Multiple ammonia-induced episodes of hepatic encephalopathy provoke neuronal cell loss in bile-duct ligated rats

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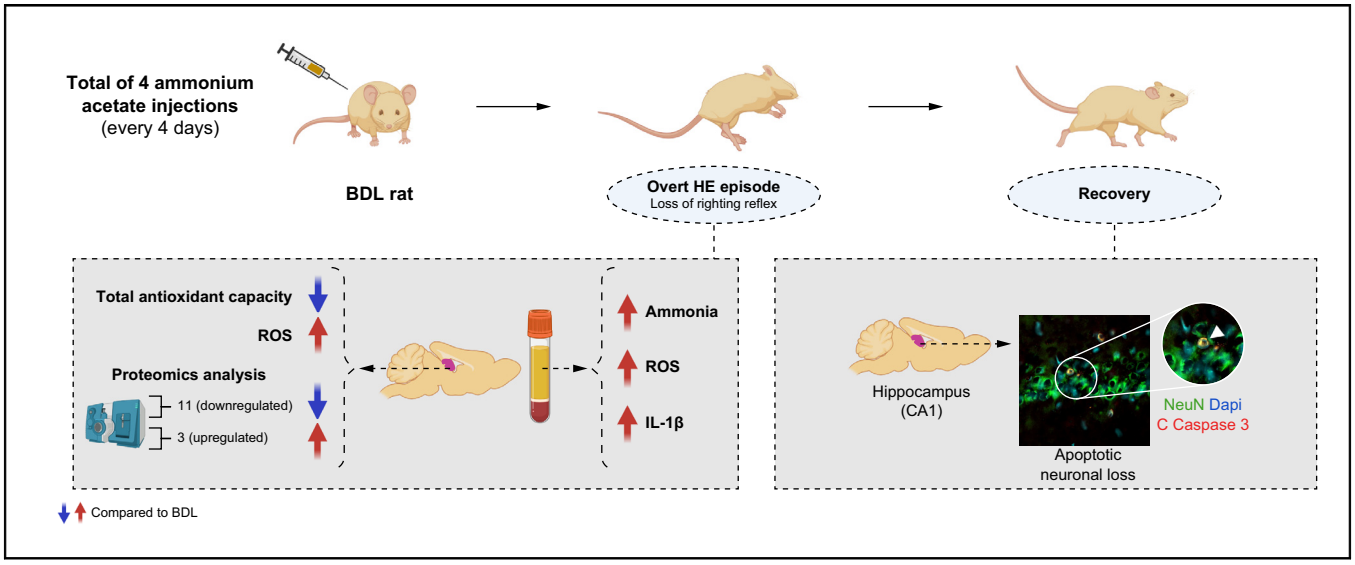
Graphical abstract



Highlights

- Multiple ammonia-induced episodes of HE cause apoptosis and a reduction in neuronal cell markers in the hippocampus.
- Higher levels of oxidative stress markers and decreased total antioxidant capacity in the hippocampus suggest oxidative stress-related apoptotic neuronal loss.
- Multiple ammonia-induced episodes of HE lead to permanent cell damage and therefore irreversibility.
- History of episodes of HE may justify persisting neurological complications observed in patients following liver transplantation.

Impact and implications

Hepatic encephalopathy (HE) is defined as a reversible neuropsychiatric syndrome resolving following liver transplantation (LT); however, ~47% of patients demonstrate neurological impairments after LT, which are associated with a previous history of overt HE pre-LT. Our study indicates that multiple episodes of overt HE can cause permanent neuronal damage which may lead to neurological complications after LT. Nevertheless, preventing the occurrence of overt HE episodes is critical for reducing the risk of irreversible neuronal injury in patients with cirrhosis.

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Research article

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Multiple ammonia-induced episodes of hepatic encephalopathy provoke neuronal cell loss in bile-duct ligated rats

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Background & Aims: Hepatic encephalopathy (HE) is defined as a reversible syndrome and therefore should resolve following liver transplantation (LT). However, neurological complications have been reported in up to 47% of LT recipients, which have been documented to be associated with a history of overt HE pre-LT. We hypothesise that multiple episodes of HE lead to permanent cell injury and exacerbate neurological dysfunction. Our goal was to evaluate the impact of cumulative HE episodes on neurological status and brain integrity in rats with chronic liver disease.

Methods: Episodes of overt HE (loss of righting reflex) were induced following injection of ammonium acetate in bile duct ligation (BDL) rats (BDL-Ammonia) every 4 days starting at week 3 post-BDL. Neurobehaviour was evaluated after the last episode. Upon sacrifice, plasma ammonia, systemic oxidative stress, and inflammation markers were assessed. Neuronal markers including neuron-specific nuclear antigen and SMI311 (anti-neurofilament marker) and apoptotic markers (cleaved caspase-3, Bax, and Bcl2) were measured. Total antioxidant capacity, oxidative stress marker (4-hydroxynonenal), and proinflammatory cytokines (tumour necrosis factor-alpha and interleukin-1β) were measured in brain (hippocampus, frontal cortex, and cerebellum). Proteomic analysis was conducted in the hippocampus.

Results: In hippocampus of BDL-Ammonia rats, cleaved caspase-3 and Bax/Bcl2 ratio were significantly increased, whereas NeuN and SMI311 were significantly decreased compared with BDL-Vehicle rats. Higher levels of oxidative stress-induced post-translational modified proteins were found in hippocampus of BDL-Ammonia group which were associated with a lower total antioxidant capacity.

Conclusions: Ammonia-induced episodes of overt HE caused neuronal cell injury/death in BDL rats. These results suggest that multiple bouts of HE can be detrimental on the integrity of the brain, translating to irreversibility and hence neurological complications post-LT.

Impact and implications: Hepatic encephalopathy (HE) is defined as a reversible neuropsychiatric syndrome resolving following liver transplantation (LT); however, ∼47% of patients demonstrate neurological impairments after LT, which are associated with a previous history of overt HE pre-LT. Our study indicates that multiple episodes of overt HE can cause permanent neuronal damage which may lead to neurological complications after LT. Nevertheless, preventing the occurrence of overt HE episodes is critical for reducing the risk of irreversible neuronal injury in patients with cirrhosis.

Introduction

Hepatic encephalopathy (HE) is a frequent neurological complication which manifests as a wide spectrum of neurological or psychiatric abnormalities ranging from cognitive impairment to coma.[1] HE is classified into two categories: covert HE (CHE) and overt HE (OHE). CHE is diagnosed using neuropsychiatric tests, whereas OHE is clinically diagnosed (symptoms) and remains one of the primary reasons for hospitalisations of patients with cirrhosis.[2]

An increase in blood ammonia plays a major role in the pathogenesis of HE.[1] Hyperammonaemia leads to an increase in blood-borne ammonia in the brain, because ammonia, both as a gas (NH_3) and an ion (NH_4^+), can easily cross the blood–brain barrier. Increased ammonia directly impacts cell metabolism, cellular pH, and membrane potential, culminating into neurotoxicity and encephalopathy.[3]

HE, defined as a metabolic disorder, is expected to completely resolve following liver transplantation (LT). However, several retrospective reports have documented that up to 47% of LT recipients have persisting neurological complications and enduring symptoms which have been documented to be associated with a history of OHE before LT.[4–6] In addition, patients with cirrhosis who have experienced multiple episodes of OHE become refractory to treatment.[7] These observations suggest repeated episodes of OHE may lead to neurological irreversibility

Keywords: Episodic hepatic encephalopathy; Ammonia toxicity; Neurodegeneration; Apoptosis; Neurological complications; Proteomics.
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and neurological damage. Therefore, the impact and the underlying mechanisms of multiple episodes of OHE on neurological integrity merits to be assessed. To do so, we first developed and characterised an animal model of OHE to subsequently evaluate the effect of repeated bouts of ammonia-induced OHE on neuronal integrity. We hypothesise multiple ammonia-induced episodes of OHE cause neuronal damage and permanent cell loss in rats with chronic liver disease (CLD).

