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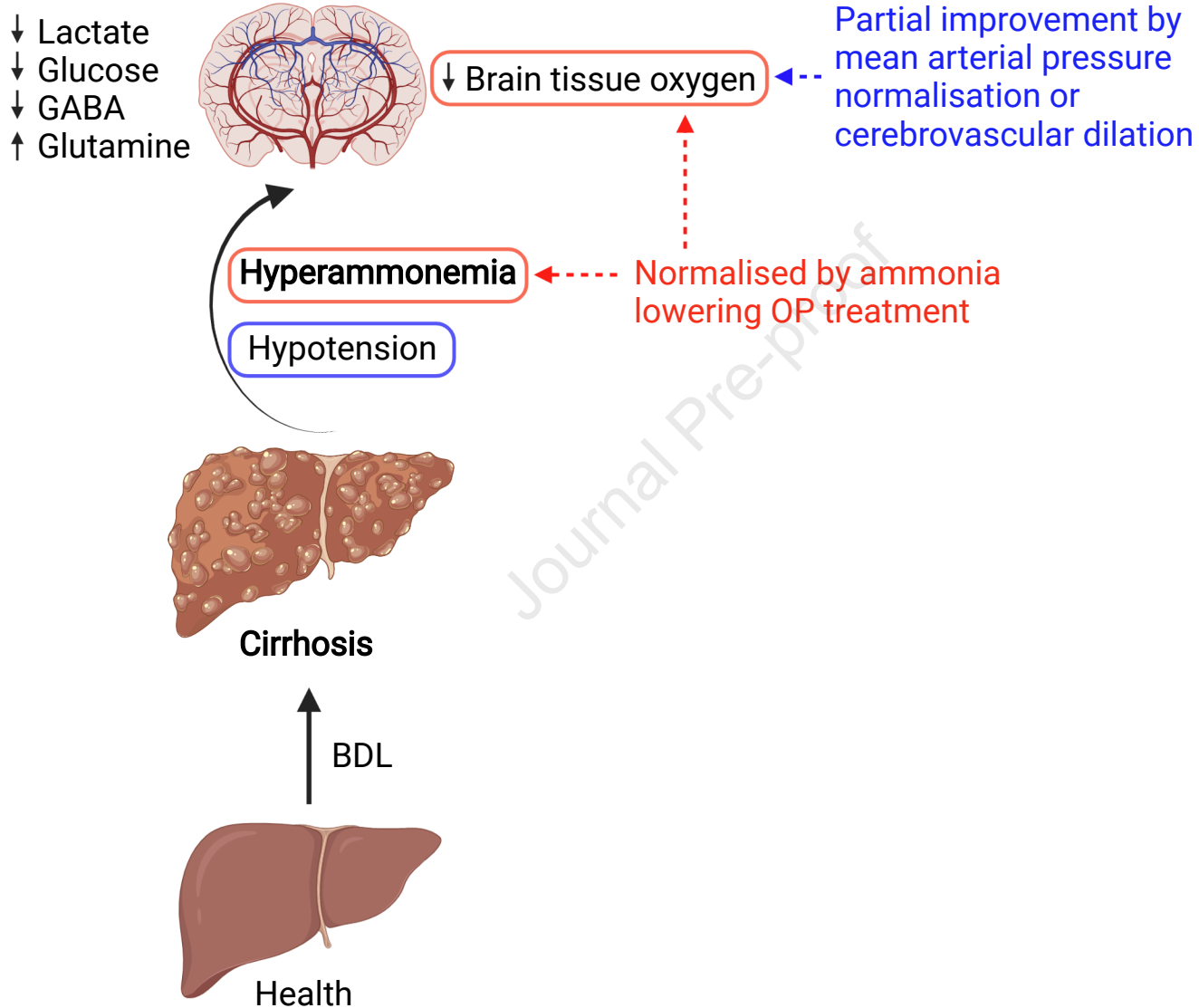
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Hepatic Encephalopathy

↓ Lactate
↓ Glucose
↓ GABA
↑ Glutamine

↓ Brain tissue oxygen ◄--- Partial improvement by mean arterial pressure normalisation or cerebrovascular dilation

Hyperammonemia ◄---- Normalised by ammonia lowering OP treatment

Hypotension

Cirrhosis

BDL

Health

Abnormal brain oxygen homeostasis in an animal model of liver disease

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Abbreviations:

HE: Hepatic encephalopathy

BDL: Bile duct ligation

CLD: Chronic liver disease

HA: Hyperammonemia

OP: Ornithine phenylacetate

CMRO2: Cerebral metabolic rate of oxygen

PO$_2$: Partial pressure of oxygen

SEM: Standard error mean

ATZ: Acetazolamide

PE: Phenylephrine

BP: Blood pressure

MAP: Mean arterial pressure

HRR: High resolution respirometry

eNOS: endothelial nitric oxide synthase

NO: Nitric oxide

1H-MRS: Proton magnetic resonance spectroscopy

Gln: Glutamine

Tau: Taurine

Asc: ascorbate

Asp: aspartate

GABA: γ aminobutyric acid

Glc: glucose

Glu: glutamate

Ins: myo-inositol

Lac: lactate

NAA: N acetylaspartate

tCho: total choline

tCr: total creatine

TCA: tricarboxylic acid

fMRI: functional magnetic resonance imaging

BOLD: blood oxygen level dependent

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Conflict of interest

Data availability statement: The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Lay Summary:

Brain dysfunction is a serious complication of cirrhosis and affects about 30% of these patients, while its treatment continues to be an unmet clinical need. This study shows that oxygen concentration in the brain of an animal model of cirrhosis is

markedly reduced. Low arterial blood pressure and increased ammonia (a

neurotoxin that accumulates in patients with liver failure) are shown to be the main

underlying causes. Experimental correction of these abnormalities restored oxygen

concentration in the brain, suggesting potential therapeutic avenues to explore.

