



C1 Esterase Inhibitor Reduces BBB Leakage and Apoptosis in the Hypoxic Developing Mouse Brain

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Abstract

Inflammatory pathways involved in blood–brain barrier (BBB) vulnerability and hypoxic brain oedema in models of perinatal brain injury seem to provide putative therapeutic targets. To investigate impacts of C1-esterase inhibitor (C1-INH; 7.5–30 IU/kg, i.p.) on functional BBB properties in the hypoxic developing mouse brain (P7; 8% O₂ for 6 h), expression of pro-apoptotic genes (BNIP3, DUSP1), inflammatory markers (IL-1 β , TNF-alpha, IL-6, MMP), and tight junction proteins (ZO-1, occludin, claudin-1, -5), and S100b protein concentrations were analysed after a regeneration period of 24 h. Apoptotic cell death was quantified by CC3 immunohistochemistry and TUNEL staining. In addition to increased apoptosis in the parietal cortex, hippocampus, and subventricular zone, hypoxia significantly enhanced the brain-to-plasma albumin ratio, the cerebral S100b protein levels, BNIP3 and DUSP1 mRNA concentrations as well as mRNA expression of pro-inflammatory cytokines (IL-1 β , TNF-alpha). In response to C1-INH, albumin ratio and S100b concentrations were similar to those of controls. However, the mRNA expression of BNIP3 and DUSP1 and pro-inflammatory cytokines as well as the degree of apoptosis were significantly decreased compared to non-treated controls. In addition, occludin mRNA levels were elevated in response to C1-INH ($p < 0.01$). Here, we demonstrate for the first time that C1-INH significantly decreased hypoxia-induced BBB leakage and apoptosis in the developing mouse brain, indicating its significance as a promising target for neuroprotective therapy.

Keywords Neonatal brain injury · S100b protein · Hypoxia · Neuroprotection · Tight junctions · Occludin · Matrix metalloproteinases · BNIP3 · DUSP1

Abbreviations

BBB	Blood–brain barrier
BNIP3	BCL2 Interacting Protein 3
C1-INH	C1 esterase inhibitor
CC3	Cleaved caspase 3
DUSP1	Dual specificity phosphatase 1
FOV	Field of view
HI	Hypoxia and ischemia

HIF	Hypoxia-inducible transcription factors
MMP	Matrix metalloproteinase
TIMP	Tissue inhibitor of metalloproteinases
ZO-1	Zona occludens protein 1
IL	Interleukin
TNF-alpha	Tumour necrosis factor-alpha

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Introduction

In preterm and term newborns, hypoxia and ischemia (HI) are the most common causes of acquired brain injury that occur during the neonatal period (Ahearne et al. 2016; Stark et al. 2016). In view of the acute and long-term consequences of HI brain injury, including high mortality rates, persisting deficits in motor, sensomotor, and cognitive development, as well as in abnormal behaviour, the development of specific neuroprotective treatments is one of the main topics of current scientific work in the field of neonatal neurology (Zou et al. 2018; Fitzgerald et al. 2018). Currently,

specific neuroprotective pharmacologic treatments are not available for clinical use (Dixon et al. 2015; Davis et al. 2016). In asphyxiated term neonates, exclusively, the supportive option of therapeutic hypothermia is commonly used based on promising experimental data (Chevin et al. 2016) and the results of international, controlled clinical studies (Azzopardi et al. 2014).

According to experimental studies, neuroprotective strategies are most promising during the immediate period of the HI insults (Hsu et al. 2014; Savard et al. 2015). However, this “window of opportunity” for any compensatory mechanisms of the neonatal brain in response to profound hypoxia is temporally limited. Among the rapidly available, endogenous neuroprotective mechanisms, hypoxia-inducible transcription factors (HIFs) (HIF-1, HIF-2) play a crucial role in the regulation of oxygen and energy homeostasis in the hypoxic developing brain (Chu and Jones 2016; Semenza 2014). HIFs modulate a variety of adaptive and regenerative processes after HI brain injury, such as vasculogenesis, cellular metabolism, neuronal and glial migration, and differentiation. This is accomplished by the transcriptional activation of specific target genes (e.g. EPO, VEGF-A, IGF-1), activation of pro-apoptotic mechanisms (Li et al. 2017), and breakdown of the BBB (Engelhardt et al. 2014) under conditions of profound HI. For example, the hypoxia-induced accumulation of the O₂-sensitive HIF- α subunit leads to increased BBB vulnerability via the selective disruption of microvascular endothelial tight junction (TJ) complexes. Presumably, this may be caused by delocalisation and activated tyrosine phosphorylation of TJ proteins (Engelhardt et al. 2014; Bonestroo et al. 2015).

The complex and cell type-specific neurotoxic mechanisms that trigger hypoxia-induced BBB breakdown in the developing brain are not fully understood (Burek et al. 2019; Hu et al. 2017; Ek et al. 2015; Chen et al. 2012). Functionally, the integrity of the BBB depends on specific endothelial junction proteins including tight junction [TJ; e.g. occludin, claudin, zona occludens protein (ZO)-1] and adherens junction proteins (e.g. α -catenin, β -catenin) (Diaz et al. 2016, Marin et al. 2012). The significance of TJ proteins in the regulation of BBB integrity was demonstrated in adult (Yang and Rosenberg 2011) and neonatal rodent (Wang et al. 2016; Diaz et al. 2016) and foetal sheep stroke models (Chen et al. 2012). In response to HI brain injury, activation of reactive oxygen species (ROS) and matrix metalloproteinases (MMP), in particular MMP-9, have been shown to mediate BBB disruption via oxidative damage and TJ dysregulation (Alluri et al. 2016; Diaz et al. 2016, Hu et al. 2017). Recently, hypoxia-induced expression of microRNA-212/132 in hypoxic mouse and human brain microvascular endothelial cells was proposed as specific target of hypoxia-induced BBB disruption through

inhibition of TJ proteins such as claudin-1 (Burek et al. 2019). Studies in adult murine models of traumatic brain injury (Albert-Weissenberger et al. 2014) and stroke (Chen et al. 2018; Heydenreich et al. 2012; Gesuete et al. 2009) have observed significant advantages of treatment with C1 esterase inhibitor (C1-INH), which has regularly and successfully been used in patients with hereditary angioedema (Valerieva et al. 2018). C1-INH, which is a member of the serine protease inhibitors (serpins) and is usually activated due to inflammatory events, inactivates its substrate by covalently binding to the reactive site. C1-INH is the only known inhibitor of the complement component 1 subcomponent r (C1r), C1s, coagulation factor XIIa, kallikrein, and coagulation factor XIa (intrinsic coagulation cascade) (Valerieva et al. 2018). Growing evidence suggests that C1-INH may reduce inflammatory destruction of the BBB as well as injury-induced focal and diffuse brain oedema (Chen et al. 2018), and this could be the result of suppression of leukocyte infiltration, expression of specific pro-inflammatory cytokines and pro-caspase 3, resulting in both an anti-inflammatory and anti-apoptotic effect (De Simoni et al. 2004; Storini et al. 2005). However, studies on exact binding sites, functional mechanisms, efficacy, and safety in neonatal HI brain injury are lacking.

We aimed to analyse the effects of C1-INH on BBB function, neuroinflammatory and apoptotic mechanisms in the developing hypoxic mouse brain. We show for the first time that C1-INH treatment reduced disruption of the developing BBB, modified cerebral occludin expression, and exerted anti-inflammatory and anti-apoptotic effects in the hypoxic developing mouse brain.

