Supplementary Materials and Methods

Protein expression and purification

Recombinant human ADAMTS13 with a C-terminal Myc/His6 tag in pCDNA3.1\textsuperscript{1} was used to generate ADAMTS13 variants with point mutations in the linker 3 region by site directed mutagenesis. Expression of ADAMTS13 was performed in CHO-K1 stable cell lines and protein was purified by FPLC using zinc-coupled HiTrap chelating columns (GE Healthcare) followed by passage over a hydroxyapatite column, to remove contaminating proteins. Purified ADAMTS13 was quantified by in house ELISA, as described previously\textsuperscript{1,2}. The α-MDTCS, α-CUB1/CUB2 and α-TSP(2-4) antibodies used in this ELISA were purified, using sepharose-immobilised recombinant domain fragments, from the total IgG isolated from rabbits immunised with full length wild type ADAMTS13. Once quantified, ADAMTS13 was concentrated to 1.5 mg/ml using 100 kDa MWCO spin columns and dialysed into 150 mM NaCl, 20 mM histidine, 2% sucrose, 0.05% Tween 80, pH 7.4. The dimeric VWF D4-CK domain fragment (VWF residues 1874-2813) in the vector pcDNA\textsuperscript{3.1/His} was transiently expressed in HEK293S, purified by FPLC and quantified by ELISA as previously described\textsuperscript{3}.

Mass spectrometry of ADAMTS13 preparation purity

Gel tops containing the entire ADAMTS13 preparations were excised and dehydrated using acetonitrile followed by vacuum centrifugation. Dried gel pieces were reduced with 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide. Gel pieces were then washed alternately with 25 mM ammonium bicarbonate followed by acetonitrile. This was repeated, and the gel pieces dried by vacuum centrifugation. Samples were digested with trypsin overnight at 37 °C. Digested samples were analysed by LC-MS/MS using an UltiMate\textsuperscript{®} 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA) coupled to an Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. Peptide mixtures were separated using a gradient from 92% A (0.1% FA in water) and 8% B (0.1% FA in acetonitrile) to 33% B, in 20 min at 300 nL min\textsuperscript{-1}, using a 75 mm x 250 μm i.d. 1.7 mM BEH C18, analytical column (Waters). Peptides were selected for fragmentation automatically by data dependant analysis. Data produced were searched using Mascot
(Matrix Science UK), against the Swissprot and Trembl database with taxonomy of 
mammalia selected. Data were validated using Scaffold (Proteome Software, Portland, OR).

**Immunoprecipitation assay of ADAMTS13 conformation**

The Thrombotic Thrombocytopenic Purpura (TTP) patient derived II-1 mAb\(^4\) was covalently coupled to M280 tosylactivated Dynabeads (Invitrogen) following manufacturer’s instructions. The coupled beads were added to 40 nM ADAMTS13 (WT or A1144V variant) and pre-incubated for 1 hour at 37 °C in the absence/presence of 100 nM VWF D4-CK domain fragment. After 4 hours incubation, the beads were washed and bound protein eluted in LDS loading buffer at 70 °C for 10 min. Inputs (pre-IP), flow through (post-IP) and eluates were analysed by SDS PAGE and anti-Myc-HRP western blot.

**ADAMTS13 activity assay**

The FRETS-VWF73 assays of ADAMTS13 activity were performed as described previously\(^5,6\) and using enzyme/substrate concentrations suggested by the manufacturer (Peptanova, Germany). Purified VWF D4-CK (100 nM) was added before a 45 min pre-incubation at 37 °C. Linear regression of data, collected over the first 30 min of the assay, was used to determine activity (slope values) which were normalised to that of WT ADAMTS13 and presented as relative activity.

**Parallel flow assays**

For parallel flow assays of platelet capture, Vena8 Fluoro+ biochips (Cellix) were coated with 200 µg/ml collagen type III (Southern Biotech) and blocked with 1% BSA, 1 mg/ml glucose in HEPES buffer. Whole donor blood was collected on PPACK (Sigma) and Enoxaparin (low molecular weight heparin from Sanofi-Aventis) and treated with 100 nM PGE1 (Sigma) and 75 mU/ml Apyrase (Sigma), to prevent platelet activation. Washed platelets were labelled with 10 µM DiOC6 (Sigma) and perfused over the collagen surface at a constant shear rate of 1500 s\(^{-1}\) (at which platelet capture is VWF dependent) for 3.5 min. Adhesion of labelled platelets was visualised by fluorescence imaging at 250 ms intervals using a 20x objective and analysed using Slidebook software (3iEurope, Germany) to determine platelet coverage (%) at 180 seconds. To determine the effect of ADAMTS13 on platelet capture the assay was also performed in the presence of wtADAMTS13 or caADAMTS13 at a range of concentrations and EC50 values were determined by dose-response curves using GraphPad Prism 9 software (GraphPad Software Inc).
Fibrinolysis assays