Materials and methods

Experimental design

Male Sprague–Dawley rats (n = 130, 175–200 g; Charles River) underwent bile duct ligation (BDL) or SHAM surgery as described.[8] All experiments were approved by the Institutional Animal Care and Use Committee at the CRCHUM (4I015049CR). Starting at Day 20 post-BDL surgery, a dose of ammonium acetate was injected in BDL rats that precipitated an episode of overt HE every 4 days (a total of four subcutaneous injections). Comparable doses of ammonium acetate were injected into the SHAM-operated control rats. Similarly, saline injections were administered as controls to BDL and SHAM rats. Three days after the last injection, a battery of behavioural assessments was performed. Blood samples were taken at three time points: while in pre-coma (during first episode and last episode) as well as upon sacrifice. Groups of rats were sacrificed during the last episode (while in pre-coma) and on Day 40, 8 days after the last injection (Fig. 1A). Upon sacrifice, plasma and brain were collected and stored at -80°C until analysis. Gastrocnemius muscle was dissected, and its weight was measured using a precision scale. A separate set of animals were perfused with saline followed by 10% formalin before tissue collection; the brain samples were placed in optimum cutting temperature compound and stored at -80°C for

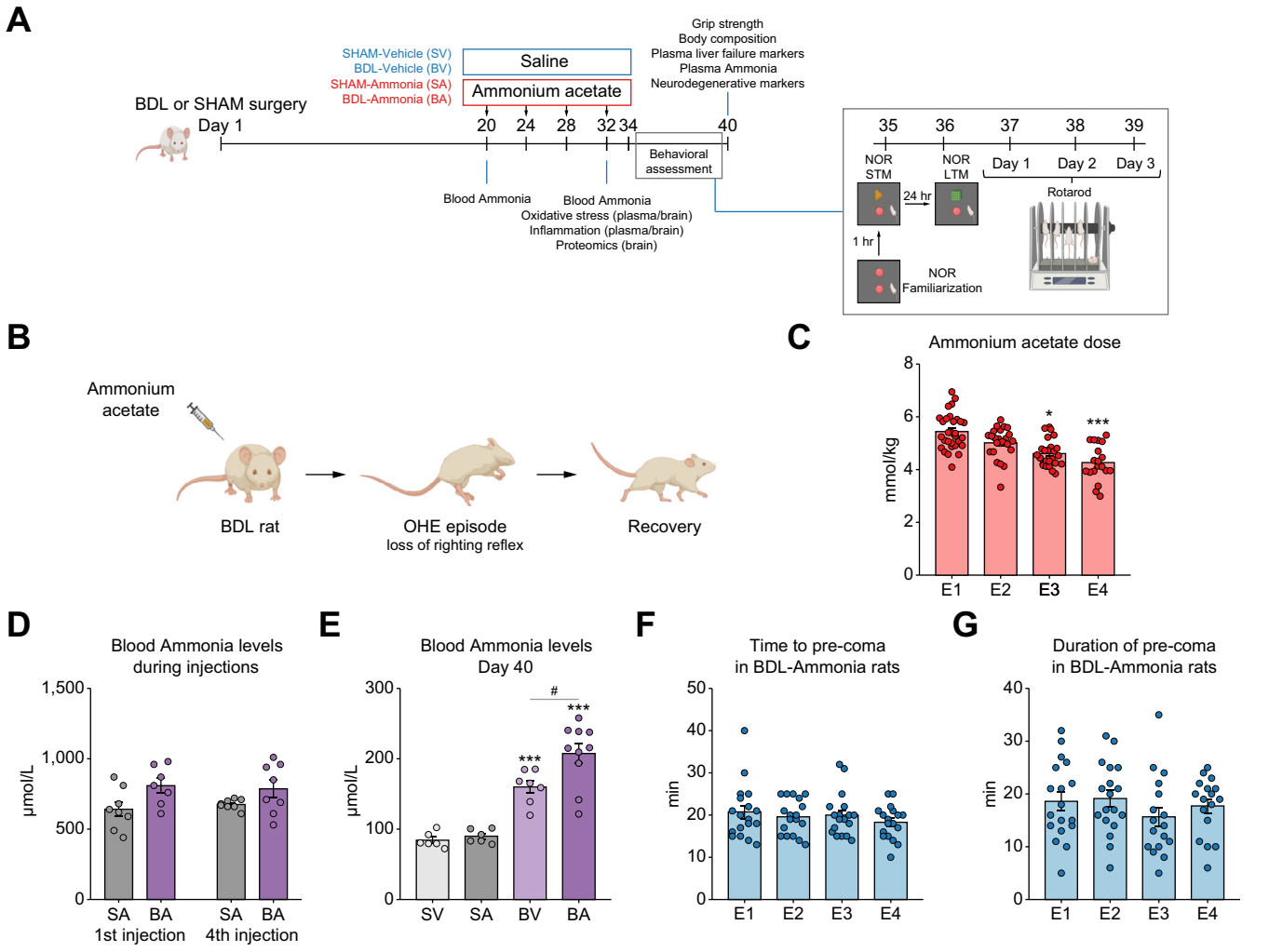

Fig. 1. Ammonia and induction of overt episode of OHE. (A) Experimental design. (B) Ammonia injection induced an OHE episode in BDL rats leading to pre-coma and followed by complete recovery. (C) Ammonium acetate dose injected to induce an episode of HE lessened with each subsequent episode. One-way ANOVA, $^{*}p$ <0.05 and $^{***}p$ <0.001 *vs.* the first episode. (D) Blood ammonia levels measured during pre-coma (first and fourth episode) in BDL rats and respective SHAMs. One-way ANOVA. (E) Blood ammonia levels at the end of the model (Day 40) were higher in the BDL-Ammonia group; however, in the SHAM-Ammonia group, these levels were not significantly different compared with the SHAM-Vehicle group. Two-way ANOVA with Tukey's multiple comparisons, $^{***}p$ <0.001 *vs.* respective controls, $^{\#}p$ <0.05 BDL-Vehicle *vs.* BDL-Ammonia. (F,G) In BDL-Ammonia rats, time to pre-coma and time in pre-coma was not altered between each induced episode. One-way ANOVA with Tukey's multiple comparisons. BDL, bile duct ligation; HE, hepatic encephalopathy; LTM, long-term memory; NOR, novel object recognition; OHE, overt hepatic encephalopathy; STM, short-term memory.

immunofluorescence analysis, and liver samples were fixed in 10% formalin for H&E staining.

Statistical analysis

Data are expressed as mean ± SEM. One-way ANOVA and two-way ANOVA analyses with Tukey's multiple comparisons test *post-hoc* were performed, and values of p <0.05 were considered statistically significant. Statistical analysis was done using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

Ammonia-induced episodes of OHE

In BDL rats, an acute ammonia injection precipitated an episode of severe lethargy and loss of righting reflex (defined as pre-coma) which was followed by a complete recovery (Fig. 1B). The time to pre-coma after ammonia injection was within 10–40 min, and the duration of pre-coma ranged from 5 to 35 min. The mean time to and duration of pre-coma did not significantly differ between subsequent episodes (Fig. 1F and G). The dose of injected ammonia required to induce an episode of OHE in BDL rats lessened with each subsequent episode and a significant decrease was found for episode 3 (4.61 ± 0.10 mmol/kg) and 4 (4.26 ± 0.15 mmol/kg) compared with episode 1 (5.44 ± 0.12 mmol/kg) (p <0.05 and p <0.001, respectively) (Fig. 1C). Levels of blood ammonia preceding the first injection were significant in BDL rats (Ammonia and Vehicle) whereas blood ammonia levels were found significantly higher in the BDL-Ammonia group before the fourth injection (Fig. S1I). Equal doses of ammonia injected into SHAM-operated controls did not lead to severe lethargy or loss of righting reflex. During the first and last (fourth episode) ammonia injections, there was no significant difference in blood ammonia levels between BDL rats and SHAM-operated controls (Fig. 1D). At Day 40, 8 days following the last ammonia injection, blood ammonia levels remained significantly higher in the BDL-Ammonia group compared with both the BDL-Vehicle and SHAM-Ammonia groups (p <0.05) (Fig. 1E).

Liver disease assessment

Plasma liver markers including alanine transaminase, aspartate transaminase, alkaline phosphatase, gamma-glutamyl transferase, and bilirubin were significantly higher in both BDL-Vehicle and BDL-Ammonia groups compared with the respective SHAM groups (p <0.01), with no significant difference found between the BDL-Ammonia *vs.* BDL-Vehicle group. Serum albumin levels were decreased in both BDL *vs.* respective SHAM groups (p <0.001) and liver histology did not reveal any differences between BDL-Ammonia and BDL-Vehicle groups (Fig. S1A–G).