Materials and Methods

Chemicals

DAKO[®] Protein Block, serum-free was purchased from Agilent (Waldbronn, Germany). Water and sodium chloride 0.9% (w/v) solution for injection were purchased from Berlin-Chemie (Berlin, Germany). Digitonin, HEPES, hexylene glycol, magnesium chloride, paraformaldehyde, Triton X-100, and xylene were purchased from Carl Roth (Karlsruhe, Germany). Calcium chloride, ethanol, methanol, and paraffin were purchased from Merck Millipore (Darmstadt, Germany). Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS w/o) was purchased from PAN-Biotech (Aidenbach, Germany). Cresyl violet, DTT, Eukitt[®] mounting medium, hydrogen peroxide, and Igepal[®] were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Animal Experiments

All animal experiments were reviewed and performed under the approval of the national care committee (Regierung Unterfranken, Germany) according to national and European laws on the protection of animals. All mice were housed with a 12-h light/12-h dark cycle and were provided with food and water ad libitum. Pups were kept together with the dam to provide normal temperature and nutrition. At the age of P7, the stage of mouse brain development corresponds to that of humans at near-term (Dobbing and Sands 1979).

C1-INH Treatment

Plasma-derived, nanofiltered human C1-INH, (pnC1-INH; Berinert[®], CSL Behring) was injected intraperitoneally (i.p.) at doses of 7.5, 15, and 30 IU/kg (injection volume 10 mL/kg, prepared according to the manufacturer's instructions). Age-matched controls were treated with C1-INH solvent (injection volume 10 mL/kg). Tolerance of C1-INH at a dose of 20 IU/kg bodyweight and in vitro data on dose-dependent efficacy have been shown in adult rodents upon systemic (i.v.) application (Albert-Weissenberger et al. 2014; Heydenreich et al. 2012; Gesuete et al. 2009). Different doses were required to define the optimal dose range in our experimental setting, especially considering immature BBB functions. To this end, pilot experiments on neonatal C57BL/6N wild-type mice under normoxia treated with C1-INH i.p. ($n = 3$ per dose) versus controls, ($n = 3$) revealed stable tolerance in the selected dose range. Comparable data from the literature are missing.

Neonatal Model of Systemic Hypoxia

Seven-day-old neonatal C57BL/6N wild-type mice (P7; Charles River Laboratories, Sulzfeld, Germany) were exposed to normoxia or acute systemic hypoxia as previously described (Trollmann et al. 2018). Briefly, pups were exposed to acute systemic hypoxia (FiO₂ 8%) for 6 h using an INVIVO₂400 hypoxia workstation (Ruskinn, Bridgend, UK). O₂ deprivation was conducted by gradually decreasing FiO₂ in steps of 2% O₂ every 10 min. Age-matched litter mates ($n = 8$ per group) were housed in the INVIVO₂400 chamber in room air (21% O₂, normoxia). At the end of hypoxia exposure, pups were treated i.p. with C1-INH at a single dose of either 7.5, 15, or 30 IU/kg. Controls were treated i.p. with vehicle (VT) or remained non-treated (NT). In summary, a total of 80 pups (P7) were randomized into 10 groups: (i) NT (normoxia, $n = 8$; hypoxia, $n = 8$); (ii) VT (normoxia, $n = 8$; hypoxia, $n = 8$); (iii) C1-INH 7.5, 15, or 30 IU/kg (normoxia, $n = 24$; hypoxia, $n = 24$). At the end of the hypoxic incubation (P7) and after a 24-h interval of regeneration, we did not find any significant differences in

the brain-to-bodyweight ratio (data not shown) between normoxic and hypoxic mice, and this was independent of C1-INH treatment.

After a regeneration period of 24 h, brains were dissected, frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$ until mRNA and protein extraction. For immunohistochemical analyses, a subgroup of pups ($n = 3$ per group) were perfused with DPBS followed by PFA 4% prior to dissection of the brains that were embedded in paraffin. Blood samples were collected in Microvette K3 EDTA tubes (Sarstedt, Numbrecht, Germany) and centrifuged at 2000 $\times g$ for 10 min. Plasma was stored at $-80\text{ }^{\circ}\text{C}$ until further use. Total plasma protein concentrations were determined at 280 nm using a NanoDrop ND 2000c (Peqlab VWR, Erlangen, Germany).

Immunohistochemistry (IHC)

Five- μm -thick coronal sections at the level of the dorsal hippocampus of PFA-embedded mouse brains were stained as described previously (Trollmann et al. 2018). For detection of cleaved caspase 3 (CC3), rabbit anti-CC3 antibody (2.5 ng/mL; Merck Millipore, Darmstadt, Germany) and biotinylated goat anti-rabbit IgG secondary antibody (3 $\mu\text{g}/\text{mL}$; Vector Laboratories, Burlingame, CA) were used. Staining was performed using the VECTASTAIN Elite ABC HRP and the DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's protocols. Slides were counterstained with cresyl violet. Co-stainings for Zonula occludens-1 (ZO-1) with Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) as well as occludin with PECAM-1 were performed using rat anti-ZO-1 (40 $\mu\text{g}/\text{mL}$; DSHB, Iowa City, USA), mouse anti-occludin and rabbit anti-PECAM-1 antibody (Santa Cruz, Heidelberg, Germany; 1 $\mu\text{g}/\text{mL}$, and 0.5 $\mu\text{g}/\text{mL}$, resp.), as well as goat anti-rat IgG (H+L) Alexa Fluor 647 antibody (20 $\mu\text{g}/\text{mL}$, Thermo Fisher Scientific, Schwerte, Germany), goat anti-mouse IgG (H+L) Alexa Fluor 647, and goat anti-rabbit IgG (H+L) Alexa Fluor 488 (10 $\mu\text{g}/\text{mL}$, Thermo Fisher Scientific, Schwerte, Germany) as secondary antibodies. Negative controls were performed by omitting the primary antibody. All stainings were performed in triplicate. Quantitative evaluation of immunohistochemical staining in the cerebral parietal cortex was performed by determining the area of ZO-1/PECAM-1 and occludin/PECAM-1 in relation to normoxic and vehicle-treated samples (2×5 visual fields, right and left hemispheres per section), using a Scope.A1 microscope (Zeiss, Jena, Germany) and ImageJ.

TUNEL Staining

To determine the degree of apoptosis-like cell death, terminal deoxynucleotidyl transferase-mediated dUTP end-labelling (TUNEL) (In Situ Cell Death Detection Kit,

Promega, Mannheim, Germany) was performed using the VECTASTAIN Elite ABC HRP and the DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories, Burlingame, CA). Quantification of TUNEL and CC3 staining ($n=3$ per group) was performed by two independent investigators who were blinded to the study groups. The quantitative evaluation of TUNEL and CC3 staining of the cerebral parietal cortex, hippocampus, and subventricular zone (SVZ) was done by counting the number of positive cells (2×5 fields of view (FOV); right and left hemispheres per region/section) using a Scope.A1 microscope (Zeiss, Jena, Germany).

