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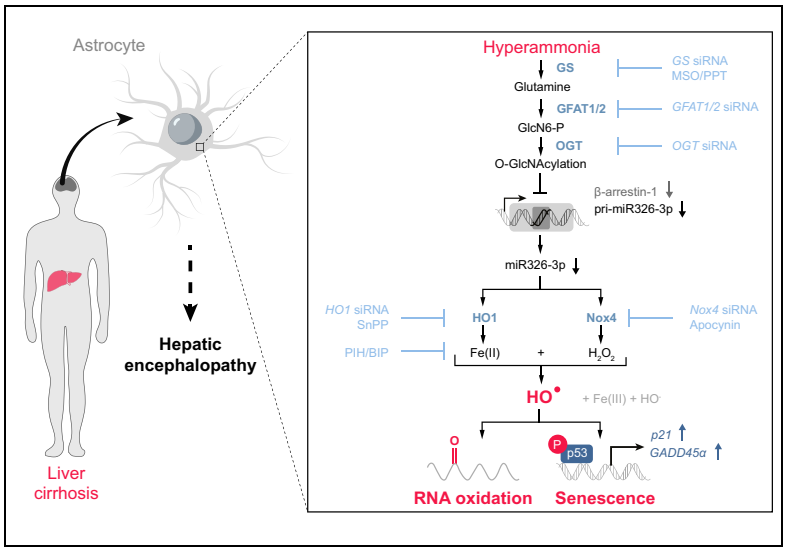
O-GlcNAcylation-dependent upregulation of HO1 triggers ammonia-induced oxidative stress and senescence in hepatic encephalopathy

Graphical abstract



Highlights

- Ammonia triggers protein O-GlcNAcylation in astrocytes via synthesis of GlcN.
- O-GlcNAc-dependent upregulation of HO1 + Nox4 induces RNA oxidation and senescence.
- Protein O-GlcNAcylation is increased in the brains of cirrhotic patients with HE.

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Lay summary

Patients with liver cirrhosis frequently exhibit hyperammonemia and suffer from cognitive and motoric dysfunctions, which at least in part involve premature ageing of the astrocytes in the brain. This study identifies glucosamine and an O-GlcNAcylation-dependent disruption of iron homeostasis as novel triggers of oxidative stress, thereby mediating ammonia toxicity in the brain.

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O-GlcNAcylation-dependent upregulation of HO1 triggers ammonia-induced oxidative stress and senescence in hepatic encephalopathy

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Background & Aims: Cerebral oxidative stress plays an important role in the pathogenesis of hepatic encephalopathy (HE), but the underlying mechanisms are incompletely understood. Herein, we analyzed a role of heme oxygenase (HO)1, iron and NADPH oxidase 4 (Nox4) for the induction of oxidative stress and senescence in HE.

Methods: Gene and protein expression in human *post-mortem* brain samples was analyzed by gene array and western blot analysis. Mechanisms and functional consequences of HO1 upregulation were studied in NH_4Cl-exposed astrocytes *in vitro* by western blot, qPCR and super-resolution microscopy.

Results: HO1 and the endoplasmic reticulum (ER) stress marker grp78 were upregulated, together with changes in the expression of multiple iron metabolism-related genes, in *post-mortem* brain samples from patients with liver cirrhosis and HE. NH_4Cl elevated HO1 protein and mRNA in cultured astrocytes through glutamine synthetase (GS)-dependent upregulation of glutamine/fructose amidotransferases 1/2 (GFAT1/2), which blocked the transcription of the HO1-targeting miR326-3p in a O-GlcNAcylation dependent manner. Upregulation of HO1 by NH_4Cl triggered ER stress and was associated with elevated levels of free ferrous iron and expression changes in iron metabolism-related genes, which were largely abolished after knockdown or inhibition of GS, GFAT1/2, HO1 or iron chelation. NH_4Cl, glucosamine (GlcN) and inhibition of miR326-3p upregulated Nox4, while knockdown of Nox4, GS, GFAT1/2, HO1 or iron chelation prevented NH_4Cl-induced RNA oxidation and astrocyte senescence. Elevated levels of grp78 and O-GlcNAcylated proteins were also found in brain samples from patients with liver cirrhosis and HE.

Conclusion: The present study identified glucosamine synthesis-dependent protein O-GlcNAcylation as a novel mechanism in the pathogenesis of HE that triggers oxidative and ER stress, as well as senescence, through upregulation of HO1 and Nox4.

Lay summary: Patients with liver cirrhosis frequently exhibit hyperammonemia and suffer from cognitive and motoric dysfunctions, which at least in part involve premature ageing of the astrocytes in the brain. This study identifies glucosamine and an O-GlcNAcylation-dependent disruption of iron homeostasis as novel triggers of oxidative stress, thereby mediating ammonia toxicity in the brain.

Introduction

Ammonia is a major toxin in the pathogenesis of hepatic encephalopathy (HE).[1] In the brain, ammonia is detoxified by glutamine synthetase (GS) in astrocytes and glutamine accumulation in astrocytes in patients with liver cirrhosis and HE triggers the development of a low grade cerebral edema[2] associated with oxidative/nitrosative stress.[3,4]

Several *in vitro* and *in vivo* studies demonstrated that ammonia induces the formation of reactive oxygen species (ROS) in astrocytes,[1] which triggers a variety of functional consequences, such as gene expression changes, protein tyrosine nitration, RNA oxidation and senescence.[1,5,6] Of note, these functional consequences were also identified in *post-mortem* brain samples from patients with liver cirrhosis and HE, but not in those without HE.[3,4,6] Of particular interest, RNA oxidation was suggested to disturb postsynaptic protein synthesis which is required for memory formation in HE.[5] Astrocyte senescence was proposed to destabilize synaptic connections, thereby explaining the clinical observation that cognitive impairment in patients with liver cirrhosis can persist after resolution of an acute episode of overt HE.[6,7] In line with this, a recent study showed that astrocyte senescence initiates and promotes cognitive decline in a mouse model of Alzheimer's disease.[8] Overall, this evidence strongly supports an important role of oxidative stress in the pathogenesis of HE, but the underlying mechanism remains poorly understood.

Oxidative stress in ammonia-exposed astrocytes and in brains from animal models of HE, or patients with liver cirrhosis and HE, is associated with an upregulation of heme oxygenase-1 (HO1).[3,9] While HO1 was shown to protect from oxidative stress in some brain pathologies such as traumatic brain injury,[10] its role in the pathogenesis of HE remains unclear. Likewise, the mechanisms underlying upregulation of HO1 by ammonia are

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only incompletely understood but may involve downregulation of HO1-targeting microRNAs.[11] Upregulation of HO1 may also be triggered by endoplasmic reticulum (ER) stress[12] but whether upregulation of HO1 by ammonia in astrocytes is a consequence of ER stress is not known.

Another functional consequence in ammonia-exposed astrocytes is the glutamine synthesis-dependent O-GlcNAcylation of individual proteins.[13] This post-translational modification depends on the synthesis of activated sugar nucleotides within the hexosamine biosynthetic pathway and the conversion of glutamine and fructose-6-phosphate to glucosamine-6-phosphate (GlcN-6-P) by GFAT1/2 is rate-controlling. Similar to phosphorylation, O-GlcNAcylation can modulate the function of many proteins in specific ways.[14] Unfortunately, the functional consequences of the ammonia-induced protein O-GlcNAcylation in astrocytes remained completely unknown.[13]

The aim of the present study was to analyze the mechanisms underlying the ammonia-induced upregulation of HO1 and its role in astrocytes. Here we identified the O-GlcNAcylation-dependent upregulation of HO1 and Nox4 as a novel mechanism that triggers ammonia-induced RNA oxidation and senescence in astrocytes.

Material and methods

Materials

A detailed list of all materials can be found in the supplementary CTAT table.

Preparation, culture and experimental treatment of rat brain astrocytes and rat and brain tissue sampling

Detailed information on the culture, treatment and viability of the astrocytes is provided in the supplementary information and in Fig. S1. Tissue sampling was approved by the responsible local authorities (Office for Nature, Environment and Consumer Protection, North Rhine-Westphalia and the Animal Facility of the Central Unit for Animal Research and Animal Welfare Affairs of the University of Düsseldorf, Germany).

Whole genome microarray

Gene expression levels were analyzed in *post-mortem* brain tissue in 2 former studies as described before[3,15] and in the supplement.

Western blot, qPCR, miQPCR, immunofluorescence analysis and semi-quantitative measurement of proliferation

Detailed information on measurement of protein, mRNA and miRNA levels and astrocyte proliferation is provided in the supplement and in.[6,11]

Detection of free ferrous iron and staining of cell organelles in vital astrocytes

For detecting free ferrous iron, vital astrocytes were stained with the fluorescent iron chelators rhodamine B-[(2,2′-bipyridine-4-yl)-aminocarbonyl]benzyl ester (RDA) or RhoNox™-1. Specific detection of ferrous iron by RDA and RhoNox™-1 in astrocytes was validated by incubating the astrocytes with $Fe(II)Cl_2$ (Fig. S2).

Analysis of results

Experiments were carried out with the indicated number of independent preparations or samples. Values are given as means ± SEM. Statistical testing was performed as indicated in the respective legends to figures and tables. A p value ≤0.05 was considered significant.