Food intake and body composition

Food consumption and weight gain were found to be less in both BDL-Ammonia and BDL-Vehicle rats compared with respective SHAMs (Fig. 2A and B). Using EchoMRI, less fat mass was found in both BDL-Vehicle and BDL-Ammonia groups compared with respective SHAMs (p <0.01 and p <0.001, respectively) (Fig. 2C). No significant difference was observed in total body water content between groups, although free water content was significantly higher in BDL-Vehicle and BDL-Ammonia groups compared with the respective controls (p <0.01 and p <0.001, respectively) with no significant differences found in the BDL-Ammonia group compared with the BDL-Vehicle group (p <0.05) (Fig. 2D and E). In addition, a significant decrease in lean mass was solely observed in the BDL-Ammonia group compared

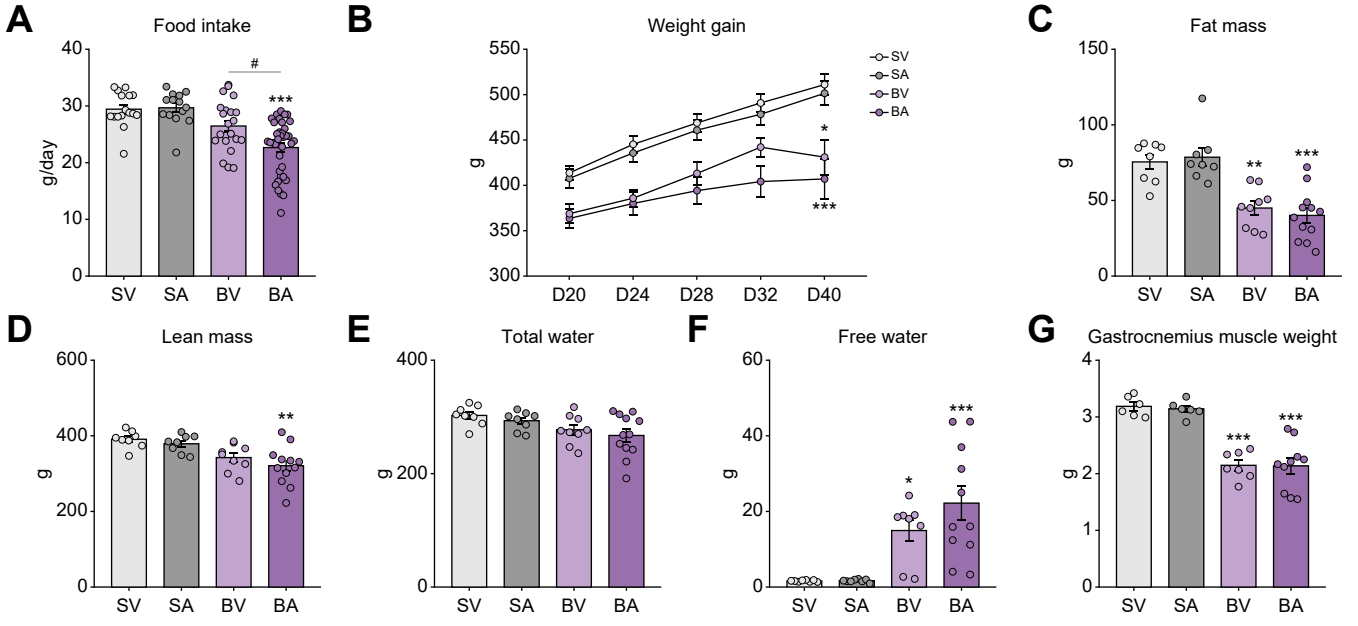

Fig. 2. Food intake and body parameters. (A) Food consumption values from Day 20 to Day 40 showed that BDL-Ammonia rats consumed less during the episodic period. (B) The weight gain remained unchanged compared to BDL-Vehicle. (C–F) Body composition analysis showed lower fat and lean mass in the BDL-Ammonia group with a higher free water content in these rats. (G) Gastrocnemius muscle weight was significantly decreased in BDL-Vehicle and BDL-Ammonia rats. Two-way ANOVA with Tukey's multiple comparisons. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 *vs.* respective controls; $^{\#}p$ <0.05 BDL-Vehicle *vs.* BDL-Ammonia. BA, BDL-Ammonia; BDL, bile duct ligation; BV, BDL-Vehicle; SA, SHAM-Ammonia; SV, SHAM-Vehicle.

with other groups (p <0.01) (Fig. 2F). However, the gastrocnemius muscle weighed significantly less in both BDL-Ammonia and BDL-Vehicle groups compared with the respective controls (p <0.001) (Fig. 2G).

Oxidative stress and inflammatory markers

To investigate the impact of acute ammonia injections on pathogenic factors, we evaluated oxidative stress and inflammatory markers during the fourth (last) ammonia-induced episode of HE. Plasma ROS levels were increased in both BDL-Vehicle and BDL-Ammonia groups compared with the respective SHAMs (p <0.01 and p <0.001, respectively); however, these levels were significantly higher in the BDL-Ammonia group compared to BDL-Vehicle (p <0.001) (Fig. 3A). Plasma levels of inflammatory marker IL-1β were found increased in BDL-Vehicle and BDL-Ammonia groups compared with the respective SHAMs (p <0.01 and p <0.001, respectively). However, IL-1β levels were significantly higher in the BDL-Ammonia group compared with the BDL-Vehicle group (p <0.001). Plasma tumour necrosis factor-alpha (TNF-α) levels also increased in both BDL-Vehicle and BDL-Ammonia groups compared with the respective controls (p <0.01 and p <0.001, respectively) (Table 1).

In the brain, total antioxidant capacity (TAC) levels were decreased in the hippocampus of both BDL-Vehicle and BDL-Ammonia groups compared with the respective SHAMs (p <0.05 and p <0.001, respectively). However, hippocampal TAC levels in the BDL-Ammonia group were significantly reduced by 40% compared with the BDL-Vehicle group (p <0.05). In the frontal cortex and cerebellum, TAC levels remained unchanged across all four groups (Fig. 3B). In addition, 4-hydroxynonenal (4-HNE), a marker of oxidative stress, was found to be increased in the hippocampus in the BDL-Ammonia group compared with all other groups. No significant difference was found between all groups in the frontal cortex and cerebellum (p <0.01) (Fig. 3C). Brain region-specific levels of IL-1β were increased in the frontal cortex and hippocampus of BDL-Vehicle and BDL-Ammonia groups (p <0.05 and p <0.001, respectively) whereas IL-1β levels in the cerebellum remained unchanged across all groups.

