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Kininogen deficiency protects from ischemic neurodegeneration in mice by reducing thrombosis, blood-brain-barrier damage and inflammation

Short title: Kininogen promotes stroke

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Abstract

Thrombosis and inflammation are hallmarks of ischemic stroke still unamenable to therapeutic interventions. High molecular weight kininogen (KNG) is a central constituent of the contact-kinin system which represents an interface between thrombotic and inflammatory circuits and is critically involved in stroke development. *Kng*$^{-/-}$ mice are protected from thrombosis after artificial vessel wall injury and lack the proinflammatory mediator bradykinin. We investigated the consequences of KNG deficiency in models of ischemic stroke. *Kng*$^{-/-}$ mice of either sex subjected to transient middle cerebral artery occlusion (tMCAO) developed dramatically smaller brain infarctions and less severe neurological deficits without an increase in infarct-associated hemorrhage. This protective effect was preserved at later stages of infarction as well as in elderly mice. Targeting KNG reduced thrombus formation in ischemic vessels and improved cerebral blood flow, and reconstitution of KNG-deficient mice with human KNG or bradykinin restored clot deposition and infarct susceptibility. Moreover, mice deficient in KNG showed less severe blood-brain-barrier damage and edema formation and the local inflammatory response was reduced compared with controls. As KNG appears to be instrumental in pathologic thrombus formation and inflammation but dispensable for hemostasis, KNG inhibition may offer a selective and safe strategy for combating stroke and other thromboembolic diseases.

Key Words: Stroke, FXII, kallikrein-kinin system, inflammation, thrombosis

Introduction

The pathology of ischemic stroke is complex and involves a myriad of distinct molecular pathways and cellular interactions. Among these, progressive thrombus formation in the cerebral microvasculature is a key process that can cause secondary infarct growth despite successful recanalization of larger proximal brain vessels both under experimental conditions as well as in humans.[1,2] We recently identified the intrinsic coagulation cascade as a novel and safe antithrombotic target for the prevention and treatment of acute ischemic stroke.[3] Genetic depletion or pharmacological blockade of coagulation factor XII (FXII), the origin of the intrinsic pathway, markedly reduced intracerebral thrombus formation and infarct growth in mice without increasing the risk of bleeding complications.[4,5] Clot formation was also significantly reduced in several in vitro models of thrombosis following FXII inhibition.[5] Current pathophysiological concepts also emphasize the importance of inflammatory mechanisms in stroke.[6] The cerebral endothelium is activated early during the course of an ischemic event leading to the up-regulation of cell adhesion molecules and successive trafficking of inflammatory cells (neutrophils, macrophages, T cells) from the blood stream into the brain parenchyma. Those cells attracted from the periphery in concert with resident cell populations (endothelial cells, microglia) secrete an array of soluble immune mediators such as cytokines and chemokines that perpetuate the inflammatory response to cause direct or indirect tissue damage. In case of persisting ischemia the structural components forming the blood-brain-barrier, such as tight-junction proteins, become disengaged leading to the formation of vascular edema.[7] Edema, in turn, harms otherwise healthy brain areas by compression and is a frequent cause of delayed functional deterioration in stroke patients. Pharmacologic strategies to combat inflammation or edema formation in acute ischemic stroke are not currently available.[8] Interestingly, however, there is increasing evidence of a

tightly regulated interplay between thrombotic and inflammatory mechanisms during stroke development and this 'thrombo-inflammation' might be accessible to specific therapeutic interventions.[9]

High-molecular-weight kininogen (KNG) is an important constituent of the plasma contact-kinin system which represents a network of serially connected serine proteases.[10] Activation of the contact-kinin system by FXII triggers cleavage of KNG by plasma kallikrein (PK) and subsequent release of the proinflammatory peptide hormone bradykinin. The contact-kinin system occupies a central position in the pathophysiology of different neurological disease models mimicking, for example, multiple sclerosis[11] or traumatic brain injury (TBI).[12] In acute ischemic stroke activation of the contact-kinin system fosters vascular permeability and stroke-related inflammation by the formation of short-lived kinins while at the same time it is linked to thrombus formation via the FXII-driven intrinsic coagulation cascade.[4,5,13] Therefore, the contact-kinin system represents a promising multifunctional target for potential stroke therapies. However, to which extent the different molecular constituents of the contact-kinin system contribute to stroke development has been largely unknown, a situation resulting from the lack of appropriate transgenic mouse models.

To address this shortcoming, we analyzed functional outcome, thrombus formation and inflammatory processes in models of acute ischemic stroke in KNG-deficient mice recently described by our group.[14]

Methods

A detailed description of the methods, the surgical procedures and the stroke study population is provided in the **Supplemental Material**.

Ischemia model. A total of 298 mice (149 C57Bl/6 wild-type mice and 149 *Kng*$^{-/-}$ mice) were included in the study which was conducted in accordance with institutional guidelines (University of Würzburg, Germany) for the use of experimental animals, and the protocols were approved by governmental authorities (Regierung von Unterfranken, Würzburg, Germany). *Kng*$^{-/-}$ mice were described previously[14] and were backcrossed for more than 10 generations into a C57Bl/6 background. Age- and sex-matched C57Bl/6 mice (Charles River) served as controls. If not otherwise mentioned, we performed 60 min transient middle cerebral artery occlusion (tMCAO) in 6 weeks old male mice weighing 20 – 25 g, as described.[4,15] To exclude age- or gender-specific effects, 6 month old male or 6 week old female mice were used in some subgroups. For permanent MCAO (pMCAO) the occluding filament was left in situ until sacrificing the animals. Mice were controlled for physiological parameters that can critically affect stroke outcome (cerebral blood flow, blood pressure, heart rate, brain structure, blood gases) (Supplementary Figures 1 – 3, Supplementary Table 1). For reconstitution experiments, high molecular weight kininogen from human plasma (Calbiochem) was injected intravenously in a subgroup of *Kng*$^{-/-}$ mice at a dosage of 4 µg/g body weight[16] immediately before the induction of tMCAO. In order to reconstitute bradykinin levels in the central nervous system (CNS) of *Kng*$^{-/-}$ mice, *Kng*$^{-/-}$ mice received an intrathecal (i.t.)[17] injection of bradykinin (Sigma, 100 ng dissolved in 5 µl PBS)[18,19] immediately after the induction of tMCAO and again at the time of reperfusion. We calculated edema-corrected infarct volumes from coronal 2,3,5-triphenyltetrazolium chloride (TTC) stained brain slices. All stroke experiments were performed in accordance with the recently published ARRIVE guidelines

(http://www.nc3rs.org/ARRIVE). Animals were randomly assigned to the operators by an independent person not involved in data acquisition and analysis. We performed surgery and evaluation of all read-out parameters while being blinded to the experimental groups.

Functional outcome tests. We assessed the Bederson score[20] and the grip test score[21] on day 1 and day 3 after tMCAO or pMCAO to monitor global neurological function, motor function and coordination in mice. Mortality rates were assessed on a daily basis until day 8 post stroke.