Results

Expression levels of HO1 and iron-related genes in *post-mortem* brain samples from patients with liver cirrhosis with or without HE

Gene expression levels were analyzed by microarray in *post-mortem* brain tissue from 2 independent patient cohorts. As shown in Fig. 1, mRNA levels of *HO1* were significantly higher in parallel with multiple expression changes of genes related to iron metabolism in *post-mortem* brain tissue from patients with liver cirrhosis and HE in a European (Fig. 1A) and an Australian (Fig. 1B) cohort. Importantly, mRNA levels of heavy and light subunits of ferritin were elevated in patients with liver cirrhosis and HE in both cohorts. Elevated expression levels of ferritin light chain positively correlated with *HO1* mRNA levels in patients with liver cirrhosis and HE (Spearmann's correlation: Australian cohort: $r^2 = 0.984$, $p = 0.016$; European cohort: $r^2 = 0.709$, $p = 0.049$). Such changes were not observed in *post-mortem* brain samples from patients with liver cirrhosis without HE (Fig. 1A, B).

These data are indicative for altered iron homeostasis in the brain of patients with liver cirrhosis and HE.

Characterization of the ammonia-induced upregulation of HO1 in rat astrocytes *in vitro*

Time- and concentration-dependent effects of ammonia on HO1 protein and mRNA expression levels were analyzed in astrocytes *in vitro*. As shown in Fig. 2A, HO1 protein levels were elevated in astrocytes exposed to NH_4Cl (5 mM) for 24 h and were still increased after 72 h. Exposure of astrocytes to NH_4Cl also upregulated *HO1* mRNA levels in a concentration-dependent way (Fig. S3A). *HO1* mRNA levels were significantly elevated in astrocytes exposed to 2.5 and 5 mM NH_4Cl for 72 h. Exposure of astrocytes to 0.5 and 1 mM NH_4Cl transiently elevated *HO1* mRNA levels at 24 h, but not at 72 h (Fig. S3A). Upregulation of HO1 protein (Fig. 2A) and mRNA (Fig. S3B) by NH_4Cl (5 mM) was largely abolished after siRNA-mediated knockdown of GS. Like NH_4Cl, also *L*-Gln (10 mM, 72 h) upregulated HO1 protein (Fig. S3B). These results suggest that ammonia upregulates HO1 in a glutamine synthesis-dependent way.

As glutamine is a substrate for glucosamine-6-phosphate (GlcN-6-P) synthesis by GFAT1/2, we analyzed levels of GlcN-6-P, GFAT1/2 protein and mRNA in NH_4Cl (5 mM)-exposed astrocytes. GlcN-6-P levels in NH_4Cl (5 mM, 48 h)-exposed astrocytes significantly increased to 1.72 ± 0.16-fold of untreated controls (n = 3, Student's t test, $p < 0.05$). Both GFAT isoforms were significantly upregulated at the mRNA and protein level by NH_4Cl (5 mM) after 48–72 h (Fig. 2B, Figs. S3A, C). Already 1 mM NH_4Cl transiently raised *GFAT1/2* mRNA levels after 24 h (Fig. S3A).

Upregulation of *GFAT1/2* mRNA by NH_4Cl (5 mM) was prevented by knockdown or inhibition of GS using siRNA or MSO (3 mM), respectively (Fig. S3C). siRNA-mediated knockdown of GFAT1/2 significantly decreased HO1 protein (Fig. 2B) and mRNA (Fig. S3B) levels in untreated astrocytes and prevented the NH_4Cl (5 mM)-induced upregulation of HO1.

GlcN (8 mM, 72 h), which is intracellularly converted to GlcN-6-P by the low-affinity hexokinase glucokinase

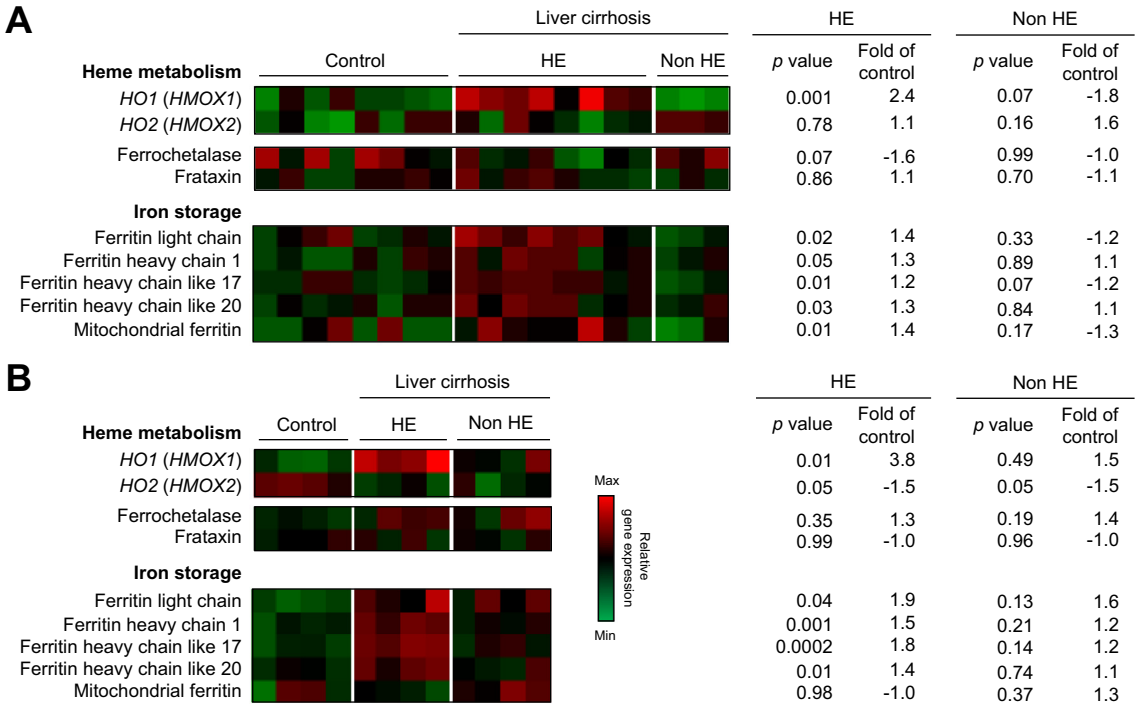


Fig. 1. Expression levels of HO1 and genes related to iron metabolism in *post-mortem* brain samples from patients with liver cirrhosis with or without HE. mRNA was prepared from cerebrocortical human brain samples from a (A) European or an (B) Australian cohort. mRNA levels were measured by microarray and are color-coded with red indicating higher and with green indicating lower individual mRNA levels compared to the median level of the respective gene. For each gene the *p*-value and fold-change in patients with liver cirrhosis with or without HE compared to controls is presented. (one-way ANOVA with Dunnett's (A) or Tukey's (B) *post hoc* test). Gene expression levels of *HO1* in (A) reproduced with permission from ([3], John Wiley & Sons, © 2013 by the American Association for the Study of Liver Diseases).

(K_m = 8 mM),[16] strongly upregulated *HO1* mRNA and protein in astrocytes (Figs. S3B, D). While NH_4Cl (5 mM) and GlcN (8 mM) elevated HO1 protein to similar extend, HO1 levels in astrocytes exposed to NH_4Cl + GlcN were not significantly higher when compared to astrocytes exposed to NH_4Cl or GlcN only (Fig. S3B).

These findings indicate that ammonia elevates HO1 protein and mRNA through glutamine synthesis-dependent upregulation of GFAT1/2 and synthesis of GlcN-6-P.

HO1 induction by ammonia is blocked by taurine via an unknown mechanism.[11] We therefore investigated the effects of taurine on the NH_4Cl-induced upregulation of GFAT1/2. As shown in Figs. S3B, C, taurine (5 mM) prevented both, the ammonia-induced upregulation of *HO1* as well as of *GFAT1/2* mRNA.

Role of endoplasmic reticulum stress for ammonia-induced upregulation of HO1 in rat astrocytes *in vitro*

Upregulation of HO1 has been suggested as a consequence of the unfolded protein response which is triggered by ER stress.[12] Whether upregulation of HO1 is the cause or a consequence of ammonia-induced ER stress in astrocytes was investigated by analyzing grp78 protein and mRNA levels by Western blot and qPCR, respectively.

NH_4Cl (5 mM) significantly upregulated grp78 protein after 48 and 72 h and *grp78* mRNA after 72 h (Fig. 2C; Fig. S3E). Already 1 mM NH_4Cl transiently elevated *grp78* mRNA levels in astrocytes after 24 h (Fig. S3A). Knockdown of *GS, GFAT1/2* or *HO1* by siRNA strongly inhibited the NH_4Cl (5 mM)-induced upregulation of grp78 protein and mRNA (Fig. 2C; Fig. S3E).

Since taurine prevented the ammonia-induced upregulation of *HO1* mRNA (Fig. S3B) we investigated effects of taurine on *grp78* mRNA levels in NH_4Cl-exposed astrocytes. As shown in Fig. S3E, upregulation of *grp78* mRNA by NH_4Cl (5 mM) in astrocytes was prevented by taurine.

Also GlcN (8 mM, 72 h), which upregulates HO1 in cultured astrocytes, elevated *grp78* mRNA (Fig. S3D).