Author contributions

Study concept and design: A.H, R.J, A.V.G and P.S.H

Acquisition of data: A.H, P.S.H, C.C and C.K

Analysis and interpretation of data: A.H, P.S.H, C.D, D.S, R.J and C.C

Drafting of the manuscript: A.H, C.C, C, K, P.S.H and R.J

Critical revision of the manuscript for important intellectual content: A.H, R.J and P.S.H

Statistical analysis: A.H, D.S and P.S.H

Obtained funding: A.V.G, A.H and R.J

Administrative, technical, or material support: A.H, C.K, C.D, K.P, Ab.H, P.S.H, D.S, A.V.G, C.C and N.D

Study supervision: A.H, P.S.H, A.V.G and R.J

Abstract

Background & Aims: Increased plasma ammonia concentration and consequent disruption of brain energy metabolism could underpin the pathogenesis of hepatic encephalopathy (HE). Brain energy homeostasis relies on effective maintenance of brain oxygenation, and dysregulation impairs neuronal function leading to cognitive dysfunction. We hypothesise that HE is associated with reduced brain oxygenation and explored the potential role of ammonia as an underlying pathophysiological factor.

Methods: In a rat model of chronic liver disease with minimal HE (mHE; bile duct ligation [BDL]), brain tissue oxygen measurement and proton magnetic resonance spectroscopy were used to investigate how hyperammonemia impacts oxygenation and metabolic substrate availability in the CNS. Ornithine phenylacetate (OP, OCR-002; Ocera Therapeutics, USA) was used as an experimental treatment to reduce ammonia concentration in plasma.

Results: In BDL animals, glucose, lactate and tissue oxygen concentrations in the cerebral cortex were significantly lower compared to sham-operated controls. OP treatment corrected the hyperammonemia and restored brain tissue oxygen. While BDL animals were hypotensive, cortical tissue oxygen was significantly improved by treatments which increased arterial blood pressure. Cerebrovascular reactivity to exogenously applied CO_2 was found to be normal in BDL animals.

Conclusions: These data suggest that hyperammonemia significantly decreases cortical oxygenation, potentially compromising brain energy metabolism. These

findings have potential clinical implications for the treatment of patients with mHE

aiming to restore normal brain blood flow alongside ammonia-reducing strategies.

Introduction

Hepatic encephalopathy (HE) in chronic liver disease (CLD) is characterised by a spectrum of neuropsychiatric symptoms that include impairment of cognitive function[1] [2]. This is a serious but potentially reversible condition that severely limits the patient's quality of life and long-term prognosis. HE can progress quickly resulting in coma with mortality rates of up to 50%[3] without liver transplantation. For some patients, despite successful transplantation, the neuropsychiatric symptoms can persist indefinitely[4]. Blood ammonia concentration is one of the main mechanisms thought to underlie the development of HE[5] and is an important therapeutic target[6]. However, the exact mechanism of how hyperammonemia leads to this complex neuropsychiatric syndrome is still unclear. What is known is that excess ammonia in the CNS impacts both astrocytic and neuronal function to impair cognitive processing in a graded, progressive fashion[7]. A more granular understanding of the pathophysiology could inform better treatment regimens and identify additional drug targets.

Cognitive impairment seen in HE may be the result of several related factors, including altered glutamatergic[8] (excitatory) and GABAergic neurotransmission[9], as well as (early) compromised brain energy metabolism[10-12], all of which are affected by or correlated with hyperammonemia. The latter will impair all aspects of brain function as neurotransmission is a particularly metabolically demanding activity[13]. A characteristic decline of whole-brain oxidative metabolism has been seen in HE patients, which implicates changes of neurons and their energy turnover, rather than a malfunction of oxidative metabolism in astrocytes[14]. Animal models using bile-duct ligation (BDL)-induced elevation in ammonia have reported mitochondrial dysfunction; a reduction in both mitochondrial membrane potential and respiratory chain enzymes, as well as

swelling of mitochondria[15]. This culminates in impaired ATP generation and oxidative stress, which in turn leads to compromised brain energy metabolism[11][16].

Oxygen is the key metabolic substrate within the CNS but only a 1 second supply remains at any one time. It is therefore necessary to tightly control delivery of this resource and mechanisms have evolved to closely regulate blood flow to match oxygen supply with demand[17][18]. Long-term impairment of cerebral blood flow (CBF) control and therefore oxygen delivery, has been linked to the development and/or progression of cognitive impairment during ageing and Alzheimer's disease[19][20]. Similarly, acute impairment can have long-term consequences on neurological function[21]. In patients with liver cirrhosis and HE, cerebral metabolic rate of oxygen (CMRO$_2$) and CBF are decreased compared to patients with cirrhosis without HE and healthy controls[14][22][23]. It has not yet been possible to determine if these derangements are associated with brain hypoxia and whether hyperammonemia contributes to this reduction[24].