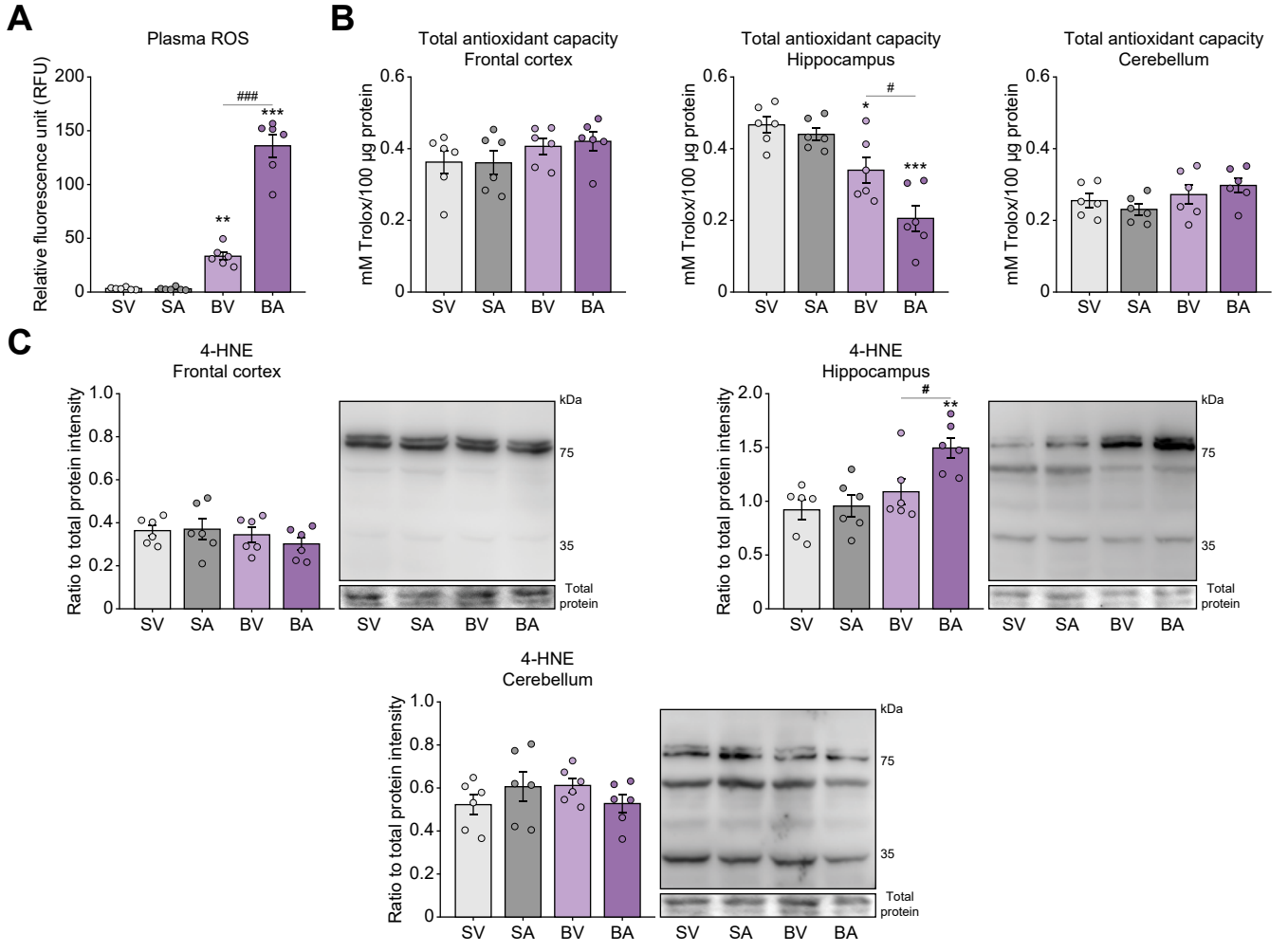

Fig. 3. Multiple ammonia induced OHE episodes and oxidative stress. (A) Plasma ROS was significantly higher in the BDL-Ammonia group when compared with other groups. (B) TAC was significantly lower in the hippocampus of BDL-Ammonia rats. (C) 4-HNE levels measured lipid peroxidation in different brain regions showed a significantly higher expression only in the hippocampus of episodic BDL rats. Two-way ANOVA with Tukey's multiple comparisons. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 *vs.* respective controls, $^{\#}p$ <0.05 and $^{\#\#\#}p$ <0.001 BDL-Vehicle *vs.* BDL-Ammonia. 4-HNE, 4-hydroxynonenal; BA, BDL-Ammonia; BDL, bile duct ligation; BV, BDL-Vehicle; OHE, overt hepatic encephalopathy; ROS, reactive oxygen species; SA, SHAM-Ammonia; SV, SHAM-Vehicle; TAC, total antioxidant capacity.

Table 1. Inflammatory markers.

	SHAM-Vehicle	SHAM-Ammonia	BDL-Vehicle	BDL-Ammonia
Plasma (ng/ml)				
IL-1β	11.15 ± 0.12	11.15 ± 0.12	14.49 ± 0.94***	21.52 ± 0.99***,‡
TNF-α	9.23 ± 0.17	9.21 ± 0.38	16.23 ± 1.76**	16.87 ± 1.35***
Brain (ng/200 μg protein)				
Frontal cortex				
IL-1β	16.94 ± 0.12	16.97 ± 0.13	17.39 ± 0.10*	17.72 ± 0.08***
TNF-α	15.00 ± 0.13	15.01 ± 0.20	15.35 ± 0.16	15.56 ± 0.15
Hippocampus				
IL-1β	18.26 ± 0.25	17.85 ± 0.20	19.27 ± 0.24*	19.64 ± 0.23***
TNF-α	14.26 ± 0.12	14.62 ± 0.12	15.27 ± 0.16**	15.53 ± 0.24**
Cerebellum				
IL-1β	23.32 ± 0.61	23.17 ± 0.66	22.73 ± 0.50	22.95 ± 0.53
TNF-α	18.25 ± 1.33	17.67 ± 0.31	19.01 ± 0.30	20.24 ± 0.19***,†

Higher levels of inflammatory cytokines (IL-1β and TNF-α) were found in the plasma of BDL rats. However, IL-1β levels were even significantly higher in episodic rats when compared to BDL controls. The levels of both cytokines were higher in the hippocampus of all BDL rats. Additionally, IL-1β levels were only increased in the frontal cortex of BDL rats. TNF-α was only increased in the cerebellum of episodic rats. Two-way ANOVA with Tukey's multiple comparisons, numbers expressed as means ± SEM.
*p <0.05, **p <0.01 and ***p <0.001 *vs.* respective control, †p <0.05 and ‡p <0.001 BDL-Vehicle *vs.* BDL-Ammonia. BDL, bile duct ligation; IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-alpha.

However, brain TNF-α levels were increased only in the hippocampus of both BDL-Vehicle and BDL-Ammonia groups compared with the respective controls (p <0.01). However, in the cerebellum, a significant increase in TNF-α levels was only found in the BDL-Ammonia group compared with the BDL-Vehicle group (p <0.001) (Table 1).

Neurological assessment

A novel object recognition test demonstrated significant decrease in performance in both short- (STM) and long-term memory (LTM) in the BDL-Vehicle and BDL-Ammonia groups compared with the respective controls (p <0.05 and p <0.001, respectively). However, LTM impairment was significantly different in the BDL-Ammonia group when compared with the BDL-Vehicle (p <0.05) (Fig. 4A and B).

Rotarod motor coordination assessment showed a significant decrease in latency to fall at Day 2 in the BDL-Ammonia group compared with the BDL-Vehicle group and the respective control group (p <0.01), whereas on Day 3 of the assessment, both BDL-Vehicle and BDL-Ammonia groups showed poor performance compared with the respective controls (p <0.01 and p <0.001, respectively) (Fig. 4C). The longitudinal comparison showed a