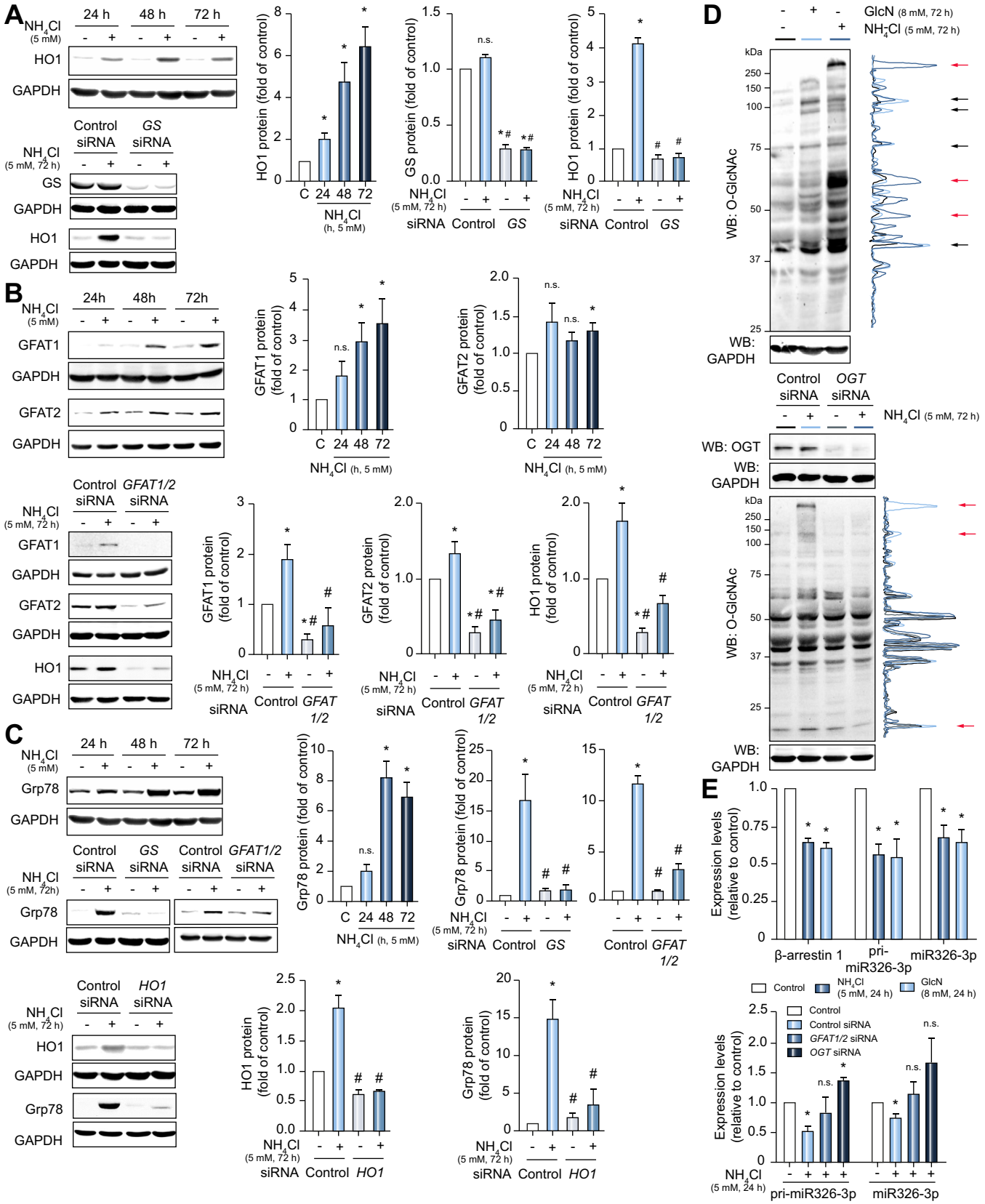
These data suggest that the glutamine and GlcN-6-P synthesis-dependent upregulation of HO1 is not a consequence but rather a trigger of ammonia-induced ER stress in astrocytes.

Ammonia inhibits the transcription of the HO1-targeting miR326-3p in an O-GlcNAcylation-dependent way

The glutamine-dependent synthesis of GlcN-6-P by GFAT1/2 determines intracellular levels of activated sugar nucleotides which are utilized by O-GlcNAc transferase (OGT) for the O-GlcNAcylation of individual proteins.[14] As ammonia triggers glutamine synthesis-dependent protein O-GlcNAcylation[13] and downregulates the HO1-targeting miR326-3p,[11] we investigated a role of GFAT-dependent GlcN-6-P synthesis and protein O-GlcNAcylation for downregulation of miR326-3p in ammonia-exposed astrocytes.

As shown in Fig. 2D, GlcN (8 mM, 72 h) and NH_4Cl (5 mM, 72 h) triggered the O-GlcNAcylation of individual proteins in astrocytes and the NH_4Cl-induced O-GlcNAcylation was prevented by siRNA-mediated knockdown of OGT. While distinct proteins were O-GlcNAcylated by GlcN and NH_4Cl, others were only O-GlcNAcylated by NH_4Cl.

Both, NH_4Cl (5 mM) and GlcN (8 mM) also downregulated β-arrestin 1 mRNA and the miR326-3p primary transcript


A
B
C
D
E
HO1
GAPDH
GS
GFAT1
GFAT2
Grp78
Control siRNA
GS siRNA
GFAT1/2 siRNA
HO1 siRNA
OGT siRNA
WB: O-GlcNAc
WB: OGT
GlcN (8 mM, 72 h)
β-arrestin 1
pri-miR326-3p
miR326-3p
pri-miR326-3p
Control
Control siRNA
GFAT1/2 siRNA
OGT siRNA
n.s.

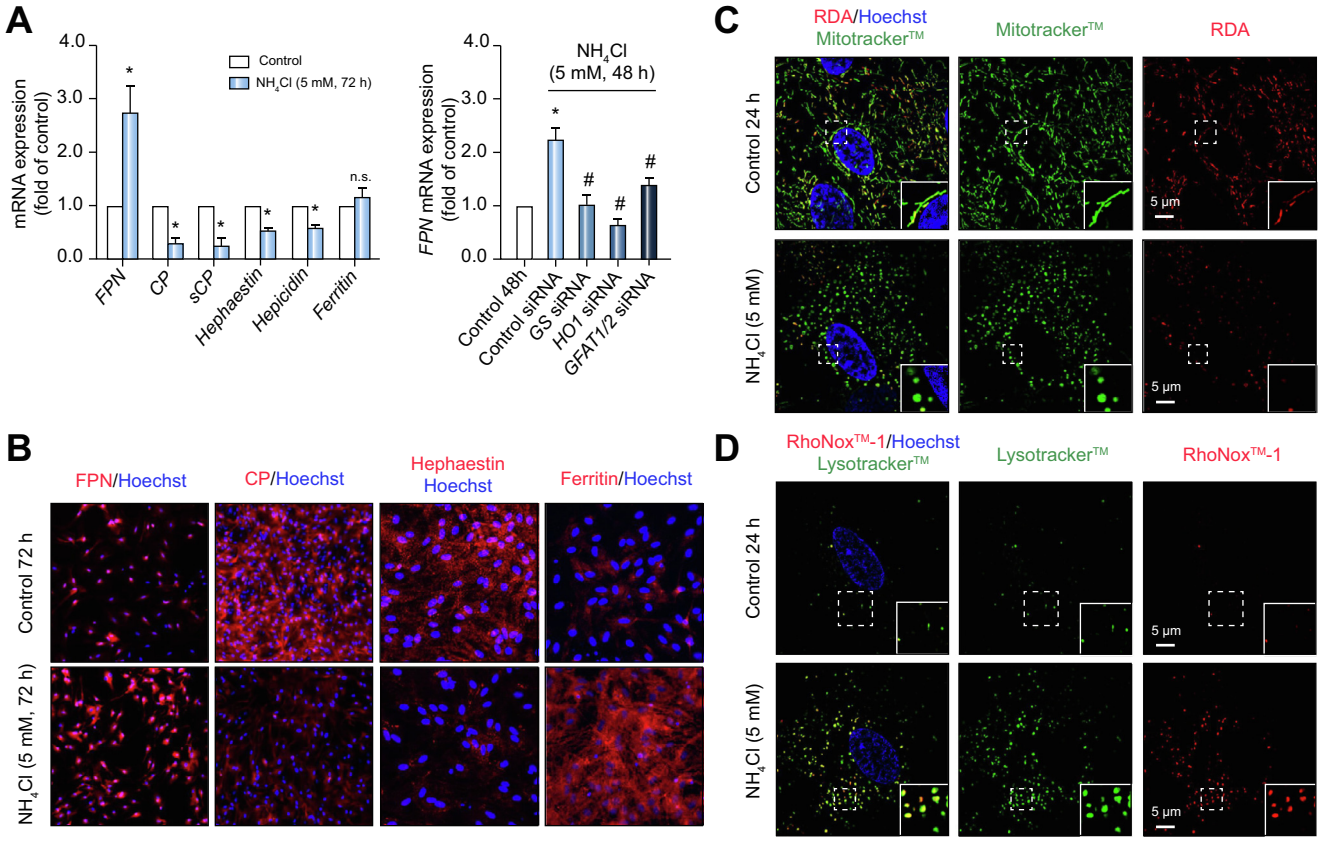


Fig. 3. Effects of NH_4Cl on expression levels of genes and proteins related to iron metabolism and intracellular levels of free ferrous iron. Astrocytes were either exposed to NH_4Cl (5 mM) or were left untreated (control) for the indicated time. Where indicated, astrocytes were exposed to the indicated siRNA. (A) Quantification of *FPN*, *CP*, *sCP*, hephaestin, hepcidin and ferritin mRNA by qPCR. mRNA levels are given relative to the respective control (untreated or siRNA without NH_4Cl). *Significantly different compared to control. #Significantly different compared to control siRNA + NH_4Cl. (n = 3–4 independent experiments; 3A left panel: Student's *t* test; 3B right panel: one-way ANOVA, Tukey's *post hoc* test). (B) Immunofluorescence analysis of FPN, CP, hephaestin and ferritin. Nuclei were counterstained using Hoechst34580. (C, D) Detection of free ferrous iron in vital astrocytes by the fluorescent iron chelators RDA or RhoNox™-1 and super-resolution microscopy. Mitochondria, lysosomes and nuclei were costained using Mitotracker™ Green, LysoTracker™ Green and Hoechst34580, respectively.

pri-miR326-3p which is co-transcribed with β-arrestin 1, as well as mature miR326-3p (Fig. 2E). Knockdown of *GFAT1/2* or *OGT* fully prevented the NH_4Cl-induced downregulation of pri-miR326-3p and miR326-3p (Fig. 2E).

