SUPPLEMENTAL MATERIALS

Intracerebral hemorrhage and thrombin-induced alterations in cerebral microvessel matrix

SUPPLEMENTAL MATERIALS AND METHODS

Materials and Methods

Animal materials

All animal protocols and experiments were reviewed and approved by the University of Washington Institutional Animal Care and Use Committee. All mice were maintained in a secured pathogen-free facility in a closed breeding colony before and during the studies. All mice were male (age 8 weeks) of the C57Bl/6 J strain (Jackson Laboratory, CA). The subjects were housed in separate cage groups at 23±2°C and 55% relative humidity on a 12-hour light/dark cycle. Non-human primate samples (male) alluded to here were obtained from archived materials, previously described, from experiments at The Scripps Research Institute (TSRI).[1-4] These studies were also performed following institutionally approved procedures complying with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. All experiments conformed to the deliberations of STAIR and the ARRIVE Guidelines.[5,6]

Reagents

Interventional agents. Purified murine α-thrombin was obtained from Haematologic Technologies (Essex Junction, VT). Murine α-thrombin was produced by the methods of Lundblad with the modifications described by Nesheim.[7] Zymography of the purified murine α-thrombin preparation on casein-containing gels demonstrated no additional cleavage activities (data not shown).

Dabigatran and dabigatran etexilate were gifts from Boehringer-Ingelheim GmbH; dabigatran was used previously for *in vitro* permeability studies.[8] The binding between dabigatran and α-thrombin is tight, but not irreversible.[8]

Immunochemicals. The sources of the antibodies used for either immunohistochemistry or immunoblots have included: i) for perlecan, the rat anti-mouse monoclonal antibody (MoAb) clone A7L6, MAB 1948P (Chemicon/Millipore) or the mouse MoAb, clone 7E12 (Chemicon); ii) for perlecan domain V, the mouse MoAb clone 268908 (R&D Systems); iii) for laminin, the rabbit polyclonal antibody (PoAb) ab128053 (Abcam), the MoAb LAM 89 (Sigma-Aldrich), and for laminin β1-chain, the rat anti-mouse MoAb LT3 clone ab44941 (Abcam); iv) for collagen IV, the rabbit PoAb ab6586 (Abcam), for collagen IV α1-chain, the mouse MoAb clone CIV22 (Sigma-Aldrich) and the rabbit PoAb (Novus Biologicals LLC), and for

collagen IV α2-chain, a rabbit PoAb; (Sigma-Aldrich). For Hgb, the rabbit anti-hemoglobin PoAb ab191183 (Abcam) was used. All reagents have been used successfully by this laboratory for the purposes intended here, and in previously published experiments.[9,10]

Solutions. The standard reagent solution for the immunohistochemistry and the stereotaxic injection experiments was PBS modified by the addition of Ca^{2+} and Mg^{2+} (DPBS, sterile filtered; nr SH30264.01, HyClone Laboratories, Logan UT). This served as the sterile diluent for α-thrombin or was used alone as the control for specific experiments. Note that α-thrombin requires both Ca^{2+} and Mg^{2+} for activity. Ringer's solution (117 mM NaCl, 4.7mM KCl, 0.8mM $MgSO_4$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5mM $CaCl_2$, 10mM D-glucose) was employed for specific experimental conditions.

Chow. Dabigatran etexilate was added to standard dietary chow for rodents (Provimi Kliba AG, Switzerland) by Boehringer Ingelheim Pharma GmbH (Biberach, Germany). The chow was supplied as standardized pressed tablets in separate vacuum-sealed pouches of equal weight for dietary consumption by the mice and was either supplemented by dabigatran etexilate (at 10mg/gm chow) or by matching placebo. The pouches were stored at -20ºC prior to thawing and use.

Trans-cardiac perfusion fluid. In preparation for tissue processing for the immunohistochemistry and immunoblot studies trans-cardiac perfusion cleared blood elements from the cerebral microvasculature. The isosmotic perfusion fluid contained Ringer's solution, with 18gm/L bovine serum albumin (BSA), and heparin (2U/ml) (Fresenius Kabi US, LLC, Lak Zurich, IL).[1,11,12]

Immunoprobe procedures

Immunohistochemistry. Antigens of interest in cerebral microvessels were identified on 10μm frozen sections of mouse cortex by immunohistochemistry, as previously described.[3] Both single label and dual label studies were performed. Where two MoAbs were tested against each other the immunohistochemical process preceded the immunofluorescent process. Fixation was achieved with the mixture 1:1 acetone: methanol (vol:vol). For the anti-laminin β1-chain antibody fixation was achieved with 2% paraformaldehyde (PFA) as the sole approach.

To demonstrate the impact of α-thrombin on microvessel structure, 10μm thick frozen coronal sections of mouse brain were incubated with 100μl of murine α-thrombin (3 U/ml) for 60 minutes in a moisture

chamber at 37°C, and then developed with specific primary antibody probes against collagen IV (α1-chain), laminin (β1-chain), or perlecan. The secondary antibodies were detected with DAB. Immunochemical "signals" were quantified from images (.jpg) captured by computer-assisted video-imaging (CVI) microscopy (see below) and transferred to Image J on a Mac platform.

To evaluate microvessel integrity, immunochemical procedures were also applied onto 10μm frozen coronal sections directly, with immunoprobes against collagen IV, laminin, and perlecan.

Quantitative video-imaging (CVI) microscopy. To quantify the immunoreactive signals on the 10μm frozen sections, randomly chosen non-contiguous full fields (1.80mm^2 images) were scanned in full color (RGB) using a modified Zeiss S100 Invert fluorescence microscope, equipped with AxioVision and KS 400 software (Zeiss, Oberkochen, Germany). The individual full-field images were rendered as the presence ("0" (black, stain)) *vs* absence ("256" (white, background)) of stain using the RGB color code with NIH ImageJ software (Bethesda, MD), and the number of "0" pixels depicted graphically. Data of microvessel matrix protein epitope expression and the extent of IgG leakage are presented numerically as "immunoreactivity (pixels)," that is a direct measure of the surface area of epitope expression.[9,10]

α-thrombin/matrix degradation assays

The impact of α-thrombin on the purified matrix proteins collagen IV, laminin, and perlecan were assayed with the purified enzyme and purified matrix substrates.

Purified collagen IV, laminin, and perlecan were obtained from Sigma-Aldrich (St. Louis). Two micrograms of each matrix protein was mixed with serial concentrations of α-thrombin (0, 1, 2, and 3 U/ml) in 20μl PBS (Ca^{+2}, Mg^{+2}), and incubated at 37°C for 1 hour. Immunoblot was used to detect thrombin cleavage effect following incubation.

Homogenization of cerebral cortex/matrix degradation assays

Matrix substrates from the cerebral microvasculature were obtained by homogenization of cortex dissected from perfused mouse brain at 4ºC with a glass tissue grinder (Dounce; Wheaton Scientific, Millville). Ringer's solution containing 0.86% NaCl, 0.03% KCl, 0.033% $CaCl_2$ was added to the homogenates which were heated at 70ºC for 60 minutes, and then centrifuged for 10 minutes at 6,000g to remove soluble matrix protein products. The pellets were washed with PBS twice with centrifugation at 6,000g each time. For

primate cortex and striatal samples, 10 cryosections (10μm each) in PBS were homogenized and centrifuged at 6,000g for 10 minutes at 4ºC. The supernatants were discarded and the pellets were stored at -80ºC.

For the matrix degradation assays, pellets of the frozen perfused murine cortex were thawed resuspended in DPBS, and aliquoted equally into Eppendorf tubes, each centrifuged at 6,000g for 10 minutes. To the individual pellets, 20μl of solution containing α-thrombin was added, and the mixture vortexed, then incubated at 37ºC for 2 hours. The supernatants were concentrated 20-fold, separated on 10% SDS–PAGE, and examined by immunoblot. Protein concentrations were measured using the bicinchoninic acid method (BCA, Thermo Scientific).