In this study, we hypothesised that HE is associated with brain hypoxia, which is a consequence of the high concentrations of circulating ammonia. Using the BDL rat model of CLD with minimal HE (mHE), we investigated the mechanism of HE- related low brain oxygen tension by manipulating peripheral and cerebral perfusion. Additionally, we sought to clarify the role of ammonia in altering brain oxygenation during HE using the drug, ornithine phenylacetate (OP, OCR-002; Ocera Therapeutics, CA, USA), which is known to reduce plasma and brain ammonia concentrations[25].

Materials and Methods

All experiments were performed in accordance with the European Commission
Directive 2010/63 (European Convention for the Protection of Vertebrate Animals
used for Experimental and Other Scientific Purposes) and the UK Animals in
Scientific Procedures Act (ASPA) 1986 (amended 2012), with project approval by the
Institutional Animal Welfare and Ethical Review Board. All experiments were
designed and reported in adherence to ARRIVE guidelines[26]. Some experiments
were performed in collaboration with the CIBM MRI EPFL AIT, Switzerland due to
the availability of proton magnetic resonance spectroscopy (1H-MRS) and were
approved by the Committee on Animal Experimentation for the Canton of Vaud,
Switzerland (VD3022.1). In both cases experimental subjects were obtained from a
commercial supplier, Charles Rivers Laboratories, Inc. Animals were group-housed
in individually ventilated cages, enriched with rails and cardboard tubes, in a room of
20–22 °C, relative moisture 50–60%, and 12 h light–dark cycle (light 7 am-7 pm).

Animal Model of HE

HE in experimental animals was induced by BDL procedure as described
previously[10 27]. Briefly, under surgical anaesthesia (5% isoflurane in oxygen for
induction, 2% isoflurane in air for maintenance) rats underwent triple ligation of the
bile duct via a small laparotomy to induce advanced chronic liver injury. Control
groups underwent a sham surgical procedure where the bile duct was exposed for
equal time, prior to closure of the incision. Body temperature was monitored via a
rectal probe and maintained at 37±0.5°C with a Homeothermic Blanket Control Unit
(Harvard). At the end of the experiments, blood was collected from the left ventricle

of the heart under anaesthesia and biochemical measurements were performed using a Cobas Integra II system (Roche Diagnostics) with plasma or PocketChemTM (BA PA-4140) with fresh blood (supplementary table 1). Plasma bilirubin was measured using Cobas Integra II system (Roche Diagnostics) or Reflotron® Plus system (F. Hoffmann-La Roche Ltd) as indicated in supplementary table 1.

Brain tissue partial pressure of oxygen (PO_2) measurements were performed in Sprague-Dawley rats at 28 days post-surgery. 1H-MRS experiments were performed in Wistar rats at 42 days post-surgery, as previous studies have shown slower progression of liver disease development[10 28]. Despite the difference in strain and duration post-surgery, the selected time points have previously been defined as the time required for each animal model to develop similar degree of severe fibrosis with manifestation of severe cholestasis, portal hypertension and cerebral dysfunction[10 29], as well as similar ammonium and bilirubin concentrations (supplementary table 1). The study overview and experimental design is schematised in Figure 1.

OP treatment

Combined doses of L-ornithine and phenylacetate (0.3g/kg; OP) were given twice daily, by intraperitoneal injections, 23 days after the surgery, ~7 hours apart for 5 days. This dosing regime has previously been shown to reduce plasma ammonia concentration by approximately 50%[30]. The rats were studied on day 28 post BDL surgery, within 3 hours of the last OP injection.

Brain tissue oxygen measurements

Brain tissue oxygen was measured *in vivo* in BDL and sham-operated animals (Figure 1). Anaesthesia was induced by isoflurane as stated above and maintained with α-chloralose (100 mg kg^{-1}, i.v.). Supplementary doses of α-chloralose (10–20 mg kg^{-1}, i.v.) were given as required. The depth of anaesthesia was assessed by the stability of cardiovascular and respiratory variables being recorded. The right femoral artery was cannulated for the measurement of blood pressure (BP) and for sampling arterial blood for analysis of pH and blood gases. Samples were collected at regular intervals and analysed using a pH/blood gas analyser (Siemens Rapidlab 248; Siemens Healthcare, Sudbury, UK). Blood gasses and pH were maintained within the physiological range (PO$_2$; 100-120mmHg, PCO$_2$; 30-40mmHg, pH 7.35-7.40 and calculated bicarbonate between 22-26 meq/L) by adjusting the rate and/or stroke volume of the ventilator, and by supplementary oxygen in the inspired room air. Body temperature was monitored via a rectal probe and maintained at 37±0.5°C with a Homeothermic Blanket Control Unit (Harvard). BP was measured using a pressure transducer (Neurolog, Digitimer, UK), and heart rate was derived electronically from the BP signal.

Animals were placed in a stereotaxic frame and a limited craniotomy was performed in order to access the somatosensory (forelimb) region of the cortex (S1FL ~0.5mm below the cortical surface, Figure 1). PO$_2$ was monitored by optical fluorescence technology that allows real-time detection of PO$_2$ *in vivo* (OxyliteTM, Oxford Optronics), as previously described[31] [32]. Following the insertion of the sensor, the craniotomy was sealed from the air with petroleum jelly, preventing diffusion of

ambient oxygen. Following a 15 min recovery period, parenchymal PO_2 sampling was started until a stable reading was achieved.