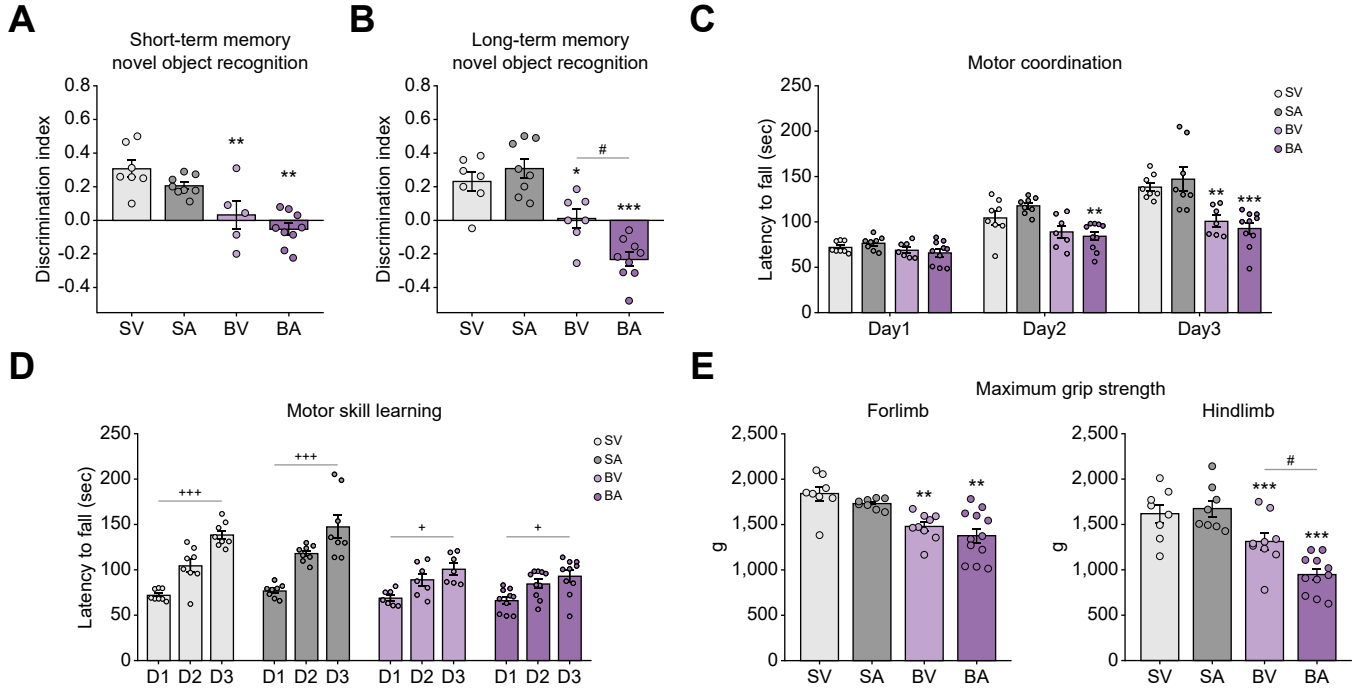

Fig. 4. Behavioural assessment. (A,B) STM and LTM performance was decreased in all BDL rats. However, this performance was further worsened in BDL-Ammonia rats. Discrimination index = ([time spent with the novel object] − [time spent with the familiar object])/([time spent with the novel object] + [time spent with the familiar object]). Two-way ANOVA with Tukey's multiple comparisons. (C,D) Motor coordination and motor skill learning performance were significantly decreased in BDL rats. One-way ANOVA with Tukey's multiple comparisons. (E) Forelimb and hindlimb maximum muscle strength was significantly decreased in all BDL rats, and the hindlimb strength showed a decreased performance in the BDL-Ammonia group *vs.* BDL-Vehicle. Two-way ANOVA with Tukey's multiple comparisons. *p <0.05, **p <0.01, and ***p <0.001 *vs.* respective controls #p <0.05 BDL-Vehicle *vs.* BDL-Ammonia. BA, BDL-Ammonia; BDL, bile duct ligation; BV, BDL-Vehicle; LTM, long-term memory; SA, SHAM-Ammonia; SV, SHAM-Vehicle; STM, short-term memory.

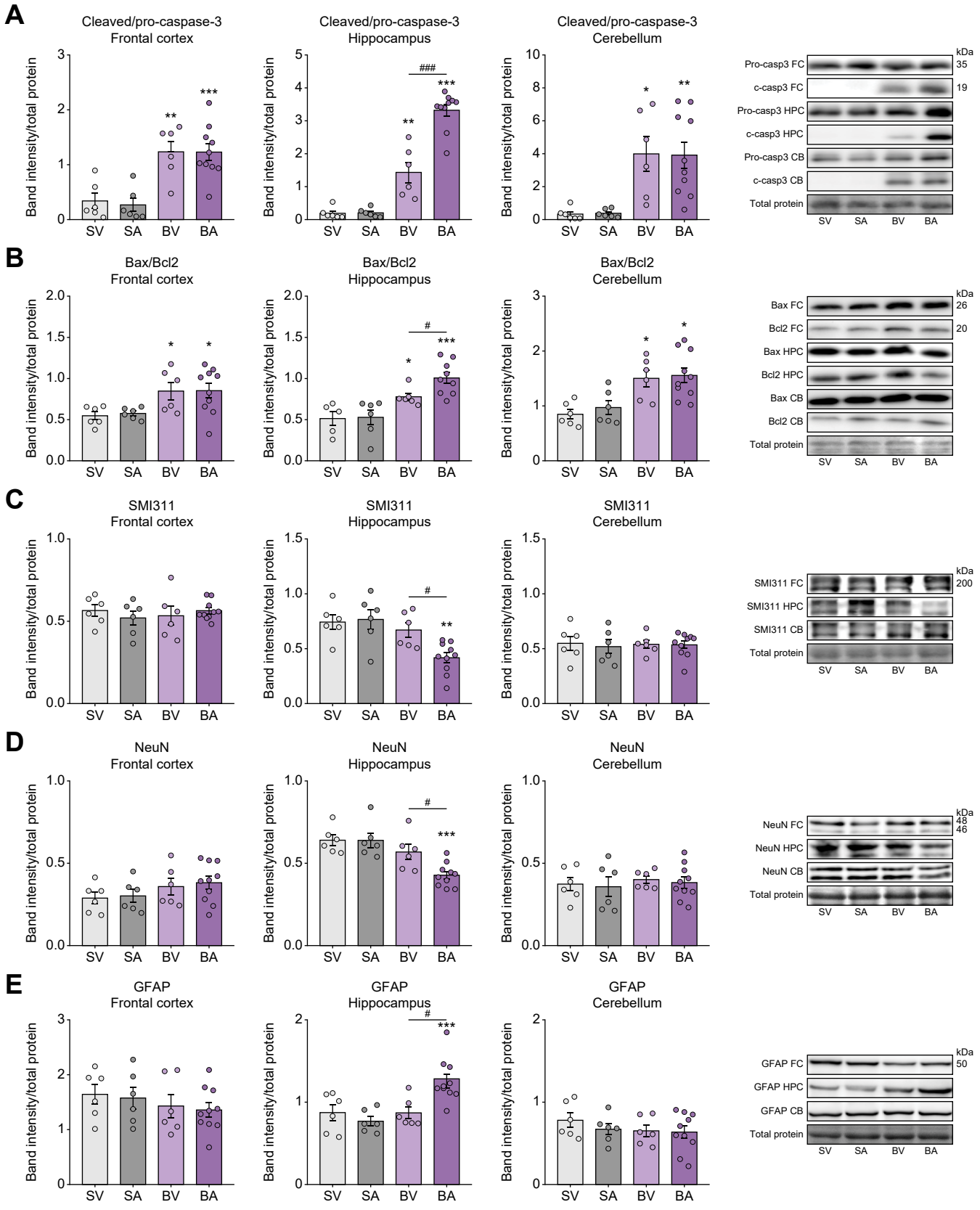

Fig. 5. Ammonia-induced episodes of OHE and neuronal loss in hippocampus. Protein expression levels of apoptotic markers (A) Cleaved/pro-caspase-3 and (B) Bax/Bcl2 increased in the frontal cortex (FC), hippocampus (HPC), and cerebellum (CB) of all BDL rats; however, BDL-Ammonia rats had a higher level when compared with BDL-Vehicle. Neuronal markers (C) SMI311 and (D) NeuN protein expression levels were only decreased in the hippocampus of BDL-Ammonia rats. (E) GFAP, an astrocytic marker, showed a higher expression in the hippocampus of the BDL-Ammonia group. Two-way ANOVA with Tukey's multiple

significantly longer latency to fall in all groups at Day 3 compared with their performance at Day 1 (Fig. 4D). However, the latency to fall in both SHAM groups increased by 93% from Day 1 to Day 3 which was significantly reduced in BDL-Vehicle and BDL-Ammonia rats 47 and 42%, respectively. Total distance travelled remained unchanged between groups (Fig. S1H).

Forelimb and hindlimb maximum muscle strength was significantly lower in BDL-Vehicle and BDL-Ammonia groups when compared with the respective SHAMs (p <0.01 for forelimb and p <0.001 for hindlimb). However, the hindlimb maximum muscle strength was significantly weaker in the BDL-Ammonia group when compared with the BDL-Vehicle group (p <0.05) (Fig. 4E).

Apoptosis and cell death

Western blot analysis showed significantly higher levels of apoptotic markers, cleaved/pro-caspase-3, and Bax/Bcl2 ratio in the frontal cortex, hippocampus, and cerebellum in both BDL-Vehicle and BDL-Ammonia groups when compared with the respective SHAMs. However, in the BDL-Ammonia group, a significantly increase of these apoptotic markers was found in the hippocampus compared with the BDL-Vehicle group (cleaved/pro-caspase-3, p <0.001 and Bax/Bcl2 ratio, p <0.05) (Fig. 5A and B). Additionally, the hippocampus of BDL-Ammonia displayed a significant decrease in both neuronal markers neuron-specific nuclear antigen (NeuN) and anti-neurofilament marker (SMI311) (p <0.001 and p <0.01, respectively) and increased levels of the astrocytic marker, glial fibrillary acidic protein (GFAP) (p <0.001) compared with the respective SHAM and BDL-Vehicle groups (Fig. 5C–E, representative immunohistochemistry images from all brain regions are shown in Fig. S3). These significant differences observed in the hippocampus of the BDL rats following four episodes were not demonstrated in BDL rats following one episode (Fig. S2).

To localize cellular cleaved caspase-3, we performed co-staining of neuronal and astrocytic markers with cleaved caspase-3. Our results demonstrate the colocalization of cleaved caspase-3 with NeuN (neuronal marker) in the CA1 region of the hippocampus of the BDL-Ammonia group (Fig. 6B). This colocalization was also observed in the cerebellum of both BDL-Vehicle and BDL-Ammonia groups with calbindin (neuronal marker specific for Purkinje neurons) (Fig. 6D). In addition, the colocalization of GFAP (astrocytic marker) and cleaved caspase-3 was found in all three studied brain regions (frontal cortex, hippocampus, and cerebellum) of both BDL-Vehicle and BDL-Ammonia groups (Fig. 6E–G).