***In vivo* permeability assay**

The whole brain (*in vivo*) blood-brain barrier permeability measurements used a pulse-chase method modified from that described by Hawkins *et al*, and adapted for mice.[13-16] Mice were anesthetized with isoflurane (1%). A ventral midline incision extended to a thoracotomy was performed. Pulse perfusion with the two fluorescent tracer molecules, fluorescein Na^+ (NF) and Evans blue albumin (EBA), in combination in Ringer's solution was performed.

Following clamping of the descending thoracic aorta, trans-cardiac perfusion via the left ventricle employed Ringer's solution with 18gm/l BSA (without heparin) by a peristaltic pump, with fluid exit by the right atrium. The initial perfusion with 15ml Ringer's solution/BSA without tracers was followed directly by 10ml Ringer's solution/BSA with NF/EBA (1gm/L each), then followed with 25ml Ringer's solution/BSA. All perfusion solutions were at 37°C.

Following perfusion, the brains were immediately removed for tissue processing, stripped of meninges, the hemispheres weighed, and then mechanically homogenized in 1ml 7.5% (w/v) trichloroacetic acid (TCA) at 4°C. The resulting homogenate was divided into two parts for the NF and EBA assays.

For the NF assay, the ratio of NF in the brain to that in the right atrial perfusate, R_{NF}, was calculated by:

$$R_{NF}\ (\mu l/gm) = [NF]_{susp} V_{susp} / [NF]_p M_{sam}$$

where $[NF]_{susp}$ was the NF concentration in the brain suspension, V_{susp} was the total volume of the brain suspension. $[NF]_p$ was the measured NF concentration in the perfusate, and M_{sam} was the wet mass of the brain tissue sampled. For the assay, 83.5µl 5N NaOH was added to 500µl of the homogenate and mixed well. An assay volume of 150µl/well of the suspension was added into a 96 well plate (n = 3 each). R_{NF} perfusate was also measured by mixing 50µl of collected perfusate to 500µl TCA, and 83.5µl of 5N NaOH was added. Samples were read on a microplate reader at 485nm excitation, with emission at 535nm for NF.

For the EBA assay, the ratio of EBA in brain tissue to that in the right atrial perfusate, R_{EBA}, was calculated by:

$$R_{EBA}\ (\mu l/gm) = [EBA]_{susp}V_{susp}/[EBA]_pM_{sam}$$

where $[EBA]_{susp}$ was the measured concentration of Evans blue albumin (EBA) in the brain suspension, V_{susp} was the total volume of the brain suspension, $[EBA]_p$ the measured concentration of EBA in the perfusate, and M_{sam} was the wet mass of the brain tissue sampled. For this assay 1ml of the brain suspension was centrifuged at 10,000g for 10 min, the supernatant was collected. R_{EBA} was measured by mixing 50µl of perfusate into 500µl TCA. An assay volume of 300µl/well (n = 3) was read directly by spectrophotometer at 600nm.

Western immunoblot

Immunoblots of matrix protein degradation products were measured as previously described.[3,9,16] Samples included purified matrix proteins, samples of homogenized cortex, products from the thrombin degradation assay. Proteins (2µg/lane) were applied to 10% Tris-glycine gels, resolved, and transferred to PVDF membranes (Millipore) that were then blocked for 1 hour with 5% milk solids in PBS.[4] The membranes were probed with the primary antibody at 4ºC overnight. Signals were detected with Pierce™ ECL Western Blotting Substrate (Thermo Scientific, USA) or Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, UK) on X-ray film (LabScientific, Inc). The individual bands were quantified using Image J gel analysis software as described.[10]

PVDF membranes were probed with antibodies to perlecan or perlecan domain V, to the collagen IV α2-chain or the laminin β1-chain at 4ºC overnight then incubated with appropriate secondary antibodies at room temperature for 1 hour. The membranes were washed in PBS/Tween 20 and the proteins identified with an

enhanced chemiluminescence detection system (with Pierce™ ECL Western Blotting Substrate (Thermo Scientific, USA) or Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, UK) on X-ray film (LabScientific, Inc) according to the manufacturer's instructions. The individual bands were quantified using Image J gel analysis software as described.[10]

Stereotaxic delivery

Under isoflurane anesthesia (1.0-2.5%), mice were placed in a stereotaxic frame (KOPF, David Kopf Instruments, Los Angeles). A 2mm skull window was opened using a dental tool, and a 33 gauge injection needle was lowered through the hole prepared in the skull into the center of the right striatum at bregma -1 mm anterior (Y); 2 mm lateral (X); to a depth of -3.5 mm (Z), and 2 µl of test agent was injected over 1 minute with a Hamilton syringe at 1µl/min. The needle was left in place for 5 minutes after completion of the injection, and then removed slowly (1mm/min) and the skull defect closed with bone wax, and incision sutured. There was 100% survival.

Following trans-cardiac perfusion with isosmotic buffer containing BSA without tracer at 24 hours post-injection the brains were then removed, placed on ice, divided in the coronal plane at the level of the injection site into anterior and posterior segments, embraced in OCT compound (Tissue-Tek, Sakura Finetek USA, Inc.), and quick frozen. Samples were stored at -80°C until sectioning and study.

Feeding protocol

Mice were housed in separate cage groups for 5 days before the diet exposure. For the blinded placebo-controlled dabigatran ingestion experiments mice were distributed into separate cages (4 mice/cage) and randomly assigned into control (placebo-containing chow) and experimental (dabigatran-containing chow) diet groups per cage. The subjects were fed continuously for 14 days prior to entry into the stereotaxic procedure and for 24 hours thereafter with their respective pellets feeding. Twenty-four hours after the injection of α-thrombin solution or vehicle control liquid each subject underwent transcardiac perfusion, with harvest of the cerebral tissues.

Statistical Analysis

Data are expressed as mean ± standard deviation of replicate experiments (with parallel duplicate or triplicate measures) on separate days. The number of measurements is shown in each figure. Data sets were blinded for analysis using the Latin square assignment method. Statistical methods routinely involved general linear models (GLM) or analysis of variance (ANOVA). We have utilized general linear models

(GLMs) followed by *post-hoc* multiple comparison procedures if warranted as our standard method of analysis. Since individual sample sizes were typically too small to assess accurately any normality assumption within groups, we chose instead to assess normality on the residuals from the GLM fits. We utilized Lilliefors corrected Kolmogorov-Smirnov tests for normality in these assessments; invariably, *p*-values were non-significant at the α=0.05 level. All analyses were undertaken either in R v.3.2 (R Core Team (2015); R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria) or SPSS v.22 (IBM Corporation, 2013). The statistician and co-authors were blinded to intervention assignment.

Group sizes were determined with the goal of ensuring a minimal power of 0.80 to detect moderate effect sizes of 0.2 to 0.25 between controls and other groups at a two-sided α of 0.05. This required cohort sizes of 6-8 iterations per condition. Statistical methods included standard non-parametric tests for comparisons of twin data sets, one-way ANOVA with appropriate post-hoc tests for comparisons of multiple groups, hierarchical linear models for comparing data collected over multiple time points, and generalized linear models to analyze rates of transport, as appropriate. Significance was set conventionally at $p \leq 0.05$.

Individual *t-tests* were performed on select unpaired data sets from individual experiments where appropriate using the statistical analysis program Prism®. Significance was set conventionally at $p \leq 0.05$.

