Factor XII protects neurons from apoptosis by epidermal and hepatocyte growth factor receptor-dependent mechanisms

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Essentials section points:

- Several studies have shown that plasma Factor XII exerts a deleterious role in cerebral ischemia and traumatic brain injury by promoting thrombo-inflammation.
- Nevertheless, the impact of FXII on neuronal cell fate remains unknown.
- FXII and FXIIa exert neuroprotective effects in the brain parenchyma *in vivo*.
- This anti-apoptotic effect of FXII is mediated by epidermal and hepatocyte growth factor receptor-dependent mechanisms.

Abstract

Background

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

Objectives

We investigated the role of FXII and FXIIa in neuronal injury and apoptotic cell-death.

Methods

We tested the neuroprotective roles of FXII and FXIIa in an experimental model of neuronal injury induced by stereotaxic intracerebral injection of N-Methyl-D-Aspartic acid (NMDA) *in vivo* and in a model of apoptotic death of murine primary neuronal cultures through serum-deprivation *in vitro*.

Results

Here, we found that exogenous FXII and FXIIa reduce brain lesions induced by NMDA injection *in vivo*. Furthermore, FXII protects cultured neurons from apoptosis through a growth factor-like effect. This mechanism was triggered by direct interaction with epidermal growth factor (EGF) receptor and subsequent activation of this receptor. Interestingly, the “proteolytically” active and two-chain form of FXII, FXIIa, exerts its protective effects by an alternative signaling pathway. FXIIa activates the pro-form of hepatocyte growth factor (HGF) into HGF, which in turn activated HGF receptor (HGFR) pathway.

Conclusion

This study describes two novel mechanisms of action of FXII and identifies neurons as target cells for the protective effects of single and two-chain forms of FXII. Therefore, inhibition of FXII in neurological disorders may have deleterious effects by preventing its beneficial effects on neuronal survival.

Keywords: Apoptosis, Contact Pathway, Intrinsic Pathway, Neuroprotection, Thromboinflammation.

Introduction

Factor XII (FXII), is a 80 kDa chymotrypsin-like serine protease with various effects in the circulation, ranging from activation of the phase contact ("intrinsic") coagulation pathway to pro-inflammatory actions[1]. FXII is primarily produced as a zymogen single-chain enzyme, secondarily activated to its two-chain form, FXIIa, by plasma kallikrein or by auto-activation when bound to some negatively charged and anionic surfaces[2-4]. Although most of the reported actions of FXII are due to the proteolytic activity of its active form FXIIa, proteolytic and non-proteolytic actions of the zymogen FXII have also been reported[5-7] . Several receptors, including uPAR and Epidermal Growth Factor (EGF) receptor (EGFR)[5, 6, 8] have been suggested to mediate these non-proteolytic effects, mediating proliferative responses in endothelial cells.

Several studies have shown that circulating FXII can exert detrimental effects in cerebral ischemia[9] and traumatic brain injury[10] by promoting thrombo-inflammation. Nevertheless, the question remains open whether FXII may impact neuronal cell fate within the brain parenchyma, independently of its effects on vascular injury. This question is important considering the recent discovery that a short isoform of FXII is expressed by neurons in the brain[11].

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 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) [12, 13]. In light of the above, we hypothesized that Factor XII could promote anti-apoptotic effects in neurons by activating EGFR signaling pathway.

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[14-16]. 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[12]. This latter condition can be mimicked *in vitro* by removing trophic support from cultured neurons in a classical paradigm termed serum deprivation (SD)[13].

Here, we report that both forms of inactive and active FXII (FXII and FXIIa) protect from neuronal injury induced by stereotaxic intracerebral injection of N-Methyl-D-Aspartic acid (NMDA) *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. Interestingly, we observe that FXIIa also protects neurons by indirect trophic effects: it cleaves the pro-form of hepatocyte growth factor (HGF) into HGF, which in turn protects neurons from apoptosis by HGFR pathway activation. Together, these data indicate that both forms of FXII promote survival of neurons by EGFR and HGRF dependent mechanisms.

Materials and methods

Materials

Corn trypsin inhibitor (CTI) and all FXII forms (single-chain FXII, FXIIa and β-FXIIa) were purchased from Enzyme Research Laboratories (UK) and presented with a certificate of analysis. As detailed by the supplier, all proteins were purified from human plasma. FXII content was determined to be >95% as judged by SDS-PAGE and showed no reduction upon incubation with 2-mercaptoethanol. FXIIa β-FXIIa were activated from homogeneous FXII using dextran sulfate (unknown concentration) that was removed thereafter. Complete activation was observed on SDS-PAGE.

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 HGF (PHG0254) were obtained from Invitrogen. Anti-HGF (sc1356) and anti-kininogen (sc-59581) antibodies were obtained from Santacruz. Anti-Actin (A2066), antibody was 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. Blocking anti-HGF (AF2207) and recombinant human HGF Propeptide (pro-HGF, 7057-HG-010) were obtained from R&D Systems. Erk Inhibitor (SCH772984) was obtained from Selleckchem. JNJ-38877605 was kindly provided by Janssen Pharmaceutica. Rabbit anti-mouse plasminogen polyclonal antibody was kindly provided by HR Lijnen (University of Leuven, Belgium). Mouse FXII total antigen assay ELISA kit came from Molecular Innovations.

Animals

Studies were conducted in male Swiss mice (DAP #2889; 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. 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_2O 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 or FXIIa (1µg; FXII and FXIIa groups respectively) or control buffer (vehicle group) was inserted (coordinates: -1mm anteroposterior; +3,3mm lateral; -0,8mm 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 for 2 minutes (by applying positive pressure with a syringe connected to the pipette through a catheter). After the injection, the pipette was left in place again for 2 minutes to ensure adequate diffusion of the solution.

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 above. 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_2O 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. Apoptotic lesion sizes were manually segmented on each section of T2-weighted images to calculate the total volume of brain lesions. These images were analyzed using ImageJ software by a blinded investigator. T2*-weighted sequences were used to control for brain hemorrhage.”

