Coagulation Factor XII protects neurons from apoptosis by triggering a crosstalk between HGFR/c-Met and EGFR/ErbB1 signaling pathways

Eugénie Gamier  
INSERM

Damien Levard  
INSERM

Carine Ali  
INSERM

Yannick Hommet  
INSERM

Tiziana Crepaldi  
Istituto per la Ricerca e la Cura del Cancro di Candiolo

Paolo Comoglio  
Istituto per la Ricerca e la Cura del Cancro di Candiolo

Marina Rubio  
INSERM

Denis Vivien  
INSERM

Sara Martinez de Lizarrondo  
INSERM

Fabian Docagne (docagne@cyceron.fr)  
“INSERM“  https://orcid.org/0000-0003-1745-0625

Research article

Keywords: Apoptosis, intrinsic pathway, Hageman factor, scatter factor, serine proteases, neuroprotection

DOI: https://doi.org/10.21203/rs.3.rs-66115/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

**Background** Factor XII (FXII) is a serine protease that participates in the intrinsic coagulation pathway. Several studies have shown that plasmatic FXII exert a deleterious role in cerebral ischemia and traumatic brain injury by promoting thrombo-inflammation. Nevertheless, the direct impact of FXII on neuronal cell fate remains unknown.

**Methods** We investigated whether FXII influenced neuronal death induced in vivo by stereotaxic injection of N-methyl-D-Aspartate (NMDA) and in vitro by serum deprivation of cultured neurons.

**Results** We found that FXII reduced brain lesions induced *in vivo* and protected cultured neurons from apoptosis through a growth factor-like effect. This mechanism was triggered by direct interaction with epidermal growth factor (EGF) receptor, activation of this receptor and engagement of anti-apoptotic intracellular pathways. Interestingly, the "proteolytically" active and two-chain form of FXII, αFXIIa, exerted additional protective effects by converting the pro-form of hepatocyte growth factor (HGF) into its mature form, which in turn activated HGF receptor (HGFR/c-Met) pathway. Lastly, the use of non-proteolytic FXII (αFXIIa-PPACK) unveiled an alternative EGFR and HGFR co-activation pathway, through co-receptor transphosphorylation.

**Conclusion** This study describes novel mechanisms of action of FXII and discloses neurons as target cells for the protective effects of single and double-chain forms of FXII.

Background

Factor XII (FXII), also known as Hageman Factor, is a 80 kDa chymotrypsin-like serine protease (EC 3.4.21.38) with various effects in the circulation, ranging from activation of the phase contact ("intrinsic") coagulation pathway to pro-inflammatory actions. FXII is primarily produced as a single-chain enzyme ("zymogen"), secondarily activated to its two-chain form αFXIIa by plasma Kallikrein or by auto-activation when bound to some biological or artificial surfaces. Although most of the reported actions of FXII are due to the proteolytic activity of its active form αFXIIa, proteolytic and non-proteolytic actions of the zymogen FXII have also been reported. Several receptors, including uPAR and Epidermal Growth Factor (EGF) receptor (EGFR) have been suggested to mediate these non-proteolytic effects.

Several studies have shown that circulating FXII can exert detrimental effects in cerebral ischemia and traumatic brain injury by promoting thrombo-inflammation. Nevertheless, the question remains open whether FXII may directly impact neuronal cell fate within the brain parenchyma, independently of its intravascular effects. This question is important considering the recent discovery that an isoform of FXII is expressed by neurons in the brain.

Chymotrypsin-like serine proteases form a family of multi-domain proteins with mosaic structures. In addition to similarities in their trypsin like protease domains, these proteins share non-proteolytic domains such as Kringle or EGF-like domains. These structural similarities suggest that, beyond their
common protease activity, serine proteases can present similarities in their non-proteolytic actions. In particular, the domain composition of the serine protease tissue-type plasminogen activator (tPA), is very similar to that of FXII: tPA displays two kringle domains and one EGF-like domain, while FXII displays one Kringle domain and two EGF-like domains. In earlier studies, we have shown that tPA, by the virtue of its EGF-like domain, induces anti-apoptotic effects in oligodendrocytes and neurons by binding to EGF receptor (EGFR)\textsuperscript{10,11}. In light of the foregoing, we hypothesized that Factor XII could promote anti-apoptotic effects in neurons by non-proteolytic actions.

Apoptosis of neurons is considered to play a significant role in several neurovascular disorders including stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease, or amyotrophic lateral sclerosis\textsuperscript{12,13,14}. In these pathological conditions, apoptosis can result from oxidative stress, exposure to pro-apoptotic factors such as apoptosis stimulating fragment (Fas) or tumor necrosis factor (TNF), or starvation from trophic factors\textsuperscript{10}. This latter condition can be mimicked \textit{in vitro} by removing trophic support from cultured neurons in a classical paradigm termed serum deprivation (SD)\textsuperscript{11}.

Here, we report that FXII protects from neuronal injury induced by stereotaxic intracerebral injection of N-Methyl-D-Aspartic acid (NMDA) \textit{in vivo}. Besides, we show that FXII rescues cultured neurons from apoptosis by non-protease actions involving the direct binding to EGFR and subsequent activation of the Erk1/2 intracellular pathway. Pharmacological inhibition of Erk1,2 phosphorylation quenches the FXII-mediated protection. In addition, we observe that αFXIIa also promotes indirect trophic effects: it converts the pro-form of hepatocyte growth factor (HGF) to its mature form, which in turn protects neurons from apoptosis. Together, these data indicate that both forms of FXII promote survival of neurons by a crosstalk between its proteolytic and non-proteolytic effects.