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SUPPLEMENTAL TABLE

Supplemental Table 1
Thrombin cleavage site consensus sequence leu-val-arg-gly-ser (LVRGS) can be found in components of the basal lamina matrix proteins collagen IV, laminin, and perlecan.

collagen IV	laminin		perlecan
α1 chain	β1 chain	α4 precursor	
1173 1419 1619	668	545 1782	461 1957 3267 3906

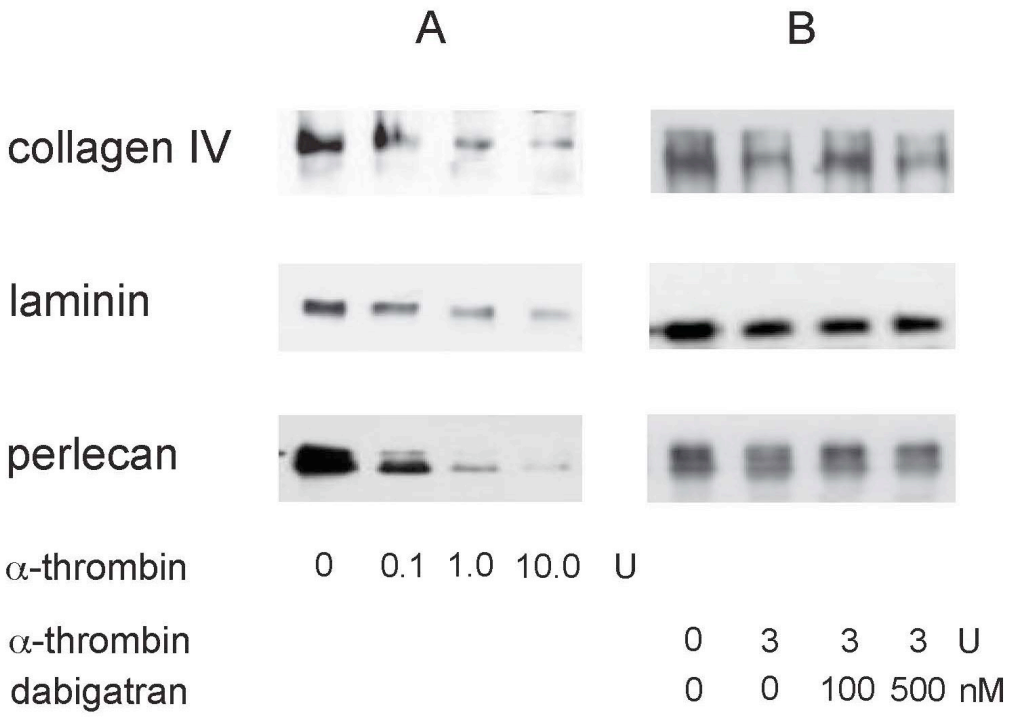
Source: derived from inputs to Expasy_Peptide_Cutter

SIB Swiss Institute of Bioinformatics
Quarter Sorge – Batiment Amphipole
1015 Lausanne
Switzerland

Portal used: https://Web.expasy.org/peptide_cutter

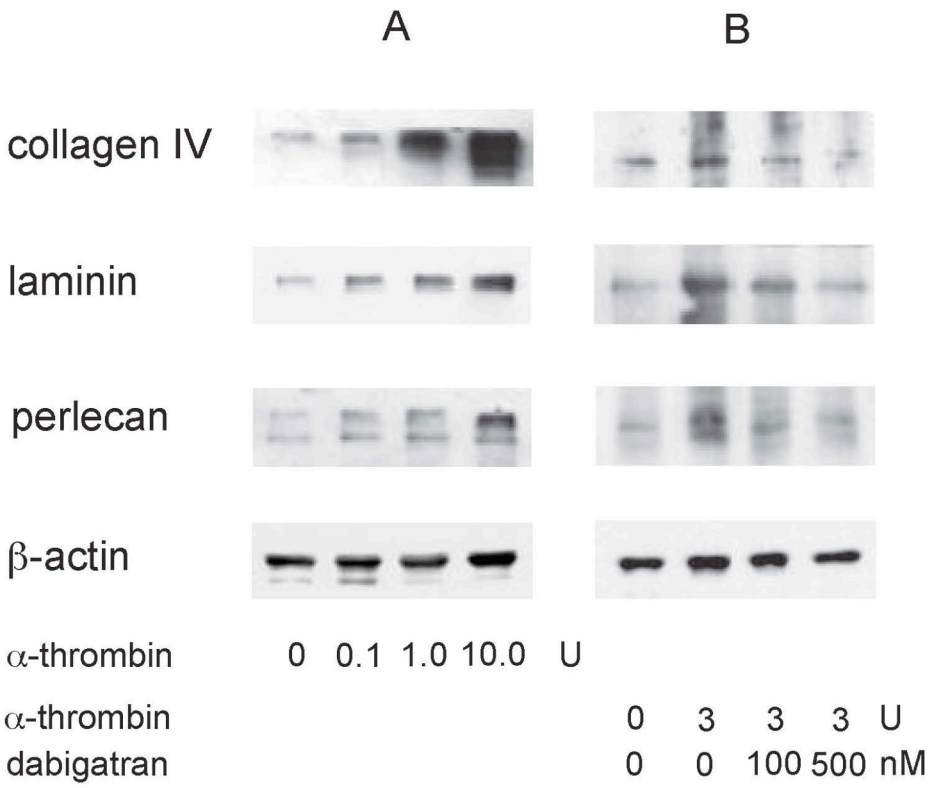
SUPPLEMENTAL FIGURES

Supplemental Figure 1



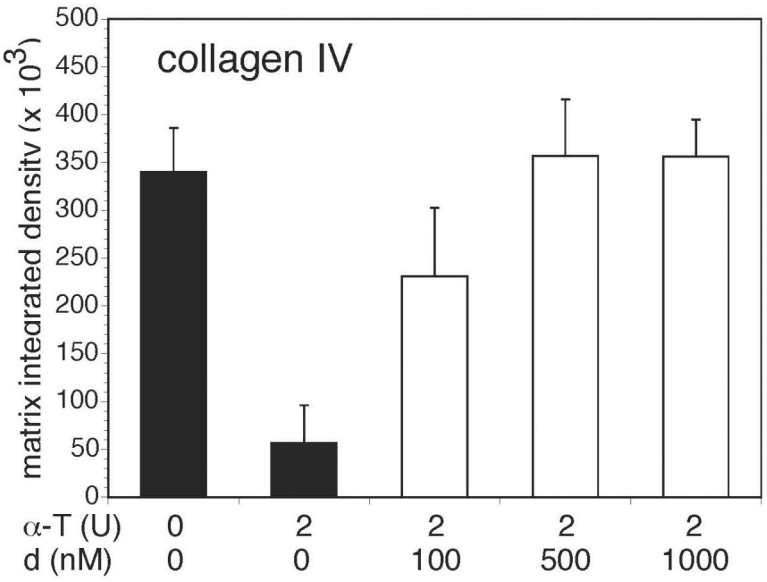
Supplemental Figure 1
Representative Western immunoblots displaying band intensities for the collagen IV α1-chain, the laminin β1-chain, and perlecan from purified collagen IV, laminin, and perlecan, under conditions of **(A)** exposure to purified murine α-thrombin (0-10 U/ml) and **(B)** exposure to increasing concentrations of dabigatran etexilate with either no or a fixed concentration of purified murine α-thrombin (3 U/ml). Each lane was loaded with 2 μg of total protein (hence no β-actin control is presented). The complete data are shown in **Figure 1**.

Supplemental Figure 2



Supplemental Figure 2
Representative Western immunoblots displaying band intensities for the collagen IV α1-chain, the laminin β1-chain, and perlecan from homogenates of freshly perfused cortical tissues from naïve adult mice, under conditions of **(A)** exposure to purified murine α-thrombin (0-10 U/ml) and **(B)** exposure to increasing concentrations of dabigatran etexilate with either no or a fixed concentration of purified murine α-thrombin (3 U/ml). The complete data are shown in **Figure 2**.