These data indicate that ammonia inhibits the transcription of the *HO1* mRNA-targeting miR326-3p in a way that depends on GlcN-6-P synthesis and protein O-GlcNAcylation.

Effects of ammonia on iron homeostasis in cultured rat astrocytes

The effects of ammonia-induced upregulation of HO1 for intracellular iron homeostasis were analyzed by measuring expression of genes and proteins related to iron export and storage.

While mRNA and protein levels of the iron exporter ferroportin (FPN) were significantly elevated, the expression of the FPN accessory factors CP, sCP and hephaestin was downregulated by NH_4Cl (5 mM, 72 h; Fig. 3A, B; Table S3). Similar effects were observed, when astrocytes were exposed to GlcN (8 mM, 72 h; Fig. S4A). Upregulation of *FPN* mRNA in NH_4Cl-exposed astrocytes was prevented by siRNA-mediated knockdown of GS, GFAT1/2 or HO1 (Fig. 3A). Furthermore, the effects of NH_4Cl on mRNA levels of *FPN*, *CP*, *sCP*, hephaestin and hepcidin were abolished by the GS inhibitors MSO (3 mM) and PPT (100 µM), by the iron chelators PIH (100 µM) and BIP (150 µM) and by taurine (5 mM) (Figs. S4B–D). While protein levels were significantly increased (Fig. 3B; Table S3), mRNA levels of the

Fig. 2. Molecular mechanisms underlying the ammonia-induced upregulation of HO1 in cultured rat astrocytes. Astrocytes were either treated with NH_4Cl (5 mM), GlcN (8 mM) or were left untreated (control, "C") for the indicated time. (A-C) Effects of NH_4Cl on GS, GFAT1/2, HO1 or grp78 protein expression were analyzed by Western blot. (A, B) Effects of siRNA-mediated knockdown of GS or GFAT1/2 on GS and HO1 or GFAT1/2 and HO1 protein expression, respectively. (C) Role of GS, GFAT1/2 and HO1 for upregulation of grp78 by NH_4Cl. Protein levels were quantified densitometrically and are given relative to respective controls (untreated, control siRNA). (D) Effects of NH_4Cl and GlcN on protein O-GlcNAcylation and role of OGT for ammonia-induced O-GlcNAcylation in astrocytes. O-GlcNAc immunoreactivities in the respective samples are plotted in a dendrogram. Red and black arrows indicate proteins O-GlcNAcylated in astrocytes by NH_4Cl only or by NH_4Cl and GlcN, respectively. (E) Effects of NH_4Cl and GlcN on β-arrestin 1 mRNA and pri-miR326-3p and miR326-3p levels and role of GlcN-6-P synthesis and protein O-GlcNAcylation for NH_4Cl-induced downregulation of pri-miR326-3p and miR326-3p. mRNA/miRNA expression levels in NH_4Cl-exposed astrocytes are given relative to the respective control (untreated, siRNA without NH_4Cl). *Significantly different to the respective control. #Significantly different compared to control siRNA + NH_4Cl. (n = 3–8 independent experiments; 2A–C time-dependency: Student's *t* test, other panels: one-way ANOVA, Tukey's *post hoc* test; 2E upper panel: one-way ANOVA, Dunnet's *post hoc* test; Fig. 2E lower panel: Student's *t* test against the respective siRNA control).

endogenous iron chelator ferritin remained unchanged (Fig. 3A) in NH_4Cl-exposed astrocytes.

While mRNA and protein levels of FPN were also upregulated by 0.5 mM NH_4Cl, CP, hephaestin and ferritin mRNA and protein levels remained unchanged (Figs. S4E, F; Table S3). However, nuclear ferritin expression significantly increased in astrocytes exposed to 0.5 mM NH_4Cl for 72 h to 1.22 ± 0.09-fold of control (Student's *t* test, n = 8) (Fig. S4F).

These findings demonstrate that ammonia concentration dependently changed the expression of genes and proteins related to iron homeostasis in astrocytes in a glutamine and GlcN-6-P synthesis, HO1 and iron-dependent way.

Upregulation of FPN and ferritin are known to reflect elevated cytosolic levels of free iron. In line with this, ferritin immunoreactivity and *FPN* mRNA were strongly enhanced after induction of an experimental iron overload in astrocytes (Fig. S4G).

We therefore analyzed effects of 0.5 and 5 mM NH_4Cl on intracellular levels of free ferrous iron by super-resolution structured illumination microscopy and epifluorescence microscopy using the fluorescent ferrous iron chelators RDA and RhoNox™-1. RDA fluorescence colocalized with Mitotracker® Green and RhoNox™-1 fluorescence overlapped with LysoTracker™ Green (Fig. 3C, D; Fig. S5A). Upon chelation of ferrous iron, RDA fluorescence decreases, whereas RhoNox™-1 fluorescence increases.

While RDA fluorescence intensity was reduced, RhoNox™-1 fluorescence was enhanced by NH_4Cl in a time and concentration-dependent manner (Fig. 3C, D; Figs. S5B, C; Table S4).