Magnetic resonance imaging (MRI). To analyze infarct dynamics and to scan for possible intracerebral bleeding serial stroke assessment by MRI was performed 24h, and again 7d after tMCAO on a 1.5 Tesla unit (Vision, Siemens) using T2-w imaging sequences and blood-sensitive T2-w gradient echo constructed interference in steady state (CISS) sequences as described.[4,22]

Laser Doppler flowmetry. Laser Doppler flowmetry (Moor Instruments) was used to monitor regional cerebral blood flow (CBF) in the territory of the right middle cerebral artery at baseline (before ischemia), immediately after insertion of the occluding filament (ischemia), immediately after removal of the occluding filament (reperfusion) and again 12h and 24h after reperfusion.[17]

Real-time PCR studies. Real-time PCR (StepOnePlus™ Real-Time PCR System, Applied Biosystems) was used to determine relative gene expression levels of inflammatory markers in the ischemic cortices and basal ganglia as described.[13]

Determination of blood-brain-barrier damage and brain edema. Blood-brain-barrier damage was quantified by photometric measurement (Fluoroskan Ascent, Thermo Scientific) of Evan's Blue dye (2% in 100 µl PBS i. v., Sigma) leaking into the brain parenchyma.[13] The free water content of the brains (edema) was calculated from the brains' wet/dry weights.[13]

Immunohistology. Staining protocols for immune cells, fibrin(ogen), occludin and the assessment of the thrombosis index are described in the **Supplemental Material**.

Western blot. Immunoreactivity for fibrin(ogen) (anti-Fibrin(ogen) pAb 1:500, cross reactive for fibrin and fibrinogen, Acris Antibodies) and occludin (anti-occludin pAB 1:1000, Abcam) was detected by Western blot and quantified by densitometry. For the detection of KNG a murine monoclonal antibody to the light chain of high molecular weight KNG was used (anti-KNG mAb 1:100, Clone C11C1, Abcam). Actin served as loading control for all Western blot experiments.

Statistics

All results were expressed as mean ± standard error of the mean (s.e.m.) except for ordinal functional outcome scales which were depicted as scatter plots including median with the 25% percentile and the 75% percentile given in brackets in the text. Numbers of animals (N = 10) necessary to detect a standardized effect size on infarct volumes ≥ 0.2 (wild-type mice versus *Kng*$^{-/-}$ mice) were determined via a priori sample size calculation with the following assumptions: $\alpha = 0.05$, $\beta = 0.2$, mean, 20% standard deviation of the mean (GraphPad Stat Mate 2.0, GraphPad Software). For statistical analysis, the GraphPad Prism 5.0 software package (GraphPad Software)

was used. Data were tested for Gaussian distribution with the D`Agostino and Pearson omnibus normality test and then analyzed by 1-way analysis of variance (ANOVA) or in case of measuring the effects of two factors simultaneously 2-way ANOVA with post hoc Bonferroni adjustment for P values. If only two groups were compared, unpaired, two-tailed Student's *t*-test was applied. Non-parametric functional outcome scores were compared by Kruskal-Wallis test with post hoc Dunn's multiple comparison test. For comparison of survival curves the log rank test was used. P-values < 0.05 were considered statistically significant.

Results

Kininogen deficiency provides sustained protection from ischemic stroke

In the first set of experiments we investigated the protein expression of KNG in the ischemic brains from wild-type mice with induced focal cerebral ischemia or sham-operated controls by immunoblot (Figure 1). Here, we chose a model of ischemic stroke in which mice are subjected to transient middle cerebral artery occlusion (tMCAO). This model induces a rapid and strong activation of the contact-kinin system leading to local inflammation and progressive microvascular thrombosis within the brain.[4,13] While native KNG was present in the brains of sham-operated mice, it was strongly down-regulated in the ischemic (ipsilateral) and contralateral hemispheres of mice with cerebral ischemia 24h after tMCAO (Figure 1). Downregulation of KNG also in the contralateral hemispheres was probably due to excessive ipsilateral infarct-related edema formation and subsequent compression of essentially 'healthy' (contralateral) brain regions under the experimental condition of 60 min tMCAO (see below). Conversely, cleaved KNG which is formed when bradykinin is released from native KNG by plasma kallikrein was abundant after stroke but was absent in brains from sham-operated animals (Figure 1). This

indicates that KNG is consumed in the cerebral circulation and/or tissue during brain ischemia and this observation would be in agreement with a major functional role for KNG in ischemic stroke.

To investigate the functional role of KNG in acute ischemic stroke, *Kng*$^{-/-}$ mice and wild-type controls of different age and gender were used (Figure 2). We first subjected 6 weeks old male *Kng*$^{-/-}$ mice to tMCAO and, after 24h, assessed infarct volumes by staining brain sections with 2,3,5-triphenyltetrazolium chloride (TTC) (Figure 2A). Infarct volumes were dramatically smaller, by approximately 75%, in male *Kng*$^{-/-}$ mice than in sex-matched wild-type controls (128.6 ± 6.6 mm^3 [wild-type] vs. 36.4 ± 8.7 mm^3 [*Kng*$^{-/-}$], $P < 0.0001$) (Figure 2A).

The smaller infarct volume was functionally relevant: Compared with wild-type mice, *Kng*$^{-/-}$ mice had significantly better overall neurological function (Bederson score: median 3.0 [2.0, 3.25] [wild-type] vs. 1.0 [1.0, 2.0] [*Kng*$^{-/-}$], $P < 0.05$) as well as basal motor function and coordination (grip test score: median 2.0 [0.0, 3.0] [wild-type] vs.4.0 [3.0, 4.0] [*Kng*$^{-/-}$], $P < 0.05$) 24h after tMCAO (Figure 2B).

To prove that the protective effect was specifically related to KNG deficiency and not to a secondary, i.e. unspecific, effect of the deficiency state, we reconstituted *Kng*$^{-/-}$ mice with human KNG in an attempt to rescue the wild-type phenotype. Intravenous infusion of human KNG (4 µg/g body weight) restored susceptibility to ischemic brain damage after tMCAO. Infarct volumes (128.6 ± 9.6 mm^3, $P > 0.05$) and neurological outcome parameters (Bederson score: 3.0 [3.0, 4.0], $P > 0.05$; grip test score: 0.5 [0.0, 1.5], $P > 0.05$) for *Kng*$^{-/-}$ mice treated with human KNG were indistinguishable from wild-type mice (Figure 2A, B).

Kng$^{-/-}$ mice are unable to produce bradykinin and blood bradykinin levels are below the detection limit in these mutants.[14] To address whether bradykinin released from KNG is indeed a critical mediator of neuronal damage after stroke, we reconstituted

Kng$^{-/-}$ mice with bradykinin. Since bradykinin has a very short half life time in the blood and in order to obtain maximum bradykinin concentrations directly within the CNS, we decided to inject bradykinin intrathecally immediately after the induction of tMCAO and again at the time point of reperfusion (100 ng per injection).[18,19] Reconstitution of *Kng*$^{-/-}$ mice with bradykinin rescued the phenotype and induced infarct volumes (121.6 ± 13.0 mm^3, $P > 0.05$) and functional deficits (Bederson score: 3.5 [3.0, 4.0], $P > 0.05$; grip test score: 0.0 [0.0, 1.5], $P > 0.05$) similar to those seen in wild-type mice on day 1 after tMCAO (Figure 2A, B).

Gender can significantly influence stroke outcome in rodents.[23] Therefore, we also subjected female *Kng*$^{-/-}$ mice to 60 min tMCAO. Consistent with the results in male mice, KNG-deficient female mice also developed significantly smaller infarctions ($P < 0.001$) and less severe neurological deficits ($P < 0.05$) compared to female controls (Figure 2A, B).

Ischemic stroke usually is a disease of the elderly and consequently, it is recommended to verify any stroke-protective effects observed in young adult laboratory animals also in an older cohort.[23] Indeed, 6 months old *Kng*$^{-/-}$ mice also had significantly smaller stroke volumes ($P < 0.05$) and a better functional outcome ($P < 0.05$, grip test score) compared to age-matched controls thereby confirming our results in young animals (Figure 2A, B).