**Methods**

**Materials.** FXII, αFXIIa and Corn trypsin inhibitor (CTI) were obtained from Enzyme Research Laboratories. NMDA, EGF receptor kinase inhibitor (AG1478) and NMDA receptor antagonist (MK801) were obtained from Tocris Bioscience. FXII chromogenic substrate (S-2302) was obtained from Werfen. Rabbit anti-pErk1/2 (#9102), anti-Erk1/2 (#9101) and anti-EGFR (#4267) antibodies were purchased from Cell Signaling. Anti-HGF (SBF5) antibody and recombinant mature HGF (PHG0254) were obtained from Invitrogen. Blocking anti-HGF (sc1356) and Anti-pMET (sc101736) antibodies were obtained from Santa cruz. Anti-Actin (A2066), Anti-Bax (sc7480) and anti-Bcl-2 (sc7382) antibodies and were purchased from Sigma. PPACK (H-D-Pro-Phe-Arg-Chloromethylketone trifluoroacetate salt) was obtained from Bachem. The biotinylation kit, EZ-link® Sulfo-NHS-LC-Biotinylation kit was purchased from Thermo Scientific. Anti-uPAR (MAB531), anti-HGFR total antibody (AF527) and recombinant human HGF Propeptide (proHGF, 7057-HG-010) were obtained from R&D Systems. Erk Inhibitor (SCH772984) was obtained from Selleckchem. JNJ-38877605 was kindly provided by Janssen Pharmaceutica.

**Animals.** Studies were conducted in male Swiss mice (age 12 weeks, weight 35–45 g; Centre Universitaire de Ressources Biologiques, Normandy University, Caen, France). Mice were housed with food and water
ad libitum access. Animals were randomized to treatment groups, and all analyses were performed by investigators blinded to group allocation. The study design and the procedures thereof were evaluated and approved by the regional committee on animal ethics (C2EA-54) and the French Ministry of Higher Education, Research, and Innovation (project licence #2889). All animal experiments were performed and reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (http://www.nc3rs.org.uk).

NMDA-induced cerebral injury. Mice were deeply anesthetized with 5% isoflurane and maintained with 1.5–2% isoflurane 30% O$_2$/70% N$_2$O during the experiment. Anesthetized mice were placed in a stereotaxic device. Then the skin was removed and a small craniotomy was performed. A glass micropipette containing 1 µl of FXII (1 µg; FXII group) or control buffer (vehicle group) was inserted (coordinates: -1 mm anteroposterior; +3.3 mm lateral; -0.8 mm depth from the Bregma) in the cortex. The pipette was left in place for 2 minutes before injection. Then the solution was pneumatically injected in the right cortex during 2 minutes. After the injection, the pipette was left in place again for 2 minutes to wait for the good diffusion of the solution and to compensate for pressure around.

Ten minutes after the injection of vehicle or FXII, a glass micropipette was inserted at the aforementioned coordinates and 0.5 µl of NMDA (40 nmol/µl; 20 nmol) was injected as described just before. Lesion volumes were quantified by Magnetic Resonance Imaging (MRI) on ImageJ software 24 hours after injection.

Magnetic Resonance Imaging. Mice were deeply anesthetized with 5% isoflurane and maintained with 1.5–2% isoflurane 30% O$_2$/70%N$_2$O during the acquisitions. Experiments were carried out on a Pharmascan 7T (Bruker, Germany). T2-weighted images were acquired using a multislice multiecho sequence: TE/TR 33 ms/2500 ms. Lesion sizes were quantified on these images using ImageJ software. T2*-weighted sequences were used to control if animals underwent hemorrhages.

TUNEL staining for apoptosis. Twenty-four h after NMDA cortical injections deeply anesthetized mice were transcardially perfused with cold heparinized saline (15 mL/min) followed by 150 mL of fixative (PBS 0.1 M. pH 7.4 containing 2% paraformaldehyde and 0.2% picric acid).

Brains were post-fixed (24 hours; 4 °C) and cryoprotected (sucrose 20% in PBS; 24 hours; 4 °C) before freezing in Tissue-Tek (Miles Scientific, Naperville, IL, USA). Cryostat-cut sections (10 µm) were collected on poly-lysine slides and stored at −80 °C before processing. Sections were permeabilized and stained with In Situ Cell Death Detection Kit, Fluorescein (Sigma Aldrich), as stated by the manufacturer instructions. After staining, washed sections were coverslipped with antifade medium containing DAPI and images were digitally captured using a Leica DM6000 microscope-coupled coolsnap camera and visualized with Metavue 5.0 software (Molecular Devices, USA) and further processed using ImageJ 1.45r software (NIH).

Primary murine neuronal cortical cultures. Murine neuronal cultures were prepared as previously described in Liot et al.$^{11}$ Neuronal cortical cultures were obtained from fetal mice at E15–E16. Cortices
were dissociated and plated on 24-well plates coated with poly-D-lysine (0.1 mg/mL) and laminin (0.02 mg/mL). Cells were cultured in DMEM supplemented with 2 mM glutamine, 5% horse serum and 5% fetal bovine serum. Cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere. Cytosine β-D-arabinoside (10 µM) was added after 3 days in vitro (DIV) to inhibit non-neuronal proliferation. All experiments were performed at DIV7.

Serum deprivation-induced apoptosis. Serum deprivation (SD) was induced by the exposure of neuronal cultures (DIV7) to a serum-free DMEM as previously described\textsuperscript{11}. Controls were maintained in serum-containing medium. MK801 (1 µM) was added to prevent secondary NMDA receptor activation. Cells under SD were treated with FXII, αFXIIa or αFXIIa-PPACK. Inhibitors were added simultaneously to treatments (unless otherwise stated in the text). Before fixation on 4% paraformaldehyde, cells were stained with 0.4% trypan blue for 15 min after 24 h of SD. Neuronal cell injury was quantified by counting trypan blue positive cells in four random fields per well. The percentage of neuronal death was determined as the number of trypan blue positive neurons after SD compared with the total neuron number. The mean values of trypan blue positive neurons in sham washed control conditions were substracted from experimental values to yield the specific effect of the tested conditions.