RNA Isolation and RT-PCR

Total cellular RNA was extracted from frozen whole forebrains using Trizol according to the manufacturer's protocol (Life Technologies, Darmstadt, Germany). Prior cDNA synthesis genomic DNA contaminations were removed by treatment with RNase-free DNase I (Promega, Mannheim, Germany). In brief, 2 μg of RNA and 2 U of DNase I in a volume of 20 μL of $1 \times$ reaction buffer were incubated at room temperature for 15 min. To stop DNase I treatment, EDTA was added to a final concentration of 2.5 mM and incubation at 65 °C for 15 min. For reverse transcription 0.4 μg of dT(16) oligonucleotides (Eurofins MWG Operon, Ebersberg, Germany), 0.6 μg of dN(6) random hexamer oligonucleotides (Roche Diagnostics, Mannheim, Germany), and DEPC water were added to a final volume of 32 μL , heated at 70 °C for 5 min, and chilled immediately on ice prior 10 μL of $5 \times$ reaction buffer, 20 U of recombinant RNasin ribonuclease inhibitor, 200 U of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Mannheim, Germany), 2.5 μL of a 10 mM each dNTP mix (Fisher Scientific, Schwerte, Germany), and DEPC-treated water were added to a final volume of 52 μL . Reaction mixture was incubated at 37 °C for 1 h, rapidly cooled on ice, and stored at -80 °C until further use. cDNA concentration was determined by UV–Vis spectroscopy and adjusted with LiChrosolv water to a final concentration of 200 ng/ μL . Quantitative PCR was performed using the qPCR Core kit with ROX according to the manufacturer's protocol (Eurogentec, Seraing, Belgium). In brief, 1 μg cDNA were assayed in the presence of 3.5–5.0 mM MgCl_2 , 0.2 mM dNTP mix with dUTP, 0.3–0.9 μM forward primer, 0.3–0.9 μM reverse primer, 0.25 U of Uracil-DNA Glycosylase (Eurogentec, Seraing, Belgium), and 0.2 μM of a gene-specific dual-labelled fluorescent probe (Biomers, Ulm, Germany) in a final volume of 25 μL . PCR oligonucleotides used in this study are listed in supplementary Table 1 (suppl_Table 1). Real-time PCR was conducted using a CFX96 Touch Real-Time PCR System (Bio-Rad, Munich, Germany) with the following thermal profile: one cycle at 50 °C for 2 min, followed by 95 °C for 10 min, 50 cycles of

95 °C for 5 s, followed by 60 °C for 60 s. The NormFinder software (Andersen et al. 2004) was used to identifying the optimal normalization gene among a set of candidate genes obtained from the literature. For hypoxia and/or C1-INH treatment, *actb* and *pbgd* were the most stable genes and therefore selected as reference genes.

Protein Extraction and Quantification

Subcellular protein fractionation was performed according to Baghirova et al. (2015). Supernatants were harvested and stored at -80 °C until further use. Protein concentrations were quantified using the Pierce™ BCA Protein Assay Kit (Life Technologies, Darmstadt, Germany) according to the manufacturer's protocol.

ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to analyse concentration of S100b (R&D Systems, Wiesbaden-Nordenstadt, Germany), Albumin (Bethyl Laboratories, Montgomery, TX, USA) in plasma and brain homogenate, as well as TNF-alpha, IL-1 β , and IL-6 (BioLegend, Fell, Germany) according to the manufacturer's protocols. Lower limits of detection were 3 pg/mL. Samples were processed in duplicates.

Statistical Analysis

Data are presented as the mean and standard error of mean (SEM). Differences between groups (normoxia vs hypoxia, NT/VT vs C1-INH) were assessed by two-way analysis of variance (ANOVA) with a Bonferroni multiple comparison post hoc test to adjust for 15 comparisons (Graph Pad 7.00, La Jolla, CA). Family-wise significance level was 0.05%. Two-tailed values of $p < 0.05$ were considered to be statistically significant.

Results

Decrease of Hypoxia-Mediated BBB Leakage in Response to C1-INH

Exposure to acute hypoxia significantly increased the concentrations of S100b protein in the brain (Fig. 1a, $p < 0.01$) and plasma (Fig. 1b, $p < 0.001$) compared to controls. The brain-to-plasma albumin ratio was also significantly elevated in hypoxia-exposed mice when compared to controls (Fig. 1c, $p < 0.01$). These data confirm the strength of the present experimental setting of acute hypoxia (FiO₂ 8%, 6 h) to cause increased permeability of the developing BBB. C1-INH significantly reduced the levels of S100b

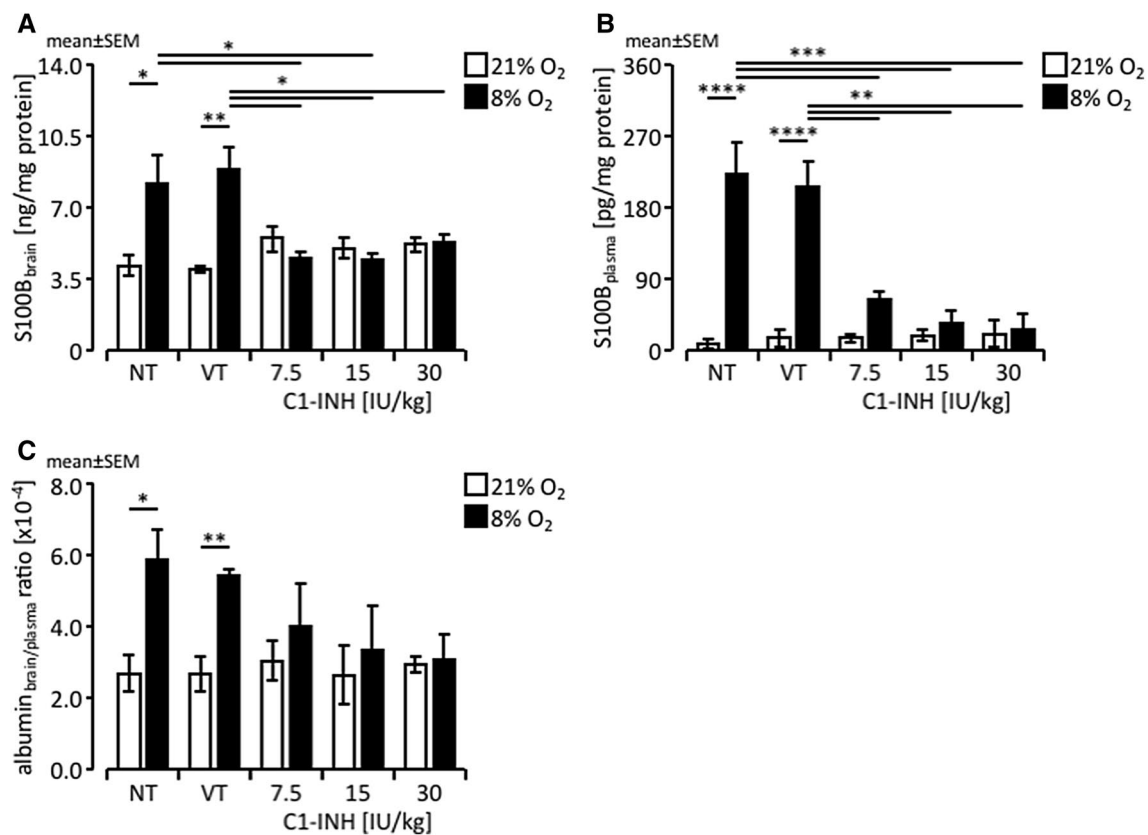


Fig. 1 Protein levels of S100b in the developing brain (a) and plasma (b) of normoxic and hypoxic mice, and c brain-to-plasma albumin ratio of neonatal mice (P7+ regeneration of 24 h) in relation to

C1-INH treatment (compared to controls). *NT* non-treated, *VT* vehicle-treated; $n = 5$ per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

protein in the hypoxic brain tissues (Fig. 1a; $p < 0.05$) and in the plasma of hypoxic pups (Fig. 1b; $p < 0.01$) in a dose-dependent manner, compared to controls. In addition, C1-INH inhibited the hypoxia-induced increase in the brain-to-plasma albumin ratio (Fig. 1c; $p < 0.05$).