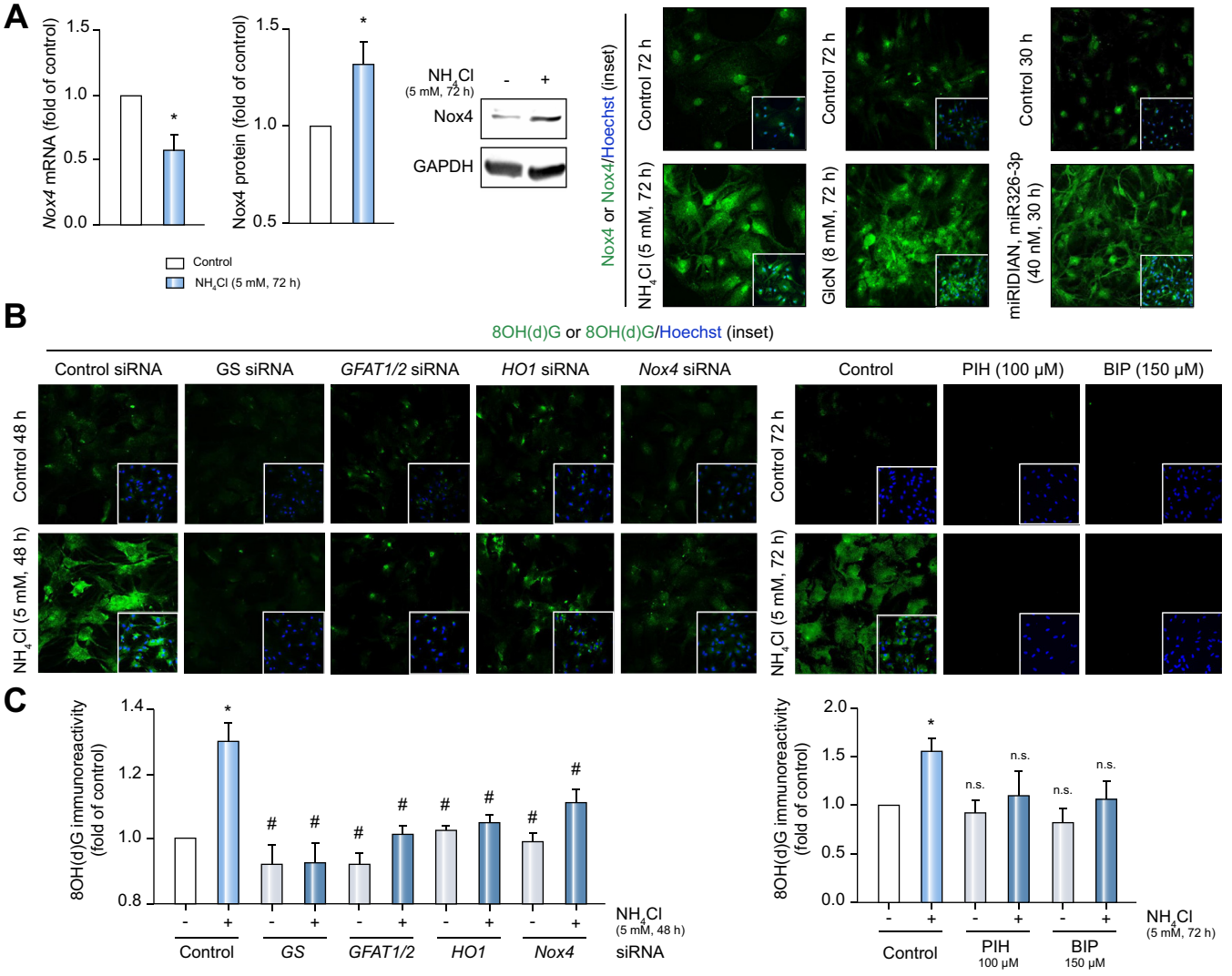


Fig. 4. Role of HO1 and Nox4 for ammonia-induced RNA oxidation in cultured rat astrocytes. Astrocytes were exposed to NH_4Cl (5 mM), GlcN (8 mM), miRIDIAN miR326-3p inhibitor or were left untreated (control). Where indicated, astrocytes were exposed to the indicated siRNA, PIH (100 µM) or BIP (150 µM). (A) Effects of NH_4Cl and GlcN and miR326-3p inhibition on Nox4 expression as measured by immunofluorescence and Western blot analysis or qPCR. (B) Role of GS, GFAT1/2, HO1, Nox4 and free iron for NH_4Cl-induced RNA oxidation. Detection of 8OH(d)G by immunofluorescence analysis. Nuclei were counterstained using Hoechst34580. (C) Quantification of anti-8OH(d)G immunoreactivity. *:Significantly different compared to the respective control (untreated or siRNA without NH_4Cl). #Significantly different compared to control siRNA + NH_4Cl (left graph). (n = 3–9 independent experiments; A: Student's *t* test; C: one-way ANOVA, Tukey's (left panel) or Dunnet's *post hoc* test (right panel)).

These findings suggest that ammonia enhances intracellular levels of free ferrous iron in astrocytes.

Role of HO1 for ammonia-induced oxidative stress

Ammonia induces RNA oxidation (8OHG) in cultured rat astrocytes in a NADPH oxidase-dependent way.[5] RNA is especially vulnerable to oxidation by hydroxyl radical anion which is formed in the so-called Fenton reaction in the presence of ferrous iron and H_2O_2 and therefore is considered a surrogate marker for oxidative stress.[17] As Nox4 is the only Nox isoform which produces H_2O_2,[18] we analyzed effects of ammonia and GlcN on Nox4 expression in astrocytes.

Although NH_4Cl (5 mM, 72 h) downregulated *Nox4* mRNA, Nox4 protein levels increased as shown by immunofluorescence and Western blot analysis (Fig. 4A). A strong increase in Nox4 immunoreactivity was already noted in astrocytes exposed to 0.5 mM NH_4Cl (Fig. S6). Also treating the astrocytes with GlcN (8 mM) or inhibiting miR326-3p, which is predicted to target *Nox4* mRNA (Table S5), upregulated Nox4 immunoreactivity in the astrocytes (Fig. 4A).

To further analyze whether the ammonia-induced upregulation of HO1 and Nox4 are triggers for ammonia-induced RNA oxidation in the astrocytes, we performed siRNA experiments. As shown in Fig. 4B, C and Fig. S6, exposure to NH_4Cl (0.5 or 5 mM) strongly increased cytosolic 8OH(d)G immunoreactivity in the astrocytes, which is indicative of RNA oxidation (8OHG). The NH_4Cl (5 mM)-induced increase of 8OH(d)G immunoreactivity in astrocytes was completely prevented by siRNA-mediated knockdown of GS, GFAT1/2, HO1, Nox4 or by iron chelation using PIH (100 μM) or BIP (150 μM) (Fig. 4B, C).

These findings demonstrate that ammonia induces RNA oxidation in cultured astrocytes in a glutamine and glucosamine synthesis-dependent way by upregulating HO1, free iron and Nox4-derived H_2O_2.

Role of HO1 and Nox4 for ammonia-induced senescence

The ammonia-induced ROS formation triggers senescence in astrocytes by activating the p53-dependent transcription of the cell cycle inhibitory genes *p21* and *GADD45α*.[6] We therefore investigated, whether upregulation of HO1 and Nox4 activates p53 and elevates *p21* and *GADD45α* mRNA levels.

As shown by immunofluorescence analysis in Fig. 5A-C, knockdown of GS, GFAT1/2, HO1 or Nox4 or iron chelation by PIH (100 μM) or BIP (150 μM) inhibited serine^{392}-phosphorylation of p53 and nuclear accumulation of p53 and p21 in NH_4Cl (5 mM, 72 h)-exposed astrocytes. Serine^{392} phosphorylation of p53 and nuclear accumulation of serine^{392}-phosphorylated p53 and p21 were also observed in $Fe(II)Cl_2$ (25 μM, 18 h)-exposed astrocytes (Fig. S7). Upregulation of *GADD45α* mRNA by NH_4Cl was significantly inhibited after knockdown of GS, GFAT1/2, HO1 or Nox4 (Fig. 5C). GS inhibition by MSO (3 mM) or PPT (100 μM) or iron chelation using PIH (100 μM) or BIP (150 μM) prevented upregulation of *GADD45α* and *p21* mRNA in NH_4Cl-exposed astrocytes (Fig. S8). Similar to NH_4Cl, also GlcN (8 mM, 72 h) and $Fe(II)Cl_2$ but not the carbon monoxide (CO) donor CORM-A1 (25 μM) or biliverdin (1 μM) upregulated *GADD45α* and *p21* mRNA levels in astrocytes (Fig. 5E).

The effects of the NH_4Cl-induced upregulation of *p21* and *GADD45α* on astrocyte proliferation were measured by fluorimetric quantification of DNA content. In line with earlier findings,[6] astrocyte proliferation was significantly inhibited by NH_4Cl (5 mM) and this effect was fully prevented by knockdown of GS, GFAT1/2, HO1 or Nox4, iron chelation by PIH (100 μM) or BIP (150 μM) or HO1 inhibition by SnPP (10 μM) (Fig. 5D).

Also exposure of astrocytes to GlcN (8 mM, 120 h), $Fe(II)Cl_2$ (25 μM, 18 h) or to hemin (1 and 5 μM, 72 h) inhibited astrocyte proliferation. Importantly, the hemin-induced proliferation inhibition was prevented by the iron chelator PIH (100 μM) indicating that the release of ferrous iron from heme inhibits astrocyte proliferation (Fig. 5F).

The data suggest that ammonia inhibits astrocyte proliferation through p53-dependent transcription of the cell cycle inhibitory factors p21 and GADD45α and nuclear accumulation of p21. This pathway becomes activated by ammonia in a way that depends on glutamine and GlcN-6-P synthesis, HO1-dependent release of ferrous iron and Nox4-derived H_2O_2.

Expression levels of grp78 and O-GlcNAcylated proteins in *post-mortem* brain samples from patients with liver cirrhosis with and without HE

A potential relevance of ammonia-induced ER stress and O-GlcNAcylation for the pathogenesis of HE was investigated in *post-mortem* brain samples from patients with liver cirrhosis with and without HE by western blot analysis.

As shown in Fig. 6A, protein levels of the ER stress marker grp78 tended to be increased in brain tissue from patients with liver cirrhosis without HE ($p = 0.13$), but were significantly ($p < 0.05$) upregulated in patients with liver cirrhosis and HE.

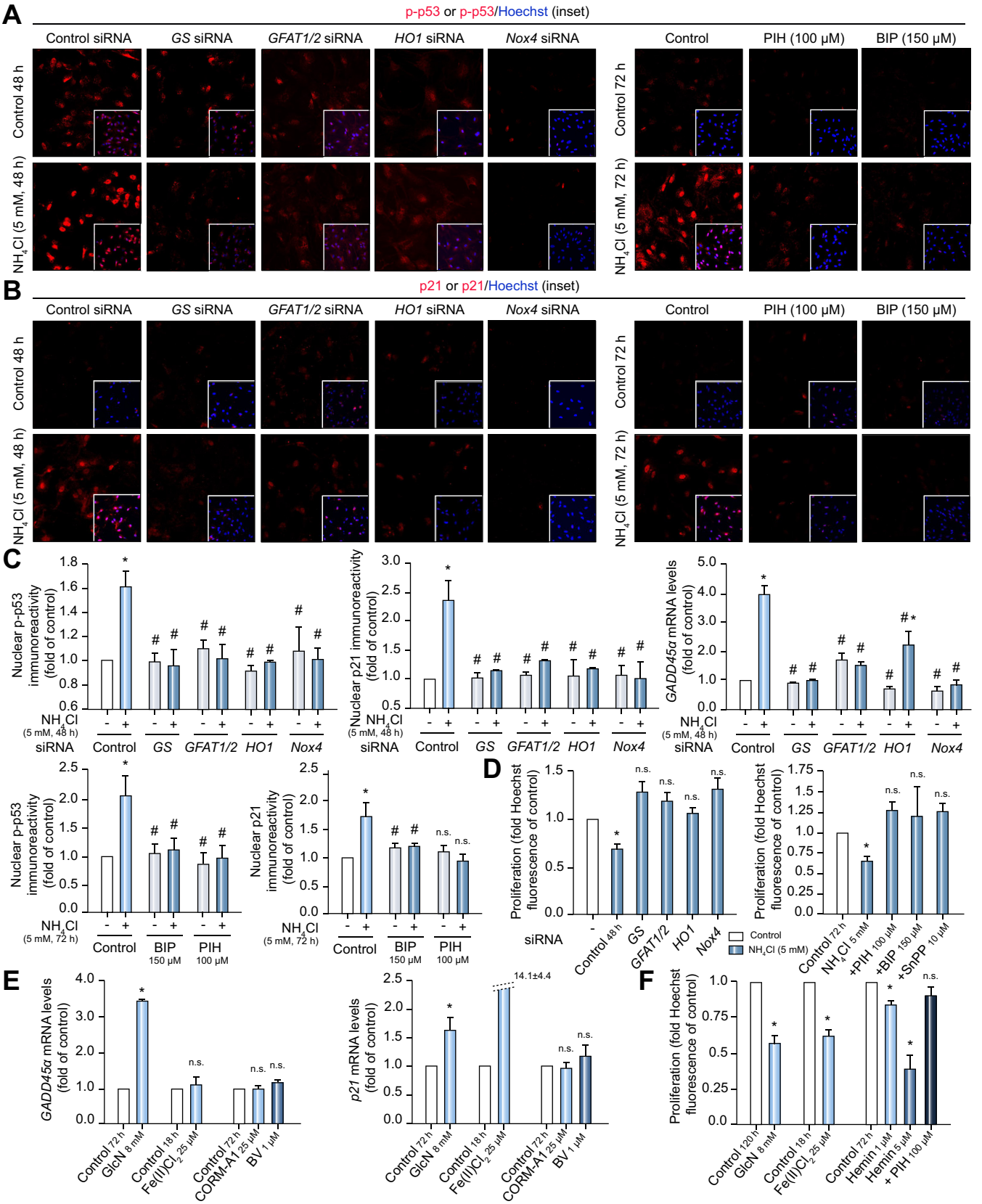
The analysis of O-GlcNAcylated proteins by western blot revealed 4 major proteins, which were prominently anti-O-GlcNAc-immunoreactive in brains from patients with liver cirrhosis and HE (Fig. 6B). Besides these, further anti-O-GlcNAc-immunoreactive proteins with lower intensity were detected (I in Fig. 6B). Out of the 4 major anti-O-GlcNAc-immunoreactive proteins, 2 were significantly higher O-GlcNAcylated in *post-mortem* brain tissue of patients with liver cirrhosis and HE, but not in those without HE (II and V in Fig. 6B).