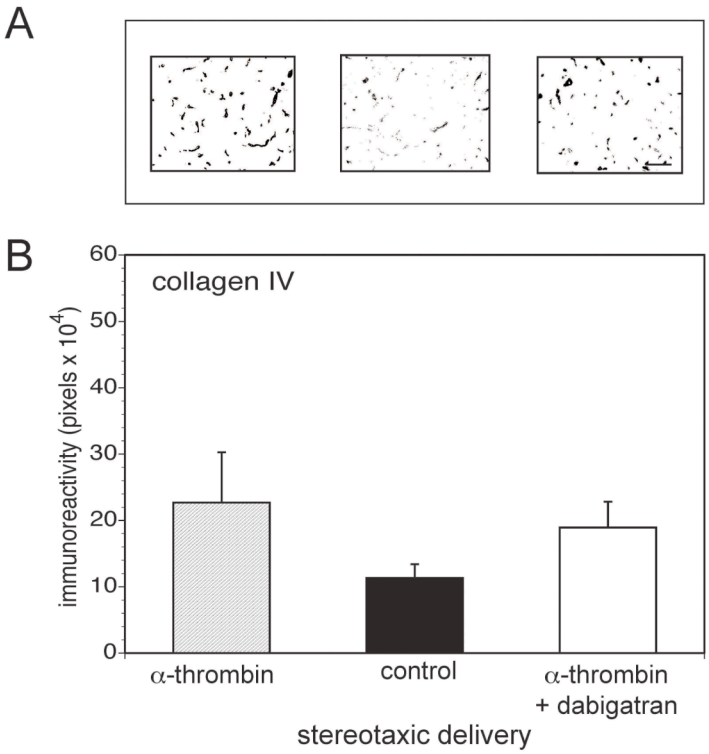
Supplemental Figure 3



Supplemental Figure 3

α-thrombin and dabigatran etexilate differentially affect microvessel basal lamina collagen IV α1-chain immunoreactivity in the striatum of the non-human primate (*Papio anubis/cynocephalus*). In these preliminary studies, collagen IV α1-chain immunoreactivity in the basal lamina of striatal microvessels was significantly decreased by exposure to α-thrombin in a concentration-dependent manner from 0.02U to 20U α-thrombin (one-way ANOVA; $F_{4,10} = 14.2$, $p=0.0004$, n = 3 each). For the experiment shown here 2U α-thrombin was chosen. Collagen IV levels differed significantly across the five groups: $F_{4,10} = 18.2$, $p=0.0001$ (one-way ANOVA, n = 3). A subsequent Bonferroni all pairwise comparisons procedure at the overall 0.05 level showed that collagen IV levels for the 2U α-thrombin group were significantly lower than the other four groups, but there were no significant differences in collagen IV levels among these latter 4 groups.

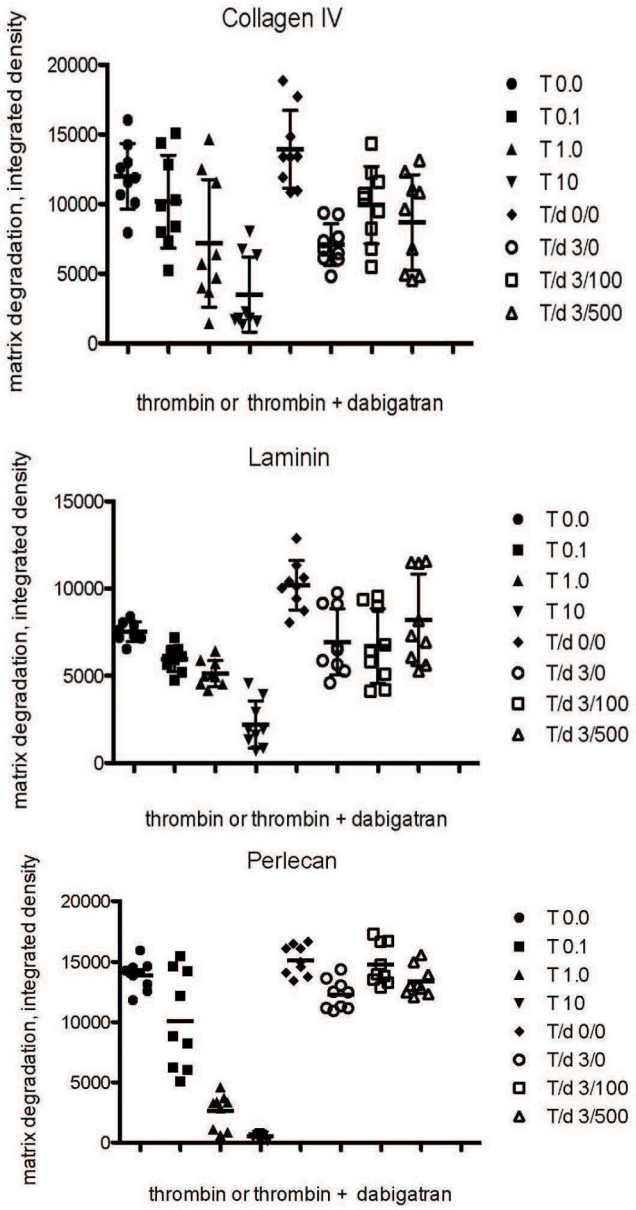
Supplemental Figure 4



Supplemental Figure 4
The α-thrombin-induced decrease in microvessel matrix collagen IV is abrogated by dabigatran etexilate *in vivo*. **Panel A.** Stereotaxic injection of 40mU α-thrombin into mouse striatum significantly decreased microvessel collagen IV α1-chain immunoreactivity at 24 hours, which was prevented by co-injection with dabigatran etexilate, as shown in respective photomicrographic fields of microvessels in binary output. Magnification bar=50μm. **Panel B**. Overall, microvessel collagen IV α1-chain immunoreactivity differed significantly among the control (gray bar), α-thrombin (black), and α-thrombin + dabigatran etexilate (white bar) cohorts ($F_{2,21}$=10.5, p=0.0007 from one-way ANOVA; n=8 each). Based on subsequent pair-wise comparisons, co-injection of α-thrombin and dabigatran restored collagen IV to control levels compared to α-thrombin alone (Bonferroni, α=0.05)**.** For the comparison [α-thrombin + dabigatran] *vs* [control], p=0.0009. For the comparison, [α-thrombin + dabigatran] *vs* [α-thrombin], p=0.1393 (NS).

FIGURES WITH DATA POINTS AND SUPPLEMENTAL FIGURE LEGENDS

Figure 1 with Data



Supplemental Legend to Figure 1

α-thrombin–mediated degradation of purified matrix proteins and inhibition of their degradation.

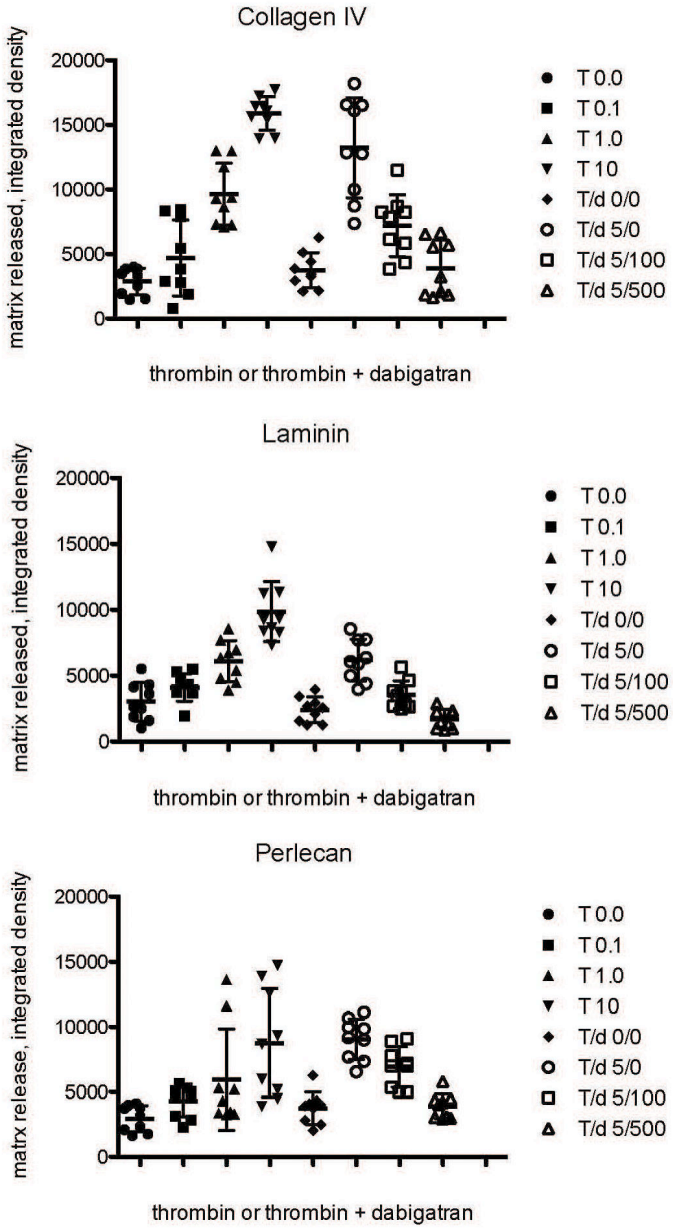
Panel A. Purified murine α-thrombin (0-10 U/ml) degraded matrix collagen IV (α1-chain), laminin (β1-chain), and perlecan in a concentration-dependent manner. Residual matrix protein content was determined