Pharmacological and blood gas manipulations

To investigate cerebrovascular reactivity and the role of peripheral and cerebral perfusion in altering cerebral cortical oxygen tension, pharmacological and blood gas manipulations were performed. Systemic hypercapnia was induced by switching the input to the ventilator from room air to a compressed gas source comprised of 21% O_2 and 10% CO_2, with the balance made of nitrogen. Animals were exposed to this gas mixture for a period of 5mins after baseline PO_2 was recorded. In a separate group of animals, sham-operated and BDL subjects (Figure 1) received the carbonic anhydrase inhibitor, acetazolamide (ATZ)[33] (10mg kg^{-1}, i.v.) dissolved in 100% DMSO (maximum volume 25μL) after baseline PO_2 was recorded. Peripheral vessel tone under anaesthesia was manipulated by infusion of the alpha1-adrenoceptor agonist, phenylephrine (PE). PE was infused at a rate of 5-10µg min^{-1} to maintain a mean arterial pressure (MAP) in the BDL subjects of ~100 mmHg for a period of approximately 10mins.

In vivo 1H-MRS at 9.4T

In order to investigate the characteristic metabolic changes known to occur in HE[7] and validate our model, sham-operated and BDL rats (Figure 1) were anesthetised with isoflurane (5% for induction, 2% for maintenance in 50% air and 50% oxygen) and underwent 1H-MRS. 1H-MRS spectra were acquired on 9.4 T system (Varian/Magnex Scientific) using the SPECIAL sequence (TE=2.8ms) as previously described[10]. Volume of interest (VOI) was selected in S1 primary somatosensory cortex (1.3x2x3

mm^3). LCModel was used for quantification using water as internal reference, allowing the quantification of a total of 18 metabolites. Body temperature of animals was monitored via a rectal probe and maintained at 37±0.5°C by means of heated MRI compatible animal cradle.

Data analysis and statistics

Physiological variables were digitised using a Power 1401 interface (CED) and stored on a PC for offline processing using Spike 2 software (CED). Statistical analysis was performed using GraphPad Prism (v9 for Mac, San Diego, CA, USA). Data are expressed as mean±SEM. Differences were ascertained by Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test or paired/unpaired t-test and Mann Whitney test, where appropriate. Differences with a *p* value of <0.05 were considered to be significant.

Results

Biochemistry

Compared to sham surgery, the BDL procedure resulted in a significant increase in plasma ammonia, alanine transaminase (ALT) and bilirubin (p<0.001), indicating impaired liver function, while albumin and total protein concentrations were significantly decreased (p<0.001). Treatment of BDL animals with OP lowered plasma ammonia concentration, which was similar to that measured in sham-operated animals (p=0.3), but had no effect on other parameters; ALT, bilirubin, albumin and total protein concentrations remained unchanged from the untreated BDL group. Plasma biochemistry, including ammonia concentration data are summarised in Supplementary Tables 1 and 2.

Brain tissue PO_2 and cerebrovascular CO_2 reactivity

Following placement of the oxygen sensor in the cerebral cortex (Figure 1), blood PO_2 and PCO_2 were measured, and no significant differences were detected between groups (Table 1). Brain PO_2 was obtained over a period of at least 5 mins of stable recording. An average of this time period revealed a significantly lower brain PO_2 (BDL: 14±1 mmHg, n=36; sham-operated controls: 27±1 mmHg, n=36; p<0.001) (Figure 2).

In order to investigate the role of hyperammonemia in brain oxygen impairment seen in our model of HE, we lowered ammonia by treating the BDL animals with OP, which had no significant effect on any other plasma biochemistry parameters (Supplementary Table 1). OP treatment of BDL rats significantly improved brain PO_2 (22±1 mmHg, n=6), increasing the oxygen tension similar to that recorded in sham-operated (27±1 mmHg; p=0.6) and sham-OP rats (27±2 mmHg, n=7; p>0.1) (Figure 2).

To evaluate the ability of cerebral vessels to respond to a known vasodilatory stimulus, hypercapnic acidosis was induced by changing the inspired gas mixture to include 10% CO_2. Hypercapnic acidosis led to a significant increase in parenchymal PO_2 from baseline in both BDL (p<0.001) and sham-operated rats (p<0.001; Figure 3A and 3C); 15±2 to 36±4 mmHg (n=6) and 26±1 to 46±3 mmHg (n=8), respectively. Despite the lower baseline PO_2 (p=0.007), CO_2 reactivity was preserved in BDL animals, with an increase in PO_2 (by 20±3 mmHg, 80% increase) not significantly different to that observed in sham-operated animals (by 21±2 mmHg, 132% increase, p=0.9; Figure 3B). This indicates that it is possible to restore brain oxygenation by cerebrovascular

dilation as there was no difference (p=0.1) between the peak tissue PO_2 measured in BDL and sham-operated controls (Figure 3A) during hypercapnic acidosis.

Brain PO_2 could also be partially restored by pharmacological agents known to specifically dilate cerebral vasculature. The carbonic anhydrase inhibitor ATZ was chosen as it known to dilate the cerebrovasculature without significant effects on arterial blood pressure. Blockade of carbonic anhydrase causes an accumulation of extracellular protons to induce smooth muscle relaxation[34][35] in the CNS. Doses of 10mg kg^{-1} were found to significantly increase brain oxygenation in BDL animals, from 16±4 to 21±5 mmHg (n=7, 28% increase, p=0.02), although this was not as effective as in sham-operated animals where it increased from 28±2 to 39±3 mmHg (n=7, 38% increase, p<0.001; Figure 3D and E).