TUNEL immunofluorescence analyses

TdT-mediated biotin-dUTP nick end labeling (TUNEL) immunofluorescence analyses were performed in control (vehicle) and FXII treated-mice (n=5/group). 6h 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 veronal buffer; 24 hours; 4°C) before freezing in Tissue-Tek (Miles Scientific, Naperville, IL, USA). Cryomicrotome-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. For detection, 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). TUNEL+ cells were calculated in both ipsilateral and contralateral cortical areas for both groups.

Primary murine neuronal cortical cultures

Murine neuronal cultures were prepared as previously described in Liot et *al[13]*. 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% CO_2 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[13]. This condition enables to eliminate all exogenous FXII coming from the serum. Controls were maintained in serum-containing medium and allow calibrating as the 0% of death. MK801 (1 µM) was added to prevent secondary NMDA receptor activation. Cells under SD were treated with FXII, FXIIa or FXIIa-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) or media supernatants (20µl) were separated by using a sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred into a polyviny-lidene difluoride membrane. Membranes were blocked for 2h 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-αEGFR, anti-pErk, anti-Erk total, anti-kininogen, anti-HGF (AF2207) and anti-Actin at 1/1000; anti-αHGF (s1256) at 1/500, anti-plasminogen 1/1500. After washes and 1h of incubation with the corresponding peroxidase secondary antibody, proteins were revealed with a chemiluminescence ECL select immunoblotting detection system (GE Healthcare).

EGFR crossed Immunoprecipitation assay

Biotinylated FXII and FXIIa (Biot-FXII and Biot-FXIIa respectively) were produced following manufacture's kit (EZ-link® Sulfo-NHS-LC-Biotinylation). After treatment during 24h with 125nM of Biot-FXII and Biot-FXIIa, 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)[12].

FXIIa-PPACK generation

To inhibit FXIIa proteolytic's activity, 250 nM of FXIIa 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 FXIIa-PPACK proteolytic activity.

FXII autoactivation by neurons during SD.

To measure the possible activation of FXII into FXIIa *in vitro*, neurons under serum deprivation (SD) were treated with 125nM of FXII over time (1h, 4h, 6h and 24h). Then, the supernatants were harvested. The possible activation of FXII into FXIIa was determined in supernatants by two techniques. FXII immunoblotting under non-reduced conditions (using FXII and FXIIa as controls), and by measuring FXIIa-mediated hydrolysis of chromogenic substrate S-2302 at 800µM. Changes in OD 405 nm were continuously monitored on a microplate reader for 2 hours. FXIIa at 125nM was used as control.

Artificial activation of FXII by the glass micropipette.

To measure the possible FXII auto-activation into FXIIa *in vivo* through the contact with the glass micropipette 1µg of FXII was processed through the glass micropipette

following the same protocol performed in the *in vivo* injection. Then the sample was retrieved and migrated in non-reduced conditions and Coomasie staining was performed. FXII and FXIIa were used as negative and positive controls, respectively.

Cleavage of pro-HGF in HGF in recombinant purified conditions

To study activation of pro-HGF into HGF, the recombinant form of pro-HGF (52nM) was incubated with either 5.2nM of FXII, FXIIa, β-FXIIa and FXIIa-PPACK for 4 hours at 37°C. At the end of the incubation, HGF Western blot which discriminates both forms of HGF was performed.

Statistical analysis

All results are expressed as mean ± SD unless otherwise stated. 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. For *in vivo* experiments, Dunn's multiple comparison test were performed.

Results

FXII and FXIIa reduces brain lesion induced by stereotaxic injection of N-Methyl-D-Aspartate (NMDA).

Our first objective was to explore the potential role of FXII in the brain parenchyma, independently of its effects on vascular injury, known to be detrimental. With this aim, a classical model of brain injury induced by the stereotaxic injection of N-Methyl-D-Aspartate (NMDA, 1µL at 40mM) in the cortex (Figure 1A) was performed. We observed that the administration of FXII and FXIIa 10min before the injection (1µg in 1µL) significantly reduced the mean lesion volume (by 45% and 37% as compared to control group) 24h after, measured by MRI (Figure 1B and corresponding quantification, Figure 1C). These data indicate that both forms of FXII exert neuroprotective effects in the brain parenchyma.

It is well-described in the literature that NMDA injection promotes apoptotic lesions that can be measured by TdT-mediated biotin-dUTP nick end labeling (TUNEL) immunofluorescence analyses [17]. Therefore, we performed TUNEL immunofluorescence analyses in a new set-up of experiments in control and FXII treated mice, confirming the anti-apoptotic effect by histological feature at an early time point (6h). As observed in Supplemental Figure 1, FXII significantly reduces apoptotic neuronal cell death in the ipsilateral cortex as compared to vehicle-treated mice (*$p<0.05$). Contralateral cortical brains were used as controls, showing a lack of significant apoptosis in both groups due to NMDA injection (Supplemental Figure 1).

FXII protects neurons from apoptosis by activating EGFR and subsequent signaling pathway.

Our next step was to investigate whether both forms of FXII display also neuroprotective effects *in vitro*. We first tested the effect of zymogen FXII in cortical neurons subjected to serum deprivation (SD), a classical and standardized model of apoptosis[13].

Interestingly, we observed that FXII exerted a dose-dependent anti-apoptotic effect (0 to 250nM) as compared to control (SD) (Figure 2). Our hypothesis to explain this effect was that FXII could act, at least in part, due to the binding to EGFR and its activation, such as previously reported for tPA[12, 13], a serine protease that presents 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 the EGFR 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[18]. We first confirmed that Erk phosphorylated levels were not significantly modified after SD compared to controls (Supplemental figure 2). Then, we studied Erk phosphorylation levels after FXII administration in SD conditions at different time-points. In this case, we observed that FXII (125nM) induced the rapid (within 5 minutes) and transient (<1h) phosphorylation of Erk1/2 (Figure 4A). Accordingly, the pre-treatment with MEK/Erk ½ 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.

FXIIa also protects neurons from apoptosis by binding EGFR but not activating downstream signaling.