Turbidity assays of fibrinolysis were performed as previously described. Briefly, normal human plasma (diluted 1:2) or 1.5 mg/ml purified human fibrinogen (Sigma) in HEPES buffer containing 20 mM CaCl₂, was incubated in a clear 96 well plate and fibrin formation, initiated by the addition of 2 nM human thrombin (Sigma), was monitored by absorbance at 405 nm using a Fluorostar Omega plate reader maintained at 37°C.

To determine the effect of wtADAMTS13 and caADAMTS13 on fibrinolysis, fibrin was formed in the presence of 100 ng/ml t-PA, alone or in combination with 50 nM ADAMTS13 or 0.67 mg/ml N-acetyl cysteine. Lysis times were calculated from the two points of 50% maximal absorbance. To determine the effect of ADAMTS13 on proteolysis of cross-linked fibrin, untreated plasma or purified fibrinogen was incubated at 37°C following the addition of 2 nM thrombin to allow fibrin cross-linking. Once maximal absorbance was achieved (at 30 min for plasma-based assay and at 10 min for pure fibrin-based assay) wells were overlaid with 1 µg/ml t-PA (Sigma), alone or combined with 50 nM ADAMTS13, and the absorbance at 405 nm was recorded at 60s intervals for 180 min.

Surgical Procedures

For all surgical procedures anaesthesia was induced with 4% isoflurane (Abbott, Berkshire, UK), and was maintained at 2% in a mixture of 30% O₂ and 70% N₂O. Body temperature was maintained using a rectal probe at 37 ± 0.5 °C with a heating blanket (Harvard Apparatus, UK) throughout surgery. At termination of studies mice were trans-cardially perfused with 0.9% saline, followed by 4% paraformaldehyde under terminal isoflurane anaesthesia.

Tail bleeding assay

Animals were injected intravenously (IV) with either vehicle (150 mM NaCl, 20 mM histidine, 2% sucrose, 0.05% Tween 80, pH 7.4.), 6 mg/kg wtADAMTS13, 6 mg/kg caADAMTS13 or 400 mg/kg N-acetyl cysteine (NAC) by tail vein catheter. These were the same doses used in MCAo protocols. After 1 h they underwent a transverse amputation of the tip (2 cm) of the tail under terminal isoflurane anesthesia. The tail was immediately submerged in a 50 ml tube containing PBS, pre-warmed and maintained at 37 °C in a water bath. Bleeding was monitored visually and the time to bleeding cessation recorded. At 15 min post-amputation, animals were humanely culled.
**Transient MCAo Surgical Procedure**

Transient (t)MCAo was used to induce focal ischemia in the left cerebral hemisphere. In brief, a midline skin incision was made at the neck of C57/BL6 mice, exposing the common carotid artery (CCA). A 6/0 silicon-coated nylon filament (Doccol, USA) was advanced into the external carotid then advanced up the internal carotid artery (ICA), causing occlusion in the MCA. The filament remained in the MCA for 30 min before being withdrawn to start reperfusion.

**Laser speckle contrast imaging (LCSI) and analysis**

Mice were anaesthetised with 4% isoflurane (Abbott, Berkshire, UK) and secured in a stereotaxic frame (World Precision Instruments, USA) positioned under a moor FLPI2 Full-Field Perfusion Imager (Moor instruments, UK). Anaesthesia was maintained at 1.5% isoflurane throughout imaging. The scalp was dissected along the midline to expose the skull, using surgical clips the skin on top of the skull was secured away to clear the region for imaging. An ultrasound gel was applied to the mouse skull before a glass coverslip was mounted on top to improve imaging quality.

Mice undergoing tMCAo underwent baseline imaging prior to surgery and were imaged during occlusion and at 20 min, 4 h and 6 h post-reperfusion (Figure S1B). LCSI was conducted for 3 min with (20 ms exposure time) and a 25 frame filter. Regions of interests (ROIs) were drawn around the contralateral and ipsilateral hemispheres of the brain using MATLAB R2020a software, and automated masks were applied around major vessels within the ROI to exclude areas with high blood flow.