by Western immunoblot with immunoprobes against the purified mouse collagen IV α1-chain, laminin β1-chain, or perlecan. The outcomes are reported as the integrated density of the major matrix protein bands. Overall, significant differences in the integrated density levels with each purified matrix protein, relative to α-thrombin concentrations were: collagen IV, $F_{3,32}$=11.01, p=0.00004; laminin, $F_{3,32}$=56.34, $p<1x10^{-10}$; perlecan, $F_{3,32}$=69.92, $p<1x10^{-10}$. With each matrix protein, there was a significant trend toward increased degradation of the matrix proteins as the concentration of thrombin increased: collagen IV, $F_{1,34}$=22.75, p=0.000034; laminin, $F_{1,34}$=88.13, $p<1x10^{-10}$; perlecan, $F_{1,34}$=29.25, p=0.000005. For select individual comparisons, α-thrombin 0U/ml *vs* 1.0U/ml: collagen IV, p=0.020; laminin, p<0.0001; and, perlecan, p<0.0001. Each bar represents n=9 independent observations.

Panel B. The anti-thrombin dabigatran etexilate inhibited the degradation of matrix collagen IV (α1-chain), laminin (β1-chain), and perlecan by 3U murine α-thrombin in a concentration-dependent manner. Overall, integrated density levels of *collagen IV* were significantly different: $F_{3,32}$=10.44, p=0.00006. However, there was no trend in degradation: the integrated density level at 0 levels of thrombin and dabigatran (left bar) was significantly greater than the other three combinations of thrombin and dabigatran, but those combinations did not differ significantly from one another (Bonferroni, α=0.05). Integrated density levels of *laminin* were also significantly different: $F_{3,32}$=5.32, p=0.004. The only significant pairwise comparisons were thrombin/dabigatran level 0/0 *vs* thrombin/dabigatran level 3/0, and thrombin/dabigatran level 0/0 *vs* thrombin/dabigatran level 3/100 (Bonferroni, α=0.05). Lastly, integrated density levels of *perlecan* differed significantly: $F_{3,32}$=8.42, p=0.0003. The only significant pairwise group comparisons were thrombin/dabigatran level 0/0 *vs* thrombin/dabigatran level 3/0, and thrombin/dabigatran level 3/0 *vs* thrombin/dabigatran level 3/100 (Bonferroni, α=0.05). For select individual comparisons, at α-thrombin (α-t) 3U/ml, dabigatran (d) 0nM *vs*100nM: collagen IV, p=0.0094; laminin, p=0.3652; and, perlecan, p=0.0040. Each bar represents n=9 independent observations.

In the **Figure 1 with Data** above, means ± standard deviations are depicted for each cohort data set.

Figure 2 with Data



Supplemental Legend to Figure 2

α-thrombin–mediated release of matrix products from homogenates of cerebral cortex, and prevention of their release by co-incubation with dabigatran etexilate.

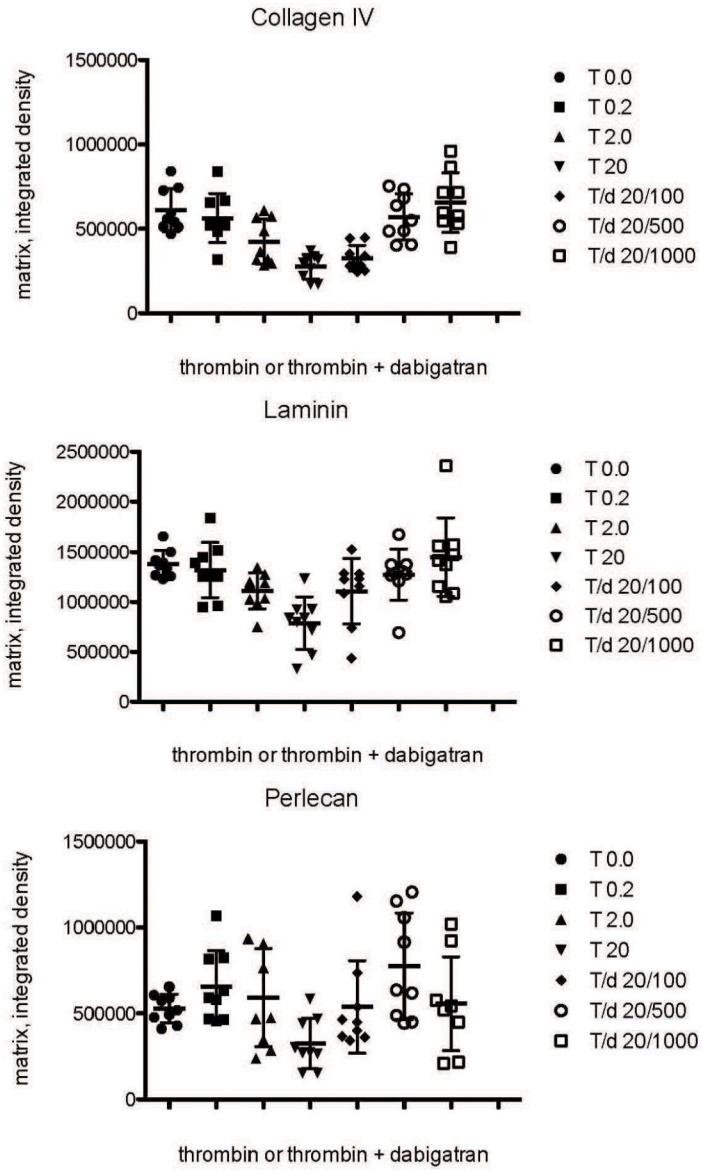
Panel A. Concentration-dependent release of products of collagen IV (α1-chain), laminin (β1-chain), and perlecan was detected by immunoblot when homogenates of freshly perfused cortical tissues from naïve adult mice were incubated with α-thrombin (0-10 U/ml). Overall, there were significant differences in the integrated density levels with each purified matrix protein, relative to α-thrombin concentrations: collagen IV, $F_{3,32}=71.54$, $p<1x10^{-10}$; laminin, $F_{3,32}=29.90$, $p<1x10^{-8}$; perlecan, $F_{3,32}=6.38$, $p=0.002$. With each matrix

protein, there was a significant trend toward increased degradation of the matrix proteins as the concentration of α-thrombin increased: collagen IV, $F_{1,34}$=17.89, p=0.0002; laminin, $F_{1,34}$=63.50, $p<1x10^{-8}$; perlecan, $F_{1,34}$=15.34, p=0.0004. . For select individual comparisons, α-thrombin 0U/ml *vs* 1.0U/ml: collagen IV, p<0.0001; laminin, p<0.0001; and, perlecan, p=0.0157. Each bar represents n=9 independent observations.

