

Ammonia mediates cortical hemichannel dysfunction in rodent models of chronic liver disease

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Abbreviations:

HE: Hepatic encephalopathy

BDL: Bile duct ligation

HA: Hyperammonemia

OP: Ornithine phenylacetate

ALF: Acute liver failure

CLD: Chronic liver disease

aCSF: Artificial cerebrospinal fluid

RT-qPCR: Quantitative real-time PCR

CBX: Carbenoxolone

NPPB: 5-Nitro-2-(3-phenylpropylamino) benzoic acid

CBF: Carboxyfluorescein

ROI: Region of interest

SEM: Standard error mean

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Potential Conflict of Interest:

Rajiv Jalan has research collaborations with Ocera and Takeda and consults with Ocera and has received speaking fees from Norgine. Rajiv Jalan is the inventor of OCR-002, which has been patented by UCL and licensed to Ocera Therapeutics. He is also the founder of Yaqrit limited, a spin out company from University College London.

Abstract

The pathogenesis of hepatic encephalopathy (HE) in cirrhosis is multifactorial and ammonia is thought to play a key role. Astroglial dysfunction is known to be present in HE. Astrocytes are extensively connected by gap junctions formed of connexins, which also exist as functional hemichannels allowing exchange of molecules between the cytoplasm and the extracellular milieu. The astrocyte-neuron lactate shuttle hypothesis suggests that neuronal activity is fuelled (at least in part) by lactate provided by neighbouring astrocytes. We hypothesised that in HE, astroglial dysfunction could impair metabolic communication between astrocytes and neurons. In this study we determined whether hyperammonemia leads to hemichannel dysfunction and impairs lactate transport in the cerebral cortex using rat models of HE (bile duct ligation [BDL] and induced-hyperammonemia [HA]) and also evaluated the effect of ammonia-lowering treatment (ornithine phenylacetate, OP). Plasma ammonia concentration in BDL rats was indeed significantly reduced by OP treatment. Biosensor recordings demonstrated that HE is associated with a significant reduction in both tonic and hypoxia-induced lactate release in the cerebral cortex, which was normalized by OP treatment. Cortical dye loading experiments revealed hemichannel dysfunction in HE with improvement following OP treatment, while the expression of key connexins was unaffected. **Conclusion.** The results of the present study demonstrate that HE is associated with CNS hemichannel dysfunction, with ammonia playing a key role. The data provide evidence of a potential neuronal energy deficit due to impaired hemichannel-mediated lactate transport between astrocytes and neurons as a possible mechanism underlying pathogenesis of HE.

Hepatic encephalopathy (HE) is a serious neuropsychiatric complication associated with liver dysfunction, diagnosed when other known brain disorders are excluded(1).

HE comprises a range of symptoms including sleep disturbances, alterations in cognitive, behavioural, fine motor and psychomotor functions, with coma and death occurring at the late stages(2). Several hypotheses regarding the pathogenesis of HE have been proposed and numerous factors have been suggested as key players in HE, including: inflammation(3), oxidative stress(4), impaired brain energy metabolism(5) and (the most common one) the neurotoxic effects of ammonia(6).

Recent evidence demonstrated that astroglial lactate production and release in cortical cultures and in the somatosensory cortex of anaesthetised rats is facilitated in the presence of ammonia(7). This appears to be due to acidification of the mitochondrial matrix resulting in a direct inhibition of mitochondrial pyruvate uptake. Increased brain lactate levels have also been reported in hyperammonemic conditions such as in acute liver failure (ALF), which is thought to be due to inhibition of the tricarboxylic acid cycle enzyme α -ketoglutarate dehydrogenase, suggesting a reduction in oxidative metabolism (8). It remains unknown whether significant changes in brain lactate metabolism develop in conditions of long-term CNS exposure to increased ammonia concentration, such as that seen during chronic liver disease (CLD) or HE.

Astrocytes, the most numerous type of glial cell in the CNS, are thought to play an important role in HE pathogenesis. The astrocytic dysfunction developing during the progression of the disease could precipitate neuronal pathology, leading to

neurological impairment. Astrocytes are extensively connected by gap junctions formed of connexins, which also exist as functional hemichannels allowing effective transfer of ions, metabolic substrates and signaling molecules across the plasma membrane(9). Under normal physiological conditions, hemichannels are either closed(10) or in a flickering state(11). In certain pathological conditions, such as epilepsy and ischaemia, significant changes in astroglial structure and function may occur, which are associated with changes in connexin hemichannel function, affecting coupling within the astroglial networks and their communication with other brain cells(12). We hypothesised, therefore, that in HE, connexin hemichannel dysfunction contributes to the development of its neurological features.

There is recent evidence that hemichannels may function as a conduit of lactate transport across the membrane(13). In this study we first investigated whether connexin hemichannel expression and hemichannel-mediated release of lactate are altered in animal models of HE. Ornithine phenylacetate (OP, OCR-002; Ocera Therapeutics, CA, USA) has been shown to reduce ammonia levels in animal models of cirrhosis and ALF. OP treatment was found to be associated with a significant reduction in the severity of brain swelling(14), improvement in neurophysiological function(15) and reduction in intracranial pressure(16). Therefore, we applied OP in this study as an experimental ammonia-lowering treatment. The data obtained demonstrate that, in HE, ammonia mediates cortical hemichannel dysfunction associated with a significant reduction in hemichannel-mediated lactate release.

Materials and Methods

All the experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA) revised according to the European Directive 2010/63/EU. All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Animal models

Male Sprague-Dawley rats (body weight ~350-400g) were obtained from a commercial supplier (Charles Rivers Laboratories, Inc.).

Bile duct ligation (BDL) surgery: Under general anaesthesia (5% isoflurane in 100% oxygen for induction, 2% isoflurane in air for maintenance) 30 rats underwent triple ligation of the bile duct via a small laparotomy to induce chronic liver injury and were studied 28 days after the surgery (17).

Non-cirrhotic hyperammonemia condition: 32 rats were administered high ammoniagenic diet (HA). The amino acid recipe used for a stock of ~100g was: 15g leucine, 7.7g phenylalanine, 7g glutamate, 10g alanine, 4.4g proline, 5.8g threonine, 11g aspartate, 5g serine, 4.8g glycine, 3.3g arginine, 9.6g lysine, 8.4g histidine, 3g tyrosine, 1.5g tryptophan and 10.6g valine. 25 g of this mix (mixed 1:5 with standard rodent chow powder) was freshly prepared daily with water in a mash form and rats

were given free access to it for 5 days. The recipe approximates the amino acid composition of a rodent haemoglobin(18), mimicking the effect of gastrointestinal bleeding, which is a known to result in systemic hyperammonemia (19).

Ornithine Phenylacetate (OP) treatment: 3 weeks after the surgery, 24 BDL-operated rats were given twice daily intraperitoneal injections of combined L-ornithine and phenylacetate (0.3g/kg; OP) ~7 hours apart for 5 days – a dosing regimen that has previously been shown to effectively reduce plasma ammonia concentration(20). The rats were studied on day 28 post BDL surgery, within 3 hours of the last OP injection.

Blood and brain tissue were collected under terminal isoflurane anaesthesia. Plasma biochemistry was performed using a Cobas Integra II system (Roche Diagnostics).

In vitro slice preparation

Rats were humanely sacrificed by isoflurane inhalation overdose. After cardiac perfusion with chilled (4°C) artificial cerebrospinal fluid (aCSF, 124mM NaCl, 3mM KCl, 2mM CaCl₂, 26mM NaHCO₃, 1.25mM NaH₂PO₄, 1mM MgSO₄, 10mM D-glucose saturated with 95% O₂, 5% CO₂, pH 7.5, *PCO*₂ 35 mmHg), with an additional 9mM Mg²⁺, the brain was rapidly removed and placed in a bath of chilled (4-6°C) aCSF. 300 µm coronal cortical slices were cut using a vibrating microtome. The slices were recovered in oxygenated (95% O₂, 5% CO₂) aCSF at room temperature for 30 min.