We know that once FXII is activated from its single-chain form (FXII) it changes into a proteolytically active two-chain form (FXIIa). Since FXIIa also significantly reduced brain lesion *in vivo* induced by injection of intracortical-NMDA (Figure 1C), our next step was to investigate if FXIIa showed the same anti-apoptotic effects than FXII *in vitro.* When applied to cortical neurons during SD, FXIIa exerted an anti-apoptotic effect in a dose-dependent manner, although at slightly higher doses than FXII (Figure 5A). Noteworthy, in contrast to what we observed for FXII, the anti-apoptotic effect of FXIIa was not reversed by the inhibitor of EGFR activation, AG1478 (5 nM, Figure 5B). Moreover, we observed that proteolytically inactive FXIIa (FXIIa-PPACK) retained residual antiapoptotic capacity that was blocked by AG1478 (Supplemental Figure 3A and B).

To test whether FXIIa is able to bind to EGFR, we repeated the immunoprecipitation studies with biotinylated FXIIa. As observed in figure 5C, biotinylated FXIIa is present among the EGFR-immunoprecipitated proteins as a ~50kDa band revealed by peroxidase-coupled avidin (Figure 5C), at the same molecular weight as biotinylated FXIIa ran in parallel. Similarly, 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 FXIIa and EGFR are part of a same protein complex in FXIIa-treated neurons and confirm that FXIIa binds to EGFR, as previously shown for FXII. Interestingly, the blockade of EGFR by AG1478 is not sufficient to block FXIIa. Because FXIIa 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 FXIIa and Corn Trypsin Inhibitor (CTI, at 10 µM) an inhibitor of its proteolytic activity. Interestingly, CTI reversed the antiapoptotic effect of FXIIa (Figure 5D). Together, these results suggest that FXIIa exerts anti-apoptotic effects on neurons mainly by a proteolytic effect.

FXIIa protects neurons from apoptosis by promoting pro-HGF cleavage, leading to HGFR-mediated anti-apoptotic effect.

The proteolytic activity of FXIIa is known, among other actions, to induce the cleavage and activation of hepatocyte growth factor (HGF) from its pro-form to its active form[19], which in turn can activate its receptor, HGFR (also known as c-Met). We thus

hypothesized that the effect of FXIIa could be mediated by the proteolytic activation of HGF and a subsequent stimulation of HGFR. When we co-treated neurons subjected to SD with FXIIa and an anti-HGF blocking antibody (2.5µg/mL) that prevents HGF and pro-HGF binding to the cells surface[20, 21], the anti-apoptotic effect of FXIIa was completely reversed (Figure 6A). An isotype of anti-HGF was used as control antibody showing no effect when added with FXIIa (Figure 6A). Interestingly, as shown on Figure 6B, the blocking anti-HGF antibody completely reverted the antiapoptotic effect of FXIIa. Moreover, it increased the quantity of free pro-HGF and HGF in the medium of SD neurons, supporting that it prevented their binding to the cellular surface (Figure 6B). JNJ, an inhibitor of HGFR phosphorylation (Figure 6C) also prevented FXIIa protective effect but had no effect on free pro-HGF and HGF in the medium (Supplemental figure 4A). These results were confirmed when adding FXIIa with another anti-HGF antibody (SC1356)[22] (Supplemental Figure 4B) whereas the anti-apoptotic effect of FXIIa-PPACK was not reversed when blocking HGF with SC1356 (Supplemental Figure 3D), showing that the serine protease domain activity is required for HGF signaling pathway. We also confirmed that only FXII forms that present active serine protease (FXIIa and β-FXIIa) can efficiently convert pro-HGF into HGF, as compared to FXII and FXIIa-PPACK (Supplemental Figure 5).

These data show that FXIIa triggers the activation of pro-HGF into HGF, which in turn binds and activates HGFR pathway to provide anti-apoptotic effects in neurons.

Lastly, we know that FXII-driven contact system start coagulation and inflammatory mechanisms via the intrinsic pathway of coagulation and the bradykinin-producing kallikrein-kinin system. Thus, we analyzed by Western Blot the levels of plasminogen and high molecular weight kininogen (HK), both precursors of the fibrinolytic and contact pathways that are activated by FXIIa in the supernatant of neurons in control, SD and SD+FXIIa conditions compared to controls. As shown on supplemental figure 6, plasminogen and HK are not readily detectable in the SD conditions. Besides, there is no apparent activation of plasminogen into plasmin nor HK cleavage in FXIIa conditions. Our results show that neither fibrinolytic nor contact pathway systems have an impact on FXIIa protective effects (Supplemental figure 6).

FXII activation into FXIIa can occur either by the action of proteases[1], or by surface-meditated auto-activation in certain conditions, such as anionic surfaces[1-4]. Thus, we wanted to study whether FXII could be auto-activated during the injection with the contact of the glass-micropipette in our *in vivo* experiments, as well as at the surface of neurons during our SD experiments in our *in vitro* set of data. For the *in vivo* experiments, we performed a control experiment to confirm that there was no auto-activation of zymogen FXII into FXIIa during the injection though the glass micro-pipette (Supplementary Figure 7). Then, we tested if FXII (at 125nM) would be activated at the surface of neurons during the 24h of SD at different time points (1h, 4h, 6h and 24h) *in vitro*. As observed on Figure 7A, FXII is only marginally converted into FXIIa at the surface of neurons at 24 by FXII immunoblot (Figure 7A). No FXIIa is present at 1h, 4h and 6h incubation nor in the SD control condition (0h). When looking at the proteolytic activity assay, FXII from supernatants also presented residual (below 4 A.U.) proteolytic activity when monitoring s-2302 cleavage as compared to FXIIa used at the same dose (>70 AU) (Figure 7B). These results confirm that there is no significant auto-activation of FXII into FXIIa at the surface of neurons in SD conditions in 24h.

Discussion

This study reveals the neuroprotective roles of FXII and FXIIa *in vivo* and describes the anti-apoptotic effects of both forms of FXII against SD-induced apoptosis in neurons. We report that their effect is due to either “growth factor-like” activity via EGFR binding (for FXII) or proteolytic activity via the activation of the HGF/HGFR pathway (for FXIIa). We propose a model in which FXII induce its effects via the direct binding to EGFR, the subsequent activation of this receptor, the triggering of Erk pathways that would lead to modulation of gens balance towards anti-apoptotic effects in neurons. In parallel, FXII once activated into FXIIa, will activate pro-HGF into HGF secreted from neurons under serum deprivation. HGF can thus bind and activate its receptor HGFR to induce anti-apoptotic effects (Figure 8).