It has been previously reported that MAP is lower in conscious BDL animals[36]. We confirmed this observation in the anaesthetised animals; BDL animals had a significantly lower MAP compared with sham-operated controls; 60±3mmHg vs. 84±8 mmHg respectively (p=0.04) (Supplementary Figure 1). In order to control for the effects of a lower MAP on brain oxygenation, an infusion of the α1-adrenergic receptor agonist PE was used to normalise MAP to that of sham-operated animals (Figure 3F and G). Increasing MAP in BDL animals significantly increased brain PO_2 by 6±1 mmHg from 14±4 to 20±4 mmHg (n=6, 45% increase, p=0.007). Inducing a corresponding change in MAP in sham-operated rats, brain oxygenation was increased by 16±5 mmHg (n=7, 55% increase, p=0.02) compared to baseline. Interestingly, BDL animals receiving OP treatment did not show a significant improvement in MAP; 69±5mmHg (n=5) compared with 60±3mmHg in untreated

animals (p=0.15). Again, arterial blood PO_2 and PCO_2 were not different between groups (Table 1).

In vivo 1H-MRS at 9.4T

The characteristic metabolic pattern of chronic HE was observed in the somatosensory cortex of BDL rat (Figure 4), characterised by a significant increase of glutamine (sham-operated: 3.8±0.7, BDL: 5.0±0.7 mmol/kg$_{ww}$, +32%, p=0.03). This increase was associated with no significant change in osmolytes. A significant decrease in lactate (sham-operated: 2.0±0.7, BDL: 0.9±0.2 mmol/kg$_{ww}$, -55%, p=0.03) was observed for BDL rats. In addition, BDL rats displayed a significant decrease in glucose (sham-operated: 3.2±0.8, BDL: 1.4±0.15 mmol/kg$_{ww}$, -56%, p=0.03) and neurotransmitter γ-aminobutyric acid (GABA) (sham-operated: 1.7±0.2, BDL: 1.1±0.2 mmol/kg$_{ww}$, -35%, p=0.02).

Discussion

Healthy brain function requires constant and sufficient supply of oxygen and other metabolic substrates. Consequently, insufficient tissue oxygenation can have deleterious effects on all cell types and their processes, which contribute to the development of severe clinical symptoms of HE in the long term. However, the exact pathway by which ammonia affects brain oxygenation remains unknown. In this study, we explored the effect of hyperammonemia on brain oxygenation in the somatosensory cortex of an animal model of advanced CLD and HE. Hyperammonemia was associated with a marked reduction in CNS tissue oxygenation, which can be considered a proxy of cerebral perfusion/blood flow at constant levels of neural activity[37]. Although several studies have reported

compromised CBF and $CMRO_2$, data regarding actual brain oxygen concentration in HE has thus far been lacking[22].

The data described herein demonstrates that by preventing circulating ammonia from accumulating in BDL animals using OP, a drug known to lower systemic and brain ammonia concentration[30], brain oxygenation is maintained within the range of control animals. This clearly implicates ammonia as the driving factor responsible for the reduction of CNS perfusion. Furthermore, we also showed for the first time a reduction in other critical metabolic substrates, such as glucose and lactate, in the somatosensory cortex of BDL animals using *in vivo* 1H-MRS. In combination, this indicates the possibility that hyperammonemia, seen in HE, has a detrimental impact on the supply of metabolic substrates.

A potential mechanism of how ammonia may impact brain oxygenation was described in a recent study which showed that hyperammonemia contributes to endothelial nitric oxide synthase (eNOS) downregulation through induction of inflammation and increased production of asymmetric-dimethylarginine, an endogenous inhibitor of eNOS[38]. Nitric oxide (NO) plays an important role in regulating functional microvascular perfusion[18] and preventing vascular and endothelial dysfunction, which could contribute to HE. Correction of hyperammonemia with OP was previously shown to restore eNOS activity resulting in improved NO metabolism[38].

We next considered the mechanism behind the apparent reduction in metabolic substrates in the CNS as their concentration is a function of consumption and delivery. First, we asked if tissue oxygenation can be improved by agents known to increase

cerebral perfusion[39]. Indeed, BDL animals responded to increased blood concentration of CO_2 in a manner indistinguishable from control animals with identical increases of tissue oxygen from their respective baselines. Additionally, ATZ was found to significantly increase tissue oxygenation from baseline in BDL animals, albeit not to the same level as controls. These observations indicate that cerebrovascular reactivity and capacity for blood vessels to dilate are intact, even during hyperammonemia, and supply of metabolic substrates is hindered by abnormal cerebral vessel tone, thereby reducing delivery.

Evidence exists pointing towards lactate as an important energy substrate[11], as well as a mediator of vasodilation[40][41]. In HE, hyperammonemia has been associated with an impaired cortical hemichannel-mediated lactate transport, contributing to the neuronal energy deficits involved in the pathogenesis of HE[11]. In this study, 1H-MRS data also revealed a significantly lower concentration of lactate, glucose and GABA in the cortex of hyperammonemic BDL rats, as well as elevated glutamine and decreased osmoregulatory myo-inositol and taurine concentrations (characteristic of HE[10][28][37]). Lactate was previously shown to be increased in the cerebellum of BDL rats mainly at 8 weeks post ligation with minor changes in the hippocampus[42], while brain glutamine showed the largest increase in the cerebellum and the smallest in the striatum of BDL rats using identical MRS measurements[42], confirming the already suspected brain metabolic regional difference in cirrhosis-induced HE[10]. In parallel, decreased glucose uptake has previously been measured *ex vivo* (brain tissue)[15] and *in vivo* (^{18}F-FDG PET[43]; plasma and cortex) using a similar animal models as used in the present study. These brain alterations indicate a dysmetabolic state and dysfunctional neurotransmission that could be arising due to impaired delivery production and/or

release of these energy substrates/neurotransmitters. Such metabolic alterations could be the cause or consequence of the reported brain hypoxia, as brain oxygenation is crucial for the production of key metabolic substrates[44]. The combination of these is expected to contribute to the development of HE-associated neuropsychiatric alterations (as seen in neurodegenerative diseases[45][46]), such as memory deficits, which have previously been reported in the same animal models and at the same time point as the present recordings[10][29].