Western blot

Proteins (30 µg) extracted from the cortices of 5 sham-operated, 6 BDL, 6 HA and 5 BDL-OP-treated rats were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on a 4% to 12% Bis-Tris NuPAGE gel (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked with 5% Bovine Serum Albumin and incubated with antibodies against connexin-43 (Cell Signaling Technology, 1:1000), connexin-36 (Santa Cruz, 1:1000), connexin-30 (Invitrogen, 1 µg/mL) and connexin-26 (Thermo Fisher Scientific, 1 µg/mL). Detection of actin (Santa Cruz, 1:1000) was used to control for protein loading. Binding of antibody was detected using a horseradish peroxidase-conjugated secondary antibody (Goat anti-rabbit or goat anti-mouse IgG-HPR, Santa Cruz, 1:10000) where appropriate and the SuperSignal Chemiluminescence Substrate for detection of horseradish peroxidase (Pierce). Densitometric analysis was performed using Kodak 1D image analysis software (Kodak, Rochester, NY).

Measurements of lactate release using microelectrode biosensors

Amperometric enzymatic biosensors were obtained from Sarissa Biomedical (Coventry, UK). The design and operation of the biosensors were described in detail previously (21, 22). All sensors were operating against a reference electrode (Ag/AgCl) and had a linear response to lactate within the concentration range recorded in this study (22, 23). Further information on the principle of operation and response time is given in the online data supplement (Supplementary Figure 1A-C). To control for the release of non-specific electroactive interferants, a dual recording configuration was used. In every recording, a “null” sensor, lacking enzymes but

otherwise identical, was used to measure current changes not associated with lactate oxidase activity, which were then subtracted from the current recorded by the lactate biosensor (24) (Figure 1).

The sensors were calibrated directly in the slice chamber immediately before and after every recording by application of 100 μM of lactate (Figure 1). To convert changes in the biosensor current to changes in lactate concentration, an average of sensor calibrations before and after the recording were used. For each of the recordings, a slice was transferred into the recording chamber and superfused with aCSF at 35°C (3 ml min⁻¹). Sensors were initially placed in the chamber having no contact with the brain slice. Once a steady-state baseline was achieved, the sensors were laid flat in direct contact with the surface of the slice (Figure 1), revealing tonic lactate release which stabilised within ~15 min. Hypoxic conditions, known to increase both lactate production due to inhibition of oxidative phosphorylation and the opening probability of astroglial connexin hemichannels (23, 25), were induced for 2-4 min by replacement of oxygen in the medium with nitrogen (perfusion of the chamber with aCSF saturated with 95% N₂/5% CO₂) (26, 27). As detection of lactate by the biosensors requires oxygen (Supplementary Figure 1B) (28), the effect of hypoxia was determined by measuring the peak lactate release upon re-oxygenation (Figure 1) as described in detail previously (23, 28). Once the baseline was restored, Ca²⁺-free aCSF (with the addition of 1 mM EGTA) was applied for 20 min as the second stimulus known to increase the opening probability of certain membrane channels, including connexin hemichannels (29). There is no prior evidence that Ca²⁺-mediated increases in mitochondrial NADH influence cytosolic NAD⁺/NADH

homeostasis and therefore lactate production(30). These stimuli were reapplied in the presence of connexin hemichannel blockers carbenoxolone (CBX, 100 μ M; Sigma) or 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, 200 μ M, Sigma). CBX and NPPB were previously shown to have no effect on lactate biosensor detection system(23).

Assessment of hemichannel function by dye loading

For the assessment of hemichannel functionality (effectiveness of channel opening and closing) we used a fluorescent dye carboxyfluorescein (CBF: 376 Da). Connexin hemichannels are permeable to CBF and can act as conduits of CBF transfer across the membrane in accord with the concentration gradient of the dye. Cortical slices from sham-operated, BDL, HA and BDL-OP-treated rats were exposed to control aCSF with an addition of CBF (200 μ M) for 9 min resulting in background connexin-mediated dye loading (Figure 5A-B), followed by perfusion with Ca^{2+} -free aCSF without CBF for 9 min resulting in CBF unloading. Ca^{2+} -free aCSF with CBF was then applied for 4 min increasing the permeability of hemichannels and therefore resulting in dye loading. Hypoxic conditions (without CBF) were next applied to unload the slice and also to demonstrate bidirectional permeability of the channel to CBF(27). The same hypoxic stimulus was then re-applied in the presence of CBF, resulting in dye loading. After application of each stimulus in the presence of CBF, a further 5 min perfusion with aCSF containing CBF, followed by a 10 min wash with normal aCSF was applied, enabling the channels to return to their physiological state. Images were taken using MiCAM-02 imaging system (SciMedia). Using ImageJ software, regions of interest (ROI) were drawn around an area of the

cerebral cortex ($\sim 3\text{cm}^2$) across layers I-III and the mean pixel intensities for the ROI were calculated. Background fluorescence was subtracted.

Statistical analysis

Western blot data were normalised using the protocol of LI-COR Biosciences (Normalization Accuracy for Western Blotting) and group data were compared using two-way ANOVA with Tukey post hoc test. Data obtained using biosensor recordings were analysed and presented non-parametrically using box and whisker plots (Figures 2A, 3A, 4A). For the comparisons between the experimental groups, Mann Whitney U test was applied. The peak hypoxia- or 0Ca^{2+} -induced lactate releases are presented as changes in release from the baseline (Figure 1). The effects of connexin blockers are presented as % changes from the control responses recorded in the absence of the blockers and Wilcoxon signed rank test was applied for comparison. *P* values in Figures 2-4B,C indicate significance level of differences between the control responses and the responses recorded in the presence of the drugs.

Data obtained in dye loading experiments were analysed using two-way ANOVA (Figure 5B, data normally distributed) followed by Tukey post hoc test or Wilcoxon signed rank test (Figures 5C-D, data not normally distributed), as appropriate. The biochemistry data was analysed using one-way ANOVA. Data are reported as mean $\pm$ SEM. Differences with *p* value of <0.05 were considered to be significant. In all the experiments the 'n' number represents the number of animals. Sample sizes were calculated using Gpower 3 v3.1.9.2 (<http://www.gpower.hhu.de/en.html>)(31);

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using a ‘means: Wilcoxon-Mann-Whitney test (two groups)’ test, with a desired power of 90% and a significance level of 5%. The effect size varied between groups according to the preliminary data acquired during the study. Statistical analysis was performed using OriginPro 9.1 (OriginLab).

Results

Biochemistry

Plasma biochemistry and ammonia concentration was assessed in all groups of animals (Supplementary Table 1). Compared to sham surgery, BDL resulted in a significant increase in plasma 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 rats with OP had no effect on these parameters. Rats fed with HA diet had similar plasma biochemistry to control rats.

Plasma ammonia concentrations were significantly higher in BDL and HA rats when compared to sham-operated animals ($p<0.001$). Treatment of BDL animals with OP lowered plasma ammonia concentration, which was similar to that measured in sham-operated animals ($p=0.3$) (Supplementary Table 1).

Release of lactate in the cerebral cortex in animal models of HE

In cortical slices of sham-operated animals, enzymatic amperometric biosensors detected tonic lactate efflux of $335\pm 10\ \mu\text{M}$ ($n=18$). Recordings from cortical slices of BDL and HA rats showed lower tonic release of lactate of $203\pm 6\ \mu\text{M}$ ($p=0.03$, $n=18$) and $178\pm 8\ \mu\text{M}$ ($p=0.005$, $n=16$), respectively (Figure 2A). Increasing the permeability of connexin hemichannels by lowering $[\text{Ca}^{2+}]_e$ (0 Ca^{2+} conditions) triggered similar increases in the release of lactate in sham-operated rats (by $43\pm 3\ \mu\text{M}$, $n=15$), BDL (by $38\pm 4\ \mu\text{M}$; $p=0.07$, $n=17$) and HA animals (by $54\pm 4\ \mu\text{M}$; $p=0.5$, $n=16$) (Figure 3A). Hypoxia facilitated release of lactate in cortical slices of sham-operated rats ($43\pm 3\ \mu\text{M}$, $n=18$), but had no effect on lactate release in slices of BDL ($1\pm 0.8\ \mu\text{M}$; $p<0.001$,

n=18) and HA animals (5 ± 2 μ M; $p < 0.001$, n=16) (Figure 4A). These results demonstrated impaired tonic and hypoxia-induced release of lactate in both animal models of HE.

