema3A-induced permeability.

We then examined morphological changes of RBEC in response to Sema3A treatment using phalloidin staining of F-actin. Sema3A treated cells exhibited a marked alteration in cell shape, an apparent loss of polarity and a distorted actin cytoskeleton over the course of treatment (30–270 min at 100 nM of Sema3A) (Fig. 3D). Intracellular cytoplasmic actin stress fibers (F-actin) began to form densely packed bundles of cortical actin filaments in the membrane ruffles, first in the shape of sawtooth-like bodies along the cell membrane (arrows in Fig. 3D), then as a thick layer of densely packed bundles of cortical actin filamentsin the membrane ruffles (Fig. 3D). This change in actin cytoskeleton and cellular morphology could lead to the disruption of intracellular junctions and increased paracellular permeability as reported by others52.

To determine the specificity of Sema3A-induced actin changes, RBECs were treated either with heat-denatured Sema3A, or a Sema3A pre-mixed with antibody to Sema3A. Both failed to produce a dramatic actin response to Sema3A (Fig. 3F), confirming the specificity of cellular response to Sema3A. RBECs were also pre-treated with specific antibodies to VEGFR1, VEGFR2, Plexin A1, NRP1, NRP2, or VEGFR inhibitors including Zm306416, Ki8751 and okadaic acid to further delineate receptors responsible for Sema3A-induced actin filament changes (Fig. 3E and F). As shown in Fig. 3E and F, blocking RBEC with antibodies to VEGFR1, NRP2 and Ki8751 ameliorated the appearance of Sema3A-induced densely packed bundles of cortical actin filaments close to the membrane. While antibodies to VEGFR2, Plexin A1, NRP1, Ki8751 and okadaic acid did not prevent the appearance of Sema3A-induced actin changes.

Together, these studies showed that Sema3A induces disruption of intracellular actin filaments and causes shrinkage of endothelial cells, through VEGFR1/NRP2 receptors.

**VEGFR1 directly interacts with Mical2 to mediate Sema3A response.** To further explore how Sema3A induces F-actin distortion, we designed and performed the following two experiments. First, ratiometric calcium concentration was measured to determine whether Sema3A directly increases intracellular calcium, which is known to modulate actin filament changes53,54. As shown in Fig. 4A, Sema3A at 10, 50, 100, and 150 nM concentrations failed to elicit any measurable changes in intracellular calcium concentration of RBEC. KCl was used as a positive control, which depolarized the cellular membrane and produced a non-specific influx of intracellular calcium (Fig. 4A). This experiment excluded the possibility that Sema3A causes F-actin disorganization through modulating intracellular calcium levels.

Secondly, experiments were performed to determine what intracellular proteins that VEGFR1 directly interacts with to mediate Sema3A response. Immunoprecipitation with an antibody to VEGFR1 was performed on Sema3A-treated RBECs followed by mass spectrometry analysis. We identified Mical2 protein as a potential intracellular target of VEGFR1 (Mascot Score = 120; peptide count = 17; cover percentage = 30.86%). Indeed, immunoprecipitation-Western blotting was performed on VEGFR1 pull-down product, which confirmed the presence of Mical2 (Fig. 4C). To validate the finding that VEGFR1 directly interacts with Mical2 in response to Sema3A treatment, Sema3A-treated RBECs were immunoprecipitated with an antibody to VEGFR1 or VEGFR2, followed by Western blotting against Mical1 (Fig. 4B) or Mical 2 (Fig. 4C). After quantification of the relative intensities of the Western blot...
Mical2 is required for Sema3A-induced actin disorganization and permeability. siRNAs to Mical1 and Mical2 were transfected into RBECs (A). Western blot was performed to determine the reduction of Mical proteins over the course of transfection (A and B). Immunostaining was performed to detect the presence of Mical proteins (C and D, green color). After 60 h or 72 h of transfection with siRNA, cells were treated with or without Sema3A for determination of formation of cortical actin filaments (arrows in D). siRNA to Mical2 inhibited Sema3A-induced Mical2 expression and prevented the accumulation of densely packed bundles of cortical actin filaments close to the membrane in Sema3A-treated cells (Fig. 5C, C', D and D'). Mical1 did not appear to increase in cortical actin filaments (Fig. 5A', A'', B and B').

Secondly, down-regulation of Mical2 prevents F-actin disorganization in response to Sema3A treatment. Specific siRNAs to Mical1 and Mical2 (two each) were transfected into RBECs. A non-specific siRNA was also used as a negative control. The siRNA1 and siRNA2 to Mical2 selectively knocked down Mical2 protein expression after 60 h of transfection in response to Sema3A treatment (Fig. 6A and B). Double immunostaining of Mical1 or Mical2 with phalloidin (F-actin) showed that the appearance of cortical actin filaments occurred in the negative siRNA and Mical1 siRNA transfected cells in response to Sema3A, but not in cells transfected with Mical2 siRNA (Fig. 6C and D), which strongly supports the hypothesis that Mical2 mediates actin filament disorganization in response to Sema3A.