We also determined the functional outcome and mortality of 6 weeks old male *Kng*$^{-/-}$ mice and matched wild-type controls over a longer time period after ischemic stroke. Four days after 60 min tMCAO, 5/10 wild-type mice (50%) had died, consistent with previous reports[17] (Figure 2C). In contrast, 10/10 *Kng*$^{-/-}$ mice (100%) survived until day 4, and 7/10 (70%) were still alive after 8 days ($P = 0.0075$). In line with these findings, KNG-deficient mice showed significantly smaller strokes ($P < 0.001$) and a better Bederson score ($P < 0.05$) than controls on day 3 (Figure 2A, B). These

observations exclude the possibility that KNG deficiency simply induces faster recovery from stroke but underlines its sustained effect on stroke outcome.

According to current experimental stroke guidelines,[23] any protective effect requires evaluation in models of both transient and permanent ischemia. We therefore subjected *Kng*$^{-/-}$ mice to filament-induced permanent middle cerebral artery occlusion (pMCAO), a procedure in which no tissue reperfusion occurs. In contrast to the striking effects observed after tMCAO, KNG deficiency did not significantly influence stroke size ($P > 0.05$) or neurological outcome ($P > 0.05$) 24h after pMCAO (Figure 2A, B).

KNG deficiency reduces thrombosis after stroke without increasing the risk of intracerebral hemorrhage

It has been shown that the *Kng*$^{-/-}$ mice used in this study produce less thrombi in models of artificial vessel wall injury[14] which is in line with the prothrombotic properties of other members of the contact-kinin system such as FXII.[4,5] We therefore analyzed whether reduced intracerebral thrombus formation underlies the remarkable stroke-protective phenotype in these animals.The amount of fibrin(ogen) (antibody cross reactive for fibrin and fibrinogen) detected by Western blot in the ischemic cortex (mean optical density: 1.0 ± 0.1 [wild-type] vs. 0.2 ± 0.1 [*Kng*$^{-/-}$], $P < 0.05$) and basal ganglia (mean optical density: 0.9 ± 0.03 [wild-type] vs. 0.4 ± 0.08 [*Kng*$^{-/-}$], $P < 0.05$) was significantly reduced in KNG-deficient mice compared with wild-type controls on day 1 post stroke, and reconstitution of *Kng*$^{-/-}$ mice with human KNG restored thrombotic activity (Figure 3A).

Immunohistochemistry consistently demonstrated intravascular fibrin(ogen) deposits occluding vessels in wild-type mice and markedly reduced fibrin(ogen) deposits in *Kng*$^{-/-}$ mice (Figure 3B). Accordingly, histological sections of infarcted brain tissue

from wild-type mice showed numerous occlusions of vessel lumina (Figure 3C). In comparison, the microvascular patency was significantly increased in mice lacking KNG (thrombosis index: 14.2 ± 1.3 [wild-type] vs. 8.6 ± 0.7 [*Kng*$^{-/-}$], P < 0.001).

To further address whether reduced clot formation in the absence of KNG also translates into better cerebral (re)perfusion following tMCAO we measured the cerebral blood flow (CBF) over time in the territory of the right middle cerebral artery by Laser Doppler flowmetry. No differences in baseline CBF (before ischemia), CBF following insertion of the occluding filament (immediately after ischemia) or CBF following removal of the occluding filament (immediately after reperfusion) were observed between wild-type mice and *Kng*$^{-/-}$ mice (P > 0.05) indicating comparable procedural conditions in both groups (Figure 3D). Twelve hours (percentage of baseline CBF: 53.9 ± 5.7% [wild-type] vs. 81.1 ± 8.7% [*Kng*$^{-/-}$], P < 0.001) and 24h (percentage of baseline CBF: 56.2 ± 9.1% [wild-type] vs. 97.9 ± 9.6% [*Kng*$^{-/-}$], P < 0.0001) after reperfusion however, CBF in the brains of KNG-deficient mice was significantly higher compared with controls (Figure 3D).

We also analyzed the impact of KNG deficiency on the dynamics of infarct development and the risk of secondary hemorrhagic transformation by serial magnetic resonance imaging (MRI) on living mice. In *Kng*$^{-/-}$ mice areas of hyperintensity on T2-weighted images typical of acute cerebral infarction were significantly smaller than in wild-type mice 24h after tMCAO (P < 0.0001) (Figure 4), confirming assessments with TTC staining. The size of infarctions assessed in individual *Kng*$^{-/-}$ mice animals by sequential MRI remained significantly smaller compared with wild-type mice on day 7 (P < 0.001) and infarctions did not increase after 1 week (P > 0.05) thus excluding delayed infarct growth (Figure 4). The alleged shrinkage in stroke size after 1 week in both groups was due to infarct maturation and subsequent 'fogging effects' on MRI[24] rather than a true reduction in infarct

volumes. In addition, the higher mortality of wild-type mice between day 1 and day 7 (Figure 4, see N numbers) probably led to a selection bias towards surviving animals with smaller infarctions on day 7. Importantly, MRI demonstrated that ischemic lesions in *Kng*$^{-/-}$ mice always presented as hyperintense lesions on T2-weighted gradient echo images, an MRI protocol for the detection of bleeding (Figure 4). Hypointense areas, which typically indicate hemorrhage, were absent in all animals, supporting the notion that KNG deficiency does not increase the risk of infarct or reperfusion-associated bleeding compared with wild-type control mice, even at more advanced stages of infarct development.

KNG deficiency reduces blood-brain-barrier damage and inflammation after stroke

The contact-kinin system is also critically involved in the regulation of vascular permeability and inflammatory processes upon tissue injury including ischemic stroke.[13] One central step in this process is the release of the proinflammatory peptide hormone bradykinin from KNG following plasma kallikrein activation. Consequently, the extent of blood-brain-barrier damage and edema formation was assessed after focal cerebral ischemia. On day 1 after tMCAO the integrity of the blood-brain-barrier, as reflected by the concentration of the vascular tracer Evan's Blue leaking into the brain parenchyma, was markedly reduced in *Kng*$^{-/-}$ mice in comparison with wild-type mice (30.5 ± 4.9 ng/mg [wild-type] vs. 13.1 ± 3.3 ng/mg [*Kng*$^{-/-}$], $P < 0.05$) (Figure 5A). This finding correlated with a similar reduction in brain water content (wet/dry weight method) in KNG-deficient mice (83.1 ± 0.5% [wild-type] vs. 80.1 ± 0.5% [*Kng*$^{-/-}$], $P < 0.0001$) (Figure 5B). Importantly, *Kng*$^{-/-}$ mice that had been reconstituted with bradykinin again displayed blood-brain-barrier breakdown similar to wild-type mice (Evan's blue leakage: 25.3 ± 1.3 ng/mg, $P > 0.05$) underlining the specificity of this detrimental bradykinin effect in stroke (Figure 5A).

We also analyzed the expression of endothelin-1 in the ischemic brains of KNG-deficient mice and controls. Endothelin-1 has been shown to be critically involved in the regulation of vascular integrity and edema formation under various pathophysiological conditions, including ischemic stroke.[25] Twenty four hours after tMCAO, endothelin-1 mRNA levels were significantly higher in the cortices and basal ganglia of wild-type mice compared with sham operated mice (relative gene expression cortex: 1.0 ± 0.1 [sham] vs. 23.8 ± 2.7 [wild-type], $P < 0.001$; relative gene expression basal ganglia: 1.1 ± 0.05 [sham] vs. 25.5 ± 4.5 [wild-type], $P < 0.0001$) (Figure 5C). Although endothelin-1 transcripts were also induced in the brains of KNG-deficient mice, expression levels remained significantly lower than in wild-type mice both in the cortex ($P < 0.05$) and in the basal ganglia ($P < 0.001$).

