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Research Paper

EPO regulates neuroprotective Transmembrane BAX Inhibitor-1 Motif-containing (TMBIM) family members GRINA and FAIM2 after cerebral ischemia-reperfusion injury

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ABSTRACT

Background and purpose: Transmembrane BAX Inhibitor-1 Motif-containing (TMBIM) family members exert inhibitory activities in apoptosis and necroptosis. FAIM2 (TMBIM-2) is neuroprotective against murine focal ischemia and is regulated by erythropoietin (EPO). Similar to FAIM2, GRINA (TMBIM-3) is predominantly expressed in the brain. The role of GRINA in transient brain ischemia, its potential synergistic effects with FAIM2 and its regulation by EPO treatment were assessed.

Methods: We performed transient (30 min) middle cerebral artery occlusion (tMCAo) followed by 72 h of reperfusion in GRINA-deficient (GRINA$^{-/-}$), FAIM2-deficient (FAIM2$^{-/-}$), double-deficient (GRINA$^{-/-}$FAIM2$^{-/-}$) and wildtype littermates (WT) mice. We administered EPO or saline 0, 24 and 48 h after tMCAo. We subjected primary murine cortical neurons (pMCN) of all mouse strains to oxygen–glucose deprivation (OGD) after GRINA and/or FAIM2 gene transfection.

Results: Compared to wildtype controls GRINA deficiency led to a similar increase in infarct volumes as FAIM2 deficiency ($p < .01$). We observed the highest neurological deficits and largest infarct sizes in double-deficient mice. EPO administration upregulated GRINA and FAIM2 mRNA levels in wildtype littermates. EPO decreased infarct sizes and abrogated neurological impairments in wildtype controls. GRINA and/or FAIM2 deficient mice showed increased expression levels of cleaved-caspase 3 and of pro-apoptotic BAX mRNA. Further, caspase 8 was upregulated in FAIM2$^{-/-}$ and caspase 9 in GRINA$^{-/-}$ mice. Overexpression of GRINA and FAIM2 in wildtype and in double deficient pMCN decreased cell death rate after OGD.

Conclusions: GRINA and FAIM2 are highly expressed in the brain and convey EPO-mediated neuroprotection after ischemic stroke involving different caspases.

1. Introduction

Ischemic stroke is a leading cause of mortality and disability worldwide (Kim et al., 2015). Its global impact is going to increase with ageing populations. Since 1995 the main aim of acute stroke treatment was early reperfusion by intravenous application of recombinant tissue plasminogen activator (rtPA). Large numbers of promising therapeutic compounds in animal stroke models resulted in negative clinical trials (Cook and Tymianski, 2011; Minnerup et al., 2014; O'Collins et al., 2006). By the end of 2014 and beginning of 2015 landmark trials showed that endovascular stroke treatment (EST) is highly efficient in patients with large vessel occluding acute ischemic stroke (AIS) (Berkhemer et al., 2015; Campbell et al., 2015; Goyal et al., 2015; Jovin et al., 2015; Saver et al., 2015). Technically successful EST (TICI ≥2b) in AIS patients and experimental transient intraluminal middle cerebral artery occlusion (tMCAo) in rodents seem to mimic each other sufficiently (Sutherland et al., 2016). Therefore, effective neuroprotection in tMCAo has become more promising to be translated into clinical practice when performed in combination with EST. Oxygen and glucose deprivation lead to energy failure and neurological damage by necrotic

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cell death in the infarct core. The adjacent tissue, however, undergoes mainly apoptotic cell death involving death receptor signaling, such as Fas/CD95, along with elevated intracellular calcium levels, excitotoxicity and inflammation (Horvath et al., 2018; Kumar et al., 2014; Lai et al., 2014). The Fas/CD95 death cascade is activated by its ligand FasL/CD95L and involves the downstream adapter protein FADD (Fas-associated death domain) and activation of caspase 8 and caspase 3. These actors of Fas/CD95 mediated apoptosis are dysregulated in animal models of stroke (Chelluboina et al., 2014; Komnig et al., 2018b; Lu et al., 2011; Reich et al., 2011). Inhibition of Fas/CD95 by transmembrane BAX inhibitor motif-containing (TMBIM)-2, also known as Fas apoptotic inhibitory molecule 2 (FAIM2), Lifeguard (LFG), LFG2, or neuronal membrane protein 35 (NMP35), prevents neuronal cell death in murine models of cerebral ischemia (Komnig et al., 2018b; Reich et al., 2011), Parkinson's disease and bacterial meningitis (Komnig et al., 2018a; Tauber et al., 2014). FAIM2 inhibits calcium release from endoplasmic reticulum (ER) upon Fas stimulation, which, for instance, reduces apoptosis in neuron-like type II apoptotic cells (Urresti et al., 2016). FAIM2 expression is regulated by phosphatidylinositol-3-kinase (PI3K) and AKT/protein kinase-B and involves ERK signaling in a model of photoreceptor cell death (Beier et al., 2005; Besirli et al., 2012; Brunet et al., 2001). The precise neuroprotective pathway of FAIM2 has not been completely resolved yet.

Another anti-apoptotic TMBIM family member is the 38 kDa TMBIM 3, also known as glutamate receptor ionotropic NMDA protein 1 (GRINA), glutamate binding protein (GBP), or LFG1. GRINA is strongly expressed in the brain and is conserved among species (Aikawa et al., 2003; Kumar et al., 1991; Nielsen et al., 2011). Its gene expression is dysregulated in various cancers (Rojas-Rivera and Hetz, 2015) and in brains of patients with major depression (Goswami et al., 2013), indicating a role in many diseases. Located at the Golgi and ER, GRINA protects from hydrogen peroxide induced apoptosis and inhibits ER calcium release by inositol trisphosphate receptors (IP_3R) (Kumar et al., 1991; Nielsen et al., 2011). GRINA is upregulated in mice after chronic exposure to ethanol (Bao et al., 2001) and after exposure to ER stress (Rojas-Rivera et al., 2012). Results from cell culture, fruit fly and zebra fish suggest that GRINA protects from calcium stress and apoptosis (Rojas-Rivera et al., 2012). GRINA-deficient mice do not show a pathological phenotype, consistent with the knock-out effects of other TMBIM family members (FAIM2, TMBIM-1 and TMBIM-6) (Chae et al., 2004; Komnig et al., 2018b; Nielsen et al., 2011). Knockdown of GRINA or TMBIM-1 is not lethal in fruit flies, whereas knockdown of both proteins reduces life span and sensitizes to ER stress (Rojas-Rivera et al., 2012). All together this highlights the role of several TMBIM family members in pathological conditions and indicates potential synergistic and or compensatory effects.

Erythropoietin (EPO) confers potent neuroprotection against cerebral ischemia-reperfusion injury through a variety of biochemical mechanisms including the modulation of inflammatory responses, reduction of glutamate toxicity and the inhibition of caspase-dependent and caspase- independent apoptosis (Agnello et al., 2002; Bernaudin et al., 1999; Digicaylioglu and Lipton, 2001; Sola et al., 2005; Villa et al., 2003).

We previously reported that neuroprotection by EPO is dose-dependent and involves FAIM2 (Komnig et al., 2018b). Given the role of FAIM2 in EPO-mediated neuroprotection after stroke along with EPO's involvement in intrinsic as well as in extrinsic apoptosis, we hypothesized that in addition to FAIM2 the TMBIM family member GRINA also contributes to neuroprotection after ischemia-reperfusion and might also be involved in EPO signaling. This hypothesis was evaluated in ischemia models (tMCAo and OGD) using FAIM2-deficient, GRINA-deficient and double-deficient mice.

2. Methods

All experimental procedures and protocols were approved by the District Government of North Rhine Westphalia in Recklinghausen, Germany (LANUV ID 84–20.04.2015.A292). All animal experiments comply with the ARRIVE guidelines.

2.1. Animals

Mice were housed and handled in accordance with the guidelines of the Federation for European Laboratory Animal Science Associations (FELASA) in a pathogen-free, temperature-controlled (20–24 °C) facility with a 12/12-h light/dark cycle and access to pelleted food and water ad libitum. $FAIM2^{-/-}$ mice were generated by cre-recombinase mediated gene knockout and had been backcrossed to C57BL/6 J background for > 8 generations (Reich et al., 2011). $GRINA^{-/-}$ mouse embryos were provided by the public Mutant Murine Regional Resource Centers (MMRRC, strain 31,871) and were generated by targeting the initiator codon situated on exon 2 of the GRINA allele with a loxP-flanked neomycin cassette as previously described by Nielsen et al., 2011 (Nielsen et al., 2011). $GRINA^{-/-}$ mice with their B6.129(FVB)-$GRINA^{tm1.1Ldh}$ background had been backcrossed to C57BL/6 J for more than ten generations. Double-deficient mice ($FAIM2^{-/-}$,$GRINA^{-/-}$) were bred by outcrossing ($FAIM2^{+/+}GRINA^{-/-}$ x $FAIM2^{-/-}GRINA^{+/+}$) and intercrossing ($FAIM2^{+/-}$ $GRINA^{+/-}$ x $FAIM2^{+/-}GRINA^{+/-}$). All mutant mice strains and their wildtype littermates were bred by the Institut für Versuchstierkunde, Universitätsklinikum RWTH Aachen University.