Thirdly, transwell assay was performed to determine if knockdown of Mical2 expression was indeed able to reduce Sema3A-induced endothelial permeability to Gr1-labeled PMNs. As shown in Fig. 6E, both siRNAs knocking down Mical2, but not Mical1, were effective in preventing Sema3A-induced permeability, presumably by reducing changes in cellular morphology due to the inhibition of actin filament disorganization. Together, these three experiments demonstrated that Mical2 is responsible for Sema3A-induced F-actin disorganization and permeability.

**Knockout Sema3A protects the brain from ischemia-induced damage.** We previously showed that Sema3A expression was elevated 6 h after transient occlusion of the middle cerebral artery (tMCAO). The high level of Sema3A expression persisted during long term reperfusion\(^\text{6,8,9}\). If Sema3A plays a role in augmenting vascular permeability during tMCAO, knockout Sema3A expression could reduce vascular permeability and provide brain protection. To test these hypotheses, two types of cerebral ischemia, i.e. tMCAO and photothermic occlusion/hemorrhagic transformation, were induced on Sema3A+/+ and Sema3A−/− littermate mice.
As Sema3A is a known inhibitor of angiogenesis and vascular patterning during development, we first compared the density of cortical cerebral microvessels of adult Sema3A+/+ and -/- littermate mice (19–21 g body weight and at least 2 weeks after weaning). Lectin staining was performed on brain sections (not shown). Although Sema3A-/- mice appeared to have a slightly higher density of cerebral microvessels, the vascular density was not significantly different from that seen in Sema3A+/+ mice brain (not shown).

Indeed, a measurement of the basal level of the middle cerebral artery blood flow for both Sema3A+/+ and -/- mice showed no significant difference (Fig. 7A, p = 0.23). Occlusion of the middle cerebral artery of both Sema3A+/+ and -/- mice produced a similar level reduction in blood flow (90%) (Fig. 7A). The blood flow was restored to almost 100% after withdrawal of the occluding thread in both Sema3A+/+ and -/- littermate mice. Interestingly, Sema3A-/- mice produced a statistically significant smaller infarct after tMCAO (Fig. 7B and C; p < 0.01), and a better neurological deficit score (Fig. 7D; p < 0.05), and forepaw pulling strength test (Fig. 7E; p < 0.05) compared with Sema3A+/+ littermate mice, suggesting that Sema3A plays a detrimental role during cerebral ischemia. To determine if Sema3A-/- mice had reduced vascular permeability, Evans blue was injected through the tail vein after tMCAO. After a thorough transcardial perfusion with saline, Sema3A-/- ischemic mouse brain had a significantly reduced level of the Evans blue compared with the Sema3A+/+ littermate mice (Fig. 7F; p < 0.01), confirming reduced vascular permeability.

To further demonstrate the role of Sema3A in vivo, another mouse stroke model, i.e. photothrombotic occlusion/hemorrhagic transformation, was used to show reduced brain injury due to the lack of Sema3A expression. As shown in Fig. 8A, this model produces vascular occlusions mostly in the cortical layer. With experimentally pre-determined illuminating light strength and duration, a well-defined and quantifiable damage volume was achieved as determined by TTC staining (Fig. 8B). On brain sections subjected to hematoxylin and eosin (H&E) staining, ischemic occlusion was followed by hemorrhagic transformation (Fig. 8C, arrowheads indicating occluded vessels, arrows indicating spilled blood cells in the brain due to hemorrhage). Sema3A-/- mice showed a significantly smaller infarct volume compared with the Sema3A+/+ mice (Fig. 8A and B; p < 0.01). Importantly, Sema3A-/- mouse brain had a significantly reduced level of hemorrhagic transformation (Fig. 8C and D; p < 0.01).

In order to confirm that Sema3A-/- mouse brain has reduced brain damage and vascular permeability during photothrombotic occlusion/hemorrhagic transformation injury, the following three experiments were performed; (1) We have previously showed that the level of CRMP3 proteolytic cleaved product p54 is associated with the degree of brain damage. Therefore, the level of p54 was determined using Western blot. The level of p54 was higher in Sema3A+/+ ischemic side of the brain compared with the Sema3A-/- brain (Fig. 8E and F), suggesting stronger brain damage in Sema3A+/+ brain. The level of residual hemoglobin (after thorough brain per-
Figure 8 | Sema3A-/- mice have reduced vascular permeability and brain damage in photothrombotic occlusion/hemorrhagic transformation model.

