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Mutation of the Kunitz-type proteinase inhibitor domain in the amyloid β -protein precursor abolishes its anti-thrombotic properties *in vivo*

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Abstract

Introduction—Kunitz proteinase inhibitor (KPI) domain-containing forms of the amyloid β -protein precursor (A β PP) inhibit cerebral thrombosis. KPI domain-lacking forms of A β PP are abundant in brain. Regions of A β PP other than the KPI domain may also be involved with regulating cerebral thrombosis. To determine the contribution of the KPI domain to the overall function of A β PP in regulating cerebral thrombosis we generated a reactive center mutant that was devoid of anti-thrombotic activity and studied its anti-thrombotic function *in vitro* and *in vivo*.

Methods—To determine the extent of KPI function of A β PP in regulating cerebral thrombosis we generated a recombinant reactive center KPI^{R13I} mutant devoid of anti-thrombotic activity. The anti-proteolytic and anti-coagulant properties of wild-type and R13I mutant KPI were investigated *in vitro*. Cerebral thrombosis of wild-type, A β PP knock out and A β PP/KPI^{R13I} mutant mice was evaluated in experimental models of carotid artery thrombosis and intracerebral hemorrhage.

Results—Recombinant mutant KPI^{R13I} domain was ineffective in the inhibition of pro-thrombotic proteinases and did not inhibit the clotting of plasma *in vitro*. A β PP/KPI^{R13I} mutant mice were similarly deficient as A β PP knock out mice in regulating cerebral thrombosis in experimental models of carotid artery thrombosis and intracerebral hemorrhage.

Conclusions—We demonstrate that the anti-thrombotic function of A β PP primarily resides in the KPI activity of the protein.

Graphical abstract

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Conflicts of Interest: None.

Keywords

amyloid β -protein precursor; Kunitz-type proteinase inhibitor; protease nexin-2; thrombosis; knock in mutation mice; hemorrhage

Introduction

The amyloid β -protein precursor (A β PP) is most often recognized as the source of amyloid β -peptides (A β) that accumulate in the brains of patients with Alzheimer's disease and related disorders [1]. Full length A β PP is a large type I integral membrane protein encoded by a gene located on chromosome 21 [2,3]. A β PP is translated from primarily three alternatively spliced mRNAs resulting in polypeptides of 695, 751 and 770 amino acids where the latter two species contain an additional Kunitz-type serine proteinase inhibitor (KPI) domain [4,5]. Secreted KPI-containing isoforms of A β PP are analogous to the previously identified cell secreted proteinase inhibitor known as protease nexin-2 (PN2) [6].

Earlier work demonstrated that PN2/A β PP is a potent inhibitor of several key proteinases involved in blood coagulation including factors XIa, IXa, Xa, and the VIIa:tissue factor complex with inhibition equilibrium constants that range in the nM to pM range [7–11]. This function suggests a role for PN2/A β PP in the regulation of thrombotic pathways leading to clot formation. Indeed, both PN2/A β PP and its purified KPI domain were shown to be effective inhibitors of the clotting of plasma *in vitro* [9,12].

The isoform of A β PP that lacks the KPI domain is primarily expressed in neurons in the brain [13]. However, the KPI-containing PN2/A β PP isoforms are also abundantly found in the central nervous system and its expression increases with age in the cerebral compartment [14,15]. Within the brain, PN2/A β PP is expressed in the parenchyma, mainly by glial cells, and in cerebral blood vessels by smooth muscle cells, microvascular pericytes and endothelial cells [16–20]. In addition to brain, PN2/A β PP is also expressed peripherally, most notably in circulating blood platelets [21]. This finding, coupled with its potent anti-thrombotic properties suggests that PN2/A β PP plays a role in the periphery in regulating thrombosis at sites of vascular injury. Moreover, the abundance of PN2/A β PP in the brain and in cerebral blood vessels suggests that this protein may serve a more local role as an intracerebral anti-thrombotic [22,23].