Increased Cerebral Occludin Expression in Response to C1-INH

To evaluate the effects of C1-INH treatment on regulatory factors of BBB integrity under conditions of acute hypoxia, we investigated the cerebral expression of selected tight junction proteins after a regeneration period of 24 h. C1-INH treatment significantly and dose-dependently increased occludin mRNA concentrations (Fig. 2a, $p < 0.05$) in normoxic and hypoxic brains. Interestingly, this was confirmed at the protein level. While hypoxia significantly reduced vascular endothelial occludin protein expression (Fig. 2b–j, $p < 0.05$), C1-INH application augmented endothelial occludin expression in a dose-dependent manner (Fig. 2b–j, $p < 0.05$). In contrast, there were no significant differences in the mRNA levels of ZO-1, claudin-1, and claudin-5 in hypoxia-exposed brains when compared with

controls (Table 1). Similarly, immunohistochemical double staining for ZO-1 and PECAM-1 (supplementary material) did not demonstrate significant differences of ZO-1 expression between C1-INH-treated brain tissues and non-treated controls.

Reduction of Cerebral Pro-inflammatory Markers in Response to C1-INH

With regard to effects of C1-INH on pro-inflammatory mediators involved in hypoxia-induced BBB breakdown and cerebral pro-apoptotic mechanisms, we analysed cerebral mRNA expression of selected MMP (Table 2) and cerebral mRNA (Fig. 3a–c) as well as plasma protein concentrations of pro-inflammatory cytokines (Fig. 3d–f). Neither hypoxia nor C1-INH treatment significantly affected the mRNA expression of MMP-2, MMP-9, and MMP-14 compared to normoxic controls (Table 2). Similarly, cerebral tissue inhibitor of metalloproteinase 2 (TIMP)-2 mRNA levels did not show any significant changes (Table 2). However, present analysis revealed a significant reduction of hypoxia-induced, elevated cerebral IL-1 β and TNF- α mRNA levels in response to C1-INH (Fig. 3a, c), and this was found even in response to

Fig. 2 a Gene expression of occludin (related to β -actin) in response to C1-INH in normoxic and hypoxic (8% O_2) brains after a 24-h period of regeneration in comparison to controls. $n=5$; **b–j** Immunohistochemical double staining for occludin and PECAM-1 of normoxic and hypoxic brains of non-treated (NT), vehicle-treated (VT), and C1-INH-treated pups; **b** quantification of occludin/PECAM-1-positive vessels in the parietal cortex. $n=3$ per group. **c–j** Representative photomicrographs of vascular occludin/PECAM-positive structures. Occludin (red), PECAM-1 (green). Magnification is given in the figures. *NT* non-treated, *VT* vehicle-treated; * $p < 0.05$

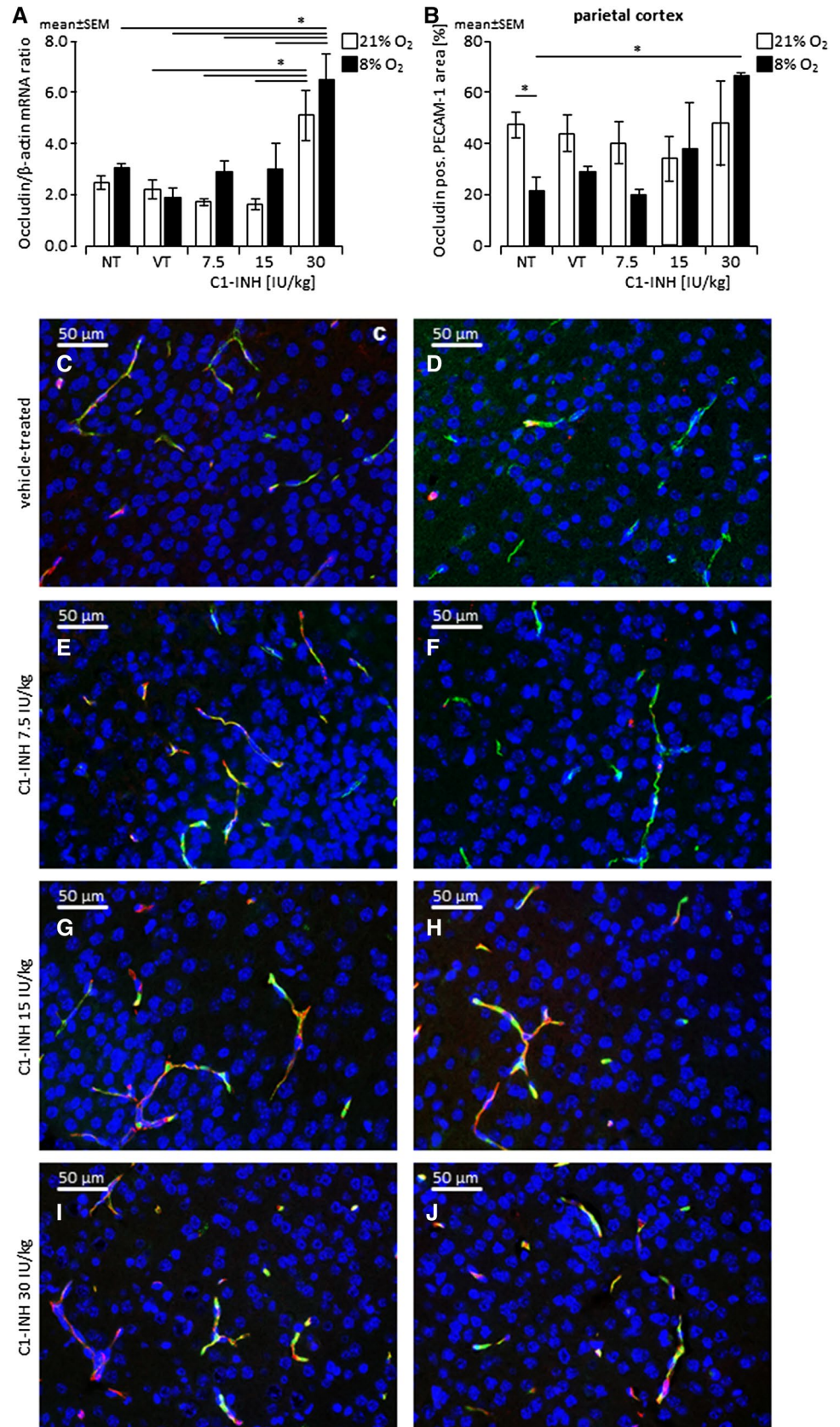


Table 1 Gene expression of tight junction proteins (related to β -actin, mean \pm SEM) in response to C1-INH in normoxic and hypoxic (8% O₂) brains after a 24-h period of regeneration in comparison to controls ($n=5$ per group)

	Non-treated		Vehicle-treated		C1-INH (7.5 IU/kg)		C1-INH (15 IU/kg)		C1-INH (30 IU/kg)	
	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
ZO-1	1.22 \pm 0.10	1.78 \pm 0.21	1.32 \pm 0.14	1.60 \pm 0.14	1.01 \pm 0.07	1.13 \pm 0.16	1.10 \pm 0.22	1.33 \pm 0.07	1.12 \pm 0.12	1.19 \pm 0.14
Claudin-1	1.15 \pm 0.15	1.36 \pm 0.08	1.22 \pm 0.26	1.14 \pm 0.12	1.17 \pm 0.18	1.16 \pm 0.20	1.77 \pm 0.19	1.33 \pm 0.15	0.83 \pm 0.09	0.80 \pm 0.09
Claudin-5	1.07 \pm 0.07	1.29 \pm 0.08	1.03 \pm 0.14	1.01 \pm 0.09	1.13 \pm 0.13	1.22 \pm 0.20	1.09 \pm 0.18	1.27 \pm 0.17	1.07 \pm 0.12	1.27 \pm 0.17