Ammonia lowering treatment restores cortical lactate release

Daily OP treatments had been shown to decrease systemic and brain ammonia concentrations in BDL animals(20). We next found that in our experiments, OP treatment of BDL rats restored tonic (374 ± 11 μ M; $p = 0.4$, n=14) and hypoxia-induced (32 ± 3 μ M; $p = 0.6$, n=14) lactate release similar to that recorded in cortical slices of sham-operated animals (Figure 2A-4A). Direct application of OP on cortical slices of sham-operated and BDL rats had no effect on lactate release (Supplementary Figure 1D). These results strongly suggest that high ammonia levels are responsible for the reduction in lactate release in the cerebral cortex of BDL animals.

Impaired hemichannel function underlies reduced cortical lactate release in animal models of HE

In cortical slices of sham-operated animals, application of hemichannel blockers CBX (n=9) or NPPB (n=9) resulted in a significant reduction in lactate tone (Figure 2B-C). Hemichannel blockade had no effect on lactate tone in cortical slices of BDL animals (Figure 2B-C). However, hemichannel blockade had an effect on lactate tone recorded in cortical slices of HA animals (CBX: n=10; NPPB: n=8) (Figure 2B-C). CBX and NPPB reduced lactate tone in cortical slices of BDL animals treated with OP (CBX: n=10; NPPB: n=10) (Figure 2B-C).

In cortical slices of sham-operated (CBX: n=8; NPPB: n=6) and HA animals (CBX: n=10; NPPB: n=8), hemichannel blockade using CBX or NPPB abolished or significantly reduced the amount of lactate release facilitated in Ca^{2+} -free conditions (Figure 3B-C). Smaller effect of hemichannel blockers on 0 Ca^{2+} -induced release of lactate was observed in cortical slices of BDL rats (CBX: n=11; NPPB: n=9) (Figure 3B-C). In conditions of OP treatment, when basal and evoked lactate release were restored in cortical slices of BDL rats, CBX (n=7) and NPPB (n=7) abolished the release of lactate triggered by 0 Ca^{2+} (CBX: n=7; NPPB: n=7); an effect similar to that observed after application of hemichannel blockers in cortical slices of sham-operated animals (Figure 3B-C).

Hypoxia-induced release of lactate recorded in cortical slices of sham-operated animals was also abolished or dramatically reduced by connexin blockade (CBX: n=9; NPPB: n=7) (Figure 4B-C). CBX and NPPB had no significant effect on the release of lactate induced by hypoxia in cortical slices of BDL (CBX: n=13; NPPB: n=9) and HA rats (CBX: n=10; NPPB: n=12) (Figure 4B-C). In cortical slices of OP-treated BDL rats, the effects of CBX and NPPB were restored. CBX (n=8) and NPPB (n=7) effectively abolished hypoxia-induced lactate release in cortical slices of BDL rats treated with OP (Figure 4B-C).

Application of MCT blocker, α -Cyano-4-hydroxycinnamic acid (4-CIN) had no significant effect on tonic release of lactate in cortical slices of sham-operated (n=5) and BDL rats (n=5) (Supplementary Figure 2A-B). Hypoxia-induced lactate release recorded in sham-operated rats (n=5) was significantly reduced by 4-CIN, as

reported previously(13). In cortical slices of BDL rats (n=5) the effect of 4-CIN on hypoxia-induced release of lactate was found to be smaller (Supplementary Figure 2A-B).

These results demonstrate that connexin hemichannel blockade has no effect on the release of lactate in the cerebral cortex of BDL rats. This implies that the function of hemichannels, which act as conduits of lactate release(23), is already compromised in the brains of these animals. These data also suggest that cortical hemichannel dysfunction in the BDL animals is likely to be due to the actions of ammonia.

Hemichannel-mediated dye loading in animal models of HE

Membrane channel-mediated dye loading experiments were next performed in cortical slices to confirm hemichannel dysfunction in HE.

In cortical slices of sham-operated rats (n=10), significant background loading (14.3 ± 0.4 A.U) was observed in control conditions when slices were perfused with aCSF containing CBF (Figure 5A-B). Increasing the permeability of hemichannels by lowering $[Ca^{2+}]_e$ in the absence of CBF reduced slice fluorescence by 6.4 ± 0.2 A.U. The same stimulus applied in the presence of CBF significantly increased fluorescence by 16.9 ± 0.6 A.U. Hypoxia-induced opening of hemichannels in the absence of CBF resulted in dye unloading with fluorescence decreasing by 12.7 ± 0.4 A.U. Addition of CBF in conditions of hypoxia increased slice fluorescence by 13.9 ± 0.4 A.U (Figure 5B).

Cortical slices from HA rats (n=10) displayed background loading (Figure 5A-B) and 0 Ca^{2+} -induced unloading of 11.2 ± 0.2 A.U ($p=0.04$) and 4.7 ± 0.08 A.U ($p=0.4$) respectively (Figure 5B). Addition of CBF in 0 Ca^{2+} conditions increased fluorescence by 11.6 ± 0.2 A.U ($p=0.004$), similar to that observed in slices of sham-operated animals. However, the effect of hypoxia was significantly reduced in slices of HA animals (unloading by 7.6 ± 0.1 A.U, $p<0.001$; loading by 7.8 ± 0.2 A.U, $p<0.001$) (Figure 5B).

In cortical slices of BDL rats (n=10) efficacy of CBF dye loading and unloading was markedly reduced under all the conditions (Figure 5A-B). In cortical slices of BDL animals treated with OP, hemichannel-mediated CBF dye loading and unloading were similar to that observed in slices of sham-operated animals (Figure 5A-B).

Hemichannel blockade with CBX or NPPB effectively abolished CBF dye loading and unloading in cortical slices of sham-operated animals (Figure 5C-D). In cortical slices of BDL and HA rats, CBX and NPPB had no significant effect on dye loading and unloading (Figure 5C-D). In cortical slices of OP-treated BDL rats, the effects of CBX and NPPB on CBF dye loading and unloading were similar to that observed in sham-operated animals. (Figure 5C-D). Figures 5C-D illustrate changes in fluorescence ($\Delta\text{Fluorescence}$) following application of the hemichannel blockers, compared to the respective changes in fluorescence recorded in the absence of blockers in slices of the same animal. Negative values show decreases in fluorescence while positive values illustrate higher fluorescence levels compared to the controls.

These data show that the background activity and stimuli-evoked opening and closure of connexin hemichannels is impaired in BDL and HA rats. The efficacy of CBF dye loading is restored by OP treatment of BDL animals suggesting that the actions of ammonia are responsible for cortical hemichannel dysfunction in HE.

Cortical connexin expression in animal models of HE

We next evaluated the expression of main astrocytic and neuronal connexins in animal models of HE used in this study. Western blots were performed on proteins extracted from the cerebral cortices of sham-operated, BDL, HA and BDL-OP animals (Figure 6). No differences in cortical connexin-43, connexin-36 and connexin-30 expression was observed between sham-operated, BDL, HA and OP-treated BDL animals. An increase in connexin-26 expression ($p=0.03$) was observed in BDL-OP rats compared to the BDL animals (Figure 6). Expression of MCT-1 was similar in all the experimental groups (Supplementary Figure 2C).

Discussion

Brain information processing requires constant and sufficient supply of oxygen and metabolic substrates. Astrocytes represent an important source of lactate which contributes to the extracellular pool of readily available metabolic substrates taken up by neurons to fuel their activity(32). While previously, lactate transport across the cell membranes was thought to be achieved solely via operation of monocarboxylate transporters (MCTs), a recent study(23) demonstrated the role of connexin hemichannels as equally important conduits of lactate release.

In animal models of ALF and patients, an increase in brain lactate concentration has consistently been reported(33). Concentrations of lactate in the cerebrospinal fluid were also found to be elevated in cirrhotic patients but only in severe cases of HE(34). The BDL and HA rats used in our experiments are models of minimal HE(35). In contrast to the existing evidence suggesting that brain lactate concentrations are increased in patients with ALF(36), our experiments demonstrated that the development of HE in rats is associated with a significant reduction in tonic and stimulated release of lactate in the cerebral cortex. Blockade of connexin hemichannels was found to be effective in reducing lactate release in sham-operated and HA animals but was ineffective in BDL rats, suggesting that the reduction in hemichannel-mediated lactate release in BDL animals is due to a combination of pathological factors (e.g. inflammation, oxidative stress).