2.2. Study protocol and animal surgery

A total of 102 male mice (10 to 12 weeks of age, weight 25-30 g) were assigned for this randomized and blinded controlled trial. We used male mice to avoid neuroprotective effects of female gonadal steroids as previously described (Habib et al., 2013; Ulbrich et al., 2012). tMCAo was performed for 30 min in 25 $FAIM2^{-/-}$, 25 $GRINA^{-/-}$, 28 GRINA and FAIM2 double-deficient ($FAIM2^{-/-}GRINA^{-/-}$), and 24 age-matched wildtype littermates followed by 72 h of reperfusion as previously described (Komnig et al., 2018b) (Fig. 1 a/b). Briefly, anesthesia was induced with 3% of isoflurane in 30% O_2 balanced with N_2O and maintained in 1% isoflurane in 30% O_2 and 69% N_2O during surgery. For measurement of the regional cerebral blood flow (rCBF), a laser doppler probe (Moor Instruments VMS-LDF2, Axminster, UK) was affixed to the skull above the left MCA territory after the overlaying temporal bone was exposed by an incision. In supine position through a midline neck incision (< 1 cm) the left common carotid artery (CCA) and the external carotid artery (ECA) were isolated and ligated. For MCA occlusion a 0.19 mm thick silicon coated filament (Doccol, #701912PK5RE) was threaded into the internal carotid artery (ICA). A sufficient occlusion was confirmed by reduction in rCBF to < 20% of the baseline (Fig. 1c). Body temperature during surgery was maintained at 37 °C ± 0.5 °C using a feedback-controlled heating pad and a heating lamp. After 30 min of tMCAo, mice were supplemented with 0.5 ml saline i.p. and placed into a temperature-controlled incubator before returning to their home cages for the postsurgical survival period of 72 h. Body weight and temperature were measured daily. The sham group (14 mice) received the same surgical procedure, except the filament insertion. Recombinant human EPO (rhEPO) (Epoetin alfa Hexal, Hexal, Holzkirchen, Germany) was diluted in 0.9% NaCl and was given intra-peritoneally at the beginning of reperfusion, 24 h and 48 h after reperfusion. We applied a EPO dose of 5000 U/kg body weight (cumulative EPO doses of 15,000 U/kg respectively), since this concentration was protective in our previous study (Komnig et al., 2018b). Controls were injected with saline only (Vehicle).

Mice would be excluded from further analysis if there was not a reduction of rCBF > 80% of baseline and a recovery of rCBF to CCAo-levels (60–70% of baseline) after 5 to 10 min reperfusion (Fig. 1c). In addition, animals with brain hemorrhage, seizures, extensive weight loss (> 20% of baseline), missing infarction in TTC-staining, and those

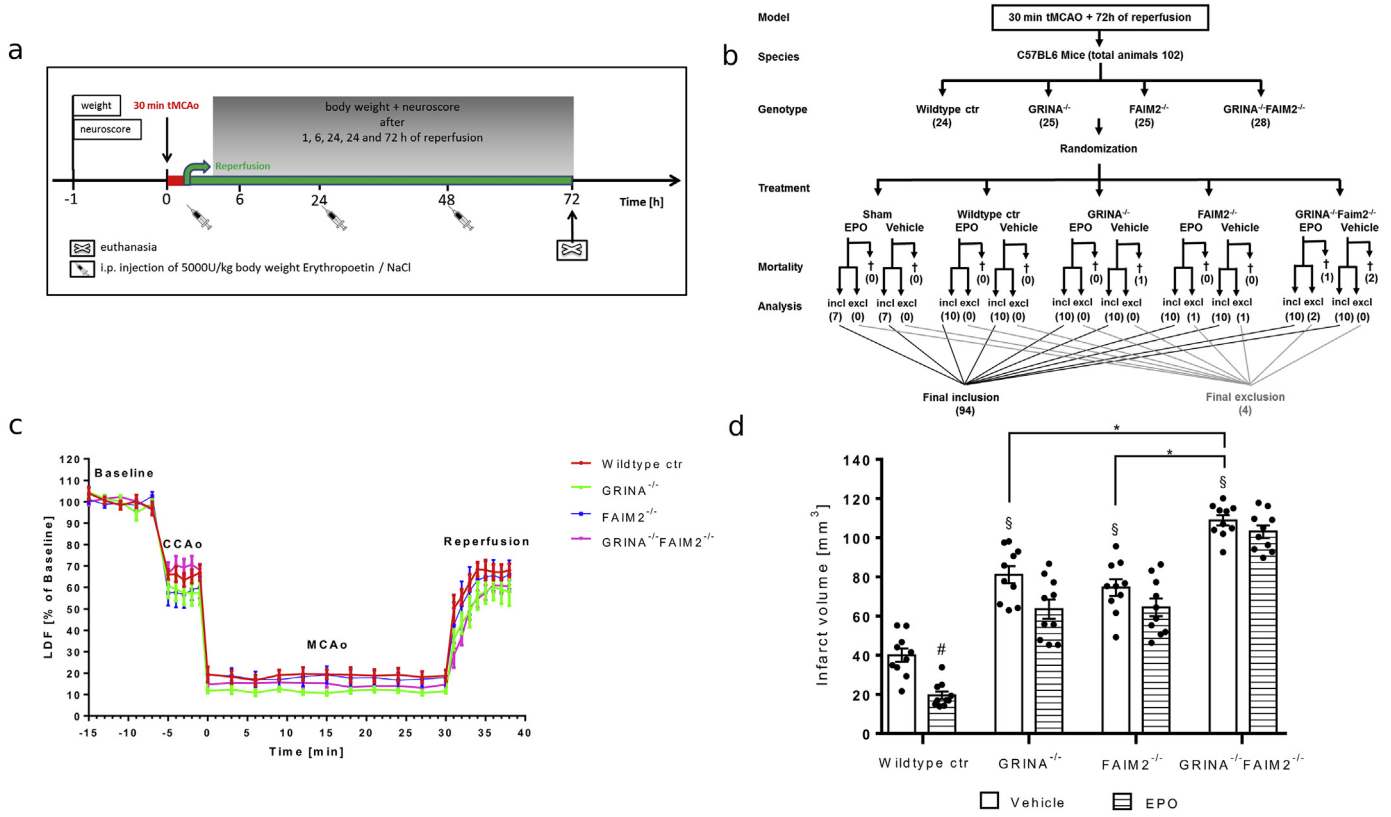

Fig. 1. FAIM2 and GRINA deficiency increases lesion sizes after ischemic stroke (a) Schematic illustration of the study protocol including time points of treatment, weight measurement and neurological assessment. (b) Protocol of preclinical randomized controlled trial (pRCT) summarizing the number of total animals (102 mice), with exclusion (excl.) per group and included animal (incl.) for final analysis. "†" indicates dead animals. Exclusion criteria are described in the material and methods section. (c) Ipsilateral Laser Doppler flowmetry changes after occlusion of CCA, MCA and reperfusion were monitored. The baseline blood flow was considered as 100% for all groups. 80% reduction of blood flow was defined as MCAo. *Abbreviations.* CCAo: Common Carotid Artery occlusion; MCAo: Middle Cerebral Artery occlusion; LDF: Laser Doppler flowmetry. (d) Infarct volumes of wildtype control, FAIM2$^{-/-}$, GRINA$^{-/-}$and FAIM2$^{-/-}$GRINA$^{-/-}$ double-deficient mice after 30 min of tMCAo followed by 72 h of reperfusion and EPO [5000 U/Kg] or saline (Vehicle) treatment were quantified. Bars represent means ± SD. *$p < .05$ between group, §$p < .05$ compared to wildtype ctr. #$p < .05$ compared to Vehicle.

that did not develop sufficient neurological deficits (mNSS < 5) were excluded. Mice that died during the observations period of 72 h were excluded from all analyses, except mortality rate between genotypes and treatment (Fig. 1b).

Surgery, examinations, data acquisition and data analysis were performed by investigators who were blinded to genotype and group assignment.