In line with a blood-brain-barrier destabilizing effect of KNG the tight junction protein occludin was strongly downregulated in cerebral vessels of wild-type mice but was preserved in *Kng*$^{-/-}$ mice 24h after tMCAO, as demonstrated by immunohistochemistry (Figure 5D). To quantify occludin protein expression in more detail we also performed Western blot analysis. Again, occludin immunoreactivity on day 1 after tMCAO was significantly weaker in the ischemic cortices ($P < 0.05$) and basal ganglia ($P < 0.05$) of wild-type mice compared with sham operated mice while no significant downregulation of occludin could be observed in KNG-deficient mice ($P > 0.05$) (Figure 5E).

As a next step we analyzed the gene expression profiles of several prototypic pro- and anti-inflammatory cytokines in the brains of wild-type mice and *Kng*$^{-/-}$ mice 24h after tMCAO (Figure 6A). The amount of IL-1β mRNA in the infarcted cortices and basal ganglia was strongly elevated in both groups compared with sham operation ($P < 0.0001$) but induction was far lower in mice lacking KNG ($P < 0.0001$). In contrast, the number of TNFα transcripts did not differ between wild-type mice and *Kng*$^{-/-}$ mice

in either brain region (P > 0.05) indicating selective regulation of distinct cytokines by KNG. In line with these findings, TGFβ-1 which can exert anti-inflammatory functions in ischemic stroke was abundantly expressed only in the ischemic brains of KNG-deficient mice but not of wild-type mice (P < 0.001 [cortex], P < 0.05 (basal ganglia]) (Figure 6A).

To further characterize the local inflammatory response, we quantified the amount of immune cells invading the ischemic hemispheres over time by immunohistochemistry. On day 1 and day 3 after the induction of tMCAO, significantly more CD11b+ macrophages/microglia cells (P < 0.0001) and neutrophils (P < 0.001 [day 1], P < 0.05 [day 3]) had entered the brains of wild-type mice compared with the brains of *Kng*$^{-/-}$ mice (Figure 6B).

Discussion

This study identifies KNG as a key mediator of ischemic neurodegeneration. Our data suggest that KNG leads to neuronal damage via different pathways related to the activation of the contact-kinin system: enhanced microvascular thrombosis, blood-brain-barrier leakage and inflammation. The extent of neuroprotection conferred by the absence of KNG in male and female *Kng*$^{-/-}$ mice was exceptional, long-lasting and preserved in older animals. Important from a translational perspective, genetic depletion of KNG protected from pathological thrombus formation during stroke but did not increase the risk of intracerebral hemorrhage.

We consider the results described here to be novel for a number of reasons. First, several reports have suggested that KNG exerts antithrombotic effects.[26-28] However, our studies clearly demonstrate that the absence of KNG protects from thrombosis in the tMCAO model. The exact reasons for these divergent findings are unclear so far. However, differences in the thrombosis models (artificial vessel wall injury models

versus in vivo stroke models) and animal species (rats versus mice) used probably play a role. Second, this apparent contradiction suggests potentially novel mechanisms by which KNG promotes thrombosis, which at this point are unknown. While these may involve FXI, which binds high-molecular-weight KNG,[29-31] whether these interactions alone account for KNG's prothrombotic activity is uncertain. Third, the degree of neuroprotection caused by KNG depletion is remarkable, and exceeds that observed in other reports of cerebral ischemia in animals deficient in members of the kallikrein-kinin system.[4,32] This is likely due to the central role of KNG in multiple processes including thrombosis, vascular permeability, and inflammation.[33] Finally, while severe deficiencies of FXI may be associated with bleeding in humans, there is no such association of bleeding with KNG deficiency. Indeed, a recent report describes a patient with plasma KNG activity of < 1% that underwent cardiopulmonary bypass under full anticoagulation without increased bleeding.[34] These results suggest a potential clinical strategy involving KNG depletion for stroke prevention and amelioration.

The observation of an anti-thrombotic effect of KNG deficiency in an in vivo model of ischemic stroke is congruent with the findings after artificial vessel wall injury. Time to occlusion of the carotid artery following Rose Bengal induced laser damage was significantly prolonged in *Kng*$^{-/-}$ mice.[14] Hematologic characterisation of *Kng*$^{-/-}$ mice revealed a significantly increased activated partial thromboplastin time (aPTT) but normal tail bleeding times[14] indicative for an anti-thrombotic but not anti-hemostatic phenotype in these animals. Indeed, the risk of stroke-related hemorrhage was not increased in mice lacking KNG in our study. Of the 151 *Kng*$^{-/-}$ mice subjected to MCAO only 9 (6.0%) showed signs of intracerebral bleeding (as macroscopically assessed during brain sampling or by blood-sensitive MRI) which was similar to the bleeding rate in wild-type mice (8 out of 167 = 4.8%; $P > 0.05$) and which ranges

within the expected bleeding frequency in this stroke model.[35] In comparison, full-intensity parenteral anticoagulation with the indirect FXa inhibitor heparin induced intracranial hemorrhages in up to 50% in rats undergoing experimental cerebral ischemia[36] and mice pretreated with the oral anticoagulant warfarin (an inhibitor of the coagulation factors II, VII, IX and X) even developed hemorrhagic transformation of brain tissue in 100% of the cases after tMCAO.[35] These numbers underpin that blocking of KNG in ischemic stroke is presumably a safer approach compared with established treatment regimens although pharmacological inhibitors that specifically suppress KNG activity are currently not available.

The phenotype of *Kng*$^{-/-}$ mice reported here is similar to the recently described phenotype in FXII-deficient mice. FXII is the primary activator of both the intrinsic coagulation and the kallikrein-kinin-system and in vivo binding of FXII to negatively charged molecules such as polyphosphates[37] or RNA[38] leads to the assembly of an activation-complex comprising FXIIa, kallikrein and high-molecular-weight KNG.[3] *FXII*$^{-/-}$ mice, like KNG-deficient mice, generate less thrombi in different in vitro and in vivo models of thrombosis including ischemic stroke but do not have an increased bleeding tendency either spontaneously or when subjected to brain ischemia.[4,5] Our findings point towards a possible involvement of KNG in pathologic thrombosis due to vascular injury, although additional studies will be required to discern whether the mechanism reflects direct enhancement of thrombus stability or other pathways, such as those directly affecting underlying endothelial cell function.[39] Interestingly, a previous study suggested that mice lacking the bradykinin receptor B2, which acts downstream of KNG, are protected from thrombosis by increased nitric oxide and prostacyclin formation.[40] Successful prevention of excessive clotting without interfering with physiological hemostasis would be a major breakthrough in the therapy of many thromboembolic disorders given that the current drugs used to

prevent or reverse thromboembolism are all associated with severe bleeding complications.[41]

We have demonstrated that KNG is consumed in the cerebral circulation or tissue following brain ischemia/reperfusion (I/R) in wild-type mice. KNG cleavage by plasma kallikrein reflects activation of the kallikrein-kinin system after stroke and subsequent bradykinin formation and can also be observed in I/R models in other organ systems such as the heart (myocardial infarction)[42] or after brain trauma.[43] Importantly, blood KNG levels are also reduced in human stroke patients.[44]