Increased lactate production by astrocytes appears to be essential for the recovery of synaptic function during re-oxygenation after hypoxia(37). We found that hypoxia-

induced lactate release was significantly lower in the cerebral cortex of BDL and HA rats compared to control animals and this was unaffected by the hemichannel blockers indicating hemichannel dysfunction. The observed decrease in extracellular lactate is likely due to impaired release from astrocytes although increased neuronal activity and therefore lactate consumption cannot be excluded. The observation of Bosoi et al.,(38) showing higher total brain lactate of BDL rats using NMR spectroscopy seems to contradict our data. Bosoi and colleagues suggested that increased lactate contributes to the pathogenesis of brain edema (cytotoxic), and may imply that the observed increase in total brain lactate is due to its intracellular accumulation. If the rate of lactate production and glymphatic clearance (39) are not affected, intracellular retention of lactate would explain higher concentration of this metabolite as measured by NMR spectroscopy (40) and would be in full agreement with our data showing impairment of hemichannel-mediated release in HE.

High concentration of ammonia can potentially generate significant pH changes, which can have various effects on many pH sensitive membrane channels, including hemichannels. The pH sensitivity of hemichannels is known as the chemical gate, which is the phenomenon of hemichannel blockade when intracellular pH (pH_i) decreases(40). This provides one potential mechanism which might be responsible for impaired hemichannel-mediated lactate release in HE.

We also examined hemichannel functionality using the dye loading method. Some differences between the data obtained using biosensor recordings and this technique could be due to the fact that CBF is not identical to the molecular structure and size

of lactate. Additionally, lactate could be released through specific connexin hemichannels, whereas CBF is small enough to pass through the majority of hemichannels expressed by both astrocytes and neurons. While induction of hypoxia targets predominantly astroglial hemichannels, the rest of the conditions are not cell specific and conclusions on affected cell types cannot be drawn from the results obtained using dye loading experiments.

The experimental stimuli used (0 Ca^{2+} and hypoxia) are known to increase the permeability of hemichannels, possibly by affecting various protein bonds resulting in conformational changes(29). Dye loading experiments clearly demonstrated a marked reduction in fluorescent dye uptake and release in cortical slices of BDL and HA rats compared to sham-operated animals (the differences were more profound when hypoxia was used as a stimulus), suggesting reduced bidirectional permeability of hemichannels in these animal models of HE. Hemichannel blockade had no effect on fluorescent dye uptake in BDL and HA rats providing additional evidence that the function of these channels is already compromised in HE. Ammonium ions may cause structural alterations to the connexin proteins, by interacting with various amino acid side chains, which could affect gating of the channel. However, since hemichannels have a relatively short life cycle and recycled frequently, the observed changes in hemichannel functionality appear to be reversible with OP treatment.

In CLD, hyperammonemia is believed to impair mitochondrial function and induce astroglial dysfunction, which is associated with altered neurotransmitter recycling

leading to neuronal damage(41). Ammonia may also interfere with cell energy metabolism in several ways. There is recent evidence that in astrocytes ammonia may divert the flux of pyruvate to lactate production, contributing to the net aerobic lactate production(7). However, the effects of chronic ammonia exposure on astrocytes are unknown. We investigated the role of ammonia by treating BDL rats with OP, a drug known to lower systemic and brain ammonia(20). OP treatment improved the neurochemical phenotype of BDL animals by restoring the tonic and stimulated connexin hemichannel-mediated lactate release. Furthermore, hemichannel blockade became effective following OP treatment, suggesting that ammonia is indeed responsible for hemichannel dysfunction observed in this model.

Cytotoxic brain edema observed in BDL animals is attenuated by ammonia-lowering treatments such as the one used in this study(20, 42). The effect of cell swelling on hemichannel function is poorly understood. Ye et al.,(43) showed that astrocytes obtained from connexin-43 knock-out animals developed cell swelling as efficiently as the wild type animals when exposed to a hypotonic solution suggesting that hemichannels do not play a significant role in this process although evidence to the contrary exists (44).

We also examined the expression profile of key astroglial and neuronal connexins in the animal models of HE used in this study. No significant differences in connexin hemichannel protein expression profile were observed suggesting that HE is associated with altered hemichannel function but not with changes in connexin expression. The upregulated expression of connexin-26 observed in the BDL-OP

animals is not prominent enough to explain the marked improvement observed in the lactate measurements and dye loading experiments. Additionally, we did not observe any changes in the expression of the main astroglial lactate transporter MCT-1 in the models of HE used in this study. No effect of 4-CIN, a MCT blocker, on lactate release was observed in cortical slices of sham-operated and BDL animals. Hypoxia-induced lactate release in sham-operated rats was significantly reduced by the application of 4-CIN, as demonstrated in the previous study (23).

Depletion of lactate as one of the key readily available metabolic substrates may have important neurological consequences particularly in patients with advanced cirrhosis given the fact that these patients display evidence of cerebral vasoconstriction(45), which is associated with impaired cerebral autoregulation, an important mechanism which ensures constant cerebral blood flow (46). Clinical consequences of this may be relevant during liver transplantation, where further reductions in cerebral blood flow have been observed during the anhepatic phase of transplantation and may contribute to post-transplant neurologic dysfunction(47). Evidence for critical reduction in cerebral oxygenation was obtained in the majority of patients with acute-on-chronic liver failure who had poor neurologic outcome supporting the hypothesis that the brain energy metabolism is critically compromised in cirrhosis and further perturbations as demonstrated in this paper may be clinically deleterious(48). Our data indicating an impaired hemichannel-mediated lactate release during tissue hypoxia, in combination with these observations could help to explain the severe neurological manifestations in patients with HE. As ammonia is central in causing this dysfunction, the potential clinical implications involve the use

of ammonia lowering treatments as the main therapeutic strategy, as well as attempts to increase cerebral oxygenation in order to preserve the neuronal function.

In conclusion, the results of the present study suggest that HE is associated with CNS hemichannel dysfunction, with ammonia playing a key role. The data provide evidence of a potential neuronal energy deficit due to impaired hemichannel-mediated lactate transport between astrocytes and neurons as a possible mechanism underlying pathogenesis of HE.

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LEGENDS TO FIGURES

Figure 1: Measuring release of lactate using microelectrode biosensors.

Representative example of changes in the net lactate biosensor current (difference in current between lactate and null sensors) during calibration (100 μ M lactate), after biosensor placement in a direct contact with the surface of the cortical slice (recording tonic lactate release), in response to a hypoxic challenge (perfusion with aCSF saturated with 95% N₂/5% CO₂), and in response to lowering extracellular [Ca²⁺]. Peak hypoxia-induced lactate release is measured upon re-oxygenation. *Inset*: Schematic drawing of the dual recording configuration of lactate and null (control) biosensors placed on the surface of the slice.

Figure 2: HE is associated with a reduction in hemichannel-mediated tonic release of lactate in the cerebral cortex. A) Summary data illustrating

tonic release of lactate in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats. *p* values indicate differences from sham-operated group. **B)**

Left: Summary data illustrating the effect of hemichannel blocker carbenoxolone (CBX, 100 μ M) on tonic release of lactate (expressed as % change from the baseline) in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats. *Right*: Representative recordings of lactate biosensor current showing changes in tonic release of lactate in response to CBX application. *p* values indicate the level of significant differences from the respective baseline. **C) Left**: Summary data illustrating the effect of 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB, 200 μ M) on tonic release of

lactate (expressed as % change from the baseline) in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats. *Right*: Representative recordings of lactate biosensor current showing changes in tonic release of lactate in response to NPPB application. *p* values indicate the level of significant differences from the respective baseline.