Finally, we considered the possibility that lower MAP, could be responsible for the apparent decrease in central perfusion rather than a CNS-intrinsic mechanism of altered central vessel tone. Restoring MAP by infusion of a peripheral vasoconstrictor agent, successfully increased brain PO_2 in BDL animals. This observation is in keeping with the effect of systemic vasoconstrictors on renal perfusion in patients with cirrhosis making terlipressin or noradrenaline the drugs of choice to treat hepatorenal syndrome[47][48]. However, increasing MAP did not completely normalise the oxygenation to the same levels recorded in sham-operated controls. This suggests that hypotension is not solely responsible for the decreased brain oxygenation observed in animals with HE and central vessel tone remains a major factor in determining the supply of metabolic substrates in conditions of hyperammonemia. Taken together, these data suggest that compromised systemic and cerebral perfusion contribute to the low brain oxygen concentration in BDL animals. The exact mechanism of the role of ammonia will need to be explored in future studies.

The limitations of the present study are that it only includes measures of tissue PO_2 and uses this to infer changes in cerebral blood flow/perfusion. Tissue oxygenation is

not solely a function of blood flow and will also be sensitive to the basal metabolic rate of oxygen. CBF measurements using functional magnetic resonance imaging (fMRI) would be the only possibility to determine brain perfusion in a way that would allow comparisons between groups of animals but were not performed in this particular study. However, CBF is known to be a significant component of brain tissue oxygenation and we have recently shown that PO$_2$ changes in the cortex of experimental animals show excellent correlation with blood oxygen level dependent (BOLD) signals obtained using an fMRI scanner[49]. Additionally, neuropsychological data was not collected from the subjects in the present study, however this has been detailed in several previous publications[10][29], including the effect of OP on cognitive performance in the BDL model[50]. In this study we observe brain hypoperfusion at time points corresponding to previous reports of cognitive task performance impairment[10][29]. We rely on the logical extension that a reduction in availability of metabolic substrates will lead to neuronal dysfunction manifesting neuropsychological impairment[51].

Supporting the results of our study, Clément and colleagues[50] recently demonstrated that in HE the brain, which is already compromised (decreased oxygenation and metabolic dysregulation) becomes susceptible to hypotensive insults resulting in neuronal cell death. Treating BDL rats with OP, which as we have shown here improves brain oxygenation, protected the brain against hypotension-induced neuronal cell degeneration. This provides the rationale to explore the role of drugs often used in clinical practice to increase brain perfusion, in combination with ammonia lowering interventions, as potential therapeutic agents for treatment of HE.

In conclusion, the results presented in this study suggest that HE is associated with reduced brain tissue PO$_2$ and corresponding reduction in other metabolic substrates driven by hyperammonemia, which can be prevented with OP treatment. While the exact mechanism of the reported phenotype is still unclear, it is proposed that ammonia could act by increasing central vascular tone, possibly via NO dysregulation. The hypoxic conditions reported in this study are sufficient to trigger astrocytic activation[52], as well as neuronal death[53], which are hypothesised to contribute to the pathogenesis of HE. This study offers the novel prospect that cerebral vascular tone could be a potential therapeutic target alongside ammonia lowering strategies to specifically target neuronal dysfunction.

	Arterial blood PO$_2$, mmHg	*Arterial blood PCO$_2$, mmHg*
Sham	121±2	32±1
BDL	114±3	33±1
Sham-OP	116±2	34±2
BDL-OP	115±5	31±2

Table 1: Arterial blood PO$_2$ and PCO$_2$ in an animal model of HE, indicating no statistically significant differences between the groups. Data expressed as mean±SEM and compared using one-way ANOVA

Figure Legends

Fig. 1: Study design overview. Schematic depicting the study design overview and animal number allocation. *Sprague-Dawley rats, **Wistar rats.

Fig. 2: Cortical PO_2 in an animal model of HE. Summary data illustrating basal PO_2 in the somatosensory cortex of sham-operated, BDL, sham-OP and BDL-OP treated animals. Data expressed as mean±SEM and compared using Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test. *p* values indicate differences from sham-operated rats.

Fig. 3: Effect of vessel tone manipulations on cortical PO_2 in BDL animals. A) Grouped data showing cortical peak PO_2 changes from baseline in BDL and sham-operated control animals in response to hypercapnic acidosis (10% inspired CO_2). **B)** Grouped data comparing the relative change in PO_2 in BDL and sham-operated control animals. Compared using the Mann Whitney-U test. **C)** Time series of cortical PO_2 changes in response to hypercapnic acidosis in BDL and sham-operated control animals. **D)** Grouped data showing cortical peak PO_2 changes from baseline in BDL and sham-operated control animals in response to carbonic anhydrase inhibition (Acetazolamide [ATZ], 10mg kg^{-1}). **E)** Example experimental trace showing the effect of ATZ on cortical PO_2 and arterial blood pressure (black line indicates MAP) after BDL. **F)** Grouped data showing cortical peak PO_2 changes from baseline in BDL and sham-operated control animals in response to increased arterial blood pressure (Phenylephrine [PE] infusion, 5-10µg min^{-1}). **G)** Example experimental trace showing the normalisation of atrial blood pressure with PE infusion after BDL and corresponding change in cortical PO_2. All grouped data is expressed as mean±SEM paired sample t-test when comparing an experimental manipulation within subject or unpaired sample t-test when comparing between groups of subjects, unless otherwise stated.