In this regard, earlier we showed that modest over-expression of PN2/A β PP in the brain or in the platelets of transgenic mice caused a significant reduction of cerebral thrombosis *in vivo* [23,24]. On the other hand, removal of PN2/A β PP in A β PP gene knock out mice increases cerebral thrombosis [23,25]. Together, these findings suggest changes in the levels of PN2/A β PP can impact cerebral thrombosis. However, in addition to the KPI domain of PN2/A β PP all isoforms of A β PP harbors many different biologically active extracellular domains that could potentially influence thrombosis. For example, all isoforms of A β PP have two high affinity heparin binding sites were identified in the N-terminal region of A β PP that may participate in substrate adhesion [26,27]. Similarly, high affinity binding sites for Zn²⁺ and Cu²⁺ binding were localized on N-terminal region of all A β PP isoforms [28–30]. It is noteworthy that with regards to PN2/A β PP both heparin and Zn²⁺ binding was shown to enhance the inhibition of factor XIa [7,31]. Further, fibrillar A β was shown to bind with high affinity to a region between residues 95-118 on all A β PP isoforms and in the case of PN2/A β PP this interaction can promote its inhibition of pro-thrombotic proteinases [32,33]. Additionally, other common domains on the extracellular portion of all A β PP isoforms can participate in cell adhesion [34,35] and regulation of Ca²⁺ homeostasis [36,37]. Therefore, it is plausible that in addition to the specific KPI-mediated inhibition of pro-thrombotic proteinases other regions of A β PP, that are common to all isoforms, might also impact cerebral thrombosis. In the present study, we mutated the reactive center arginine residue of the KPI domain to evaluate its impact on the ability of A β PP to regulate cerebral thrombosis.

Materials and Methods

Reagents and Chemicals

Human factors XIa, Xa, IXa and VIIa were obtained from Enzyme Research Laboratories (South Bend, IN). Bovine trypsin, *p*-nitrophenyl guanidinobenzoate, tosyl-Gly-Pro-Arg-*p*-nitroanilide, N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and methoxycarbonyl-D-cyclohexylGly-Gly-Arg-*p*-nitroanilide were from Sigma (St. Louis, MO). Pyro-Glu-Pro-Arg-*p*-nitroanilide and reagents for the activated partial thromboplastin time (APTT) were purchased from Helena Laboratories (Beaumont, TX). Recombinant tissue factor was from Haematologic Technologies (Essex Junction, VT).

Expression and Purification of Recombinant KPI Domains of PN2/A β PP

The construction of *Pichia pastoris* vector, pKPI200, encoding the wild-type KPI sequence has been described [38]. To replace the reactive center arginine at position 17 with isoleucine, we utilized a previously described two-step PCR mutagenesis process to introduce the site-specific mutation to replace the arginine codon (AGA) with an isoleucine codon (ATA) [39]. The expression and purification of the wild-type KPI domain and the mutant KPI^{R13I} domain were performed as previously described [38,39].

Proteinase Inhibition Measurements

The active site of trypsin was titrated by the method of Chase and Shaw [40] using the burst titrant *p*-nitrophenyl guanidinobenzoate. This trypsin was used to titrate the proteinase inhibitory activity of the purified wild-type KPI domain as previously described [38,39].

Inhibition equilibrium constants (K_i) for the purified KPI domains and the proteinases were determined by the method of Bieth [41] as we previously described [11,38,39]. Graphical analysis yielded an apparent K_i ($K_{i,app}$). Since the inhibition is reversible a correction must be made for the K_m of the protease-substrate reaction. The K_m for each proteinase and its corresponding chromogenic substrate was determined independently and the true K_i was calculated using the following equation:

$$K_i = \frac{K_{i,app}}{1 + [S]/K_m}$$

APTT assay

Microtiter plate APTT coagulation assays were conducted as previously described [42]. Briefly, 30 μ l of either pooled normal, human citrated-plasma or mouse citrated-plasma, 30 μ l of APTT reagent, and 30 μ l of Tris buffered-saline were incubated in triplicate microtiter plate wells in the absence or presence of increasing concentrations of the purified KPI domains for 10 min at 22° C. The APTT assay was initiated by the addition of 30 μ l of 25 mM $CaCl_2$ and the time to clot formation was monitored by the change in absorbance at 405 nm in a Spectramax kinetic microtiter plate reader (Molecular Devices, Sunnyvale, CA). The absorbance readings were recorded every 5 sec for 3 to 5 min.