In the absence of substrate, *Kng*$^{-/-}$ mice are completely unable to produce bradykinin.[14] This lack of bradykinin most likely underlies the strong anti-inflammatory phenotype observed in the context of brain ischemia. The blood-brain-barrier was highly maintained in the absence of KNG after stroke, an effect that could be ascribed to preserved occludin expression. As a consequence mice without KNG developed significantly less brain edema which is known to be a frequent cause of secondary infarct growth and deterioration of neurological symptoms. Importantly, reconstitution of *Kng*$^{-/-}$ mice with bradykinin fully restored edema formation after tMCAO underpinning the specificity of this detrimental bradykinin effect. By analogy, degradation of occludin and other tight junction proteins via a bradykinin-dependent pathway destabilized the blood-brain-barrier in different tumor models.[45] Endothelin-1 levels were also lower in the ischemic brains of *Kng*$^{-/-}$ mice compared with controls. Endothelin-1 has been shown to be critically involved in regulating vascular integrity and edema formation under various pathophysiological conditions including ischemic stroke.[46] Mice overexpressing endothelin-1 developed more brain edema and larger cerebral infarctions after tMCAO.[47] Moreover, the pharmacological blockade of the endothelin type A receptor attenuated ischemic brain injury, edema formation, and blood-brain-barrier disruption in rats[48] and high serum levels of endothelin-1 have

recently been shown to predict malignant edema in patients with acute ischemic stroke receiving recombinant tissue plasminogen activator (tPA).[49]

Only very few immune cells such as neutrophils invaded the brains of KNG-deficient mice after tMCAO. Neutrophils have been shown to be involved in stroke development by producing free radicals and other neurotoxic factors.[6] Moreover, neutrophils can impair tissue reperfusion after transient brain ischemia by interacting with platelets and endothelial cells, a phenomenon commonly referred to as 'no reflow'.[50,51] Accordingly, CBF in the cerebral microvasculature of *Kng*$^{-/-}$ mice was significantly enhanced during the reperfusion phase after tMCAO, although less thrombus formation probably contributed to this effect as well. The number of macrophages or activated microglia cells was also reduced in the ischemic brains of *Kng*$^{-/-}$ mice. These cell types, which cannot be differentiated by morphology or immunohistochemical markers, can release a myriad of potentially harmful mediators such as reactive oxygen species, proinflammatory cytokines, or matrix metalloproteinases.[6]

Finally, the expression of soluble immune mediators was altered in the absence of KNG. KNG mutant mice expressed less IL-1β in the cortices and basal ganglia after tMCAO, whereas the amount of TGFβ-1 was increased compared to wild-type controls. IL-1β is regarded a prototypic proinflammatory cytokine known to aggravate ischemic brain damage.[52] In contrast, TGFβ-1 exerts pleiotropic immune functions and has been shown to mediate neuroprotection during stroke.

The 24h gap between the onset of ischemia and the first outcome analysis to some extent limits the interpretation of our findings. In particular, the question of whether reduced thrombosis, blood-brain-barrier damage and inflammation are the cause or the consequence of infarct protection in *Kng*$^{-/-}$ mice cannot be definitely answered in the absence of earlier time points. However, the fact that reconstitution of *Kng*$^{-/-}$ mice

with KNG or bradykinin fully restored thrombus formation and edema formation clearly argues for a causative rather than a merely correlative relationship.

Another unresolved issue is whether KNG acts detrimental in tMCAO mainly during the phase of ischemia or during reperfusion. The observation that protection from stroke in *Kng*$^{-/-}$ mice was lost after permanent MCAO suggests that KNG is of particular relevance for mediating reperfusion injury. Of note, the findings reported here are in full accordance with our studies in FXII-deficient mice which are likewise protected from transient but not permanent ischemia.[53] In these mice, restoration of blood flow in the middle cerebral artery enhanced cortical reperfusion between 2h and 24h as assessed by serial CBF measurements and this effect was related to reduced microvascular thrombosis.[4,53] However, one has to bear in mind that permanent ischemia in contrast to transient ischemia represents a maximal noxious stimulus to the brain and therefore, any results obtained in these two stroke models cannot be easily compared.

The phenotype of *Kng*$^{-/-}$ mice reported here is consistent with the phenotype of bradykinin receptor B1 (B1R)-deficient mice. B1R is another key member of the kallikrein-kinin-system which acts downstream of KNG. Blocking of B1R dramatically reduced inflammatory processes and edema formation in models of acute ischemic stroke[13], traumatic brain injury[12] and multiple sclerosis.[11] The corresponding findings in different mouse models bearing genetic defects in the contact-kinin system suggest that thrombosis and inflammation are closely intertwined during focal cerebral ischemia. This goes congruent with the novel concept of ischemic stroke being a 'thrombo-inflammatory' disease rather than a pure vessel-occlusive disease.[9]

In summary, inhibition of KNG prevents ischemic neurodegeneration by combined antithrombotic and anti-inflammatory mechanisms. Importantly, neuroprotection through targeted depletion of KNG did not increase bleeding after ischemic stroke.

Blocking of distinct members of the kallikrein-kinin-system has the potential to become an effective and safe strategy to combat this devastating neurologic disorder and other cardiovascular diseases such as myocardial infarction. However, the true pathophysiological relevance of the kallikrein-kinin system in stroke patients still needs to be established and findings from animal studies should not be uncritically transferred to the human situation. Moreover, additional mechanisms than those reported here could account for the detrimental KNG effects in stroke such as impaired fibrinolysis or cerebrovascular contractility. Further studies in relevant disease models are warranted to clarify these open issues.

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Authorship

Contributions: C.K. and K.R.M. conceived, directed and funded the entire study, designed experiments, analyzed data and drafted the manuscript; F.L., E.G., P.K., C.G., and K.G. designed and performed experiments, analyzed data, and contributed to manuscript writing; J.S. and M.Br. performed invasive hemodynamics and blood gas analysis and analyzed the data; X.H., M.P., M.B., and P.J. provided specific input to MRI experiments including experimental design and data analysis; G.S., S.G.M., and B.N. funded the study, designed experiments and contributed to manuscript writing.

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Figure legends

Figure 1: The kallikrein-kinin-system is activated in the ischemic mouse brain after stroke as indicated by KNG consumption. Immunoblot from the cortex and basal ganglia of a mouse subjected to transient middle cerebral artery occlusion (tMCAO) using an antibody against kininogen (KNG). The 24h post stroke time point is depicted. A sham-operated mouse served as control. Immunoreactivity against native KNG was markedly down-regulated in the brain of the mouse with cerebral ischemia. In contrast, cleaved KNG, which is produced when native KNG is consumed by plasma kallikrein to form bradykinin, was up-regulated in the ipsilateral (i), i.e. ischemic cortex and basal ganglia of the mouse with stroke but not in corresponding regions of the brain of the sham-operated mouse or the healthy contralateral (c) hemisphere. Immunoblots were independently replicated from 3 different animals. One representative sample is shown.