As far as we know, 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 neurons[13] and oligodendrocytes[12]. While non-proteolytic, growth factor-like effects were reported before for tPA in neurons[23], the present work is the first one to show such effects for both forms of 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. Apoptosis is regulated by a balance between pro- and anti-apoptotic factors that control downstream protease activity of effector caspases and subsequent cell death.

We report here that FXII activates MAPK/Erk pathway in neurons by a growth-factor like effect, which corroborates previous studies in endothelial cells[6] and aortic smooth muscle cells[24]. In line with this, tPA, via EGFR activation, activates Bcl-2 and inhibits Bax[12]. 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. Nevertheless, the signaling pathways underlining the neuroprotective effect of FXII and FXIIa, such as the regulation of pro and anti-apoptotic genes, remain to be studied.

We have confirmed that FXII and FXIIa forms bind EGFR by co-immunoprecipitation studies. Our data suggest that FXIIa binds EGFR, but in contrast to FXII, its neuroprotective effects are not dependent on EGFR signaling pathway. Maybe the

change of the 3D structure of FXIIa might be sufficient to block its ability to activate EGFR without blocking its ability to bind it. Nevertheless, this hypothesis remains to be confirmed. The determination of the affinity or kinetics of this interaction remains to be assessed in further studies, for instance by surface plasmon resonance to help understanding the nature of the binding at the molecular level. Besides, since it has been shown that FXII activate EGFR signaling though uPAR[6], it remains to be tested on further studies if our results are dependent on uPAR activation or directly operated by EGFR binding.

In addition to this direct growth factor-like effect due to binding to EGFR, we report that FXII, in its proteolytic form FXIIa, displays anti-apoptotic effects by promoting the activation of pro-HGF into 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 structurally similar to plasminogen, it contains four kringle domains and a serine protease homology domain that lacks proteolytic activity[25]. It stimulates migration and survival of endothelial cells to repair blood vessels[26]. Here, we provide evidence on a proteolytic action of FXII on HGF/HGFR leading to neuroprotection against cell death. Similar indirect trophic effects have been reported for tPA, although in a different context in which by the activation of plasmin, tPA can convert heparin-bound HGF into free HGF, leading to subsequent HGFR signaling.

Overall, these aforementioned studies 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 domains, such as urokinase or HGF activator (HGFA)[27]. 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 proteases 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.

Of note, it has previously been demonstrated that the ligand/receptor binding ability of FXII relies on sufficiently high ambient concentration of free Zinc (Zn) [4, 8]. Nevertheless, we were not able to test this hypothesis because Zn is toxic to neurons at concentrations relevant for FXII activity [28-30].

On the other hand, a deleterious role was attributed to circulating FXII/FXIIa in models of cerebral ischemia[9] and brain trauma[10], in which thrombosis are respectively a primary or a secondary cause of brain damage. In contrast, both forms of FXII turned out to be neuroprotective in the brain lesion model used in our study, probably because thrombosis plays 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 opposite 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 be due to an isoform so far only identified in neurons[11]. This isoform ($FXII_{297-596}$) is shorter than the liver-derived form and contains the proline-rich domain and the catalytic domain of FXII[11]. Interestingly, it can convert pro-HGF in mature HGF[11], 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 (BBB) via either active processes or passive transfer, as previously described for other serine proteases[31, 32]. Second, FXII is produced by neutrophils[5] and these cells are known 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. In line with these results, we detected low levels of FXII in the brain cortex by ELISA but not by immunoblotting (Supplementary Figure 8). Notably, in pathological conditions involving BBB leakage, FXII that is present in the plasma at higher concentrations could reach the brain and increase the local

parenchymal concentration. This point should be addressed in future studies. Besides, further cell-specific FXII knock-out mice may help deciphering the respective roles of the distinct cellular origins of FXII during brain injury.

In neuronal-cell culture system, we have observed that FXII exerts anti-apoptotic effects by binding to EGFR and FXIIa by activating pro-HGF. Nevertheless, especially *in vivo*, we cannot exclude that the neuroprotective effect is also mediated by other pathways.

FXII has been referred to as a "mysterious" protease, and the question of the real function of FXII has even been asked[33]. 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.

Besides apoptosis, our *in vivo* results suggest that FXII and FXIIa protect from NMDA induced neuronal cell death. This might be relevant to several neurological diseases involving excitotoxicity such as ischemic stroke, Alzheimer disease or multiple sclerosis[34-36].

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[12, 23, 37, 38] independently of its effect in bloodstream. Several studies in animal models of brain diseases have reported deleterious effects of FXII[10, 39-41]. 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 (12.5nM to 125nM in the present study, versus 375nM in plasma)[42]. Thus, in case of BBB 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 on

how FXII acts in brain diseases as a unique serine-protease at the interface of thrombosis, inflammation and cell survival.

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Authorship contributions: E.G. D.L. performed experiments analyzed the data and participated in manuscript redaction, M.R. C.A; S.M.D.L. performed experiments and analyzed the data, C.A, Y.H., M.G., 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, I.B., F.D., and S.M.D.L. designed the study, analyzed the data and wrote the manuscript.

Conflict of Interest Disclosures: The authors have nothing to disclose.

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Figure Legends

Figure 1. Factor XII and Factor XIIa (FXII and FXIIa respectively) reduce brain lesions induced by stereotaxic injection of N-Methyl-D-Aspartate (NMDA). A: Experimental design. B: Representative T2-weighted (top) and T2*-weighted MRI images, showing respectively the NMDA-induced lesion in vehicle and FXII/FXIIa treated mice 24 hours after NMDA intracortical injection. T2 (top) and T2* (bottom) C: Lesion volume quantification. n=30 mice per group. Data are presented as mean ± SD and individual values. Dunn's multiple comparisons test, ***p<0.001, *p<0.05.

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 (12,5–250nM). Error bars represent the mean ± SD. Symbols indicate significantly different from serum deprivation by Mann Whitney (***p<0.001, n=16-28 from 4-7 different experiments). CTRL:control.