Generation of A β PP/KPI^{R13I} mutant knock in mice

The targeting strategy was aimed at introducing two point mutations in the arginine codon (CGC to ATC) on a 10 kb long arm fragment 300 bp downstream of exon 7 of the mouse *APP* gene which would effectively introduce the R13I substitution in the KPI domain. The short arm (a 1.4 kb DNA fragment) was amplified with primers APPSA2, 5' CCCAGATATCCAGGAAAGGG 3' and APPSA1, 5' CTTCTGACTGCTTCTTTACAC 3' and inserted into PspOMI site of Neo gene cassette, flanked by lox P sites. The targeting vector was subsequently linearized by Not I and electroporated into 129/Sv(ev) embryonic stem cells. The resulting colonies were subsequently treated with G418 for selection. PCR analysis using primers APPSA2, 5' AAGTGGAGTTATCTCCTAGGAGACC 3' (located 100 bp downstream from APPSA2), and Neo 1, 5' TGCGAGGCCAGAGGCCACTTGTGTAGC 3', (located within the 5' promoter region of the Neo gene cassette) resulting in a 1.5 kb PCR fragment, was performed on stably transfected clones to identify successful homologous recombination.

The correctly targeted ES cell lines were injected into 3.5 day-old C57Bl/6 blastocysts that were transferred into the uteri of pseudopregnant Cd1 foster mothers. Chimeric mice were generated and screened for germline transmission of the A β PP point mutation. For genotyping purposes the wild-type *APP* allele is identified with primer LOX98AN9, 5' TAGAGGATCCCCGCGCTGGACCT 3', and antisense APPKI7, 5' CACCATGATAGCACCTGAGGTGAC 3', resulting in a 300bp PCR product. Heterozygous offspring, positive for the A β PP/KPI^{R13I} point mutation, were crossbred with Jackson EIIa transgenic mice to remove the Neo gene cassette. Identification of Neo deletion was analyzed by PCR analysis using primers APPKI2, 5' TGTGGCCATGGTGTCTCTTCACAG 3' which is 360 bp downstream of APPSA1 and

APPKI1, 5' CAACTCGACTCTAGCCTAGGATGC 3' located upstream of APPSA1 with some vector sequence resulting in a 500 bp PCR product. Homozygous A β PP/KPI^{R13I} mutant knockin mice were identified with a 600 bp PCR product using primer APPKI8, 5' TGCTTCCTCCGTTTCAGCCCAGGTC 3', and antisense primer LOX98AN9, 5' GCTAATTCCGATCATATTCAATAACCC 3', and no PCR fragment with LOX98AN9/APPKI7 primers. The A β PP/KPI^{R13I} mutant knock in mice were backcrossed for ten generations onto a C57/Bl6 background.

Animals

All work with mice was approved by the Stony Brook University Institutional Animal Care and Use Committee and in compliance with the guidelines established by the Public Health Service Guide for the Care and Use of Laboratory Animals.

Carotid Artery Thrombosis Assay

This assay was performed essentially as described by Eitzman et al. [43] and induces a fibrin- and platelet-rich clot. Briefly, mice (8–12 weeks of age) were prepared for surgery and anesthetized by i.p. injection of sodium pentobarbital (70 mg/kg). A midline neck incision was made to expose the common carotid artery, which was then cradled by a miniature flowmeter probe to record blood flow rate and ultimately determining presence of thrombosis. Then 0.1 ml of rose bengal [4,5,6,7-tetrachloro-3',6'-dihydroxy 2',4',5',7' tetraiodospiro[isobenzofuran 1(3H),9[9H]xanthen]-3-one dipotassium salt] (50 mg/kg in 0.9% saline) was injected through the tail vein; the dye is activated by laser light (540 nm) to generate a superoxide anion. The superoxide anion leads to endothelial cell damage with a transient thrombus and subsequent neointima formation. Blood flow rate is monitored within the laser specific area and the time to vessel occlusion and cessation of blood flow was documented.