Figure 3: HE is associated with a reduction of lactate release in response to lowering extracellular $[Ca^{2+}]_e$, which promotes hemichannel opening. **A)** Summary data illustrating peak changes in lactate release in response to lowering $[Ca^{2+}]_e$ in cortical slices of sham-operated, BDL, HA and BDL-OP-treated rats. *p* values indicate differences from the responses in sham-operated animals. **B) Left**: Summary data illustrating the effect of CBX (100 μ M) on the release of lactate facilitated in response to 0 $[Ca^{2+}]_e$ (expressed as the % of the amount of lactate released in response to 0 $[Ca^{2+}]_e$ in the absence of CBX) in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats. *Right*: Representative recordings of lactate biosensor current showing the effect of CBX on 0 $[Ca^{2+}]_e$ -induced release of lactate. *p* values indicate differences between the responses recorded in the absence and presence of CBX. **C) Left**: Summary data illustrating the effect of NPPB (200 μ M) on the release of lactate facilitated in response to 0 $[Ca^{2+}]_e$ (expressed as the % of the amount of lactate released in response to 0 $[Ca^{2+}]_e$ in the absence of NPPB) in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats. *Right*: Representative recordings of lactate biosensor current showing the effect of NPPB on 0 $[Ca^{2+}]_e$ -induced release of

lactate. *p* values indicate differences between the responses recorded in the absence and presence of NPPB.

Figure 4: HE is associated with a reduction of hypoxia-induced release

of lactate. A) Summary data illustrating peak changes in lactate release in

response to hypoxia (aCSF saturated with 95% N₂/5% CO₂) in cortical slices

of sham-operated, BDL, HA and BDL-OP-treated rats. *p* values indicate

differences from the responses in sham-operated animals. **B) Left:** Summary

data illustrating the effect of CBX (100 μM) on the release of lactate facilitated

in response to tissue hypoxia (expressed as the % of the amount of lactate

released in response to hypoxia in the absence of CBX) in cortical slices of

sham-operated, BDL, HA and BDL-OP treated rats. *Right:* Representative

recordings of lactate biosensor current showing the effect of CBX on hypoxia-

induced release of lactate. Decrease in O₂ availability reduces biosensor

current followed by a positive signal upon re-oxygenation, which is used to

estimate hypoxia-induced lactate release. *p* values indicate differences

between the responses recorded the absence and presence of CBX. **C) Left:**

Summary data illustrating the effect of NPPB (200 μM) on the release of

lactate facilitated in response to hypoxia (expressed as the % of the amount

of lactate released in response to hypoxia in the absence of NPPB) in cortical

slices of sham-operated, BDL, HA and BDL-OP-treated rats. *Right:*

Representative recordings of lactate biosensor current showing the effect of

NPPB on the hypoxia-induced release of lactate. *p* values indicate differences

between the responses recorded in the absence and presence of NPPB.

Figure 5: Impaired hemichannel-mediated dye loading reveals cortical**hemichannel dysfunction in HE. A)** Representative images of background

loading with carboxyfluorescein (CBF) dye in cortical slices of sham-operated, BDL, HA and BDL-OP-treated rats. White dashed line depicts the edge of the cortical slice. **B)** Fluorescence intensity changes in cortical slices of sham-

operated, BDL, HA and BDL-OP treated rats in response to 0 $[Ca^{2+}]_e$ and hypoxia in the absence and presence of CBF in the medium. Application of 0

$[Ca^{2+}]_e$ aCSF or hypoxia in the presence of CBF results in dye loading and increase in fluorescence, while application of these stimuli in the absence of

CBF results in dye unloading and decrease in fluorescence. *Insets:* Schematic drawings of connexin hemichannel mediated dye loading and unloading. *

$p < 0.05$, ** $p < 0.001$ significant differences from the sham-operated group. **C)** Summary data illustrating the effect of CBX (100 μ M) on fluorescence

intensity changes (Δ Fluorescence) in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats induced by 0 $[Ca^{2+}]_e$ and hypoxia in the absence

and presence of CBF in the medium. The data are presented as differences in fluorescence following CBX application compared to the respective

fluorescence recorded in the absence of CBX. * $p < 0.05$ significant effect of CBX on CBF loading and unloading. **D)** Summary data illustrating the effect of

NPPB (200 μ M) on fluorescence intensity changes (Δ Fluorescence) in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats induced by 0

$[Ca^{2+}]_e$ and hypoxia in the absence and presence of CBF in the medium. The data are presented as differences in fluorescence following NPPB application

compared to the respective fluorescence recorded in the absence of NPPB. *
 $p < 0.05$ significant effect of NPPB on CBF loading and unloading.

Figure 6: Cortical connexin expression is not affected in HE. Summary data illustrating means $\pm$ SE of the densitometry of connexin-43, connexin-36, connexin-30 and connexin-26 protein expression normalized to the expression of Actin, in cell lysates of the cerebral cortices of sham-operated, BDL, HA and BDL-OP treated rats. *Bottom:* Representative immunoblots showing connexin-43, connexin-36, connexin-30 and connexin-26 protein expression in cerebral cortices of sham-operated, BDL, HA and BDL-OP treated rats. p value indicates difference in expression level between BDL and BDL-OP groups.

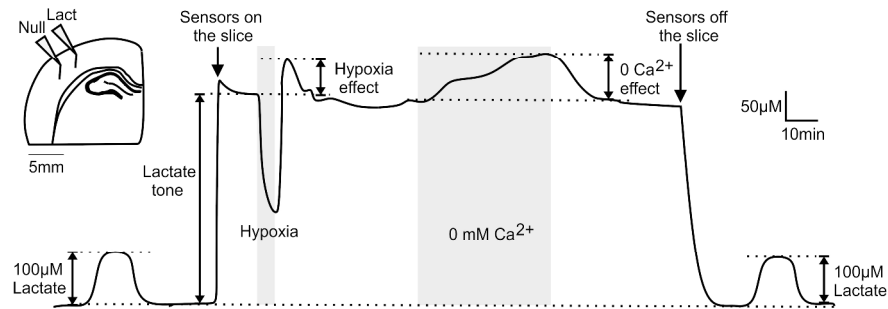


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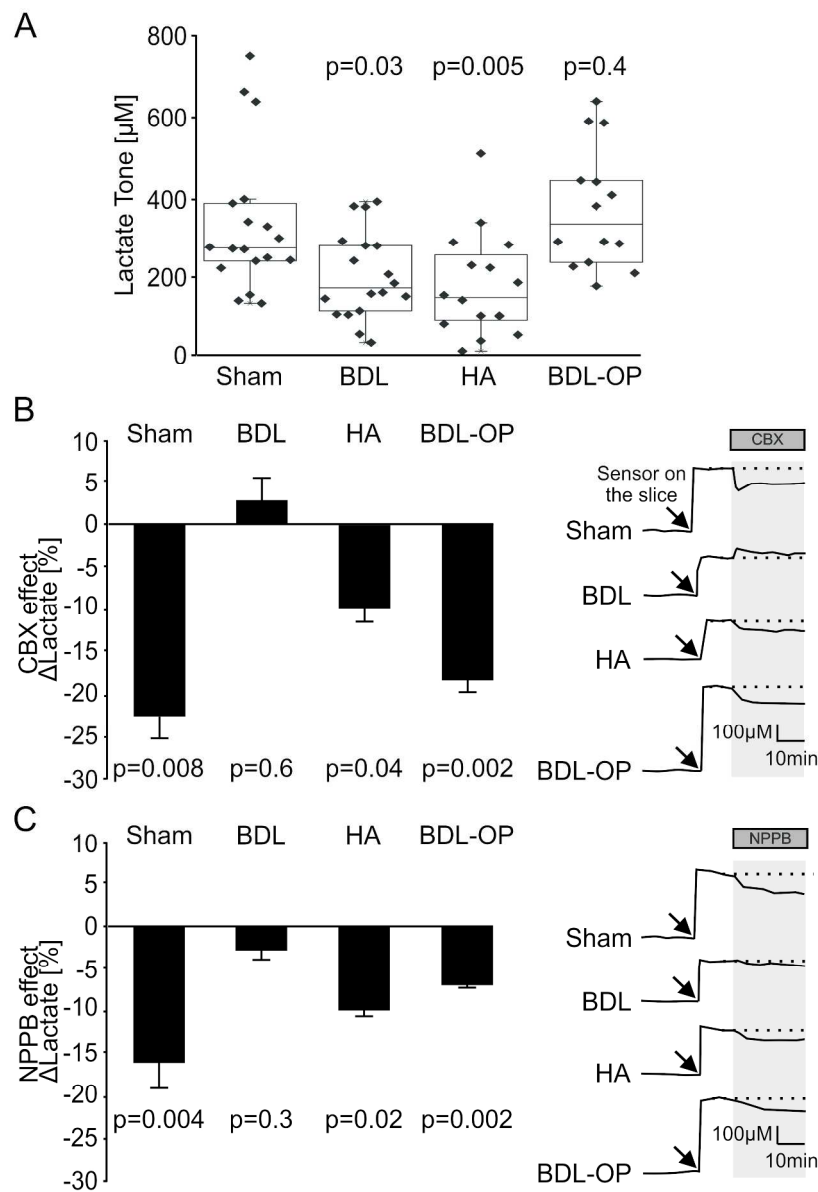