Figure 3. FXII interacts with and activates Epithelial Growth Factor Receptor (EGFR) to mediate its antiapoptotic effects in neurons. A: 100µg 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 Factor XIIa are indicated as FXII or FXIIa, 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 or SD in the presence of 125nM FXII with or without 5 µM of the EGFR kinase inhibitor, AG1487 (mean ± SD; n=12 in 3 different experiments). *** and ###, p<0.001 significantly different from SD and FXII respectively. CTRL:control.

Figure 4. Antiapoptotic effect of FXII implicates activation of Erk1/2 intracellular pathway. A: Immunodetection of total and phosphorylated Erk1/2 forms (denoted with a prefix, pErk and Erk total respectively) in 20µg of total proteins from lysates of neurons

subjected to SD in the presence of 125nM FXII after 5', 30' and 1h of incubation. Actin was used as a loading control. Total forms of Erk1/2 were 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 24h of either SD alone or SD in the presence of 125nM FXII with or without 5μM Erk1/2 inhibitor, SCH772984 (mean ± SD; n=12 in 3 different experiments). *, p<0,05 significantly different from SD.

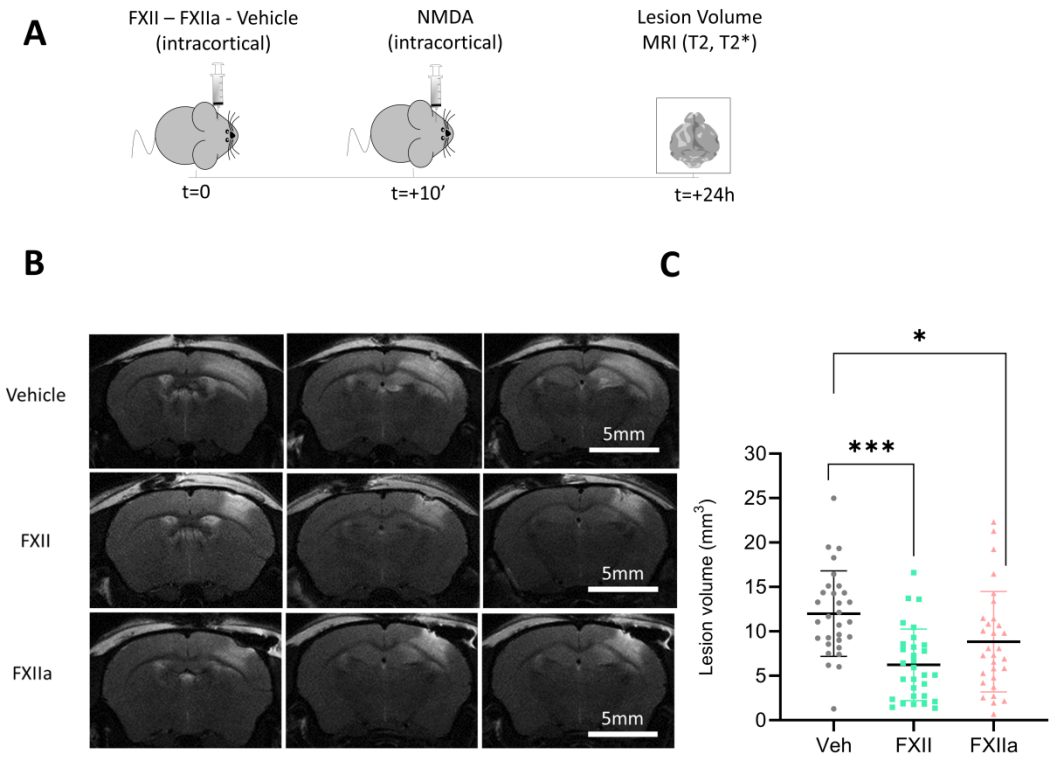
Figure 5. Factor XIIa (FXIIa) rescues neurons from serum deprivation-induced apoptosis. The mechanism is dependent on EGFR binding but not EGFR signalling pathway activation. A: Quantification of neuronal death following 24h of either SD alone or SD in the presence of recombinant FXIIa (12,5–125nM). Error bars represent the mean ± SD. Symbols indicate significantly different from SD by Mann Whitney (** p<0.01, n=12 in 3 different experiments). B: Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125nM FXIIa with or without 5μM EGFR kinase inhibitor, AG1478 (mean ± SD; n=16 in 4 different experiments). ***, p<0,001 and no significantly different from SD and FXIIa respectively. C: 100 μg of total proteins from lysates of untreated (SD) or biotinylated FXIIa (Biot-FXIIa)–treated mouse neurons (125nM) or purified Biot-FXIIa 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). 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 125nM FXIIa with or without 10μM FXIIa inhibitor, Corn trypsin inhibitor, CTI (mean + SD; n = 16 in 4 different experiments). *, p<0.05 and #, p<0.05 significantly different from SD and FXIIa respectively. CTRL: Control.

Figure 6. The antiapoptotic effect of FXIIa is also dependent on HGF conversion and HGFR activation. A: Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125nM FXIIa with or without an IgG and/or blocking antibody of HGF (mean + SD; n = 10 in 4 different experiments). *** p < 0,001, **, p <

0,01 and ###, p < 0,001 significantly different from SD and FXIIa respectively. B: Representative western blot image of the HGF conversion blocking in the supernatants by the treatment of cells with 125nM FXIIa with or without an IgG and/or blocking antibody of HGF (4 different experiments). C: Quantification of neuronal death following 24h of either SD alone or SD in the presence of 125nM FXIIa with or without 500nM JNJ, an inhibitor of HGFR phosphorylation (mean ± SD; n=16 in 4 different experiments). **, p<0.01 significantly different from SD.

Figure 7. FXII is not activated into FXIIa by neurons during SD. A: Representative FXII Immunoblot of Supernatants of neurons subjected to SD in the presence of 125 nM FXII at different time points (1h, 4h, 6h and 24h). FXII and FXIIa were used as controls. 4 independent experiments are presented. B: FXII proteolytic activity measured in those same samples after incubation with 800 µM chromogenic substrate S-2302. Changes in OD 405 nm were continuously monitored on a microplate reader for 2 hours. FXIIa at 125nM was used as control. p<0.05 compared to SD condition. 4 independent experiments are presented.