2.3. Neurological outcome

In order to evaluate the general status and focal neurologic dysfunction after tMCAo, a modified neurologic severity score (mNSS) has been applied (Li et al., 2000). It separately graded motor function (body asymmetry, muscle status, abnormal movement and gait), sensory function (visual, tactile and proprioceptive) and reflexes (corneal reflex, pinna reflex, whisker response to light touch, startle reflex) (Supplementary Fig. S1c). Each animal testing was performed by two individual investigators 1 h before and 1, 6, 24, 48 and 72 h after tMCAo or sham surgery. The score ranges from 0 (no deficits) to 15 points representing the poorest performance in all items and is calculated as the sum of the general and focal deficits. For the inability to perform the task, abnormal performance, or for lack of a tested reflex one point was awarded. The authors defined a score of 1 to 5 as mild, 6 to 10 as moderate, 11 to 15 as severe neurological deficit.

2.4. Infarct volumetry and hematology

The infarct volume was measured using the 2,3,5-triphenyltetrazolium chloride staining method as described earlier (Ulbrich et al., 2012). In brief, 72 h after surgery mice were deeply anesthetized and EDTA blood of each animal was taken transcardially for a blood count using the Celltac α MEK-6450 K (Nihon Kohden Europe, Rosbach, Germany) in the Institute for Experimental Animal Science and Central Laboratory for Experimental Animals (RWTH Aachen) (Supplementary Fig. S4). The brain was removed immediately and sliced coronally. The 1-mm thick brain sections were incubated in 2% TTC (Sigma Aldrich) for 10 min at 37 °C followed by a fixation in 10% formaldehyde in phosphate-buffered saline (PBS). The stained sections were photographed and evaluated in a blinded manner using ImageJ software (NIH, Bethesda, Md., USA). The infarct volume was corrected for brain edema with following equation: Corrected ischemic lesion = measured ischemic lesion – (Ipsilateral hemisphere – contralateral hemisphere). Total infarct volumes were calculated by adding the mean area of each section and multiplied by the thickness of the sections.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Apoptosis/necrosis after 30 min of tMCAo or sham surgery followed by 72 h of reperfusion was measured by TUNEL assay using In Situ Cell Death Detection Kit (Roche) and DAPI (Roth, Karlsruhe, Germany) according to the manufacturer's instructions. For detailed information

Table 1
Antibodies for immunocytochemistry, immunohistology and primers for RT-qPCRs.

Antibody	Manufacturer	Catalog number	Dilution
GRINA	Genetex	GTX51232	1:100
FAIM2	Novusbio	NBP2–24713	1:500
c-Myc	Santa Cruz	sc-40	1:500
GFP	Santa Cruz	sc-8334	1:200
GFAP	Santa Cruz	sc-6170	1:100
Iba1	Abcam	ab107159	1:200
NeuN	Millipore	MAB377	1:1000
Caspase 8	proteintech	13,423–1-AP	1:100
Caspase 9	Santa Cruz	sc-7885	1:200
Cleaved Caspase 3	Cell signaling	9661	1:100
Goat Anti-Rabbit IgG	Vector	BA-1000	1:200
Horse Anti-Mouse IgG	Vector	BA-2001	1:200
Goat anti-Mouse IgG, Alexa Fluor 594	Invitrogen	A11005	1:500
Goat anti-Rabbit IgG, Alexa Fluor 488	Invitrogen	A11008	1:500
Blocking			
Normal Goat Serum	Vector	S-1000	3% in PBS
Normal Horse Serum	Vector	S-2000	3% in PBS

Primer	Forward	Reverse	BP	AT[°C]	Species
HPRT	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC	249	62	mouse
GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA	77	65	mouse
Bax	GGCAGACAGTGACCATCTTT	AGTGGACCAGAGGTTTATTG	227	59	mouse
Bcl2	CGATCAATCAAAGCCAAGCA	AGCCTTCAGGCAAGTTCAGG	181	62	mouse
EPO-R	GGACACCTACTTGGTATTGG	GACGTTGTAGGCTGGAGTCC	451	60	mouse
GRINA	GTGCTTCCCTCTCTCTCTGGTG	CCATAGAGGGTCCAGACGTT	99	62	mouse
FAIM2	CTGGTCATCATCACGGCTCTCG	GGGTCATGAGCAGCACAAAC	106	62	mouse
Caspase 3	GGGCCTGTTGAACTGAAAAA	CCGTCCTTTGAATTTCTCCA	242	60	mouse
Caspase 8	TGCTTGGACTACATCCCAGAC	TGCAGTCTAGGAAGTTGACCA	168	60	mouse
Caspase 9	GACCAATGGGACTCACAGCA	CACCACTGGGGTGAGGTTTC	86	60	mouse

Primary antibodies were incubated overnight at 4 °C, second antibodies 1 h at room temperature.
BP = base pairs, AT = annealing temperature.

please see the supplement Material and Methods section.

2.6. Reverse-transcription quantitative PCR (RT-qPCR)

Gene expression analyses were performed with tissue from the peri-infarct area (Bregma 0 ± 1 mm) and the corresponding contralateral hemisphere using a stereomicroscopic approach. A detailed description is available in the supplement Material and Methods section.

2.7. Immunocytochemistry and immunohistochemistry

After removal of the medium, cells were fixed with 3,7% PFA in PBS for 30 min at room temperature (RT) and washed three times with PBS. Permeabilization was achieved by incubating the cells with 0,2% Triton X-100 in PBS for 10 min at RT. Next, cells were blocked with IFF buffer for 1 h. The primary antibody diluted in PBS was applied and incubated overnight at 4 °C. The following day the second antibody (Table 1) diluted in PBS was applied for 1 h at RT. In addition, cell nuclei were stained with Hoechst. Purity of the cultures was checked with immunocytochemical staining using antibodies against glial fibrillary acidic protein (GFAP) to mark astrocytes, NeuN to label neuronal cells and IBA1 to detect microglia. To evaluate the transfection rate c-Myc-tagged GRINA and GFP-tagged FAIM2, anti-c-Myc and anti-GFP antibodies (Table 1) were used.

Results were evaluated with a Leica fluorescence microscope (Leica, Wetzlar, Germany). For immunohistochemistry 5 µm paraffin embedded coronal brain sections of all genotypes were used. Deparaffinization, rehydration, antigen-retrieval as well as the washing steps were performed as previously described (Komnig et al., 2018b). The primary and secondary antibodies are given in Table 1.

2.8. Transfection of primary murine cortical neurons (pMCN)

For in vitro experiments pMCN from C57BL/6 or TMBIM-deficient mice were prepared at postnatal day 1. Cell culture preparation was performed as previously described (Gold et al., 2015). Five days after culturing pMCN were transfected with a GRINA expression plasmid (Grina Mouse cDNA ORF Clone Myc-DDK-tagged, NM_023168, BioCat, Heidelberg,Germany) and FAIM2 plasmid (Faim2 GFP-tagged ORF Clone, BC032278, ORIGENE) using Magnetofection™ with its magnetic plate (OZ Biosciences, Marseille, France) according to the manufacturer's instructions. Briefly, pMCN were incubated at 37 °C and 5% CO_2 for 5 days (DIV 5). Before transfection the magnetic plate was stored in the incubator at 37 °C and 5% CO_2. NeuroMag reagent was thoroughly vortexed and mixed with plasmid DNA and in Neurobasal Medium without serum and antibiotics. This stock solution was incubated for 15 min at 21 °C. The mixture was carefully mixed and dropwise added to the cells in the following concentrations: In 6 well plate: 2 g/µl plasmid cDNA and 1, 5 µl NeuroMag per well with half of the ingredient's concentration in the next smaller well plate size. The plates were placed on the magnetic plate in the incubator for 15 min and further incubated solely for 72 h before using the transfected cells for further experiments.

2.9. Oxygen glucose deprivation (OGD) and cell viability

Oxygen glucose deprivation was performed using a self-constructed cube-shaped hypoxia chamber (28 × 14 × 26 cm). The chamber was flooded with inert nitrogen gas to replace aerial oxygen as previously described (Habib et al., 2013; Habib et al., 2014). To analyze the OGD induced cell death we determined cell vitality using CTB (CellTiter-Blue® assay, Promega, Germany) and LDH (CytoTox 96® Non-

Radioactive Cytotoxicity Assay, Promega, Germany) according to the manufacturer's protocol as previously described (Habib et al., 2013). Fluorescence or absorption was measured using a microplate reader (Tecan GmbH, Switzerland). In both assays, lysed cells (Triton X-100) served as internal positive controls, and pure cells without treatment as negative controls.

2.10. Statistical analysis

All statistical tests were performed using JMP(R), Version 10. SAS Institute Inc., Cary, NC, 1989–2007. Residuals were analyzed for normal distribution using the Shapiro-Wilk normality test. In case the normality test was significant, values were BOX-COX-transformed after calculation of the optimal lambda and used for statistical analysis according to the Handbook of Biological Statistics (McDonald, 2014). Intergroup differences were tested by ANOVA two-way followed by Tukey post-hoc test (multiple groups). Data are given as arithmetic means ± SD. The level of significance was set as $p < .05$. Asterisks indicating significant between group differences, "#" compares EPO vs. Vehicle and "§" compares wildtype controls vs. genotypes. The individual data of each experiment are shown in the legends.