Proteomic analysis

To explore protein alterations occurring during an overt episode in the affected brain region (hippocampus), proteomic analysis identified 1,202 proteins from which 438 proteins were significantly altered between all four groups. Among 438 proteins, 14 proteins were significantly changed between BDL-Vehicle and BDL-Ammonia, 275 and 73 proteins were significantly altered in the BDL-Vehicle and BDL-Ammonia groups, respectively, when compared with the respective SHAM groups. Additionally, 199 proteins were changed in the SHAM-Ammonia *vs.* SHAM-Vehicle groups. Two proteins were identified that were specifically altered in the BDL-Ammonia group when compared with the BDL-Vehicle and SHAM-Ammonia groups (Fig. 7).

Discussion

In this study, for the first time, we developed and characterised an animal model of episodic HE using the BDL rat; a well described and recognized animal model of CHE associated with CLD.[9] An animal model of episodic OHE was lacking and remained an unmet research gap to properly evaluate the impact of multiple OHE episodes on neuronal integrity.

Our results demonstrate that multiple ammonia-induced episodes of OHE lead to neurodegeneration, primarily in the hippocampus. With several studies demonstrating patients with the history of OHE leading up to LT are associated with neurological complications post-LT,[5,10–12] neuronal cell loss, hence irreversibility, strongly suggests multiple episodes of OHE impact brain integrity. This would also apply to patients, who following multiple bouts of OHE, become refractory to HE treatment. Additionally, MRI analysis in patients who have experienced multiple episodes of OHE demonstrated impaired brain connectivity in different brain regions when compared with patients without a history of OHE.[13] Furthermore, brain atrophy and the reduction in the neuronal marker *N*-acetylaspartate (indicating neuronal loss), have been reported in patients who have experienced episodes of OHE.[11,14] These findings are supported by studies showing patients without a history of OHE improve with HE treatments[15] and rarely develop neurological complications after LT.[5]

A rise in blood ammonia remains a primary factor in the pathogenesis of OHE which is principally caused by precipitating events of HE, including gastrointestinal bleeding, protein overload, and constipation.[1] In our study, we injected ammonia to trigger an episode of OHE (loss of righting reflex) in BDL rats, an ammonia dose which did not induce an episode in SHAM rats. Interestingly, less ammonia was required to induce each ensuing episode in BDL rats, possibly because of an elevated degree of hyperammonaemia preceding each episode or equally suggesting the brain becomes sensitised to subsequent ammonia insults which could explain why episodes of OHE lead to a higher risk of additional bouts.[16–18]

To study the long-term impact of multiple episodes of OHE, central nervous system (CNS) function (battery of behavioural tests) and neuronal integrity were assessed 1 week following the last injection of ammonia. We have previously shown BDL rats develop impaired STM and LTM performance.[19] In the present study, a significant difference was found within the discrimination index for LTM between BDL rats following multiple episodes of OHE and BDL-Vehicle. However, because of several limitations of the method,[20] this may entail that in both groups LTM is maximally impaired and therefore, the impact of ammonia-induced episodes on LTM impairment requires further evaluation. Interestingly, motor coordination and motor skill learning performance were not significantly impacted by multiple

comparisons. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 *vs.* respective controls, $^{\#}p$ <0.05 and $^{\#\#\#}p$ <0.001 BDL-Vehicle *vs.* BDL-Ammonia. BA, BDL-Ammonia; BDL, bile duct ligation; BV, BDL-Vehicle; GFAP, glial fibrillary acidic protein; NeuN, neuron-specific nuclear antigen; OHE, overt hepatic encephalopathy; SA, SHAM-Ammonia; SMI311, anti-neurofilament marker; SV, SHAM-Vehicle.

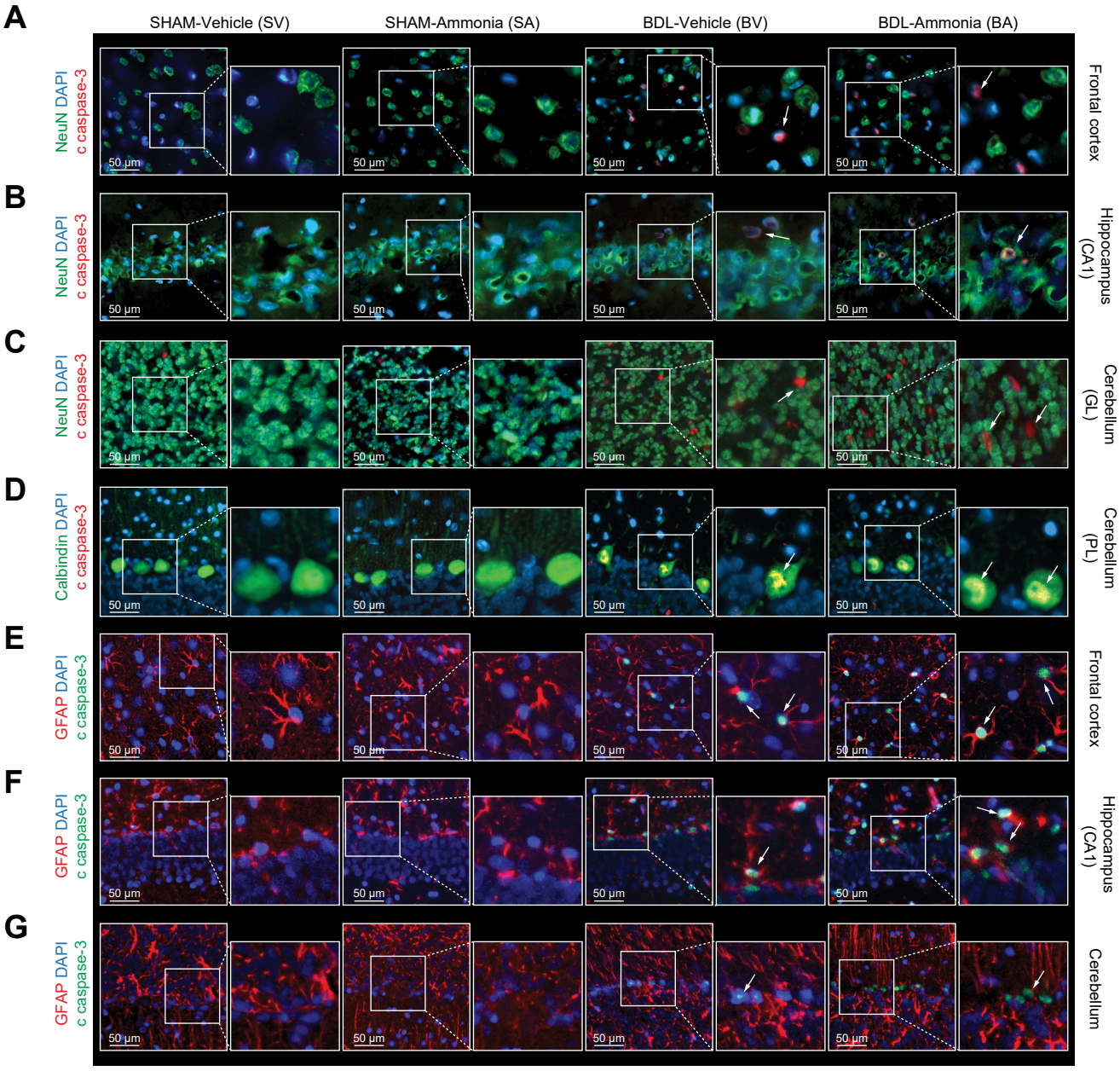

Fig. 6. Immunohistochemistry. (A–C) Co-staining of cleaved caspase-3 (apoptosis marker) and NeuN (neuronal marker) in the frontal cortex and granular layer (GL) of cerebellum did not show any colocalization of apoptotic and neuronal markers in BDL-Vehicle and BDL-Ammonia; however, immunofluorescence showed colocalization of cleaved caspase-3 (apoptosis marker) and NeuN (neuronal marker) in the CA1 region of the hippocampus of BDL-Ammonia rats. This colocalization was not observed in other groups. (D) Colocalization of cleaved caspase-3 (apoptosis marker) and calbindin (Purkinje neuron marker) was observed in the Purkinje layer (PL) of the cerebellum in BDL-Vehicle and BDL-Ammonia groups. (E–G) Co-staining of cleaved caspase-3 and GFAP (astrocytic marker) were conducted in all three brain regions, the results showed colocalization of apoptotic marker with astrocytes in both BDL-Vehicle and BDL-Ammonia groups. BDL, bile duct ligation; GFAP, glial fibrillary acidic protein; NeuN, neuron-specific nuclear antigen.

episodes of OHE. However, both BDL rats (with or without ammonia injections) remained affected compared with the respective SHAM controls.