Experimental Intracerebral Hemorrhage

This procedure was performed as described by Clark et al. [44]. Briefly, mice (8–12 weeks of age) were prepared for surgery and anesthetized by i.p. injection of sodium pentobarbital (70 mg/kg). A sagittal incision was made caudal to rostral, allowing the scalp to be retracted and held in place with microclips. A small hole, 1.0 mm posterior and 3.0 mm lateral of bregma, was drilled to perforate the skull. A 1- μ l Hamilton syringe was used to deliver 500 nl of bacterial collagenase (150 units/ml)/saline to the caudate putamen at a depth of 4.0 mm unilaterally. After the injection of collagenase/saline ($\approx$ 30 sec), the needle remained in place for another 2 min to prevent reflux of fluid. The surgery was concluded with the closing of the scalp skin by using 4-0 nylon sutures. Twenty-four hours after initiation of hemorrhage, the mice were perfused with PBS, the brains were harvested, and 14- μ m sections were prepared by using a cryostat and mounted on glass slides. Sections were stained with hematoxylin, and an Olympus (Melville, NY) BX60 upright systems microscope with a digital camera was used to capture images. The hemorrhage volume was measured by using the STEREOLOGER software system (Alexandria, VA).

Statistical Analysis

Mean differences between groups were analyzed using *t* test. Analysis of data on the time course of carotid artery blood flow was by repeated measures ANOVA. Significance was set at $p < 0.05$ and the results of which are reported in the corresponding figure legends.

Results

Mutation of the KPI reactive center arginine to isoleucine of PN2/A β PP abolishes its anti-thrombotic properties

A recombinant reactive center mutant KPI domain (KPI^{R13I}) and wild-type KPI domain of A β PP was expressed in yeast and purified. The purified KPI domains were analyzed by SDS-PAGE and both migrated at ≈ 6 kDa (Fig. 1). The mutant KPI^{R13I} domain and wild-type KPI domain were then compared for their inhibition of trypsin and pro-thrombotic proteinases. As summarized in Table 1, the wild-type KPI domain of PN2/A β PP was a potent inhibitor of trypsin as well as the pro-thrombotic enzymes Factors XIa, IXa, Xa and tissue factor:VIIa as previously reported [7–11]. In contrast, the mutant KPI^{R13I} domain exhibited no detectable inhibition of any of these proteinases consistent with the requirement of the arginine residue at position 13 to dictate this trypsin-like specificity.

We next determined the activity of the mutant KPI^{R13I} domain towards inhibiting the clotting of pooled human plasma *in vitro*. As shown in Fig. 2, wild-type KPI domain showed a dose-dependent prolongation of clot formation in the APTT assay with 50 μ M increasing this time by six-fold ($p < 0.001$). On the other hand, mutant KPI^{R13I} domain exhibited no effect on *in vitro* clot formation consistent with its lack of ability to inhibit pro-thrombotic enzymes.

Generation of A β PP/KPI^{R13I} knock in mutant mice

We next sought to determine how mutation and inactivation of the KPI domain of endogenous A β PP affects cerebral thrombosis *in vivo*. The targeting strategy was designed to introduce two point mutations in the reactive center arginine codon (CGC to ATC) of the KPI domain of the *APP* gene effectively converting this residue to isoleucine, the same amino acid change that was introduced to the recombinant KPI domain as described above. Homozygous A β PP/KPI^{R13I} knock in mutant mice were identified by a unique 600 bp PCR product using primers to target this mutation (Fig. 3A). The A β PP/KPI^{R13I} knock in mutant mice were then backcrossed for ten generations onto a C57Bl/6 background to maintain a consistent background with our other mouse models. The generated mice were fully viable, fertile and exhibited no overt behavioral phenotype (data not shown). As shown in Fig. 3, A β PP/KPI^{R13I} knock in mutant mice expressed comparable levels of endogenous A β PP protein in brain compared to wild-type mice as assessed by quantitative immunoblotting. Thus, mutating the reactive center arginine residue in the KPI domain did not have any appreciable effect on A β PP levels in brain. Further, pooled plasma collected from A β PP/KPI^{R13I} knock in mutant mice and wild-type mice showed the same time to clot formation in an APTT assay (Fig. 3D). This is consistent with previous findings that platelet-poor plasma contains very little A β PP [45].