**Tissue processing**

Brains were removed and post-fixed in 4% paraformaldehyde for 24 h before being cryoprotected in a 20% sucrose solution at 4 °C for up to 2 days. Coronal sections (30 μm) were cut on a sledgemicrotome (Leica, Milton Keynes, UK) with freezing stage (Bright Instruments, Huntingdon, UK) then stored in antifreeze solution (30% ethylene glycol and 20% glycerol (Sigma, Gillingham, UK) in phosphate-buffered saline (PBS) at −20 °C, for histological staining.

**Lesion Volume**
Coronal brain sections (30 μm) were stained with 1% cresyl violet and cover-slipped with DPX mounting medium. Areas of cell death were identified using images collected by a 3D Histec Pannoramic 250 slide scanner. Lesion volume was then calculated by measuring the areas of cell death at 3-9 coronal levels for distal MCAo and at 4-5 coronal levels for proximal tMCAo.

**Immunofluorescence (IF) and immunohistochemistry (IHC)**

Brain sections were mounted on super-frost slides (Thermos fisher Scientific) for 24 h before antigen retrieval. Slides were submerged in Tris-EDTA pH 8.6 solution for 20 min in a water bath set to 97.5 °C. Section were then incubated with the following primary antibodies: rabbit-anti-Iba1 (1:500, EPR16588, Abcam), goat-anti-IL-1α (1:200, AF-400, RnDSystems). No Antigen retrieval were needed for rat-anti-CD41 (1:100, MWRReg30, BD Pharmingen), Sheep-anti-VWF (1:200, Abcam), rat-anti-Ly6G (1:200, RB6-8C5, Abcam), chicken-anti-GFAP (1:1000, Abcam). Primary antibody diluted in (1 % BSA, 0.3 % Triton-X-100, 0.05 % sodium azide, PBS) and incubated on sections overnight at 4 °C. Sections were further incubated with Secondary antibodies in secondary antibody buffer (0.1 % bovine serum albumin in tris-buffered saline) for 1.5 h at RT. Secondary antibodies used were:

- donkey-anti-rabbit Alexa Fluor® 647 (1:200, Abcam), goat-anti-rat Alexa Fluor® 488 (1:500, Thermos fisher Scientific), donkey-anti-sheep Alexa Fluor® 488 (1:500, Thermos fisher Scientific), donkey-anti-rat Alexa Fluor® 594 (1:500, Thermos fisher Scientific), donkey-anti-Chicken Alexa Fluor® 594 (1:500, Thermos fisher Scientific). The Tyramide SuperBoostTM (B40936, ThermoFisher) kit was used In order to amplify low signals of IL-1α epitopes, protocol was followed as per manufacturer’s instructions with the secondary antibody biotinylated horse-anti-goat IgG (1:200, BA-9500, Vector). Slides were washed with wash buffer (0.1 % tween in Tris-buffered saline) then dried overnight in RT prior to coverslip in ProLong diamond anti-fade mountant (Thermos fisher Scientific).

For IgG staining, endogenous peroxidase was quenched using 1% H₂O₂, followed by blocking step with 5% goat serum. Sections were then incubated in biotinylated goat anti-horse (1:500, Vector Laboratories) secondary antibody. To amplify the signal Vectastain ABC-HRP kit (Vector Laboratories) was used and followed by 3’3-diaminobenzidine (DAB) (Sigma-Aldrich) to allow detection of positive staining.
Microscopy

Images were collected on a 3D Histech Pannoramic 250 slide scanner (3D Histec, Hungary). For histological quantification, Images were processed and analysed using QuPath v0.1.2 and Fiji ImageJ softwares. For platelets, neutrophils and IL-1α analysis, large areas of interests were analysed by automated counting on QuPath software, while the percentage area of VWF, astrocytes and the manual cell count of microglia activation were calculated using Fiji ImageJ in 4-5 fields of view per mouse.

Measurement of coagulation factors in mouse plasma by ELISA

Plasma was prepared from whole, anticoagulated blood taken by cardiac perfusion and stored at -80°C. Plasma from all experimental animals was compared to stored plasma samples from age and sex matched naive animals. The concentration of VWF antigen in mouse plasma was determined using an in house ELISA as previously described. A modified VWF ELISA, in which the capture antibody was replaced with 5 μg/ml human collagen type III (Sigma, product code C4407), was used to determine VWF collagen binding activity (VWF:CBA) which was normalised to VWF antigen concentrations (VWF:Ag) in each sample and are presented as a ratio (VWF:CBA/VWF:Ag). The concentration of fibrinogen in mouse plasma was determined using a commercial ELISA kit following the manufacturer’s protocol (Abcam, product code AB213478).