Panel B. Dabigatran etexilate inhibited matrix collagen IV, laminin, and perlecan degradation in homogenates of murine cerebral cortex by 10U murine α-thrombin in a concentration-dependent manner. Overall, integrated density levels of *collagen IV* were significantly different: $F_{3,32}$=25.91, $p<1x10^{-7}$. However, there was no trend in degradation: the only significant differences were between the integrated density levels of α-thrombin/dabigatran 5/0 and each of the other three combinations of thrombin and dabigatran (Bonferrroni, α=0.05). Integrated density levels of *laminin* were also significantly different: $F_{3,32}$=27.54, $p<1x10^{-8}$. All pairwise comparisons among the four thrombin/dabigatran combinations were significant, with the exception of the 0/0 *vs* 5/500 comparison (Bonferroni, α=0.05). Integrated density levels of *perlecan* also differed significantly: $F_{3,32}$=32.20, $p<1x10^{-9}$. The multiple comparison pattern was identical to that with laminin: all pairwise comparisons among the four thrombin combinations were significant, with the exception of the 0/0 *vs* 5/500 comparison (Bonferroni, α=0.05). For select individual comparisons, at α-thrombin (α-t) 5U/ml, dabigatran (d) 0nM *vs*100nM: collagen IV, p=0.0019; laminin, p=0.0004; and, perlecan, p=0.0071. Each bar represents n=9 independent observations.

In the **Figure 2 with Data** above, means ± standard deviations are depicted for each cohort data set.

Figure 3 with Data



Supplemental Legend to Figure 3

α-thrombin and dabigatran etexilate differentially affect microvessel matrix immunoreactivity

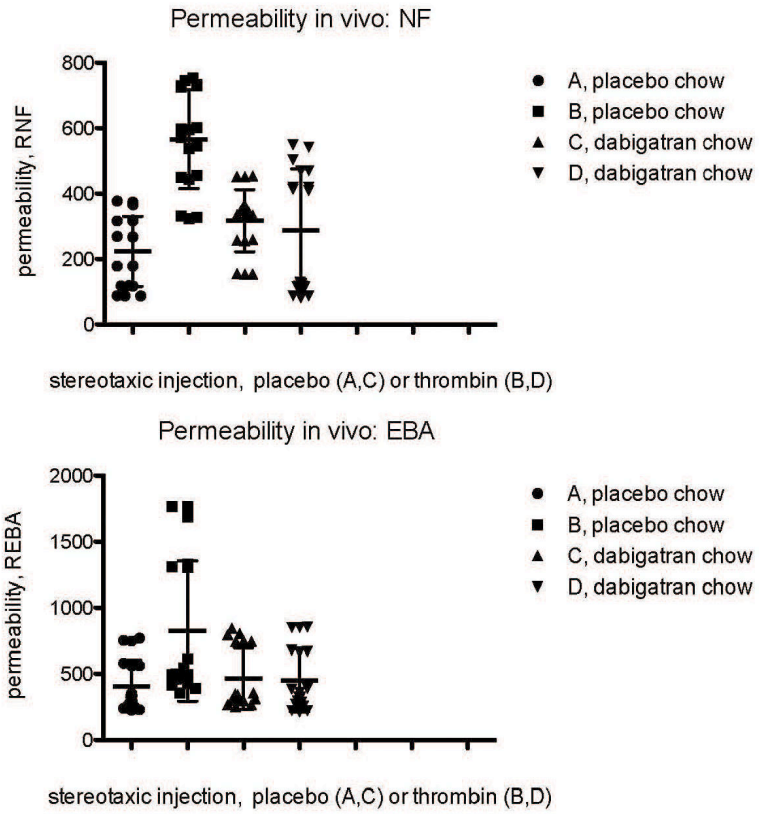
Panel A. Immunohistochemistry. Panels demonstrate decreased immunoreactivity of the collagen IV α1-chain, but not the entire molecule, in cortical microvessel basal lamina by α-thrombin. The concentration-dependent loss in α1-chain immunoreactivity was prevented when dabigatran was co-incubated with 2U α-thrombin. Magnification bar = 50μm.

Panel B. Immunoblots. Collagen IV. From a general linear model (GLM) analysis with main effects dabigatran (at 0 (placebo), 100, 500, or 1000nM) and α-thrombin (0, 0.2, 2.0, or 20U), both dabigatran and thrombin were significant factors: α-thrombin, $F_{3,56}$=18.45, p=2x10^{-8}; dabigatran, $F_{3,56}$=12.32, p=3x10^{-6}.

Subsequent pair-wise comparisons involving all dabigatran x thrombin combinations identified three homogeneous subgroups: (i) dabigatran-thrombin combinations {100-20, 0-0, 500-20, 0-0.2}; (ii) combinations {0-0, 500-20, 0.2-0, 2-0}; and (iii) combinations {0-2, 100-20, 0-20} (Bonferroni, α=0.05). **Laminin.** From a similar GLM analysis, both dabigatran and thrombin were significant factors: α-thrombin, $F_{3,56}$=8.58, p<0.0001; dabigatran, $F_{3,56}$=9.55, p=2x10^{-5}. Subsequent pair-wise comparisons involving all dabigatran x thrombin combinations found two homogeneous subgroups: (i) dabigatran-thrombin combinations {1000-20, 0-0, 0-0.2, 500-20, 0-2}, and (ii) combinations {0-2, 100-20, 0-20} (Bonferroni, α=0.05). **Perlecan.** From a similar GLM analysis both dabigatran and thrombin were significant factors: α-thrombin, $F_{3,56}$=3.27, p=0.028; dabigatran, $F_{3,56}$=5.35, p=0.003. Subsequent pair-wise comparisons involving all dabigatran x thrombin combinations found only one significant difference in immunoreactivity: the 20-0 subgroup had significantly lower reactivity than the 20-500 subgroup. All other pair-wise comparisons were not significant, at the α=0.05 level, per Bonferroni. For select individual comparisons, at α-thrombin (α-T) 0U *vs* 20U: collagen IV, p<0.0001; laminin, p<0.0001; and, perlecan, p=0.0028. At α-thrombin (α-T) 20U, dabigatran (d) 0nM *vs* 500nM: collagen IV, p<0.0001; laminin, p=0.0028; and, perlecan, p=0.0004 (MWU). **NB.** Non-human primate data is presented in **Supplemental Figure 3**.

In the **Figure 3 with Data** above, means ± standard deviations are depicted for each cohort data set.

Figure 4 with Data



Supplemental Legend to Figure 4

The effect of dabigatran ingestion on whole brain permeability following stereotaxic injection of α-thrombin.

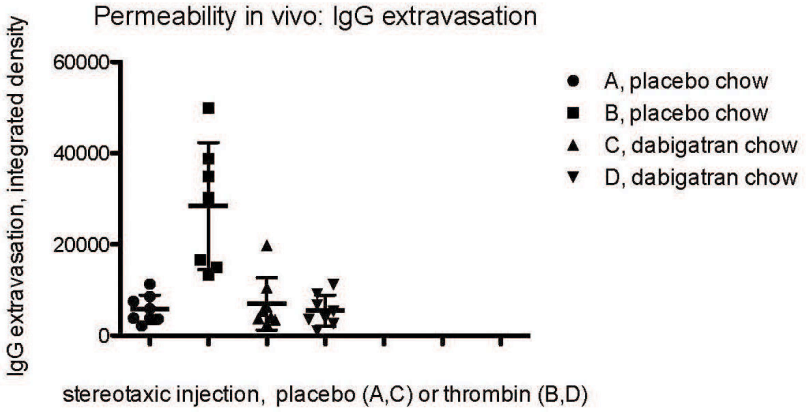
In a balanced factorial design, 24 animals received placebo-containing chow or dabigatran-containing chow for 14 days, and then received intra-striatal injections of either placebo or α-thrombin. Whole (global) brain permeability was measured at 24 hours.