3. Results

In this preclinical randomized and blinded controlled trial (pRCT), a total of 102 male 10 to 12 weeks old mice were subjected to 30 min tMCAo or sham surgery followed by 72 h of reperfusion. Four animals (1 GRINA$^{-/-}$, 3 GRINA$^{-/-}$FAIM2$^{-/-}$) died during or shortly after surgery and 4 animals (2 FAIM2$^{-/-}$, 2 GRINA$^{-/-}$FAIM2$^{-/-}$) were excluded from the study since they did not comply with the inclusion criteria (hemorrhage and weight loss > 20% of initial weight). Hence, 94 animals followed the entire study protocol with 7–10 mice per genotype and treatment group (Fig. 1b).

3.1. Lack of GRINA and FAIM2 increases infarct volume and abolishes lesion size reduction after EPO treatment

MCA was occluded for 30 min as shown by laser doppler flowmetry during surgery. A reduction of nearly 40% of cerebral blood flow of the ipsilateral MCA after occlusion of CCA and at least 80% of baseline after intraluminal occlusion of the ipsilateral cerebral media artery proofed a successful MCAo. After reperfusion the baseline of CCA perfusion was reached in each genotype after 5–10 min. The lack of differences in cerebral blood flow indicated that no obvious vascular abnormalities between the genotypes existed (Fig. 1c).

Computer-assisted evaluation of TTC stained brain slices showed significant higher infarct volumes in GRINA$^{-/-}$ (mean = 81.1 mm^3, SD = 13.8) and FAIM2$^{-/-}$ (mean = 74.5 mm^3, SD = 13.4) mice when compared with wildtype littermates (mean = 39.9 mm^3, SD = 10.8) ($p < .01$). The lesion sizes of GRINA$^{-/-}$ mice did not differ significantly from FAIM2$^{-/-}$ mice The double-deficient mice revealed nearly 30 mm^3 larger infarct sizes (mean = 108.9 mm^3, SD = 8.3) compared to GRINA$^{-/-}$ or FAIM2$^{-/-}$ mice ($p < .01$).

The application of three times 5000 U/kg EPO reduced the infarct sizes by half (19.5mm^3, SD = 6.3) in the wildtype mice only (Fig. 1d). EPO treatment did not modify infarct sizes in GRINA- and/or FAIM2-deficient mice. Surgical procedure, representative TTC-stained images, demographics and hematology of all genotypes and treatment groups are shown in Supplementary Fig. S1 and in Table 1.

3.2. GRINA and FAIM2 deficiency worsens clinical outcome after stroke

The modified neurologic severity score (mNSS) was assessed at different time points (−1 h, 1 h, 6 h, 24 h, 72 h) to grade various aspects of neurologic functions (Supplementary Fig. S1c). The score is a composite of the motor (abnormal movement, muscle status), sensory (visual, proprioceptive and tactile), and reflex tests. Comparing Sham surgery (mild injury, 0–4 points) to wildtype controls (moderate injury 5–9 points; Fig. 2a), the later showed a flexion of forelimb and or hindlimb when raising the mice by the tail and showed a weaker cage grasp of the contralateral limb. In addition, wildtype controls after revealed cycling towards the paretic side and deficits in the sensory system and reflexes (missing visual placing, whisker response to light touch and missing pinna and corneal reflex) 1to 6 h after stroke. EPO administration reduced neurological deficits at each time point in wildtype littermates. In all animals, the most severe neurological deficits were observed 1 h after surgery, which decreased during the observation period of 72 h. Early after surgery (1 to 6 h) GRINA$^{-/-}$ and FAIM2$^{-/-}$ mice revealed higher neurological impairments compared to wildtype littermates and did not benefit clinically from EPO administration during the observation period. No significant differences in single knock-out mice could be detected after 6 h compared to wildtype littermates (Fig. 2b/c). Main column effect analysis revealed an EPO dependent benefit on neurological outcome in wildtype mice over the observation period of 72 h ($p \leq .01$; Fig. 2d).

3.3. FAIM2 and GRINA are mainly expressed in the brain and might compensate each other after ischemia

In order to examine the cellular mRNA expression levels and distribution of FAIM2 and GRINA, tissue from brain, heart, liver, testes and gastrocnemic muscle of six wildtype mice was used for RT-qPCR analyses. GRINA mRNA was most abundant in the brain but also present in testes, heart, liver, and gastrocnemic muscle. FAIM2 mRNA was nearly exclusively expressed in the brain (Supplementary Fig. S2a/b).

To evaluate the expression patterns in different cerebral regions, biopsies from cortex, striatum, cerebellum and hindbrain were used for RT-qPCR. GRINA was expressed at approximately the same level in all four major brain regions tested, in line with findings from Nielsen et al. (2011) (Supplementary Fig. S2c) (Nielsen et al., 2011). Within the tested brain regions a marked difference in distribution of FAIM2 mRNA was not detected (Supplementary Fig. S2d). Recently, we demonstrated an upregulation of FAIM2 mRNA after EPO administration in mice (Komnig et al., 2018b). However, a potentially EPO-mediated regulation of GRINA and FAIM2 after ischemia-reperfusion injury is not known. Since both TMBIM family members are mainly expressed in the brain (Supplementary Fig. S2a/b), a synergistic or compensatory mechanism potency is conceivable.

In the peri-infarct zone of wildtype mice, the level of GRINA mRNA expression decreased 39% compared to the contralateral hemisphere ($p = .0097$). This effect was abrogated by EPO administration ($p = .0087$). Remarkably, a 44% higher level of GRINA mRNA level was detected ($p = .0089$) in FAIM2$^{-/-}$ compared to wildtype mice (Fig. 3a). Furthermore, FAIM2 mRNA level was decreased after ischemia ($p = .0075$) and increased after EPO application ($p = .0045$). Comparable to the increase of GRINA mRNA expression pattern in FAIM2-deficient mice, FAIM2 mRNA expression was increased in GRINA$^{-/-}$ mice compared to wildtype littermates (p = .0089; Fig. 3b). The mRNA levels of EPO-receptor in the peri-infarct zone were increased in all genotypes after 30 min of tMCAo, followed by 72 h of reperfusion. EPO administration further increased the EPO-receptor mRNA levels ($p < .001$, Fig. 3c). Moreover, mRNA levels of apoptotic BAX and anti-apoptotic BCL-2 were analyzed in the peri-infarct zone. Here, in all genotypes a statistically significant higher BAX/BCL-2 mRNA ratio was noticed when compared to the contralateral hemisphere and Sham group. Single and double deficient mice showed a higher ratio compared to wildtype littermates ($p < .01$). The highest ratio could be measured in double deficient mice. In all groups, the ratio was shifted to anti-apoptotic pattern after application of EPO (p < .001; Fig. 3d and Supplementary Fig. S3).