Fig. 4: ***In vivo*** **brain 1H-MRS results obtained in the somatosensory cortex of BDL and sham-operated animals. A)** Grouped data comparing changes in the main metabolites and osmolytes in the somatosensory cortex of BDL and sham-operated control animals. **B)** Representative 1H-MRS spectra measured in BDL and sham-operated animals with the corresponding voxel location (top panel). Metabolite changes are visual in the spectra (i.e. Gln, GABA, Lac) and are highlighted in grey. Data expressed as mean±SEM and compared using unpaired sample t-test. Asc: ascorbate; Asp: aspartate; GABA: γ aminobutyric acid; Glc: glucose; Gln: glutamine; Glu: glutamate; Ins: myo-inositol; Lac: lactate; NAA: N acetylaspartate; Tau: taurine; tCho: total choline; tCr: total creatine.

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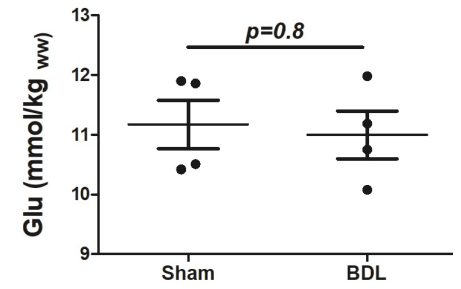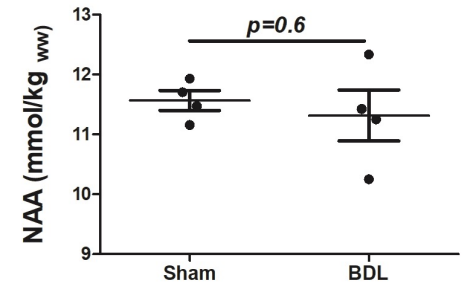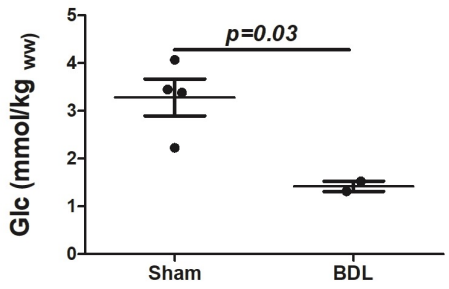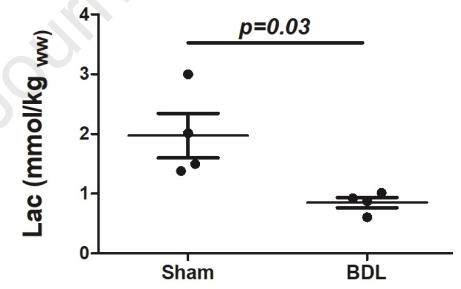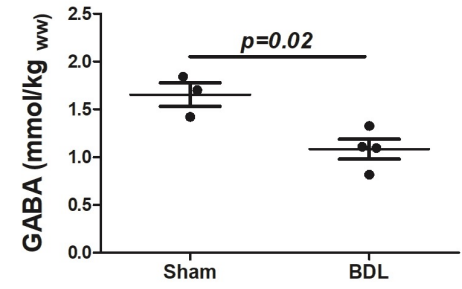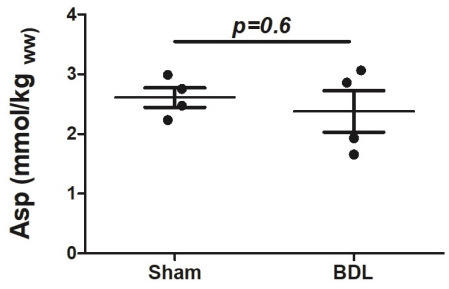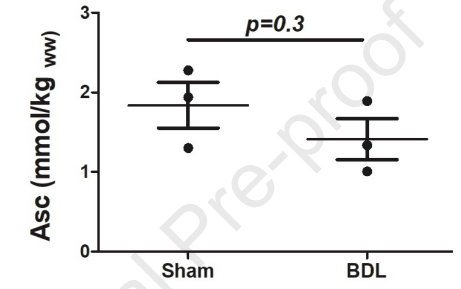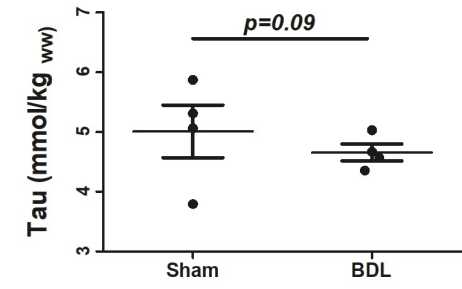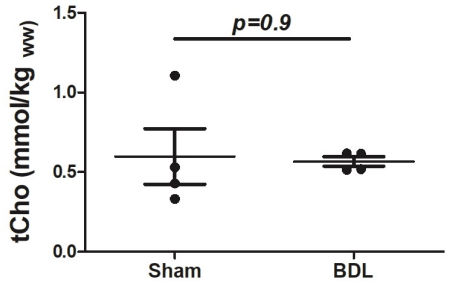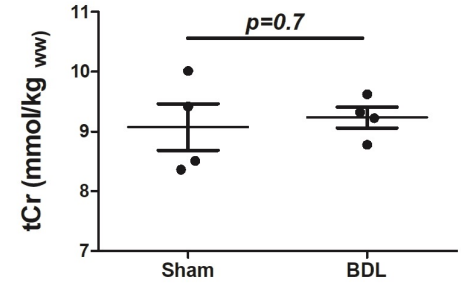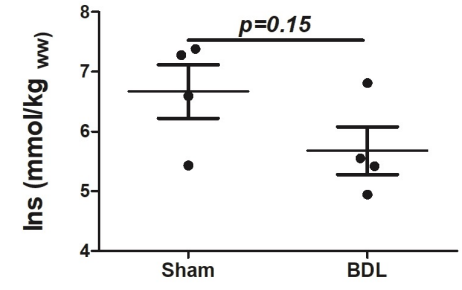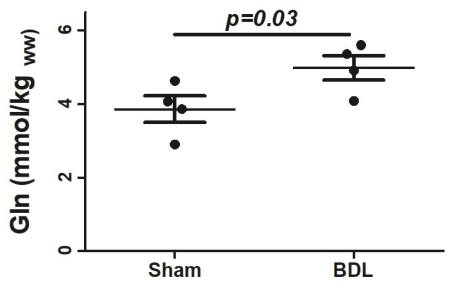
In vivo ¹H – MR spectroscopy