Discussion

The present study suggests an important and previously unrecognized role of O-GlcNAcylation-dependent upregulation of HO1 and ferrous iron release for induction of oxidative stress in HE.

In line with this, *HO1* mRNA levels and multiple genes involved in iron homeostasis were elevated in *post-mortem* brain tissue from patients with liver cirrhosis and HE from 2 independent patient cohorts. Of particular importance, ferritin mRNA levels were significantly upregulated in patients with liver cirrhosis and HE in both cohorts (Fig. 1A, B) and a strong correlation between expression levels of ferritin and *HO1* mRNA was found in both cohorts. Since expression levels of ferritin reflect cytosolic levels of free iron in astrocytes,[19] these data are indicative of elevated levels of free iron in brain of patients with liver cirrhosis and HE.

The mechanisms underlying the ammonia-induced upregulation of HO1 and its functional consequences for oxidative stress and senescence were further analyzed in cultured astrocytes. Our data show that treating the astrocytes with glutamine upregulates HO1 and that siRNA-mediated knockdown of GS fully abolished the ammonia-induced upregulation of HO1 (Fig. 2A, Fig. S3B). Moreover, the ammonia-induced synthesis of glutamine elevated HO1 protein and mRNA by upregulating GFAT1/2 and consequently the synthesis of GlcN-6-P. In line


A
p-p53 or p-p53/Hoechst (inset)
Control siRNA
GS siRNA
GFAT1/2 siRNA
HO1 siRNA
Nox4 siRNA
Control
PIH (100 μM)
BIP (150 μM)
Control 48 h
NH4Cl (5 mM, 48 h)
Control 72 h
NH4Cl (5 mM, 72 h)
B
p21 or p21/Hoechst (inset)
C
Nuclear p-p53 immunoreactivity (fold of control)
Nuclear p21 immunoreactivity (fold of control)
GADD45α mRNA levels (fold of control)
NH4Cl (5 mM, 48 h)
siRNA
Control
GS
GFAT1/2
HO1
Nox4
NH4Cl (5 mM, 72 h)
BIP 150 μM
PIH 100 μM
D
Proliferation (fold Hoechst fluorescence of control)
Control 48 h
Control 72 h
NH4Cl 5 mM
+PIH 100 μM
+BIP 150 μM
+SnPP 10 μM
Control
NH4Cl (5 mM)
E
p21 mRNA levels (fold of control)
Control 72 h
GlcN 8 mM
Control 18 h
Fe(II)Cl2 25 μM
Control 72 h
CORM-A1 25 μM
BV 1 μM
14.1±4.4
F
Control 120 h
GlcN 8 mM
Control 18 h
Fe(II)Cl2 25 μM
Control 72 h
Hemin 1 μM
Hemin 5 μM
+ PIH 100 μM

with a role of glucosamine for the upregulation of HO1, both, ammonia as well as GlcN increased HO1 protein and mRNA (Fig. 2A; Figs. S3A, B). Interestingly, lower ammonia concentrations upregulated *GFAT2, grp78* and *HO1* mRNAs transiently. This may relate to the activation of counter-regulatory mechanisms which either terminate the *de novo* synthesis or which enhance the degradation of the respective mRNAs.

Upregulation of HO1 in neurological disorders was demonstrated to be a consequence of ER stress and HO1-derived CO was even suggested to ameliorate ER stress.[12] Unexpectedly, we found that upregulation of HO1 is not a consequence but a trigger of ER stress in ammonia-exposed astrocytes (Fig. 2C, Fig. S3E). The underlying mechanisms are currently unclear, but since HO1 resides at the outer ER membrane, ferrous iron release may trigger oxidative stress close to the ER membrane, thereby disturbing protein synthesis.

In search of the mechanism by which enhanced synthesis of GlcN-6-P upregulates HO1 mRNA and protein in ammonia-exposed astrocytes (Fig. 2A, Fig. S3B), we found that elevating intracellular GlcN-6-P levels either by exposure to GlcN or NH_4-Cl markedly enhanced the O-GlcNAcylation of specific proteins. Knockdown of OGT inhibited both, the ammonia-induced O-GlcNAcylation of individual proteins (Fig. 2D) as well as the transcriptional inhibition of pri-miR326-3p which is under β-arrestin 1 promoter control (Fig. 2E). Thus, downregulation of the HO1-targeting miR326-3p by ammonia was due to a GlcN-6-P synthesis and protein O-GlcNAcylation-mediated inhibition of the transcription of pri-miR326-3p (Fig. 2E). Unfortunately, the precise mechanism by which O-GlcNAcylation represses the transcription of pri-miR326-3p remains unclear. A likely explanation could be that transcription factors recruit OGT to the β-arrestin 1 promoter, where OGT inhibits the initiation of transcription through O-GlcNAcylation of RNA polymerase II.[20,21] This mechanism was recently identified to underlie the inhibition of nuclear factor κB (NFκB)-dependent proinflammatory gene transcription by the glucocorticoid receptor (GR).[21] However, whether GR recruits OGT to the β-arrestin 1 promoter in ammonia-exposed astrocytes needs to be determined.

Upregulation of HO1 by ammonia or GlcN was associated with expression changes of proteins and/or genes reflecting elevated intracellular free iron levels, such as upregulation of the iron exporter FPN and the iron chelator ferritin (Fig. 3A, B; Table S3; Fig. S4A). Interestingly, ammonia also triggered a nuclear accumulation of ferritin in astrocytes (Fig. S4F) which may be triggered by O-glycosylation[22] and which may protect DNA from oxidative damage.[23] This may explain why ammonia triggers oxidation of RNA, but not of DNA in astrocytes.[5]

Importantly, our data indicate that HO1 activity substantially affects iron homeostasis in ammonia-exposed astrocytes. This is evidenced by the observations that knockdown of GS, GFAT1/2 and HO1 fully prevented the upregulation of *FPN* mRNA by ammonia (Fig. 3A). Upregulation of FPN may be easily explained by iron-dependent activation of the metal-responsive transcription factor (MTF) 1 in ammonia-exposed astrocytes.[24,25] In line with this, exposure of astrocytes to Fe(II)Cl_2 upregulated *FPN* mRNA levels (Fig. S4G). Elevated FPN protein levels in ammonia-exposed astrocytes (Fig. 3B; Table S3) may also reflect diminished degradation of FPN by hepcidin (Fig. S4D). Interestingly, upregulation of FPN and ferritin was accompanied by downregulation of CP and hephaestin mRNA and protein (Fig. 3A, B; Table S3). As FPN activity relies on physical association with CP[26] or hephaestin, our data may indicate that ammonia could impair the export of iron from the astrocytes. This assumption is further supported by our findings showing that free ferrous iron levels were markedly enhanced in mitochondria and lysosomes in ammonia-exposed astrocytes (Fig. 3C, D) which may relate to ammonia-induced autophagy, mitochondrial dysfunction[27] and mitophagy.[28] While autophagy releases iron ions from ferritin,[29] mitophagy may elevate cytosolic heme levels.[30]

A functional consequence of elevated cytosolic free iron levels in the presence of H_2O_2 is the formation of highly reactive hydroxyl radical anions in the so-called Fenton reaction, which may contribute to ammonia-induced oxidative stress in astrocytes. As one potential source for H_2O_2 we identified Nox4, which is upregulated by ammonia via a post-transcriptional mechanism (Fig. 4A) most likely through downregulation of Nox4-targeting (Table S5) miR326-3p (Fig. 2E, 4A).