Panel A. The permeability to fluorescein Na (R_{NF}) was assessed with 3 replicate readings per animal. A general linear model analysis of permeability (with main effects dabigatran and α-thrombin) found a significant thrombin effect ($F_{1,68}$=22.46, p=1.2x10^{-5}), a significant dabigatran effect ($F_{1,68}$=7.86, p=0.007), and a large α-thrombin x dabigatran interaction ($F_{1,68}$=31.74, p=4x10^{-7}). Subsequent pair-wise comparisons found that injection of α-thrombin was associated with increased permeability, and that dabigatran chow lowered permeability compared to placebo chow. The significant interaction owes to the significantly higher permeability of the placebo chow–α-thrombin group than any of the other three groups (Bonferroni, α=0.05). **Panel B**. Permeability to albumin (R_{EBA}) was also assessed with 3 replicate readings per animal. A general linear model analysis of permeability (with main effects dabigatran and α-thrombin) found a

significant thrombin effect ($F_{1,68}$=6.77, p=0.011), a significant dabigatran effect ($F_{1,68}$=4.17, p=0.045), and a large α-thrombin x dabigatran interaction ($F_{1,68}$=7.68, p=0.007). Subsequent pair-wise comparisons yielded results qualitatively identical to those in Panel A: injection of α-thrombin was associated with increased permeability, dabigatran chow lowered permeability compared to placebo chow, and the significant interaction owes to the significantly higher permeability of the placebo chow–α-thrombin group than any of the other three groups (Bonferroni, α=0.05).

In the **Figure 4 with Data** above, means ± standard deviations are depicted for each cohort data set.

Figure 5 with Data



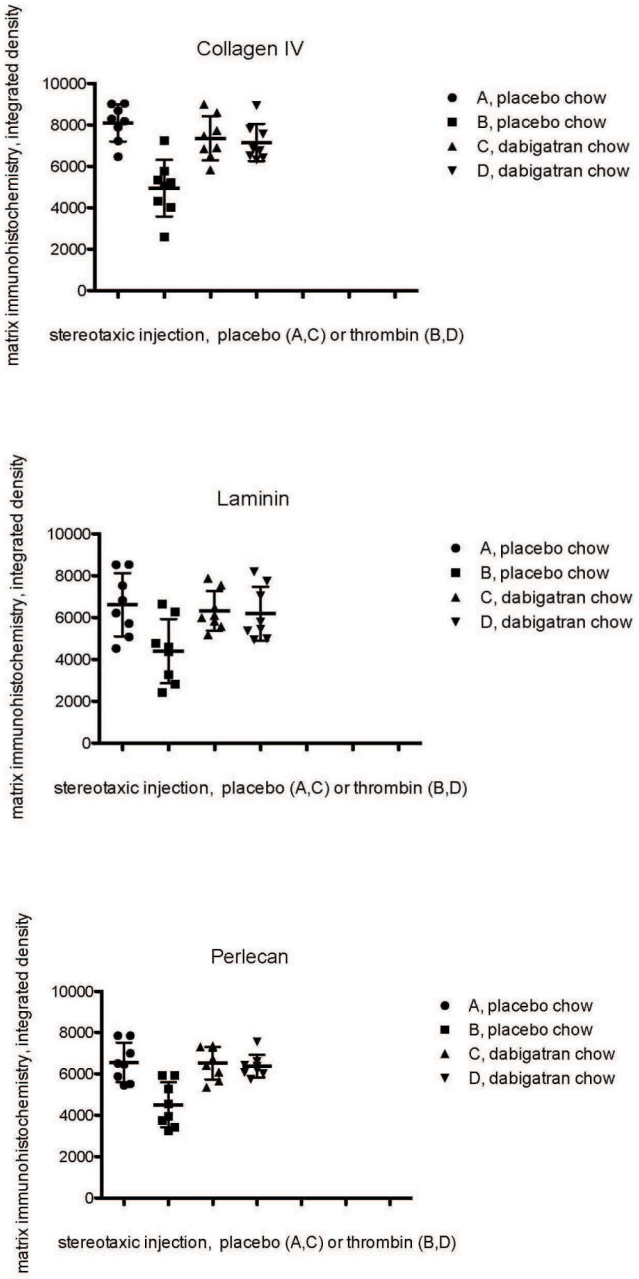
Supplemental Legend to Figure 5

Anti-thrombin dabigatran prevents α-thrombin-induced increase in microvessel permeability to IgG

Significantly increased IgG extravasation into the neuropil from the microvasculature was captured in animals receiving α-thrombin and placebo chow (see **inset** (arrow) photomicrographs of immunoreactive IgG in matched fields). Dabigatran-containing chow significantly reduced IgG extravasation compared to the placebo chow. A general linear model analysis of IgG extravasation (with main effects dabigatran and α-thrombin) found a significant α-thrombin effect ($F_{1,27}$=15.04, p=0.001), a significant dabigatran effect ($F_{1,27}$=16.08, p<0.001), and a large α-thrombin x dabigatran interaction ($F_{1,27}$=19.61, p=0.0002). The significant interaction is attributable to the fact that the placebo chow–α-thrombin regimen significantly increased IgG extravasation relative to the other 3 regimens (Bonferroni, α=0.05). A total of 31 animals received placebo-containing chow (n=15) or dabigatran-containing chow (n=16 bar). Magnification bar=100μm.

In the **Figure 5 with Data** above, means ± standard deviations are depicted for each cohort data set.

Figure 6 with Data



Supplemental Legend to Figure 6

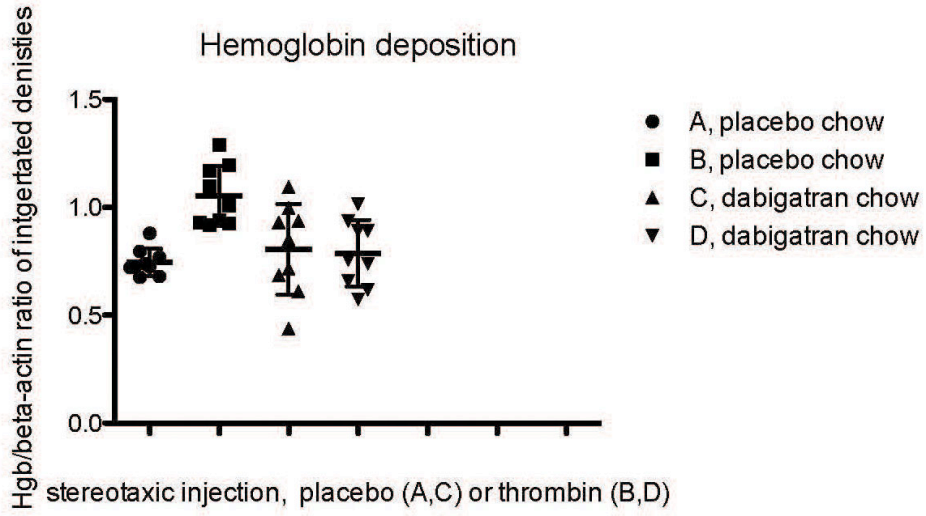
Dabigatran prevents α-thrombin-induced loss of microvessel basal lamina matrix proteins *in vivo*.