Sema3A +/+, -/- littermate mice were subjected to the same surgical procedure to produce ischemic occlusion/hemorrhagic transformation in the brain as described in the Methods section. As shown in A (top panels), blood left in the brain (red color) was visible even after perfusion (scale bar = 10 mm). Thick coronal sections (2 mm) were stained with TTC to show brain infarction (white area in the lower panels of A, scale bar = 15 mm). This infarction in Sema3A +/+ mice were significantly larger than that of Sema3A-/- mice (B, ** indicates p < 0.01). Brain sections were stained with H&E to show elevated presence of blood cells in the brain parenchyma (arrows in C) indicating increased hemorrhagic transformation in this model. Arrowheads in C show occluded, but non-hemorrhagic vessels. The number of broken blood vessels were counted and plotted in D to indicate significantly reduced vascular damage in Sema3A-/- mouse brain. (E) Brain tissue was subjected to protein isolation and Western blot to detect proteolytic cleaved CRMP3 (54 kD band, p54), and the residual hemoglobin (Hgb-α) in the brain. GAPDH was used as an internal control. I = ipsilateral side of the brain, while C = contralateral side of the brain. The cropped blots are shown in the figure and the full-length blots are presented in supplementary figure S6. (F) Image J was used to quantify Western blot band intensities from panel E. ** indicates p < 0.01. (G) Gelatin zymography was performed on brain protein samples to detect pro-MMP2/9 (98 and 70 kD, respectively) and activated MMP2/9 (80 and 60 kD, respectively). (H) The band intensity was measured using Image J. ** indicates p < 0.01. (I) Evans blue was injected into mouse brain 15 min before sacrifice. After thorough transcardial perfusion with saline, mouse brain was taken out and residual Evans blue was quantified using methods described in the Methods section. Sema3A -/- mice showed significantly reduced level of Evans blue in the ischemic brain (** indicates p < 0.01; n = 3).
fusion with saline), indicating hemorrhagic transformation, were also measured using Western blotting (Fig. 8E and F). Sema3A-/mouse brain showed reduced presence of residual hemoglobin compared with a Sema3A+/+ brain (Fig. 8E and F; p < 0.01), demonstrating reduced brain damage in the Sema3A/-/- mouse. (2) Gelatin zymography was performed to show reduced activation of MMP2 and MMP9, both are important markers for vascular damage and permeability. As shown in Fig. 8G and H, the levels of activated MMP2 and 9 increased significantly in the ischemic side of the brain of Sema3A+/+ mouse (p < 0.05), but not in the Sema3A/-/- mouse brain, lending further support to the notion that Sema3A modulates vascular permeability. (3) Ischemic mice were injected with Evans blue after 7 d of ischemic surgery. After a thorough transcardial perfusion, residual Evans blue in the brain was quantified as shown in Fig. 8I. Sema3A/-/- mice had a significantly lower level of residual Evans blue in the brain (p < 0.01), which again demonstrated that Sema3A plays a role in augmenting vascular permeability in the ischemic brain.

Collectively, these studies provided in vitro and in vivo evidence to show that Sema3A indeed modulates vascular permeability to affect brain damage after cerebral ischemia. Ischemic brain tissue secretes Sema3A which binds to the VEGFRR1/NRP2 complex to trigger the activation of Mical2. Mical2 co-localizes with actin filaments and disorganizes F-actin bundles into densely packed bundles of cortical actin filaments close to the membrane leading to the alteration of endothelial cell morphology. The cellular integrity of endothelial cells is critical in guarding the vascular permeability.

Discussion

In the present study, we provided evidence to demonstrate that Sema3A is a vascular permeability factor and contributes to ischemic brain damage. By interacting with the VEGFRR1/NRP2 receptor complex, Sema3A triggers the activation of Mical2 to cause disorganization of F-actin, alteration of cellular morphology, and elevation of the endothelial permeability. It is especially important that the Sema3A/-/- mouse has reduced vascular permeability in response to two kinds of stroke, in vitro, all resulting in a better protection of the brain tissue and function in comparison with the Sema3A+/+ littermate mouse. Collectively, these data strongly support the notion that Sema3A induces stroke brain damage through elevation of vascular permeability.

We employed Mile’s assay to show that exogenous Sema3A elevated the permeability of both peripheral and cerebral vasculatures. Importantly, exogenous Sema3A injected into mice lacking of endogenous Sema3A (Sema3A/-/-) also evoked elevated permeability of cerebrovasculature (Fig. 2A), confirming the specificity of Sema3A’s action. Comparing Sema3A’s effect in vascular permeability with that caused by VEGF1.165 using the Mile’s assay, it became apparent that Sema3A is as potent as VEGF in the degree of inducing vascular permeability. This is perhaps not surprising in light of the fact that Sema3A is as potent as VEGF in the degree of inducing vascular permeability. Comparing Sema3A’s effect in vascular permeability with that caused by VEGF1-165 using the Mile’s assay, it became apparent that Sema3A is at least as potent as VEGF in the degree of inducing vascular permeability. This is perhaps not surprising in light of the fact that Sema3A is as potent as VEGF in the degree of inducing vascular permeability.