Statistical analysis

All data were analysed with GraphPad Prism 9 (GraphPad Software Inc) using the appropriate tests. Equal variance and normality were assessed with the Shapiro–wilk test. Data are presented as mean ± standard deviation (SD). Student’s t-test was used for single comparisons and one-way or two-way analysis of variance (ANOVA) were followed by Sidak’s or Tukey’s post-hoc tests for multiple comparisons. Data with non-normal distributions was analysed with Kruskal-Wallis test followed by Dunn’s test for multiple comparisons. Statistical significance was accepted at *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
| Species          | Identified Protein                                                                 | No. of matched peptides | Wild Type | Ala1144Val |
|------------------|--------------------------------------------------------------------------------------|-------------------------|-----------|------------|
| Bos taurus       | Alpha-2-macroglobulin                                                               | 30                      | 36        |            |
| Bos taurus       | Serum albumin                                                                        | 16                      | 17        |            |
| Bos taurus       | Alpha-1-antiproteinase                                                               | 10                      | 11        |            |
| Bos taurus       | Alpha-2-HS-glycoprotein                                                              | 6                       | 6         |            |
| Bos taurus       | Serpin A3-7                                                                          | 4                       |           |            |
| Cricetulus griseus | Chondroitin sulfate proteoglycan 4                                                  | 79                      | 76        |            |
| Cricetulus griseus | Basement membrane-specific heparan sulfate proteoglycan core protein                | 16                      | 17        |            |
| Cricetulus griseus | Prolow-density lipoprotein receptor-related protein 1                               | 68                      | 65        |            |
| Cricetulus griseus | Laminin subunit alpha-5                                                              | 32                      | 27        |            |
| Cricetulus griseus | Laminin subunit beta-1                                                               | 32                      | 32        |            |
| Cricetulus griseus | EMILIN-1                                                                             | 30                      | 23        |            |
| Cricetulus griseus | Lyososomal alpha-glucosidase                                                         | 19                      | 22        |            |
| Cricetulus griseus | Prostaglandin F2 receptor negative regulator                                         | 23                      | 23        |            |
| Cricetulus griseus | Nidogen-1                                                                           | 4                       | 4         |            |
| Cricetulus griseus | Calsyntenin-1                                                                         | 21                      | 20        |            |
| Cricetulus griseus | Galectin-3-binding protein                                                           | 17                      | 14        |            |
| Cricetulus griseus | Fibronectin                                                                          | 25                      | 20        |            |
| Cricetulus griseus | Cation-independent mannose-6-phosphate receptor                                     | 22                      | 25        |            |
| Cricetulus griseus | Peroxidasin-like                                                                     | 17                      | 14        |            |
| Cricetulus griseus | Uncharacterized protein                                                               | 18                      | 18        |            |
| Cricetulus griseus | Tubulointerstitial nephritis antigen-like                                            | 9                       | 8         |            |
| Cricetulus griseus | Amine oxidase                                                                        | 13                      | 12        |            |
| Cricetulus griseus | Granulins                                                                            | 7                       | 8         |            |
| Cricetulus griseus | CD109 antigen isoform 2                                                              | 13                      | 11        |            |
| Cricetulus griseus | Alpha-N-acetylglucosaminidase                                                         | 13                      | 11        |            |
| Cricetulus griseus | Sulfhydryl oxidase                                                                   | 9                       | 10        |            |
| Cricetulus griseus | Laminin subunit gamma-1                                                              | 14                      | 8         |            |
| Cricetulus griseus | Clusterin                                                                            | 9                       | 9         |            |
| Cricetulus griseus | Purine nucleoside phosphorylase-like protein                                         | 9                       | 10        |            |
| Cricetulus griseus | Inositol-3-phosphate synthase 1-like protein                                         | 10                      | 9         |            |
| Cricetulus griseus | N-sulphoglucosamine sulphohydrolase                                                  | 8                       | 7         |            |
| Cricetulus griseus | Neutral alpha-glucosidase AB isoform 1                                              | 10                      | 7         |            |
| Cricetulus griseus | Alpha-mannosidase                                                                    | 9                       | 8         |            |
| Cricetulus griseus | Inter-alpha-trypsin inhibitor heavy chain HS                                         | 9                       | 7         |            |
| Cricetulus griseus | N-acetylglucosamine-1-phosphotransferase subunit gamma                               | 5                       | 7         |            |
| Cricetulus griseus | Neuropilin                                                                           | 9                       | 7         |            |
| Cricetulus griseus | Agrin-like isoform 1                                                                 | 6                       | 8         |            |
| Cricetulus griseus | Legumain                                                                             | 5                       | 4         |            |
| Cricetulus griseus | Nucleobindin-2-like protein                                                           | 6                       | 5         |            |
| Cricetulus griseus | Extracellular matrix protein 1                                                       | 6                       | 6         |            |
| Cricetulus griseus | Vasorin                                                                              | 5                       | 6         |            |
| Cricetulus griseus | Aminoacylase-1A-like protein                                                          | 5                       | 7         |            |
| Cricetulus griseus | Tissue alpha-L-fucosidase                                                            | 6                       | 5         |            |
| Cricetulus griseus | Low-density lipoprotein receptor                                                     | 5                       | 4         |            |
Table S1. Contaminating proteins identified in ADAMTS13 preparations (wild type or Ala1144Val) used in in vivo experiments. Proteins unique to the wild type preparation are highlighted in red and those unique to the Ala1144Val preparation are highlighted in blue. Human ADAMTS13 is highlighted in grey.
Supplementary Figures and Legends