A β PP/KPI^{R13I} knock in mutant mice exhibit increased cerebral thrombosis

We next performed experiments to determine the consequences of the A β PP/KPI^{R13I} knock in mutation in mice on carotid artery thrombosis. Fig. 4A shows representative experiments measuring the time to vessel occlusion and loss of carotid artery blood flow in mice of the different genotypes. Measurement of carotid artery thrombosis in wild-type mice revealed a mean time of 52.0 ± 10.0 min to cessation of blood flow (Fig. 4B). In contrast, A β PP knock out mice, which are absent of this protein, showed decreased times for vessel occlusion compared to wild-type mice (34.5 ± 4.0 min, $p < 0.0001$). A β PP/KPI^{R13I} knock in mutant mice, which express comparable amounts of A β PP protein as wild-type mice (Fig. 3), but lack a properly functioning KPI domain, also exhibited a pronounced decrease in time to vessel occlusion (38.5 ± 3.4 min, $p < 0.001$). It was noted that vessel occlusion was slightly, yet significantly, faster in A β PP gene knock out mice ($p < 0.05$). This *in vivo* thrombosis assay indicates that the anti-thrombotic activity of A β PP is primarily controlled through the activity of its KPI domain.

We next determined if the A β PP/KPI^{R13I} knock in mutation might comparably influence cerebral thrombosis during a brain vascular injury event such as intracerebral hemorrhage. A β PP/KPI^{R13I} knock in mutant mice and A β PP gene knock out mice similarly presented with smaller hematomas compared to wild-type mice in experimentally induced intracerebral hemorrhage (Fig. 5). Quantitative measurement of hematoma volumes showed that compared to wild-type mice the hematomas of A β PP/KPI^{R13I} knock in mutant mice and A β PP gene knock out mice were markedly reduced ($p < 0.001$ and $p < 0.001$, respectively). However, the hematomas of the A β PP/KPI^{R13I} knock in mutant mice were significantly smaller than those observed in the A β PP gene knock out mice ($p < 0.05$). Together, these data demonstrate that targeted inactivation of the KPI domain of A β PP was largely comparable to deletion of the entire A β PP protein to produce significant effects on cerebral thrombosis during hemorrhagic brain injury.

Discussion

In the present study, we show that the *in vivo* anti-thrombotic properties of PN2/A β PP appear to largely reside within the function of the KPI domain. The physiological functions of all A β PP isoforms, including KPI-containing and KPI-lacking, have yet to be clearly defined. However, earlier studies have shown that over-expression of PN2, the KPI-containing isoforms of A β PP, in transgenic mice, either in circulating blood platelets or in brain, resulted in significantly decreased cerebral thrombosis in experimental models of carotid artery thrombosis and intracerebral hemorrhage [23–25]. These findings indicate that the KPI function of A β PP influences cerebral thrombosis. On the other hand, the absence of PN2/A β PP was predicted to result in a pro-thrombotic phenotype. Indeed, previous studies have shown that A β PP knock out mice exhibited a significantly reduced time to vessel occlusion in carotid artery thrombosis, significantly reduced hematoma volumes in intracerebral hemorrhage and larger infarcts in ischemic stroke [23–25]. However, in A β PP knock out mice all isoforms of A β PP, both KPI-containing and KPI-lacking, are lacking. Therefore, it is possible that other regions common to all A β PP isoforms could also participate in regulating thrombotic events in these conditions.

In this study, we selectively inactivated the KPI function of A β PP so that we could determine if other activities of the protein also influence cerebral thrombosis. Previously, we showed that arginine 13 residue of the KPI domain dictated its specificity towards trypsin-like pro-thrombotic proteinases and that mutating this residue to lysine enhanced its plasmin inhibitory [39]. Therefore, we mutated this reactive center basic arginine 13 residue of the KPI domain to the similarly sized, but hydrophobic, isoleucine. Indeed, this mutation effectively abolished the inhibitory activity towards trypsin-like pro-thrombotic proteinases and our findings indicate that the anti-thrombotic role of A β PP is primarily served through the function of the KPI domain.