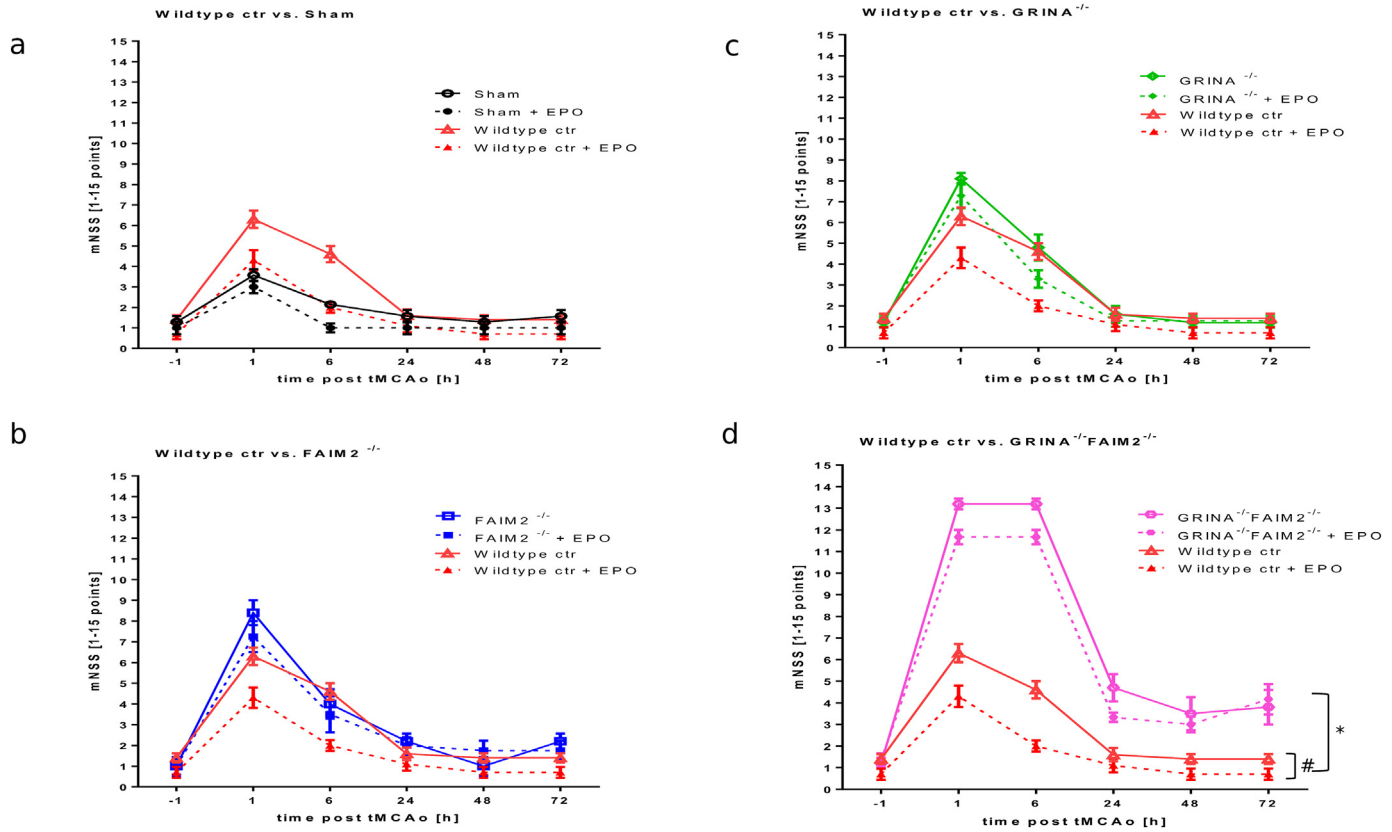

Fig. 2. FAIM2 and GRINA double-deficiency reveals additive neurologic deficits, which is not responsive to EPO administration.
Modified neurologic severity score (mNSS) in a time-dependent manner (−1 h to 72 h) was assessed to grade various aspects of neurologic function after 30 min of tMCAo followed by 72 h of reperfusion. Neurological outcomes of all genotypes were compared to the same wildtype vehicle and EPO treated wildtype groups. (a) Sham ($n = 7$) vs. wildtype control ($n = 10$). (b) FAIM2$^{-/-}$(n = 10) vs. wildtype control (n = 10). (c) GRINA$^{-/-}$(n = 10) vs. wildtype control (n = 10). (d) GRINA$^{-/-}$FAIM2$^{-/-}$(n = 10) vs. wildtype control (n = 10). Bars represent means ± SD. §p < .05 compared to wildtype ctr. #p < .05 compared to the vehicle group.

3.4. Deficiencies of TMBIM family members GRINA and FAIM2 increase the level of apoptosis and distinct caspases after cerebral ischemia

The involvement of TMBIM family members GRINA and FAIM2 in the extent of apoptosis after ischemic stroke was analyzed by TUNEL assay. MCAo for 30 min followed by 72 h of reperfusion induced an increase in the number of TUNEL-positive/apoptotic cells in GRINA and in FAIM2-deficient mice compared to wildtype littermates. In line with the volumetric evaluation of infarct volumes, the largest number of TUNEL-positive cells were detected in double-deficient mice. The absence of TUNEL-positive cells in the respective contralateral hemisphere indicated that the apoptosis was specific to ischemic brain tissue. Interestingly, EPO-administration reduced the amount of apoptotic cells in all genotypes, but not in double-deficient mice (Fig. 4a/b).

To gain further information about the apoptotic initiator and effector caspases after cerebral ischemia, mRNA levels of caspases 3, 8 and 9 in ipsi-and contralateral brain hemispheres of all genotypes (Sham and tMCAo) and treatment groups (saline and EPO) were measured by RT-qPCR and immuno-histological staining (representative images in Fig. 5a). Three days after tMCAo surgery, caspase 3 mRNA levels and cleaved-caspase 3 positive cells were elevated in all groups after ischemia. In single and double-deficient mice had higher caspase 3 cell count and caspase 3 mRNA levels were increased compared to wildtype littermates. EPO lead to a decreased caspase 3 mRNA level in all groups (Fig. 5b). Similarly, caspase 8 mRNA levels and cell counts were increased after tMCAo in all groups and EPO lowered this effect. FAIM2$^{-/-}$ mice however, showed the highest upregulation of caspase 8 ($p = .0085$; Fig. 5c). The mRNA expression levels and the immunohistological cell count of caspase 9 were elevated in all tMCAo groups and was mitigated by EPO administration. In case of caspase 9, the highest rate was measured in GRINA$^{-/-}$ mice (Fig. 5d). In contrast to the gene expression analysis, EPO administration did not significantly lower caspases 3,8 and 9 positive cell counts in double deficient mice (Fig. 5b–d).

3.5. Reintroduction of FAIM2 and GRINA in primary cortical neurons reversed knockout-induced aggravation of OGD stress

In order to examine the neuroprotective potency of FAIM2 and GRINA in primary murine cortical neurons, they were overexpressed in wildtype pMCN and re-expressed in FAIM2 and GRINA double-deficient pMCN. The transfected cells were subjected to 30 min of OGD and LDH-release was determined after 1 h and 72 h of reperfusion (Fig. 6a). Prior to experiments, the transfection rate was routinely evaluated using immunofluorescence staining and showed mean rates of 73.6% viable FAIM2-transfected and 69.9% of viable GRINA-transfected cells (Fig. 6b). An increased cell death in wildtype pMCN was detected 1 h after a 30 min period of OGD compared to normoxic conditions. The OGD-induced cell death could be slightly reduced after FAIM2 or GRINA transfection alone or in combination with EPO administration [1 U/ml]. After 72 h of reperfusion, the OGD-induced LDH release was reduced statistically significant by EPO administration ($p = .0237$). Furthermore, FAIM2 or GRINA over-expression solely or in combination with EPO administration reduced cell death to the level of corresponding normoxic controls (Fig. 6c). For examining the “rescue” potency of TMBIM family members, FAIM2 and GRINA were transfected