Western Blot. After solubilization in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100) containing protease and phosphatase inhibitor cocktails (1/100), cell lysates were centrifuged for 20 min at 12 000 g and supernatants were harvested. Protein concentrations were calculated by using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Protein samples (20 µg) were separated by using a sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred into a polyvinylidene difluoride membrane. Membranes were blocked for 2 h in Tween 20-Tris Base Solution with 1% BSA and incubated overnight at 4 °C with the specific primary antibody at the following concentrations: for anti-Bax and anti-Bcl-2 at a dilution of 1/200; anti-αEGFR, anti-pErk, anti-Erk total and anti-Actin at 1/1000; anti-MET total and anti-αHGF at 1/500; anti-pMET at 1/250. After washes and 1 h of incubation with the corresponding peroxidase secondary antibody, proteins were revealed with a chemiluminescence ECL select immunoblotting detection system (GE Healthcare).

EGFR crossed Immunoprecipitation assays. Biotinylated FXII and αFXIIa (Biot-FXII and Biot-αFXIIa respectively) were produced following manufactured's kit (EZ-link® Sulfo-NHS-LC-Biotinylation). After treatment during 24 h with 125 nM of Biot-FXII and Biot-αFXIIa, lysed cultured cortical neurons (DIV 7) (100 µg of total protein) were incubated overnight at 4 °C with an antibody anti-EGFR (6 µg, Cell Signaling, #4267) and then coupled to protein G–Sepharose. Then, immunoprecipitated proteins were separated by 7.5% SDS-PAGE, and immunoblots were revealed with Extravidin peroxidase (1/2000)\textsuperscript{10}.

αFXIIa-PPACK preparation. To inhibit αFXIIa proteolytic activity, 250 nM of αFXIIa was incubated with PPACK (H-D-phe-pro-arg-chloromethylketone; 1000 nM) for 30 minutes at room temperature in Hepes-buffer (25 mM Hepes, pH = 7.4, 150 mM NaCl, 1 mg/ml BSA). After incubation, free PPACK was extensively dialyzed. We then used FXII chromogenic substrate s-2302 to confirm the complete blockade of αFXIIa-PPACK proteolytic activity.
FXII auto-activation at the surface of neurons. Neurons under serum deprivation (SD) were treated with 125 nM of single-chain FXII for several durations (1 h, 3 h, 6 h and 24 h). Then, the supernatants were collected. The conversion of FXII into the two-chain form was determined using FXII chromogenic substrate s-2302.

Statistical analyses. All results are expressed as mean ± SD. For in vitro experiments, the n value corresponds to n different well pools derived from independent dissections. For group comparison, Kruskal-Wallis tests were used followed by Mann–Whitney U-tests as post-hoc tests.

Results

FXII protects from stereotaxic brain lesion

Our first objective was to explore the potential role of FXII in brain lesions induced by experimental process independent of the circulatory system. For this, we used a classical model of brain injury induced by the stereotaxic injection of NMDA (Figure 1). We observed that the administration of FXII (1 µg) reduced the mean lesion volume by 46% (Figure 1B and corresponding quantification, Figure 1C). These data indicate that FXII could exert direct neuroprotective effects in the brain parenchyma.

FXII protects neurons from apoptosis by activating EGFR and subsequent signaling pathways

Our next step was to decipher whether FXII may also display neuroprotective effects in vitro. Considering that the in vivo paradigm of brain lesion induces neuronal apoptosis (Supplemental Figure 1), we tested the effect of single-chain FXII in cortical neurons subjected to serum deprivation (SD), a classical model of apoptosis. We observed that FXII exerted a dose-dependent anti-apoptotic effect on cortical neurons (Figure 2). Our hypothesis to explain this effect was that FXII could act, at least in part, via binding to EGFR and activation of this receptor, such as previously reported for tPA, a serine protease presenting homologous EGF-like domains. To address this question, we treated cortical neurons with biotinylated FXII, extracted the proteins and subjected them to immunoprecipitation (IP) using an anti-EGFR antibody (Figure 3A). We detected biotinylated FXII among the EGFR-immunoprecipitated proteins as a ~80kDa band revealed by peroxidase-coupled avidin (Figure 3A), at the same molecular weight as biotinylated FXII ran in parallel. FXII was absent in untreated cells (SD). This data show that FXII and EGFR are part of a same protein complex in FXII-treated neurons.

Then, we asked whether the interaction of FXII with EGFR, and its subsequent activation could be responsible for the anti-apoptotic effect of FXII on neurons. In line with this hypothesis, the inhibitor of EGF receptor kinase, AG1478 (5 µM), reversed the effect of FXII on neurons during SD, while it showed no effect when applied alone (Figure 3B).
EGFR activation can trigger several signaling cascades, including mitogen-activated protein kinase/extracellular regulated kinase (MAPK/Erk). Moreover, Tyr1068 in EGFR, the residue phosphorylated upon FXII treatment, is involved in the transduction of EGF signal through Erk pathway\textsuperscript{15}. Here, we observed that single-chain FXII (125 nM) induced the rapid (within 5 minutes) and transient (<1h) phosphorylation of Erk1/2 (Figure 4A). Accordingly, the pre-treatment with MEK/Erk1/2 inhibitor (SCH7772984, 5 µM) reversed the anti-apoptotic effect of FXII (Figure 4B). Together, these data show that the activation of EGFR by FXII triggers Erk activation, and that this pathway actively participates in the anti-apoptotic effects of FXII in neurons.

Apoptosis is regulated by a balance between pro- and anti-apoptotic factors that control downstream protease activity of effector caspases and subsequent cell death. Here, we studied whether the pro-apoptotic factor Bax and the anti-apoptotic factor Bcl-2 are regulated by FXII during SD. We observed that after 24h of SD, as compared to 1h, treatment with FXII (125 nM) significantly decreased Bax expression, while it induced the up-regulation of Bcl-2 (Figure 4C), thus reducing the ratio between pro- and anti-apoptotic factors. These data show that single-chain FXII triggers anti-apoptotic pathways in neurons subjected to SD.