Figure 2: KNG deficiency confers long-term neuroprotection and reduces mortality after acute ischemic stroke in young and aged mice of either sex. (A) (upper panel) Representative 2,3,5-triphenyltetrazolium chloride (TTC) staining of three corresponding coronal brain sections of (left to right): 6 week old male wild-type (WT) mouse, 6 week old male *Kng*$^{-/-}$ mouse, 6 week old male *Kng*$^{-/-}$ mouse reconstituted with human kininogen (KNG), 6 week old male *Kng*$^{-/-}$ mouse reconstituted with bradykinin (BK), 6 week old female WT mouse, 6 week old female *Kng*$^{-/-}$ mouse as well as 6 month old male WT mouse and 6 month old male *Kng*$^{-/-}$ mouse on day 1 after tMCAO. The ischemic infarcts (white) appear smallest in the *Kng*$^{-/-}$ mice (white arrows) of either age or sex, and this result was confirmed by infarct volumetry (lower panel) (N = 6 – 10/group). Note that reconstitution of *Kng*$^{-/-}$

mice with human KNG or BK fully restored infarct susceptibility underlining the specificity of the KNG effect. Infarctions in KNG-deficient mice remained small on day 3 after tMCAO thereby excluding secondary infarct growth. In contrast, protection from stroke was lost in *Kng*$^{-/-}$ mice after permanent (p) MCAO. (B) Neurological Bederson score (upper panel) and grip test score (lower panel) on day 1 or day 3 after tMCAO or day 1 after pMCAO in the twelve mouse groups indicated above. The reduction of infarct size in *Kng*$^{-/-}$ mice after tMCAO also translated into better functional outcome (N = 6 – 10/group). (C) Mortality in 6 week old male *Kng*$^{-/-}$ mice and WT controls between day(d)0 and d8 after tMCAO (N = 10/group). a, b, ***P < 0.0001, **P < 0.001, *P < 0.05, 1-way ANOVA followed by Bonferroni's multiple comparison test (infarct volumes) or Kruskal-Wallis test followed by Dunn's multiple comparison test (neurological scores) compared with the respective WT groups. c, survival curve: **P = 0.0075, log rank test compared with WT mice.

Figure 3: KNG deficiency reduces intracerebral thrombosis and improves cerebral blood flow after stroke. (A) Accumulation of fibrin(ogen) in the infarcted (i) and contralateral (c) cortices and basal ganglia (BG) of wild-type (WT) mice, *Kng*$^{-/-}$ mice and *Kng*$^{-/-}$ mice reconstituted with human kininogen (KNG) was analyzed by immunoblotting 24h after tMCAO (upper panel) and bands were quantified by densitometry (lower panel) (N = 3 – 4/group). One representative immunoblot of each group is shown. AU: arbitrary units. (B) Immunohistochemical localization of fibrin(ogen) in the lumina of brain microvessels (stained with the endothelial marker CD31) 24h after tMCAO in the infarcted hemispheres of WT mice or *Kng*$^{-/-}$ mice. Hoechst staining (blue) depicts cell nuclei. One representative panel per group out of 3 independent experiments is shown. Bar represents 50 µm. (C) (left) Representative H&E staining from the infarcted hemispheres of WT mice and *Kng*$^{-/-}$ mice on day 1

after tMCAO. Thrombotic vessels were abundant in WT mice (arrows) while the microvascular patency was significantly increased in *Kng*$^{-/-}$ mice (arrowheads) and this was confirmed by calculation of the thrombosis index (right) (N = 5/group). Bar represents 100 µm. (D) Reduced intracerebral thrombosis in *Kng*$^{-/-}$ mice improved cerebral blood flow (CBF) in the territory of the right middle cerebral artery 12h and 24h after reperfusion compared with WT mice as determined by serial Laser Doppler flow measurements. No differences in CBF were detectable at baseline (before ischemia), immediately after insertion of the filament (ischemia) or immediately after reperfusion (removal of the filament) (N = 8/group and time point). a, ***P < 0.0001, *P < 0.05, 2-way ANOVA, followed by Bonferroni's multiple comparison test, group comparisons as indicated in the figure. c, **P = 0.0054, unpaired Student's *t*-test. d, ***P < 0.0001, **P < 0.001, 2-way ANOVA, followed by Bonferroni's multiple comparison test compared with WT mice.

Figure 4: KNG deficiency provides sustained protection from ischemic stroke and does not increase the risk of intracranial hemorrhage. (upper panel) Serial coronal T2-weighted gradient echo MR images show extensive hyperintense (bright) ischemic lesions (white arrows) on day 1 and day 7 after tMCAO in wild-type (WT) mice while the infarctions are mainly restricted to the basal ganglia in *Kng*$^{-/-}$ mice. Hypointense (dark) areas indicative of intracerebral hemorrhage were always absent in both groups. One representative panel per group is depicted. (lower panel) MRI-based infarct volumetry confirms significantly smaller infarct volumes and excludes delayed infarct growth in *Kng*$^{-/-}$ mice after tMCAO (N = 3 – 8 per group and time point). Alleged shrinkage of strokes between day 1 and day 7 is due to fogging effects during infarct maturation. Note that 5/8 WT mice died between day 1 and day 7 after tMCAO and were no longer available for the second MRI examination. ***P <

0.0001, **P < 0.001, ns: not significant, 2-way ANOVA followed by Bonferroni's multiple comparison test compared with WT mice or *Kng*$^{-/-}$ mice.

Figure 5: KNG deficiency has profound blood-brain-barrier stabilizing and anti-edematous effects in ischemic stroke. (A) (left) Representative corresponding coronal brain sections from a wild-type (WT) mouse, a *Kng*$^{-/-}$ mouse, and a *Kng*$^{-/-}$ mouse reconstituted with bradykinin (BK) on day 1 after tMCAO following injection of the vascular tracer Evan's blue. Vascular leakage was significantly decreased in the absence of KNG after stroke as confirmed by the concentration of Evan's blue detectable in the brain parenchyma (right). BK reconstitution restored edema formation in *Kng*$^{-/-}$ mice (N = 7 -8/group). (B) Edema formation as reflected by the brain water content in the ischemic hemispheres of WT mice and *Kng*$^{-/-}$ mice on day 1 after tMCAO (N = 6 – 9/group). (C) Relative gene expression of endothelin-1 (*Edn-1*) in the cortices and basal ganglia of WT mice and *Kng*$^{-/-}$ mice 24h after tMCAO or sham operation (N = 4/group). (D) Expression of occludin on day 1 after tMCAO in the hemispheres of WT mice and *Kng*$^{-/-}$ mice. Immunohistochemistry suggests that occludin is predominately located in the gaps between vascular endothelial cells (indicated by the marker CD31). Occludin expression was markedly reduced in WT mice but preserved in mice lacking KNG. Hoechst staining (blue) depicts cell nuclei. One representative panel per group out of 3 independent experiments is shown. Bar represents 50 µm. (E) (upper panel) Occludin expression in the cortex or basal ganglia (BG) of WT mice or *Kng*$^{-/-}$ mice on day 1 after tMCAO or sham operation as determined by immunoblot. One representative immunoblot of each group is shown. (lower panel) Densitometric quantification of occludin immunoreactivity in the mouse groups indicated above (N = 4/group). i: ipilsateral (ischemic) hemisphere, c: contralateral (healthy) hemisphere. a, b, ***P = 0.0008, *P = 0.0138, unpaired

Student's *t*-test. c, ***P < 0.0001, **P < 0.001, *P < 0.05, $^{\#\#}$P < 0.001, $^{\#}$P < 0.05, 1-way ANOVA followed by Bonferroni's multiple comparison test compared with sham-operated mice (* symbol) or WT mice ($^{\#}$ symbol). e, *P < 0.05, 2-way ANOVA, followed by Bonferroni's multiple comparison test, group comparisons as indicated in the figure.

Figure 6: KNG deficiency reduces inflammation after stroke. (A) Relative gene expression of interleukin-1β (*Il-1β*), tumor necrosis factor alpha (*Tnfα*), and transforming growth factor beta-1 (*Tgf-β1*) in the cortices and basal ganglia of wild-type (WT) mice and *Kng*$^{-/-}$ mice 24h after tMCAO or sham operation (N = 4/group). (B) Cellular inflammatory response in the ischemic hemispheres of WT mice or *Kng*$^{-/-}$ mice on day 1 and day 3 after tMCAO. (upper panel) Representative immunohistochemical staining against CD11b+ macrophages/microglia cells (arrows) on day 1. Scale bar represents 100 µm. (lower panel) Quantification of immune cell infiltration (CD11b+ macrophages/microglia, Ly-6B.2+ neutrophils) on day 1 and day 3 post stroke (N = 5/group and time point). ***P < 0.0001, **P < 0.001,$^{\#\#\#}$P < 0.0001, $^{\#}$P < 0.05, 2-way ANOVA, followed by Bonferroni's multiple comparison test compared with WT mice on day 1 (* symbol) or WT mice on day 3 ($^{\#}$ symbol).