In vitro evidence to demonstrate that Sema3A is a vascular permeability factor and contributes to ischemic brain damage. By interacting with the VEGFRR1/NRP2 receptor complex, Sema3A triggers the activation of Mical2 to cause disorganization of F-actin, alteration of cellular morphology, and elevation of the endothelial permeability. It is especially important that the Sema3A/-/- mouse has reduced vascular permeability in response to two kinds of stroke, in vivo, all resulting in a better protection of the brain tissue and function in comparison with the Sema3A+/+ littermate mouse. Collectively, these data strongly support the notion that Sema3A induces stroke brain damage through elevation of vascular permeability.

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The molecular mechanism for Sema3A-mediated vascular permeability is through activation of Mical2 protein, which in turn causes F-actin disorganization. This process appears to be selective, since Mical1 expression did not become elevated in response to Sema3A treatment. Down-regulation of Mical1 using siRNA also did not show any effect on endothelial cell F-actin disorganization and permeability. Mical2 protein, on the other hand, is required in shaping endothelial cell F-actin organization, confirming a previous study in fruit fly, in that Mical2 links Semaphorin to F-actin disassembly. However, our finding that Mical2 protein-mediated actin changes affects human endothelial permeability is novel. F-actin plays such an important role in maintaining tight and adherence junction proteins in place. Ample studies have clearly shown that the disruption of cytoskeleton, including actin filaments, will increase BBB permeability. Therefore, it is reasonable to conclude that Sema3A enhances endothelial permeability as a result of Mical2-mediated disorganization of F-actin and the loss of endothelial cell shape and polarity.

Our in vitro data predicts that Sema3A secreted in the ischemic brain exacerbates vascular permeability. Indeed, mice lacking the expression of functional Sema3A (Sema3A-/-) showed a significant reduction in vascular permeability and brain damage in two models of cerebral ischemia. Sema3A is the only member of the semaphorin family as a secreted polypeptide. Based on our previous published studies, Sema3A expression in ischemic brain during reperfusion is relatively evenly diffused in the ischemic core and its adjacent area, which may account for the striking reduction in vascular permeability of the ischemic hemisphere shown in Fig. 7 and 8. It is especially interesting to see Sema3A-/- mice also having reduced hemorrhagic transformation in the brain, which further supports an important role of Sema3A as a vascular permeability factor. Future studies are required to determine how exactly Sema3A causes hemorrhagic transformation.

Nevertheless, brain protection seen in Sema3A/-/- mice can also be attributed to direct neuroprotection. Our previous studies showed that Sema3A can directly interact with NRP1 receptors, but not NRP2 receptors, expressed on neurons to cause neuronal death both in vitro and in vivo in ischemic brain. Perhaps, Sema3A interacts with different receptor complexes to discretely impact neurons and the vasculature. Indeed, studies showed that the cellular target of Sema3A is very selective, as it only inhibits the outgrowth of a specific set of neurons such as spinal motor neurons and neurons in the embryonic dorsal root ganglion and sympathetic ganglia, lending further support to the notion that Sema3A’s effect on vascular permeability is separate from its effect on neuronal death possibly through interacting with different receptor complexes. In fact, Sema3A released from ischemic neuron has been reported to inhibit vascular regeneration. Moreover, Sema3A inhibition alleviates ischemia-reperfusion-induced acute kidney injury. Therefore, Sema3A plays a very important role in a diverse disease conditions through affecting vascular homeostasis.

Collectively, these studies revealed a previously unknown function of Sema3A in mediating vascular permeability in ischemic brain. The possible mechanism is through its selective interaction with VEGFRR1/NRP2 receptor complex to cause F-actin disorganization and alteration of endothelial cell shape, polarity and permeability.
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Author contributions
S.T.H. conceived and designed the experiments, analyzed the data and wrote the manuscript. L.N. performed Evans blue dye brain injection and assay, L.N. performed brain tissue sectioning and immunostaining; X.S.L. performed the Mile’s assay and quantification; brain surgery, RBEC treatment, acquired images and analyzed some of the data; J.S. performed tMCAO stroke surgery and S.X.J. performed neurological deficit analysis; A.A. performed IP and siRNA studies; S.G. performed PMN transmigration assay using RBECs. S.X.J. and R.M. performed calcium imaging and photothrombotic model work.

Additional information
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