**FXII promotes HGF maturation, leading to HGFR-mediated anti-apoptotic effects**

FXII can be activated from a single-chain form (FXII) to a more proteolytically active two-chain form (αFXII\textsubscript{a}) (Figure 5A). Our next step was to investigate if αFXII\textsubscript{a} showed the same anti-apoptotic effects than its single chain form, FXII. When applied to cortical neurons during SD, αFXII\textsubscript{a} exerted an anti-apoptotic effect, although at slightly higher doses than FXII (Figure 5A). Noteworthy, in contrast to what we observed for FXII, the anti-apoptotic effect of αFXII\textsubscript{a} was not completely reversed by the inhibitor of EGFR activation, AG1478 (5 nM, Figure 5B). Moreover, we observed that proteolytically inactive αFXII\textsubscript{a} (αFXII\textsubscript{a}-PPACK) still retained an antiapoptotic capacity (Supplemental Figure 2A). Thus, we wanted to study if αFXII\textsubscript{a} also acts via binding to EGFR. We repeated the immunoprecipitation studies with biotinylated αFXII\textsubscript{a}, confirming that we detected biotinylated αFXII\textsubscript{a} among the EGFR-immunoprecipitated proteins as a ~50kDa band revealed by peroxidase-coupled avidin (Figure 5C), at the same molecular weight as biotinylated αFXII\textsubscript{a} ran in parallel. In addition, the above immunoprecipitated material showed a band at approximately 175 kDa corresponding to EGFR, when revealed with anti-EGFR antibodies (Figure 5C). These data show that αFXII\textsubscript{a} and EGFR are part of a same protein complex in αFXII\textsubscript{a}-treated neurons. Interestingly, the blockade of EGFR by AG1478 is not sufficient to block αFXII\textsubscript{a}. Because αFXII\textsubscript{a} differs from FXII by its proteolytic activity, we wondered whether this activity could be involved in its anti-apoptotic function. To address this question, we co-treated neurons subjected to SD with αFXII\textsubscript{a} and Corn Trypsin Inhibitor (CTI, at 10 µM) an inhibitor of its proteolytic activity. Interestingly enough, CTI reversed the antiapoptotic effect of αFXII\textsubscript{a} (Figure 5D). Together, these results suggest that αFXII\textsubscript{a} exerts anti-apoptotic effects on neurons by a combination of proteolytic and non-proteolytic (“growth factor-like”) effects.
The proteolytic activity of αFXIIa is known, among other actions, to induce the activation of hepatocyte growth factor (HGF) from its pro-form to its active form\(^{16}\), which in turn can activate its receptor, HGFR (also known as c-Met). We thus hypothesized that the effect of αFXIIa could be mediated by the proteolytic activation of HGF and a subsequent stimulation of HGFR. When we co-treated neurons subjected to SD with αFXIIa and sc1356 (100ng/mL), a blocking antibody of HGF\(^{17}\), the anti-apoptotic effect of αFXIIa was completely reversed (Figure 6A). The same result was obtained when using JNJ, an inhibitor of HGFR phosphorylation (Figure 6B). These data show that αFXIIa triggers the activation of HGF, which in turn acts on HGFR to provide anti-apoptotic effects in neurons.

FXII activation into αFXIIa can occur either by the action of proteases\(^{1}\), or by surface-mediated auto-activation in certain conditions\(^{1,2}\). Thus, we wanted to study whether FXII could be auto-activated at the surface of neurons. By the use of a chromogenic substrate specific for αFXIIa activity, we observed that FXII underwent activation into its two-chain form at the surface of neurons (Figure 7A) and not in the absence of cells (DMEM + FXII condition). Considering the protective effect of αFXIIa described above (Figure 6), we hypothesized that protease-mediated mechanisms could also be involved in FXII anti-apoptotic effects. Indeed, the anti-apoptotic effect of FXII was also reversed by CTI (Figure 7B).

Furthermore, in purified conditions, incubation of pro-HGF with either FXII or αFXIIa led to an increase in the amount of mature HGF (Supplemental Figure 3). That may account in our conditions for an increased activation of pro-HGF in mature HGF by the protease activity which comes up from the activation of FXII into its protease-active form (Figure 7A). As previously observed with αFXIIa, FXII effects were also reversed by JNJ (Figure 7C) and sc1356 (Figure 7D). Finally, incubation of neurons with FXII led to phosphorylation of HGFR (Figure 7E), which reflects its activation. These data indicate that FXII can protect from apoptosis by protease-mediated, HGF-mediated effects, in addition to its protease-independent, EGFR mediated effects (Figure 3-4). In accordance with this, we observed that proteolytically-inactive PPACK-αFXIIa showed a residual, protease-independent, anti-apoptotic effect (Supplemental Figure 2A) reversed by AG1478 (Supplemental Figure 2B). Interestingly enough, this anti-apoptotic effect was also blocked by JNJ, showing that HGFR pathway is activated (Supplemental Figure 2C). We hypothesized that EGFR and HGFR could be active via a co-receptor crosstalk. To test if EGFR could transphosphorylate and activate HGFR independently of mature HGF generation, as reported previously, we added sc1356 (100 ng/mL) to block a putative interaction between HGFR and an extracellular ligand. The EGFR-dependent anti-apoptotic effect of αFXIIa-PPACK was not reversed when blocking the binding of HGF to HGFR (Supplemental Figure 2D), whereas – as described above - it was reversed when the phosphorylation of HGFR was blocked using JNJ. These results support the transphosphorylation of HGFR by the activation of EGFR by FXII. Finally, since it has been shown FXII activates EGFR signaling through uPAR\(^{4}\) we also tested this hypothesis using a uPAR blocking antibody. As presented in Supplemental Figure 4, FXII antiapoptotic effect appears independent of uPAR activation in this context.