Most importantly, all maneuvers preventing either upregulation of HO1 or Nox4 such as knockdown of GS, GFAT1/2, HO1 and Nox4 as well as chelation of free iron prevented the ammonia-induced RNA oxidation (Fig. 4B, C). These findings clearly demonstrate an important role of both, HO1-derived ferrous iron and Nox4-derived H_2O_2 for the induction of oxidative stress in ammonia-exposed astrocytes.

Our study further identified the ammonia-induced upregulation of HO1 and Nox4 as the mechanism underlying the induction of astrocyte senescence.[6] This is evidenced by the findings that the ammonia-induced activation of p53 (Fig. 5A, C), upregulation and nuclear accumulation of p21 (Fig. 5B, C), enhanced transcription of *GADD45α* (Fig. 5C) and inhibition of proliferation (Fig. 5D) are prevented by knockdown of GS, GFAT1/2, HO1 and Nox4 or iron chelation. In line with this, incubation of astrocytes with GlcN or ferrous iron upregulated *GADD45α* and *p21* mRNA levels and inhibited astrocyte proliferation. Importantly, astrocyte proliferation was also inhibited by hemin in an iron-dependent way (Fig. 5E, F).

Another novel finding of the present study is that taurine blocks ammonia-induced ER stress by inhibiting the ammonia-induced upregulation of *GFAT1/2* and *HO1* (Figs. S3B, C, E). In line with this, taurine depletion in brains from taurine transporter (*TauT*) knockout mice is associated with

Fig. 5. Role of HO1 and Nox4 for ammonia-induced astrocyte senescence. Astrocytes were either exposed to NH_4Cl (5 mM), GlcN (8 mM), Fe(II)Cl_2 (25 or 500 μM), hemin (5 μM) or were left untreated (control) for the indicated time. Where indicated, cells were exposed to the indicated siRNA, PIH (100 μM), BIP (150 μM) or SnPP (10 μM). Immunofluorescence analysis of p^{Ser392}-p53 (A) or p21 (B). Nuclei were counterstained using Hoechst34580. (C) Quantification of nuclear p^{Ser392}-p53 and p21 immunoreactivity and *GADD45α* mRNA levels as analyzed by qPCR. n = 3–5 independent experiments. (D) Role of glutamine and GlcN-6-P synthesis, HO1, Nox4 and free iron for the NH_4Cl-induced proliferation inhibition. (E, F) Effects of GlcN, Fe(II)Cl_2, CORM-A1 and biliverdin (BV) on *GADD45α* and *p21* mRNA expression and astrocyte proliferation. mRNA levels of *GADD45α* and *p21* and proliferation are given relative to the respective control (untreated, inhibitor or the respective siRNA only). *Statistically significantly different to control siRNA or untreated astrocytes. #Statistically significantly different compared to control siRNA + NH_4Cl or NH_4Cl. [n = 3–5 (E) or n = 3–12 (F) independent experiments; C: one-way ANOVA, Tukey's *post hoc* test; D-F: Student's *t* test when comparing 2 groups, otherwise one-way ANOVA, Dunnett's *post hoc* test].

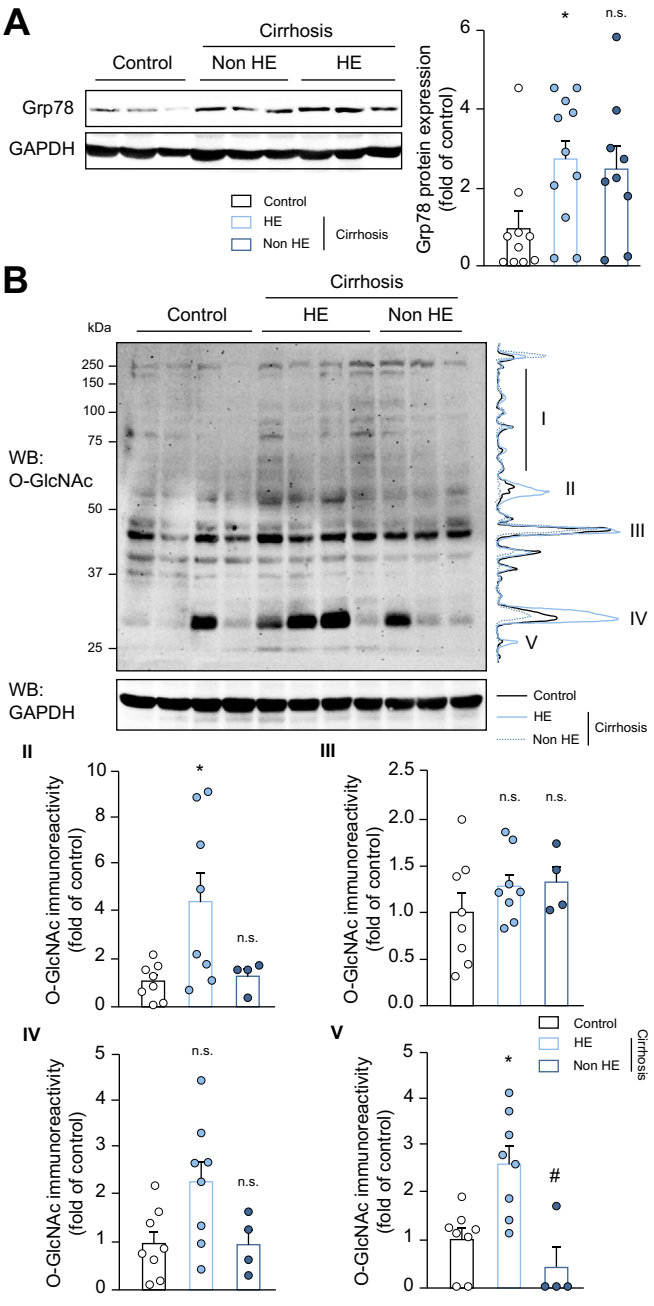


Fig. 6. Expression levels of the ER stress marker grp78 and O-GlcNAcylated proteins in *post-mortem* brain tissue from controls and patients with liver cirrhosis with or without HE. Protein lysates were prepared from human *post-mortem* brain samples from controls and patients with liver cirrhosis with or without HE. (A) Western blot analysis and densitometric quantification of grp78 protein (Australian cohort). (B) Western blot analysis and densitometric quantification of O-GlcNAcylated proteins (European cohort). Median anti-O-GlcNAc immunoreactivities of the respective groups are plotted in a dendrogram. Major anti-O-GlcNAc-immunoreactive bands indicated by roman letters I-IV were quantified by densitometric analysis. *Significantly different compared to control. #Significantly different compared to HE. (n = 4–11 independent samples one-way ANOVA, Tukey's *post hoc* test).

upregulation of HO1 and senescence biomarkers[11] and high levels of free iron ions (Fig. S9A). Cerebral iron levels were also strongly elevated in aged rats (Fig. S9B). These data further strengthen a role of iron and taurine deficiency for development of senescence.

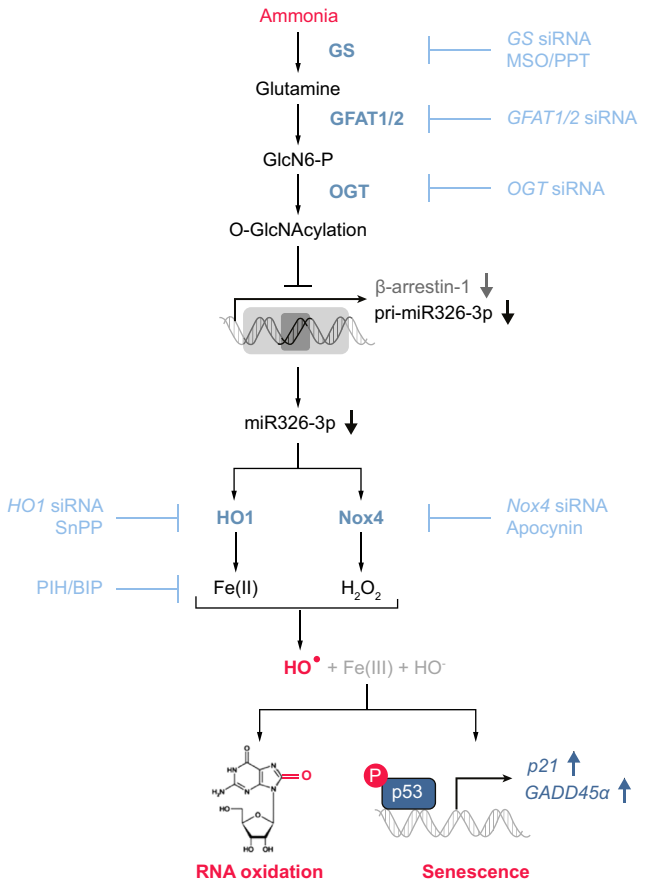


Fig. 7. Mechanisms of ammonia-induced RNA oxidation and astrocyte senescence. Ammonia upregulates HO1 through glutamine and GlcN-6-P synthesis and O-GlcNAcylation-dependent inhibition of pri-miR326-3p transcription. Downregulation of miR326-3p upregulates HO1 and Nox4 which trigger the formation of hydroxyl radical anion in the so-called Fenton reaction. As a consequence, RNA is oxidized and astrocytes become senescent in a p53, p21 and GADD45α-dependent way.