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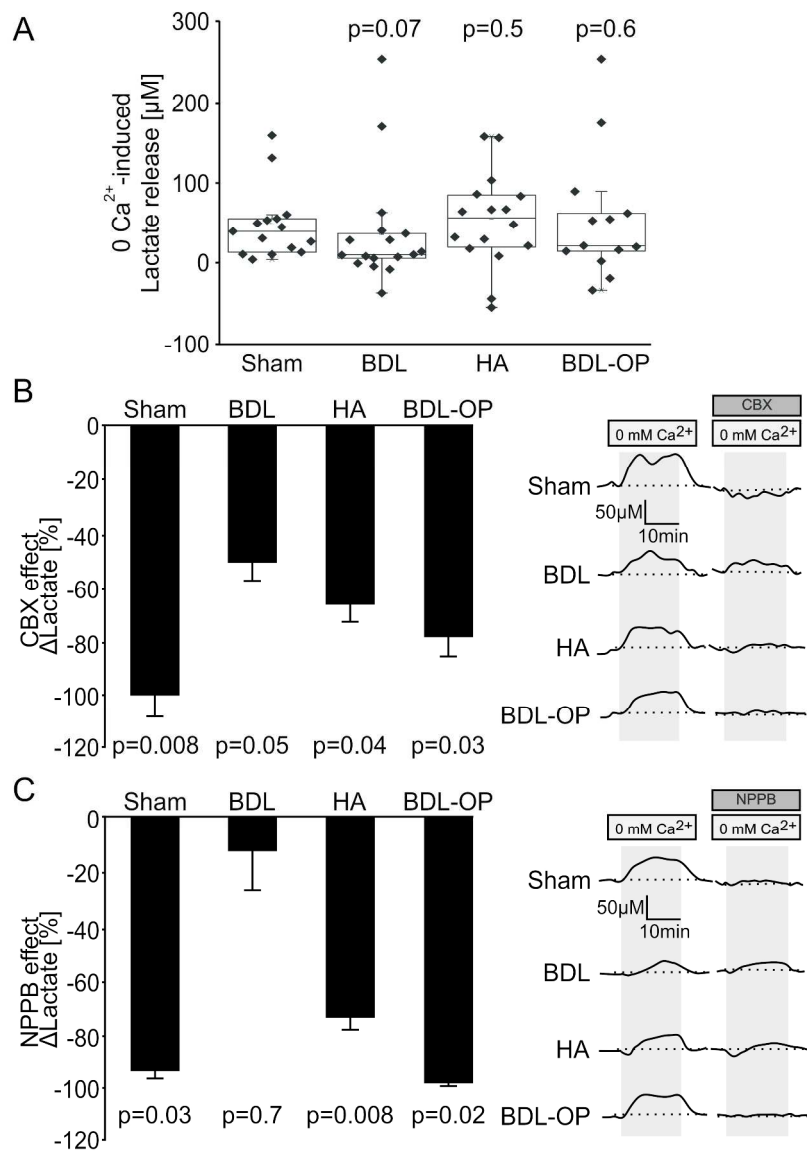


Figure 3: HE is associated with a reduction of lactate release in response to lowering extracellular $[\text{Ca}^{2+}]$, which promotes hemichannel opening. **A**) Summary data illustrating peak changes in lactate release in response to lowering $[\text{Ca}^{2+}]_e$ in cortical slices of sham-operated, BDL, HA and BDL-OP-treated rats. p values indicate differences from the responses in sham-operated animals. **B**) *Left:* Summary data illustrating the effect of CBX (100 μM) on the release of lactate facilitated in response to 0 $[\text{Ca}^{2+}]_e$ (expressed as the % of the amount of lactate released in response to 0 $[\text{Ca}^{2+}]_e$ in the absence of CBX) in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats. *Right:* Representative recordings of lactate biosensor current showing the effect of CBX on 0 $[\text{Ca}^{2+}]_e$ -induced release of lactate. p values indicate differences between the responses recorded in the absence and presence of CBX. **C**) *Left:* Summary data illustrating the effect of NPPB (200 μM) on the release of lactate facilitated in response to 0 $[\text{Ca}^{2+}]_e$ (expressed as the % of the amount of lactate released in response to 0 $[\text{Ca}^{2+}]_e$ in the absence of NPPB) in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats. *Right:* Representative recordings of lactate biosensor current showing the effect of NPPB on 0 $[\text{Ca}^{2+}]_e$ -induced release of lactate. p values

indicate differences between the responses recorded in the absence and presence of NPPB.

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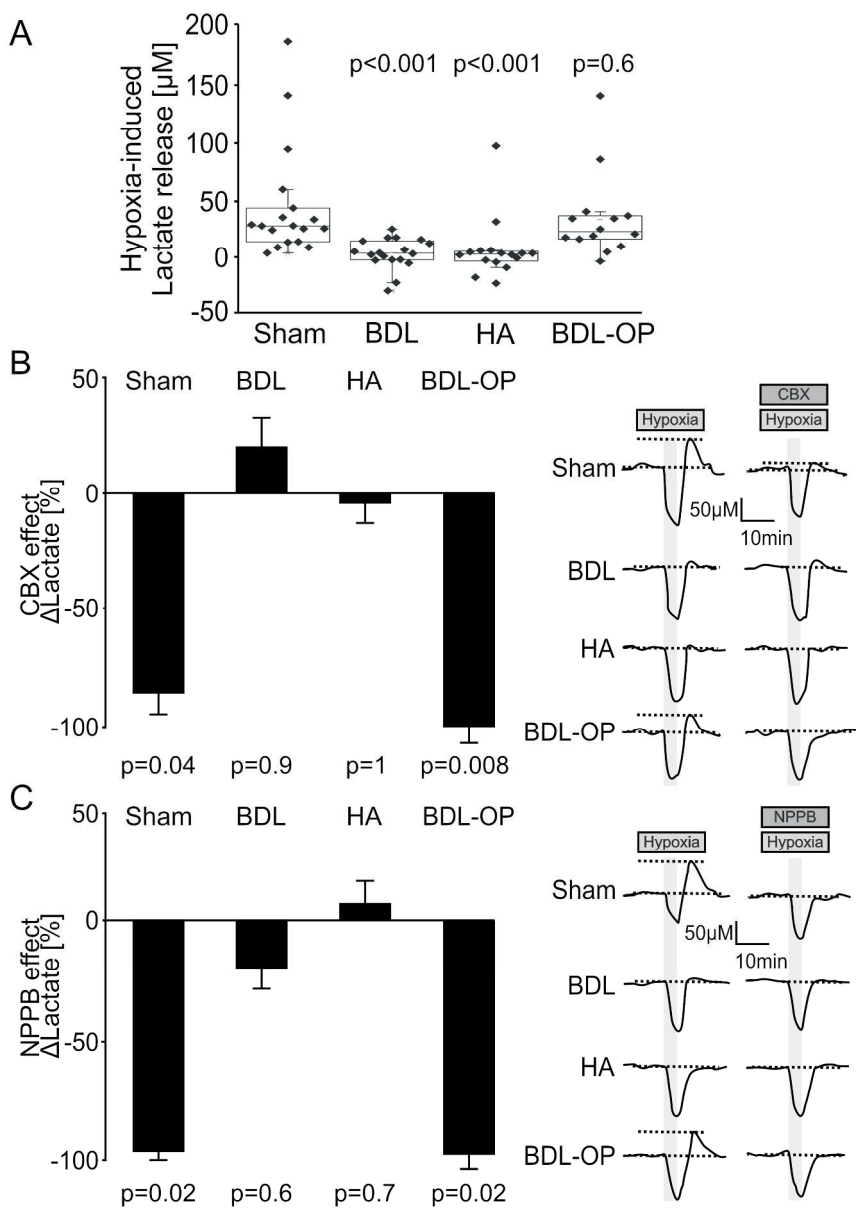


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lactate biosensor current showing the effect of NPPB on the hypoxia-induced release of lactate. *p* values indicate differences between the responses recorded in the absence and presence of NPPB.