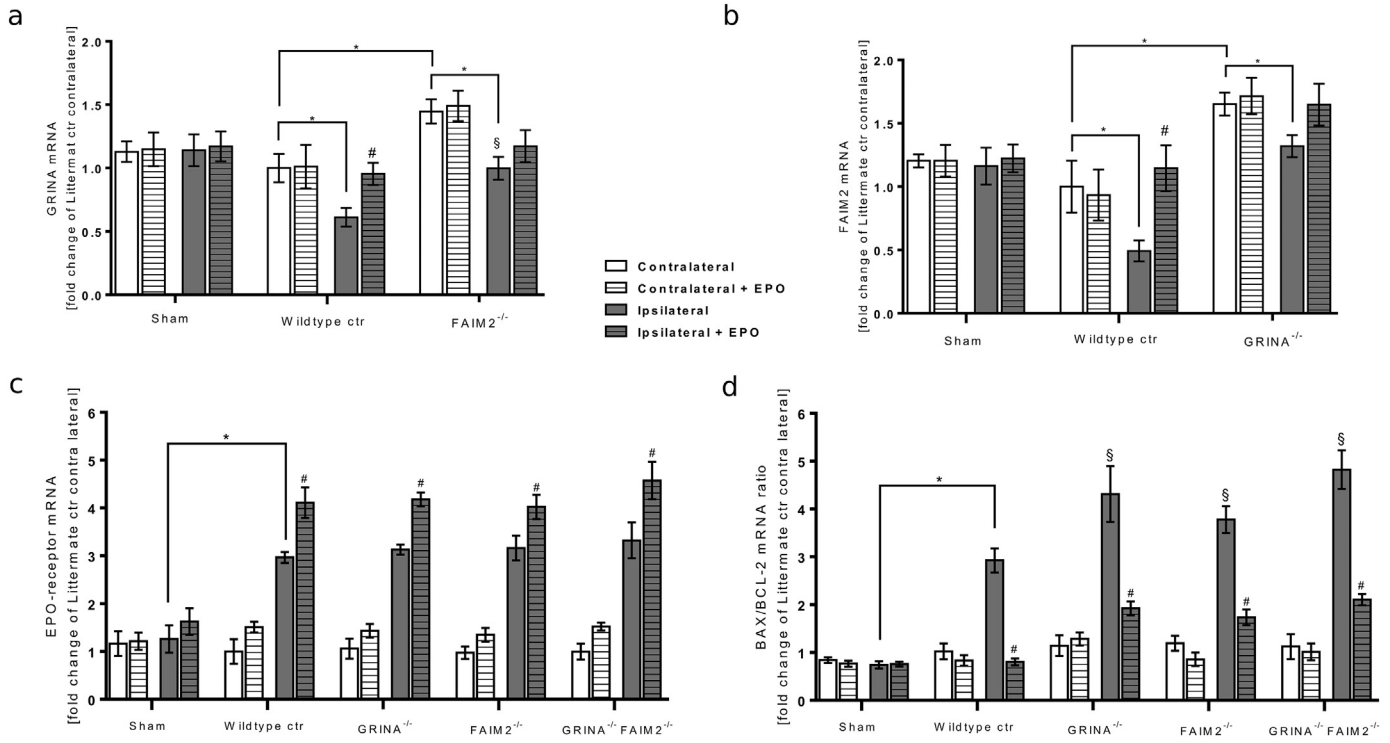

Fig. 3. Gene expression analysis of FAIM2, GRINA, EPO-receptor and pro- and anti-apoptotic (BAX, BCL2) genes after 30 min of tMCAo followed by 72 h reperfusion. (a, b) GRINA and FAIM2 mRNA levels were decreased nearly by half after tMCAo. EPO abrogates this effect in both TMBIM family members. GRINA mRNA is increased in FAIM2$^{-/-}$ mice and vice versa. (c) mRNA levels of EPO-receptors were elevated in all genotypes after ischemia. EPO administration contributes to a further increase of mRNA levels, but only in ischemic brain tissue. (d) BAX/BCL2 mRNA-ratio is increased after tMCAo in all genotypes and is decreased after EPO administration in all groups. Bars represent means ± SD. $N = 6$ with three technical replicates. *p < .05 intergroup comparison, §p < .05 compared to wildtype ctr. #p < .05 compared to Vehicle.

to FAIM2 and GRINA double-deficient pMCN. Here, 30 min of OGD induced 40% higher LDH release after 1 h and 36% higher LDH release after 72 h in double-deficient pMCN compared to wildtype pMCN. The re-expression of FAIM2 or GRINA alone decreased cell death for about 13% after 1 h and for about 8% after 72 h of reperfusion compared to double-deficient pMCN. However, the combined re-expression of TMBIM family members lowered the LDH release to the level of wildtype controls (Fig. 6d) .

4. Discussion

This study evaluated the neuroprotective potency of the TMBIM family members GRINA and FAIM2 and their regulation by exogenous EPO after transient cerebral ischemia. We have demonstrated that both TMBIM family members are highly expressed in the murine brain and that the lack of GRINA or FAIM2 similarly resulted in an increased infarct sizes compared to wildtype littermates. The double-deficient genotype further aggravated the infarct volumes and showed the worst clinical outcome. EPO administration decreased infarct sizes and abrogated the neurological deficits in wildtype mice only. In line with these findings, the ischemic tolerance in vitro of FAIM2 and GRINA-deficient cortical neurons was impaired statistically significant compared to neurons from wildtype littermates. The overexpression of GRINA and FAIM2 in wildtype neurons and re-introduction of both family members solely and in combination abrogated OGD-induced cell death in double deficient neurons.

Fas/CD95 and its ligand FasL/CD95L play a pivotal role in apoptosis in the ischemic core and in the penumbra and involve both caspase-dependent and caspase-independent components (Chelluboina et al., 2014; Lu et al., 2011; Reich et al., 2011). Fas-mediated apoptosis can be inhibited by FAIM2 (Beier et al., 2005; Brunet et al., 2001; Fernandez et al., 2007; Schweitzer et al., 1998; Somia et al., 1999). FAIM2 expression is regulated by the PI3K/AKT pathway which beyond others is activated by EPO (Bond and Rex, 2014; Vauzour et al., 2007). The neuroprotective potency of FAIM2 was demonstrated in models of stroke, bacterial meningitis and Parkinson's disease. Furthermore, an increased expression of FAIM2 mRNA was found in the peri-infarct zone of human brains (Komnig et al., 2016; Reich et al., 2011; Tauber et al., 2014). Moreover, EPO in a low dose (5.000 U/kg) conveyed neuroprotection and regulated FAIM2, whereas a high dose of EPO (90.000 U/kg) was not beneficial (Komnig et al., 2018b). This study confirmed previous findings that FAIM2 deficiency leads to aggravated infarct sizes and mitigates EPO-mediated neuroprotection. While FAIM2 is exclusively expressed in the brain, GRINA shows the highest expression of mRNA levels in the brain, but is also abundant in testes, liver, heart and muscles. Rojas-Rivera and colleagues reported that GRINA synergizes with TMBIM6, the best characterized member of the TMBIM family, in the modulation of ER calcium homeostasis and apoptosis by direct interactions with inositol trisphosphate receptors. However, compared to GRINA and FAIM2, TMBIM6 is poorly expressed in the brain (Rojas-Rivera et al., 2012). Similar to FAIM2, the lack of GRINA caused higher infarct volumes, elevated apoptosis levels and worse clinical outcome compared to wildtype littermate mice suggesting a crucial role of GRINA in the brain. Moreover, analogous to FAIM2 deficiency, a lack of GRINA abrogated EPO-mediated reduction of infarct sizes and neurological impairment, consistent with the hypothesis that both TMBIM family members convey, at least partly, neuroprotection via EPO. In order to investigate whether GRINA and FAIM2 are directly involved in cell death mechanisms and not only show a higher cell death rate earlier than the wildtype, it is crucial to investigate whether the infarct sizes of wildtype mice differ from those of knockout mice during a longer ischemic period (1 h). It is possible that the infarct sizes of wildtype and GRINA and FAIM2 knockout mice might converge in the course of a longer occlusion period. Future

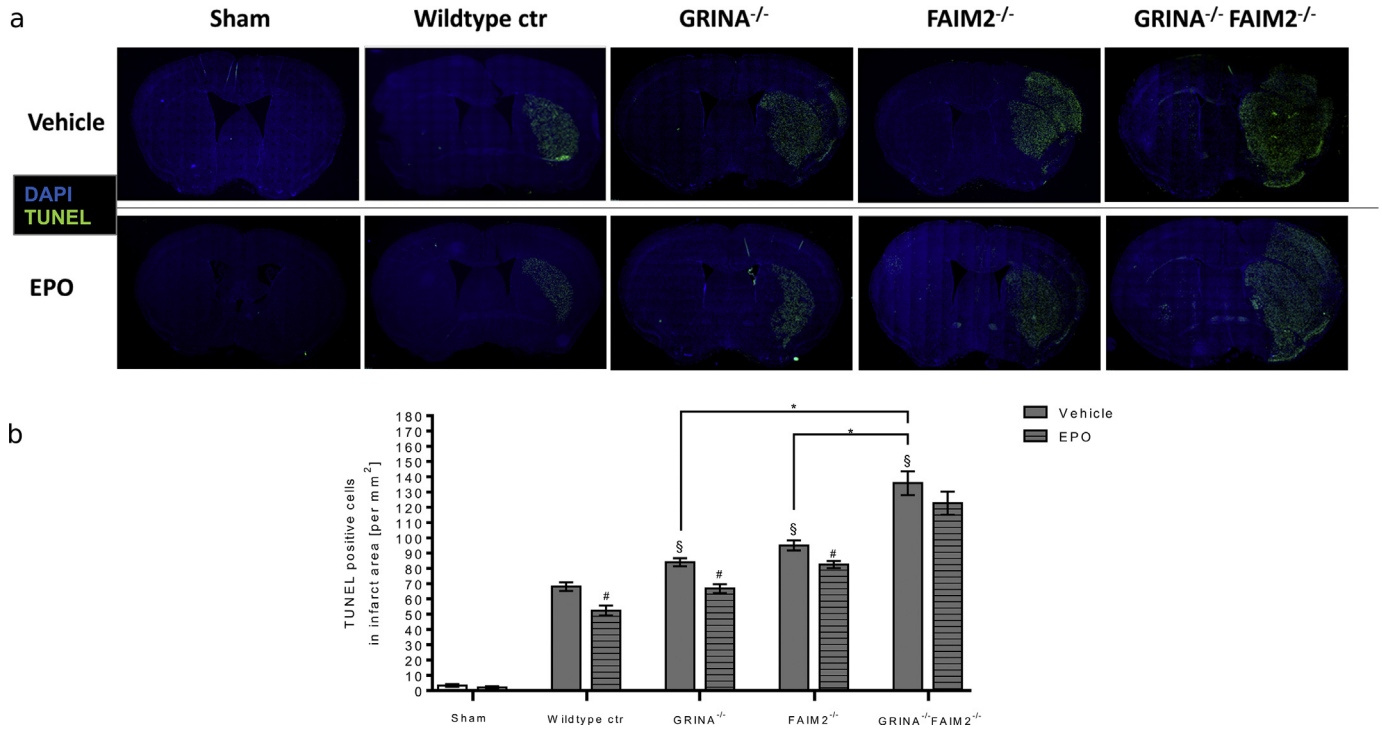

Fig. 4. FAIM2- and GRINA-deficiency increases the number of TUNEL-positive cells count in post-ischemic mouse brain after 30 min of MCAo followed by 72 h of reperfusion.