There were no statistically significant differences between the groups (two-way ANOVA, $p > 0.05$)

Table 2 Gene expression of MMP and TIMP-2 (related to β -actin, mean \pm SEM) in response to C1-INH in normoxic and hypoxic (O₂ 8%) brains after a 24-h period of regeneration in comparison to controls ($n=5$ per group)

	Non-treated		Vehicle-treated		C1-INH (7.5 IU/kg)		C1-INH (15 IU/kg)		C1-INH (30 IU/kg)	
	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
MMP-2	1.10 \pm 0.05	1.08 \pm 0.14	0.94 \pm 0.10	0.93 \pm 0.09	0.97 \pm 0.12	1.03 \pm 0.13	1.07 \pm 0.09	1.02 \pm 0.14	0.93 \pm 0.15	1.19 \pm 0.10
MMP-9	0.85 \pm 0.09	1.32 \pm 0.06	0.71 \pm 0.09	1.27 \pm 0.19	0.68 \pm 0.06	0.77 \pm 0.17	1.07 \pm 0.11	1.04 \pm 0.22	0.77 \pm 0.25	1.17 \pm 0.08
MMP-14	0.81 \pm 0.10	0.86 \pm 0.18	0.61 \pm 0.08	0.70 \pm 0.05	0.66 \pm 0.06	0.66 \pm 0.10	0.77 \pm 0.09	0.60 \pm 0.07	0.80 \pm 0.13	0.79 \pm 0.11
TIMP-2	0.73 \pm 0.08	0.66 \pm 0.06	0.79 \pm 0.26	0.70 \pm 0.05	0.72 \pm 0.05	0.60 \pm 0.05	0.62 \pm 0.08	0.77 \pm 0.09	0.91 \pm 0.11	0.75 \pm 0.08

There were no statistically significant differences between the groups (two-way ANOVA, $p > 0.05$)

low-dose treatment. Additionally, similar significant effects of C1-INH application were observed on IL-1 β , IL-6, and TNF-alpha plasma concentrations compared to non-treated and vehicle-treated hypoxic controls (Fig. 3d–f).

Reduction of Cerebral Apoptosis in Response to C1-INH

To determine the impact of C1-INH treatment on early markers of cerebral apoptosis, hypoxia-sensitive pro-apoptotic factors (BCL2 Interacting Protein (BNIP)-3, and dual-specificity phosphatase (DUSP)-1) were analysed at the mRNA level after a 24-h interval of regeneration. Hypoxia led to significant increases in cerebral BNIP3 (Fig. 4a, $p < 0.05$) and DUSP1 mRNA levels (Fig. 4b, $p < 0.05$) compared to controls, but in response to C1-INH treatment, both BNIP3 (Fig. 4a, $p < 0.01$) and DUSP1 levels (Fig. 4b, $p < 0.05$) were markedly lower in hypoxia-exposed brains compared to non-treated hypoxia-exposed and normoxic controls. As cerebral activation of pro-apoptotic mechanisms may develop after a latency period of up to several days, we used CC3-IHC (Fig. 5) and TUNEL staining (Fig. 6) to examine the degree of apoptotic cell death in developing brains after a short recovery period (21% O₂) of 24 h. A comparative analysis of CC3-positive cell numbers in the cerebral parietal cortex, hippocampus, and subventricular zone (SVZ) of hypoxia-exposed brains between vehicle-treated and C1-INH-treated pups demonstrated that the degree of apoptotic cell death in response to C1-INH decreased significantly (Fig. 5a–c, $p < 0.05$). Compared to controls, significantly

higher numbers of CC3-positive cells were detected in all of the highly vulnerable brain regions selected (Fig. 5a–c) in response to hypoxia. However, hypoxic brains exposed to C1-INH showed markedly lower CC3 positivity compared to non- and vehicle-treated hypoxic controls (Fig. 5a–c, $p < 0.05$). Representative photomicrographs are shown in Fig. 5d, demonstrating the degree of CC3 positivity in the SVZ of developing brains of the different treatment groups. Similar results were obtained with TUNEL staining (Fig. 6).

Discussion

In order to mimic functional disruption of the immature BBB due to acute systemic hypoxia, the present study used a mouse model of neonatal brain injury and demonstrated that C1-INH markedly improved hypoxia-induced BBB leakage and significantly reduced apoptotic developmental brain damage. C1-INH significantly inhibited a hypoxia-induced increase in brain-to-plasma albumin ratio and S100b protein accumulation, and suppressed the hypoxia-induced upregulation of pro-inflammatory and pro-apoptotic mechanisms in the hypoxic neonatal mouse brain, indicating the reduction of hypoxia-induced apoptosis. Additionally, after a post-hypoxic regeneration period of 24 h, C1-INH augmented cerebral occludin expression, indicating it has modulatory effects on specific TJ proteins in the hypoxic developing mouse brain.

Disruption of the BBB plays a pivotal role in the activation of neurotoxic mechanisms, as shown in rodent models