In a balanced factorial design, employing 32 animals, stereotaxic striatal injection of 40mU α-thrombin significantly deceased microvessel basal lamina collagen IV (α1-chain) immunoreactivity compared to placebo in subjects fed placebo-containing chow, while dabigatran ingestion prevented this decrease. **Upper panel. Collagen IV.** A general linear model analysis of subsequent microvessel basal lamina collagen IV (α1-chain) immunoreactivity, with main effects α-thrombin *vs* dabigatran, found a significant α-thrombin

effect ($F_{1,28}$=19.61, p=0.001), a borderline significant dabigatran effect ($F_{1,28}$=3.75, p=0.063), and a large α-thrombin x dabigatran interaction ($F_{1,28}$=15.10, p=0.001). Subsequent pair-wise comparisons of the main effects and interaction terms found that injection of α-thrombin was overall associated with decreased collagen IV immunoreactivity, and the significant interaction is attributable to the fact that the placebo chow–α-thrombin group had a significantly lower level of collagen IV immunoreactivity than any of the other three groups (Bonferroni, α=0.05). **Middle panel. Laminin, β1.** A similar analysis of laminin β1-chain immunoreactivity found a significant α-thrombin effect ($F_{1,28}$=6.18, p=0.019), a non-significant dabigatran effect ($F_{1,28}$=2.50, p=0.125), and a significant α-thrombin x dabigatran interaction ($F_{1,28}$=4.86, p=0.036). Injection of α-thrombin was overall associated with a slight decrease in laminin β1-chain immunoreactivity, from 6467±335 to 5291±335, and the significant interaction is attributable to the fact that the placebo chow–α-thrombin group had a significantly lower level of laminin β1-chain immunoreactivity than any of the other three groups (Bonferroni, α=0.05). **Lower panel. Perlecan.** Similarly, microvessel perlecan immunoreactivity found significant main effects (α-thrombin: $F_{1,28}$=11.92, p=0.002, and, dabigatran: $F_{1,28}$=8.12, p=0.008), and a significant α-thrombin *vs* dabigatran interaction ($F_{1,28}$=9.52, p=0.005). Dabigatran chow significantly increased microvessel perlecan immunoreactivity compared to placebo chow, whereas injection of α-thrombin significantly decreased microvessel perlecan immunoreactivity. The significant interaction is attributable to the fact that the placebo chow-α-thrombin regimen significantly decreased microvessel perlecan immunoreactivity relative to the other three regimens (Bonferroni, α=0.05).

In the **Figure 6 with Data** above, means ± standard deviations are depicted for each cohort data set.

Figure 7 with Data



Supplemental Legend to Figure 7

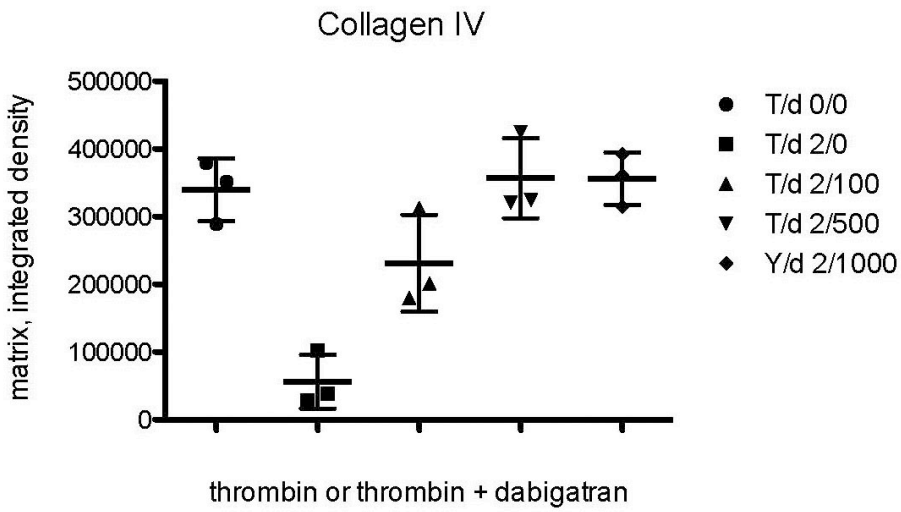
Dabigatran prevents α-thrombin-induced loss cerebral hemorrhage *in vivo*.

In a balanced factorial design, a total of 36 animals received placebo-containing chow or dabigatran-containing chow, and stereotaxic striatal injection of α-thrombin or placebo (n=9 each). **Upper panel.** Coronal sections demonstrate hemoglobin deposition at 24 hours along the injection track in the placebo chow–α-thrombin group only. All sections are scanned wet-mounts of immunohistochemistry preparations for hemoglobin at no magnification (normal image size), which explains the apparent lack of expected detail. **Middle Panel.** Evidence of increased hemoglobin immunoreactivity on Western immunoblot in the placebo chow–α-thrombin group compared to the other groups. **Lower panel.** Immunoreactive hemoglobin relative to β-actin from immunoblots in the middle panel. A general linear model analysis of the hemoglobin disposition found a significant α-thrombin effect ($F_{1,32}$=8.79, p=0.006), a dabigatran effect of borderline significance ($F_{1,32}$=3.75, p=0.062), and a large α-thrombin x dabigatran interaction ($F_{1,32}$=11.19, p=0.002). Subsequent pair-wise comparisons of the main effects and interaction terms found that injection of α-thrombin was associated with increased hemoglobin disposition, where the significant interaction owes to the significantly higher hemoglobin disposition in the placebo chow–α-thrombin group than any of the other three groups which were not different among themselves (Bonferroni, α=0.05).

In the **Figure 7 with Data** above, means ± standard deviations are depicted for each cohort data set.

SUPPLEMENTAL FIGURES WITH DATA POINTS

Supplemental Figure 3 with Data

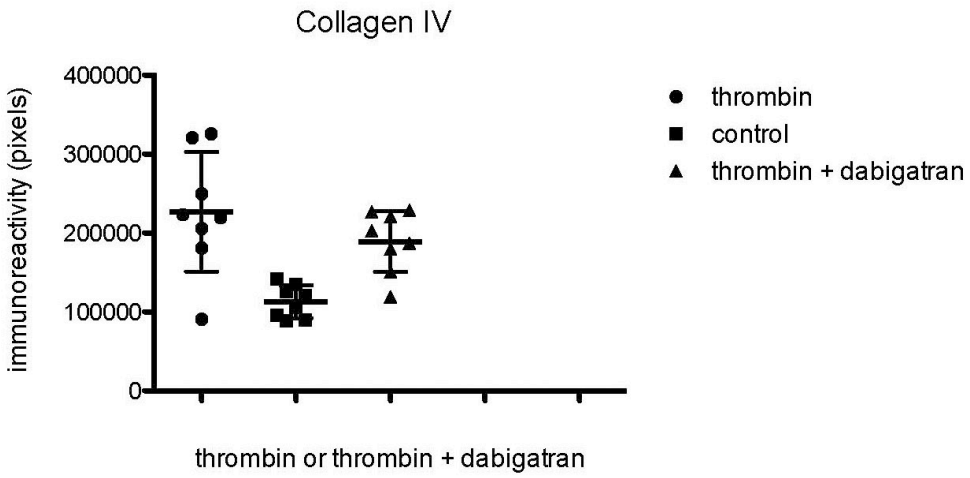


Supplemental Figure 3 (see above for the full Legend)

α-thrombin and dabigatran etexilate differentially affect microvessel basal lamina collagen IV α1-chain immunoreactivity in the striatum of the non-human primate (*Papio anubis/cynocephalus*). In these preliminary studies, collagen IV α1-chain immunoreactivity in striatal microvessels was significantly decreased by exposure to α-thrombin in a concentration-dependent manner from 0.02U to 20U α-thrombin. For the experiment shown here 2U α-thrombin was chosen. Collagen IV levels differed significantly across the five groups (n = 3 each).

In the **Supplemental Figure 3 with Data** above, means ± standard deviations are depicted for each cohort data set.

Supplemental Figure 4 with Data



Supplemental Figure 4 (see above for the full Legend)

The α-thrombin-induced decrease in microvessel matrix collagen IV is abrogated by dabigatran etexilate *in vivo*.
Panel B. Microvessel collagen IV α1-chain immunoreactivity differed significantly among the control, α-thrombin, and α-thrombin + dabigatran etexilate cohorts (n=8 each).

In the **Supplemental Figure 4 with** Data (panel B) above, means ± standard deviations are depicted for each cohort data set.