(a) TUNEL assay on paraffin-embedded coronal brain sections of mice sacrificed 72 h after 30 min of tMCAo. Green fluorescence in the ischemic brain regions of the representative images indicates TUNEL-positives cells. Blue fluorescence indicates DAPI staining of the nuclei. (b) Quantification of TUNEL-positive cells in peri-infarct zones of $n = 4$ mice. TUNEL-positive cells in six slices per mice (Bregma 0) with 12×0.01 mm^2 grids per slice were counted. Bars represent means ± SD. $N = 4$ of every genotype and treatment group. *p < .05 intergroup comparison, §p < .05 compared to wildtype controls. #p < .05 compared to Vehicle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

studies should address with the help of imaging techniques (e.g. MRI) the progression of infarction in both half-hour and one-hour of MCA occlusion over a longer period of reperfusion-time (e.g. 7–14 days).

Gene expression analyses revealed ischemia-dependent reduction of GRINA and FAIM2 mRNA in the ipsilateral hemisphere. EPO administration increased the expression of both TMBIM family members in the peri-infarct zone. Remarkably, a statistically significant higher level of GRINA mRNA in FAIM2$^{-/-}$ mice and vice versa compared to wildtype was detected (Fig. 3a), suggesting a compensatory effect of both family members. A regulation of protein levels of both TMBIM family members would support the assumption based on our mRNA results, which we were not able to provide due to the lack of specific murine antibodies.

It is well known that subsequent to cerebral ischemia and reperfusion multiple and overlapping cell signaling processes, including both extrinsic and intrinsic apoptotic pathways are activated. Upon initiation of these pathways, downstream caspases are activated to execute cell death. In our in vitro and in vivo stroke models an increased amount of TUNEL-positive cells, LDH-release and high activation of executioner caspase 3 in GRINA$^{-/-}$ and FAIM$^{-/-}$ and further augmentation in double-deficient mice were observed. Interestingly, the initiator caspases (caspase 8 and caspase 9) showed a distinct expression pattern in the absence of TMBIM family members. Caspase 8 mRNA and caspase 8 positive cell count showed highest levels in FAIM2$^{-/-}$ mice, which confirms an inverse correlation between FAIM2 and pro-apoptotic proteins, FAS and Caspase 8 (Besirli et al., 2012; Kang et al., 2016; Reich et al., 2011). In GRINA$^{-/-}$ mice however, caspase 9 was detected at highest levels. Notably, EPO administration decreased the expression of caspases in GRINA and in FAIM2-deficient mice emphasizing its pleiotropic neuroprotective potency. However, EPO-dependent regulation of initiator and executioner caspases was missing in double TMBIM deficiency, which underlines the role of GRINA and FAIM2 in EPO-mediated neuroprotection. While activated caspase 8 is propagated to mediate the extrinsic apoptotic pathway by cleaving executioner caspases, caspase 9 is known to initiate the intrinsic or mitochondrial apoptosis pathway (Spencer et al., 2009). Based on our results, FAIM2 might be involved in the extrinsic and GRINA in the intrinsic apoptotic pathway.

There is overwhelming evidence to suggest that apoptosis in the surrounding penumbra region of the ischemic core can be counteracted by EPO to avoid the progress of infarction. In order to evaluate the efficacy and safety of EPO, a double-blind, placebo-controlled, randomized phase II/III German Multicenter EPO Stroke Trial enrolled 460 patients treated with either EPO or placebo within 6 h of symptom onset. Here, EPO treatment did not show any benefit in Barthel Index at day 90 (primary endpoint). Especially patients who received EPO after rtPA had a higher rate of intracerebral hemorrhages (Ehrenreich et al., 2009). Here, the enrolling investigators reported a high number of patients (> 60%) who received rtPA, although approximately 50% of them had contraindications to rtPA. The authors suggested a potential rtPA-EPO interaction being responsible for serious complication in the EPO arm. Subgroup analysis and re-examination of data revealed beneficial effects in the absence of thrombolysis (Ehrenreich et al., 2011). This demonstrates that EPO's interactions and its mechanism of neuroprotection are not completely understood yet. Moreover, EPO is still considered as safe and beneficial with respect to neurological recovery in patients non-qualifying for rtPA after AIS and is already widely used in clinical practice, especially in patients with anemia associated with chronic kidney disease. More basic research on EPO function in the brain is required to understand the acting mechanisms of anti-apoptotic regulators such as GRINA and FAIM2 before translation into clinical implementation can be performed.

A revolution in the treatment of large vessel occlusion in the

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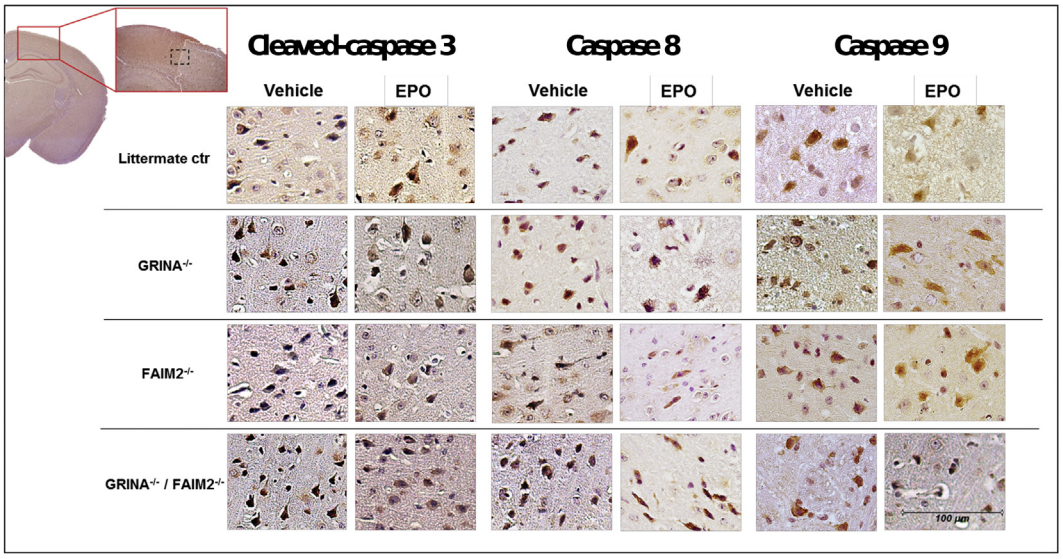

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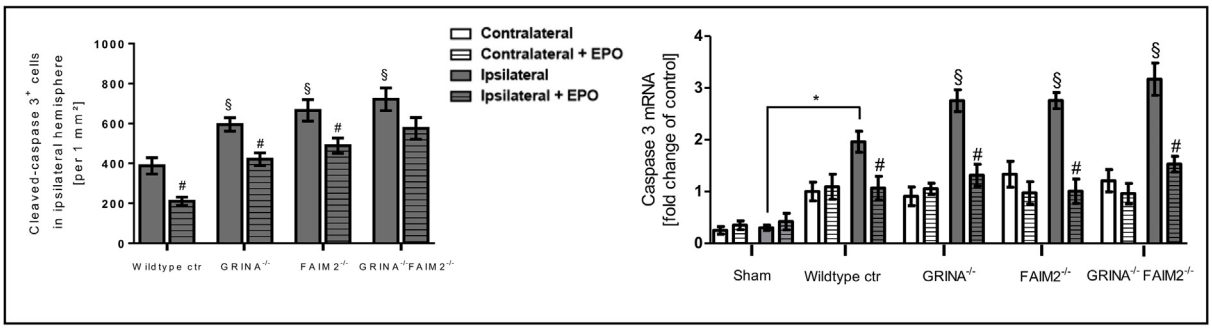

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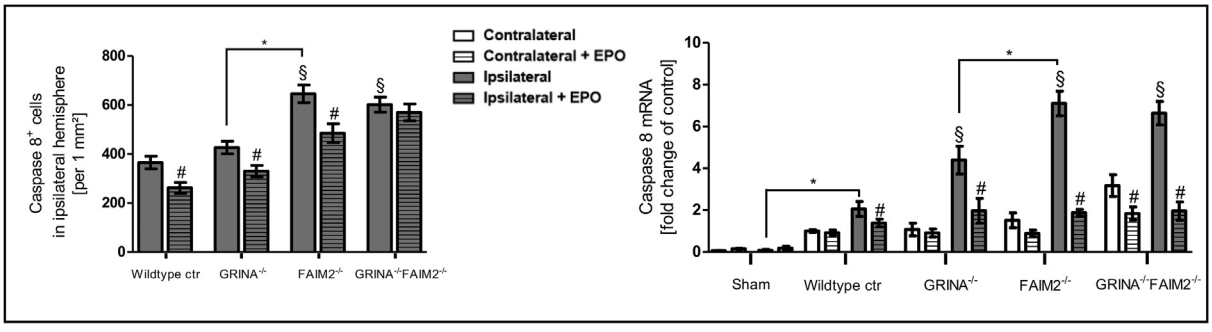