Figure 8. FXII and FXIIa protect neurons from apoptosis by EGFR and HGFR dependent mechanisms.



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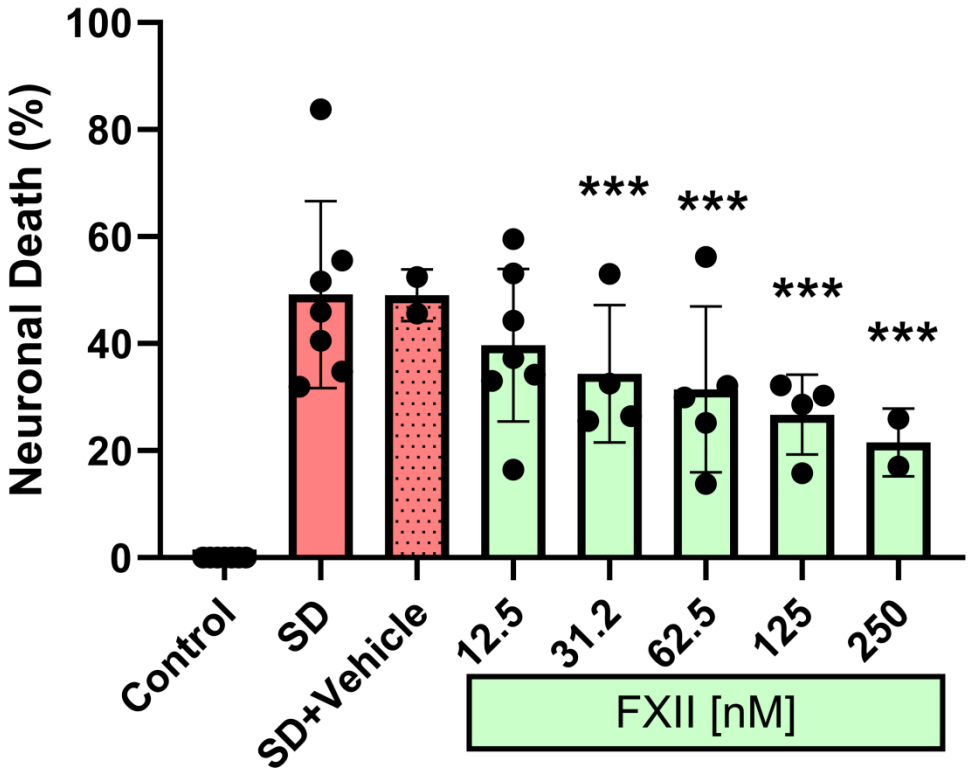

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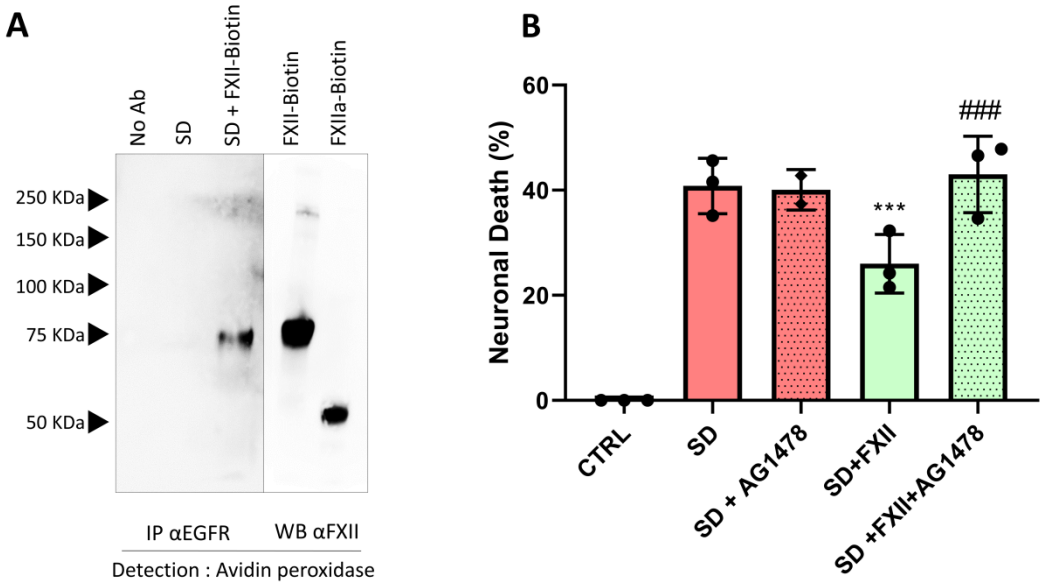

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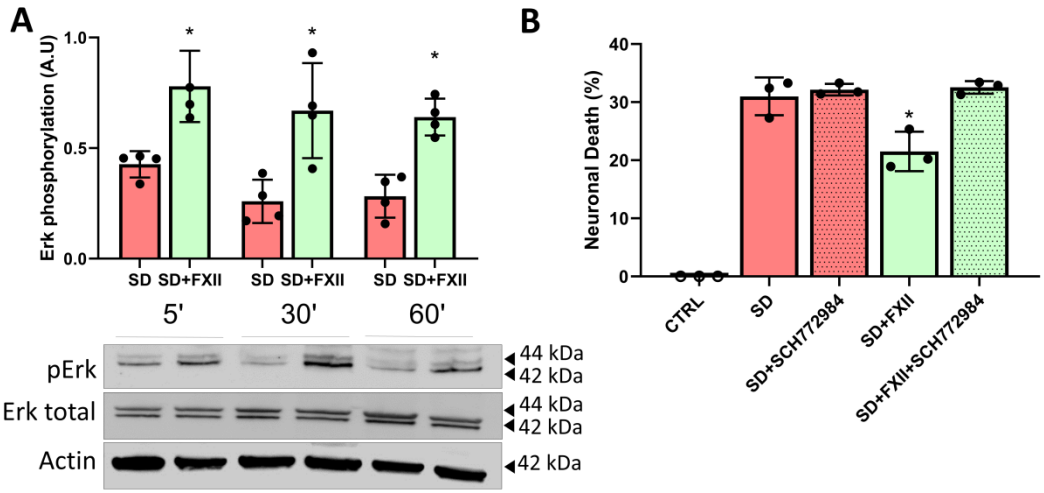