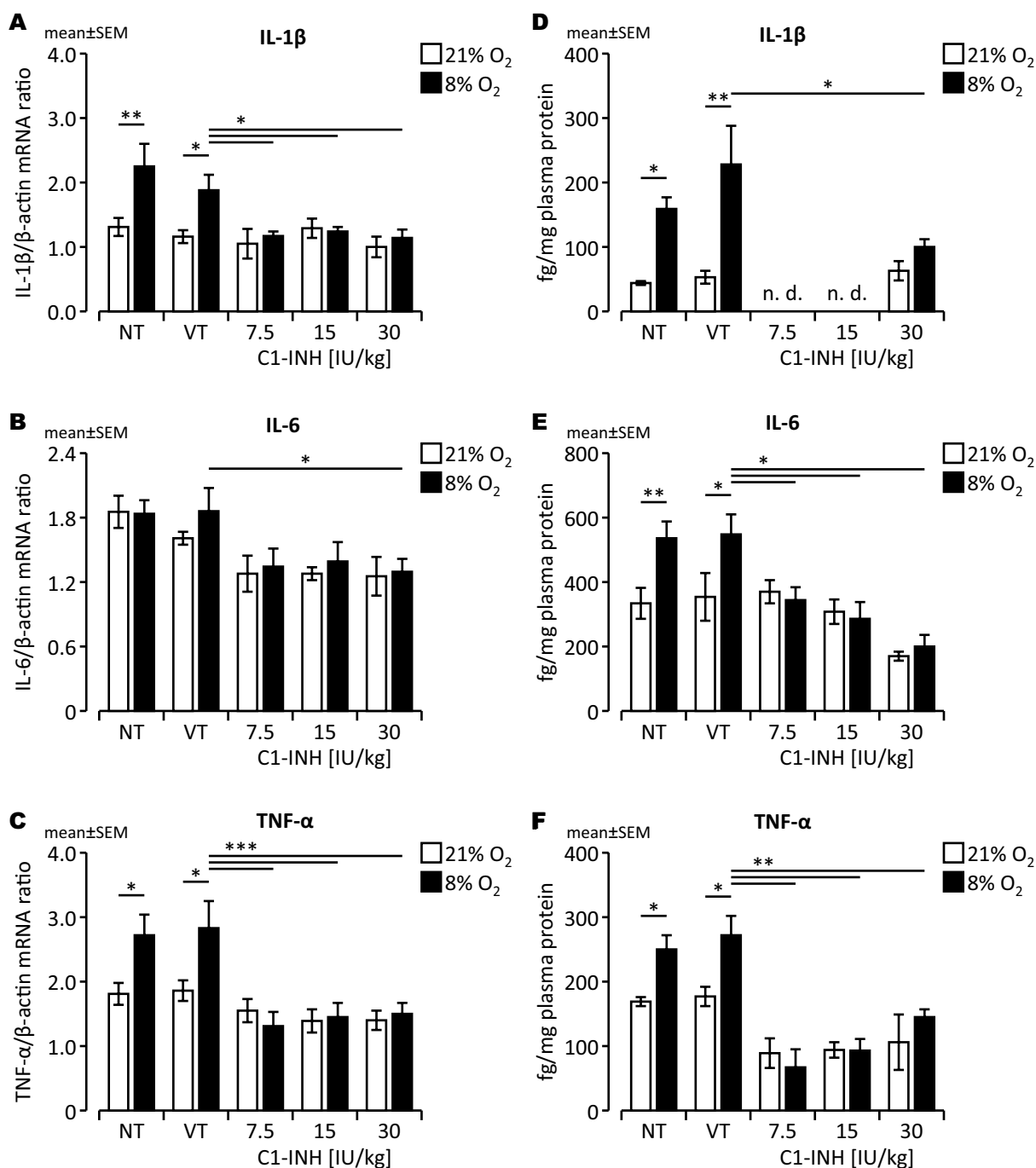


Fig. 3 Cerebral gene expression (a–c) and plasma protein concentrations (d–f) of pro-inflammatory cytokines in response to C1-INH in normoxic and hypoxic (8% O₂) neonatal brains (P7) after a regenera-

tion period of 24 h in comparison to non-treated (NT) and vehicle-treated (VT) controls. a–c $n=5$ per group; d–f: $n=3$ per group; n.d. not detected; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

of developing brain injury, such as hypoxic-ischemic (Diaz et al. 2016; Hsu et al. 2014), traumatic, haemorrhagic, and inflammatory injury models (Wang et al. 2014; Diaz et al. 2016). Using an established mouse model of neonatal hypoxic brain injury, we have clearly demonstrated BBB damage in response to acute hypoxia, as assessed by brain-to-plasma albumin ratios (Tibbling et al. 1977) and S100b

concentrations in plasma and brain, with elevated S100b levels indicating marked astroglial damage under conditions of acute hypoxia (Thelin et al. 2017). S100b protein (molecular weight 9–14 kDa) belonging to a family of intracellular, calcium-binding proteins, is mainly present in perivascular astrocytes and in low concentrations in oligodendrocytes and neural progenitor cells (Donato et al. 2013). The functions

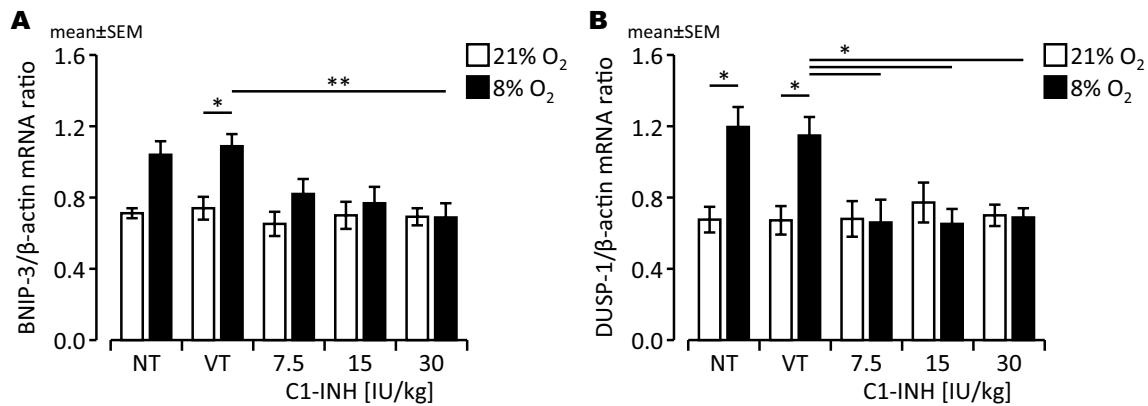


Fig. 4 Gene expression of BNIP3 (a) and DUSP1 (b) in response to C1-INH in normoxic and hypoxic (8% O₂) neonatal brains (P7) after a regeneration period of 24 h in comparison to non-treated (NT) and vehicle-treated (VT) controls. $n = 5$ per group; * $p < 0.05$, ** $p < 0.01$

of S100b differ in a concentration-dependent manner, with neurotrophic effects occurring at the nanomolar level. However, supraphysiologically increased levels, due to acute brain injury, have been shown to induce neuronal dysfunction and apoptosis as a result of astroglial and microglial pro-inflammatory cytokine production and activation of cytotoxic nitric oxide (Thelin et al. 2017). Similar to our observations, S100b that is released into the serum due to hypoxia-induced BBB disruption has been proposed as a useful biomarker of BBB permeability by several research groups (Kleindienst et al. 2010; Kapural et al. 2002).

Various triggers for the breakdown of the BBB have been suggested; however, data from developing brain injury are limited. With regard to HI events, inflammatory cytokines, oxidative stress, and reactive oxygen species (ROS) mediate BBB damage, disturbance of TJ integrity, and activation of MMPs (Wang et al. 2014; Alluri et al. 2016). Using a preclinical rat model of LPS+HI-induced neonatal encephalopathy, Savard et al. (2015) stressed the neurotoxic significance of IL-1 β and MMP-9 activation in immature brain injury. Evidence has emerged that an HI-induced inflammatory cascade crucially modifies BBB tight junction function (Chen et al. 2012; Diaz et al. 2016) and consequently white matter integrity (Hu et al. 2017). In response to acute hypoxic injury, here we have demonstrated increased apoptotic cell death in the parietal cortex, SVZ, and hippocampus, as expected. Although, we did not find any significant changes in expression of selected TJ, there was a significant increase of inflammatory cytokines in plasma and brain. These differences are mainly caused by different experimental procedures, with more extended brain lesions in ischemic (Chen et al. 2012; Diaz et al. 2016) or LPS-sensitized HI injury models (Savard et al. 2015) and different regeneration intervals. Chen et al. (2012) examined effects of ischemia and reperfusion on the regulation of TJ proteins in the foetal sheep brain. These authors observed a relatively rapid

regeneration of BBB functions shown by lower occludin and claudin-5 protein levels exclusively after a 4-h interval compared to non-ischemic controls, however, values are similar to the non-ischemic group 24 and 48 h after ischemia. Our data are in line with the observations of Diaz et al. (2016) who showed HI-induced decreased occludin protein levels at P22 and P60 (together with low levels of β -catenin and GFAP) in the hippocampus of neonatal rats after HI injury (Levine–Rice model). However, no differences were observed at P8, indicating the significance of maturational aspects of BBB vulnerability.