Discussion
This study reveals a neuroprotective role of FXII in vivo and describes the anti-apoptotic effects of FXII against SD-induced apoptosis in neurons. We report that this effect is due to a combination of direct “growth factor-like” effects via the EGFR and proteolytic effects via the activation of the HGF/HGFR pathway. Interestingly enough, we also observed a crosstalk between EGFR and HGFR in the absence of HGF maturation. We propose a model in which FXII can induce part of its effects via the direct binding to EGFR, the subsequent activation of this receptor, the triggering of Erk pathways and the modulation of Bcl-2/Bax balance towards anti-apoptotic effects in neurons. In parallel, FXII can be activated to its two-chain, proteolytically active form αFXIIa which in turn activates proHGF into mature HGF. Mature HGF can then activate its receptor HGFR to induce additional anti-apoptotic effects.

This is the first description that FXII, in addition to its largely described effects in thrombosis and inflammation, can induce anti-apoptotic effects on neurons. It adds to previous reports on anti-apoptotic effects of the structurally related serine protease tPA on neurons11 and oligodendrocytes10. While non-proteolytic, growth factor-like effects were reported before for tPA in neurons18, the present work is the first one to show such effects for FXII in neurons. The presence of EGF-like domains in both proteins (one in tPA, two in FXII) could explain why both proteins can induce these similar trophic effects by directly binding to EGFR and activating it.

We report here that FXII activates MAPK/Erk pathway in neurons by a growth-factor like effect, which corroborates previous studies in endothelial cells4 and aortic smooth muscle cells19. In line with this, tPA, via EGFR activation, activates Bcl2 and inhibits Bax10, similarly to what reported here for FXII. Together, these studies seem to indicate that growth factor-like activity of serine proteases such as FXII and tPA trigger in neurons the same anti-apoptotic pathways than genuine growth factors.

In addition to this direct growth factor-like effect due to binding to EGFR, we report that FXII, in its proteolytic form αFXIIa, induces additional indirect growth-factor like effects by promoting the maturation of HGF, which in turn activates its receptor, HGFR. HGF is linked to the blood coagulation and fibrinolytic system not only structurally but also functionally. In fact HGF is similar to plasminogen, contains four kringle domains and a serine protease homology domain that lacks proteolytic activity20. It stimulates migration and survival of endothelial cells to repair blood vessels21. Thus, it is rational that blood coagulation system triggers activation of a growth factor that promotes angiogenesis. Here, we provide fresh knowledge on proteolytic action of FXII on HGF/HGFR leading to neuroprotection against cell death. Similar indirect trophic effects were reported before for tPA, although in a different context: by activating plasmin, tPA can convert heparin-bound HGF into free HGF, leading to subsequent HGFR signaling.

Surprisingly, we observe that even in the absence of HGF maturation, HGFR pathway can be activated by EGFR transphosphorylation. This crosstalk between EGFR and HGFR has been reported previously in cancer cell survival pathways. However, is the first time to our knowledge to be addressed in neurons under apoptotic conditions22,23.
Overall, these aforementioned works suggest that serine proteases with growth factor-like domains could in specific conditions substitute trophic molecules such as cytokines or growth factors to promote survival of brain cells. In addition to tPA and FXII, several other serine proteases contain growth-factor like domain, such as urokinase or HGF activator (HGFA)\(^{24}\). The conservation of these domains in several of these mosaic proteins is intriguing on an evolutionary point of view. Some of the functions of these proteases are redundant, while others are specific, which may explain their maintenance over evolution. Strikingly, these different studies point out the fact that serine protease such as tPA or FXII, and growth factors such as EGF or HGF are redundant and pleiotropic actors which take part in interrelated networks, in which serine proteases can facilitate growth factor maturation and activate their receptors to induce trophic effects.

In previous works, a deleterious role was attributed to circulating FXII in models of cerebral ischemia\(^{7}\) and brain trauma\(^{8}\), in which thrombosis is respectively a primary or a secondary cause of brain damage. In contrast, FXII turned out to be neuroprotective in the brain lesion model used here, where thrombosis plays very limited if any role in the development of lesions. Together, these sets of studies suggest a dual role of FXII in acute brain diseases such as stroke or head trauma: a deleterious pro-thrombotic activity in the circulation and a beneficial anti-apoptotic effect within the brain parenchyma. These opposing effects not only occur at distinct sites but are also likely to appear with distinct timings in the injured brain.

While the pro-thrombotic activity is commonly attributed to liver-derived FXII, it is tempting to hypothesize that its anti-apoptotic effects may due to an isoform so far only identified in neurons\(^{9}\). This isoform (FXII\(^{297-596}\)) is shorter than the liver-derived form and contains the proline-rich domain and the catalytic domain of FXII\(^{9}\). Interestingly, it can convert pro-HGF in mature HGF\(^{9}\), and may thus display the proteolytic, HGFR/c-Met-mediated anti-apoptotic effects described here in cultured neurons.

Neuron-derived FXII\(^{297-596}\) does not contain EGF domain and is therefore unlikely to display the “growth-factor” like effect mediated by direct EGFR activation described here. However, the full length form of FXII, able to display this effect, may reach the CNS parenchyma by at least two ways: First, its structure and size are compatible with its transport through the blood-brain barrier via either active processes or passive transfer, as previously described for other serine proteases\(^{25,26}\). Second, FXII is produced by neutrophils\(^{3}\) and these cells are known to infiltrate the injured CNS. In addition, it cannot be ruled out that CNS cells other than neurons are able to produce isoforms of FXII containing the EGF domain. This point should be addressed in future works. Besides, further studies in cell-specific FXII knock-out mice may help deciphering the respective roles of the distinct cellular origins of FXII during brain injury.