Figure S1. Study design for distal MCAo (A) and tMCAo I/R injury (B) models.
Figure S2. Biochemical characterisation of ADAMTS13 variants. A, SDS PAGE analysis of purified wild type ADAMTS13 (wt) and Ala1144Val ADAMTS13 (ca). Coomassie staining suggests a similar level of purity of both preparations which has been confirmed by mass spectrometry (see Table S1). The black arrow indicates a predominant band at ~170 kDa confirmed to be ADAMTS13 by western blot using antibodies against ADAMTS13 or the His\textsuperscript{6} tag. B, an in house ELISA was used to determine ADAMTS13 concentrations which corresponded well with western blot band intensities. Capture and detection antibodies used in the ELISA were polyclonal preparations targeting regions distinct from the location of the linker 3 mutations. C and D, the relative activities of ADAMTS13 variants (presented in Figure 1C) were determined by FRETS-VWF73 assay. The linearity of the raw data was interrogated using a runs test, with a p value of <0.05 indicating deviation of the data from the linear regression. Over the full duration of the assay (55 min) the data collected in assays of variants with enhanced activity deviated from linearity, presumably due to substrate depletion. However, the data collected over the first 30 min, from which the relative activities were derived, was linear in all assays.
Figure S3. Assessment of bleeding risk associated with treatment groups in distal MCAo and tMCAo models. A, hematoxylin and eosin stained coronal brain sections showing the largest post-MCAo bleeds observed in each experiment group in the distal MCAo study. Each field of view is shown at 2 magnifications with bleeds identified by black arrows in the higher magnification image. Scale bars = 500 μm or 100 μm. B, the length of the largest bleed in each animal was measured using Slidescanner software. Ordinary one-way ANOVA showed no significant difference between groups. C, the time taken for bleeding cessation after transverse tail tip amputation. Again, ordinary one-way ANOVA showed no significant difference between groups. D, E and F, determination of VWF antigen concentration, VWF collagen binding activity and fibrinogen concentration in mouse plasma showed no significant difference between treatment groups or when compared to an unrelated cohort of naive CD1 mice. G, representative cresyl violet stained coronal brain sections from animals in tMCAo study. Each field of view is shown at 2 magnifications with bleeds identified by black arrows in the higher magnification image. Scale bars = 1000 μm or 50 μm.
Figure S4. Relationships between key IF measurements and other measured stroke parameters. A-F, for each pair of parameters a two-tailed Pearson correlation analysis was performed with 95% confidence intervals (dotted lines) to generate correlation coefficients (r) and p values. Data points corresponding to sham animals are shown in green (n=4) and animals treated with caADAMTS13 shown in blue (n=7).
Figure S5. Delayed administration of caADAMTS13 following tMCAo and I/R injury does not significantly reduce VWF deposition or neutrophil recruitment in the mouse brain. A, deposition of VWF and recruitment of neutrophils to the ischaemic tissue were visualised by IF staining for VWF and for the neutrophil marker Ly-6G. Each representative field of view is shown at 2 magnifications with parenchymal neutrophils identified by white arrows in the higher magnification image. Scale bars = 100 μm or 50 μm. B, thresholded single channel images were used to quantify the total area of VWF positive staining in ImageJ. C, the number of Ly-6G positive particles, with an area greater than 80 μm², was measured using automated particle counting applied to thresholded single channel images in ImageJ. Error bars represent mean ± S.D. Single comparisons were performed using an unpaired t-test (*p<0.05, **p<0.01, ***p<0.001).
1 **Supplementary References**

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