Recombinant C1-INH, which belongs to the group of serine protease inhibitors, is successfully used in children and adults with hereditary angioedema (Valerieva et al. 2018; Riedl et al. 2016). Physiologically activated during inflammatory processes, C1-INH is the only known inhibitor of the activated complement component 1 subcomponent r (C1r). (Valerieva et al. 2018). Consequently, incorporation into the C1qrs complex is prevented. Although the exact cerebral effector mechanisms are still unknown, growing evidence suggests that C1-INH may reduce inflammatory destruction of the BBB, injury-induced focal and diffuse brain oedema, and may additionally prevent secondary brain injury by antithrombotic effects. Heydenreich et al. (2012) showed a marked reduction of stroke volume and improved neurological outcome in response to C1-INH in an adult rodent stroke model, and observed lower degrees of BBB damage, oedema formation, inflammation, and strong antithrombotic effects in C1-INH-treated animals compared to controls. Due to their observations of significant reductions in C3b and TLR2 levels in an adult murine stroke model, Chen et al. (2018) posited the neuroprotective effects of high-dose C1-INH application through its effects on complement lytic activity and TLR signalling. Similarly, the neuroprotective effects of C1-INH have been shown in rodent models of traumatic brain injury (Albert-Weissenberger et al. 2014, Longhi

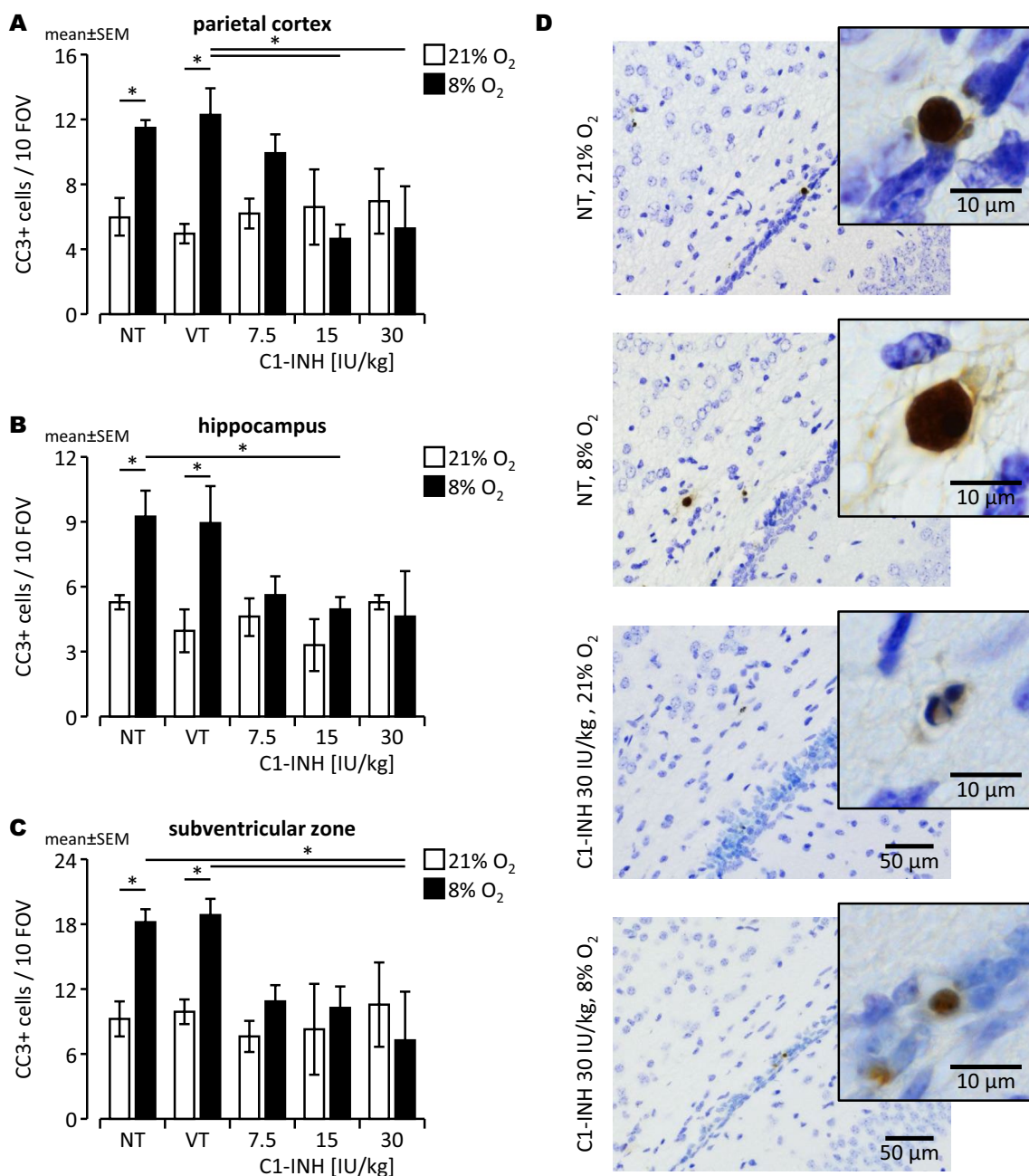


Fig. 5 Immunohistochemical analysis of the degree of apoptosis in normoxia- and hypoxia (8% O₂)-exposed brains of non-treated (NT), vehicle-treated (VT), and C1-INH-treated neonatal mice. **a–c** Quantification of CC3-positive cells in the parietal cortex, the hippocam-

pus, and subventricular zone (SVZ). $n=3$ per group; $*p<0.05$. **d** Representative photomicrographs of the subventricular zone. CC3+ (brown), Nissl (blue)

et al. 2009). However, therapy regimens markedly differed between these studies; e.g. in the rodent injury model, C1-INH dosage (150 IU/kg, Chen et al. 2018; 100 IU/kg, Tomasi et al. 2011; 20 IU/kg, Heydenreich et al. 2012) and time-point of treatment (60–90 min post injury, Heydenreich et al. 2012, De Simoni et al. 2003, Longhi et al. 2009; 3 h, Chen et al. 2018; up to 18 h post-ischemia, Gesuete et al. 2009). Recently, Chen et al. (2018) observed comparable

protective effects of combination therapy with low-dose intravenous immunoglobulin (IVIg) and C1-INH, administered either 3 h or 1 h after cerebral ischemia in adult mice. However, these authors used C1-INH at a dose of 150 IU/kg, whereas here, we used a lower C1-INH dose of 30 IU/kg i.p. in neonatal mice based on previous reports (Heydenreich et al. 2012; Albert-Weissenberger et al. 2014) and our own dose–effect studies, which confirmed a significant