FXII has been referred to as a “mysterious” protease, and the question of the real function of FXII has even been asked\(^{27}\). In addition to the two major physiological functions attributed to FXII -maintenance of thrombus stability and regulation of vascular permeability- its anti-apoptotic action should emerge as an important function and may help explaining the evolutionary maintenance of this protease.
The present study should inspire further works concerning the effects of FXII on neuronal death in *in vivo* models of brain injury. First, a thorough description of FXII expression in brain cells is still lacking and should be the purpose of further studies using appropriate and reliable tools. Considering that it exerts common mechanisms of action with tPA, FXII may induce protective effects in models where tPA has already been shown to do so. Indeed, the growth factor-like effects of tPA (produced by brain cells or exogenously administered) have been shown to induce protection to brain cells in several *in vivo* animal models of brain injury. Noteworthy, these deleterious effects are attributed to pro-thrombotic or pro-inflammatory effects of FXII. The protective effects of FXII described here may have been masked in those conditions. Nevertheless, is important to note that the doses used in this study are well below the plasma concentrations of FXII (12.5 nM to 125 nM in the present study, versus 375 nM in plasma). Thus, in case of blood brain barrier leakage, the deleterious effects of FXII observed in different brain models could be modulated by its beneficial effects. Besides, the FXII benefits may be unveiled in models where the impact of apoptosis is superior to those of thrombosis or inflammation. These studies should thus help further understanding how FXII acts in brain diseases as a unique serine protease at the interface of thrombosis, inflammation and cell survival.

**Conclusion**

This work shows that FXII can protect neurons from cell death by acting directly on these cells. This neuroprotective effect occurs by two complementary pathways sustained respectively by “growth factor-like” and proteolytic actions. The discovery that FXII can promote neuroprotection is crucial to understand its role in neurological diseases: FXII has to be seen as a multifaceted factor which global effect will result from a balance between its respective effects on thrombosis, inflammation and neuronal survival. Increasing evidence describe that proteins primarily identified for their role in blood homeostasis can also act directly on neural cells. Our study identifies FXII as a potentially important actor of this finely regulated crosstalk occurring at the blood-brain interface.

**Abbreviations**

EGF: Epidermal Growth Factor, FXII: Factor XII, HGF: Hepatocyte Growth Factor, NMDA: N-Methyl-D-Aspartate, SD: Serum deprivation, TNF: Tumor Necrosis Factor, tPA: tissue-Plasminogen Activator.

**Declarations**

**Ethics approval:** The study design and the procedures thereof were evaluated and approved by the regional committee on animal ethics (C2EA-54) and the French Ministry of Higher Education, Research, and Innovation (project licence #2889). All animal experiments were performed and reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (http://www.nc3rs.org.uk).
Consent for publication: Not applicable.

Availability of Data and Materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest Disclosures: The authors declare that they have no competing interests.

Funding: This work was supported by the “Institut National de la Santé Et de la Recherche Médicale” (INSERM), the University of Caen Normandy, the “Etablissement Français du Sang” (EFS) and the Normandy Regional Council. T.C. and P.C were supported by FISM Onlus-“Fondazione Italiana Sclerosi Multipla”.

Authorship contributions: E.G. and D.L. performed experiments, analyzed the data and participated in manuscript redaction, M.R. S.M.D.L. performed experiments and analyzed the data, C.A, Y.H., T.C, P.C. M.R. and D.V participated in data acquisition, provided reagents and critically reviewed the manuscript, D.V and F.D secured funding of the study, S.M.D.L. and F.D. designed the study, analyzed the data and wrote the manuscript.

Acknowledgments: The authors thank Elodie Hedou for her help in pro-HGF conversion experiments.

References

1. Maas C, Renne T. Coagulation factor XII in thrombosis and inflammation. 2018, 131(17): 1903–1909.
2. Samuel M, Pixley RA, Villanueva MA, Colman RW, Villanueva GB. Human factor XII (Hageman factor) autoactivation by dextran sulfate. Circular dichroism, fluorescence, and ultraviolet difference spectroscopic studies. J Biol Chem. 1992;267(27):19691–7.
3. Stavrou EX, Fang C, Bane KL, Long AT, Naudin C, Kucukal E, et al. Factor XII and uPAR upregulate neutrophil functions to influence wound healing. J Clin Invest. 2018;128(3):944–59.
4. LaRusch GA, Mahdi F, Shariat-Madar Z, Adams G, Sitrin RG, Zhang WM, et al. Factor XII stimulates ERK1/2 and Akt through uPAR, integrins, and the EGFR to initiate angiogenesis. Blood. 2010;115(24):5111–20.
5. Ivanov I, Matafonov A, Sun MF, Cheng Q, Dickeson SK, Verhamme IM, et al. Proteolytic properties of single-chain factor XII: a mechanism for triggering contact activation. Blood. 2017;129(11):1527–37.
6. Mahdi F, Madar ZS, Figueroa CD, Schmaier AH. Factor XII interacts with the multiprotein assembly of urokinase plasminogen activator receptor, gC1qR, and cytokeratin 1 on endothelial cell membranes. Blood. 2002;99(10):3585–96.
7. Kleinschnitz C, Stoll G, Bendszus M, Schuh K, Pauer HU, Burfeind P, et al. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. J Exp Med. 2006;203(3):513–8.
8. Hopp S, Albert-Weissenberger C, Mencl S, Bieber M, Schuhmann MK, Stetter C, et al. Targeting coagulation factor XII as a novel therapeutic option in brain trauma. Ann Neurol. 2016;79(6):970–82.

9. Zamolodchikov D, Bai Y, Tang Y, McWhirter JR, Macdonald LE, Alessandri-Haber N. A Short Isoform of Coagulation Factor XII mRNA Is Expressed by Neurons in the Human Brain. Neuroscience. 2019;413:294–307.

10. Correa F, Gauberti M, Parcq J, Macrez R, Hommet Y, Obiang P, et al. Tissue plasminogen activator prevents white matter damage following stroke. J Exp Med. 2011;208(6):1229–42.

11. Liot G, Roussel BD, Lebeurrier N, Benchenane K, López-Atalaya JP, Vivien D, et al. Tissue-type plasminogen activator rescues neurons from serum deprivation-induced apoptosis through a mechanism independent of its proteolytic activity. J Neurochem. 2006;98(5):1458–64.

12. Radi E, Formichi P, Battisti C, Federico A. Apoptosis and oxidative stress in neurodegenerative diseases. J Alzheimers Dis. 2014;42(Suppl 3):125–52.