Body composition analysis using EchoMRI showed a decrease in lean and fat mass in BDL rats as we have previously reported.[19] Multiple spikes in hyperammonaemia (episodes of OHE) did not impact alterations in lean and fat mass. Similarly, the gastrocnemius muscle weight which we previously found to be decreased in BDL-Vehicle,[21] was not impacted in the BDL-Ammonia group. However, the hindlimb maximum muscle strength was significantly weaker in BDL rats with multiple episodes of OHE compared with BDL-Vehicle rats, suggesting high acute doses of ammonia can impact the quality and function of muscle. It has been demonstrated that the toxicity of ammonia can impinge on other organs aside the brain.[22] Ammonia toxicity has been shown to act on the muscle with ammonia causing contractile dysfunction including reduced twitch force and decreased rate of force development and relaxation, as well as nitration of myosin heavy chain, a major contractile protein in the skeletal muscle.[23] Muscle contraction requires energy and impaired mitochondrial function

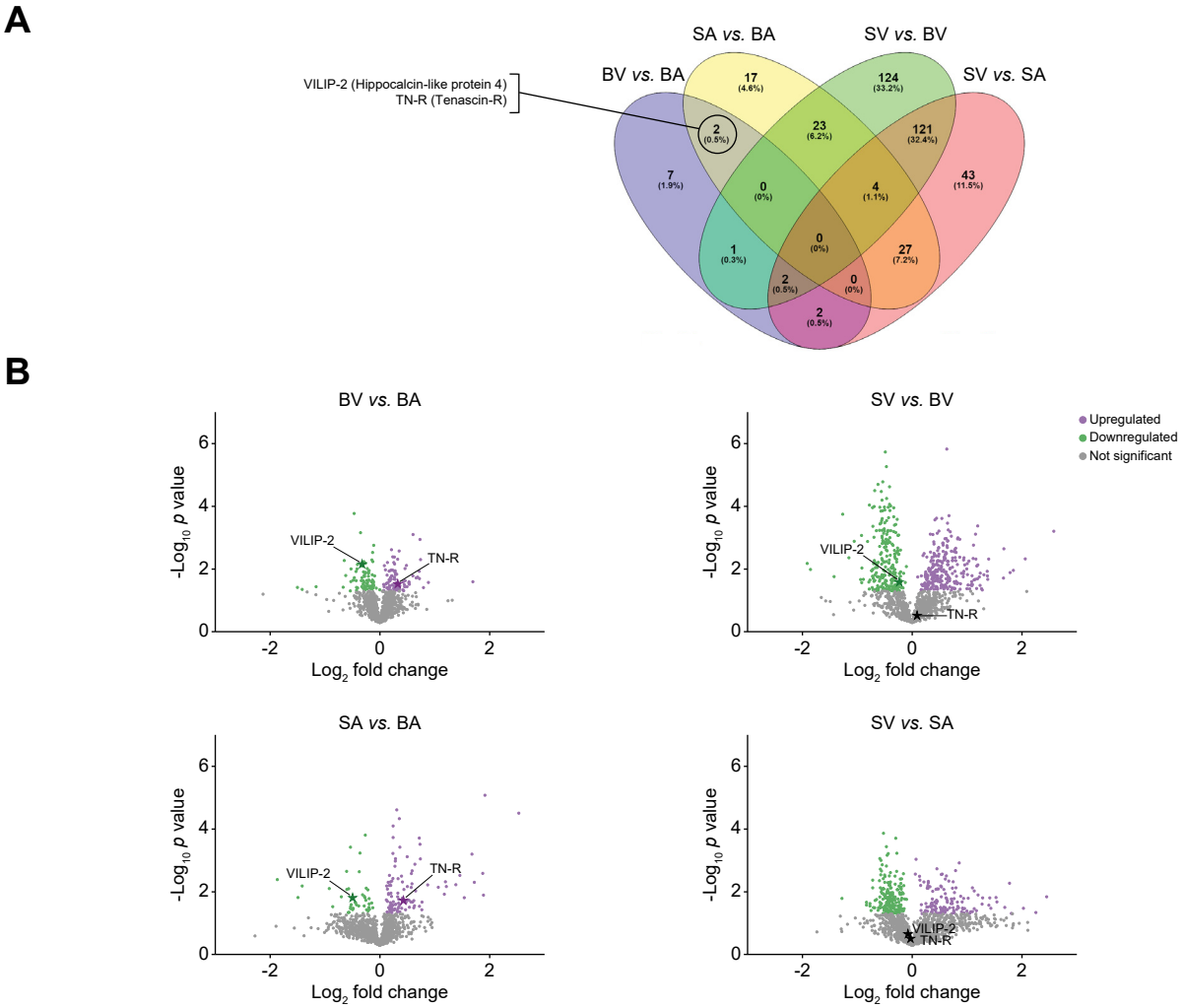

Fig. 7. Proteomic analysis. In the hippocampus, (A) shows the Venn diagram used to compare and determine shared proteins with significant alteration among four comparison groups. (B) The volcano plots demonstrating no significant change as well as up- and downregulation of the expression of proteins between two groups. Two-way ANOVA with Tukey's multiple comparisons for Venn diagram and t-test for Volcano plots. BA, BDL-Ammonia; BDL, bile duct ligation; BV, BDL-Vehicle; SA, SHAM-Ammonia; SV, SHAM-Vehicle; TN-R, Tenascin-R; VILIP-2, visinin-like protein 2.

and ATP content have been reported in cases of hyperammonaemia.[24] Additionally, ammonia is known to cause post-translational modification including protein nitration and oxidative stress-induced carbonylation of contractile proteins, which can lead to impaired actomyosin interactions. Together, these results show ammonia impacts muscle function, causing muscle weakness independent of muscle mass.[23]

Several apoptotic markers were used to evaluate the impact of cumulative OHE on brain integrity. Caspase-3 and Bax/Bcl2, involved in the apoptosis signalling cascade, are widely used as apoptosis biomarkers in evaluating neurodegeneration.[25,26] Interestingly, an increase in apoptotic markers was found in all three brain regions in all BDL rats *vs.* all other groups. However, in the BDL-Ammonia rats, we identified even higher levels of apoptosis selectively in the hippocampus when compared with BDL-Vehicle rats. This observation was not found in other regions studied (frontal cortex or cerebellum). At the cellular level, using immunofluorescence, we demonstrated the colocalization of cleaved caspase-3 with neurons using NeuN (neuronal marker) in the CA1 region of the hippocampus of BDL-Ammonia rats, which suggests apoptosis in these neurons. Such colocalization was not found in neurons of other regions (frontal cortex or granular layer of cerebellum). Interestingly, Angelova *et al.*[27] have reported, with immunohistochemistry, a decrease in the neuronal marker beta III tubulin in the hippocampus in BDL rats.[27] This finding in non-episodic BDL rats may be explained, aside using a different neuronal marker, by the fact that older rats were used in the study, a risk factor for both HE and neurodegeneration.[28,29] Furthermore, apoptosis and neuronal cell death was not investigated. The hippocampus is a critical structure for memory function[30] and damage in this area is believed to be the primary cause of memory loss in several neurodegenerative diseases such as Alzheimer's disease.[31,32] Interestingly, Purkinje neurons of the cerebellum in both BDL-Vehicle and BDL-Ammonia groups were found to express the cleaved caspase-3 protein. These findings have also been observed in the portacaval anastomosis model of hyperammonaemia[33,34] and patients with or without OHE.[35] It remains unclear what are the underlying reasons for the apoptotic Purkinje neurons, as well as the impact, but chronic hyperammonaemia may play a role.[36]