Elevated protein levels of the ER stress surrogate marker grp78 were also found in *post-mortem* brain samples from patients with liver cirrhosis with and without HE (Fig. 6A). This suggests that besides HO1-derived iron, other factors such as ethanol[31] may also contribute to ER stress in the brains of patients with liver cirrhosis. As ER stress is considered multifactorial, ammonia and ethanol may have additive effects on the expression of grp78.

The relevance of our findings for the pathogenesis of HE in humans is further strengthened by our data showing enhanced O-GlcNAcylation in *post-mortem* brain samples from patients with liver cirrhosis and HE, but not in those without HE (Fig. 6B). We cannot rule out an impact of confounding factors on the results obtained from human *post-mortem* brain samples. However, due to the similar gene expression changes observed in the 2 independent patient cohorts, we are confident in the observed effects.

The findings of our study are at variance with protective effects of HO1 in neurological disorders, such as traumatic brain injury, which may be attributed to the anti-oxidative actions of biliverdin and bilirubin.[32] The findings of our study suggest that an insufficient sequestration of ferrous iron by ferritin triggers oxidative stress in ammonia-exposed astrocytes. In line with this, a sustained transgenic overexpression of HO1 in astrocytes elevates free ferrous iron levels and triggers oxidative stress, mitophagy and senescence.[32]

It is a limitation of the present study, that the ammonia concentrations used in the *in vitro* experiments exceed the ammonia levels found in blood of patients with liver cirrhosis and HE (range: 48–343 μmol/L, mean: 146.0 ± 42.6, n = 6). However, significantly elevated levels of *HO1* mRNA and O-GlcNAcylated proteins were observed in *post-mortem* brain samples from patients with liver cirrhosis and HE, supporting the *in vivo* relevance of these findings. However, it remains to be established whether our findings are relevant not only for patients with severe hyperammonemia, but also for those with liver cirrhosis and HE.

Conclusions

The present study identified the O-GlcNAcylation-dependent upregulation of HO1 and Nox4 as a novel mechanism underlying ammonia-induced RNA oxidation and astrocyte senescence (Fig. 7). Given the important role of oxidative stress for the pathogenesis of HE, and the inherent limitations of a broad antioxidant therapy, our study warrants further research on the suitability of HO1 or Nox4 inhibitors or iron chelators for the treatment of HE.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

BG, AK, ES, MP, ASc, ASh, MC and HJB performed research and analyzed data. BG and DH designed the study and wrote the manuscript. All authors approved the manuscript.

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Supplementary data

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References

Author names in bold designate shared co-first authorship

[1] Görg B, Schliess F, Häussinger D. Osmotic and oxidative/nitrosative stress in ammonia toxicity and hepatic encephalopathy. Arch Biochem Biophys 2013;536:158–163.

[2] Häussinger D, Laubenberger J, vom Dahl S, Ernst T, Bayer S, Langer M, et al. Proton magnetic resonance spectroscopy studies on human brain myo-inositol in hypo-osmolarity and hepatic encephalopathy. Gastroenterology 1994;107:1475–1480.

[3] Görg B, Bidmon HJ, Häussinger D. Gene expression profiling in the cerebral cortex of patients with cirrhosis with and without hepatic encephalopathy. Hepatology (Baltimore, MD) 2013;57:2436–2447.

[4] Görg B, Qvartskhava N, Bidmon HJ, Palomero-Gallagher N, Kircheis G, Zilles K, et al. Oxidative stress markers in the brain of patients with cirrhosis and hepatic encephalopathy. Hepatology (Baltimore, MD) 2010;52:256–265.

[5] Görg B, Qvartskhava N, Keitel V, Bidmon HJ, Selbach O, Schliess F, et al. Ammonia induces RNA oxidation in cultured astrocytes and brain in vivo. Hepatology (Baltimore, MD) 2008;48:567–579.

[6] Görg B, Karababa A, Shafigullina A, Bidmon HJ, Häussinger D. Ammonia-induced senescence in cultured rat astrocytes and in human cerebral cortex in hepatic encephalopathy. Glia 2015;63:37–50.

[7] Bajaj JS, Schubert CM, Heuman DM, Wade JB, Gibson DP, Topaz A, et al. Persistence of cognitive impairment after resolution of overt hepatic encephalopathy. Gastroenterology 2010;138:2332–2340.

[8] **Bussian TJ**, **Aziz A**, Meyer CF, Swenson BL, van Deursen JM, Baker DJ. Clearance of senescent glial cells prevents tau-dependent pathology and cognitive decline. Nature 2018.

[9] Warskulat U, Görg B, Bidmon HJ, Müller HW, Schliess F, Häussinger D. Ammonia-induced heme oxygenase-1 expression in cultured rat astrocytes and rat brain in vivo. Glia 2002;40:324–336.

[10] Fukuda K, Richmon JD, Sato M, Sharp FR, Panter SS, Noble LJ. Induction of heme oxygenase-1 (HO-1) in glia after traumatic brain injury. Brain Res 1996;736:68–75.

[11] Oenarto J, Karababa A, Castoldi M, Bidmon HJ, Görg B, Häussinger D. Ammonia-induced miRNA expression changes in cultured rat astrocytes. Sci Rep 2016;6:18493.

[12] Kim HP, Pae HO, Back SH, Chung SW, Woo JM, Son Y, et al. Heme oxygenase-1 comes back to endoplasmic reticulum. Biochem Biophys Res Commun 2011;404:1–5.

[13] Karababa A, Görg B, Schliess F, Häussinger D. O-GlcNAcylation as a novel ammonia-induced posttranslational protein modification in cultured rat astrocytes. Metab Brain Dis 2014;29:975–982.

[14] Copeland RJ, Han G, Hart GW. O-GlcNAcomics-Revealing roles of O-GlcNAcylation in disease mechanisms and development of potential diagnostics. Proteomics Clin Appl 2013;7:597–606.

[15] **Sobczyk K**, **Jördens MS**, Karababa A, Görg B, Häussinger D. Ephrin/Ephrin receptor expression in ammonia-treated rat astrocytes and in human cerebral cortex in hepatic encephalopathy. Neurochem Res 2015;40:274–283.

[16] Oguchi M, Miyatake Y, Ayabe J, Akamatsu N. Phosphorylation of D-glucosamine by rat liver glucokinase. J Biochem 1975;77:1117–1121.

[17] Kong Q, Lin CL. Oxidative damage to RNA: mechanisms, consequences, and diseases. Cell Mol Life Sci 2010;67:1817–1829.

[18] Takac I, Schröder K, Zhang L, Lardy B, Anilkumar N, Lambeth JD, et al. The E-loop is involved in hydrogen peroxide formation by the NADPH oxidase Nox4. J Biol Chem 2011;286:13304–13313.

[19] Dang TN, Bishop GM, Dringen R, Robinson SR. The metabolism and toxicity of hemin in astrocytes. Glia 2011;59:1540–1550.

[20] Lewis BA, Burlingame AL, Myers SA. Human RNA polymerase II promoter recruitment in vitro is regulated by O-linked N-acetylglucosaminyltransferase (OGT). J Biol Chem 2016;291:14056–14061.

[21] Li MD, Ruan HB, Singh JP, Zhao L, Zhao T, Azarhoush S, et al. O-GlcNAc transferase is involved in glucocorticoid receptor-mediated transrepression. J Biol Chem 2012;287:12904–12912.

[22] Surguladze N, Patton S, Cozzi A, Fried MG, Connor JR. Characterization of nuclear ferritin and mechanism of translocation. Biochem J 2005;388:731–740.

[23] Thompson KJ, Fried MG, Ye Z, Boyer P, Connor JR. Regulation, mechanisms and proposed function of ferritin translocation to cell nuclei. J Cell Sci 2002;115:2165–2177.

[24] Kruczek C, Görg B, Keitel V, Bidmon HJ, Schliess F, Häussinger D. Ammonia increases nitric oxide, free Zn(2+), and metallothionein mRNA expression in cultured rat astrocytes. Biol Chem 2011;392:1155–1165.

[25] Troadec MB, Ward DM, Lo E, Kaplan J, De Domenico I. Induction of FPN1 transcription by MTF-1 reveals a role for ferroportin in transition metal efflux. Blood 2010;116:4657–4664.

[26] Jeong SY, David S. Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. J Biol Chem 2003;278:27144–27148.

[27] Rama Rao KV, Norenberg MD. Glutamine in the pathogenesis of hepatic encephalopathy: the trojan horse hypothesis revisited. Neurochem Res 2014;39:593–598.
[28] Polletta L, Vernucci E, Carnevale I, Arcangeli T, Rotili D, Palmerio S, et al. SIRT5 regulation of ammonia-induced autophagy and mitophagy. Autophagy 2015;11:253–270.
[29] Zhang Y, Mikhael M, Xu D, Li Y, Soe-Lin S, Ning B, et al. Lysosomal proteolysis is the primary degradation pathway for cytosolic ferritin and cytosolic ferritin degradation is necessary for iron exit. Antioxid Redox Signal 2010;13:999–1009.
[30] Khan AA, Quigley JG. Control of intracellular heme levels: heme transporters and heme oxygenases. BBA 2011;1813:668–682.
[31] Yang F, Luo J. Endoplasmic reticulum stress and ethanol neurotoxicity. Biomolecules 2015;5:2538–2553.
[32] Schipper HM, Song W, Tavitian A, Cressatti M. The sinister face of heme oxygenase-1 in brain aging and disease. Prog Neurobiol 2019;172:40–70.