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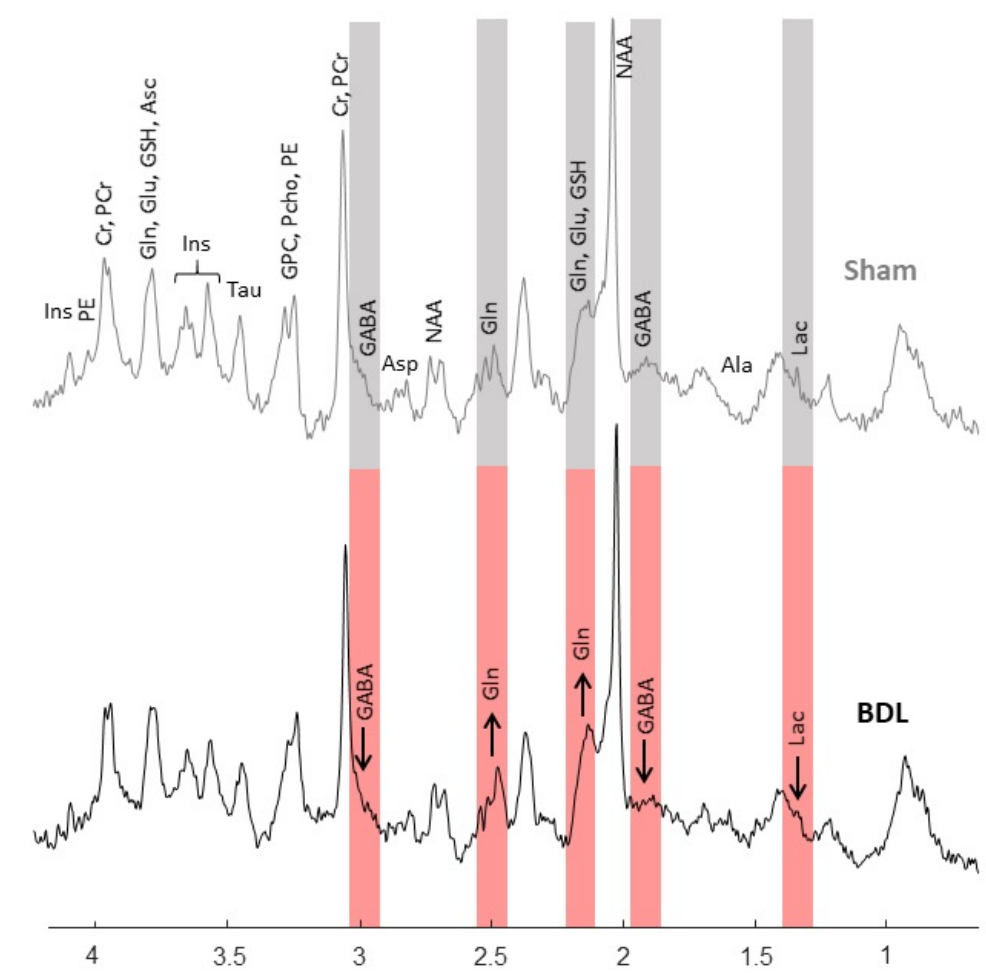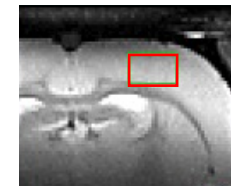
Highlights:

- Using a rat model, HE is shown to be associated with reduced brain tissue $P\text{O}_2$ and other metabolic substrates
- Cerebrovascular dilating agents and ammonia lowering treatment reduced effect on brain oxygenation indicating that ammonia could act by increasing central vascular tone
- Cerebral vascular tone could be a potential therapeutic target alongside ammonia lowering strategies