Insults to the brain trigger astrogliosis and one of the features of astrocytic activation and proliferation is the upregulation of the GFAP which has been vastly reported that astrocyte activation is associated with a broad range of neuropathologies such as stroke, trauma, haemorrhage, and neurodegenerative diseases including Alzheimer's disease, amyotrophic lateral sclerosis and multiple sclerosis. Reactive astrogliosis (astrocyte scar), a defensive reaction that aims at restoring the tissue homeostasis and restricting the tissue damage occurs following CNS insults and neuronal cell death and loss.[37] We found significantly higher levels of GFAP in the hippocampus (not frontal cortex or cerebellum) of BDL-Ammonia rats compared with all other groups. These results suggest astrogliosis, which leads to changes in morphology and function by altering expression of many genes, including GFAP. However, in addition, using immunohistochemistry, we demonstrated astrocytes in the frontal cortex, hippocampus, and cerebellum of BDL-Vehicle and BDL-Ammonia groups express the apoptotic marker cleaved caspase-3. Collectively, these findings suggest astrocyte apoptosis might coexist with reactive astrogliosis; however, this remains to be thoroughly investigated. Irrespective, this supports the important pathological role of astrocytes in HE. Astrocytes have been well documented to be affected in HE since several *in vivo* and *in vitro* studies have shown increased ammonia leads to swelling, Alzheimer type II, reactive astrogliosis, senescence, and death.[38–41] Healthy astrocytes are critical for supporting neuronal homeostasis and any alternations in astrocyte–neuron communication can lead to neuropathological states, including HE.[42]

To further investigate the underlying causes of an ammonia-induced episode, we explored the pathogenic environment which develops during an episode. We investigated oxidative stress and inflammation which have been suggested to be involved in the pathogenesis of HE[43] as it has been previously reported that hyperammonaemia influences oxidative stress and inflammation.[44,45] Our results show that an acute increase in blood ammonia triggers a further increase in systemic ROS in BDL-Ammonia *vs.* BDL-Vehicle rats. Interestingly, a similar acute increase in blood ammonia in the SHAM-Ammonia group did not lead to an increase in systemic ROS. These results indicate a lower systemic antioxidant capacity exists in BDL rats, possibly as a result of a decrease in plasma antioxidants including albumin, catalase, glutathione reductase, glutathione and glutathione/oxidised glutathione ratio as we have previously reported.[45] To evaluate oxidative stress status in the brain, we measured 4-HNE and TAC in the frontal cortex, hippocampus, and cerebellum. The results showed a significant accumulation of 4-HNE only in the hippocampus of the BDL-Ammonia group. The 4-HNE is the end-product of lipid peroxidation, which is capable of binding to proteins and forming stable adducts. Changes in protein structure leads to protein malfunction and damage to different tissues and cells, including loss of membrane integrity, cytotoxicity, cell dysfunction, and apoptotic cell death.[46,47] Also, lower TAC was detected in the hippocampus of BDL rats, a finding which was not observed in other regions. More importantly, TAC was found to be significantly lower in the BDL-Ammonia group compared with BDL-Vehicle rats, suggesting the hippocampus is highly vulnerable to ROS damage. Interestingly, an increase in hippocampal ROS has been documented in non-episodic BDL rats *vs.* respective controls using older Wistar rats, however, neuronal cell integrity was not evaluated.[48] Intriguingly, higher levels of ROS have been reported in autopsied brain tissue from patients with cirrhosis who died with OHE. However, these results are limited to cerebral cortex and the history of OHE episodes in these patients was not reported.[49]

Plasma levels of IL-1β and TNF-α were significantly higher in both BDL-Vehicle and BDL-Ammonia groups than controls; however, only IL-1β was significantly higher following multiple episodes of OHE. Elevated plasma levels of IL-1β and TNF-α have been previously reported in patients with HE[50] with plasma levels of TNF-α correlating with severity of HE.[51] In the brain, levels of IL-1β and TNF-α were found to be higher in the hippocampus of both BDL-Vehicle and BDL-Ammonia groups compared with respective controls. Also, elevated levels of IL-1β were found in the frontal cortex in both BDL-Vehicle and BDL-Ammonia groups compared with respective controls. This same significant change was not observed in the cerebellum where only TNF-α levels were found to be increased in BDL-Ammonia rats compared with BDL-Vehicle rats. These results suggest an independent response of pro-inflammatory cytokines arises in different brain regions following acute hyperammonaemia.

Hippocampus proteomic analysis in the hippocampus revealed alterations of multiple proteins. Out of 1,202 analysed, when controlling for liver disease (BDL) and ammonia-injections, two proteins were identified to be significantly altered in the BDL-Ammonia group when compared with the BDL-Vehicle group and the respective SHAM groups. A significant downregulation of VILIP-2/HPCAL-4 (visinin-like protein 2/hippocalcin-like protein 4) was demonstrated whereas a trend was found for VILIP-1 (visinin-like protein 1) and HPCA (neuron-specific calcium-binding protein/hippocalcin). These proteins belong to the subfamily of visinin-like proteins, shown to play neuroprotective and neurotoxic roles, which have been implicated in several neurodegenerative diseases.[52] Interestingly, the downregulation VILIP-1 has been previously reported in post-mortem brains of patients with Alzheimer's disease, including the hippocampus area.[53,54] Hippocalcin has been shown to contribute to activity-dependent plasticity, neuronal excitability, and memory formation, and is most abundantly found in pyramidal cells of the hippocampal CA1 region.[55] It has been shown mice lacking hippocalcin develop mild deficits in spatial and associative memory.[56] Moreover, hippocampal neurons from hippocalcin-deficient mice have been shown to be more vulnerable to degeneration induced by excitotoxicity caused through glutamate receptor agonists.[57] Interestingly, downregulation of hippocalcin has been reported in neurodegenerative diseases such as Huntington's.[58] An upregulation of Tenascin-R (TN-R) was also identified. TN-R is one of the major extracellular matrix components of the perineuronal nets.[59] The function of TN-R is dependent on its presented physical form; growth-promoting *vs.* inhibiting. TN-R may decrease or increase after CNS injury, influencing microglial and astrocytic reaction and contributing to neurodegeneration or alternatively, neuroprotection. TN-R has also been shown to contribute to astroglial scar formation.[60]

Finally, we demonstrated that the number of episodes plays an essential role in influencing neuronal integrity. We found that a single episode of ammonia-induced OHE does not lead to any detectable neuronal loss or injury and have comparable findings to those found in BDL rats without episodes of OHE (Fig. S2). However, our study does not determine whether two or three episodes are required to induce neuropathological consequences and in addition merits to be validated in another animal model of

CLD.[9] Finally, the goal of this study was not to determine the number of episodes required to induce neurological damage, as clinically, the duration, frequency of episodes, and number of episodes will vary considerably between patients. These clinical details remain to be explored and merit investigation.

Conclusions

In conclusion, this new animal model of episodic OHE reveals the importance of cumulative OHE episodes on irreversible neuronal cell degeneration. Moreover, this model represents an excellent approach to explore the further pathological mechanisms arising from cumulative episodes, and an invaluable platform to investigate novel therapies to prevent or treat episodic OHE. Thus, evaluating and preventing the occurrence of OHE episodes may have a crucial impact on reducing the risk of irreversible neuronal injury in patients with cirrhosis, causing untreatable neurological complications and poor quality of life after LT.

Abbreviations

4-HNE, 4-hydroxynonenal; BDL, bile duct ligation; CHE, covert hepatic encephalopathy; CLD, chronic liver disease; CNS, central nervous system; GFAP, glial fibrillary acidic protein; HE, hepatic encephalopathy; HPCA, hippocalcin; HPCAL-4, hippocalcin-like protein 4; IL-1β, interleukin-1β; LT, liver transplantation; LTM, long-term memory; NeuN, neuron-specific nuclear antigen; OHE, overt hepatic encephalopathy; ROS, reactive oxygen species; SMI311, anti-neurofilament marker; STM, short-term memory; TAC, total antioxidant capacity; TNF-α, tumour necrosis factor-alpha; TN-R, Tenascin-R; VILIP-1, visinin-like protein 1; VILIP-2, visinin-like protein 2.

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Conflicts of interest

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

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

Authors' contributions

Study concept and design: FT, RO, MT, CFR. BDL surgery: MO, CB. Proteomics analysis: CL, ML, LS. Acquisition of data: FT, RO, KD. Analysis and interpretation of data: FT, MT, CFR. Writing of the manuscript: FT. Critical revision of the manuscript: MT, CFR. Study supervision, obtained funding, and approval of the final version of manuscript: CFR.

Data availability statement

The data that support the findings of this study are available from the corresponding author, CFR, upon request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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