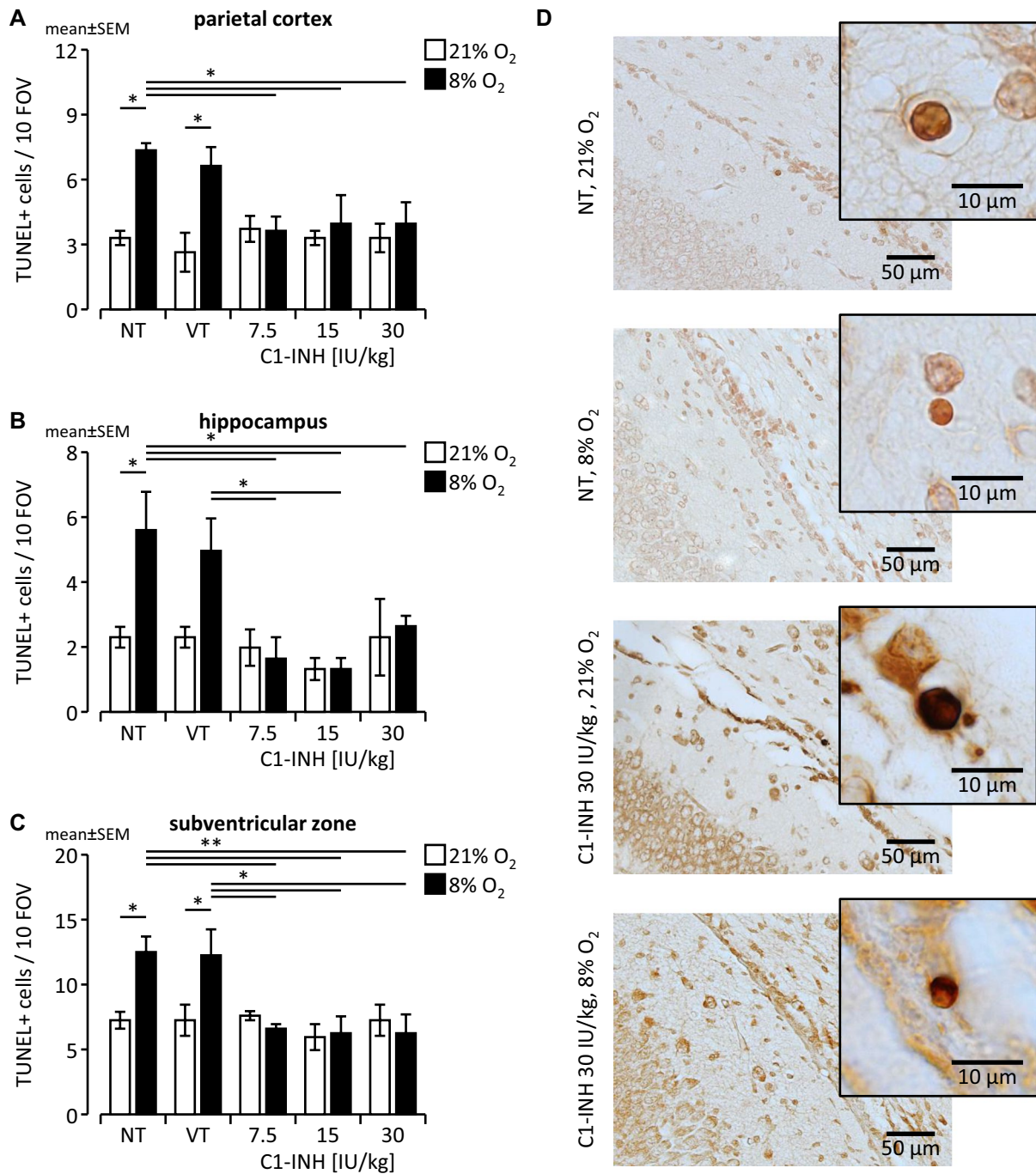


Fig. 6 Immunohistochemical analysis of the degree of apoptosis in normoxia- and hypoxia (8% O₂)-exposed brains of non-treated (NT), vehicle-treated (VT), and C1-INH-treated neonatal mice. **a–c** Quanti-

fication of TUNEL-positive cells in the parietal cortex, the hippocampus, and subventricular zone (SVZ), *n*=3 per group; **p*<0.05. **d** Representative photomicrographs of the SVZ

reduction in BBB leakage in response to low-dose C1-INH, as assessed by albumin and S100b protein plasma and tissue concentrations. As this is the first data to demonstrate the reduction of BBB breakdown and apoptosis due to C1-INH treatment in the hypoxic developing brain, there are no comparable treatment regimens in the literature. Due to the immature BBB functions in developing mice, we decided

to apply C1-INH at a physiological dose range (7.5–30 IU/kg) (Riedl et al. 2016). In line with our treatment regimen, Heydenreich et al. (2012), who found a significantly reduced infarct size in an adult rat stroke model (tMCAO), applied C1-INH at a dose of 20 IU/kg i.v. 90 min after ischemic insult. Studies on the optimal dosing of C1-INH in rodents (Heydenreich et al. 2012; Albert-Weissenberger et al. 2014)

were based on in vitro analyses measuring the activity of exogenous C1s in mouse plasma after adding C1-INH at different doses between 1 and 20 IU for every 1 mL of mouse plasma. Of note, the application of high doses of 7.5–15 IU per animal, as used in adult rodent stroke models (Chen et al. 2018; Tomasi et al. 2011), is technically not a practical method in neonatal mice because of the large injection volumes (e.g. 50 IU/mL, Berinert). In addition, side effects due to procoagulatory actions of high-dose C1-INH (> 100 IU/kg) have been demonstrated in an adult pig model of ischemic myocardium, whereas C1-INH at the dose of 20–40 IU/kg significantly protected ischemic tissue from reperfusion damage (Horstick et al. 2001).

C1-INH inhibited the hypoxia-induced activation of the pro-apoptotic factors BNIP3 and DUSP-1 (Bernaudin et al. 2002; Althaus et al. 2006) and counteracted the increase in CC3- and TUNEL-positive cells in the hypoxic neonatal mouse brain, indicating reduction of hypoxia-induced apoptosis. The anti-apoptotic effects of C1-INH are known from adult rodent stroke (Chen et al. 2018), transient ischemia–reperfusion injury (Storini et al. 2005), and traumatic brain injury models (Albert-Weissenberger et al. 2014); however, specific mechanisms of neuroprotective action in the immature brain are unknown. Anti-inflammatory effects, including diminished activation of microglia/macrophages (Storini et al. 2005), down-regulation of specific adhesion molecules (P-selectin, ICAM-1) (Storini et al. 2005), pro-inflammatory cytokines (TNF-alpha, IL-18), TLR-2 (Chen et al. 2018), as well as antithrombotic effects (Heydenreich et al. 2012) have been suggested from studies on adult rodent models of transient ischemia–reperfusion brain injury (Storini et al. 2005) and ischemic stroke (Chen et al. 2018). C1-INH increased the mRNA levels of the protective cytokines IL-2 and IL-10 (Storini et al. 2005) and prevented the increase of complement lytic activity (C3b) (Chen et al. 2018). Here, we demonstrated the significant reduction of cerebral mRNA concentrations (IL-1 β , TNF-alpha) as well as plasma protein levels of pro-inflammatory cytokines (IL-1 β , TNF-alpha, IL-6) in response to C1-INH in hypoxic brain injury indicating the significance of anti-inflammatory effects of C1-INH in the developing brain. Furthermore, we observed protective effects of C1-INH on the expression of HI inducible pro-apoptotic factors BNIP3/DUSP1, the degree of apoptosis, as well as the modulation of occludin expression at a very early stage of brain development. The TJ protein occludin is crucially involved in the maturation of BBB functions; however, diminished regulatory functions and cell type-specific responses in the neonatal rodent brain have been demonstrated (Diaz et al. 2016; Engelhardt et al. 2014, 2015). This might be due to immaturity of the BBB, and could explain our observations of missing effects of hypoxia on occludin, ZO-1, claudin-1, and claudin-5 mRNA expression. Of note, recently the close

relationship between the TLR4/PKC α /occludin signalling pathway and BBB damage has been shown in vitro in a brain microvascular endothelial cell (BMEC) model (Tang et al. 2018). Thus, our ongoing research will focus on further understanding the effects of C1-INH on TJ regulation in the developing brain.

As this was the first in vivo study of C1-INH effects at a very early stage of brain development, we are aware of the limitations of the present study, e.g. the region- and cell-specific analyses and additional functional studies at the protein level that will be in the scope of our future work. Extending the present knowledge on the molecular mechanisms underlying reduced BBB damage in response to C1-INH provides basic information for future development of novel neuroprotective options such as a combinatory treatment with C1-INH and therapeutic hypothermia during the immediate post-hypoxic period.

Conclusions

Our results demonstrate for the first time that C1-INH has the potential to prevent hypoxia-induced BBB leakage and to reduce apoptotic cell death in the neonatal mouse brain at the early developmental stage. In addition, C1-INH treatment seemed to modulate occludin expression in the developing mouse brain. Further cell type-specific and protein analyses are necessary to confirm the significance of C1-INH as a promising target for neuroprotective therapy in hypoxic-ischemic neonatal brain injury.

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Compliance with Ethical Standards

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

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