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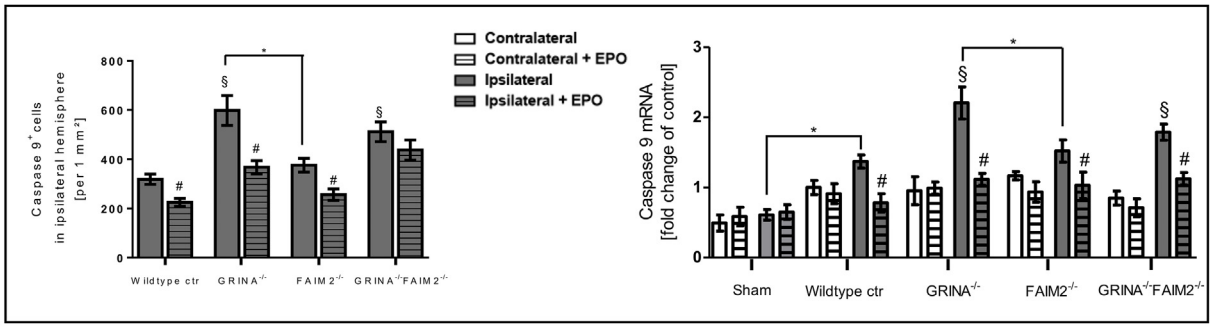

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Fig. 5. TMBIM family members FAIM2 and GRINA are involved in the regulation of caspases after ischemic stroke.
Cleaved-caspase 3, caspase 8 and caspase 9 staining of brain slices and RT-qPCR of brain biopsies (Bregma 0 ± 1 mm) from mice subjected to 30 min tMCAo or sham surgery followed by 72 h of reperfusion was performed. Caspases positive cells in 12 × 0.01 mm^2 grids from the peri-infarct zone were counted by two individual investigators. (a) Representative images of cleaved-caspase 3, caspases 8 and 9 positive cells in 40 x magnitude in the peri-infarct zones of all genotypes and treatment groups are shown. (b) Cleaved-caspase 3 positive cells in the peri-infarct zone and caspase 3 mRNA levels, (c) The number of caspase 8 positive cells in the peri-infarct and caspase 8 mRNA and (d) caspase 9 positive cells in the peri-infarct area plus caspase 9 mRNA expression are shown. Bars represent means ± SD. N = 6 of every genotype and treatment. *p < .05 between group, §p < .05 compared to wildtype controls. #p < .05 compared to Vehicle.

anterior circulation came with the endovascular mechanical thrombectomy, when EST was proven to be an effective maneuver for salvaging ischemic brain injuries in nine positive RCTs since November 2014. Despite its efficacy, the morbidity and mortality rate of patients who underwent EST ranged from 29 to 67% after 90 days, emphasizing the need for the implementation of adjunct neuroprotective strategies to EST after ischemic stroke (Elgendy et al., 2015; Palaniswami and Yan, 2015). The tMCAo model sufficiently mimics EST, since changes in cerebral blood flow and pathophysiological characteristics seems to highly resemble each other and can reliably be used to explore cerebral ischemia/reperfusion injury and neuroprotective agents (Sutherland et al., 2016).

5. Conclusion

Our current study uncovered a crucial role of the TMBIM family member GRINA in the control of apoptosis after cerebral ischemia. While FAIM2 is involved in the extrinsic apoptotic pathways, GRINA appears to regulate the intrinsic apoptotic pathway. Similar to FAIM2, GRINA is also regulated by EPO. In addition, our observations suggest a synergistic or partly compensatory action of GRINA and FAIM2 (Graphical Abstract). Further research is needed to evaluate the cellular and subcellular distribution and to examine potential functional and physical interaction of both TMBIM family members. In the context of successful endovascular reperfusion treatment of patients with large vessel occluding acute ischemic stroke, the therapeutic potential of EPO should be reconsidered.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2019.112978.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving animals were in accordance with the ARRIVE guidelines and the ethical standards of the District Government of North Rhine Westphalia in Recklinghausen, Germany (LANUV ID 84–20.04.2015.A292). All institutional guidelines for the care and use of animals were followed.

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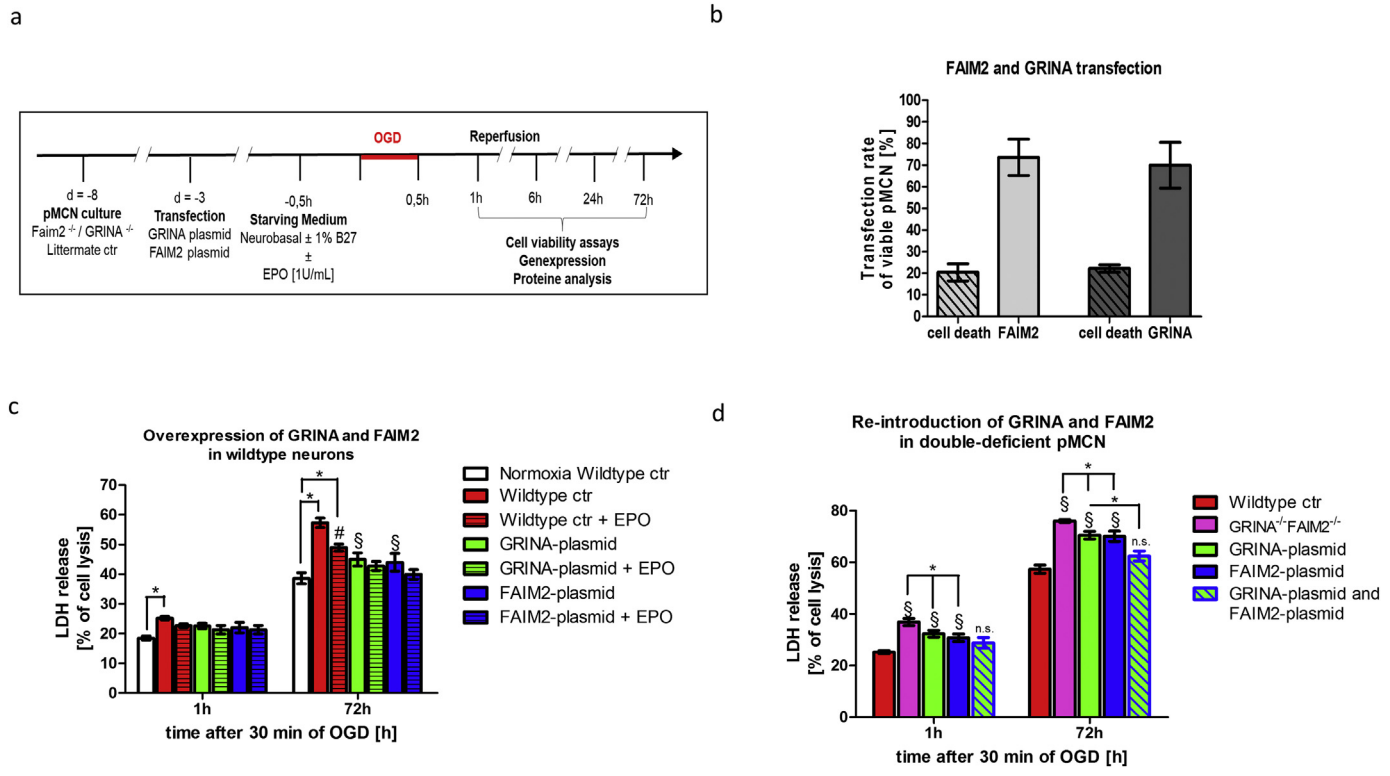

Fig. 6. FAIM2 and GRINA reduce cell death of pMCN after 30 min OGD followed by 72 h of reperfusion.
(a) Schematic illustration of pMCN culture preparation, transfection and OGD-procedure is illustrated. (b) Immunofluorescence staining of pMCN was performed after transfection with GFP-tagged FAIM2-plasmid and Myc-tagged GRINA-plasmid. Mean rates of GRINA transfected cells (69,94%) and FAIM2 transfected viable pMCN (73,57%) are shown. (c) LDH-release in GRINA and FAIM2 transfected wildtype pMCN subjected to 30 min of OGD followed by 1 h and 72 h of reperfusion is shown. (d) LDH-release in GRINA and FAIM2 transfected double deficient pMCN subjected to 30 min of OGD followed by 1 h and 72 h of reperfusion is shown. Bars represent means ± SD of 4 individual experiments with three technical replicates in each experiment. *p < .05 between group, §p < .05 compared to wildtype control. #p < .05 compared to Vehicle.

Medicine, RWTH Aachen University. The funding body had no influence on the design of the study, data acquisition or on analyses and interpretation of data.

Declaration of competing interests

None.

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