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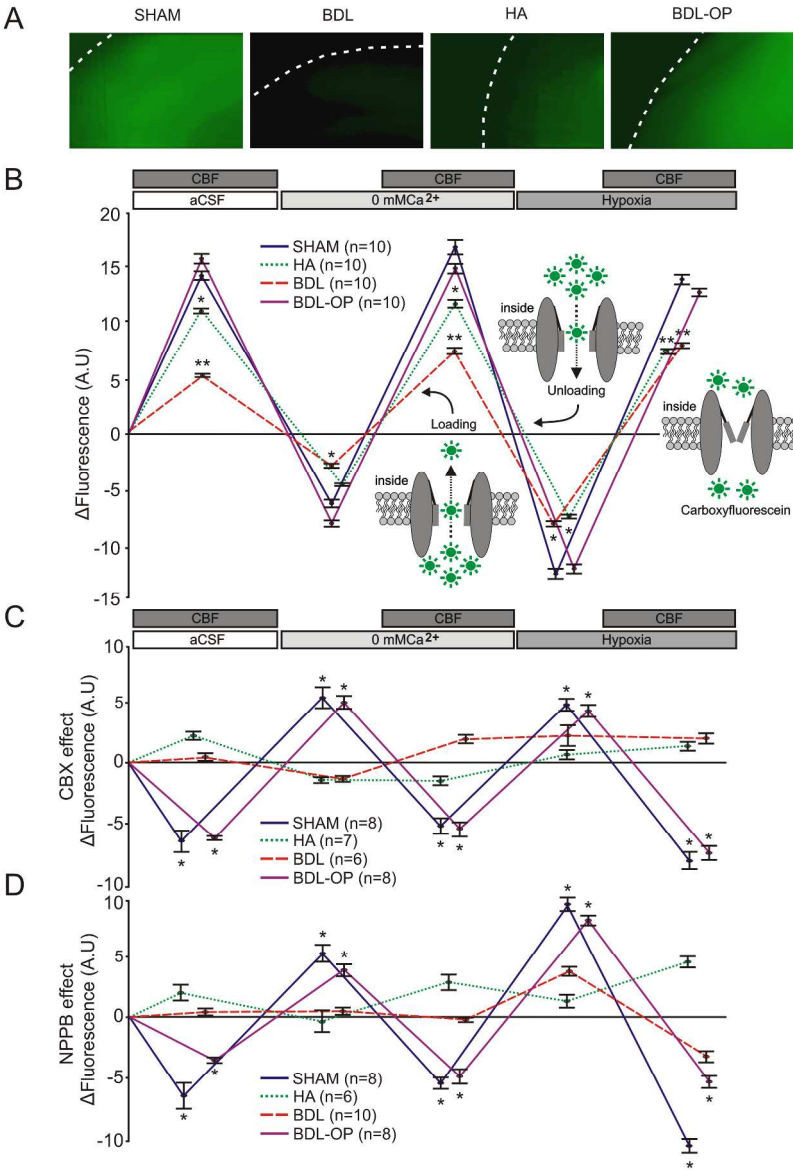


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and unloading. **D)** Summary data illustrating the effect of NPPB (200 μM) on fluorescence intensity changes ($\Delta\text{Fluorescence}$) in cortical slices of sham-operated, BDL, HA and BDL-OP treated rats induced by 0 $[\text{Ca}^{2+}]_e$ and hypoxia in the absence and presence of CBF in the medium. The data are presented as differences in fluorescence following NPPB application compared to the respective fluorescence recorded in the absence of NPPB. * $p < 0.05$ significant effect of NPPB on CBF loading and unloading.

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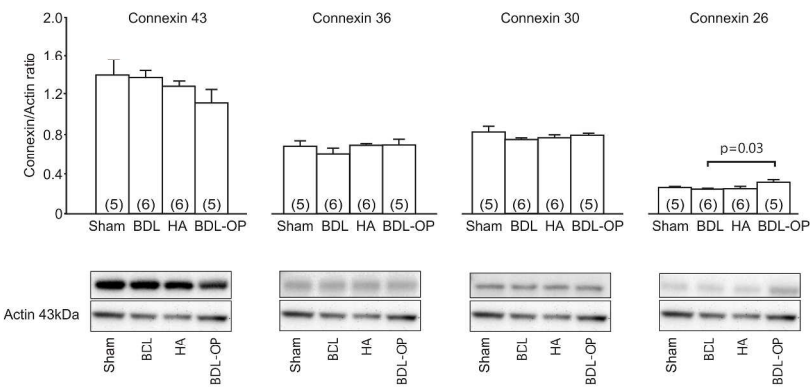


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SUPPLEMENTARY MATERIAL**Supplementary Figure 1: Response time, principles of lactate biosensor operation and the effect of OP on lactate detection system. *A)***

schematic drawing of the lactate biosensor assembly showing the enzymatic biolayer, which surrounds the tip of the platinum (Pt) wire. **B)** Enzymatic reaction taking place in the biolayer of the sensor. **C)** Expanded portion of the lactate calibration trace illustrating the response characteristics of the lactate biosensor. Note that the sensor responds immediately when lactate solution is starting to enter the calibration chamber. **D)** Raw traces illustrating lactate biosensor current responses to lactate (100 μ M) when calibrated in control artificial cerebrospinal fluid (aCSF), and in the presence of ornithine phenylacetate (OP, 1:50 dilution of the stock solution of 0.1g/ml; concentration applied is similar to the concentration estimated to be reached in a 300 g rat following IP injections). *Bottom:* Representative trace from the recordings obtained in the cerebral cortex of a BDL rat showing the effect of OP on lactate tone. Grey shading indicates period of drug application.