13. Radak D, Katsiki N, Resanovic I, Jovanovic A, Sudar-Milovanovic E, Zafirovic S, et al. Apoptosis and Acute Brain Ischemia in Ischemic Stroke. Curr Vasc Pharmacol. 2017;15(2):115–22.

14. Behrens MI, Koh JY, Muller MC, Choi DW. NADPH diaphorase-containing striatal or cortical neurons are resistant to apoptosis. Neurobiol Dis. 1996;3(1):72–5.

15. Rojas M, Yao S, Lin YZ. Controlling epidermal growth factor (EGF)-stimulated Ras activation in intact cells by a cell-permeable peptide mimicking phosphorylated EGF receptor. J Biol Chem. 1996;271(44):27456–61.

16. Shimomura T, Miyazawa K, Komiyama Y, Hiraoka H, Naka D, Morimoto Y, et al. Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor Xlla and hepatocyte growth factor activator. Eur J Biochem. 1995;229(1):257–61.

17. Nayeri F, Nayeri T, Aili D, Brudin L, Liedberg B. Clinical impact of real-time evaluation of the biological activity and degradation of hepatocyte growth factor. Growth Factors. 2008;26(3):163–71.

18. Vivien D, Gauberti M, Montagne A, Defer G, Touze E. Impact of tissue plasminogen activator on the neurovascular unit: from clinical data to experimental evidence. J Cereb Blood Flow Metab. 2011;31(11):2119–34.

19. Gordon EM, Venkatesan N, Salazar R, Tang H, Schmeidler-Sapiro K, Buckley S, et al. Factor XII-induced mitogenesis is mediated via a distinct signal transduction pathway that activates a mitogen-activated protein kinase. Proc Natl Acad Sci U S A. 1996;93(5):2174–9.

20. Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. Nat Rev Mol Cell Biol. 2010;11(12):834–48.

21. Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, et al. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol. 1992;119(3):629–41.

22. Breindel JL, Haskins JW, Cowell EP, Zhao M, Nguyen DX, Stem DF. EGF receptor activates MET through MAPK to enhance non-small cell lung carcinoma invasion and brain metastasis. Cancer Res. 2013;73(16):5053–65.
23. Reznik TE, Sang Y, Ma Y, Abounader R, Rosen EM, Xia S, et al. Transcription-dependent epidermal growth factor receptor activation by hepatocyte growth factor. Mol Cancer Res. 2008;6(1):139–50.

24. Shia S, Stamos J, Kirchhofer D, Fan B, Wu J, Corpuz RT, et al. Conformational lability in serine protease active sites: structures of hepatocyte growth factor activator (HGFA) alone and with the inhibitory domain from HGFA inhibitor-1B. J Mol Biol. 2005;346(5):1335–49.

25. Benchenane K, Berezowski V, Ali C, Fernández-Monreal M, López-Atalaya JP, Brillault J, et al. Tissue-type plasminogen activator crosses the intact blood-brain barrier by low-density lipoprotein receptor-related protein-mediated transcytosis. Circulation. 2005;111(17):2241–9.

26. Benchenane K, Berezowski V, Fernández-Monreal M, Brillault J, Valable S, Dehouck MP, et al. Oxygen glucose deprivation switches the transport of tPA across the blood-brain barrier from an LRP-dependent to an increased LRP-independent process. Stroke. 2005;36(5):1065–70.

27. de Maat S, Maas C. Factor XII: form determines function. J Thromb Haemost. 2016;14(8):1498–506.

28. Lemarchand E, Maubert E, Haelewyn B, Ali C, Rubio M, Vivien D. Stressed neurons protect themselves by a tissue-type plasminogen activator-mediated EGFR-dependent mechanism. Cell Death Differ. 2016;23(1):123–31.

29. Fredriksson L, Lawrence DA, Medcalf RL. tPA Modulation of the Blood-Brain Barrier: A Unifying Explanation for the Pleiotropic Effects of tPA in the CNS. Semin Thromb Hemost. 2017;43(2):154–68.

30. Zamolodchikov D, Chen ZL, Conti BA, Renne T, Strickland S. Activation of the factor XII-driven contact system in Alzheimer's disease patient and mouse model plasma. Proc Natl Acad Sci U S A. 2015;112(13):4068–73.

31. Chen ZL, Revenko AS, Singh P, MacLeod AR, Norris EH, Strickland S. Depletion of coagulation factor XII ameliorates brain pathology and cognitive impairment in Alzheimer disease mice. Blood. 2017;129(18):2547–56.

32. Kleinschnitz C, Stoll G, Bendszus M, Schuh K, Pauer HU, Burfeind P, et al. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. J Exp Med. 2006;203(3):513–8.

33. Stavrou E, Schmaier AH. Factor XII: what does it contribute to our understanding of the physiology and pathophysiology of hemostasis & thrombosis. Thromb Res. 2010;125(3):210–5.

Figures
Factor XII (FXII) reduces brain lesions induced by stereotaxic injection of N-Methyl-D-Aspartate. A: Experimental design. B: Representative T2-weighted (top) and T2*-weighted MRI images, showing respectively the NMDA-induced lesion and the absence of hemorrhage, in vehicle and FXII treated mice 24 hours after NMDA ic injection. T2 (top) and T2* (bottom) C: Lesion volume quantification. n=20 mice per group. Data are presented as mean ± S.E.M and individual values. Mann Whitney’s U test, ***p<0.001.
Figure 2