Previously, two distinct proteins were identified that share a high degree of structural homology with A β PP and are known as A β PP-like protein-1 (APLP1) and APLP2, [46–48]. APLP1 does not contain a KPI domain [46]. However, APLP2 does harbor a KPI domain that is highly homologous and has overlapping proteinase inhibitory properties with the KPI domain contained in A β PP [47–49]. Like A β PP knock out mice, knock out mice for each A β PP-like protein exhibited no overt phenotype [50]. However, when challenged APLP2 knock out mice exhibit a similar pro-thrombotic phenotype to A β PP knock out mice in experimental models of cerebral vascular thrombosis [25,51]. The tight functional redundancy between A β PP and APLP2 may abrogate any thrombotic alteration observed in the animal due to the loss of one or the other protein. Interestingly, A β PP/APLP1 double knock out mice are viable without an overt phenotype. In this case, the double knock out mice still possess APLP2, which has KPI activity. On the other hand, A β PP/APLP2 double knock out mice, which lack the shared KPI domain function, are post-natal lethal with the animals dying several hours after birth [50]. Although the reason for the lethality in the A β PP/APLP2 double knockout remains unclear it indicates that some redundant function between A β PP and APLP2 is required for viability. The most striking difference between A β PP and APLP2 compared to APLP1 is the presence of a KPI domain. This suggests that in addition to its anti-thrombotic activity, the KPI domain of A β PP, and perhaps APLP2, could serve other essential proteolytic regulatory functions in the brain.

Although the A β PP/KPI^{R13I} knock in mutant mice and A β PP gene knock out mice were markedly different compared to wild-type mice with respect to experimental carotid artery thrombosis and intracerebral hemorrhage they were more subtly, yet significantly, different when compared to each other. For example, the A β PP gene knock out mice exhibited a slightly shorter time to vessel occlusion compared to the A β PP/KPI^{R13I} knock in mutant mice in the carotid artery thrombosis model. This is consistent with earlier studies showing that A β PP gene knock out mice present with larger photo-induced thrombotic lesions and decreased cerebral blood flow and increased risk of mortality in response to global cerebral ischemia as compared to wild-type mice [25,52]. This supports that other regions or fragments of A β PP, such as the vasoactive A β peptide, can also contribute to ischemic damage. On the other hand, in the intracerebral hemorrhage model A β PP/KPI^{R13I} knock in mutant mice had somewhat smaller hematoma volumes compared to the A β PP gene knock out mice suggesting that the latter are slightly more pro-thrombotic and have a lesser tendency to bleed. In any case, the present data indicate that the anti-thrombotic function of A β PP primarily resides with the KPI domain but that other regions and/or fragments of the A β PP can also participate in the maintenance of cerebral hemostasis and blood flow.

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Highlights

- The recombinant KPI domain of A β PP inhibits pro-thrombotic proteinases
- Recombinant mutant KPI^{R13I} domain does not inhibit pro-thrombotic proteinases
- A β PP/KPI^{R13I} mutant mice were deficient in regulating cerebral thrombosis in vivo
- The anti-thrombotic function of A β PP largely resides within its KPI activity

Figure 1. SDS-PAGE analysis of wild-type KPI domain and mutant KPI^{R13I} domain
Wild-type KPI domain (lane 1) and mutant KPI^{R13I} domain (lane 2) were expressed at high levels in the *P. pastoris* system, purified and analyzed by SDS-PAGE.

Figure 2. Mutant KPI^{R13I} domain does not inhibit the coagulation of plasma *in vitro*
 Increasing concentrations of purified recombinant wild-type KPI domain (gray bars) or mutant KPI^{R13I} domain (blue bars) were tested for their abilities to prolong coagulation using a microtiter plate APTT assay. The data presented are the mean $\pm$ S.D. of triplicate measurements from three separate experiments. * $p < 0.0001$.