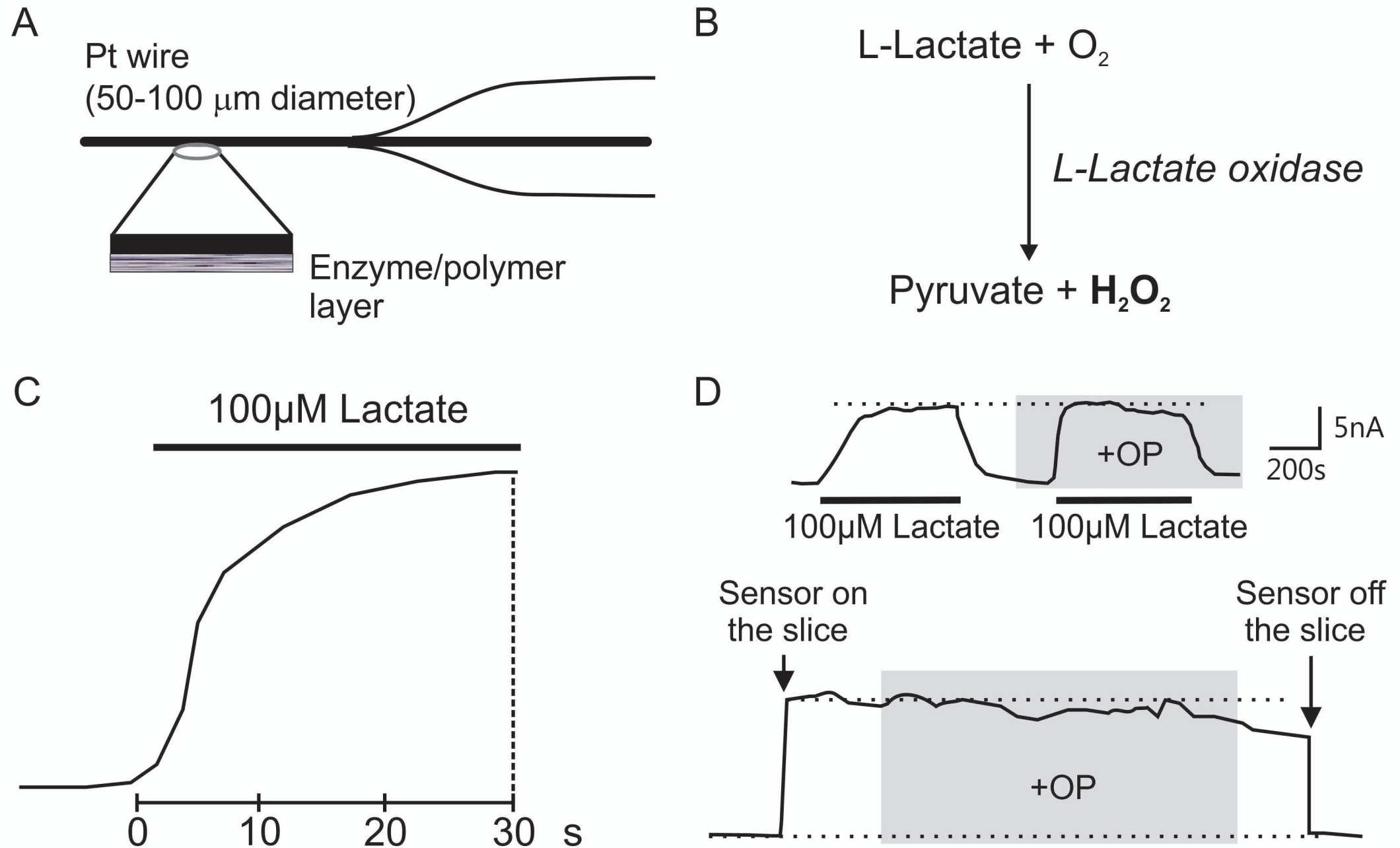
Supplementary Figure 2: Monocarboxylate transporter (MCT) protein expression and functionality in cortical slices of sham-operated and BDL rats. *A)* Summary data illustrating the effect of MCT blocker, 4-CIN (250 Mm, Sigma) on tonic release of lactate (expressed as % change from the baseline) and release of lactate facilitated in response to tissue hypoxia and 0 $[Ca^{2+}]_e$ (expressed as the % of the amount of lactate released in response to hypoxia/0 $[Ca^{2+}]_e$ in the absence of 4-CIN) in cortical slices of sham-operated,

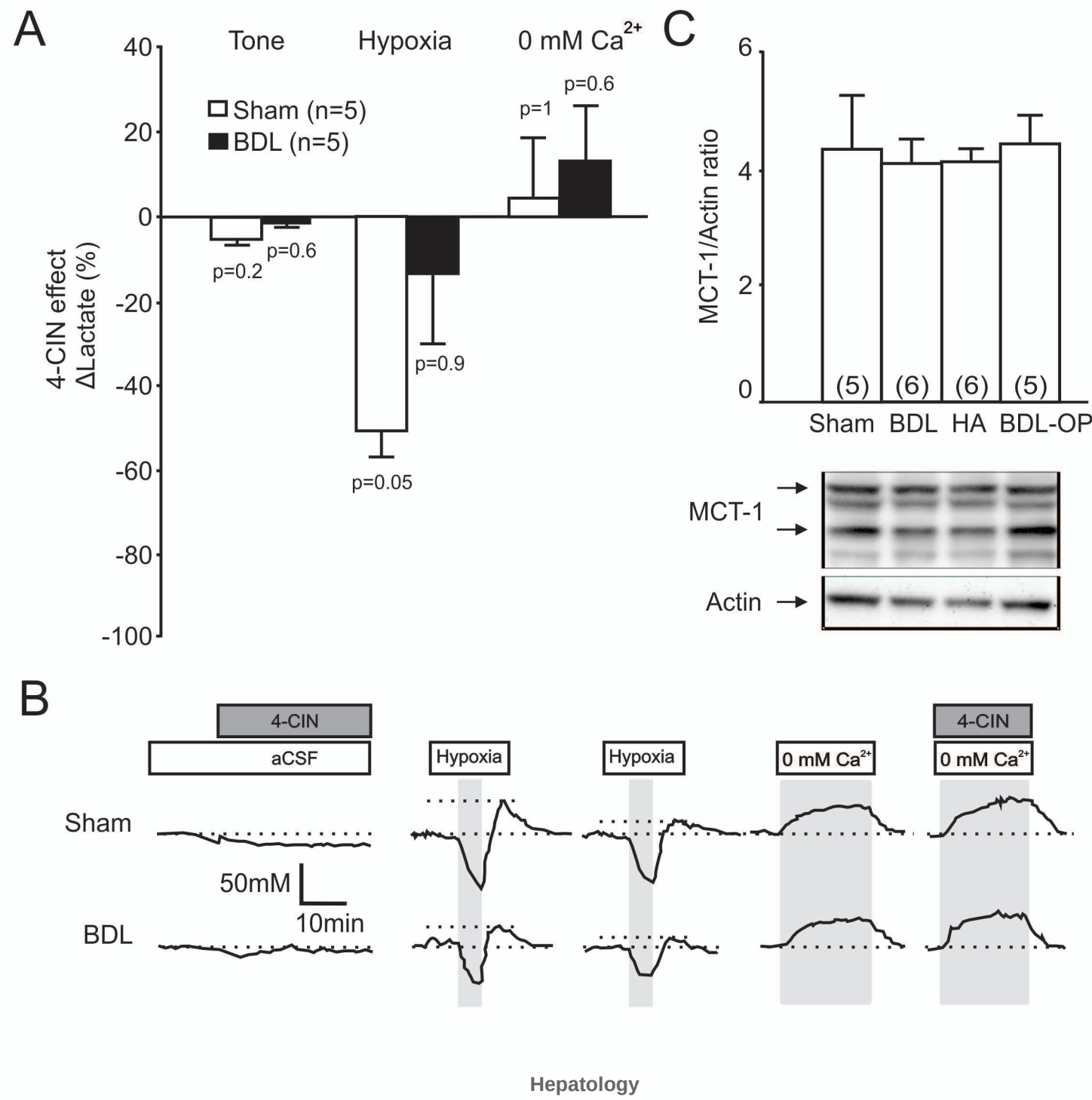
BDL, HA and BDL-OP treated rats. *p* values indicate differences between responses recorded in the absence and presence of 4-CIN. **B)** Representative recordings of lactate biosensor current showing the effect of 4-CIN on tonic as well as hypoxia and 0 $[Ca^{2+}]_e$ -induced release of lactate. **C)** Summary data illustrating means $\pm$ SE of the densitometry of MCT-1 protein levels, mainly expressed in astrocytes (Chemicon International, 0.5 μ g/mL) normalized to the expression of Actin, in cell lysates of the cerebral cortices of sham-operated, BDL, HA and BDL-OP treated rats. *Bottom:* Representative immunoblots showing MCT-1 protein expression in cerebral cortices of sham-operated, BDL, HA and BDL-OP treated rats.

Supplementary Table 1: Plasma biochemistry.

Bile duct ligated (BDL), hyperammonemic (HA), ornithine phenylacetate treated BDL rats (BDL-OP); *ALT*, alanine aminotransferase.

Data expressed as means $\pm$ SEM, ** $p < 0.001$ compared to sham group using one-way ANOVA.





Parameters	Sham	BDL	HA	BDL+OP
N numbers	22	26	22	14
Ammonia, $\mu\text{mol/L}$	56 $\pm$ 3	141 $\pm$ 4**	121 $\pm$ 9**	60 $\pm$ 2
Albumin, g/L	35 $\pm$ 4	23 $\pm$ 0.4**	30 $\pm$ 0.8	23 $\pm$ 1
Total protein, g/L	50 $\pm$ 0.3	38 $\pm$ 0.5**	51 $\pm$ 3	40 $\pm$ 1
Bilirubin, $\mu\text{mol/L}$	5 $\pm$ 0.5	205 $\pm$ 3**	4 $\pm$ 0.6	161 $\pm$ 6
ALT, U/L	11 $\pm$ 0.4	130 $\pm$ 2**	17 $\pm$ 4	87 $\pm$ 4