Figure 1

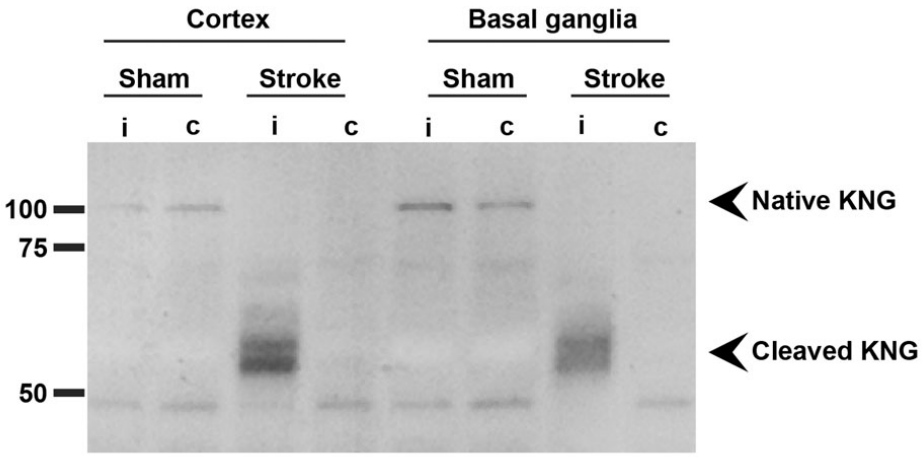
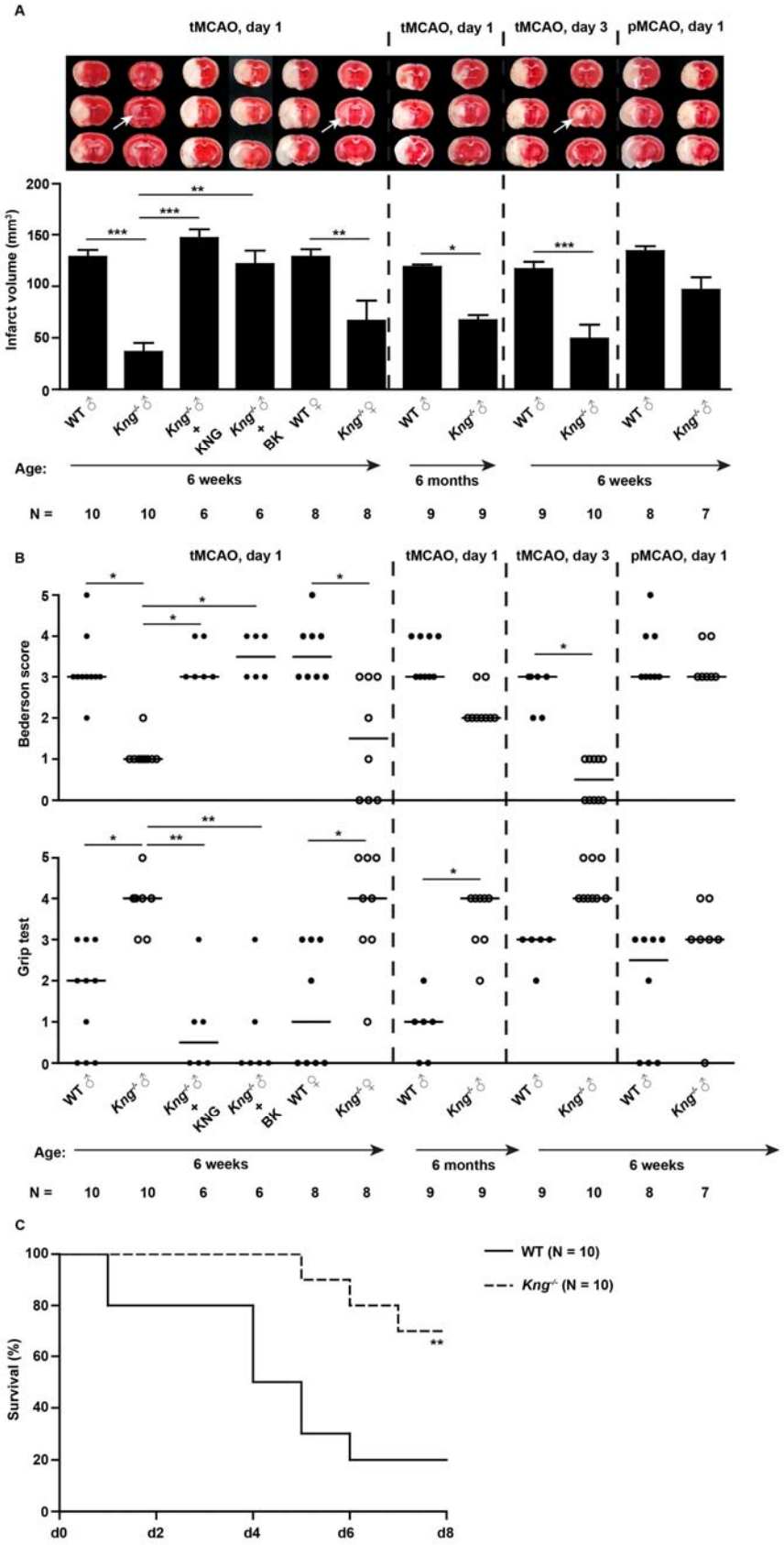