Figure 3. Characterization of AβPP/KPI^{R13I} knock in mutant mice
(A) The AβPP/KPI^{R13I} knock in mutant mice are distinguished from wild-type mice based on differential PCR analysis using the primer APPKI8, 5' TGCTTCCTCCGTTTCAGCCCAGGTC 3', and antisense primer LOX98AN9, 5' GCTAATTCGATCATATTCAATAACCC 3' generating 300 and 600 bp products, respectively. DNA from wild-type mice (lanes 2 and 3) and homozygous AβPP/KPI^{R13I} mutant mice (lanes 4 and 5) were screened with primer sets for the wild-type *APP* allele (lanes 2 and 4) or the *APP/KPI^{R13I}* allele (lanes 3 and 5). Lane 1, 100 bp ladder standard.
(B) Representative immunoblots for AβPP (top panel) and actin (bottom panel) in brain homogenates from wild-type and AβPP/KPI^{R13I} knock in mice. **(C)** Quantitation of AβPP levels in the brains of wild-type mice (black bar) and AβPP/KPI^{R13I} knock in mice (blue bar). Data presented are the mean ± SD from four mice of each genotype; n.s. = not specific.
(D) The clotting of pooled plasma collected from n = 6 wild-type mice (black bar) and n = 8 AβPP/KPI^{R13I} knock in mice (blue bar) was determined using a microtiter plate APTT assay. The data presented are the mean ± S.D. of four replicate measurements from three separate experiments; n.s. = not specific.

Figure 4. AβPP/KPI^{R13I} mutant mice exhibit increased carotid artery thrombosis

(A) Mice of different genotypes were injected with the photoactivated dye Rose Bengal at $t = 0$ and the carotid artery was exposed to a laser light. Blood flow through the carotid artery was monitored with a flow probe. Blood flow ceased due to thrombus formation in the vessel. Measurement of carotid artery blood flow from $n = 8$ wild-type mice; (black line), $n = 10$ AβPP^{-/-} mice (red line) and $n = 10$ AβPP/KPI^{R13I} mutant mice (blue line). The data presented are the mean $\pm$ S.E.M. Repeated measures ANOVA confirmed significant main effects of genotype ($p < 0.03$) and genotype $\times$ time point interaction ($p < 0.0001$). (B) Measurement of the time to carotid artery occlusion in $n = 8$ wild-type mice; (●), $n = 10$ AβPP^{-/-} mice (●) and $n = 10$ AβPP/KPI^{R13I} mutant mice (●). Bars represent the mean. * $p < 0.05$; ** $p < 0.001$, *** $p < 0.0001$.

Figure 5. AβPP/KPI^{R13I} mutant mice exhibit decreased hematoma volumes in experimental intracerebral hemorrhage

Mice were stereotactically injected with collagenase in the caudate/putamen. Twenty four hours later the brains were removed and analyzed for hematomas. Stained brains sections from AβPP^{-/-} mice (A), wild-type mice (B) and AβPP/KPI^{R13I} mutant mice (C).

Quantitation of hematoma volume from n = 14 wild-type mice (●), n = 8 AβPP^{-/-} mice (●) and n = 12 AβPP/KPI^{R13I} mutant mice (●) at 24 h. Bars represent the mean. **p* < 0.05; ***p* < 0.001.

Table 1
Proteinase inhibition constants for mutant KPI^{R13I} domain and wild-type KPI domain of PN2/AβPP

The proteinase inhibition equilibrium constants (K_i) were determined as described in “Materials and Methods”. The data represent the mean $\pm$ SD of 4 separate determinations.

Proteinase	KPI ^{R13I}	wild-type KPI
K_i (M)		
Trypsin	not detected	$2.7 \pm 1.5 \times 10^{-10}$
Factor XIa	not detected	$2.5 \pm 1.2 \times 10^{-10}$
Factor IXa	not detected	$2.8 \pm 1.7 \times 10^{-8}$
Factor Xa	not detected	$1.3 \pm 0.9 \times 10^{-8}$
Factor VIIa + tissue factor	not detected	$6.9 \pm 0.5 \times 10^{-8}$