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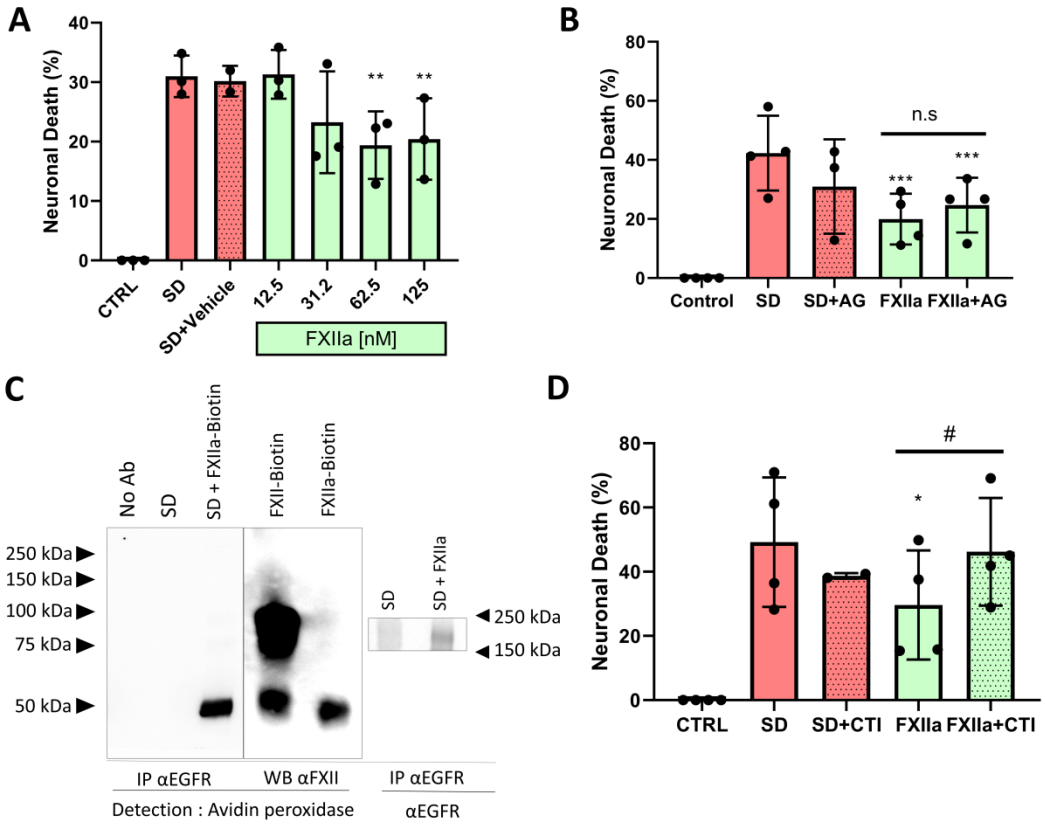

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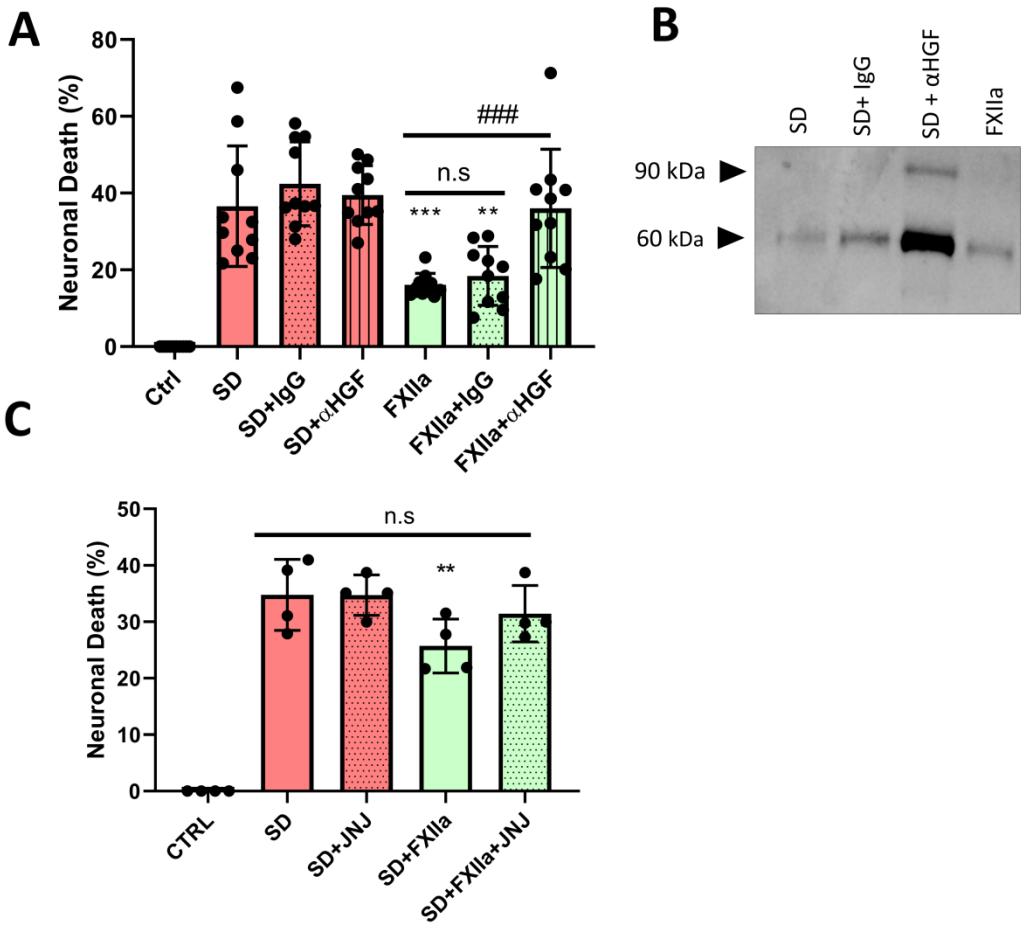