FXII rescues neurons from serum deprivation-induced apoptosis. Quantification of neuronal death following 24h of serum deprivation (SD) alone or in the presence of recombinant FXII (12.5–250 nM). Error bars represent the mean ± SEM. Symbols indicate significantly different from SD by Mann Whitney (*** p < 0.001, n = 28 from 7 different experiments). CTRL: control.
FXII interacts with and activates Epithelial Growth Factor Receptor (EGFR) to mediate its antiapoptotic effects in neurons. (A) 100 µg of total proteins from lysates of untreated (SD) or biotinylated FXII (Biotin-FXII)–treated mouse neurons (125nM) or purified Biotin-FXII were subjected to immunoprecipitation (IP) using α-EGFR antibody followed by detection with either peroxidase-coupled avidin or with α-EGFR. As a control, the same procedure was performed by omitting the α-EGFR (No Ab). Factor XII and alpha-Factor
XIIa are indicated as FXII or αFXIIa, respectively. Representative images of immunoblots from three individual experiments are presented. Numbers indicate molecular mass of standard proteins in kilodaltons (kDa). (B) Quantification of neuronal death following 24h of either SD alone (blue) or SD in the presence of 125 nM FXII (yellow) with or without 5 µM of the EGFR kinase inhibitor, AG1487 (green; mean ± SEM; n = 16 in 4 different experiments). *** and ###, p < 0.001 significantly different from SD and FXII respectively. CTRL: control.

**Figure 4**

The antiapoptotic effect of FXII implicates activation of Erk1/2 intracellular pathway and targets anti-apoptotic pathways. (A) Immunodetection of total and phosphorylated Erk1/2 forms (denoted with a prefix, tot and p respectively) in 20 µg of total proteins from lysates of neurons subjected to SD in the presence of 125 nM FXII after 5', 30' and 1h of incubation. Actin was used as a loading control. Total forms of Erk1/2 was used as a control. Representative images of four independent experiments are presented. *, p < 0.05 significantly different from corresponding SD. (B) Quantification of neuronal death following 24 h of either SD alone or SD in the presence of 125 nM FXII (yellow) with or without 5 µM Erk1/2 inhibitor, SCH772984 (green; mean ± SEM; n = 16 in 4 different experiments). *, p < 0.05 and #, p < 0.05 significantly different from SD. (C) Immunodetection of Bax and Bcl-2 in 20 µg of total proteins from lysates of neurons subjected to SD in the presence of 125 nM FXII at different times (1h and 24h).
Representative images from three individual experiments are presented. Actin was used as a loading control.

**Figure 5**

Active alpha-Factor XII (αFXIIa) rescues neurons from serum deprivation-induced apoptosis, but the mechanism is only partially dependent on EGFR activation. (A) Quantification of neuronal death following 24h of either SD alone or SD in the presence of recombinant αFXIIa (12.5–125 nM). Error bars represent the mean ± SEM. Symbols indicate significantly different from SD by Mann Whitney (** p < 0.01, n = 16 in 4 different experiments). (B) Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125 nM αFXIIa (orange) with or without 5 µM EGFR kinase inhibitor, AG1478 (green; mean ± SEM; n = 16 in 4 different experiments). ***, p < 0.001 and no significantly different from SD and αFXIIa respectively. (C) 100 µg of total proteins from lysates of untreated (SD) or biotinylated αFXIIa (Biot-αFXIIa)–treated mouse neurons (125nM) or purified Biot-αFXIIa were subjected to immunoprecipitation (IP) using α-EGFR antibody followed by detection with either peroxidase-coupled avidin or with αEGFR. As a control, the same procedure was performed by omitting the α-EGFR (No Ab).
Factor XII and alpha-Factor XII are indicated as FXII or α-FXIIa, respectively. Representative images of immunoblots from three individual experiments are presented. Numbers indicate molecular mass of standard proteins in kilodaltons. (D) Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125 nM αFXIIa (orange) with or without 4 or 10 µM FXIIa inhibitor, Corn trypsin inhibitor, CTI (green; mean + SEM; n = 16 in 4 different experiments). ***, p < 0.001 and #, p < 0.05 significantly different from SD and αFXIIa respectively. CTRL: Control.

Figure 6

The antiapoptotic effect of αFXIIa is also dependent on HGF conversion and HGFR/c-MET activation. (A) Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125 nM αFXIIa (orange) with or without a blocking antibody of HGF (green; mean + SEM; n = 16 in 4 different experiments). **, p < 0.01 and #, p < 0.05 significantly different from SD and αFXIIa respectively. (B) Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125 nM αFXIIa (orange) with or without 500 nM JNJ, an inhibitor of MET phosphorylation (green; mean + SEM; n = 16 in 4 different experiments). *, p < 0.05 and #, p < 0.05 significantly different from SD and αFXIIa respectively.
Figure 7

FXII auto-activation during SD triggers HGF maturation and resultant mature HGF interacts with HGFR/c-MET to mediate its antiapoptotic effects in neurons. (A) Supernatants of neurons subjected to SD in the presence or not of 125 nM FXII during 1h to 24 h were incubated with 800 µM chromogenic substrate S-2302 (yellow; conditions with neuron). In control 125 nM FXII in DMEM was incubated during 1h to 24h at 37°C (green; condition without neurons). Changes in OD 405 nm were continuously monitored on a microplate reader. (B) Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125 nM FXII (yellow) cotreatment with or without 4 µM αFXIIa inhibitor, corn trypsin inhibitor, CTI (green; mean ± SEM; n = 16 in 4 different experiments). *** and ###, p < 0.001 significantly different from SD and FXII respectively. (C) Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125 nM FXII (yellow) with or without 500 nM JNJ, an inhibitor of MET phosphorylation (green; mean ± SEM; n = 16 in 4 different experiments). *, p < 0.05 and no significantly different from SD and FXII respectively. (D) Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125 nM FXII (yellow) with or without 50-100 ng/mL sc1356, a blocking antibody of HGF (green; mean ± SEM; n = 16 in 4 different experiments). *, p < 0.05 and ###, p < 0.001 significantly different from SD and FXII. (E) The phosphorylated form of MET was immunodetected in 20
µg of total proteins 20 µg of total proteins from lysates of neurons subjected to SD in the presence of 125 nM FXII. Total MET was used as a control. Representative images from four individual experiments are presented and the relative quantification. *, p < 0.05 different from SD and FXII.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalMaterialGarnieretal.docx