Figure 2



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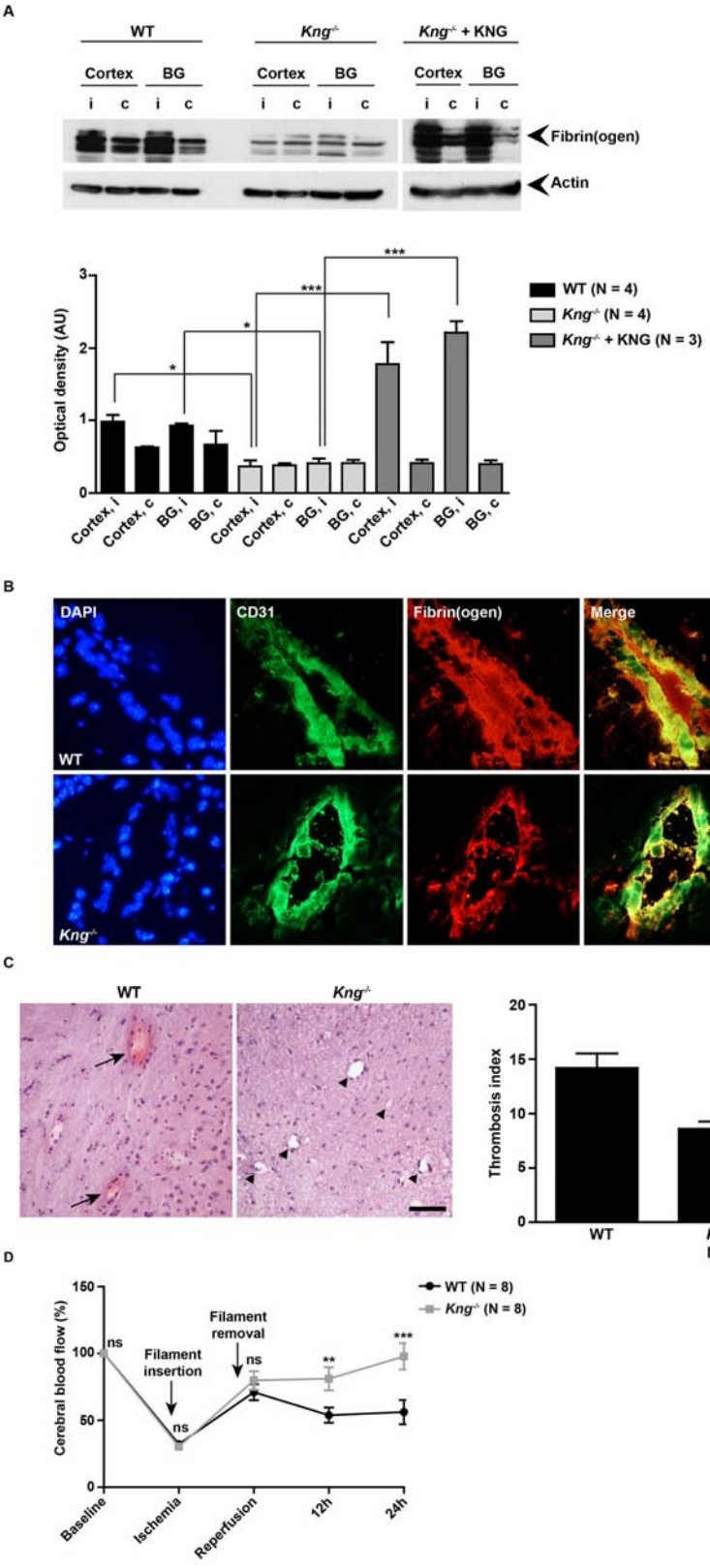

Figure 4

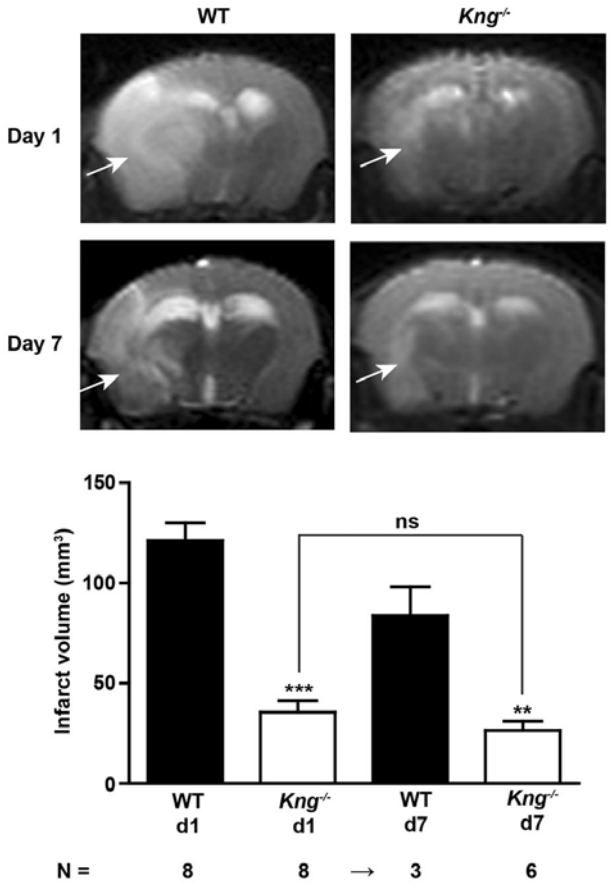

Figure 5

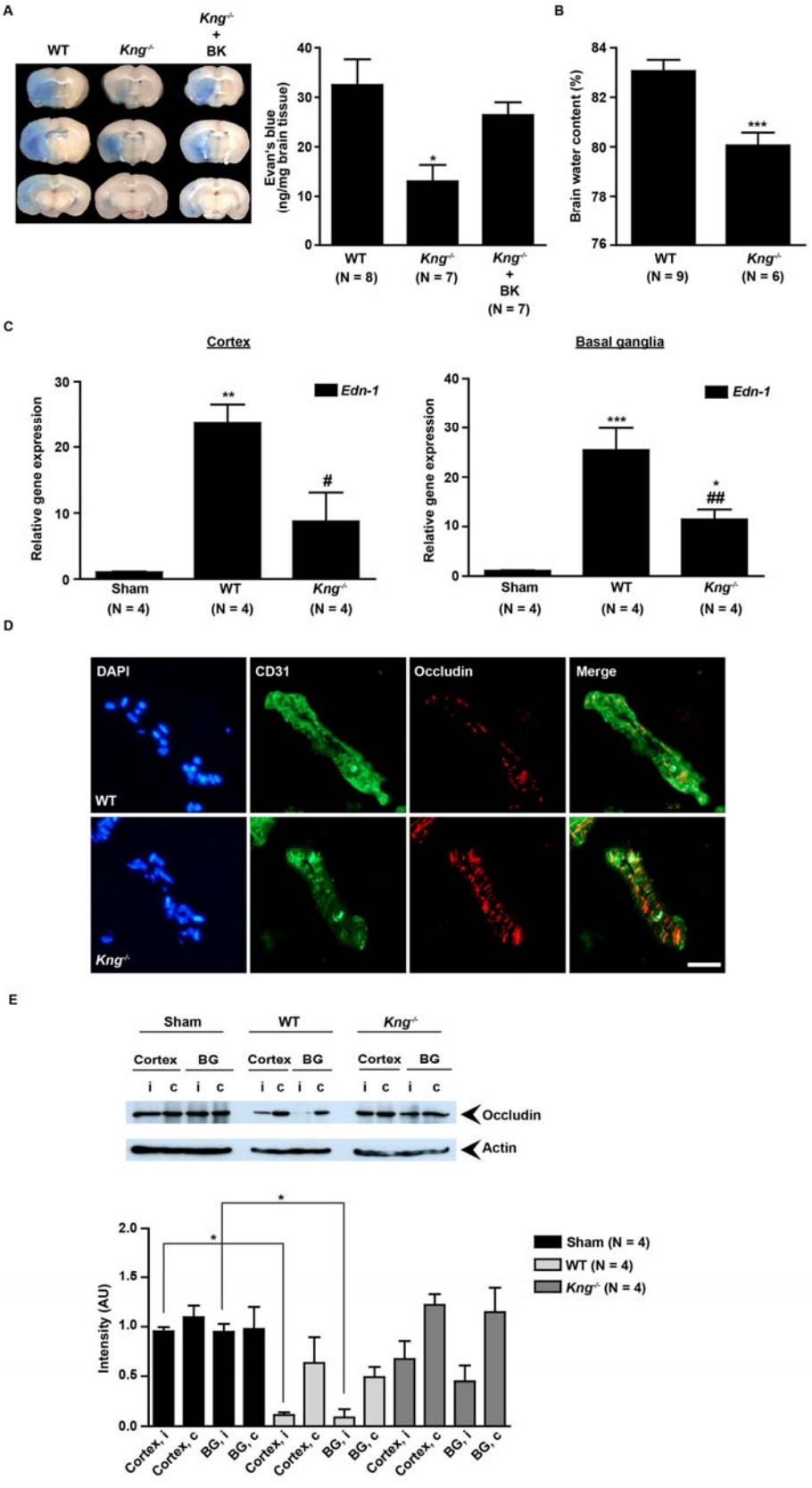

Figure 6