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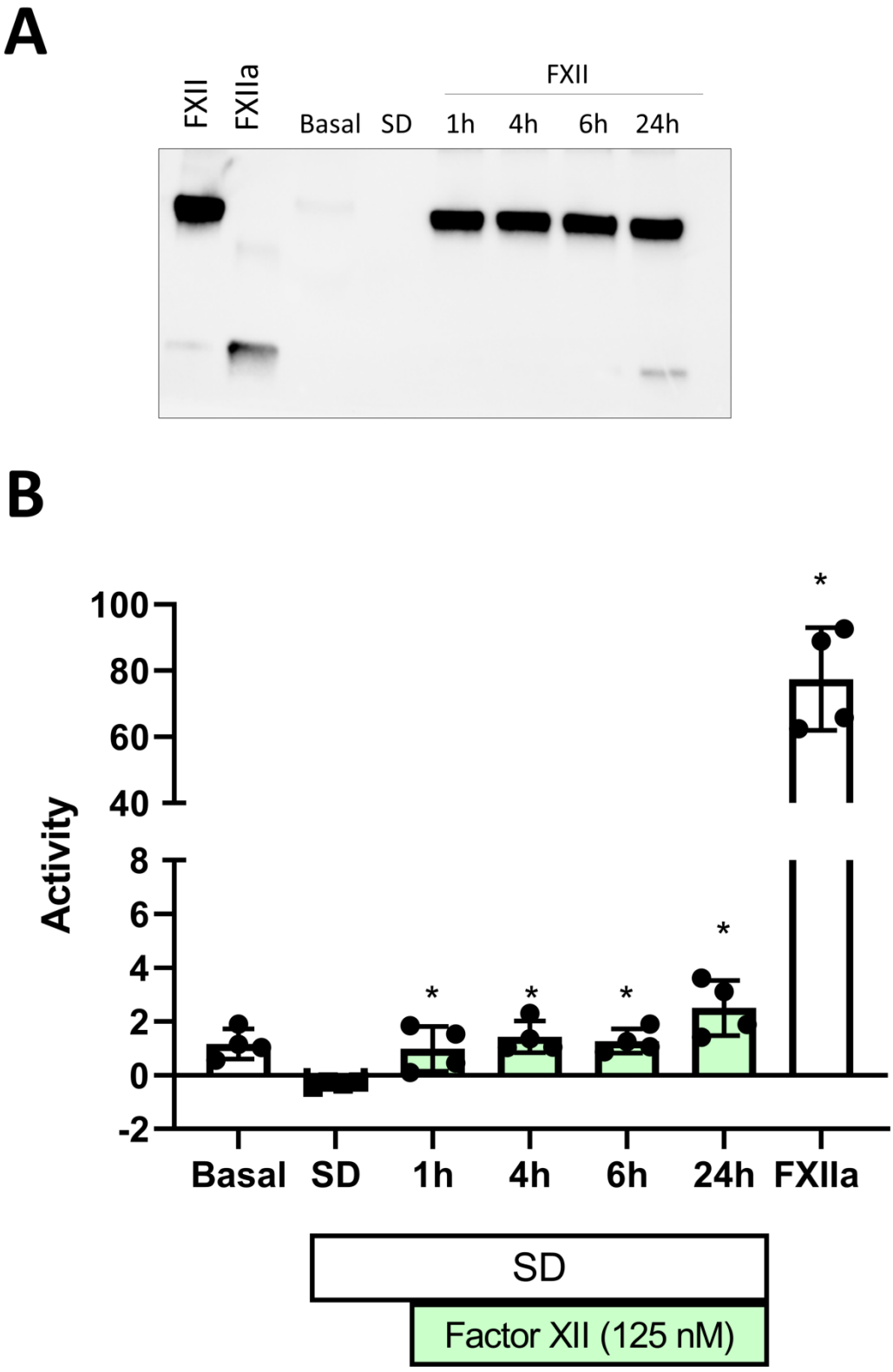

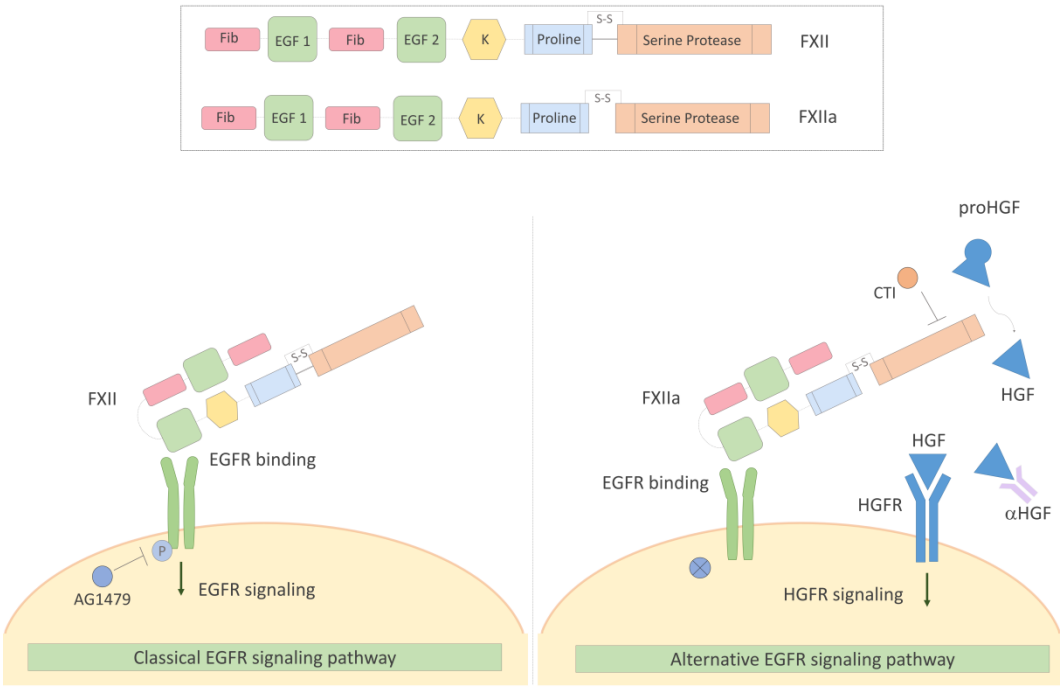

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